VITAMIN D SUPPLEMENTATION AND MYOKINE RESPONSE IN EARLY PUBERTAL ADOLESCENTS

by

STEPHANIE MORGAN FOSS

(Under the Direction of Richard D. Lewis)

ABSTRACT

Vitamin D may play a role in muscle function. The effects of changes in serum vitamin D on myokines in 64 male and female, black and white adolescents (9-13 years) was determined following 12 weeks of vitamin D supplementation. Serum 25-hydroxyvitamin D {25(OH)D}, 1,25-dihydroxyvitamin D {$1,25(OH)_2D$}, myostatin, follistatin, and insulin-like growth factor 1 (IGF-I)] were measured at baseline and 12 weeks. At baseline, black males had significantly higher myostatin concentrations than white males. Concentrations of 25(OH)D and $1,25(OH)_2D$ increased significantly over 12 weeks, while myostatin, follistatin, and IGF-I did not change. This data suggests that large changes in serum 25(OH)D over 12 weeks does not alter myokines, but the differences in myostatin concentrations by race warrants further investigation.

INDEX WORDS: Vitamin D, Adolescents, Supplementation, Myostatin, Follistatin, IGF-I

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DEDICATION

This thesis is dedicated to my loving family, friends, and all the people of the Lewis Lab. Dad, I am so thankful for all the support, guidance, last minute trips to Athens, and love you have provided me during the past two years. Mom, I appreciate you always being there to talk over my day with me and providing love and encouragement when I needed it. Stephen, although you have been away during most of my time at UGA I am so thankful to have a brother that shows support and love from so far away. Christian, you have made this last year of graduate school possible and kept me motivated even when I was struggling. To my church family at the Episcopal Center and my awesome bible study, I couldn't have made it through grad school without your support and prayers. Internship cohort, it has been such a pleasure getting to know you all and I am excited to have you all as life long friends. Dr. Lewis, thank you for your guidance in school in life, your willingness to go above and beyond to provide me with an amazing research experience UGA, and for being willing to put up with a Gator in your lab! Lewis Lab, words can't express how much I have enjoyed being part of this family and how thankful I am to have had a place to call my home these past two years.

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CHAPTER 1

INTRODUCTION

Vitamin D deficiency exists in a significant portion of children and adolescents in the United States. According to recent NHANES data, 21% and 27% of nine to 13 year old males and females, respectively, have serum 25(OH)D concentrations < 50 nmol/L signifying they are at risk for vitamin D deficiency and/or inadequacy (Looker et al 2011). Additionally, vitamin D deficiency is found in a great proportion of the non-Hispanic Black and Mexican-American populations (Looker et al 2011). Low serum vitamin D concentrations could have significant health consequences in adults and children. In addition to the skeletal roles of vitamin D (refs), vitamin D has been linked with other health outcomes including some cancers, cardiovascular disease, insulin resistance and type 2 diabetes mellitus, and multiple sclerosis (IOM 2010; DeLuca 2004; Zofková 2008).

Over the past decade there has been an increasing interest in vitamin D influencing muscle function and strength (Rauch et al 2004). Older men and women with low vitamin D status have been shown to have poor short physical performance battery scores, weaker handgrip strength (Houston et al 2007, Houston et al 2011), postural instability defined as low limits of stability and high center of pressure under eyes closed on foam, slower gait velocity (Boersma et al 2011), and longer sit-to-stand and 8-foot walk time intervals (Bischoff-Ferrari et al 2004). Vitamin D intervention trials in older adults have provided additional support for this claim showing that an increase in 25(OH)D concentration with supplementation is associated with

improved muscle strength and composition (Bischoff-Ferrari et al 2009; Zhu et al 2010; Ceglia and Harris 2013).

Less is known regarding the relationship between vitamin D and muscle in children. If vitamin D has a positive effect on muscle during youth, this could be important for skeletal health since bone development and strength are partially driven by muscle forces applied to bones as described by the mechanostat theory (Fricke et al 2010). A major determinant of bone mineral accrual during growth is muscle and throughout pubertal growth spurts peak lean tissue mass accrual precedes the increase in bone mineral content (Rauch et al 2004, Jackowski et al 2009). Cross-sectional studies have shown that handgrip strength, muscle power, Esslinger Fitness Index, jump height and velocity, and muscle force were positively associated with vitamin D status in adolescent females aged 12 to 15 years (Foo et al 2008, Ward et al 2009). Findings from two supplementation trials in adolescent females found that jump velocity significantly increased in females with the lowest baseline 25(OH)D concentrations after one year (Ward et al 2010) and lean body mass increased among in premenarcheal females with relatively low 25(OH)D at baseline (El-Hajj Fuleihan et al 2006).

There is an emerging interest in the mechanism of how vitamin D impacts muscle, specifically through muscle-derived factors known as myokines (Hamrick 2010). Several myokines have been discovered in the muscle-bone interface that play a role in bone formation and can significantly impact bone metabolism and repair through paracrine and endocrine signaling pathways (Hamrick 2010). Myostatin is a muscle biomarker that has a catabolic effect in type-II muscle fibers, inhibiting muscle cell growth and differentiation (Hamrick et al 2010). Decreased or low serum myostatin concentrations have been associated with aerobic exercise (Hittel et al 2010), while increased concentrations have been correlated with muscle wasting

conditions including cancer, acquired immunodeficiency syndrome-related cachexia, and disuse atrophy (Elkasrawy and Hamrick 2010, Hamrick 2010). Follistatin, another biomarker of interest, acts as an antagonist of myostatin leading to decreased muscle catabolism (Hill et al 2002). A recent in vitro study showed that when skeletal muscle cells were treated with active $1,25(OH)_2D_3$, the expression of myostatin and follistatin were decreased and increased, respectively, after 4 and 7 days of incubation (Garcia et al 2011). To date, only one human study in older males has evaluated the relationship between vitamin D status and myostatin and showed a positive correlation between 25(OH)D and serum myostatin (Szulc et al 2012). Similar studies have not yet been conducted in younger age groups, nor has myostatin been evaluated in response to vitamin D supplementation. Information on the relationships of vitamin D with myostatin and follistatin in humans is lacking.

This thesis is an ancillary study to a vitamin D clinical trial in black and white males and females in the early stages of puberty receiving vitamin D supplementation for 12 weeks (Lewis et al, in preparation). Chapter 2 provides background information on vitamin D metabolism, assessment, and status, interactions between muscle and bone, the current data available on vitamin D and muscle, descriptions of myokines, and details about a cell study that provides some rationale for the current project. The purpose of this study presented in Chapter 3 was to assess serum myostatin, follistatin, and IGF-I responses to 12-weeks of vitamin D supplementation. A secondary aim was to examine associations between myokines, vitamin D metabolites and muscle outcomes. The primary hypothesis is that vitamin D supplementation will decrease serum myostatin concentrations and increase serum follistatin and IGF-I concentrations in males and females in the early stages of puberty. We further hypothesize that myostatin will be inversely correlated and follistatin, positively correlated with vitamin D

metabolites and muscle outcomes. Because of the availability of glucose and insulin data from the primary clinical trial we also decided to examine the relationships with glycemic control and that is also reported in chapter 3. In closing, Chapter 4 summarizes and discusses the study's finding and addresses the implications for future research.

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CHAPTER 2

LITERATURE REVIEW

Introduction

In adults and children vitamin D supplementation trials have demonstrated positive effects on muscle function. Low vitamin D status has been associated with poor Short Physical Performance Battery scores, weaker handgrip strength (Houston et al 2007, Houston et al 2011), postural instability defined as low limits of stability and high center of pressure under eyes closed on foam, slower gait velocity (Boersma et al 2011), and longer sit-to-stand and 8-foot walk time intervals (Bischoff-Ferrari et al 2004), Vitamin D intervention trials in older adults have provided additional support for this claim showing that with an increase in 25 hydroxyvitamin D (25[OH]D) concentration there was an association with improved muscle strength and composition (Bischoff-Ferrari et al 2009; Zhu et al 2010; Ceglia and Harris 2013). Cross-sectional studies have shown that muscle strength (Foo et al 2008), power, and force (Ward et al 2009) were positively associated with vitamin D status in adolescent females while vitamin D supplementation studies have demonstrated significant increases in jump velocity (Ward et al 2010), premenarcheal female lean body mass, and non-significant increases in handgrip strength (El-Hajj Fuleihan et al 2006). Cell culture studies provide a possible mechanism for how vitamin D may influence muscle through regulation of muscle proteins, specifically myostatin, follistatin, and IGF-I (Garcia et al 2011). The following sections review vitamin D metabolism, vitamin D status in children, muscle and bone interactions and relationships between vitamin D, myostatin, follistatin, and insulin like growth factor-1 (IGF-I).

Vitamin D metabolism

Vitamin D is a fat-soluble secosteroid that functions as a prohormone (DeLuca 2004). There are two main forms of vitamin D: vitamin D_2 or ergocalciferol and vitamin D_3 or cholecalciferol (Lips 2006). Vitamin D_3 can be synthesized in the skin with ultraviolet light from the sun that irradiates a cholesterol derivative, 7-dehydrocholesterol, or from nutritional sources that include salmon, mackerel, sardines, cod liver oil, and egg yolk (Lips 2006, Holick 2007). Vitamin D_3 production in the skin decreases with age, darker pigmentation of skin, the use of sunscreen, and clothing and is estimated to provide 80-100% of vitamin D required daily (Lips 2006; Kimlin 2008). Vitamin D_2 is derived from plants, plant materials, or foods that have been irradiated, especially mushrooms (Lips 2006). Most individuals acquire the recommended daily amount of vitamin D through sunlight and/or supplements (Macdonald 2012). Vitamin D does not occur naturally in many foods at high concentrations and when it is fortified into foods it is not at a high enough dose to meet daily recommendations (Macdonald 2012). Breakfast cereal, butter, cheese, margarine, milk, orange juice, yogurt, and infant formula are fortified with vitamin D_3 in the United States (Holick 2007). Vitamin D fortification in the United States and other countries has improved dietary intakes of vitamin D and increased serum 25(OH)D concentrations (O'Donnell et al 2008; Black et al 2012).

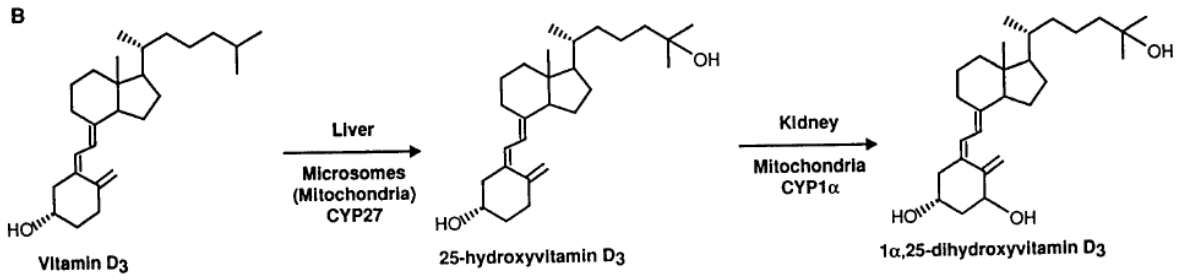

Figure 2.1 – Jones et al 2008

Vitamin D_2 and D_3 are not active in the body and must go through two hydroxylations to become active (Lips 2006). 7-dehydrocholesterol in skin is irradiated by the sun's ultraviolet light (280-320 nm range) and is converted to vitamin D_3 (Lips 2006; Kimlin 2008). The first hydroxylation, regulated by 25-hydroxylase, occurs in the liver and converts vitamin D_3 to 25(OH)D (Figure 2.1) (Lips 2006). The second hydroxylation converts 25(OH)D to the active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$), and this hydroxylation takes place in the kidney (Figure 2.1) (Lips 2006). The second hydroxylation is regulated by the enzyme1α-hydroxylase (DeLuca 2004). Plasma levels of inorganic phosphate, IGF-I, parathyroid hormone (PTH), and growth hormone (GH) regulate the activity of 1α-hydroxylase (Gomez 2006). The body regulates the concentration of $1,25(OH)_2D$ by decreasing the synthesis of PTH, which consequently decreases the conversion of 25(OH)D to $1,25(OH)_2D$ (Holick 2007). Once in the active form, vitamin D has many functions such as reducing inflammation, modulating cell growth and targeting a variety of tissues including the intestine, skeleton, parathyroid gland, reproductive and immune systems, skin, liver, breast tissue, and striated muscle (IOM 2010; DeLuca 2004; Zofková 2008).

Vitamin D assessment, status, and recommendations

Currently the most acceptable method to measure vitamin D status is through serum 25(OH)D (DeLuca 2004). Serum 25(OH)D is an ideal indicator of overall vitamin D status because it reflects dietary intake and cutaneous production, has a half-life of 15 d, and is not regulated as tightly as $1,25(OH)_2D$ (IOM 2010; Jones 2008). Alternatively, even though $1,25(OH)_2D$ is the biologically active form of vitamin D it has a relatively short half-life of 15 h and serum concentrations of $1,25(OH)_2D$ are tightly regulated by parathyroid hormone, phosphate, and calcium (IOM 2010; Jones 2008). Over the past few decades there have been

advances in technology and our understanding of vitamin D metabolism that led to various techniques of assessing vitamin D for both research and clinical purposes. Radioimmunoassay (RIA), high performance liquid chromatography (HPLC), competitive protein binding assay (CPBA), enzyme-linked immunosorbent assay (ELISA), and liquid chromatography linked with mass spectrometry (LC-MS) are all acceptable methods for assessing 25(OH)D and the method used for assessment should be decided based on resources and the specifics of what the investigator is interested in researching (Holick 2008). Additionally, the Vitamin D External Quality Assessment Scheme (DEQAS) has been a monitoring tool used since 1989 to assess the validity of varying vitamin D assays and the inter-laboratory coefficient of variation of these assays have greatly improved from 32.0% in 1994 to 15.3% in 2009 (Carter et al 2010).

There is a debate over what is the optimal serum level of vitamin D. The most recent guideline set by the Institute of Medicine (IOM) in October 2010 concluded that a serum 25(OH)D concentration less than 30 nmol/L is associated with vitamin D deficiency and concentrations between 30-49 nmol/L are associated with vitamin D insufficiency (IOM 2010). At a deficient 25(OH)D concentration an infant or child would be at risk for rickets and adults at risk for osteomalacia while vitamin D insufficiency means inadequate intake to support bone and overall health (IOM 2010). Based on the IOM report, serum 25(OH)D concentrations greater than or equal to 50 nmol/L are adequate to support bone and overall health in 97.5% of the population (IOM 2010). Table 1 shows the current serum 25(OH)D concentrations and their associated health status as determined by the IOM (IOM 2010). Serum concentrations of 25(OH)D are reported in both nmol/L and ng/mL. The conversion factor for nmol/L to ng/mL is 1 nmol/L = 0.4 ng/mL. The Endocrine Society released vitamin D recommendations in 2011 that were different from the IOM guidelines. According to the Endocrine Society a serum 25(OH)D

concentration of less than 50 nmol/L and 50 – 72.5 nmol/L defines vitamin D deficiency and insufficiency, respectively (Endocrine Society 2011).

Table 2.1: Serum 25(OH)D Status and Related Health Concerns (IOM 2010 Report)

nmol/L	**ng/mL**	**Health Status**
<30	<12	Considered **vitamin D deficiency**– Associated with rickets in infants and children and osteomalacia in adults
30-49	12-19	Generally accepted as **vitamin D insufficiency**– Inadequate concentration to support bone and overall health
≥50	≥20	Generally accepted as **vitamin D sufficiency**–Adequate concentration to support bone and overall health
>125	>50	Current research shows potential negative effects at concentrations this high

Vitamin D status in children

The Recommended Dietary Allowances (RDAs) for children ages 1-18 years is 600 IU/d (IOM 2010). The Endocrine Society has the same recommendations but add that it takes approximately 1,000 IU/d to raise 25(OH)D concentrations above 75 nmol/L (Endocrine Society 2011). The upper limit for 9 – 13 y olds is 4,000 IU/day (IOM 2010). According to NHANES data, 2% and 5% of 9 to 13 year old males and females, respectively, are at risk of vitamin D deficiency defined as serum 25(OH)D < 30 nmol/L (Looker et al 2011). In the same population, 19% and 22% of males and females, respectively, are at risk for vitamin D inadequacy defined as

serum 25(OH)D of 30 - 49 nmol/L (Looker et al 2011). Overall approximately one quarter of adolescents are at risk for either vitamin D deficiency or inadequacy and this is a significant portion of the population. Looker et al (2011) also reported that when examining race after adjusting for age and season the prevalence of being at risk for vitamin D deficiency and inadequacy in participants from NHANES over the age of one year was 3% and 13% in non-Hispanic Whites, 32% and 41% in non-Hispanic Blacks, and 9% and 33% in Mexican-Americans, respectively.

Findings from recent studies report that there is an inverse relationship between vitamin D status in adolescents and adiposity (Rajakumar et al 2011; Alemzadeh et al 2008). Rajakumar et al (2011) conducted a study in 237 children, mean age 12.7 ± 2.2 years who are 43% male, 47% obese, and 47% black and found that blacks were more likely to be vitamin D deficient, defined as 25(OH)D < 20 ng/ml. In addition plasma 25(OH)D was inversely associated with BMI, BMI percentile, percentile of total body fat, subcutaneous adipose tissue, and visceral adipose tissue while it was positively associated with HDL cholesterol in the entire study sample (Rajakumar et al 2011). Castaneda et al (2012) evaluated the response of obese and non-obese Caucasian adolescents to 2,000 IU/d of vitamin D_3 supplementation for 12 weeks and found that there was a significantly lower increase in serum 25(OH)D in obese adolescents following supplementation than in non-obese adolescents. The article concludes that the dose of vitamin D needed to treat hypovitaminosis D in obese children needs to be higher than for non-obese children (Castaneda et al 2012). Currently the Endocrine Society recommends a higher dose of vitamin D for obese adults but to-date, such a recommendation has not been made for children (Holick et al 2011).

Muscle and bone interaction

Vitamin D supplementation has been associated with BMC gains in children (El-Hajj Fuleihan et al 2006; Winzenberg et al 2011) and it may be linked to the effect of muscle on bone given that a study reported an increase in fat-free mass with 2000 IU of vitamin D supplementation daily for one year (El-Hajj Fuleihan et al 2006). The mechanostat theory states that bone development and strength are partially driven by increased muscle forces applied to the bones (Frost 1987). This theory uses the minimum effect strain (MES) mechanism to show that there is first mechanical loading on the bone that generates a primary signal that can be detected by other cells that then send a secondary signal to impact bone modeling and remodeling (Frost 1987). Non-mechanical factors impact the MES mechanism and since a vitamin D receptor has been found in skeletal muscle cells it could also have an effect on this model (Frost 1987; Bischoff-Ferrari et al 2004). The overall pathway for the mechanostat theory, as described by Frost (1987), starts with mechanical usage, physical loads and motions from physical activity, that impacts bone. The impact on bone then leads to the MES mechanism that stimulates bone modeling and remodeling and has an effect on bone mass. The mechanostat feedback loop acts like a home thermostat and turns this mechanism on when the bone mass is not great enough to endure the mechanical forces being applied to the skeleton (Frost 1987). If the bone does not gain enough strength to endure the impact imposed on it the bone is at risk of fracture.

Based on the ideals of the mechanostat theory, an increase in muscle strength should occur before an increase in bone strength (Rauch et al 2004). Rauch et al (2004) conducted a longitudinal study in boys and girls during the pubertal development (eight to 14 years of age) and measured their height and weight every six months and their fat-free mass and bone mineral content (BMC) with dual-energy X-ray absorptiometry (DXA) annually. The results from this

study showed that peak velocity in LBM accretion preceded the total body peak velocity in BMC in both boys and girls (Rauch et al 2004). Another longitudinal study was conducted in males and females aged 8 to 18 years, following participants for six years (Jackowski et al 2009). This study found that the age of peak lean tissue mass accrual precedes both bone peak cross sectional area velocity and peak section modulus velocity when accounting for sex (Jackowski et al 2009). Both of these studies support the mechanostat theory and the idea that muscle development plays an important role in affecting bone strength.

In addition to the physical effect that muscle has on bone there are also hormonal controls that affect the muscle-bone system. Some of the major hormones that affect the muscle-bone system are somatotropin, IGF-I, androgens, including testosterone, estrogen, leptin, and vitamin D (Zofková 2008). For this review the hormonal affects of IGF-I and vitamin D will be highlighted.

Vitamin D and muscle

Recent studies have investigated the relationship between vitamin D and muscle. Published observational studies performed in active and inactive older adults have found that there is a positive association between vitamin D status and muscle function (Bischoff-Ferrari et al 2004; Wicherts et al 2007). Cross-sectional studies have shown that older adults with low vitamin D status have poor Short Physical Performance Battery scores, weaker handgrip strength (Houston et al 2007, Houston et al 2011), postural instability defined as low limits of stability and high center of pressure under eyes closed on foam, slower gait velocity (Boersma et al 2011), and longer sit-to-stand and 8-foot walk time intervals (Bischoff-Ferrari et al 2004). A meta-analysis was conducted by Muir et al (2011) to determine the effect of vitamin D supplementation in older adults, ≥60 year old, on muscle strength, gait, and balance. The study

concluded that when older adults were supplemented with vitamin D doses of 100 to 1,000 IU per day there were positive effects on muscle strength and balance, but that association did not extend to gait (Muir et al 2011). Vitamin D intervention trials in older adults have provided additional support for this claim showing that with an increase in 25(OH)D concentration there was an association with improved muscle strength and composition (Bischoff-Ferrari et al 2009; Zhu et al 2010; Ceglia and Harris 2013). Although the effect of vitamin D on muscle function has been well studied in older adults there are limited studies published on the effect of vitamin D on muscle in adolescents, and only two that have studied vitamin D supplementation and muscle.

Adolescent cross-sectional data

A cross-sectional study conducted by Foo et al (2008) investigated the effect of low vitamin D status on muscle strength in healthy Chinese adolescent girls, 15 years of age. The participants that had serum 25(OH)D concentrations that were < 50 nmol/L had significantly lower muscle strength than the girls with adequate 25(OH)D concentrations (Foo et al 2008). These studies in adolescents provide a basis upon which further research needs to be conducted to determine the relationship between vitamin D and muscle and potential mechanisms. Another study performed by Ward et al (2009) in 99 post-menarchal 12 to 14 year old females examined the association between vitamin D status and muscle function. The research group determined that after controlling for weight there was a positive relationship between 25(OH)D and jump velocity, jump height, power, Esslinger Fitness Index, and force (Ward et al 2009).

Vitamin D supplementation trials

To date there have been two published studies investigating vitamin D supplementation and muscle function in adolescents (Ward et al 2010; El-Hajj Fuleihan et al 2006). Ward et al

(2010) conducted a vitamin D supplementation trial in healthy, postmenarchal 12 to 14 year old females who received either 150,000 IU of D_2 or placebo four times over 12 months. Muscle function was measured by jumping mechanography and grip strength (Ward et al 2010). In addition to the expected increase in 25(OH)D in the treatment group, there was a significant increase in muscle efficiency in the vitamin D supplementation group compared to the placebo and there was a marginal increase in the muscle parameters that measures power, jumping velocity, and height (Ward et al 2010). Another vitamin D supplementation trial was conducted by El-Hajj Fuleihan et al (2006) who studied healthy girls ages ten to 17 years who were given either placebo, 200 IU or 2,000 IU/d of vitamin D_3 for 12 months. This study found that there was a significant increase in fat-free mass in both vitamin D supplementation groups compared to the controls ($p<0.05$) but there was no increase in grip strength (El-Hajj Fuleihan et al 2006). When the researchers examined only the premenarcheal girls they also found a significant increase in lean mass ($p<0.04$) but not in grip strength comparing both supplementation groups to the control group (El-Hajj Fuleihan et al 2006).

Myokines

Although muscle is most commonly thought to affect bone strength and geometry through the forces it applies on bone, recent studies have shown that muscle may additionally impact bone through muscle-derived factors known as myokines (Hamrick 2010). There have been several myokines discovered in the muscle-bone interface that play a role in bone formation (Hamrick 2010). These myokines can significantly impact bone metabolism and bone repair through paracrine and endocrine signaling pathways (Hamrick 2010). A few of the myokines known to have this effect on bone are outlined in the following sections.

Myostatin

Myostatin is a member of the transforming growth factor-β superfamily (TGF-β) of proteins (McPherron et al 1997; Hamrick 2010). It is primarily secreted from skeletal muscle cells (McFarlane et al 2011). The active form of myostatin is formed when the initial precursor protein that contains a signal sequence, an N-terminal propeptide domain, and a C-terminal domain (the active ligand) is proteolytically processed between the propeptide domain and the C-terminal domain releasing mature myostatin (McPherron et al 1997; Hill et al 2002; Lee and McPherron 2001). Myostatin can form disulfide–linked dimers at the C-terminal end (McPherron et al 1997; Lee and McPherron 2001). Myostatin propeptide and follistatin can bind to myostatin and inhibit its function (Lee and McPherron 2001; Zimmer et al 2002). Myostatin has a catabolic effect in type-II muscle fibers because of its ability to inhibit muscle cell growth and differentiation (Hamrick 2010). The production of myostatin is controlled by the Mstn gene (Hamrick 2010). Studies conducted in mice have shown that when there is a homozygous deletion of the Mstn gene there is a greater increase in skeletal muscle mass due to both hypertrophy and hyperplasia (Lui et al 2011). Similar effects have also been observed when myostatin production has been blocked (Lui et al 2011). Studies performed in cattle, sheep, dogs, pigs, mice, and humans suggest that evolutionarily myostatin may serve as a negative regulator of skeletal muscle mass (Lui et al 2011).

Serum myostatin levels have been found to be elevated in patients with cancer, acquired immunodeficiency syndrome-related cachexia, and disuse atrophy (Elkasrawy and Hamrick 2010; Hamrick 2010). The opposite was found as serum myostatin levels decreased with the addition of aerobic exercise (Hittel et al 2010). A study conducted in six to 16 years olds by Ehehalt et al (2011) showed that after a six month lifestyle intervention program in obese

children both muscle mass and serum myostatin concentrations increased. Before the intervention, the subjects under ten years of age had lower myostatin serum levels than the older children (14.1±5.1 ng/mL vs. 19.4±8.3 ng/mL, p=0.04) but after the six-month intervention period there was no difference between the two age groups (19.0±8.7 vs. 21.9±8.2) (Ehehalt et al 2011). The researchers also concluded that the participants that had a significantly lower increase in muscle mass had the largest rise in serum myostatin (Ehehalt et al 2011). The researchers believe their is reason to believe that there is a negative feedback loop between myostatin and muscle tissue (Ehehalt et al 2011). A study conducted by Castro-Gago et al (2007) found that there was no difference in myostatin expression in children with muscle diseases, including congenital fiber type I disproportion, neurogenic muscular atrophy, myotonia congenital, infantile glycogenosis type II, and Prader-Willi syndrome, in comparison with age-matched controls. Another study performed in children with congenital heart disease found that myostatin expression was increased in participants with a left ventricular orthotopic heart transplant compared to participants with a right ventricular orthotopic heart transplant or right ventricular outflow tract (Bish et al 2011). It was also found that with a decrease in ventricular function there was an increased myostatin/IGF-I ratio (Bish et al 2011).

The studies presented above provide evidence that serum myostatin concentrations are elevated in certain disease states. Some but not all conditions show promise in disease state improvement with decreased serum myostatin concentrations. Vitamin D may decrease serum myostatin and aid in disease management (Garcia et al 2011).

Relationship of vitamin D and myostatin

To our knowledge a single prior study looked at the relationship between serum 25(OH)D and serum myostatin concentrations in humans. This prospective cohort study was

conducted in 1121 males aged 20 to 87 years from the STRucture of the Aging Men's Bones (STRAMBO) cohort, which consists of males who have private health insurance coverage with Mutelle de Travailleurs de la Région Lyonnaise (Szulc et al 2012). Serum samples were collected and a competitive ELISA was used to measure serum myostatin and RIA was used to measure serum 25(OH)D concentrations (Szulc et al 2012). After analyzing the data the researchers found a split in the association between myostatin and age at the age of 57 (Szulc et al 2012). In the 296 males aged 57 or younger serum myostatin concentrations correlated positively with age whereas in males older 57 years serum myostatin significantly decreased with age (Szulc et al 2012). The rest of the analyses conducted for the study were analyzed separately based on the two age categories (Szulc et al 2012). There was no correlation between serum myostatin and 25(OH)D concentrations (Szulc et al 2012). There was a significant positive correlation between serum myostatin and 25(OH)D concentrations in the males over 57 who were not taking a vitamin D supplement (Szulc et al 2012). There was an increase of 0.13 SD in myostatin per 10 ng/mL increase in 25(OH)D (Szulc et al 2012). Circannual variability was seen in the serum myostatin with the highest concentrations found in the spring (Szulc et al 2012). According to Szulc et al (2012) 25(OH)D concentrations and season contributed to the variability of the serum myostatin concentrations. The authors discuss that the decrease in serum myostatin which is produced by muscle may reflect age-related loss of muscle mass or the body's method of attempting to minimize muscle mass (Szulc et al 2012). This study was performed on male volunteers who may be healthier than the general population but it provides a starting observation for researchers to expand upon.

Myostatin and insulin resistance

Diabetes affects 25.8 million people in the United States of all ages, which is equivalent to 8.3% of the United States. population based on data from 2010 (CDC Diabetes Fact Sheet 2011). Of the 25.8 million people affected, 18.8 million have been diagnosed and a predicted seven million are undiagnosed with the greatest percentage of these people being over the age of 65 years (CDC Diabetes Fact Sheet 2011). It is estimated that the total cost of diabetes for the United States in 2007 was $174 billion and this number continues to rise (CDC Diabetes Fact Sheet 2011). A majority of the previous diabetes research has examined the association between insulin resistance, obesity, and the endocrine function of adipokines (Saremi and Parastesh 2011). More recent research has indicated that skeletal muscle might also have a role in this relationship through the secretion of the muscle protein myostatin (Saremi and Parastesh 2011).

Wang et al (2012) investigated the relationship between plasma myostatin in patients with type 2 diabetes to better understand myostatin's role in the clinical pathophysiological changes associated with type 2 diabetes. The study evaluated 42 patients who where diagnosed with type 2 diabetes and 20 age-matched controls (Wang et al 2012). Wang et al (2012) found that the female controls had higher plasma myostatin concentrations than the male controls. There was a significant increase in plasma myostatin in the patients with type 2 diabetes compared to the healthy controls and like the healthy controls, the females with type 2 diabetes had higher plasma myostatin concentrations than the men with type 2 diabetes (Wang et al 2012). They also found that in addition to sex, increased BMI and triglyceride levels had a significant relationship with higher plasma myostatin concentrations, which indicates that the pathophysiological changes associated with type 2 diabetes may be linked to plasma myostatin concentrations (Wang et al 2012). Plasma myostatin concentrations in the patients with type 2

diabetes were negatively correlated with BMI and fasting plasma glucose but were positively associated with homeostasis model assessment of insulin resistance (HOMA-IR) (Wang et al 2012).

A study conducted by Brandt et al (2012) looked at the associations of myostatin with insulin resistance, fitness, low-grade inflammation, and lean body mass by quantifying the amount of circulating myostatin and myostatin expression in skeletal muscle of patients with type 2 diabetes to evaluate the relationship between myostatin and metabolism. The researchers enrolled 168 subjects, which included 92 healthy controls and 76 patients with type 2 diabetes. The myostatin mRNA from the skeletal muscle was 1.4 fold ($P<0.05$) greater in the patients with type 2 diabetes compared to the healthy controls and this remained statistically significant after controlling for gender and age (Brandt et al 2012). The difference in plasma myostatin was only significant after adjusting for age and gender and the researchers found that patients with type 2 diabetes had higher concentrations (Brandt et al 2012). There was a positive correlation between myostatin mRNA in skeletal muscle and fasting insulin, HOMA2-IR, plasma interleukin 6 (IL-6), C-reactive protein (CRP), body mass index (BMI), and triglycerides and a negative correlation with maximal oxygen uptake when analyzing all participants together (Brandt et al 2012). There was a positive correlation in healthy controls between plasma myostatin and skeletal muscle myostatin mRNA (Brandt et al 2012). Overall this study showed that myostatin mRNA in skeletal muscle is elevated in patients with type 2 diabetes compared to healthy controls and that a higher expression of myostatin mRNA is associated with impaired metabolism obesity, systemic inflammation, and lower fitness levels in healthy controls (Brandt et al 2012). The researchers believe that these associations were not found in the patients with

type 2 diabetes because the metabolic effects of myostatin are overtaken by other contributing factors once an individual develops type 2 diabetes (Brandt et al 2012).

Saremi and Parastesh (2011) conducted a 12-week study to explore the effects of an exercise intervention on serum myostatin concentrations in relationship with insulin resistance. The study participants were 19 overweight-obese women who had a mean age of 23.2 ± 3.4 years and a BMI ≥25 kg/m^2 who were randomized to either a control or exercise intervention group. All participants were instructed to continue their usual eating behaviors. The exercise intervention group participated in resistance training about 60 minutes a day, three days a week (on nonconsecutive days), for 12 weeks. The researchers hypothesized that there would be an improvement in insulin resistance and a reduction in myostatin following 12 weeks of resistance training. The results confirmed the researchers' hypothesis as they found improvement in insulin resistance and a decrease in myostatin concentrations in the exercise intervention group (Saremi and Parastesh 2011). Although this study has confirmed the relationship between myostatin and insulin resistance it is unable to determine if the decrease myostatin is directly or indirectly linked to insulin resistance (Saremi and Parastesh 2011). Some researchers have found evidence that myostatin can have a direct effect on insulin resistance by inhibiting the activation of the Akt enzyme that is key for glucose metabolism or by increasing the expression of adiponectin and peroxisome proliferator-activated receptor gamma (PPAR-γ) which can improve insulin resistance (Zhao et al 2005; Akpan et al 2009; Suzuki et al 2007). This study does confirm that patients with diabetes should be encouraged to exercise regardless of whether myostatin has a direct or indirect affect on insulin resistance (Saremi and Parastesh 2011).

Follistatin

Follistatin (FST), a glycoprotein, is known regulator of activins and antagonist of many members of the TGF-β superfamily, including myostatin (Schenyer et al 2004). The regulatory function of follistatin is to modulate paracrine and autocrine functions and the process of development and differentiation. Follistatin regulates activin in many tissues including the pituitary, gonads, muscle and bone, and tissue differentiation in embryological development (Welt et al 2002). One of the main topics of research in terms of follistatin is its relationship to myostatin. Follistatin is an inhibitory binding protein of myostatin causing negative regulation (Figure 2.2) (Hill et al 2002). It acts a negative regulator by preventing myostatin from binding to its receptor (Hill et al 2002). A study conducted Kocamis et al (2004) demonstrated that the mRNA for myostatin and follistatin were expressed in C_2C_{12} cells and with the addition of follistatin to the cell cultures there was an effect on the myostatin mRNA expression. Amthor et al (2004) examined the expression of myostatin and follistatin during chick development. The researchers found that myostatin and follistatin directly interact and follistatin binds to myostatin with a high affinity (Amthor et al 2004). Myostatin mediated inhibition of Pax-3 (plays a role in striated muscle development) and MyoD (a protein that regulates muscle differentiation) expression is suppressed when follistatin is present (Amthor et al 2004). This suggests that there is a direct protein interaction between follistatin and myostatin that makes myostatin unable to inhibit muscle development (Amthor et al 2004). Another study by Lee and McPherron (2001) found that mice treated with follistatin had similar results in terms of increases in muscle mass as seen in myostatin knockout mice. Overall these studies suggest that follistatin may be a possible agent for use to enhance muscle mass or decrease the extent of muscle catabolism in humans.

Role of IGF-I

Insulin-like growth factors are involved in the growth and function of most of the organs in the body (Le Roith 1997). The synthesis of the peptide IGF-I, a product of somatotropin, primarily occurs in the liver and is released when growth hormone interacts with its hepatic receptor to stimulate release (Le Roith 1997; Zofková 2008). The concentration of IGF-I in the body parallels the concentration of growth hormone secreted (Le Roith 1997). It is also synthesized in the bone and its production is regulated by growth hormone, parathyroid hormone, and sex steroids (Zofková 2008; Le Roith 1997). The IGF-I produced by the bone directly stimulates the formation of bone once it binds to a specific receptor (Zofková 2008).

The action of IGF-I has been shown to be promoted by $1,25(OH)_2D$ by increasing the number of IGF-I receptors (Gómez 2006). IGF-I can also increase the concentration of $1,25(OH)_2D$ because it increases the rate of hydroxylation of 25(OH)D to $1,25(OH)_2D_3$ by regulating the 1α-hydroxylase enzyme (Gómez 2006). The vitamin D-myostatin-muscle link to bone may be mediated by IGF-I through this relationship and others (Garcia et al 2011). IGF-I regulates skeletal muscle hypertrophy that is induced by mechanical loading (Machida et al 2004). IGF-I aids in the regulation of skeletal muscle hypertrophy through at least three processes that include increased satellite cell proliferation, increased skeletal α-actin mRNA expression, and increased protein synthesis (Machida et al 2004). IGF-I stimulated proliferation of satellite cells is associated with the activation of PI3K/Akt signaling pathway, the up-regulation of a cyclin-dependent kinase 2 kinase activity, and the down regulation of $p27^{Kipl}$, a cell-cycle inhibitor, as demonstrated in transgenic mice that were over-expressing IGF-I (Chakravarthy et al 2000).

Concentrations of serum IGF-I are low at birth and substantially increase during growth and through puberty and a decline is seen around the third decade (Le Roith 1997). A cross-sectional study of healthy nine to 17 y olds performed by Kanbur et al (2005) found that serum IGF-I levels reached a peak in girls at an age of 12 years and at age 14 to 16 years in boys. When analyzing the data based on pubertal stage the investigators found that the maximum mean values for girls were found at pubertal stages three and four and for boys the maximum mean values occurred at stage four (Kanbur et al 2005). Furthermore, there was a significant increase in serum IGF-I concentrations in both boys and girls between stage one and two and between stage two and three (Kanbur et al 2005).

Breen et al (2011) used baseline data from a longitudinal study conducted in four to eight year old females that were measured for up to nine years to determine the influence of IGF-I and 25(OH)D on BMC accrual. The researchers found that during growth, IGF-I concentrations were associated with a heightened rate of BMC accrual and 25(OH)D did not have any additional predictive effects on BMC accrual after accounting for IGF-I (Breen et al 2011). This relationship may be mediated by muscle since peak BMC accrual has been shown to be preceded by increases in muscle growth (Rauch et al 2004). Another study completed by Bogazzi et al (2011) was conducted in healthy subjects with a mean age of 49.2 ± 14.4 years. The researchers identified a positive relationship between serum IGF-I and 25(OH)D concentrations (Bogazzi et al 2011). Subjects who were vitamin D deficient, defined by a serum 25(OH)D concentration < 20 ng/ml, had lower IGF-I concentrations compared to the subjects with mild to absent vitamin D deficiency.

Vitamin D supplementation effects on myostatin, follistatin, and IGF-I

The mechanism explaining how $1,25(OH)_2D$ promotes myogenic differentiation at the cellular or molecular level is not understood (Garcia et al 2011). A recent study was conducted to examine the effect of treating C_2C_{12} skeletal muscle cells with $1,25(OH)_2D_3$ on myogenic differentiation (Garcia et al 2011). Garcia et al (2011) used real-time qPCR and Western blots to examine the effect of treatment with active $1,25(OH)_2D_3$ on skeletal muscle cells for four and seven days on myostatin, follistatin, and IGF-I expression. The results from the real-time PCR measured after four and seven days of supplementation showed a decrease in myostatin expression by 2.5- and ten-fold, respectively, when compared to controls (Garcia et al 2011). Western blottings were used to evaluate myostatin expression at the protein level and showed a decrease in myostatin expression using densitometric analysis that was completed in triplicate (Garcia et al 2011). This decrease in myostatin could improve muscle strength by reducing muscle catabolism, and consequently improve bone strength (Garcia et al 2011). Follistatin was measured to determine if it plays a role in the mechanism through which $1,25(OH)_2D_3$ promotes muscle growth knowing that follistatin is an inhibitor of myostatin activity (Garcia et al 2011). Garcia et al (2011) found that after incubation with $1,25(OH)_2D_3$ for four days the follistatin expression was significantly increased by 2.5-fold when compared to controls. Since there was a significant decrease in myostatin expression at the mRNA level after seven days with $1,25(OH)_2D_3$ incubation the researchers tested to see if the protein level of follistatin was up-regulated knowing that the protein level of myostatin was down-regulated (Garcia et al 2011). There was an up-regulation in follistatin at the protein level and this suggests that myogenic differentiation related to incubation with $1,25(OH)_2D_3$ could be a result of increased expression of follistatin and its ability to inhibit myostatin (Garcia et al 2011). This study also examined

IGF-I expression and found a three-fold decrease in the expression of IGF-I at the steady-state mRNA level after four days of incubation with $1,25(OH)_2D_3$ using quantitative real-time PCR when compared to controls (Garcia et al 2011). To confirm these results, Western blottings were conducted with their respective densitometric analysis done in triplicate to show that at the protein level there was also a decrease in IGF-I expression (Garcia et al 2011). Both of the tests used to examine IGF-I expression were also conducted after seven days and these results also showed a decrease in IGF-I expression (Garcia et al 2011).

Figure 2.2 is a representation of the effect of vitamin D supplementation on myostatin, follistatin, and IGF-I and how changes in the expression of these biomarkers affect muscle growth and differentiation (Garcia et al 2011). This figure shows how mesodermal stem cells become myotubules and the role that $1,25(OH)_2D_3$ plays in decreasing cell proliferation and enhancing myogenic cell differentiation by regulating the expression of myostatin, follistatin, and IGF-I (Garcia et al 2011).

An additional study examined the effect of treating mesenchymal multipotent cells (MMCs) with active $1,25(OH)_2D_3$ (Artaza and Norris 2009). One of the results found with the treatment of $1,25(OH)_2D_3$ was an increased expression of follistatin (Artaza and Norris 2009). This was determined using both real-time PCR arrays and DNA microarrays which showed increased expression of follistatin at 24 hours and four days post-treatment with $1,25(OH)_2D_3$ (Artaza and Norris 2009).

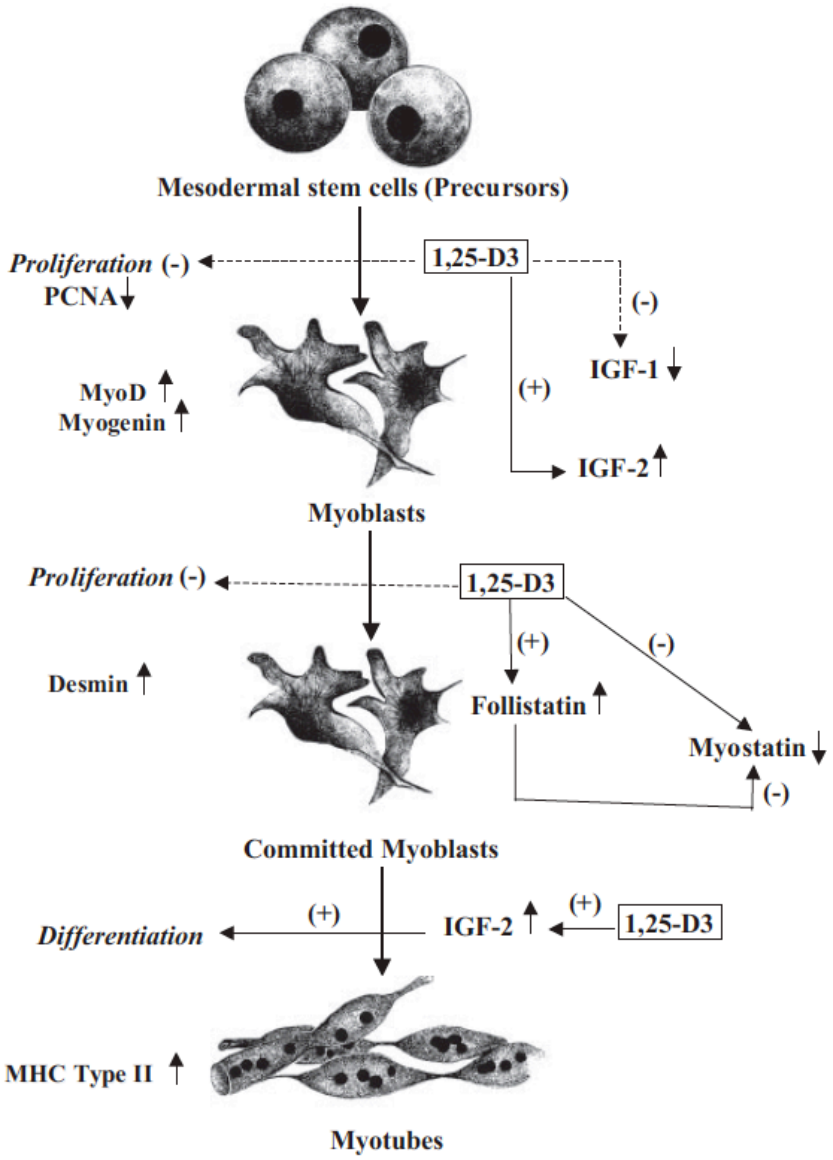

Figure 2.2 - Garcia et al 2011

Summary

A significant proportion of the adolescents between 9 and 13 y old are at risk of vitamin D deficiency or inadequacy. The mechanostat theory and studies showing that peak LBM precedes peak BMC accrual provide justification for evaluating muscle function and muscle biomarkers as a method for improving bone strength and potentially reducing the risk of

fractures later in life. The literature review showed that vitamin D trials conducted in adults and children have found positive effects on muscle function, but not in all studies. No studies have examined serum myostatin and follistatin concentrations following vitamin D supplementation. The cell culture study by Garcia et al (2011) showed that myostatin expression decreased and follistatin expression increased in skeletal muscle cells with $1,25(OH)_2D_3$ supplementation which provides a potential mechanism for how vitamin D may influence muscle through regulation of muscle proteins. Since IGF-I regulates the activation of vitamin D and Breen et al (2011) found a positive association between peak BMC accrual and IGF-I, there is reason to believe that IGF-I may mediate the link between vitamin D-myostatin-muscle and bone. Increasing our understanding of how myokines and growth factors such as myostatin, follistatin, and IGF-I are associated with vitamin D and muscle development may lead to novel approaches to reduce the risk of falls and bone fractures later in life. Elevated serum myostatin concentrations are associated with insulin resistance and exercise-training programs have been shown decrease myostatin concentrations. Data from the current project will help us better understand the links between myostatin and glycemia.

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CHAPTER 3

VITAMIN D SUPPLEMENTATION AND MYOKINE RESPONSE IN EARLY PUBERTAL ADOLESCENTS

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Abstract

Background: Vitamin D status has been linked with muscle function. Treatment of skeletal muscle cells in vitro with the active form of vitamin D changed the expression of the myokines myostatin and follistatin and IGF-I. Not much is known about the myokine response to vitamin D in humans

Objective: In this ancillary study of a larger vitamin D dose-response trial, we examined whether changes in vitamin D metabolites following 12 weeks of vitamin D supplementation were associated with changes in myostatin, follistatin, and IGF-I in adolescents.

Design: Participants were selected from 323 early pubertal males (aged 10 to 13 years) and females (aged 9 to 12 years) who were enrolled in a previous 12-week vitamin D intervention trial (Lewis et al, in preparation). This smaller ancillary study included 64 participants with the lowest (n=32) and highest (n=32) changes in vitamin D concentrations following 12 weeks of supplementation. An equal number of subjects from each race and sex were included in this sampling procedure. Biochemical markers [25-hydroxyvitamin D (25(OH)D), 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), myostatin, follistatin, and insulin-like growth factor 1 (IGF-I)] were measured at baseline and 12 weeks.

Results: At baseline, black males had significantly higher myostatin concentrations than white males. There was a negative correlation between 25(OH)D and myostatin at baseline. Serum 25(OH)D and $1,25(OH)_2D$ significantly increased over 12 weeks, while myostatin, follistatin, and IGF-I did not change.

Conclusion: This data suggests that large increases in serum 25(OH)D over 12 weeks does not alter myokines in adolescents, but the variation in myostatin concentrations by race warrants further investigation.

Introduction

Over the past decade there has been an increasing interest in the nonskeletal roles of vitamin D. In older adults, vitamin D status has been linked to muscle function. Men and women with low vitamin D status have been shown to have poor short physical performance battery scores, weaker handgrip strength (Houston et al 2007, Houston et al 2011), postural instability defined as low limits of stability and high center of pressure under eyes closed on foam, slower gait velocity (Boersma et al 2011), and longer sit-to-stand and 8-foot walk time intervals (Bischoff-Ferrari et al 2004). Vitamin D intervention trials in older adults have provided additional support for this claim showing that an increase in 25(OH)D concentration with supplementation is associated with improved muscle strength and composition (Bischoff-Ferrari et al 2009; Zhu et al 2010; Ceglia and Harris 2013).

Less is known regarding the relationship between vitamin D and muscle in children. If vitamin D has a positive effect on muscle during youth, this could be important for skeletal health since bone development and strength are partially driven by muscle forces applied to bones as described by the mechanostat theory (Fricke et al 2010). A major determinant of bone mineral accrual during growth is muscle and throughout pubertal growth spurts, peak lean tissue mass accrual precedes the increase in bone mineral content (Rauch et al 2004, Jackowski et al 2009). Cross-sectional studies have shown that handgrip strength, muscle power, Esslinger Fitness Index, jump height and velocity, and muscle force were positively associated with vitamin D status in adolescent females aged 12 to 15 years (Foo et al 2008, Ward et al 2009). Findings from two supplementation trials in adolescent females found that jump velocity significantly increased in females with the lowest baseline 25(OH)D concentrations after one

year (Ward et al 2010) and lean body mass increased among in premenarcheal females with relatively low 25(OH)D at baseline (El-Hajj Fuleihan et al 2006).

There is an emerging interest in the mechanism of how vitamin D impacts muscle, specifically through muscle-derived factors known as myokines (Hamrick 2010). Several myokines have been discovered in the muscle-bone interface that play a role in bone formation and can significantly impact bone metabolism and repair through paracrine and endocrine signaling pathways (Hamrick 2010). Myostatin is a muscle biomarker that inhibits muscle cell growth and differentiation (Hamrick et al 2010). Decreased or low serum myostatin concentrations have been associated with aerobic exercise (Hittel et al 2010), while increased concentrations have been correlated with muscle wasting conditions including cancer, acquired immunodeficiency syndrome-related cachexia, and disuse atrophy (Elkasrawy and Hamrick 2010, Hamrick 2010). Follistatin, another biomarker of interest, acts as an antagonist of myostatin leading to decreased muscle catabolism (Hill et al 2002). A recent in vitro study showed that when skeletal muscle cells were treated with active $1,25(OH)_2D_3$, the expression of myostatin and follistatin were decreased and increased, respectively, after 4 and 7 days of incubation (Garcia et al 2011). To date, only one human study in older males has evaluated the relationship between vitamin D status and myostatin and showed a positive correlation between 25(OH)D and serum myostatin (Szulc et al 2012). Similar studies have not yet been conducted in younger age groups, nor has myostatin been evaluated in response to vitamin D supplementation.

Because information on the relationships of vitamin D with myostatin and follistatin in humans is lacking, the purpose of this study was to assess serum myostatin, follistatin, and IGF-I responses to 12-weeks of vitamin D supplementation. A secondary aim was to examine associations between myokines, vitamin D metabolites and muscle outcomes. The primary

hypothesis is that vitamin D supplementation will decrease serum myostatin concentrations and increase serum follistatin and IGF-I concentrations in males and females in the early stages of puberty. We further hypothesize that myostatin will be inversely correlated and follistatin, positively correlated with vitamin D metabolites and muscle outcomes.

Methods

Study design

Participants in this study were selected from 323 early pubertal males (aged 10 to 13 years) and females (aged 9 to 12 years) who were enrolled in a previous 12-week vitamin D intervention trial (Lewis et al, in preparation). In this study participants were living in Georgia, 34°N, and Indiana, 40°N, USA. This smaller ancillary study included 64 participants with the lowest (n=32) and highest (n=32) changes in vitamin D concentrations. An equal number of subjects from each race and sex were included in this sampling procedure.

Screening Protocol

All methods and procedures were approved by the Institutional Review Board for Human Subjects at the University of Georgia. The screening of participants was conducted in two phases. First, potential participants who were in the appropriate age range were screened over the telephone using a medical questionnaire that assessed basic information including race/ethnicity, medication and supplement use, and medical history. Exclusion criteria included achievement of menarche, existing muscle or bone disorders (e.g., intestinal malabsorption, cerebral palsy), growth disorders, or use of medications/supplements that may influence vitamin D metabolism. Subjects agreed to provide blood samples, avoid taking any vitamin, mineral or herbal supplements, and maintain their normal diet and physical activity patterns for the duration of the 12-week intervention. A washout period of 4 weeks was an option for subjects that met all

requirements except current supplement use. During the second screening phase, subjects were mailed a sexual maturation self-assessment form that they were to complete at home and mail back to the proper testing location. Males and females who reported their pubertal stage (Tanner 1962) as 2 or 3 for genitalia and breast development, respectively, were enrolled into the study.

Biochemical assays

All samples were stored at < -70°C until analysis. All biochemical assays were conducted using a block design, such that all samples from the same subject at each time point were assayed at one time by the same investigator using the same kit. The investigators were blinded to the race, latitude, and dose of each sample and a repeat analysis was conducted when duplicated samples differed by >10%. The analyses presented here include blood samples from the baseline and 12-week testing sessions.

Serum 25(OH)D was assayed using a 2-step radioimmunoassay (DIASORIN, 25 hydroxyvitamin D^{125} 1 RIA kit no. 68100, Stillwater, MN, 100 tube kit). The inter- and intra-assay coefficients of variation in a sample of black and white children, were 7.3% - 10.5% and 5.9% - 7.0%, respectively (N = 83). $1,25(OH)_2D$ was assessed using a 2-step radioimmunoassay (DIASORIN, 1,25- dihydroxyvitamin D^{125} 1 RIA kit, no. 65100E, Stillwater, MN, 100 tube kit). The inter- and intra- assay coefficients of variation for $1,25(OH)_2D$ assay were 11.6% and 14.4% - 16.9%, respectively. Serum iPTH was assayed using an immunoradiometric assay (DIASORIN, N-tact PTH SP kit, no. 26100, Stillwater, MN, 100 beads/kit). The intra- and inter-assay CVs range from 3.1% - 9.4% and 0.5% - 3.0%, respectively.

Serum myostatin concentrations were assayed using ELISA as instructed by the manufacturer (R&D Systems). The samples were read using a microtiter plate reader at 450 nm. A four-parameter algorithm was used to calculate the standard curve against which the

concentrations of myostatin in human samples were determined. The coefficient of variation was 37.4 at baseline and 38.6 at 12 weeks. Serum follistatin was assessed using ELISA (R&D Systems) according to the manufacturer's protocol. The coefficient of variation at baseline was 47.8 and 42.4 at 12 weeks. Serum IGF-I concentrations were assessed using recombinant human IGF-I quantitative sandwich immunoassay technique (R&D Systems, Human IGF-I Immunoassay, #890773, Minneapolis, MN, 96 test kit). The samples of serum were pretreated with: A) an acidic disassociation solution and B) a buffered protein with blue dye and preservatives; lyophilized, to release IFG-I from its binding proteins. The inter- and intra- assay coefficients of variation were 7.5% - 8.3% and 3.5% - 4.3%, respectively. Serum glucose concentrations were measured using a microtiter modification of the enzymatic Autokit Glucose method (Wako Chemicals USA, Richmond, VA). The limit of detection for this assay is 0 – 500 mg/dL. Serum insulin was measured by Human Insulin Specific RIA (HI-14K; St. Charles, MO) and the detection limit is 3.125 – 100 uU/mL.

Surrogate Measures of Insulin Sensitivity

A surrogate for insulin resistance was determined by incorporating fasting glucose and insulin values into the homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR = [(fasting glucose [mg/dL] x fasting insulin [uU/mL] / 405] (Matthews et al 1985). Quantitative insulin sensitivity check index (QUICKI) was used as a surrogate measure for insulin sensitivity. QUICKI = 1/[log(fasting glucose{mg/dL}) + log(fasting insulin {uU/mL})]. There is a strong correlation (r = 0.6 – 0.8) between HOMA-IR and QUICKI with the gold standard euglycemic hyperinsulinemic clamp technique (Wallace et al 2004; Gungor et al 2004).

Statistical Analyses

All analyses were performed using SPSS version 21 (Chicago, IL). Descriptive statistics

were generated and range, as well as normality checks, were performed. A *P*-value < 0.05 was considered statistically significant for all analyses. The dependent variables for this study included serum myostatin, follistatin, and IGF-I. The independent variables were serum 25(OH)D and 1,25$(OH)_2$D, iPTH, age, sex, and race. Independent samples *t*-tests were conducted to determine differences in baseline characteristics between groups. In addition, bivariate correlations were conducted to identify relationships between dependent and independent variables at baseline. Due to potential confounding by age, race, and sex on the primary dependent variables, these factors were considered as covariates and thus controlled for in the mixed effects model. This model produced group (i.e., treatment, 0 and 4,000 IU), time (i.e., 0 and 12 weeks), and group x time effects.

Results

Baseline and Participant Characteristics

Baseline characteristics of the participants are presented in Table 3.1. Black males had greater weight, BMI-for-age, and myostatin at baseline in comparison to white males, but had significantly lower serum 25(OH)D. Black females had significantly lower serum 25(OH)D and serum IGF-I in comparison to white females. When controlling for baseline variances in lean mass, the differences in myostatin among the males remained statistically significant (p=0.049), however QUICKI was no longer significant. Participants in the placebo group had significantly greater serum 25(OH)D than participants in the 4,000 IU group at baseline. None of the participants were meeting the recommended dietary intake for vitamin D of 600 IU/day by diet alone. 11% of the participants had serum 25(OH)D concentrations < 50 nmol/L. Of the 64 participants, 38 were considered normal weight as defined by a BMI-for-age < 85%, 11 were

overweight as defined 85 to 95% BMI-for-age, and 15 were obese as defined by a BMI-for-age > 95%.

Baseline Correlations

Table 3.2 depicts simple bivariate correlations at baseline. At baseline, myostatin was positively correlated with height, lean mass, 25(OH)D, PTH, arm MCSA, leg MCSA, TBBMC, and TBBMD while it was negatively correlated with serum 25(OH)D and serum glucose. Follistatin was positively associated with QUICKI and negatively associated with height, weight, BMI-for-age, lean mass, leg MCSA, arm IMAT, leg IMAT, TBBMC, and TBBMD at baseline. There were positive correlations between IGF-I and height, weight, lean mass, serum $1,25(OH)_2D$, serum insulin, HOMA-IR, and TBBA, whereas IGF-I was negatively correlated with QUICKI.

Intervention Outcomes

Table 3.3 summarizes the comparisons between groups over the 12-week intervention. Serum 25(OH)D and $1,25(OH)_2D$ increased significantly ($p < 0.001$) and PTH decreased ($p < 0.001$) in the 4,000 IU treatment group compared to the placebo group following 12-weeks of supplementation. There were no significant differences between the treatment groups for myostatin, follistatin, IGF-I, serum glucose, serum insulin, HOMA-IR, and QUICKI.

Discussion

To our knowledge, this is the first examination of serum myostatin and follistatin responses to vitamin D supplementation. The primary findings from this study indicate that despite significant increases in 25(OH)D and $1,25(OH)_2D$ in children following 12-weeks of vitamin D supplementation, there were no associated changes in myostatin, follistatin, or IGF-I. Though a supplementation effect on myokines was not detected, there were significant baseline

correlations of interest, for example, a significant negative correlation between 25(OH)D and myostatin and positive correlations between IGF-I and several variables ($1,25[OH]_2D$, height, weight, lean mass, serum insulin, HOMA-IR, and QUICKI, a surrogate measure of insulin sensitivity). Moreover, serum glucose was negatively associated with myostatin concentrations and QUICKI was positively associated with follistatin. Positive correlations between myostatin and arm MCSA, leg MCSA, TBBMC, and TBBMD were observed, while follistatin was negatively correlated with leg MCSA, arm IMAT, leg IMAT, TBBMC, and TBBMD. Black males and females had lower serum 25(OH)D concentrations at baseline compared to white males and females while black males had significantly greater myostatin at baseline than white males.

Since the active form of vitamin D decreases the expression of myostatin in cell culture (Garcia et al 2011), we expected that significant increases in serum $1,25(OH)_2D$ following vitamin D supplementation would be associated with decreases in serum myostatin. This was not the case. The lack of changes in myostatin with increasing serum vitamin D levels may be explained by the length of our intervention. Garcia et al (2011) noted that the decrease in myostatin expression in cell culture after treatment with $1,25(OH)_2D$ was more pronounced after seven days than four days. These results are consistent with findings by Artaza et al (2002), which demonstrated that myostatin expression was more pronounced in later vs. earlier stages of myoblast cell differentiation. One of the few human studies assessing myostatin found improvements in muscle function and decreased serum myostatin in overweight children following six months of strength and aerobic exercise training (Ehehalt et al 2011). In the current project, vitamin D supplementation over 12 weeks was not a sufficient stimulus to elicit significant muscle changes (Wright et al, in preparation).

The treatment group was receiving the upper limit for vitamin D for the age group of the study population. While it is difficult to know if our intervention dose was comparable to the dose given by Garcia et al (2011), 4,000 IU/day was probably high enough to elicit significant change in myostatin although this was not observed. Vitamin D supplementation for a year increased lean mass in ten to 17 year old females with doses equivalent to 200 IU/day and 2,000 IU/day (El-Hajj Fuleihan et al 2006). Another study found similar results after vitamin D supplementation for a year in 12 to 13 year old females who were supplemented with 150,000 IU every three months, which is approximately 1600 IU/day (Ward et al 2010). Both of these supplementation trials supplemented with lower doses of vitamin D than our study and saw positive results in muscle function providing justification to predict that we supplemented our participants with an appropriate dose.

There is evidence to suggest that season effects myostatin concentrations (Szulc et al 2012), but we controlled for season by only conducting our trial during the winter months. Seasonal variations in myostatin expression have been reported in animals but the outcomes varied based on the species and muscle evaluated (Swanson et al 2009; Braulke et al 2010). In the pectoralis muscle of small birds, myostatin mRNA expression was decreased in the winter (Swanson et al 2009). Decreased myostatin leads to increased muscle mass which is needed by these small house sparrows to keep them warm and increase the heat produced from shivering in the winter (Swanson et al 2009). A shift in myostatin expression was also noted as an adaptation for survival during the winter in ground squirrels (Nowell et al 2011). In muscles that were needed for respiration myostatin expression was decreased but in muscles that were not necessary for survival during hibernation, like skeletal muscles, myostatin didn't decrease (Nowell et al 2011). To date only one study has examined this relationship in humans (Szulc et al

2012). Serum myostatin concentrations were increased in the spring for both younger and older men and the relationship was not associated with 25(OH)D concentrations (Szulc et al 2012).

A previous study in adult males found conflicting data where people with higher serum 25(OH)D concentrations had higher serum myostatin (Szulc et al 2012). These significant correlations were only present in older males who were not taking supplements (Szulc et al 2012). Lakahman et al (2009) observed that younger men, mean age 26.5 ± 4.6 years, had significantly higher concentrations of serum myostatin (8.0 ± 0.3 ng/mL versus 7.0 ± 0.4 ng/mL, p=0.03) than older men, mean age 66.4 ± 4.7 years. Another study that examined younger and older men found no difference in myostatin between the two age groups (Ratkevicius et al 2011). Not only has this relationship been seen in comparison with vitamin D concentrations but a similar trend has been noted in the relationship between muscle mass and myostatin. It has been coined the "myostatin paradox" because one would expect to find lower myostatin concentrations in people with greater muscle but instead researchers have been finding just the opposite (Kim et al 2012). This suggests that other factors are regulating myostatin and it is believed that vitamin D may be a contributing factor (Kim et al 2012; Garcia et al 2011).

Myostatin has been shown to have growth inhibiting actions mediated through the attenuation of IGF-I induced Akt phosphorylation (Ji et al 2008). We found no significant correlations between IGF-I and either myostatin or follistatin at baseline or over time. IGF-I acts as a major regulator of adult muscle tissue and plays a major role in muscle growth and regeneration (Florini et al 1993). Kanbur et al (2005) noted that serum IGF-I concentrations reached a peak in girls at an age of 12 years and at age 14 to 16 years in boys. Prior to the six month exercise intervention, Ehehalt et al (2011) found that children under the age of ten had lower serum myostatin concentrations than the older children but following the intervention there

was no difference between the two age groups. This suggests that interventions may have a greater impact on adolescents during various stages of puberty when the myokines are more susceptible to change.

The relationship in the present study between myostatin and serum glucose indicates that there may exist a relationship in relatively healthy adolescents with myostatin and insulin resistance. MSTN-null mice exhibit immunity to dietary-induced obesity and insulin resistance (Gonzalez-Cadavid et al 2004). Human studies have also reported a link between myostatin concentration and insulin resistance (Hittel et al 2010). Compared to healthy controls, individuals with type 2 diabetes have significantly increased plasma myostatin concentrations and skeletal muscle myostatin mRNA (Wang et al 2012; Brandt et al 2012). Our study was performed in overall healthy adolescents, which may provide an additional explanation as to why we didn't observe myostatin decrease with vitamin D supplementation. In our ancillary study, four of the 64 participants were identified as prediabetic after enrollment into the study based on parameters examined after the completion of the study but they did not have a different response to supplementation than the other participants. A few studies reported a decrease in plasma and muscle myostatin following an exercise intervention in patients with type 2 diabetes or insulin resistance (Hittel et al 2010; Saremi and Parastesh 2011). Some researchers have found evidence that myostatin can have a direct effect on insulin resistance by inhibiting the activation of the Akt enzyme that is key for glucose metabolism or increasing the expression of adiponectin and peroxisome proliferator-activated receptor gamma (PPAR-γ) which can improve insulin resistance (Zhao et al 2005; Akpan et al 2009; Suzuki et al 2007).

There are no published studies evaluating the difference in myostatin and follistatin concentrations by race. Our study found that black males have higher serum myostatin

concentrations than whites males. Even when we statistically corrected for fat-free mass, the significant differences remained. Hull et al (2011) reported black males and females had greater fat-free mass than white males and females and that blacks had the lowest percentage change in muscle over the lifespan. This is important since myostatin is produced in skeletal muscle cells (McPherron et al 1997). Some studies suggest that blacks have different muscle composition, in terms of fiber type, than whites but the evidence is conflicting (Suminski et al 2002; Duey et al 1997). Additionally, myostatin may play a role in determining skeletal muscle composition. Myostatin knockout mice display a greater proportion of fast twitch type 2 muscle fibers and a decreased proportion of slow twitch type 1 muscle fibers (Girgenrath et al 2004).

This study had several strengths. It was the first clinical vitamin D intervention trial that examined myostatin and follistatin and it was performed in black and white male and female adolescents in the early stages of puberty. Secondly, we supplemented with a safe dose of 4,000 IU/day which generated significant increases in serum 25(OH)D and $1,25(OH)_2D$. The current study also had some limitations. There is a considerable amount of variation in the assays for myostatin and follistatin. Secondly, the length of the intervention trial may not have been of sufficient duration to elicit muscle changes. Lastly, interpretation of our data is complicated by the fact that this is a novel area of study with little comparable data.

In summary, the hypothesized decrease in serum myostatin and increase in follistatin following vitamin D supplementation was not observed. However, there was a negative correlation at baseline between 25(OH)D and myostatin. Additionally, black males had significantly higher myostatin concentrations than white males at baseline. Although prior research studies that were longer in length showed increases in muscle, there are no indications from our data that if our study was extended we could expect to see our hypothesized changes in

myostatin and follistatin. Further research is warranted to evaluate the observed differences in baseline myostatin concentrations between black and white males.

Table 3.1 Baseline Characteristics Overall, by Race, Sex, and Treatment

Overall Characteristics[2]		Characteristics by Vitamin D dose						
Variable[1]	**N**	**Overall (N = 64)**	**White Male (n = 16)**	**Black Male (n = 16)**	**White Female (n = 16)**	**Black Female (n = 16)**	**Placebo (n = 32)**	**4,000 IU (n = 32)**
Age (yrs)	64	11.3 ± 1.3	11.9 ± 1.1	11.8 ± 1.4	10.9 ± 1.0	10.7 ± 1.3	11.3 ± 1.4	11.3 ± 1.1
Anthropometry								
Weight (kg)	64	47.2 ± 13.4	42.0 ± 12.4	56.4 ± 15.6*	44.4 ± 9.1	45.8 ± 12.1	47.3 ± 12.7	47.0 ± 14.3
Height (cm)	64	149 ± 10	152 ± 9.9	153 ± 10.3	147 ± 9.1	147 ± 10.4	150 ± 10.8	149 ± 9.4
BMI-for-age (%)	64	68.5 ± 29.2	41.9 ± 29.2	86.8 ± 13.1*	72.1 ± 24.5	73.1 ± 28.3	68.6 ± 29.7	68.4 ± 29.1
Body Composition[3]								
Fat mass (kg)	61	16.3 ± 8.2	14.8 ± 5.5	16.9 ± 9.7	18.3 ± 10.1	14.9 ± 6.4	17.4 ± 8.6	15.1 ± 7.7
Percent body fat	61	32.4 ± 9.5	26.5 ± 8.6	32.9 ± 11.1	35.7 ± 7.5	33.8 ± 8.7	32.2 ± 9.4	32.6 ± 9.7
Lean mass (kg)	61	29.9 ± 7.0	29.5 ± 6.3	34.7 ± 8.2	27.0 ± 4.7	28.3 ± 6.2	30.6 ± 7.3	29.3 ± 6.8
Biochemical[4]								
25(OH)D (nmol/L)	64	70.8 ± 18.3	84.5 ± 14.8	60.3 ± 10.0*	78.0 ± 16.3	60.5 ± 14.0*	75.3 ± 19.3	66.3 ± 16.5*
$1,25(OH)_2D$ (pmol/l)	64	143 ± 47.8	135 ± 46.0	142 ± 49.4	152 ± 60.6	142 ± 34.6	148 ± 45.2	137 ± 50.4
iPTH (pg/mL)	64	26.4 ± 10.6	21.9 ± 9.3	29.2 ± 12.7	25.1 ± 9.1	29.6 ± 9.8	25.7 ± 11.1	27.2 ± 10.2
Myostatin (pg/mL)	60	1143 ± 428	986.2 ± 372	1284 ± 386*	1012 ± 349	1312 ± 527	1109 ± 384	1177 ± 472
Follistatin (pg/ml)	60	2443 ± 1182	2873 ± 1354	2195 ± 848	2241 ± 992	2481 ± 1458	2216 ± 1132	2670 ± 1207
IGF-1 (ng/mL)	64	283.4 ± 195	245.3 ± 161	272.6 ± 133	419.4 ± 289	196.3 ± 60.3*	285.9 ± 159	280.9 ± 229
Serum glucose (mg/dL)	64	89.9 ± 6.5	90.3 ± 6.2	89.1 ± 5.5	92.0 ± 8.2	88.0 ± 5.7	88.9 ± 6.6	90.8 ± 6.3
Serum insulin (uU/mL)	64	22.4 ± 15.6	19.9 ± 20.5	22.0 ± 6.7	19.8 ± 11.0	28.1 ± 19.7	26.0 ± 19.4	18.9 ± 9.5
HOMA-IR	64	5.02 ± 3.7	4.53 ± 5.1	4.86 ± 1.6	4.53 ± 2.7	6.17 ± 4.5	5.80 ± 4.7	4.25 ± 2.3
QUICKI	64							

		0.31 ± 0.02	0.32 ± 0.03	0.31 ± 0.01	0.31 ± 0.02	0.30 ± 0.02	0.31 ± 0.02	0.32 ± 0.02
Dietary Intake (/day)								
Vitamin D (IU)	61	158 ± 95.8	155 ± 95.1	186 ± 106	163 ± 90.2	128 ± 93.8	136 ± 93.2	181 ± 94.9

[1] Values are presented as means ± SD.

[2] Overall characteristics represent data collapsed across the 2 treatment groups.

[3] Body composition measures assessed using dual energy X-ray absorptiometry.

[4] Abbreviations: 25(OH)D = 25-hydroxyvitamin D; $1,25(OH)_2D$ = 1,25-dihydroxyvitamin D; iPTH = intact parathyroid hormone; IGF-1 = insulin-like growth factor 1; HOMA-IR = homeostasis model of assessment – insulin resistance; QUICKI = quantitative insulin sensitivity check index.

[*] Statistical significance determined by independent samples *t*-tests is indicated by $p \leq 0.05$.

Table 3.2 Baseline Correlations

Variables	Myostatin		Follistatin		IGF-1	
	r	*P*	r	*P*	R	*P*
Height	0.401	**0.002**[1,2]	-0.246	**0.059**[1,2]	0.295	**0.018**[1,2]
Weight	0.234	0.073[1]	-0.245	**0.059**[1,2]	0.287	**0.021**[2]
BMI-for-age	0.045	0.733	-0.286	**0.027**[1,2]	0.180	0.155[2]
Lean mass	0.379	**0.004**[1,2]	-0.271	**0.042**[1,2]	0.289	**0.024**[1,2]
25(OH)D	-0.250	**0.054**[1]	0.083	0.527	-0.027	0.833
$1,25(OH)_2D$	-0.126	0.338	0.160	0.223	0.395	**0.001**[1,2]
iPTH	0.245	**0.059**[1]	0.099	0.454	0.042	0.744
Serum glucose	-0.285	**0.027**[1]	-0.027	0.836	0.147	0.246
Serum insulin	0.114	0.384	-0.174	0.184	0.298	**0.017**[2]
HOMA-IR	0.081	0.540	-0.159	0.224	0.318	**0.010**[2]
QUICKI	-0.139	0.290	0.318	**0.013**[1,2]	-0.310	**0.013**[2]
Arm MCSA	0.313	**0.015**[1,2]	-0.241	0.064[1,2]	0.098	0.441
Leg MCSA	0.325	**0.012**[1,2]	-0.292	**0.025**[1,2]	0.179	0.161
Arm IMAT	0.226	0.098	-0.302	**0.025**[1,2]	0.010	0.942
Leg IMAT	0.231	0.090[1]	-0.425	**0.001**[1,2]	0.099	0.457
TBBA	0.178	0.185	-0.073	0.588	0.385	**0.002**[1,2]
TBBMC	0.414	**0.001**[1,2]	-0.399	**0.002**[1,2]	0.133	0.306
TBBMD	0.389	**0.003**[1]	-0.499	**0.000**[1,2]	-0.211	0.103

Values are presented as simple bivariate correlations (r) and statistical significance (p).
Bold variables are statistically significant at $p \leq 0.05$.
[1] Variables are statistically significant after controlling for sex.
[2] Variables are statistically significant after controlling for race.
Abbreviations: 25(OH)D = 25-hydroxyvitamin D; $1,25(OH)_2D$ = 1,25-dihydroxyvitamin D; iPTH = intact parathyroid hormone; IGF-1 = insulin-like growth factor 1; HOMA-IR = homeostasis model of assessment – insulin resistance; QUICKI = quantitative insulin sensitivity check index; MCSA = muscle cross-sectional area; IMAT = intermuscular adipose tissue; TBBA = total body bone area; TBBMC = total body bone mineral content; TBBMD = total body bone mineral density

Table 3.3 Treatment by Time Interactions

Biochemical variable[1]	4,000 IU		Placebo		Treatment[2] x time[3]
	Baseline	12-weeks	Baseline	12-weeks	(p)[4]
25(OH)D (nmol/L)	77.4 ± 2.9	61.2 ± 5.6	69.5 ± 2.9	170 ± 5.6	0.001
$1,25(OH)_2D$ (pmol/L)	151 ± 10.1	148 ± 11.0	143 ± 10.3	207 ± 11.2	0.001
iPTH (pg/mL)	25.5 ± 1.8	30.3 ± 2.2	27.0 ± 1.9	23.5 ± 2.2	0.001
Myostatin (pg/mL)	1069 ± 72	1021 ± 67	1136 ± 73	1045 ± 68	0.653
Follistatin (pg/mL)	2211 ± 233	2227 ± 207	2605 ± 236	2475 ± 210	0.550
IGF-1 (ng/mL)	325 ± 37	345 ± 36	323 ± 37	350 ± 36	0.658
Serum glucose (mg/dL)	89.1 ± 1.3	88.7 ± 1.4	91.0 ± 1.3	90.7 ± 1.5	0.979
Serum insulin (uU/mL)	24.7 ± 3.0	24.9 ± 3.0	17.7 ± 3.1	21.5 ± 3.0	0.200
HOMA-IR	5.5 ± 0.7	5.5 ± 0.8	4.0 ± 0.7	4.9 ± 0.8	0.217
QUICKI	0.31 ± 0.00	0.31 ± 0.00	0.32 ± 0.01	0.31 ± 0.01	0.529

[1] Values are adjusted by sex and race and are presented as estimated marginal means ± SE.
Abbreviations: 25(OH)D = 25-hydroxyvitamin D; 1,25(OH)2D = 1,25-dihydroxyvitamin D; iPTH = intact parathyroid hormone; IGF-1 = insulin-like growth factor 1; HOMA-IR = homeostasis model of assessment – insulin resistance; QUICKI = quantitative insulin sensitivity check index.
[2] Treatment groups = 0 or 4,000 IU Vitamin D_3/day
[3] Time = 0 and 12 weeks
[4] Statistically significant treatment by time effects are based mixed model analyses and are significant at $p \leq 0.05$.

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CHAPTER 4

SUMMARY AND CONCLUSIONS

Over the past decade there has been an increasing interest in the nonskeletal roles of vitamin D. In older adults, vitamin D status has been linked to poor muscle function (Houston et al 2007, Houston et al 2011, Boersma et al 2011, Bischoff-Ferrari et al 2004). Vitamin D intervention trials in older adults have provided additional support for this claim showing that an increase in 25(OH)D concentration with supplementation is associated with improved muscle strength and composition (Bischoff-Ferrari et al 2009; Zhu et al 2010; Ceglia and Harris 2013).

Less is known regarding the relationship between vitamin D and muscle in children. If vitamin D has a positive effect on muscle during youth, this could be important for skeletal health since bone development and strength are partially driven by muscle forces applied to bones as described by the mechanostat theory (Fricke et al 2010). Cross-sectional studies have shown increased muscle size and strength with higher vitamin D status in adolescent females aged 12 to 15 years (Foo et al 2008, Ward et al 2009). Findings from two supplementation trials in adolescent females found that jump velocity significantly increased in females with the lowest baseline 25(OH)D concentrations after one year (Ward et al 2010) and lean body mass increased among in premenarcheal females with relatively low 25(OH)D at baseline (El-Hajj Fuleihan et al 2006).

There is an emerging interest in the mechanism of how vitamin D impacts muscle, specifically through muscle-derived factors known as myokines (Hamrick 2010). Several myokines have been discovered in the muscle-bone interface that play a role in bone formation

and can significantly impact bone metabolism and repair through paracrine and endocrine signaling pathways (Hamrick 2010). Myostatin is a muscle biomarker that inhibits muscle cell growth and differentiation (Hamrick et al 2010). Follistatin, another biomarker of interest, acts as an antagonist of myostatin leading to decreased muscle catabolism (Hill et al 2002). A recent in vitro study showed that when skeletal muscle cells were treated with active $1,25(OH)_2D_3$, the expression of myostatin and follistatin were decreased and increased, respectively, after 4 and 7 days of incubation (Garcia et al 2011). To date, only one human study in older males has evaluated the relationship between vitamin D status and myostatin and showed a positive correlation between 25(OH)D and serum myostatin (Szulc et al 2012). Similar studies have not yet been conducted in younger age groups, nor has myostatin been evaluated in response to vitamin D supplementation. The current study sought to examine serum myostatin, follistatin, and IGF-I responses to 12-weeks of vitamin D supplementation to fill in gaps in the literature.

To our knowledge, this is the first examination of serum myostatin and follistatin responses to vitamin D supplementation. The primary findings from this study indicate that despite significant increases in 25(OH)D and $1,25(OH)_2D$ in children following 12-weeks of vitamin D supplementation, there were no associated changes in myostatin, follistatin, or IGF-I. Though a supplementation effect on myokines was not detected, there were significant baseline correlations of interest, for example, a significant negative correlation between 25(OH)D and myostatin and positive correlations between IGF-I and several variables ($1,25[OH]_2D$, height, weight, lean mass, serum insulin, HOMA-IR, and QUICKI, a surrogate measure of insulin sensitivity). Moreover, serum glucose was negatively associated with myostatin concentrations and QUICKI was positively associated with follistatin. Positive correlations between myostatin and arm MCSA, leg MCSA, TBBMC, and TBBMD were observed, while follistatin was

negatively correlated with leg MCSA, arm IMAT, leg IMAT, TBBMC, and TBBMD. Black males and females had lower serum 25(OH)D concentrations at baseline compared to white males and females while black males had significantly greater myostatin at baseline than white males.

Since the active form of vitamin D decreases the expression of myostatin in cell culture (Garcia et al 2011), we expected that significant increases in serum $1,25(OH)_2D$ following vitamin D supplementation would be associated with decreases in serum myostatin. This was not the case. The lack of changes in myostatin with increasing serum vitamin D levels may be explained by the length of our intervention. Garcia et al (2011) noted that the decrease in myostatin expression in cell culture after treatment with $1,25(OH)_2D$ was more pronounced after seven days than four days. These results are consistent with findings by Artaza et al (2002), which demonstrated that myostatin expression was more pronounced in later vs. earlier stages of myoblast cell differentiation. One of the few human studies assessing myostatin found improvements in muscle function and decreased serum myostatin in overweight children following six months of strength and aerobic exercise training (Ehehalt et al 2011). In the current project, vitamin D supplementation over 12 weeks was not a sufficient stimulus to elicit significant muscle changes (Wright et al, in preparation).

The treatment group was receiving the upper limit for vitamin D for the age group of the study population. While it is difficult to know if our intervention dose was comparable to the dose given by Garcia et al (2011), 4,000 IU/day was probably high enough to elicit significant change in myostatin although this was not observed. Vitamin D supplementation for a year increased lean mass in ten to 17 year old females with doses equivalent to 200 IU/day and 2,000 IU/day (El-Hajj Fuleihan et al 2006). Another study found similar results after vitamin D

supplementation for a year in 12 to 13 year old females who were supplemented with 150,000 IU every three months, which is approximately 1600 IU/day (Ward et al 2010). Both of these supplementation trials supplemented with lower doses of vitamin D than our study and saw positive results in muscle function providing justification to predict that we supplemented our participants with an appropriate dose.

There is evidence to suggest that season effects myostatin concentrations (Szulc et al 2012), but we controlled for season by only conducting our trial during the winter months. Seasonal variations in myostatin expression have been reported in animals but the outcomes varied based on the species and muscle evaluated (Swanson et al 2009; Braulke et al 2010). In the pectoralis muscle of small birds, myostatin mRNA expression was decreased in the winter (Swanson et al 2009). Decreased myostatin leads to increased muscle mass which is needed by these small house sparrows to keep them warm and increase the heat produced from shivering in the winter (Swanson et al 2009). A shift in myostatin expression was also noted as an adaptation for survival during the winter in ground squirrels (Nowell et al 2011). In muscles that were needed for respiration myostatin expression was decreased but in muscles that were not necessary for survival during hibernation, like skeletal muscles, myostatin didn't decrease (Nowell et al 2011). To date only one study has examined this relationship in humans (Szulc et al 2012). Serum myostatin concentrations were increased in the spring for both younger and older men and the relationship was not associated with 25(OH)D concentrations (Szulc et al 2012).

A previous study in adult males found conflicting data where people with higher serum 25(OH)D concentrations had higher serum myostatin (Szulc et al 2012). These significant correlations were only present in older males who were not taking supplements (Szulc et al 2012). Lakahman et al (2009) observed that younger men, mean age 26.5 ± 4.6 years, had

significantly higher concentrations of serum myostatin (8.0 ± 0.3 ng/mL versus 7.0 ± 0.4 ng/mL, p=0.03) than older men, mean age 66.4 ± 4.7 years. Another study that examined younger and older men found no difference in myostatin between the two age groups (Ratkevicius et al 2011). Not only has this relationship been seen in comparison with vitamin D concentrations but a similar trend has been noted in the relationship between muscle mass and myostatin. It has been coined the "myostatin paradox" because one would expect to find lower myostatin concentrations in people with greater muscle but instead researchers have been finding just the opposite (Kim et al 2012). This suggests that other factors are regulating myostatin and it is believed that vitamin D may be a contributing factor (Kim et al 2012; Garcia et al 2011).

Myostatin has been shown to have growth inhibiting actions mediated through the attenuation of IGF-I induced Akt phosphorylation (Ji et al 2008). We found no significant correlations between IGF-I and either myostatin or follistatin at baseline or over time. IGF-I acts as a major regulator of adult muscle tissue and plays a major role in muscle growth and regeneration (Florini et al 1993). Kanbur et al (2005) noted that serum IGF-I concentrations reached a peak in girls at an age of 12 years and at age 14 to 16 years in boys. Prior to the six month exercise intervention, Ehehalt et al (2011) found that children under the age of ten had lower serum myostatin concentrations than the older children but following the intervention there was no difference between the two age groups. This suggests that interventions may have a greater impact on adolescents during various stages of puberty when the myokines are more susceptible to change.

The relationship in the present study between myostatin and serum glucose indicates that there may exist a relationship in relatively healthy adolescents with myostatin and insulin resistance. MSTN-null mice exhibit immunity to dietary-induced obesity and insulin resistance

(Gonzalez-Cadavid et al 2004). Human studies have also reported a link between myostatin concentration and insulin resistance (Hittel et al 2010). Compared to healthy controls, individuals with type 2 diabetes have significantly increased plasma myostatin concentrations and skeletal muscle myostatin mRNA (Wang et al 2012; Brandt et al 2012). Our study was performed in overall healthy adolescents, which may provide an additional explanation as to why we didn't observe myostatin decrease with vitamin D supplementation. In our ancillary study, four of the 64 participants were identified as prediabetic after enrollment into the study based on parameters examined after the completion of the study but they did not have a different response to supplementation than the other participants. A few studies reported a decrease in plasma and muscle myostatin following an exercise intervention in patients with type 2 diabetes or insulin resistance (Hittel et al 2010; Saremi and Parastesh 2011). Some researchers have found evidence that myostatin can have a direct effect on insulin resistance by inhibiting the activation of the Akt enzyme that is key for glucose metabolism or increasing the expression of adiponectin and peroxisome proliferator-activated receptor gamma (PPAR-γ) which can improve insulin resistance (Zhao et al 2005; Akpan et al 2009; Suzuki et al 2007).

There are no published studies evaluating the difference in myostatin and follistatin concentrations by race. Our study found that black males have higher serum myostatin concentrations than whites males. Even when we statistically corrected for fat-free mass, the significant differences remained. Hull et al (2011) reported black males and females had greater fat-free mass than white males and females and that blacks had the lowest percentage change in muscle over the lifespan. This is important since myostatin is produced in skeletal muscle cells (McPherron et al 1997). Some studies suggest that blacks have different muscle composition, in terms of fiber type, than whites but the evidence is conflicting (Suminski et al 2002; Duey et al

1997). Additionally, myostatin may play a role in determining skeletal muscle composition. Myostatin knockout mice display a greater proportion of fast twitch type 2 muscle fibers and a decreased proportion of slow twitch type 1 muscle fibers (Girgenrath et al 2004).

This study had several strengths. It was the first clinical vitamin D intervention trial that examined myostatin and follistatin and it was performed in black and white male and female adolescents in the early stages of puberty. Secondly, we supplemented with a safe dose of 4,000 IU/day which generated significant increases in serum 25(OH)D and $1,25(OH)_2D$. The current study also had some limitations. There is a considerable amount of variation in the assays for myostatin and follistatin. Secondly, the length of the intervention trial may not have been of sufficient duration to elicit muscle changes. Lastly, interpretation of our data is complicated by the fact that this is a novel area of study with little comparable data.

In summary, the hypothesized decrease in serum myostatin and increase in follistatin following vitamin D supplementation was not observed. However, there was a negative correlation at baseline between 25(OH)D and myostatin. Additionally, black males had significantly higher myostatin concentrations than white males at baseline. Although prior research studies that were longer in length showed increases in muscle, there are no indications from our data that if our study was extended we could expect to see our hypothesized changes in myostatin and follistatin. Further research is warranted to evaluate the observed differences in baseline myostatin concentrations between black and white males.

In conclusion, this study indicated that there was no relationship between changes in vitamin D metabolites following supplementation and the response of serum myostatin, follistatin, and IGF-I. However this study was the first vitamin D intervention trial to provide insight into the relationship between vitamin D metabolites and myokines in early pubertal

adolescents of varying race and sex. Results from this study encourage future studies examining the difference in serum myostatin concentration between blacks and whites.

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APPENDICES

APPENDIX A

Telephone screening questionnaire

Supplemental Vitamin D in Early Adolescence
Telephone Screening Questionnaire

This interview should take approximately ten minutes:

Date: _____ Time: _____ Screen completed by: _____

1. A. Is your child male_____ or female_____?

1. B. (*If Female*) Has your child started her menstrual cycles? YES ____ NO ____

1. C. How would you describe your child's ethnicity/race?

Ethnicity:	Hispanic or Latino
	Non-Hispanic or Latino
Race:	American Indian or Alaska Native
	Asian
	Black or African American
	Native Hawaiian or other Pacific Islander
	White
	any combination of the above

Parents of participants may indicate one or more of the following (mixed racial heritage should be indicated by selecting more than one category):

2. We would also like to know the ethnicity/race of the child's biological parents and grandparents. How do you describe your child's mother and father?

	Child's Mother	***Child's Father***
Ethnicity:	Hispanic or Latino	Hispanic or Latino
	Non-Hispanic or Latino	Non-Hispanic or Latino
Race:	American Indian or Alaska Native	American Indian or Alaska Native
	Asian	Asian
	Black or African American	Black or African American
	Native Hawaiian/ Pacific Islander	Native Hawaiian/other Islander
	White	White
	any combination of the above	any combination of the above

How do you describe your child's grandmothers and grandfathers?

	Child's Grandmothers	***Child's Grandfathers***
Ethnicity:	Hispanic or Latino	Hispanic or Latino
	Non-Hispanic or Latino	Non-Hispanic or Latino
Race:	American Indian or Alaska Native	American Indian or Alaska Native
	Asian	Asian
	Black or African American	Black or African American
	Native Hawaiian/ Pacific Islander	Native Hawaiian/other Islander
	White	White
	any combination of the above	any combination of the above

3. How old is your child? _____Years; DOB: _____/_____/_____
mm dd yy

4. What grade does he/she attend in school? _____ What county?_____

5. Has your child lost or gained weight in the past 3 months? YES _____NO _____

If yes, how much? _____lbs

6. Has your child ever been diagnosed with any of the following diseases/conditions?

Bone Disease	YES _____NO _____
Diabetes	YES _____NO _____
High Blood Pressure	YES _____NO _____
High Cholesterol	YES _____NO _____
Renal Disease or Kidney Stones	YES _____NO _____
Cerebral Palsy	YES _____NO _____
Intestinal Malabsorption	YES _____NO _____
Juvenile Rheumatoid Arthritis	YES _____NO _____
Growth Disorders	YES _____NO _____
Thyroid Disease	YES _____NO _____
Psychological Illness	YES _____NO _____

6. Because vitamin D is synthesized in the skin from direct sunlight, we are asking that your child limit extra sun exposure during the course of this 12-week study.

Do you anticipate your child traveling outside of Georgia during the course of this study? If so, where?_____

Are you willing to restrict travel outside of Georgia while your child is enrolled in the study?_____
(*determinations will be made on a case by case basis*)

7. Is your child currently taking any medications? YES _____NO _____

If yes, what medication(s)? _____
(*check approved medication list: determinations may need to be made on a case by case basis*)

8. Is your child taking an herbal, vitamin or mineral supplement? YES ____ NO ____

If yes, how much and how often? _____

If yes, would your child be willing to stop taking the supplement? YES ____ NO ____
(*child would be eligible to enroll in the study after a 4-week washout period*)

9. All participants must be willing to drink a small cup of orange juice. Is your child willing to do this? YES _____NO _____

10. In this study, all participants must provide blood and urine samples (at the start and after every 3 weeks). Is your child willing to do this? YES _____NO _____

11. Before initiation of this study, we will ask your child to give a self-assessment of pubertal maturation. We will send you the form for your child to complete. Would your child be willing to fill out a self-assessment of sexual maturation form and mail it back in a self-addressed envelope we will provide? YES _____ NO _____

12. If your child meets our criteria for pubertal maturation, then he/she will come to our laboratory for the initial testing session. Would your child be willing to come to our laboratory? YES_____NO_____

If the caller is still interested, explain more about the study and why we are doing it and collect the following information:

Parent's name: _____

Child's name: _____

Address: _____

Zip Code: _____

Daytime Phone Number: _____ (home or work?)

Email Address: _____

Is it okay to call in the evening? If yes, evening phone: _____

How did you hear about the study? _____

If selected to participate, what mornings during the week would you be available to come to the UGA Bone and Body Composition Lab, located in Dawson Hall, for testing?
M_____ T_____ W_____ Th_____ F_____ Sat_____ Sun _____

"This is the end of our telephone screening. We will review this and determine your child's eligibility for the study. We will get back to you with in one week to let you know the status of his/her eligibility. Do you have any additional questions for me?"

Make sure the potential volunteer has contact numbers for future questions.

If child is eligible based on the telephone screen, notify parent that we will be sending them the maturation questionnaire and consent forms by mail within the next week. Once mailed, we will make a follow-up phone call to check status of maturation questionnaire and child's eligibility.

APPENDIX B

Anthropometric data sheet

Supplemental Vitamin D in Early Adolescence

Participant Information Sheet
Anthropometrics/DXA/pQCT

Subject ID: _____ Visit Date:_____

Race/Ethnicity: _____ Sex: _____

DOB: Month _____ Day _____ Year _____

Weight (kg): _____ _____ _____

Measure 1 Measure 2 Average of 1 and 2

Height (cm): _____ _____ _____

Measure 1 Measure 2 Average of 1 and 2

Sitting Ht (cm): _____ _____ _____

Measure 1 Measure 2 Average of 1 and 2

BMI (g/cm^2): _____

Maturity Offset (years): _____ (Eligible to participate if -1.5 to 0 years)

Hand Grip: _____ _____ _____ _____ _____

Grip 1 Grip 2 Grip 3 Average SD

DXA operator use	PQCT operator use
❑ Total Body Scan date: _____ Completed by: _____ initials of operator	**Non-Dominant Limb: R L** circle one ❑ Arm Length _____ and MCSA _____ ❑ Leg Length _____ and MCSA _____ Scan date: _____ Completed by: _____ initials of operator

APPENDIX C

Sexual maturation questionnaires

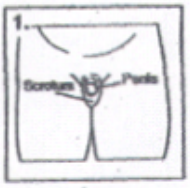
Lewis, Richard D.

SEXUAL MATURATION QUESTIONNAIRE (BOYS)

Subject ID#: _____
Date: _____

We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.

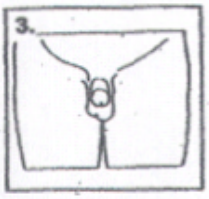
1. Scrotum and Penis same size as when you were younger.



2.

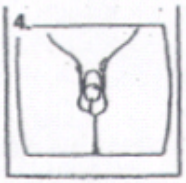
The Scrotum has lowered a bit and the Penis is a little larger.

3.



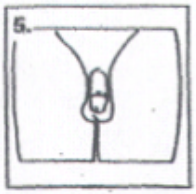
The Penis is longer the Scrotum is larger.

4.



The Penis is longer and wider the Scrotum is darker and bigger than before

5.



The Penis and Scrotum are the size and shape of an adult.

Thank you for answering this question. Please send this questionnaire back to the researcher in the stamped envelope provided.

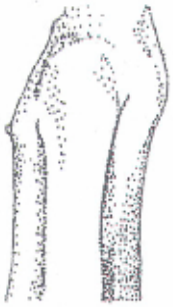
S.J.C. Taylor et al. Pediatric and Perinatal Epidemiology 2001. 15, 88-94.

Lewis, Richard D.

SEXUAL MATURATION QUESTIONNAIRE (GIRLS)

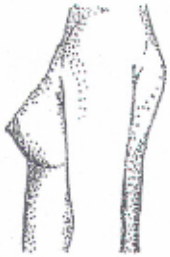
Subject ID#: _____
Date: _____

We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.



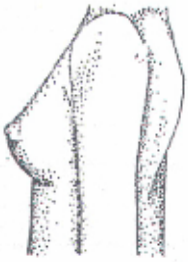
Stage 1: Elevation of papilla only.

Stage 2: Elevation of breast and papilla as small mound, areola diameter enlarged.



Stage 3: Further enlargement without separation of breast and areola.

Stage 4: Secondary mound of areola and papilla above the breast.



Stage 5: Recession of areola to contour of breast.

Thank you for answering this question. Please send this questionnaire back to the researcher in the stamped envelope provided.

J. Tanner (1962) Growth and Adolescence, 2nd ed. Blackwell Scientific Publications, Oxford.

APPENDIX D

3-day diet record

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.

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

6. **Write down the first foods you ate for that day. Write down:**

8. The time of day you ate the food(s).

9. Each food that you ate.

10. How the food was prepared (baked, boiled, fried, microwaved).

11. How much you ate (cup, 1/2 cup, pieces, tablespoons, teaspoons).

7. **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?**

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

   _____

5. **About how many glasses of water do you drink each day? _____**

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.

Time Eaten	Foods Eaten	Preparation Methods	Amount (cup, 1/2 cup, piece, etc)