VITAMIN D SUPPLEMENTATION AND MUSCLE RESPONSE

IN EARLY PUBERTAL ADOLESCENTS

by

CHRISTIAN STEPHEN WRIGHT

(Under the Direction of Richard D. Lewis)

ABSTRACT

We determined the effect of changes in vitamin D metabolites on muscle in 323 children (9-13 y), from US latitudes 34°N and 40°N, and randomly assigned to either: 0, 400, 1,000, 2,000, or 4,000 IU/d vitamin D_3 for 12 weeks (in winter). Muscle parameters [fat-free mass, muscle cross-sectional area (MCSA), muscle density (MD), intermuscular adipose tissue (IMAT), and forearm strength] and serum [25-hydroxyvitamin D (25(OH)D), 1,25-diydroxyvitamin D ($1,25(OH)_2D$), and intact parathyroid hormone (iPTH)] were measured at baseline and 12 weeks. 25(OH)D and $1,25(OH_2D$, but not iPTH, increased significantly over 12 weeks. Significant correlations existed between $1,25(OH)_2D$ and arm MCSA, and between iPTH and leg MCSA, leg MD, and leg IMAT. These data suggest that 25(OH)D is not a sensitive biomarker of muscle development, and iPTH may play a key role in adolescent muscle growth. Whether these observations with respect to iPTH and muscle are clinically relevant should be further explored.

INDEX WORDS: Vitamin D, Adolescents, Muscle cross-sectional area, Muscle density, Intermuscular adipose tissue, Supplementation, Fat-free soft tissue

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by

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DEDICATION

This thesis is dedicated to my loving mother, Tammy Lynn Wright, a source of Godly strength and wisdom, and to Dr. Lewis and the Lewis Lab. Mom, you have been a constant source of comfort, strength, and support throughout my life; I would simply not be the man I am today without you. Dr. Lewis, thank you for your guidance and direction, your patience and diligence, but most of all, thank you for your friendship. Lewis Lab, thank you for being my family these past two years and for always putting a smile on my face.

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CHAPTER 1

INTRODUCTION

Hypovitaminosis D is common amongst older adults (Haroon et al 2010; Holick et al 2006) and is becoming increasingly recognized even in pediatric populations (Looker et al 2011; Roth et al 2005; Outila et al 2001; El-Hajj Fuleihan et al 2001). Poor vitamin D status is associated with decreased calcium absorption and impaired skeletal mineralization (Deluca 2004), and if untreated increases the risk of fractures (Lips et al 2001), osteoporosis, and rickets in children (Holick et al 2006). Beyond its skeletal roles, there is an increasing interest in the non-skeletal roles of vitamin D. Low vitamin D status has been associated with an increased risk of cardiovascular diseases (Van der Schueren et al 2012, Pittas et al 2010), an impaired immune system (Cantorna et al 2004; Hewison M 1992) and an increased risk of mortality (Autier et al 2007). Possessing both a skeletal and non-skeletal role, as defined by the mechanostat theory (Frost 1990), the association between vitamin D and muscle function (Yoshikawa et al 1979; Bischoff-Ferrari et al 2004; Ceglia et al 2009, Wicherts et al 2007, Bischoff-Ferrari et al 1999, Visser et al 2003) has been of particular interest since first identified in clinical observations of muscle myopathy in osteomalacic patients and rickets in children (Prineas et al 1965). Vitamin D deficiency myopathy results in a weakness of the proximal muscles; mostly of the extension, flexion, and abduction of the hips as well as the flexion and extension of the knees (Pfeifer et al 2002), which can cause difficulty in mobility such as rising from a seated or squatted position or walking up stairs (Cegila 2008). Based upon the

mechanostat theory, wherein an increase in muscle contraction and muscle forces exerted on bone increases bone mineral content and adaptation, the associated muscle weakness of vitamin D deficiency myopathy could result in a decrease in bone quality and strength (Frost 1990). Other clinical signs of vitamin D deficiency myopathy in both older adults and adolescents include a waddling gait, muscle wasting (Schott and Wills 1976), and muscle pain (Van der Heyden et al 2004; Gloth et al 1991).

Cross-sectional studies and vitamin D intervention trials conducted in older adults support an association between vitamin D and muscle. The associated proximal muscle weakness and decrease in physical performance seen with vitamin D deficiency points to an alteration in muscle composition, with muscle biopsies showing an atrophy of the type II muscle fibers, an infiltration of fat, fibrosis, and glycogen granules (Yoshikawa et al 1979; Bischoff-Ferrari et al 2001). It has also been reported that older adults with low vitamin D status (<35 nmol/L) have inferior gait speed, poor balance, weak thigh muscle strength (Gerdhem et al 2005), poorer Short Physical Performance Battery scores, weaker handgrip strength (Houston et al 2011; Houston et al 2007), longer sit-to-stand time intervals (Bischoff-Ferrari et al 2004), and weaker knee extension power (Kwon et al 2007). Several vitamin D intervention trials in older adults have provided additional evidence of a link between vitamin D and muscle. Vitamin D supplementation resulting in an increase in serum 25(OH)D was associated with improved muscle composition (Sorensen et al 1979; Sato et al 2005; Cegila 2009) and strength (Michael et al 2010; Bischoff-Ferrari et al 2010; Dawson-Hughes et al 2010), which often improved or

diminished the negative skeletal muscle effects occurred during the deficient period (Glerup et al 2000; Zhu et al 2010).

Similar associations between vitamin D and muscle have been shown in adolescents. Cross-sectional studies have shown that vitamin D deficiency is associated with muscle-fat infiltration (Gilsanz et al 2010) and a decrease in jumping velocity, jump height, muscle power, Esslinger Fitness Index, muscle force (Ward et al 2008), and handgrip strength (Foo et al 2009). In comparison to older adults, only two intervention trials to date have examined the relationship between vitamin D and muscle in adolescents (El-Hajj Fuleihan et al 2006; Ward et al 2010). Lebanese adolescent females (N = 168, aged 10-17 years) were supplemented with oral vitamin D_3 (cholecalciferol) doses of 1,400 IU (200 IU/d), 14,000 IU (2,000 IU/d), or placebo every week for one year. Following the one-year intervention, increases in total body lean muscle mass were observed in pre-menarcheal females only (n=34), with both pre- and post-menarcheal females also having non-significant increases in handgrip strength as measured by pressure gauge (El-Hajj Fuleihan et al 2006). A predominately South Asian female adolescent population (N = 69, aged 12 to 14 years) was supplemented with an oral vitamin D_2 (ergocalciferol) dose of 150,000 IU or placebo every three months (~1,600 IU/d) for one year. Following the one-year intervention, significant improvements in muscle efficiency, shown by jumping velocity, were observed in addition to non-significant increases in handgrip strength and muscle cross-sectional area (MCSA) as measured by handgrip dynamometry and peripheral quantitative computed tomography (pQCT), respectively (Ward et al 2010).

Though the association between vitamin D and muscle is well supported in older adults (Yoshikawa et al 1979; Bischoff-Ferrari et al 2001; Gerdhem et al 2005; Houston et al 2011; Houston et al 2007; Bischoff-Ferrari et al 2004; Kwon et al 2007; Sorensen et al 1979; Sato et al 2005; Cegila 2009; Michael et al 2010; Bischoff-Ferrari et al 2010; Dawson-Hughes et al 2010; Glerup et al 2000; Zhu et al 2010), current pediatric data, particularly intervention trials, are limited. Findings from the previously mentioned vitamin D intervention trials suggest that vitamin D supplementation positively impacts adolescent muscle (El-Hajj Fuleihan et al 2006; Ward et al 2010); however, these studies are limited to adolescent females who were mostly of Caucasian and South Asian descent. Due to the high prevalence of hypovitaminosis D amongst non-Hispanic black adolescents (Mansbach et al 2009; Wolff et al 2008; Holick et al 2006), understanding the role of low circulating 25(OH)D and muscle function in this population is essential. Additionally, there are no published studies that have addressed this association of vitamin D and muscle in male adolescents. Data surrounding the effect of age, race, sex, and maturation on changes in vitamin D metabolites and muscle parameters are also limited.

Chapter 2 provides background information on the metabolism and function of vitamin D, the prevalence of vitamin D deficiency and factors affecting vitamin D status, and the potential role of vitamin D in muscle. The purpose of the study presented in Chapter 3 was to determine how changes in vitamin D metabolites over a 12-week vitamin D intervention affected changes in muscle parameters in early pubertal adolescents through the measurement of fat-free soft tissue (FFST), fat mass, and body

fat percent by dual energy X-ray absorptiomerty (DXA), MCSA, muscle density (MD), and intermuscular adipose tissue (IMAT) by pQCT, and forearm strength by handgrip dynamometry, and whether this effect was dependent upon race and sex. The current study was a secondary project of the main University of Georgia, Purdue University and Indiana University School of Medicine (GAPI) vitamin D intervention trial. Our primary hypotheses were that changes in vitamin D metabolites are associated with changes in FFST, MCSA, MD, IMAT and handgrip strength and that race and/or sex would modify this relationship. The current study is unique because it addresses the relationship of vitamin D and muscle through the use pQCT to assess MCSA, MD, and particularly IMAT, where data surrounding its use are limited in pediatric populations, and also as it investigates these relationships in both black and white male and female adolescents. In closing, Chapter 4 summarizes the study's findings and describes the implications for future research.

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CHAPTER 2

REVIEW OF LITERATURE

Introduction

It has been well established that vitamin D plays an essential role in the regulation of calcium absorption and in bone mineralization and repair (Deluca 2004). However in the past few decades there's been increasing evidence that vitamin D plays an essential role in muscle metabolism. Although low serum 25(OH)D is well known to be associated with muscle atrophy and weakness (Prineas et al 1965), it is only recently that possible mechanisms of action have been proposed (Ceglia 2008). In order to treat and deter the onset of deficiency, vitamin D supplementation has become a common practice; giving way to several clinical intervention trials to determine the optimal dosage and identify any other positive benefits of vitamin D supplementation on muscle. However the vast majority of these intervention trials have solely been conducted with older adults, resulting in insufficient pediatric data. The proposed research project therefore seeks to contribute to the limited data surrounding vitamin D supplementation and muscle metabolism in adolescents.

The next few sections of this literature review will highlight the physical and metabolic properties of vitamin D, hypovitaminosis D myopathy, and the current literature surrounding clinical vitamin D intervention trials.

Vitamin D: Metabolism and regulation

Vitamin D, a fat-soluble vitamin and a secosteroid hormone, is a vital component of many physiological systems and essential to life itself. Production of vitamin D largely occurs in the skin after a cholesterol derivative, 7-dehydrocholesterol pro-vitamin D3, is exposed to ultraviolent radiation (270-300 nm range). Once pro-vitamin D3 is exposed to ultraviolent radiation, a photolytic process creates previtamin D3 which is then slowly isomerized to vitamin D3 (cholecalciferol) (Velluz et al 1949) (Fig. 1). This cutaneous production of vitamin D can readily supply the body with 80-100% of its daily requirement of vitamin D (Webb et al 1990). Alternatively vitamin D in the form of vitamin D2 (ergocalciferol), a source found mostly in plants and yeast, or vitamin D3, found mostly in fatty fish, eggs, and dairy products, can be acquired through dietary intake. However due to the fact that very few foods actually contain and/or are fortified with vitamin D, a majority of individuals acquire their daily intake through supplementation or sunlight exposure. Foods that do naturally contain vitamin D often only have it in very small amounts; an exception would be fatty fish (i.e. cod, mackerel, tuna, and salmon). Fatty fish can actually contain between 200 and 1,600 IU of vitamin D per 100 grams, which is far greater than other dietary sources of naturally occurring vitamin D (eggs/20IU, beef liver/25IU, cheese/12IU) (Moore et al 2004). Vitamin D

fortification of food items now provides the mass majority of an individual's dietary intake, with items like fortified breakfast cereals, milk, and juice (Moore et al 2004).

The metabolism of vitamin D and the creation of its active form were not clearly understood until the early 1960s. Prader et al (1961) showed that by themselves vitamin D3 and D2 (Fig. 2) are biologically inert, that is until they are acted upon by the hepatic enzyme 25-hydroxlase. Prader showed that individuals with a genetic defect in 25-hydroxylase also displayed clinical signs of vitamin D deficiency despite normal intakes. Blunt et al (1968) further discovered that once vitamin D is cutaneously produced or consumed it is quickly bound to a specialized multifunctional, highly expressed, vitamin D-binding protein (DBP) and then transported to either 1). adipose tissue for storage purposes or 2). the liver for further processing. In the liver vitamin D3 and D2 undergo their first step towards activation, known simply as 25-hydroxylation. The two enzymes responsible for this hydroxylation are 25-hydroxylase and a cytochrome *P*-450 enzyme, as shown in Figure 3. The cytochrome *P*-450 proposed to be involved in this hydroxylation step, which is thought to be found in either the mitochondria or microsomal fractions of the liver, is CYP27 or simply *P*-450c27; however other cytochrome *P*-450s have been proposed to be involved in this hydroxylation step (DeLuca 2004). This hydroxylation creates 25-hydroxyvitamin D [25(OH)D], which is considered to be an excellent indicator of total vitamin D status for 3 reasons (Webb et al 1990). 1). 25(OH)D has a half-life of 2 to 4 weeks depending upon circulating parathyroid horomone (PTH) and calcium levels. 2). 25(OH)D is reflective of all sources of vitamin D, both from cutaneous production and dietary intake. 3). conversion of

vitamin D to 25(OH)D is a very rapid reaction, almost completely unregulated. After 25-hydroxylation, 25(OH)D is once again bound to DBP and put into circulation. At some point, the bound 25(OH)D will be transported to the kidney for further hydroxylation, creating the biologically active form of vitamin D, 1,25-hydroxyvitamin D [1,25(OH)D]. The enzyme 25-hydroxyvitamin D-1α-hydroxylase is responsible for this highly regulated reaction. One important regulatory step in this reaction is the interaction between 1α-hydroxylase and PTH through a cAMP/phosphatidylinsitol 4,5-bisphosate-mediated signal transduction mechanism (DeLuca 2004). This regulatory step is classified as a positive feedback mechanism, where in 1α-hydroxylase is up regulated, increasing the rate of conversion of 25- to 1,25(OH)D, when PTH levels are high. However, much like other complicated regulatory systems in the human body, once 1,25(OH)D reaches a certain concentration it decreases its own synthesis by decreasing the synthesis of PTH through a negative feedback mechanism (Holick et al 2007).

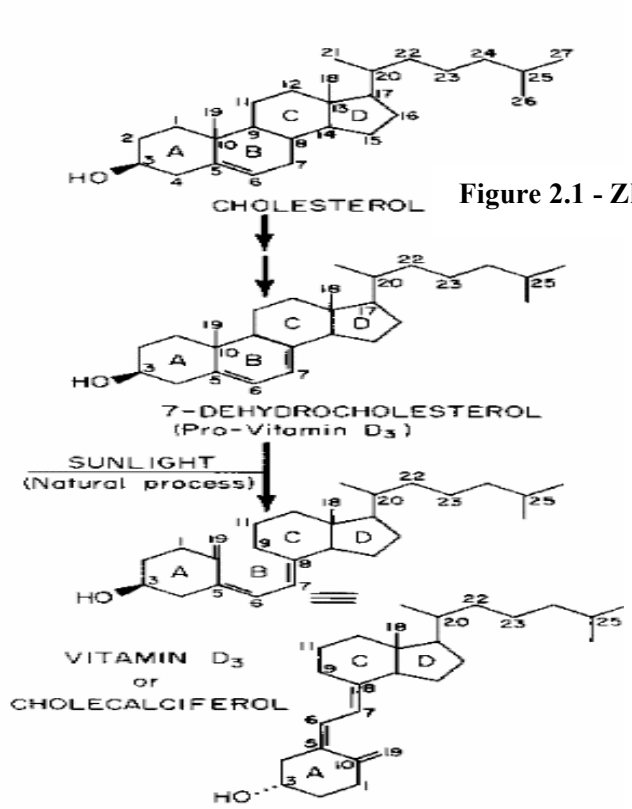


Figure 2.1 - Zhu et al 1995

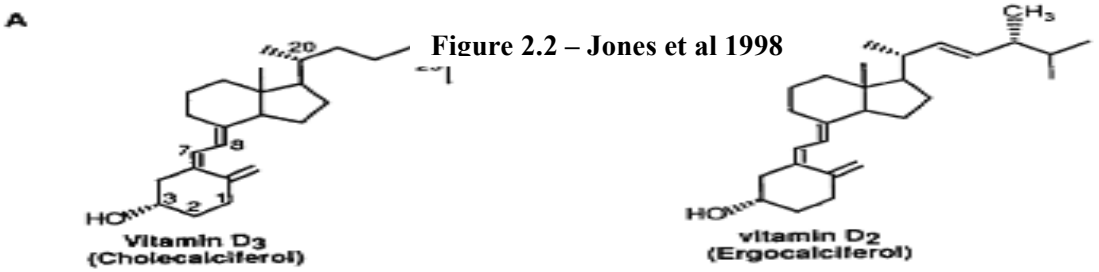


Figure 2.2 – Jones et al 1998

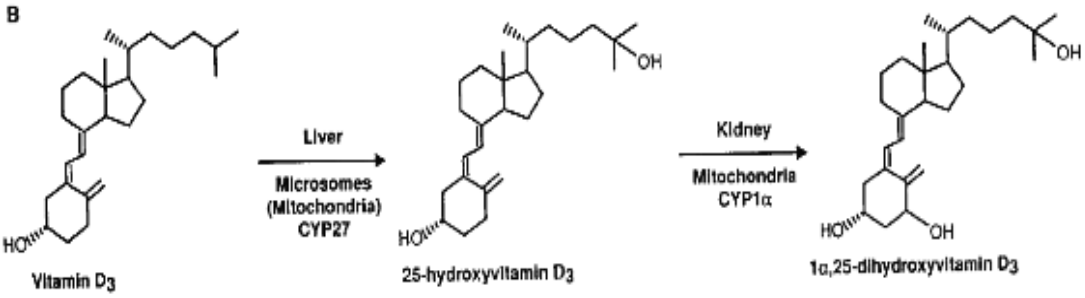


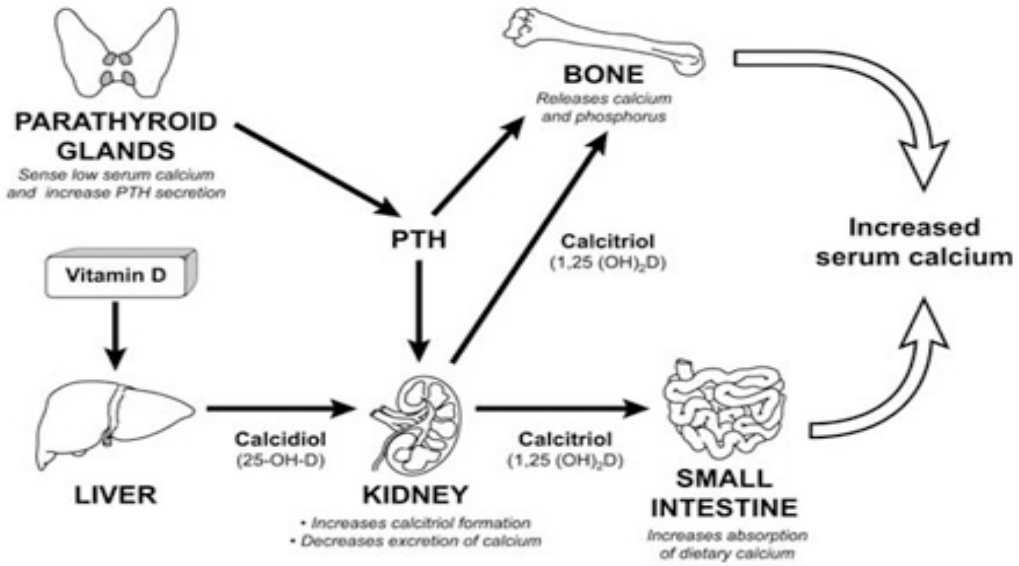
Figure 2.3 - Zhu et al 1995

Vitamin D and parathyroid hormone: Calcium regulation

Representations of vitamin D's role in the regulation of calcium absorption and in bone mineralization and repair, and how vitamin D interacts with PTH, can be seen in Figures 4 & 5. These relationships and the following discussion have predominately been investigated in adult and older adult populations. Due to the interactions between PTH and 1,25(OH)D, plasma concentrations of calcium are closely maintain at a constant level. 1,25(OH)D maintains the plasma concentration of calcium in 3 different ways. First, 1,25(OH)D, in the absence of intestinal calcium absorption, stimulates the osteoblasts to produce a receptor activator nuclear factor-kB ligand (RANKL) (Suda et al 2002). After RANKL is fully formed, it then stimulates osteoclastogenesis, which causes osteoclast precursors to fuse together and create new osteoclasts, which ultimately enhance the process of bone resorption. This mechanism allows for the calcium stored in bones to be released into the blood stream. Secondly, 1,25(OH)D increases reabsorption of calcium at the distal renal tubule. On average 7 grams of calcium is filtered through the body on a daily basis, and thus represents a huge opportunity to maintain calcium levels (Yamamoto et al 1984). And lastly but most importantly, 1,25(OH)D binds to an intestinal vitamin D receptor (VDR), a vital transcription factor, to increase the transcription of proteins involved in the active intestinal absorption of calcium and phosphate. This last mechanism of action is considered to be vitamin D's primary role, a substance responsible for the intestinal absorption of calcium; however PTH also plays a vital role in all three of these reactions. Highly sensitive to changes in calcium levels due to the calcium sensing receptors on the parathyroid gland, the slightest decrease of serum

calcium levels causes the parathyroid glands to releases PTH. Once released, PTH not only helps regulate calcium levels by interacting with 1,25(OH)D but also helps increase free calcium in the plasma (Friedlander et al 1994). 1,25(OH)D does in fact increase bone resorption by stimulating osteoblasts to create RANKL, however this mechanism will not occur unless PTH binds to the osteblasts beforehand. PTH also acts on the renal ducts to increase calcium and magnesium reabsorption while at the same time increasing phosphate secretion; which ultimately results in a net loss of plasma phosphate and an increase in free calcium. By decreasing phosphate levels, the calcium:phosphate ratio is increased, which ultimately frees up more calcium for circulation. But most importantly PTH acts on the renal enzyme 25-hydroxyvitamin D-1α-hydroxylase to increase the conversion of 25- to 1,25(OH)D, which increases calcium levels by 3 previously mentioned 1,25(OH) D reactions.

Figure 2.4 – WellSphere 2008

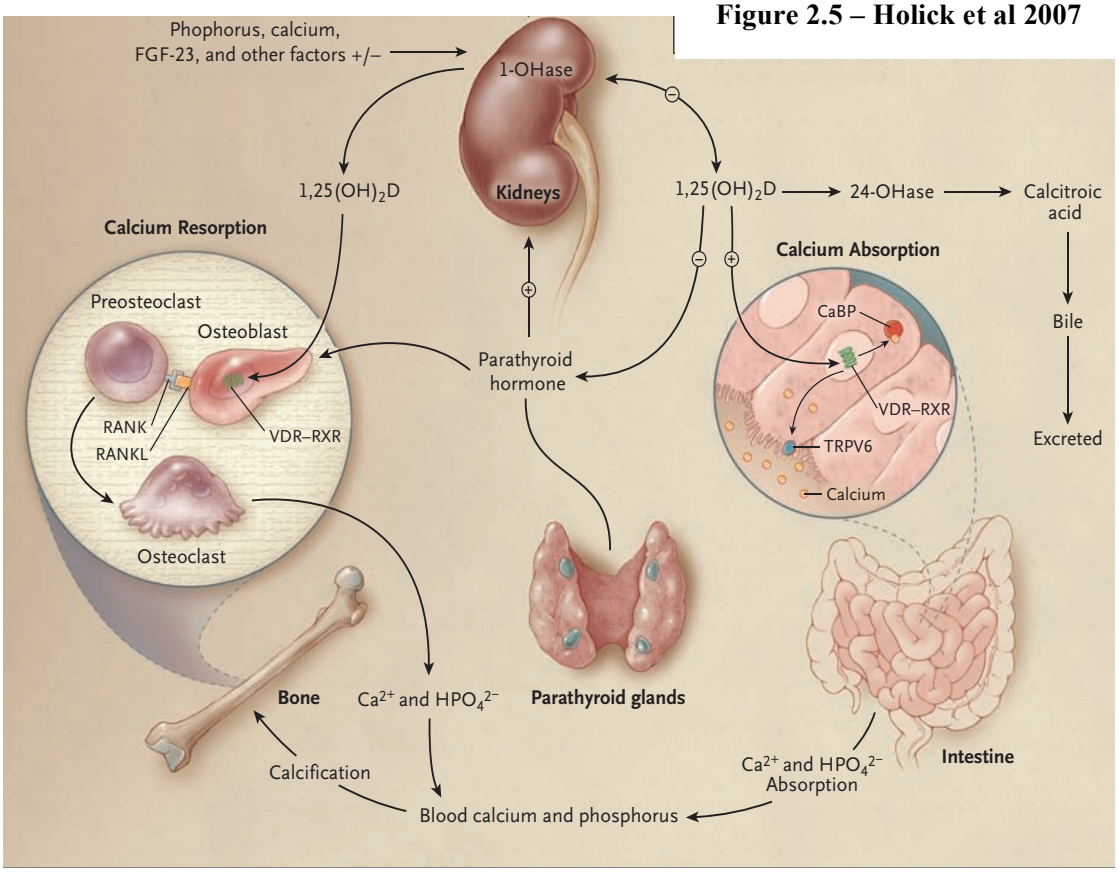


The effect of vitamin D and PTH on bone

As illustrated in Figures 4 and 5, the interactions between vitamin D and PTH help regulate calcium levels and also bone mineralization and resorption. When calcium levels are relatively low bone resorption is favored, causing calcium to be released from the bone into circulation. However this push towards bone resorption is hindered by an increase in PTH levels, which causes an increase in the transcription of proteins involved in the active intestinal absorption of calcium and phosphate. But in the absence of intestinal calcium absorption, due to low dietary intake, PTH levels will remain high which can result in significant health implications. Due to its role in the conversion of 25- to 1,25(OH)D, when PTH levels remain high in absence of calcium absorption it decreases the half-life and availability of 25(OH)D. Although PTH does increase the availability of 1,25(OH)D, the active form of vitamin D, if intakes of calcium and vitamin D remain low while serum PTH levels remain high the conversion of 25- to 1,25(OH)D will essentially remove much of the available vitamin D from the system; converting the more stable 25(OH)D, with half–life of 2 to 4 weeks, into the less stable 1,25(OH)D, with a half-life of 4 to 8 hours (Wootton 2005). Therefore if PTH levels remain high certain health implications will follow, such as vitamin D deficiency (Haroon et al 2010), a decrease in bone mineral content, an increase in bone fragility (Pasco et al 2004), muscle dysfunction, muscle wasting (Garber 1983), and an increase in the risk of bone fractures (Chapuy et al 1992). But in the presences of adequate calcium and vitamin D intake (25(OH)D levels $\geq$ 75 nmol/L), research has shown maxima

suppression of circulating PTH levels, high bone mineral accrual, high calcium absorption, and a decrease rate of bone loss and fractures (Haroon et al 2010).

Figure 2.5 – Holick et al 2007



Assessment of vitamin D status

Measurement of serum 25(OH)D is widely accepted to be the only true way to properly assess vitamin D status. Due to its almost completely unregulated conversation rate and a half-life of 2-4 weeks (Webb et al 1990), 25(OH)D is a dependable indicator of total vitamin D status because it is reflective of all sources of vitamin D, both from cutaneous production and dietary intake. Although 1,25(OH)D is the most biologically

active form of vitamin D, due to its relatively short half-life of 4 to 6 hours it is an unsuitable indicator of total vitamin D status (Cranney et al 2007). In addition, the measurement of 1,25(OH)D can be misleading to a physician and/or research scientist as it is often only at low levels (less than 10 pg/ml) in incidences of severe vitamin D deficiency. Serum 1,25(OH)D is most often normal or elevated, even in cases of vitamin D deficiency and secondary hyperparathyroidism (Holick et al 2008).

In the past four decades there have been several significant advances in our understanding of vitamin D's metabolism, which have given way to many technological advances that have allowed for the mainstream clinical assessment of vitamin D. As such, several fundamentally different methods are now available to assess circulating 25(OH)D concentrations, including radioimmunoassay (RIA), high performance liquid chromatography (hplc), competitive protein binding assays (CPBA), liquid chromatography linked with mass spectrometry (LC-MS) and enzyme-linked immunosorbent assay (ELISA). CPBA, one of the first assays widely used, was first introduced over three decades ago. With 85% of 25(OH)D being bound to a vitamin D-binding protein (DBP) (Bikle et al 1986), CPBA uses DBP as its primary binding agent, which is then tagged by the tracer element 3H-25(OH)D3 (Haddad et al 1971). However CPBA proved to be an overly cumbersome method, which ultimately gave way to the simplified non-chromatographic RIA method. RIA uses the antigen/antibody binding method, wherein a specific antigen causes the creation of antibodies to be specific to both $25(OH)D_2$ and $25(OH)D_3$ (Hollis et al 1985). Originally using $3H\text{-}25(OH)D_3$ as the tracer element until ^{125}I proved to be more effective (Hollis et al 1993), a known amount of

25(OH)D is first tagged with the tracer element and then mixed together with a sample of unknown 25(OH)D. Later on, the antibody specific to both $25(OH)D_2$ and $25(OH)D_3$ is added to this sample containing both untagged and tagged 25(OH)D. This solution is then allowed to incubate for a set period of time wherein the 25(OH)D being analyzed, the amount in the blood sample, competes with the tagged 25(OH)D for the antibody. After the incubation period, the amount of the tagged 25(OH)D associated with the antibody is measured by way UV detection and then used to extrapolate the amount of 25(OH)D in the blood sample being analyzed (Chard 1995).

Adipose tissue: Storage site

The majority of vitamin D_3 or D_2 that makes its way into circulation is quickly bound by DBP and transported to the liver for its first hydroxylation step. However often, particularly when 25(OH)D levels are already normal or above, this bound vitamin D_3 or D_2 is put into storage. The major storage site of vitamin D is adipose tissue, as shown in the Rosenstreich et al (1971) study wherein vitamin D-deficient weanling rats collected large amounts radio-labeled vitamin D_3-4-^{14}C in various sites of adipose tissue after 2 weeks of oral dosing. Due to this ability to store vitamin D, adipose tissue was originally believed to help prevent vitamin D toxicity (Mawer et al 1972) and/or maintain vitamin D levels in times of low intake (Rosenstreich et al 1971). In order to investigate these potential properties, Brouwer et al (1998) conducted a study where vitamin D-deficient weanling rats were given large oral doses of vitamin D_3 (37.5 μg/day) for 14 days and then deprived for up to 88 days. After the initial spike in adipose tissue content and

circulating 25(OH)D levels during the 14-day period, the adipose tissue content slowly began to decrease with a half-life of 97.4 days while circulating 25(OH)D levels quickly decreased with a half-life of 15-30 days, similar to other studies (Webb et al 1990; Mawer et al 1972). According to this study, the adipose tissue vitamin D cannot be regarded as a slow-releasing pool of vitamin D as the 14 day "storage" period was unable to alter the decline of 25(OH)D after vitamin D was removed. But the study did show that a group of rats, treated with high doses of vitamin D for the same 14-day period and then deprived of food 3 days afterwards, actually had their 25(OH)D levels increase during the 3-day fastening period, possibly due to the mobilization of vitamin D from adipose tissue. Even though adipose tissue is a major storage site of vitamin D, the body still requires weekly intakes of vitamin D in order to maintain 25(OH)D levels.

Defining optimal vitamin D status

What is the optimal level of vitamin D and what level constitutes as vitamin D insufficiency are two questions heavily debated about amongst several scientists around the globe. According to a recent Institute of Medicine (IOM) report in October 2010, a serum 25(OH)D concentration below 30 nmol/L (12 ng/ml) is associated with vitamin D deficiency while 30-50 nmol/L (12-20 ng/ml) is associated with vitamin D insufficiency, meaning inadequate for both bone and overall health (Institute of Medicine 2010). Most of the published literature would also agree with these two cut-off points, that are associated with vitamin D deficiency and an overall inadequate support of both bone and general health. However the perception of what level of circulating vitamin D is enough

to provide maximal suppression of the circulating PTH, maximum calcium absorption, greatest bone mineral density (BMD), reduced rate of bone loss, reduced rate of falling, and a reduced rate of fractures is still highly debated upon amongst scientists today. The IOM states that a 25(OH)D concentration at or above 50 nmol/L (20 ng/ml) can be consider adequate for both bone and overall health. However there is sufficient data in the literature that negates the IOM cut-off points, stating that vitamin D deficiency should be defined as a 25(OH)D concentration at or below 50 nmol/L (20 ng/ml), vitamin D insufficiency as 51-74 nmol/L (21-29 ng/ml), and vitamin D sufficiency at or above 75 nmol/L (30 ng/ml) (Holick et al 2007; Chapuy et al 1996; Dawson-Hughes et al 2005; Malabanan et al 1998; Holick et al 2005; Thomas et al 1998). The establishment of this optimal level of 25(OH)D was based upon several factors including maximum calcium absorption, showing a 65% increase in calcium absorption once 25(OH)D levels were increased to average level of 87.5 nmol/L (35 ng/ml) from an average level of 50 nmol/L in postmenopausal women (Heaney et al 2003), and a maximum suppression of PTH levels with 25(OH)D concentrations between 75-100 nmol/L (30-40 ng/ml) (Chapuy et al 1996; Holick et al 2005; Thomas et al 1998). The determination of this potentially optimal concentration of 25(OH)D was also based upon the positive association between 25(OH)D and total hip BMD at levels above 75 nmol/L in 13,432 men and women (age $\geq$20)(Bischoff-Ferrari et al 2004), and a decreased in the rate of bone loss of the spine and hip during the winter-months as 25(OH)D concentrations increased from 60 to 90 nmol/L in postmenopausal women (Dawson-Hughes et al 1991; Dawsom-Hughes et al 1995). Even though similar research has not been conducted in children, most researchers assume a child's vitamin D requirements are similar to that of an adult (Holick et al

2008). Although the values for deficient, insufficient and sufficient 25(OH)D concentrations are still considered to be highly controversial, for the focus of this paper and the study conducted, cut-off points will be based upon the Institute of Medicine 2010 Vitamin D Report, showing that a serum 25(OH)D concentration below 50 nmol/L is associated with hypovitaminosis D and below 20 nmol/L with vitamin D deficiency. An illustration of these vitamin D cut-off points can be seen in Table 1 below.

Table 2.1. Vitamin D Status*

nmol/L**	ng/mL	Health Status
< 30	< 12	**Vitamin D deficiency** – Associated with rickets in infants and children and osteomalacia in adults
31-49	12.4-19.6	**Vitamin D insufficiency** – Inadequate support of bone and overall health
>50	>20	**Vitamin D sufficiency** – Supports adequate bone health

*Levels based upon the study by the Institute of Medicine 2010 report.

**Serum concentrations of 25(OH)D are reported in both nanomoles per liter (nmol/L) and nanograms per milliliter (ng/mL). 1nmol/L = 0.4 ng/mL

US Vitamin D intakes

The most recent data concerning vitamin D intakes from both food and dietary supplements comes from the 2005-2006 National Health and Nutrition Examination Survey (NHANES). According to the survey, the average vitamin D intake of males from

food alone was 204 to 288 IU/day while females had an average intake of 144 to 276 IU/day; wherein fluctuations were dependent upon life stage group. Considering vitamin D intake from food alone, only males ages 1-3 and 4-8 years old, and females ages 1-3, 4-8, 9-13 years old actually met the adequate intake (AI) for their specific age group. However these AIs established for infants and adolescents are only estimates extrapolated from adult AIs. Therefore, the lack of direct evidence supporting the establishment of these vitamin D AIs for infants and adolescents could potentially imply that the above age groups are in fact below their requirement of vitamin D. Even more shocking was the vitamin D intake from food alone in adults 51 years or older, with less than 7% of both males and females actually meeting the AI. According to the survey 37% of the U.S. population used some form of dietary supplement containing vitamin D, once the vitamin D supplements were finally taken into account the average intakes of both males and females increased significantly from 264 to 428 IU/day for males and 200 to 404 for females. Once dietary supplements were taken into account the prevalence of those meeting the AI was also much higher for all age groups, except for adults older than 71 years old and females 14 to 18 years old (Bailey et al 2010). However useful it is to assess the average vitamin D intake of the U.S. population, one can not properly assess circulating 25(OH)D concentrations of the U.S. population from dietary sources alone, that is without taking into account cutanenously produced vitamin D. Due to the fact that cutaneously produced vitamin D can readily supply the body with 80-100% of its daily requirement of vitamin D (Webb et al 1990), serum 25(OH)D levels will often be higher than predicted solely from total dietary intake (Institute of Medicine 2010).

Vitamin D status: US and the World

Although in recent years the number of vitamin D fortified foods and supplements have greatly increased in the global market, the prevalence of vitamin D insufficiency and deficiency in the U.S. and across the world is incredibly high, with every race and age group across the globe being affected. Several studies have shown that the prevalence vitamin D insufficiency (<50 nmol/L [20 ng/ml]) is very high even amongst uncommon populations. The Tangpricha et al (2002) study, conducted at the Boston Medical Centre, showed that in a group of 165 healthy young medical students and doctors (18 - 29 years of age) approximately 36% of them were vitamin D insufficient. Other studies even show more shocking results in both heavily clothed or darker skinned individuals and older adults. In Saudi Arabi, where most women are heavily clothed and/or veiled, it was discovered that out of 47 women (ages 13-46 years old) 77 % were vitamin D deficient (<20 nmol/L) (Al-Said et al 2009). African Americans and darker skinned individuals are another high risk population for vitamin D deficiency, with researchers estimating 50% of the US African American population to be vitamin D insufficient (Holick MF 2004). Such claims were further supported in the literature as seen by 84% of free-living African American adults over the ages of 65 being vitamin D deficient (Holick MF 2002) and by 42% of black women ages 15 to 49 years old being vitamin D insufficient (Nesby-O'Dell et al 2002). It has also been thoroughly documented that older adults, with their decreased cutaneous production of vitamin D and poor dietary intake, have a very high prevalence of vitamin D deficiency or insufficiency. In a 2005 systematic review of vitamin D status in post-menopausal

women, the vast majority of studies (22 out of 30) reported a prevalence of vitamin D deficiency of at least 25%, with overall prevalence of vitamin D deficiency ranging from 1.6 to 86% in all 30 studies (Gaugris et al 2005). Earlier studies showed similar results where out of 116 indoor dwelling older adults, 54% homebound and 38% of nursing home older adults were vitamin D deficient (Gloth et al 1995). In a cohort of 290 hospitalized patients of the Massachusetts General Hospital, 57% of older adults were vitamin D deficient with 27% being classified as "severely" vitamin D deficient (Thomas et al 1998). Hospitalized patients continue to show this trend with 41% of outpatients ages 49 to 83 years old being vitamin D insufficient (Malabana et al 1998). Such levels of vitamin D insufficiency are often even higher in other countries. In Europe the numbers are staggering, with 28-100% of healthy and 70-100% of hospitalized adults being vitamin D insufficient (Isaia et al 2003; Mckenna et al 1992).

Since the mid 1600s, physicians have noted of the high prevalence of vitamin D deficient rickets in children (Eliot et al 1938). However for more than 300 years vitamin D deficient rickets was an untreatable and widespread disease, affecting many industrialized countries such as various parts of Europe and the United States. However following the Steenbock et al (1924) study, governments and industries alike discovered how to fortify foods, particularly milk, and the prevalence of vitamin D deficient rickets quickly diminished. But despite these practices, vitamin D deficiency and insufficiency is still very common in children, affecting children of all ages and even infants across the world. Infants and toddlers, due to the low concentrations of vitamin D in breast milk and limited availability of vitamin D fortified formulas, are particularly at risk of

developing vitamin D deficiency and the diseases associated with low vitamin D status. Newborns are not excluded from this category as shown in a study that consisted of 40 pregnant women, wherein 81% of their newborns were vitamin D deficient, despite the women's average daily intake of 600 IU (Lee et al 2007). Infants continue show similar numbers both abroad and in the United States. In rural China it was shown that 65.3% of 12 to 24-month old infants were vitamin D deficient (Strand 2007), while in the U.S. 12.1% and 40% of otherwise healthy infants and toddlers ($N = 380$) were vitamin D deficient and insufficient, respectively (Gordon et al 2008). Outside of newborns and toddlers, young children and adolescents also have a high prevalence of vitamin D deficiency or insufficency. In Europe it was shown that 52% of adolescents (aged 10-16 y) living in Lebanon were vitamin D insufficient (El-Hajji Fuleihan et al 2001), while 61.8% of adolescent females (aged 14-16 y) were vitamin D deficient in Finland (Outila et al 2001). The United States is also battling with this problem, as indicated by 48% of adolescent females (aged 9-11 y) living in Maine (Sullivan et al 2005) and 42% of black and Hispanic teenagers living in Boston found to be vitamin D insufficient (Gordon et al 2004). The Roth et al (2005) study also showed similar findings in 90 otherwise healthy children ages 2 and 16 years old, with 34% of the subjects being vitamin D insufficient and 6% vitamin D deficient. An even more interesting aspect of this study was the incredibly high prevalence of vitamin D insufficiency amongst 9 to 16 year adolescents, with 69% of the subjects being insufficient and 35% being deficient (Roth et al 2005). These findings were further supported in 2011, when the Centers for Disease Control and Prevention (CDC) reported in their 2001-2006 NHANES study that approximately 9-11%

of children ages 1-8 years old, 19-22% of children ages 9-13 years old, and 22% of children ages 14-18 years old were vitamin D deficient (Looker et al 2011).

Factors affecting 25(OH)D concentrations

Outside of dietary intake, several factors can dramatically affect circulating 25(OH)D levels including age, sex, latitude, season, melanin content of the skin, physical activity, use of sun block, extent of clothing covering, and adiposity (Holick et al 2007; Cannell et al 2008).

Age

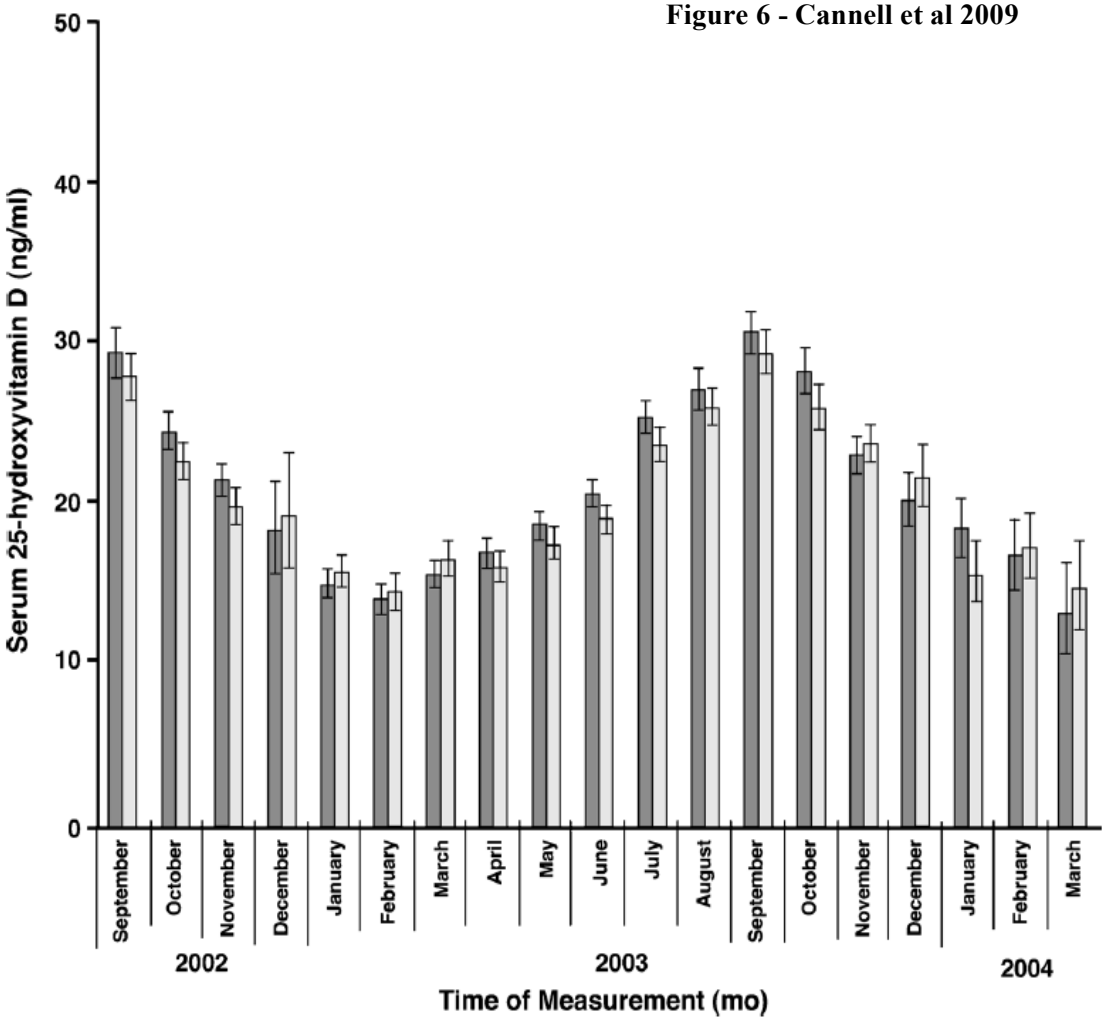
Age is a huge contributing factor to circulating serum 25(OH)D levels; compared to younger adults, individuals older than 70 years old produce 30% less vitamin D when exposed to the same amount of UVB radiation (Holick et al 1989). This age-related decline of 25(OH)D has also been shown to be a gender-dependent variable, wherein women see a decline in cutaneous production shortly after 50 years old versus in men after 70 years old (Maggio et al 2005). A similar age-related decline of 25(OH)D has also been shown in adolescents, showing that as children progress through early adolescence there is a subsequent decrease in 25(OH)D with increasing age (Willis et al 2007).

Latitude and season

Time of year and location of UVB exposure are two important variables to circulating 25(OH)D levels. Seasonal variations in 25(OH)D occurs in every race and in every age group across the globe. On average 25(OH)D levels are significantly higher in the summer in comparison to winter (Fig. 6), as shown in northern states such as Maine (Rovner et al 2008), southern states such as Georgia (Willis et al 2007), and even in different countries such as China (Arguelles et al 2009). The location and/or latitude of exposure is also a major determining factor of circulating 25(OH)D, with northern locations commonly having lower 25(OH)D levels in comparisons to more southern locations, as seen in vitamin D deficient children living in Philadelphia, Pennsylvania (latitude: 40°N), Pittsburgh, Pennsylvania (40°N), Cleveland, Ohio (41°N), Boston, Massachusetts (42°N), and Maine (44°N) (Rovner et al 2008). However there is increasing evidence that in despite of their southern location and subtropical climate, people living in lower latitudes are still at risk of becoming vitamin D deficient as seen in healthy adults living in Miami, FL (26ºN) (Levis et al 2005) and in adolescents living in Houston, Texas (30°N) (Abrams et al 2005). Even the time of day can have an influence on circulating 25(OH)D concentrations. For example when the sun is low on the horizon the atmospheric ozone, the water vapors, and especially the air pollution will retard the ultraviolent radiation exposure, which ultimately decreases if not eliminates the cutaneous production of vitamin D during the early morning and late afternoon (Cannell et al 2008). All these factors, the latitude, time of day, and season can have a compound effect on circulating 25(OH)D levels. For example, at latitude 42° N (Boston, MA, USA), sunlight is incapable of producing vitamin D between the months of November and

February. In more northern sites (Edmonton, Canada 52° N), this period of non-cutaneous production of vitamin D is extended to include the months of October through March (Holick et al 1994).

Figure 6 - Cannell et al 2009



Melanin

Skin pigmentation, primarily due to the amount of melanin in the skin, also greatly affects circulating 25(OH)D levels. Higher melanin in the skin, as seen in darker skinned individuals, helps protect the body from sunlight exposure by decreasing the

absorption of solar radiation (UVB radiation), which also decreases cutaneous production of 25(OH)D. Thus in comparison to lighter skinned individuals, darker skinned individuals produce less 25(OH)D when exposed to the same amount of sunlight (Rajakumar et al 2011) and therefore commonly have lower 25(OH)D in comparison to lighter skinned individuals independent of season (Willis et al 2007).

Sun block & clothing

Sun block is an incredibly important component in the fight against skin cancer such as melanoma. However in the process of protecting an individual from various forms of skin cancer, sun block can also essentially remove a large available source of vitamin D. Although sun block does prevent the excessive damage that ultraviolent radiation might have on an individual's DNA, in the process of protecting the skin it also decreases the cutaneous production of vitamin D. In fact sun block with a sun protection factor of 8 can reduce cutaneous produce of vitamin D by over 95% (Matsuoka et al 1987). Extent of covering the body with clothes has also been shown to decrease cutaneous production of vitamin D (Gloth et al 1995; Haroon et al 2008). However in spite of the decrease in vitamin D production, several recent studies have shown that people that wear sun block have 25(OH)D concentrations similar to controls, stating that although sun block may decrease cutaneous production it does not completely shutdown the production of sufficient vitamin D concentrations (Marks et al 1999; Ferrerons et al 2001; Kimlin et al 2007).

Adiposity

Although for some time now the negative association between adiposity and circulating 25(OH)D concentrations has been well-known (Arunabh et al 2003), recently more research has been placed on this association in different age groups and also in different races. In the Looker et al (2005) study, researchers looked at the relationship between serum 25(OH)D concentrations and body fat percent in 3,567 non-Hispanic white females and 2,475 non-Hispanic black females age 12 and older. Results showed that there was both an age and race-related association, showing that the negative association between serum 25(OH)D concentrations and adiposity was significantly stronger in non-Hispanic white females versus non-Hispanic black females, and also significantly stronger in younger females versus older females once race was controlled for. Very similar results have also been found in other studies conducted in adolescents. In the Dong et al (2010) study, 559 adolescents, ages 14 to 18 years old, showed a significantly strong negative association between 25(OH)D concentrations and all measurements of adiposity, including BMI percentile, waist circumference, total fat mass, body fat percent, visceral adipose tissue, and subcutaneous abdominal adipose tissue. This was further proven in the Rajakumar et al (2011) study that examined 237 white and black adolescents (mean age 12.7 $\pm$ 2.2 years), wherein they found low levels of 25(OH)D concentrations to be associated with higher adiposity measurements and lower HDL. This study not only showed an age-related association between adiposity and 25(OH)D concentrations, but also a race-related association between adiposity, 25(OH)D concentrations, and fat distribution. It was shown that low levels of 25(OH)D concentrations were associated with higher rates of visceral adipose tissue in white

adolescents, where as in blacks lower concentrations of 25(OH)D was associated with higher subcutaneous adipose tissue concentration (Rajakumar et al 2011).

Vitamin D supplementation

Given the negative health consequences of low circulating 25(OH)D levels, vitamin D supplementation has become a common practice and should be utilized by all high risk individuals. Even though most individuals do consume a diet that contains some form of vitamin D-fortified foods and are often exposed to some form of sunlight during the day, obtaining sufficient vitamin D is often a very difficult task and thus for many individuals should be supplemented appropriately.

Older adults

Evident in several cross-sectional studies, older adults are at a very high risk of developing vitamin D deficiency, mostly due to their decreased cutaneous production (Holick et al 1989) and inadequate intake of vitamin D fortified items (Institute of Medicine 2010). Vitamin D supplementation is thus often highly recommended for older adults (Institute of Medicine 2010).

People with limited sun exposure

Sunlight can almost exclusively provide an individual with their entire daily requirement of vitamin D, and therefore sunlight is often viewed as the predominate source of vitamin D (Webb et al 1990). However for people who are homebound, women who wear long robes and/or head coverings for religious reasons, and people with

occupations that limit sunlight exposure are at an increased risk of developing vitamin D deficiency in comparison to the rest of the population. It is therefore unlikely that this population will be able to obtain an adequate vitamin D concentration without supplementation (Webb et al 1988; Webb et al 1990).

Higher melanin content

Skin pigmentation is a result of the melanin content in the epidermal layer of an individual's skin. Higher amounts of the pigment melanin reduces the skin's ability to produce vitamin D from sunlight (Rajakumar et al 2011), which is why darker skinned individuals have lower circulating 25(OH)D concentrations in comparison to lighter skinned individuals independent of season (Willis et al 2007). For these reasons, supplementation is often recommended for darker skinned individuals (Institute of Medicine 2010).

Fat malabsorption

Fat malabsoprtion is often associated with several medical conditions including but not limited to some forms of liver disease, cystic fibrosis, and Crohn's disease. Since vitamin D is a fat-soluble vitamin, individuals with fat malabsorption have an overall reduced ability to absorb vitamin D, and thus often require vitamin D supplementation in to order meet daily requirements (Holick et al 2006).

The obese

Individuals with a body mass index (BMI) of greater than or equal to 30 are classified as obese and will often have lower 25(OH)D concentrations in comparison to

non-obese individuals (Institute of Medicine 2010). Although they do not differ in their concentrations of 7-dehydrocholesterol or previtamin D3, obese individuals have lower 25(OH)D concentrations in comparison to non-obese individuals because of the increased storage rate of vitamin D by the excess adipose tissue, which ultimately decreases the bioavailability of vitamin D (Wortsman et al 2003).

Effectiveness of cholecalciferol (D_3) over ergocalciferol (D_2)

Vitamin D supplements are readily available in two distinct forms, cholecalciferol (vitamin D_3) and erfocalciferol (vitamin D_2). Cholecalciferol, vitamin D_3, can be considered the natural form of vitamin D or the biologically identical form of vitamin D found in the human body. Erfocalciferol, vitamin D_2, however is a synthetic version of vitamin D, structurally different from vitamin D_3 (Figure 2). Both forms of vitamin D are produced commercially by exposing certain reagents to ultraviolent radiation, with one reagent being a fungus/yeast-derived source (D_2) and the other being an animal-derived source (D_3). Vitamin D_2 is created by exposing a compound called ergosterol, a type of mold, to ultraviolent radiation. Vitamin D_3 on the other hand is commercially produced by exposing 7-dehydrocholesterol, collected from the extracts of animal skins (cow, pig or sheep), or exposing cholesterol, collected from the lanolin of sheep wool, to ultraviolent radiation (Norman 1979).

Although produced from different sources and structurally different (Figure 2), it has long been thought that these two forms of vitamin D are equivalent and therefore interchangeable (Institute of Medicine 1997). However several research trials have shown

cholecalciferol to be both more potent and more stable than ergocalciferol. In the Armas et al (2004) study, a single dose of 50,000 IU of vitamin D_3 or vitamin D_2 was given to 20 healthy male volunteers. After administrating the dose, the researchers followed the serum 25(OH)D concentrations of each volunteer over a 28-day period. Results showed that both sources of vitamin D produced similar spikes in 25(OH)D following administration, however this would be the only similarity the two forms of vitamin D would share. Three days later, 25(OH)D concentrations from the D_3 group continue to rise on a daily basis, peaking all the way up to day 14 post-intervention. However 25(OH)D concentrations from the D_2 group fell dramatically after day 3 and by day 14 were identical to their baseline levels. The researchers then concluded that the potency vitamin D_2 is less than one third that of vitamin D_3, suggesting that a 50,000 IU dose of vitamin D_2 is equivalent to a 15,000 IU dose of vitamin D_3 (Armas et al 2004). Similar results have also been shown in the Romagnoli et al (2008) study, where researchers concluded that vitamin D_3 was twice as potent in raising 25(OH)D concentrations in comparison to vitamin D_2. The Heaney et al (2011) study continued to display this trend in their single blind randomized clinical trail, wherein 33 healthy individuals were given an oral dose of 50,000 IU/wk of ether cholecaliferol or ergocalciferol for 12 weeks and assessed at baseline and weeks 2, 4, 6, 8, 10, 12, and 17. Subcutaneous fat was also collected at baseline and then again at week 12. Results showed that cholecaliferol was 87% more potent in raising and maintaining serum 25(OH)D concentrations in comparison to an equimolar amount of ergocalciferol and also produced a 2 to 3 times greater storage rate of vitamin D (Heaney et al 2011).

The only recent study that actually argues in favor of ergocalciferol is the Holick et al (2008) study. According to Holick et al (2008), no significant differences were discovered between daily doses of vitamin D_2 and vitamin D_3 in an 11-week double-blind randomized placebo controlled vitamin D trail. However convincing these results may sound, it was later pointed out that the actual dose of vitamin D given to each subject was not adequate to create significant rises in 25(OH)D concentrations. In the Holick et al (2008) study, subjects were only given a 1000 IU dose of vitamin D_3 or vitamin D_2 per day, which would only produce a small increase in serum 25(OH)D concentrations (approximately 6 – 10 ng/ml for 1000 IU/d) (Heaney et al 2011). Therefore the minute variability of the increases in 25(OH)D concentrations from each vitamin D source would make assessing the differences between the two sources an overwhelming statistical task. All in all, there is an ever-evolving debate between cholecaliferol and ergocalciferol, which will most likely continue until an overwhelming amount of evidence is produced for ether side of the argument. Until then, it is up to the discretion of the researcher/physician to choose which form of vitamin D is most suitable for their needs.

Dosing frequency

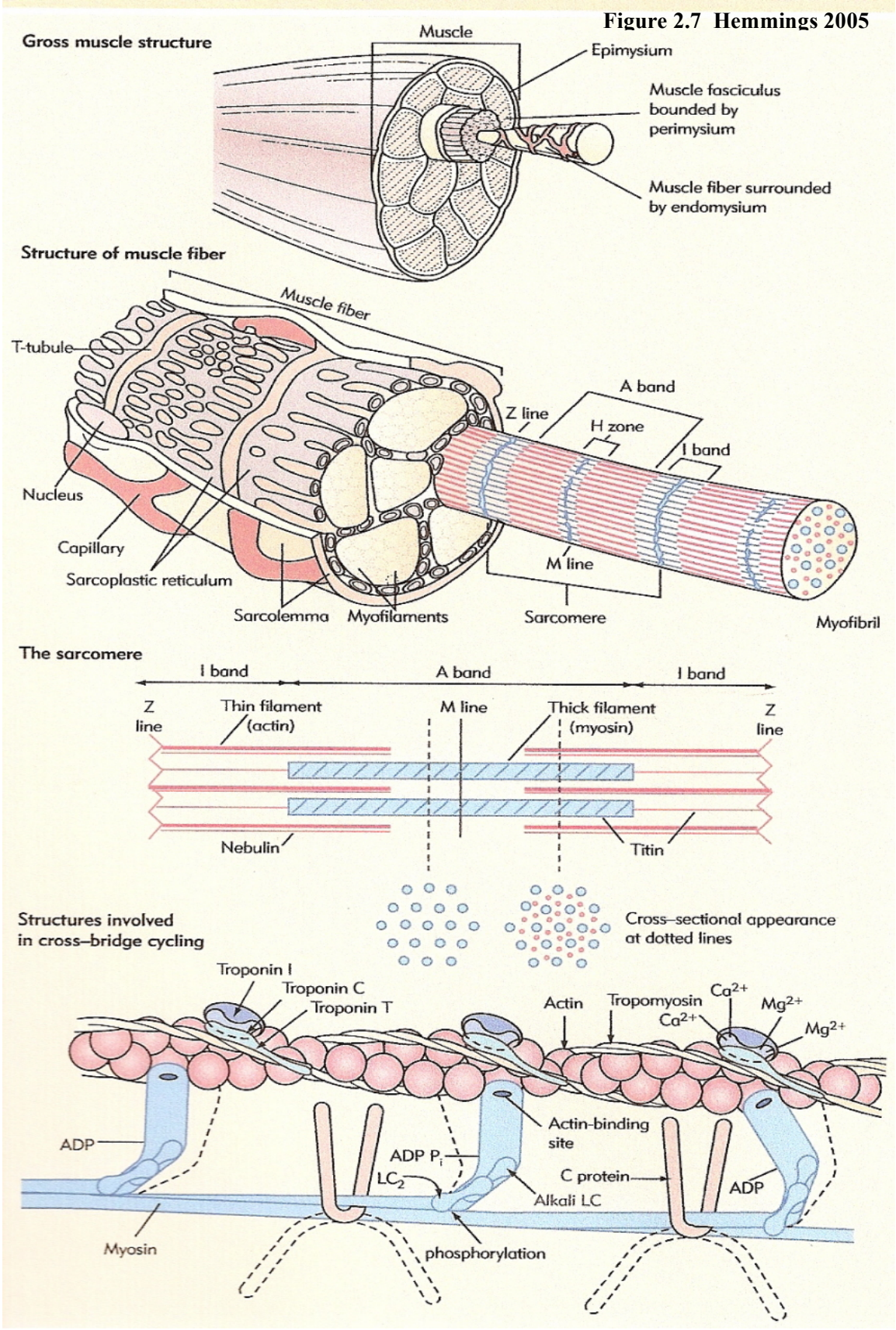
Outside of what type of vitamin D to use in the treatment of various disorders and in clinical trials, the frequency at which to administer the dose has also been highly debated. Vitamin D has been supplemented in a variety of ways, either yearly, monthly, bi-monthly, weekly, bi-weekly, and also daily; all of which have been effective in increasing 25(OH)D concentrations. However in the treatment of low vitamin D status,

particular < 20 nmol/L, daily supplementation appears to be the most effective dosing frequency. In the Saadi et al (2007) study, researchers administrated oral vitamin D2 doses of either 2,000 IU/day or 60,000 IU/month in 178 vitamin D deficient Arab and Indian women. After 3-months of supplementation, 35% of the daily supplemented group and 20% of the monthly supplemented group achieved 25(OH)D concentrations higher than 50 nmol/L. Similar results were found in a study comparing oral vitamin D3 doses of either 600 IU daily, 4,200 IU weekly or 18,000 IU monthly in individuals living in a nursing home with a mean 25(OH)D concentration of 25 ± 10 nmol/L. After 4 months of supplementation, only 11% of patients in the daily or weekly supplemented group had concentrations below 50 nmol/L, this is in comparison to the 36% of patients in the monthly supplemented group (Chel et al 2008). Although slightly different in their methods and population, both studies found that daily supplementation increased and sustained 25(OH)D concentrations more than monthly supplementations. In a recent meta-analysis, daily supplementation of vitamin D was also been shown to be more effective in preventing and improving physical performance measurements in older adults in comparison to weekly or monthly equivalent vitamin D doses (Muir et al 2011). In addition, currently there are no such no such reports in the literature that suggest daily supplementation of vitamin D could lead to toxicity, with studies showing that daily supplementation of less than 40,000 IU of vitamin D for 12 consecutive weeks to be non-toxic in an adult population (Hansen et al 2008).

Skeletal muscle

Skeletal muscles, a name derived from its connection to the skeletal by way of fibrous connective tissues called tendons, are one of three different types of muscles in the human body. Skeletal muscles are mutli-nuclear striated muscles under voluntary control that pull on the skeleton causing motion of the body. Skeletal muscles are composed of several single muscle cells or fibers grouped together in a bundle by way a connective tissue called the epimysium. The microstructure of a whole skeletal muscle and the components of each muscle fiber can be seen in Figure 7.

Figure 2.7 Hemmings 2005



As Figure 7 clearly points out, a series of muscle fibers bundle together by way of connective tissue to form a whole skeletal muscle. Examining Figure 7 more closely, each muscle fiber can be viewed as a single cell, with a cell wall (sarcolemma) encasing organelles: mitochondrias, nucleuses, sarcoplasmic reticulum, and myofibrils. The sarcoplasmic reticulum, the endoplasmic reticulum of a muscle fiber, is the storage center for calcium ions. The myofibrils are organelles responsible for the movement of the body, composed of several overlapping myofilaments that slide across each other to create movement. Myofilaments are composed of thick and thin filaments. The thick filaments are composed of proteins called myosin, a protein which is composed of two different but very large polypeptides; one forming the stalk of the thick filaments and the other forming its globular heads. The thin filaments are composed are proteins called actin. Actin is a globular protein which becomes polymerized into two separate single G-actin strands, these G-actin strands are then later twisted together in a double helix formation in order to form a single actin fiber, F-actin.

In the process of locomotion, these thick and thin filaments slide across each other causing motion of the body, i.e. causing the muscles to pull on the skeletal. In order for this sliding mechanism to occur, cross-bridges must first be formed between the two filaments and then a power stroke must occur. Cross-bridges are formed when the globular heads of myosin bind to the myosin-binding sites located on each actin protein. These myosin-binding sites located on each actin protein however are normally covered by tropomyosin, a regulatory protein wrapped around the F-actin, which prevents the globular heads of myosin from binding to the F-actin and preventing the formation a

cross-bridge. If however calcium is present in the system, calcium will bind to the C-subunit (calcium-subunit) of troponin, another regulatory protein associated with the F-actin and tropomyosin, and cause a conformational change in the I-subunit (Inhibitory subunit) of troponin. Under normal circumstances troponin prevents the movement or translocation of tropomyosin, which ultimately prevents the exposure of the myosin-binding sites and the formation of a cross-bridge. If however a conformational change has occurred in the I-subunit of troponin, which can be caused by calcium binding to the C-subunit, tropomyosin is moved from its resting position allowing the myosin-binding sites to be exposed. Now that the myosin-binding sites are exposed, adenosine triphosphate (ATP) can cause the second phase of this sliding mechanism, the power stroke, to occur. Once the myosin-binding sites are exposed, the globular heads of myosin will securely fashion themselves onto the F-actin. These globular heads will remain tightly bond to the F-actin until ATP binds to the ATP-binding site located on the globular heads. Once ATP attaches itself to the globular head it is quickly hydrolyzed into adenosine diphospate and an inorganic phosphate, which causes the globular heads to loose their affinity to the myosin-binding site and detach. Now detached from the F-actin and with the energy that accompanies the hydrolysis of ATP, the globular heads of myosin now have enough energy to move and reattach itself to a new myosin-binding site. If calcium is present in system and the myosin-binding sites are exposed, the globular heads of myosin will reattach to a new actin protein and cause the adenosine diphosphate to immediately loose its affinity for the globular heads. As soon as adenosine diphosphate detaches itself from the globular heads, a dramatic change occurs in the configuration of the myosin protein. This change ultimately causes the thick filament to

pull on the thin filament, causing a power stroke. Coupling this power stroke with several other power strokes will then cause the whole muscle to pull on the skeleton resulting in movement (Bsechle et al 2000).

In order for calcium to be present in the sarcoplasma, the sacroplasmic reticulum must first be excited upon by a motor neuron. Motor neurons are associated with skeletal muscles and cause the excitation of their motor end plate. The excitation of a motor end plate then causes the formation of an action potential, which then spreads to each muscle fiber by way of the transverse tubules (T-tubules). As this action potential spreads through the entire muscle, a conformational change occurs in two different receptors associated with the T-tubules and the sarcoplasmic reticulum, the dihydropyridine receptor and the ryanodine receptor. Once excited, the two receptors cause the release of calcium ions into the sarcoplasma from the lateral sacs of the sarcoplasmic reticulum (Bsechle et al 2000).

Muscle and bone unit: The mechanostat theory

The actual shape of a bone is structurally designed to fulfill a specific mechanical function, whether that is to support an individual's body weight or protect their vital organs, it serves a purpose. In the late 1900s, Julius Wolff, the world's first orthopaedic surgeon, discovered something that was previously left unnoticed in respects to bone formation; that is the ability of the bone to adapt to certain environmental factors (Wolff et al 1899). It wasn't until much later that Wolff's theories were further investigated,

showing that these structural adaptations to certain environmental factors are driven by forces exerted on the bone, which caused Harold Frost to propose a negative feedback system called the mechanostat theory (Frost et al 1987). Outside of gravity, forces exerted on the bone are largely due to the pulling and pressing of the muscle-bone unit, which means that increases in muscle forces exerted on bone result in an increase in bone adaptation, primary in the area of bone formation (Frost et al 1990). This proposes that as a muscle increases in strength there is also an increase in bone strength. This relationship of the muscle-bone unit has been shown in the Schönau et al (1996) study, showing increases in bone strength (across the pediatric age range) wherein the bones of the child adapted to the increasing muscle forces exerted on them as they grew. Research has also shown that this increase in muscle strength supersedes bone development by a few months (Rauch et al 2004), which strongly supports the idea that muscle plays an integral role in bone accrual and in general the mechanostat theory. This theory is further supported by observations that increases in bone strength followed increases in daily physical activities (Kohrt et al 2004), and decreases in cross-sectional muscle area (a surrogate of muscle strength) resulted in decreases in bone mineral content (Schönau et al 2002).

Muscle assessment in vitamin D research

In order to accurately assess changes in muscle parameters, either mass, density, or strength, one must use the most appropriate method for their specific research question and target population.

Muscle mass and composition

Assessing both muscle mass and composition has become an area of interest in several vitamin D research trials (Muir et al 2011), as serum 25(OH)D has been shown to affect muscle quality and composition (Cegila et al 2009). However due to the shear number of ways in which to assess muscle mass and composition, a researcher must determine the most appropriate assessment method for their specific population, environment, and budget. Imaging techniques such as magnetic resonance imaging (MRI) and computer tomography (CT) are on the forefront of advance imagery technology. With the ability to assess large 3-dimensional spaces in high contrast resolutions, MRI and CT have been both used extensively in the detection of various cancers and in chest and brain imaging. Despite how important of a role they play in certain medical diagnosis, MRI and CT are not frequently used to assess muscle mass or composition as they are very expensive instruments to both operate and maintain. Furthermore, the analysis required to properly analyze MRI and CT images are both labor intensive and requiring of great expertise. However these instruments can be used to validate other reliable instruments. Dual energy x-ray absorptiometry (DXA) is widely used to assess bone densitometry and various soft tissue compartments such as fat-free soft tissue. Unlike monoenergetic x-ray devices, DXA uses two x-ray beams at distinct energy levels, one at a lower energy and one at a higher energy, to determine the composition of a 3-dimensional object. Once the x-rays pass over a specific region, absorption or deflection of each specific tissue compartment is calculated due to the differences detected in the sensor between the two energy beams. This energy difference is then used to interpret the exact composition of the bone and/or soft tissue area. The use of DXA in

the assessment of muscle size and composition has been validated using MRI as a reference model, showing only a .5% difference in the assessment of thigh muscle volume (Elia et al 2000). Furthermore, the use of DXA has been validated as both a reliable and accurate assessment of muscle in older adults (N = 101, aged 50-79 ys; Chen et al 2007), adults (N = 100, aged 18-87 ys; Madsen et al 1997) and even in children (N = 22, aged 8-11 ys; Bridge et al 2011). Peripheral quantitative computed tomography (pQCT) is also a widely used instrument for the assessment of muscle density and muscle cross-sectional area (MCSA), a marker of muscle mass. Similar to DXA, pQCT uses x-ray beams of varying energy levels to determine the composition of a specific region of the body based upon the amount of absorption each tissue compartment attenuates. Unlike DXA however, pQCT uses twelve different x-ray beams at varying energy levels to determine the density and composition of each specific region. Due to the fact that bone has a higher attenuation point in comparison to muscle, pQCT can distinguish and measure muscle separately from bone or any other structure outside of muscle's specific attenuation point; the same applies to adipose tissue which has a lower attenuation point in comparison to bone but higher than muscle. The reliability and validity of pQCT in the assessment of MSCA and muscle density, a marker of intermuscular adipose tissue (IMAT) concentrations, has been shown in several recent studies (Sazbo et al 2011; Briggs et al 2010). In the assessment of MSCA at the 66% site of the tibia and radius, pQCT appears to be a widely used and reliable assessment measure in many pediatric populations (Ward et al 2010; Foo et al 2009; Rauch et al 2008). Recently pQCT has also shown capabilities in the accurate assessment of IMAT, a marker of muscle density, by using certain strong smoothing filters as validated by MRI (Sherk et al 2011). This

accurate assessment of IMAT has been shown in older adult males (N = 3300, aged 40-65 ys; Miljkovic-Gacic et al 2009), diabetic patients (N = 1249, aged $\geq$ 40 ys; Miljkovic-Gacic et al 2008), and also in adolescent females (N = 444, aged 9-12 ys; Farr et al 2011). Both DXA and pQCT have consistently shown to be a reliable and accurate assessment measures of muscle composition, MSCA, and muscle density, respectively.

Muscle strength

Since the vast majority of vitamin D research trials are conduct in older adults, most assessments of muscle strength look primarily at functional outcomes and/or overall physical performance outcomes in comparison to more exercise-based muscle measurements such as the 1RM leg press and bench press (ACSM 1985). The Short Physical Performance Battery (SPPB) test is a series of physical performance measurements developed and used in the Guralnik et al (1994) study to measure balance, gait, strength, and endurance in older adults. The SPPB consists of a 8-foot walking test to measure walking speed, a chair stand test to determine their ability to stand from a chair, and a standing balance test to determine their ability to maintain balance in progressively more challenging standing positions. Each physical performance measurement is then categorized into a five-level scoring system; wherein 0 represents an inability to do the test while a 4 represents the highest level of performance. The three scores are then added together to create a physical performance measurement ranging from 0 (worst) to 12 (best). Since being published in 1994, the 8-foot walking speed test, balance test, and chair stand test of the SPPB have been used in other vitamin D research trials, both in cross-sectional (Houston et al 2007; Boxer et al 2008; Bischoff-Ferrari et al

2004) and intervention studies (Burnout et al 2006). When directly assessing muscle strength, many researchers have used specialized equipment such as a strain gauge or a dynamometer. A strain gauge is a device used to measure the strain an object experiences as forces are exerted upon it. Strain gauges essentially take advantage of the physical properties of certain metals and their electrical conductance to convert mechanical motion or force into an electronic signal (Shull 1992). Using this change in electrical conductance, researchers can therefore assess muscle strength in a wide variety of motions and directions. In the Zhu et al (2010) study, researchers were able to measure ankle dorsiflexion, knee flexor, knee extensor, hip abductor, hip flexor, hip extensor, and hip adductor strength in 302 postmenopausal women (aged 70 – 90 ys) using a strain gauge. The older women were instructed, after one practice run, to exert maximal muscle contraction against the strain gauge, during which the electrical signal was then used to assess subject muscle strength. Similarly the Gleruo et al (1999) study used a strain gauge to measure the maximum isometric strength of the non-dominate leg in 55 vitamin D deficient veiled Arab women. In comparison to a strain gauge, a dynamometer does not measure the electrical differences between two points but the actual force, moment of force (torque), or power exerted on a pump or lever. A handgrip dynamometry is widely used in the medical field and also in several research trails to assess forearm strength and total body strength. Although it may seem to be only applicable to assessing forearm strength, handgrip dynamometry has been shown to be both a reliable indicator of forearm strength (Rantanen et al 1999) and total body strength (Aadahl et al 2011; Kim et al 2001), particularly in children and adolescents (Wind et al 2010; Perry et al 1997). Because it's a reliable indicator of muscle strength and also an affordable and easy to

administer test, several vitamin D cross-sectional (Valtuena et al 2012; Marantese et al 2011; Houston et al 2011; Boxer et al 2008; Houston et al 2007; Kwon et al 2007; Visser et al 2003) and intervention trials (Ward et al 2010; Burnout et al 2006; Elhajji et al 2006; Gleruo et al 1999) have included handgrip strength, measured by handgrip dynamometry, as an outcome measurement.

Hypovitaminosis D myopathy

Overview

The association between vitamin D and muscle was first identified in clinical observations of muscle weakness in osteomalacic patients. Shortly thereafter, muscle weakness and hypotonia became characteristic symptoms of vitamin D deficient rickets in children (serum 25(OH)D <30 nmol/l) (Prineas et al 1965). In adults, this muscle weakness was predominately of the proximal muscles, of the extension, flexion, and abduction of the hips as well as the flexion and extension of the knees (Pfeifer et al 2002), resulting in difficulties of mobility such as rising from a seated or squatted position or walking up stairs (Cegila 2008). Other clinical signs of vitamin D deficiency myopathy include a waddling gait, muscle wasting (Schott and Wills 1976), and muscle pain (Gloth et al 1991).

Muscle morphology

The muscle weakness associated with vitamin D deficiency is related to the actual composition of the muscle and the ratio of muscle fibers. Muscle biopsies in adults with

profound vitamin D deficiency showed an atrophy of the type II muscle fibers with enlarged interfibrillar spaces, an infiltration of fat, fibrosis, and glycogen granules (Yoshikawa et al 1979; Bischoff-Ferrari et al 2001). Since the type II muscle fibers are classified as fast-twitch muscle fibers, recruited first to exert maximum force (McComas 1996), they are essential in the prevention of falling, which may explain why vitamin D deficient older adults often have a tendency of falling (Snijder et al 2006), and potentially an overall decrease in muscle strength (Bischoff-Ferrari et al 1999). Similar symptoms of muscle weakness (Foo et al 2009; Ward et al 2008) and muscle pain (Van der Heyden et al 2004) have also been seen in adolescent children.

Physical performance

Given the extensive research conducted in the area of hypovitaminosis D myopathy among older adults and adolescents, cross-sectional studies have discovered a direct association between 25(OH)D status and parameters of physical performance, particularly when levels are below 50 nmol/L (Cegila 2009). In a US population of 60 to 90 year older adults ($N = 4100$), 25(OH)D levels above 40 nmol/L were associated with a quicker 8-foot walk and a faster sit-to-stand time interval in comparison to lower status individuals (Bischoff-Ferrari et al 2004). In another cross-sectional study, 319 participants (103 women, 216 men), with a mean age of 74.2 years for women and 76.7 years for men, found that leg extension power was significantly associated with higher 1,25(OH)D levels in both men and women ($p = .004$ and $p = .034$, respectively) (Bischoff-Ferrari et al 1999). Such findings are very similar to other cross-sectional studies, wherein low vitamin D status (<35 nmol/L) has been associated with inferior gait

speed, lower physical activity levels, poor balance, weak thigh muscle strength (Gerdhem et al 2005), a shorter 6-minute walking distance (Boxer et al 2008), poorer SPPB scores, and weaker grip strength (Houston et al 2011; Houston et al 2007). In the Longitudinal Study of Aging Amsterdam (LASA), 25(OH)D concentrations above 60 nmol/L was determined to be the threshold for improvements in physical performance (Visser et al 2003). Wicherts et al (2007) went on to show, in a prospective analysis of Visser's study, that older adults with 25(OH)D levels below 50 nmol/L had an increased risk of declining physical performance over the next threes years in comparison to higher status individuals.

This association between low vitamin D status and physical performance is not only limited older adults as three recent studies conduct in adolescent males and females also suggest a similar association. Valtuena et al (2012) showed in a cross-sectional study of 100 Spanish adolescents (aged 12.5 -17.5 y) that 25(OH)D sufficiency (>75 nmol/L) was associated with a significantly higher performance in the standing broad jump test in comparison to individuals with an insufficient 25(OH)D concentration (<75 nmol/L). Ward et al (2008) conducted a cross-sectional analysis of 99 post-menarchal females (aged 12 - 14 y) from a single secondary school in the United Kingdom who had an average 25(OH)D concentration of 21.3 nmol/L. Overall analysis revealed a significant positive correlation between 25(OH)D concentrations and jumping velocity ($P = 0.002$), jump height ($P = 0.005$), muscle power ($P = 0.003$), Esslinger Fitness Index ($P = 0.003$), and muscle force ($P = 0.05$). Shortly there after, another cross-sectional study conducted in 301 Chinese adolescent females, with a mean age of 12 years old and 25(OH)D

concentrations of 34 nmol/L, discovered a positive association between 25(OH)D concentrations and handgrip strength, even after adjusting for physical activity (Foo et al 2009).

Hypovitaminosis D myopathy & vitamin D intervention

Older adults

In order to treat and deter the signs of vitamin D deficiency including an atrophy of the type II muscle fibers, an increase risk of falling, an increase risk of fractures and an overall decrease in physical performance, vitamin D supplementation has become a common practice. Vitamin D supplementation in vitamin D deficient older adults has been shown to make significant improvements in overall muscle fiber composition. Treatment with 1-α-hydroxyvitamin D and calcium for 3 to 6 months in vitamin D deficient older females revealed significant improvements in fiber composition and an increase in the area of the type II muscle fibers (Sorensen et al 1979). In a double-blind, randomized, placebo-control vitamin D trial, supplementation of 1,000 IU of vitamin D2 in 48 older adults over a 2 year period showed significant improvements in percentage of and mean diameter size of the type II muscle fibers (Sato et al 2005). This association between 25(OH)D status and muscle composition was shown at both baseline and after the two-year follow-up. However, despite Sato's findings, at present the association between serum 25(OH)D concentrations and muscle composition remains a mystery. Scientist are still unclear whether this increase in the type II muscle fibers is due to the formation of newly synthesized type II muscle fibers or is the result of a transition

from existing muscle fibers; that is a transition from type I muscle fibers to type II (Cegila 2009). Although the mechanism of reaction may still be a mystery, vitamin D supplementation does however show to have a significant impact on muscle composition, which would explain why vitamin D supplementation has also been shown to decrease the risk of falling in older adults. In a systematic review of 9 randomized placebo-controlled vitamin D trials, the U.S. Preventive Services Task Force concluded that vitamin D supplementation results in an overall decreased risk of falling (risk ratio, .083 [CI, 0.77 to 0.89])(Michael et al 2010). In a 2010 meta-analysis of 9 different double-blind randomized placebo-controlled vitamin D trials, supplementation with daily doses of vitamin D (700 – 1000 IU) decreased the risk of falling among older adults by 19%; this risk was further decreased to 23% if 25(OH)D levels were increased above 65 nmol/L (Bischoff-Ferrari et al 2010). Such findings have led the International Osteoporosis Federation to recommend a daily vitamin D dose of 700 IU in older adults in order to help decrease the risk of falling (Dawson-Hughes et al 2010). Vitamin D supplementation has also been shown to improve muscle strength. In the Glerup et al (2000) study, scientists looked at the association between vitamin D concentrations and muscle power amongst 55 vitamin D deficient veiled Arab women living in Denmark. Baseline data revealed that all parameters of muscle function in the veiled Arab women, including maximal voluntary contraction (MVC - a measure of muscle power), were significantly lower than in Danish controls. However after treatment with an intramuscular injection of 100,000 IU of vitamin D2 every month and daily oral doses of 1200 mg of calcium and 400 IU of vitamin D2, there was a significant increase in mean MVC, showing a 13% increase after 3 months and a 24% increase after 6 months. In a

more recent one-year population-based, double-blind, randomized, placebo controlled vitamin D trial, Zhu et al (2010) evaluated the effects of vitamin D supplementation on muscle strength and mobility in 320 Australian older females (ages 70 to 90 years old) with 25(OH)D levels $\leq$ 60 nmol/L. Participants were given either a daily oral vitamin D2 dose of 1,000 IU or a placebo control (1 g calcium citrate). Muscle assessment was conducted at baseline and at the one-year follow-up, assessing lower limb muscle strength and mobility. Ankle dorsiflexion, knee flexor, knee extensor, hip abductor, hip flexor, hip extensor, and hip adductor strength were assessed using a strain gauge for overall lower limb muscle strength, while mobility was measured by Timed Up and Go test (TUAG). After follow-up, there was a significant association between 25(OH)D concentrations and muscle strength at the hip extensor and adductor among the treatment groups in comparison to controls, with the most dramatic increases seen individuals with the weakest and slowest baseline measurements. This association was also shown in the mobility of subjects treated with vitamin D, showing a 17.5% improvement in TUAG at follow-up.

Adolescents

Unlike the intervention trials conducted in older adults, at present, the majority of the research surrounding vitamin D supplementation in adolescents is concerned with the prevention of rickets in children (Gartner and Greer 2003) and/or the effect of supplementation on bone (Winzenberg et al 2011). To date, there have only been two vitamin D clinical intervention trials that examine the effect of vitamin D supplementation on various muscle parameters. In a double-blind, randomized, placebo-

controlled vitamin D trial, El-Hajj Fuleihan et al (2006) assessed the effects of supplementation on musculoskeletal health in adolescent Lebanese females (N = 168, ages 10-17 years old). Participants were randomly assigned to receive weekly oral vitamin D3 doses of 1,400 IU (200 IU/d, low dosage group), 14,000 IU (2,000 IU/d, high dosage group), or 0 IU (placebo control) for one full year. After the one-year follow-up period, analysis revealed a significant increase in lean muscle mass (both treatment groups, P = 0.04; high dosage group, P = .001), but no significant increases in grip strength (P = 0.16). However after grouping for maturation (34 premenarcheal and 134 postmenarcheal), the significant increase in lean muscle mass previously discovered was only significant in premenarcheal females. In the Ward et al (2010) study, 69 postmenarcheal multiracial females (aged 12 – 14 y) with 25(OH)D concentrations less than 37.5nmol/L were recruited for a one year randomized, double-blind, placebo-controlled vitamin D trial. Participants consumed orally either placebo or a vitamin D2 dose of 150,000IU every three months (~1600 IU/day), over a one-year period. After the one year follow-up, analysis revealed a significant increase in 25(OH)D concentrations (56.0 ($\pm$8.9) nmol/L) and a significant increase in muscle efficiency, which translated into a 5% increased in movement efficiency shown by improvements in jumping velocity. However, no significant improvements were seen in grip strength or cross-sectional muscle area.

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CHAPTER 3

VITAMIN D SUPPLEMENTATION AND MUSCLE RESPONSES IN EARLY PUBERTAL ADOLESCENTS

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Vitamin D Supplementation and Muscle Responses in Early Pubertal Adolescents

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Abbreviations used: 25-hydroxyvitamin D [25(OH)D], 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], Radioimmunoassay (RIA), Intact Parathyroid Hormone (iPTH), Immunoradiometric assay (IRMA), Dual-energy X-ray Absorptiometry (DXA), Peripheral Quantitative Computed Tomography (pQCT), Fat-free Soft Tissue (FFST), Muscle Cross-Sectional Area (MCSA), Muscle Density (MD), Intermuscular Adipose Tissue (IMAT)

Abstract

Background: Though studies in older adults suggest that vitamin D supplementation improves muscle mass and strength, pediatric data are scant and equivocal.

Objective: In this secondary study of a larger vitamin D dose-response study, we examined whether changes in vitamin D metabolites over a 12-week vitamin D_3 intervention were associated with changes in muscle parameters in adolescents in the early stages of puberty (i.e., early pubertal).

Design: A double-blind, randomized, placebo-controlled vitamin D intervention trial was conducted in 324 early pubertal (9-13 y), male and female, black and white children who were recruited from US latitudes 34°N and 40°N, and randomly assigned to five experimental conditions: 0, 400, 1,000, 2,000 or 4,000 IU/d vitamin D_3 for 12 weeks (in winter). Vitamin D metabolites [25(OH)D and $1,25(OH)_2D$ by RIA, intact parathyroid hormone (iPTH) by IRMA] and muscle parameters [Fat-free soft tissue (FFST) by dual energy X-ray absorptiometry, muscle cross-sectional area (MSCA), muscle density (MD), and intermuscular adipose tissue (IMAT) by peripheral quantitative computed tomography, forearm strength by hand-grip dynamometry] were measured at baseline and 12 weeks.

Results: 25(OH)D and $1,25(OH)_2D$ increased significantly following the 12-week intervention, iPTH however did not statistically change. FFST, handgrip strength, arm and leg MCSA all changed significantly, increasing over the 12-week intervention. Changes in 25(OH)D, adjusted for race, sex, age, and location, were not significantly correlated with changes in any of the muscle parameters. Changes in $1,25(OH)_2D$ were

positively correlated to changes arm MCSA, and changes in iPTH were positively correlated to changes in leg MCSA and leg IMAT, and negatively correlated to leg MD. **Conclusion:** If vitamin D does indeed impact muscle composition and function in adolescents, 25(OH)D may not be an appropriate biomarker to assess these changes. The link between iPTH and muscle in adolescents warrants further investigation to ascertain the role of iPTH in muscle, and whether serum levels provide any clinical relevance.

Introduction

Hypovitaminosis D is common amongst older adults [1-2] and is becoming increasingly recognized even in pediatric populations [3-6]. Although the focus of poor vitamin D status in adults is mostly centered on decreased calcium absorption and impaired skeletal mineralization [7], there is an increasing interest in the association of vitamin D and muscle function [8-13], and the potential skeletal significance of muscle [14].

Cross-sectional studies and vitamin D intervention trials conducted in older adults support an association between vitamin D and muscle. It has been reported that older adults with low vitamin D status (<35 nmol/L) have inferior gait speed, poor balance, weak thigh muscle strength [15], poorer Short Physical Performance Battery scores, weaker handgrip strength [16-17], longer sit-to-stand time intervals [18], and weaker knee extension power [19]. Several vitamin D intervention trials in older adults have provided additional evidence of a link between vitamin D and muscle. Vitamin D

supplementation resulting in an increase in serum 25(OH)D was associated with improved muscle composition [20-22] and strength [23-25].

Similar associations between vitamin D and muscle have been shown in adolescents. Cross-sectional studies have shown that vitamin D deficiency is associated with a decreased jumping velocity, jump height, muscle power, Esslinger Fitness Index, muscle force [26], and handgrip strength [27]. Only two vitamin D intervention trials to date have examined the relationship between vitamin D and muscle in adolescents [28-29]. Lebanese adolescent females (N = 168, aged 10-17 years) were supplemented with oral vitamin D_3 (cholecalciferol) doses of 1,400 IU (200 IU/d), 14,000 IU (2,000 IU/d), or placebo every week for one year. Following the one-year intervention, increases in total body lean muscle mass were observed in pre-menarcheal females only (n=34), with both pre- and post-menarcheal females also showing a non-significant increase in handgrip strength as measured by pressure gauge [28]. In another study of predominately South Asian female adolescents (N = 69, aged 12 to 14 years), subjects received oral vitamin D_2 (ergocalciferol) dose of 150,000 IU or placebo every three months (~1,600 IU/d) for one year. Following the one-year intervention, significant improvements in muscle efficiency, as shown by jumping velocity, were observed in addition to a non-significant increase in handgrip strength and muscle cross-sectional area (MCSA) as measured by handgrip dynamometry and peripheral quantitative computed tomography (pQCT), respectively [29].

Though the association between vitamin D and muscle is well supported in older adults [8-13, 15-24], current pediatric data, particularly intervention trials, are limited. Findings from the previously mentioned vitamin D intervention trials suggest that vitamin D supplementation positively impacts adolescent muscle [28-29]; however, these studies are limited to adolescent females with insufficient to low baseline 25(OH)D concentrations and were mostly of Caucasian and South Asian descent. Due to the high prevalence of hypovitaminosis D amongst non-Hispanic black adolescents [30-32], understanding the role of low circulating 25(OH)D and muscle function in this population is essential. Additionally, there are no published studies that have examined the relationships between vitamin D and muscle in male adolescents. Therefore, the purpose of this study was to determine how changes in vitamin D metabolites over a 12-week vitamin D intervention affected changes in muscle parameters in early pubertal adolescents, recruited from US latitudes 34ºN and 40ºN, through the measurement of fat-free soft tissue (FFST), fat mass, and body fat percent by dual energy X-ray absorptiometry (DXA), MSCA, muscle density (MD), and intermuscular adipose tissue (IMAT) by pQCT, and forearm strength by handgrip dynamometry, and whether this effect is dependent upon race and sex.

Materials and methods

Study Participants and Design

Males and females ($N = 324$) in the early stages of puberty were recruited from both northern (latitude 40ºN) and southern (latitude 34ºN) locations as part of the

University of Georgia, Purdue University, and Indiana University School of Medicine (GAPI) 12-week vitamin D intervention trial. Northern and southern recruitment locations were chosen to account for variations in serum 25(OH)D amongst children in different latitudes. Within each of the strata, defined by race [white (n = 159) and black (n = 165)], sex [males (n = 162) and females (n = 162)] and location [Latitude 34ºN (n = 161) and 40ºN (n = 163)], five groups were assigned to a daily oral vitamin D_3 [Douglas Laboratories (Pittsburgh, PA)] of either 0 (placebo), 400, 1,000, 2,000, 4,000 IU (*n* = 4 per sex/group/stratum/cohort). Researchers also sought information on any adverse events. Testing was conducted at baseline and at 12 weeks during the winter months (October to February). Serum 25(OH)D, $1,25(OH)_2D$, iPTH, body composition (Fat-free soft tissue [FFST], fat mass, % fat), MCSA, MD, IMAT, and forearm strength were assessed at each time point. Dietary intake and physical activity information was also collected for use as possible covariates in the analyses. This study was approved by all three of the university's Institutional Review Boards on Human Subjects.

Screening

Screening occurred in two separate phases. First, participants were screened for the appropriate age range (ages 10 to 13 years for males and 9 to 12 years for females) and race. Exclusion criteria included achievement of menarche (for females), existing muscle or bone disorders (cerebral palsy, intestinal malabsorption), growth disorders, or medications/supplements that might influence vitamin D metabolism. Subjects had to be willing to provide blood samples, maintain their normal diet and physical activity patterns, and not take any vitamin, mineral or herbal supplement during the 12-week

intervention. Eligible subjects taking supplements prior to the trial were enrolled after a 4-week washout period. In the second phase, potential subjects were mailed a sexual maturation self-assessment form to complete at home and then mail back to their respective testing location. Males and females who selected stage 2/3 for genitalia and for breast development, respectively, were then enrolled into the study, scheduled for their first testing session, and randomly assigned to one of the five treatment groups.

Biochemical Indices

Approximately 30ml of blood was drawn by a trained phlebotomist. Blood samples were left to sit for 30-60 minutes to coagulate before centrifuging at 4ºC 1800 RPMs for 15 minutes. All samples were then prepared for storage and frozen at < -70ºC until further analysis. All assays were conducted in duplicate using a block design, such that all the samples (from each recruitment site) from the same subject were assayed at the same time by the same investigator with the same kit and blinded to subject race, location, and dose. Repeat analyses were conducted if duplicated samples differed by >10%.

Serum 25(OH)D

Serum concentrations of 25(OH)D were assessed using a 2-step radioimmunoassay (DIASORIN, 25 hydroxyvitamin D I RIA kit, no. 68100, Stillwater, MN, 100 tube kit). Data from our lab show that the inter- and intra-assay coefficients of variation, in a sample of black and white children, were 7.3 to 10.5% and 5.9 to 7.0%, respectively (N=83) [33]. Analytical reliability of 25(OH)D assays were further

monitored through participation in DEQAS (Vitamin D External Quality Assessment Scheme).

Serum 1,25-dihydroxyvitamin D [1,25(OH)$_2$D]

Serum concentrations of 1,25(OH)$_2$D were assessed using a 2-step radioimmunoassay (DIASORIN, 1,25-dihydroxyvitamin D^{125} I RIA kit, no. 65100E, Stillwater, MN, 100 tube kit). The procedure involved a preliminary extraction and subsequent purification of the vitamin D metabolites from each sample using C_{18}OH columns, followed by assay of duplicated aliquots of the extract using a competitive RIA procedure. The specific antibody used was able to detect both 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$. Data from our lab show that the inter- and intra-assay coefficients of variation were 11.6% and 14.4 to 16.9%, respectively.

Serum Intact Parathyroid Hormone (iPTH)

Serum concentrations of iPTH were assessed using an immunoradiometric assay (DIASORIN, N-tact iPTH SP kit, no. 26100, Stillwater, MN 100 beads/kit). Data from our lab show that the inter- and intra-assay coefficients of variation were 0.5 to 3.0% and 3.1 to 9.4%, respectively.

Muscle Outcomes

Dual energy X-ray absorptiometry

Total body FFST (g), fat mass (g), and body fat percent were measured using DXA [Delphi A; S/N 70467; Hologic Inc., Bedford, MA (Georgia site) and GE

Healthcare Lunar iDXA; GE Medical Instruments, Madison, WI (Indiana sites)] at baseline and 12 weeks. For consistency, the same technician at each study site performed all analyses using the same software. DXA quality control for soft tissue was assured by standard protocols. The one-way random effects model single measure intraclass correlation coefficients (ICCs) were calculated in young females 5 to 8 years of age (n = 10) scanned twice in our laboratory during a 7-day period for measures of body composition ($R \geq 0.98$). Short- and long-term DXA precision at the Indiana sites demonstrates errors of less than 2%. Instruments from all three sites were cross-calibrated and study-specific conversion equations were developed to standardize the baseline and 12-week change data [34].

Peripheral quantitative computed tomography

Non-dominant tibia and radius MCSA (mm^2), MD (g/cm^2), and IMAT (mm^2) at the 66% site were measured at all study locations by pQCT [XCT-2000; Stratec Medizintechnic, Pforzheim, Germany] at baseline and 12 weeks. Subject MCSA, MD, and IMAT were each assessed through multiple analyses of the same image. At the 66% site, MCSA of the forearm and lower leg have both been accepted as surrogate markers of muscle force [35-36]. Contour Mode 1 and Peel Mode 1 with a threshold of 34 mg/cm^3 were used to obtain the area of muscle plus bone from the area of subcutaneous fat. Next, the analysis was repeated with a threshold of 710 mg/cm^3 to determine area of bone. MCSA was then determined by subtracting bone area from area of muscle plus bone. To assess MD, cortical bone area was assessed with a threshold of 149 mg/cm^3 and then compared to the cortical bone area assessed with a threshold of 710 mg/cm^3. MD was

then calculated using the results from these two analyses in a specific MD algorithm. To assess IMAT, Contour Mode 3 and Peel Mode 2 non-filtered analyses with a threshold of -101 mg/cm^3 and an inner threshold of 40 mg/cm^3 was used to separate total fat from muscle. Subcutaneous fat (threshold 40 mg/cm^3, inner threshold 40 mg/cm$^{3)}$ and bone marrow (threshold 710 mg/cm^3, inner threshold -101 mg/cm^3) were then subtracted to produce an IMAT value. Subject movement was a concern with both MD and IMAT analyses. Both positive and negative movements, which increase or decrease attenuation values within each image, were discovered in some images at baseline and at 12-weeks (radius, n = 46; tibia, n = 40). Scans with movement measurements greater than 100 (Pmove $\geq$ 100) were not included in any MD and IMAT analyses. Comparability of muscle data from each study site/scanner was achieved by scanning a cortical bone phantom with known properties a minimum of 20 times per scanner. Quality assurance was reassured with daily scans of phantom. Test-retest measurements were performed on five females, aged 18 to 24 years, to determine reliability of the pQCT in our laboratory. The one-way random effects model, ICCs for all pQCT measurements were calculated as R $\geq$ 0.97 [37]. Similar reliability measures were shown at Indiana sites, with a root mean square coefficients of variation (RMS-CVs) of <1% for bone density, mass, and structure, and <1.5% for MCSA in 30 healthy individuals scanned six times with interim repositioning [38].

Handgrip dynamometer

Maximum isometric strength of the non-dominant hand and forearm was assessed using a handgrip dynamometer (Jamar Plus+ Digital Hand Dynamometer; Patterson

Medical, Bolingbrook, IL) by the same technician at baseline and 12 weeks. At baseline, the technician adjusted the handle of the dynamometer for the most comfortable gripping position for each subject (1st and 2nd position for children), noted the subject's specific setting, and repeated the same setting at 12 weeks. Three measurements were taken 30 seconds apart and the average of the three tests was used as an indicator of forearm strength. Test-retest measurements on 11 children (4 white males, 3 white females, 3 black males, and 1 black female) aged 9 to 13 years were conducted to determine the ICC. The test-retest measurements were completed in a single day (3 hours apart) with 30-second rest periods between each of the three assessments. Using a one-way random effects model, the average measured ICC for handgrip strength in our lab was R=0.98. The correlation coefficient of the handgrip dynamometer in assessing hand and forearm strength of the dominant and non-dominant hand in children has been reported as 0.97 (95% confidence interval: 0.95 to 0.98) and 0.95 (95% confidence interval: 0.92 to 0.96), respectively [39].

Statistics

Statistical analyses were performed using the SAS statistical package [SAS System for Windows, v 9.2 (SAS Institute, Cary, NC)]. Baseline differences between race (black vs. white) and sex (male vs. female) groups were performed using independent samples *t*-tests. Data were tested for normality and outliers by the Shapiro-Wilk and the Dixon and ESD test, respectively, and revealed non-normal distribution of change data and the presence of outliers. Therefore, Spearman rank order and partial correlation analyses, adjusted for race, sex, age, and location, were performed to assess

relationships between baseline and 12-week changes in vitamin D metabolites and baseline and 12-week changes in muscle parameters. Additional correlations between vitamin D metabolites and age, physical activity, and diet were performed. Also of interest were relationships between 12-week changes in IMAT and changes in other muscle variables.

Results

Baseline and Subject Characteristics

Baseline subject characteristics are found in Table 1. Blacks had greater BMI-for-age, arm MCSA, leg and arm IMAT, handgrip, and serum iPTH in comparison to whites, but had significantly lower leg MD and serum 25(OH)D. Black males had significantly greater serum $1,25(OH)_2D$ in comparison to white males. Black females had significantly greater body weight, maturity offset, and fat mass, but were significantly younger and exhibited lower arm MD, calcium intake, and METs in comparison to white females. Sexual differences were also present within each race. White males had significantly greater age, height, maturity offset, FFST, arm MCSA, leg IMAT, and handgrip, but significantly lower BMI-for-age, percent body fat, and serum $1,25(OH)_2D$ in comparison to white females. Such sexual differences, excluding serum $1,25(OH)_2D$, BMI-for-age and leg IMAT, were observed in black males and females, with females having greater fat mass and black males having greater leg MCSA and arm IMAT. At baseline, mean circulating 25(OH)D concentrations were 69.9 ± 18.5 nmol/L, with black females having

the lowest concentration at 59.2 ± 18.0 nmol/L. Only 1% of the total baseline population had circulating 25(OH)D levels below 30 nmol/L.

12-week Change Data

In the original sample of 324 early pubertal adolescents, 299 completed the 12-week intervention trial, yielding a 93% retention rate. Mean serum 25(OH)D (p <.0001) and 1,25$(OH)_2$D (p <.0001) increased significantly by 24.97 ± 37.29 nmol/L and 10.27 ± 17.90 ng/L, respectively, from baseline to 12 weeks. No statistically significant change in iPTH was observed. FFST (p <.0001), handgrip (p = .0002), arm (p <.0001) and leg MCSA (p = .024) all increased from baseline to 12 weeks. Changes in FFST differed by race, with whites having greater increases than blacks (p = .012).

Correlations and Group Analyses

Spearman correlations of baseline vitamin D metabolites and muscle outcomes are presented in Table 2. After adjusting for age, race, sex, and location, serum 25(OH)D was positively correlated to leg MD, while serum 1,25$(OH)_2$D was positively correlated to FFST. Baseline serum iPTH, once adjusted for age, race, sex, and location, was positively correlated to FFST, arm and leg MCSA, and leg IMAT.

Comparisons between the placebo and 4,000 IU groups assessed by analysis of covariance are found in Table 3. After adjusting for race, sex, and location, FFST increased over 12 weeks (time effect; P < 0.001). There were significant interactions for 25(OH)D (P< 0.001), 1,25$(OH)_2$D (P< 0.037) and iPTH (P< 0.003), such that these serum metabolites increased more in the 4,000 IU dose group vs. placebo.

Spearman correlations for 12-week change in vitamin D metabolites and muscle outcomes are found in Table 4. There were no significant correlations between serum 25(OH)D and muscle outcomes unadjusted or adjusted for age, race, sex, and location. Changes in serum $1,25(OH)_2D$ were positively correlated with changes in arm IMAT, but once adjusted for covariates, were positively correlated with changes in arm MCSA and not arm IMAT. Although total serum changes in iPTH were not statistically significant, changes that did occur in serum iPTH were positively correlated with changes in leg IMAT. After adjusting for age, race, sex and location, changes in serum iPTH not only remained positively correlated with changes in leg IMAT, but also negatively correlated to leg MD and positively correlated to leg MCSA. Age was also positively correlated with changes in FFST ($r = 0.18$, $p = .0017$) and arm MCSA ($r = 0.13$, $p = .029$). Unadjusted dietary analyses revealed significant correlations between dietary vitamin D intake (VD) and total caloric intake (Kcal), showing VD at 12-weeks to be positively correlated to changes leg MD ($p = .029$) and negatively correlated to changes in leg IMAT ($p = .048$), and showing Kcal at 12-weeks to be negatively correlated to changes in iPTH ($p = .030$). During the 12-week intervention trial physical activity had no affect on changes in serum or muscle parameters.

Spearman correlations of 12-week change in arm and leg IMAT and muscle outcomes are found in Table 5. Leg IMAT was inversely correlated with Leg MD and positively correlated to Leg MCSA. Arm IMAT was inversely correlated to arm MD but positive correlated to arm MCSA and leg MCSA.

Discussion

The primary findings from this study indicated that despite significant increases in 25(OH)D following the 12-week vitamin D intervention, changes in 25(OH)D were not related with changes in any of the muscle parameters. Additionally, $1,25(OH)_2D$ significantly increased following the 12-week vitamin D intervention and was positively correlated with changes in arm MCSA. Though iPTH did not change following the 12-week intervention, changes in iPTH, after adjusting for age, race, sex, and location, were significantly and positively correlated with changes in leg MCSA and leg IMAT, and negatively correlated with leg MD. The lack of an association between changes in 25(OH)D and changes in muscle parameters in the current study are contradictory to several vitamin D intervention [40-43] and cross-sectional [44-47] studies in adults, wherein improvements in serum 25(OH)D or high 25(OH)D concentrations were associated with greater muscle function and composition. Such findings in comparison to the current study findings beg the question, does vitamin D supplementation affect adolescent muscle, and if so is 25(OH)D a sensitive biomarker of the action of vitamin D on muscle in pediatrics as it is in adults?

Whether vitamin D supplementation does indeed affect adolescent muscle can be described by the current study and, to our knowledge, the only two vitamin D intervention trials conduced in adolescents that examine muscle [28-29]. In these studies [28-29], the vitamin D intervention groups had significant increases in total body FFST and muscle efficiency, respectively, in comparison to placebo. The current study however

did not show similar findings in muscle mass or efficiency with vitamin D supplementation, which may be explained by the shorter intervention duration of our intervention, as well as the age, maturation and vitamin D status of the population selected. For example, both the El-Hajj Fuleihan et al [28] and Ward et al [29] studies were 12-month vitamin D intervention trials, in comparison to our study duration of 12 weeks. Although shorter by comparison, our 12-week vitamin D intervention showed significant increases in 25(OH)D, $1,25(OH)_2D$, FFST, handgrip, arm and leg MCSA. Due to the rapid period of growth experienced by adolescents in the early stages of puberty [48], it is possible within a 12-week time period to have significant changes in muscle parameters.

Age and maturation are other distinguishing factors between each of the study's populations. Findings from the Ward et al [29] study were produced in exclusively post-pubertal adolescent females (mean age 14 yrs), whereas the current study consisted of adolescents in the early stages of puberty. Moreover, the associated increase in total body FFST in the El-Hajj Fuleihan et al [28] study was only significant in pre-pubertal adolescent females, and not post-pubertal. Although the El-Hajj Fuleihan et al [28] study had a similar age distribution in comparison to the current study (both had a mean age of 11 yrs), discrepancies in the findings between the two previous vitamin D intervention trials and the current study could be further explained by the vitamin D status of each the study's population. The population group selected in the El-Hajj Fuleihan et al [28] and Ward et al [29] studies had considerably lower baseline 25(OH)D concentrations, of 35 ± 20 nmol/L and 18 ± 8 nmol/L, respectively, in comparison to the current study baseline

25(OH)D concentration of 70 ± 19 nmol/L. Vitamin D deficiency has been shown to be associated with muscle weakness [49-51]and pain [52] in adolescents. By improving the vitamin D status of previously vitamin D deficient populations through supplementation, improvements in muscle parameters are more likely to occur and therefore could differ from the current study's findings. Though results vary, all three studies illustrated that vitamin D supplementation does appear to have an effect on adolescent muscle in both deficient and relatively healthy populations. However, whether 25(OH)D is a sensitive biomarker of the action or effect of vitamin D on muscle in pediatrics is uncertain.

To date, there is a lack of evidence that shows 25(OH)D has an influence or is an ideal biomarker of the action or effect of vitamin D on muscle in pediatrics. While the two previous vitamin D intervention trials conducted in adolescents showed a significant increase in 25(OH)D [28-29] and 1,25$(OH)_2$D [28] following supplementation and that vitamin D supplementation positively affects muscle in comparison to placebo [28-29], neither study tested whether associations existed between the individual vitamin D metabolites and their respective muscle outcomes. Whether 25(OH)D is a suitable biomarker in adolescents has also been questioned with respect to calcium absorption. A vitamin D intervention trial in white and black adolescent females (N = 105; aged 11-15 yrs) showed that 25(OH)D was not associated with calcium retention. In fact, blacks with lower 25(OH)D status in comparison to whites had significantly higher calcium retention rates [53]. Furthermore, in the study by Abrams et al (2005), 1,25$(OH)_2$D and iPTH, but not 25(OH)D, were positively correlated with calcium retention [54]. Outside of being an

ideal marker of dietary and cutaneous vitamin D inputs, both studies including the current study question the exact role 25(OH)D plays in early adolescences.

Unlike 25(OH)D, changes in $1,25(OH)_2D$ were positively correlated with changes in arm MCSA. With a short half-life of 4-8 hours [55], $1,25(OH)_2D$ is not traditionally considered a reliable biomarker of vitamin D status [56-57]. Therefore, in addition to being a difficult assay to conduct, $1,25(OH)_2D$ is often not assessed in many vitamin D research trials. However the limited data available still suggest a link between $1,25(OH)_2D$ and muscle [58]. A hormone, $1,25(OH)_2D$ has been shown to display other functions outside of calcium regulation [7,59-60], including a potential role in muscle growth and development [58,61]. In the current study, baseline $1,25(OH)_2D$ concentrations were significantly higher in black males who were also stronger and had larger muscles in comparison white males. Similar associations of a higher $1,25(OH)_2D$ [62] and muscle parameters [63-64] have been shown in other cross-sectional studies in non-Hispanic blacks. Due to the rapid period of growth associated in adolescents in the early stages of puberty [48], the active form of vitamin D may be needed in much higher amounts in comparison to adults. Although the synthesis of $1,25(OH)_2D$ is tightly regulated and largely dependent upon serum calcium and iPTH concentrations in adults [7,59-60,65], perhaps in early adolescents the synthesis of $1,25(OH)_2D$ is less dependent upon calcium and iPTH concentrations and more on the body's overall metabolic demand during growth. Such a theory is further supported by the study's significant increase in $1,25(OH)_2D$ over the 12-week intervention trial in a calcium sufficient population, the study's lack of statistical change in iPTH, and the associated decrease in 25(OH)D with

increasing age in early adolescents [66-67]. However, currently there is no research addressing the conversation rates of 25(OH)D into $1,25(OH)_2D$ in early adolescents. Therefore $1,25(OH)_2D$ should be monitored more closely in future vitamin D research trials in order to understand the exact role of $1,25(OH)_2D$ plays in early adolescence and its potential role in muscle growth and development.

Despite the present study's significant increase in 25(OH)D and $1,25(OH)_2D$, iPTH did not statistically change during the 12-week intervention trial, which could be explained by the study's predominately vitamin D sufficient baseline population (~70 nmol/L). Although a negative correlation between iPTH and 25(OH)D is shown in other pediatric populations [28,54,68-69], findings are mixed whether a maximal suppression of iPTH exists in this population. Studies in male adolescents (aged 11-16 yrs) [69] and older adults [70] have shown that a maximal suppression of iPTH is associated with a 25(OH)D concentration of 60 to 80 nmol/L; however, other studies have shown a lack of an inflection point between iPTH and 25(OH)D [71]. Whether there is a 25(OH)D concentration associated with a maximum suppression of iPTH in a pediatric population is still unclear; however, it is evident in cross-sectional studies [72-74], intervention trials [75-77], and in the current study that iPTH plays a role in the growth and development of adolescent bone, and potentially muscle.

This study is one of the first studies to report these positive correlations between iPTH and muscle in adolescents. Baseline values of iPTH, adjusted for age, race, sex, and location, were positively correlated to baseline FFST, arm and leg MCSA, and leg IMAT.

Although changes in iPTH were not statistically significant following the 12-week vitamin D intervention, changes in iPTH, after adjusting for covariates, were positively correlated with changes in leg MCSA and leg IMAT, and negatively correlated with leg MD; suggesting an overall bigger and fatter muscle with higher iPTH levels. Based upon adult data, such findings would suggest an impaired muscle function and lower quality [78-79] in the study's population. In addition, it has also been documented in adults that increases in IMAT are correlated with decreases in insulin sensitivity and the prevalence of diabetes [80-81]. Based solely upon adult data, increases in IMAT, as a result of an increase in serum iPTH, would result in certain negative health outcomes. However, current data supporting this association between IMAT and these metabolic disorders are greatly limited in pediatric populations. To our knowledge, there are no studies that address the effect of IMAT on muscle function and quality in adolescents; whether it elicits a negative effect or is part of the normal growth process. Similarly, it is also important to note that a transient diabetic-like state of insulin resistance occurs during the normal growth process in adolescents [82-83]. Therefore it is possible that transient insulin resistant state and this iPTH-induced increase in IMAT coincide with each other to promote muscle growth and development in early adolescences. Such a notion would suggest that increases in iPTH resulting in increase in IMAT are potentially beneficial to adolescent muscle, either acting as a switch for the insulin resistant state seen in early adolescents or potentially as a possible immediate source of dense energy. More research is needed in order to understand this role of iPTH and IMAT in adolescent muscle and their potential interaction during the transient insulin resistant state of the growth process.

Overall findings from the current study suggest that an environment of higher iPTH is associated with greater changes in muscle in adolescents in the early stages of puberty. It is important to note that higher concentrations of serum iPTH result in an increase in 25-hydroxyvitamin D-1α-hydroxylase activity [58], which subsequently increases serum $1,25(OH)_2D$. Whether an environment of higher iPTH or an increase in $1,25(OH)_2D$ synthesis is responsible for this change in muscle parameters is unclear; however, future vitamin D research in adolescents should include these metabolites in the design.

The current study admittedly had some limitations. There were a significant number of subjects with movement in pQCT-derived muscle outcomes that led to exclusion of their muscle data (arm, n = 46; leg, n = 40). Secondly, the length of the intervention trial may not have been of sufficient duration. Though there were significant changes in serum and muscle outcomes following the 12-week intervention, a longer intervention trial may have generated larger changes in outcome variables.

The strengths of the current study are numerous. This study was the first clinical vitamin D intervention trial that examined muscle outcomes in both black and white male and female adolescents in the early stages of puberty. The 4,000 IU vitamin dose safely generated large changes in 25(OH)D. Furthermore, the study's daily supplementation appears to be the most effective dosing frequency [84-86] and cholecalciferol (vitamin D_3) appears to be superior in raising and maintaining 25(OH)D concentrations in comparison to ergocalciferol (vitamin D_2) [87-89]. Due to increased nutrient needs [48],

adolescents in the early stages of puberty were ideal our intervention trial. The study's two recruitment sites increased generalizability of our findings and the population diversity. In addition, DXA [90-91], pQCT [92-93], handgrip dynamometry [94-95], and RIA [96] are all reliable markers of muscle and serum vitamin D status. To our knowledge, this study was the first study to measure IMAT through the use of pQCT in a pediatric population. Though a relatively novel approach in pediatric populations, the assessment of IMAT by way of pQCT has been previously docusmented in adult populations [80-81,97]. Validation of this assessment of IMAT was further made by the study's significant correlations between IMAT and muscle outcomes. Future research is warranted in order to further validate this measurement in pediatric populations.

In summary, both iPTH and $1,25(OH)_2D$, but not 25(OH)D, were positively correlated with changes in muscle parameters. If vitamin D supplementation does indeed have an affect on muscle in a relatively healthy adolescent population, it is not reflected by changes in serum 25(OH)D but rather $1,25(OH)_2D$ and iPTH. Based upon the current study and previous adolescent trials [53-54], 25(OH)D appears to be a suitable biomarker of vitamin D input rather than of effect in adolescents. Although the lack of change in iPTH could be explained by our vitamin D sufficient population, due to the potential interaction of iPTH in adolescent growth [72-77], a minimal concentration of iPTH may be required for optimal growth. However, other muscle metabolites such as IGF-1 [98], myostatin [99], and various myokines [100] have also shown to be influenced by vitamin D, suggesting an indirect effect of vitamin D on muscle outcomes. Additional research is needed to clarify the involvement of $1,25(OH)_2D$ and iPTH, in addition to 25(OH)D, on

muscle in both male and female adolescent populations, and whether other metabolites are involved in this relationship.

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LIST OF TABLES

Table 3.1

Baseline Subject Characteristics[1]

			Males			Females			[2]White	Black
Variable	N	Total	White n = 80	Black n = 82	*P*	White n = 79	Black n = 83	*P*	M vs. F	M vs. F
Age (yrs)	323	11.3 ± 1.2	12.1 ± 1.0	11.8 ± 1.2	.055	11.0 ± 1.0	10.5 ± 1.0	.004*	<.001*	<.001*
Weight (kg)	323	47.4 ± 12.2	47.8 ± 13.8	49.4 ± 13.0	.461	44.2 ± 9.3	47.8 ± 11.8	.033*	.058	.399
Height (cm)	323	150.7 ± 9.3	153.5 ± 9.7	151.2 ± 9.6	.301	149.0 ± 8.8	148.3 ± 8.2	.560	.003*	.009*
Maturity Offset (yr)[3]	323	-1.1 ± 1.2	-1.4 ± 1.2	-1.7 ± 1.2	.059	-.4 ± 1.0	-.8 ± 0.8	.007*	<.001*	<.001*
BMI-for-age (%)	323	68.0 ± 29.2	56.6 ± 32.2	72.1 ± 26.1	.001*	65.8 ± 27.4	76.7 ± 27.3	.012*	.045*	.267
Body Composition[4]										
Fat Mass (kg)	306	14.9 ± 7.5	13.6 ± 8.0	13.9 ± 7.5	.827	14.7 ± 6.3	17.2 ± 7.6	.012*	.322	.006*
Percent Body Fat	306	31.2 ± 9.5	28.2 ± 9.4	28.2 ± 9.5	.980	32.9 ± 8.1	35.4 ± 8.7	.030*	.001*	<.001*
Fat-free Soft Tissue (kg)	320	30.4 ± 6.9	32.2 ± 7.8	32.5 ± 7.6	.775	28.2 ± 8.1	28.6 ± 5.4	.084	<.001*	<.001*
Muscle Variables[5]										
Arm MCSA (mm2)	321	2119 ± 494	2085 ± 522	2430 ± 534	<.001*	1825 ± 307	2117 ± 375	<.001*	<.001*	<.001*
Arm density (g/cm2)	321	74.6 ± 13.9	78.5 ± 26.8	72.8 ± 2.9	.057	75.1 ± 1.8	72.2 ± 3.0	<.001*	.269	.174
Arm IMAT (mm2)	297	273.6 ± 165.7	202.7 ± 92.3	379.1 ± 194.5	<.001*	181.4 ± 134.7	301.8 ± 133.9	.010*	.742	.018*
Leg MCSA (mm2)	318	4827 ± 1051	4972 ± 1191	5062 ± 1118	.625	4666 ± 909	4607 ± 903	.679	.083	.005*
Leg Density (g/cm2))	318	73.3 ± 3.2	75.3 ± 1.7	71.9 ± 3.2	<.001*	75.2 ± 1.7	71.2 ± 3.2	<.001*	.746	.160
Leg IMAT (mm2)	311	1090 ± 499	949.9 ± 419.9	1306 ± 626	.026*	917.4 ± 288.5	1159 ± 485	<.001*	.035*	.280
Handgrip (kg)[6]	306	20.1 ± 13.9	15.4 ± 7.4	29.5 ± 18.4	<.001*	12.8 ± 6.4	21.6 ± 12.6	<.001*	.026*	.002*
Dietary Intake[7]										
Energy Intake (kcal)	307	2001 ± 556	2143 ± 541	2025 ± 602	.203	1971 ± 540	1853 ± 505	.165	.056	.058
Calcium Intake (mg)	307	900.9 ± 395.0	963.5 ± 417.0	848.7 ± 401.1	.083	962.8 ± 416.0	824.7 ± 320.8	.023*	.992	.685
Vitamin D Intake (IU)	307	169.3 ± 124.4	188.3 ± 163.4	168.9 ± 94.8	.364	158.0 ± 120.8	162.0 ± 105.3	.828	.179	.673
Physical Activity[8]										
Activity (METs/day)	304	62.2 ± 19.9	63.4± 9.0	61.3 ± 14.6	.205	64.8 ± 10.1	59.3 ± 8.7	<.001*	.368	.216
Biochemical Variables										
25(OH)D (nmol/L)	318	69.9 ± 18.5	80.5 ± 13.4	61.1 ± 14.6	<.001*	79.7 ±16.5	59.2 ± 18.0	<.001*	.672	.460
iPTH (pg/ml)	318	27.4 ± 10.8	24.2 ±9.0	28.4 ± 11.3	.009*	25.5 ± 9.3	31.1 ± 12.1	<.001*	.294	.143
$1,25(OH)_2D$ (pg/ml)	318	55.4 ± 16.5	50.2 ± 13.3	56.3± 17.9	.014*	56.6 ± 18.3	58.5 ±14.8	.474	.016*	.401

1. Statistical differences between groups at baseline were preformed using independent samples *t*-test. *Significantly different from group, $P < 0.05$. Mean $\pm$ SD.
2. Statistical differences of sex within each race at baseline were performed using independent samples *t*-test.
3. Maturity Offset calculated (reference)
4. Measured by dual-energy x-ray absorptiometry
5. Measured by peripheral quantitative computed tomography; MCSA, muscle cross-sectional area; MD, muscle density; IMAT, intermuscular adipose tissue.
6. Measured by handgrip dynamometer
7. Dietary intake measured by 3-day diet record and analyzed by The Food Processor SQL version 9.7.3
8. Physical Activity quantified by 3-day physical activity recall [103]

Table 3.2

Spearman Correlations of Baseline Serum and Muscle Outcomes[1]

	25(OH)D (n = 318)		$1,25(OH)_2D$ (n = 318)		iPTH (n = 318)	
	Unadjusted	Adjusted (N = 272)	Unadjusted	Adjusted (N = 272)	Unadjusted	Adjusted (N = 272)
FFST (n = 320)	rs = -0.12, p = 0.04*	rs = -0.07, p = 0.22	rs = 0.14, p = 0.01*	rs = 0.14, p = 0.02*	rs = 0.18, p < 0.01*	rs = 0.19, p < 0.01*
Handgrip (n = 306)	rs = -0.21, p < 0.01*	rs = 0.05, p = 0.43	rs = 0.15, p = 0.01*	rs = 0.10, p = 0.09	rs = 0.21, p < 0.01*	rs = 0.07, p = 0.25
Arm MCSA (n = 321)	rs = -0.30, p < 0.01*	rs = -0.06, p = 0.35	rs = 0.14, p = 0.02*	rs = 0.12, p = 0.05	rs = 0.20, p < 0.01*	rs = 0.18, p < 0.01*
Leg MCSA (n = 318)	rs = -0.12, p = 0.03*	rs = -0.09, p = 0.13	rs = 0.13, p = 0.02*	rs = 0.12, p = 0.05	rs = 0.20, p < 0.01*	rs = 0.19, p < 0.01*
Arm MD (n = 321)	rs = 0.34, p < 0.01*	rs = 0.12, p = 0.06	rs = -0.03, p = 0.61	rs < -0.01, p = 0.98	rs = -0.11, p = 0.05	rs < -0.01, p = 0.99
Leg MD (n = 318)	rs = 0.42, p < 0.01*	rs = 0.14, p = 0.02*	rs = -0.02, p = 0.75	rs = 0.03, p = 0.60	rs = -0.16, p < 0.01*	rs = -0.04, p = 0.56
Arm IMAT (n = 304)	rs = -0.36, p < 0.01*	rs = -0.05, p = 0.42	rs = 0.08, p = 0.16	rs = 0.05, p = 0.38	rs = 0.16, p < 0.01*	rs = 0.09, p = 0.12
Leg IMAT (n = 301)	rs = -0.25, p < 0.01*	rs = -0.09, p = 0.16	rs = 0.11, p = 0.05*	rs = 0.09, p = 0.14	rs = 0.17, p < 0.01*	rs = 0.12, p = 0.04*

1. Spearman correlations of unadjusted and adjusted (race, sex, age, location) vitamin D metabolites and muscle outcomes. *Significantly correlated, $P < 0.05$.
2. FFST, fat-free soft tissue; MCSA, muscle cross-sectional area; MD, muscle density; IMAT, intermuscular adipose tissue

Table 3.3

Baseline and 12-Week Differences in Muscle Outcomes Between the 4,000 IU and Placebo Dose Groups[1]

	Baseline		12-Week		Group[2]		Time[3]		Group x Time[4]	
	Placebo	4,000 IU	Placebo	4,000 IU	*P*	R^2	*P*	R^2	*P*	R^2
FFST (kg)	30268 ± 767	29954 ± 787	31426 ± 791	31130 ± 787	.785	.001	<.001*	.163	.914	.000
Handgrip (kg)	21.7 ± 1.5	20.8 ± 1.5	22.1 ± 1.4	20.9 ± 1.4	.596	.003	.907	.000	.520	.004
Arm MCSA (mm^2)	2105 ± 51.7	2077 ± 53.9	2180 ± 55.4	2210 ± 57.8	.995	.000	.162	.017	.123	.021
Leg MCSA (mm^2)	4806 ± 122	4674 ± 126	4877 ± 117	4805 ± 121	.550	.003	.678	.002	.297	.010
Arm MD (g/cm^2)	74.2 ± .3	73.6 ± .3	74.2 ± .3	73.6 ± .3	.159	.017	.552	.003	.852	.000
Leg MD (g/cm^2)	73.7 ± .3	73.1 ± .3	73.8 ± .3	73.1 ± .3	.127	.021	.326	.009	.688	.001
Arm IMAT (mm^2)	265.2 ± 16.3	266.2 ± 17.1	270.9 ± 16.3	282.1 ± 18.8	.799	.001	.594	.003	.423	.006
Leg IMAT (mm^2)	1142 ± 58.6	1055 ± 58.6	1084 ± 52.3	1031 ± 52.8	.339	.008	.171	.017	.599	.003
25(OH)D (nmol/L)	72.3 ± 1.9	69.4 ± 2.0	63.4 ± 4.2	147.3 ± 4.3	<.001*	.482	<.001*	.146	<.001*	.716
iPTH (pg/ml)	26.5 ± 1.3	26.5 ± 1.2	24.4 ± 1.4	29.5 ± 1.4	.134	.019	.985	.000	.003*	.072
$1,25(OH)_2D$ (pg/ml)	56.1 ± 2.2	54.6 ± 2.3	55.4 ± 2.4	74.9 ± 2.5	.003*	.072	.037*	.037	<.001*	.306

1. Analysis of covariance controlling for sex, race, and location. Values are means ± SD
2. Between-subject group differences, *$P < .05$
3. Within-subject time differences, *$P < .05$
4. Within-subject group x time interactions, *$P < .05$

Table 3.4

Spearman Correlation of 12-week Change in Serum and Muscle Outcomes[1]

	25(OH)D (n = 318)		$1,25(OH)_2D$ (n = 318)		iPTH (n = 318)	
	Unadjusted	Adjusted	Unadjusted	Adjusted	Unadjusted	Adjusted
FFST (n = 320)	rs = -0.04, p = 0.47	rs = -0.03, p = 0.64	rs = 0.04, p = 0.53	rs = 0.06, p = 0.30	rs = 0.03, p = 0.65	rs = 0.05, p = 0.37
Handgrip (n = 306)	rs = 0.02, p = 0.78	rs = 0.06, p = 0.33	rs = 0.08, p = 0.16	rs = 0.07, p = 0.28	rs = 0.04, p = 0.55	rs = 0.03, p = 0.65
Arm MCSA (n = 321)	rs = 1.01, p = 0.84	rs = 0.04, p = 0.53	rs = 0.09, p = 0.12	rs = 0.15, p = 0.01*	rs = -0.02, p = 0.68	rs = 0.04, p = 0.55
Leg MCSA (n = 318)	rs = -0.00, p = 0.99	rs < 0.01, p = 0.94	rs = 0.05, p = 0.42	rs = 0.06, p = 0.31	rs = 0.11, p = 0.05	rs = 0.14, p = 0.02*
Arm MD (n = 321)	rs = -0.01, p = 0.78	rs = 0.05, p = 0.40	rs = -0.04, p = 0.54	rs < -0.01, p = 0.98	rs = 0.11, p = 0.06	rs = -0.08, p = 0.22
Leg MD (n = 318)	rs = -0.02, p = 0.68	rs < 0.01, p = 0.94	rs = 0.01, p = 0.88	rs = 0.01, p = 0.82	rs = -0.11, p = 0.06	rs = -0.13, p = 0.04*
Arm IMAT (n = 304)	rs = 0.02, p = 0.68	rs = -0.06, p = 0.34	rs = 0.15, p = 0.01*	rs = 0.08, p = 0.17	rs = 0.07, p = 0.24	rs = 0.11, p = 0.07
Leg IMAT (n = 301)	rs = -0.01, p = 0.95	rs = 0.08, p = 0.17	rs = 0.01, p = 0.81	rs = 0.05, p = 0.46	rs = 0.11, p = 0.05*	rs = 0.14, p = 0.02*

1. Spearman correlations of unadjusted and adjusted (race, sex, age, location) vitamin D metabolites and muscle outcomes. *Significantly correlated, $P < 0.05$.
2. FFST, fat-free soft tissue; MCSA, muscle cross-sectional area; MD, muscle density; IMAT, intermuscular adipose tissue

Table 3.5

Spearman Correlation of 12-week change in IMAT and Muscle Outcomes [1]

	Arm IMAT (n = 304)		Leg IMAT (n = 301)	
	Unadjusted	Adjusted (N = 275)	Unadjusted	Adjusted (N = 275)
FFST (n = 300)	rs = 0.20, $p < 0.01$*	rs = 0.22, $p < 0.01$*	rs = -0.03, $p = 0.56$	rs = -0.02, $p = 0.80$
Handgrip (n = 287)	rs = 0.07, $p = 0.26$	rs = 0.06, $p = 0.36$	rs = -0.02, $p = 0.80$	rs = -0.02, $p = 0.68$
Arm MCSA (n = 299)	rs = 0.62, $p < 0.01$*	rs = 0.64, $p < 0.01$*	rs = 0.04, $p = 0.48$	rs = 0.05, $p = 0.37$
Leg MCSA (n = 296)	rs = 0.15, $p = 0.01$*	rs = 0.16, $p = 0.01$*	rs = 0.70, $p < 0.01$*	rs = 0.72, $p < 0.01$*
Arm MD (n = 299)	rs = -0.29, $p < 0.01$*	rs = -0.28, $p < 0.01$*	rs = -0.02, $p = 0.72$	rs = -0.03, $p = 0.62$
Leg MD (n = 296)	rs = -0.01, $p = 0.81$	rs = 0.03, $p = 0.68$	rs = -0.35, $p < 0.01$*	rs = -0.36, $p < 0.01$*

1. Spearman correlations of unadjusted and adjusted (race, sex, age, location) vitamin D metabolites and muscle outcomes. *Significantly correlated, $P < 0.05$.
2. FFST, fat-free soft tissue; MCSA, muscle cross-sectional area; MD, muscle density; IMAT, intermuscular adipose tissue

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CHAPTER 4

SUMMARY AND CONCLUSION

Since the 1600s, the prevalence of vitamin D deficiency and its negative skeletal and musculoskeletal affects in children across various backgrounds has been well documented (Eliot et al 1938; Holick et al 2006; Das et al 2006; Gordon et al 2008; Wolff at al 2008; Mansback et al 2009; Arguelles et al 2009). Although an adequate vitamin D status aids in the development of a healthy skeletal system and the prevention of certain skeletal disorders such as rickets, the role of vitamin D in the growth and development of skeletal muscle in children may also be critically important due to its potential role in functional performance (Foo et al 2009; Ward et al 2009; Valtuena et al 2012) and bone development (Frost et al 1990). In older adults, vitamin D supplementation resulting in an increased serum 25(OH)D has been associated with improved muscle composition (Sorensen et al 1979; Sato et al 2005; Cegila 2009) and strength (Michael et al 2010; Bischoff-Ferrari et al 2010; Dawson-Hughes et al 2010). Whether vitamin D supplementation plays a similar role in muscle metabolism and function in pediatric populations is uncertain. Currently vitamin D intervention trials conducted in adolescents that examine muscle are limited, confined to predominately Caucasian or South Asian female populations. In the study by Ward et al (2010), vitamin D supplementation significantly increased muscle efficiency, as shown by jumping velocity, in post-menarcheal females (mean aged 14 yrs). In the study by El-Hajj

Fuleihan et al (2006), vitamin D supplementation significantly increased total body lean muscle mass in pre-menarcheal females only (mean aged 11 yrs), and not post-menarcheal females (mean aged 14 yrs). The results from these two studies suggest that during this period of rapid growth the muscle of adolescents in the early stages of puberty maybe more sensitive to changes in 25(OH)D. The current study sought to examine how changes in vitamin D metabolites affect changes in muscle parameters in white and black male and female adolescents in the early stages of puberty, where data concerning vitamin D and muscle are limited.

The primary findings from this study indicated that despite significant increases in 25(OH)D following the 12-week vitamin D intervention, changes in 25(OH)D were not related with changes in any of the muscle parameters. Additionally, $1,25(OH)_2D$ significantly increased following the 12-week vitamin D intervention and was positively correlated with changes in arm MCSA. Though iPTH did not change following the 12-week intervention, changes in iPTH, after adjusting for age, race, sex, and location, were significantly and positively correlated with changes in leg MCSA and leg IMAT, and negatively correlated with leg MD. The lack of an association between changes in 25(OH)D and changes in muscle parameters in the current study are contradictory to several vitamin D intervention (Sorensen et al 1979; Sato et al 2005; Bischoff-Ferrari et al 2010; Glerup et al 2000) and cross-sectional (Wicherts et al 2007; Cegila 2009; Bischoff-Ferrari et al 2004; Gerdhem et al 2005) studies in adults, wherein improvements in serum 25(OH)D or high 25(OH)D concentrations were associated with greater muscle function and composition. Such findings in comparison to the current study findings beg

the question, does vitamin D supplementation affect adolescent muscle, and if so is 25(OH)D a sensitive biomarker of the action of vitamin D on muscle in pediatrics as it is in adults?

Whether vitamin D supplementation does indeed affect adolescent muscle can be described by the current study and, to our knowledge, the only two vitamin D intervention trials conduced in adolescents that examine muscl (El-Hajj Fuleihan et al 2006; Ward et al 2010). In the studies by El-Hajj Fuleihan et al (2006) and Ward et al (2010), subjects assigned to a vitamin D intervention group had significant increases in total body FFST and muscle efficiency, respectively, in comparison to placebo. The current study however did not show similar findings in muscle mass or efficiency with vitamin D supplementation, which may be explained by the shorter intervention duration of our intervention, as well as the age, maturation and vitamin D status of the population selected. For example, both the El-Hajj Fuleihan et al the El-Hajj Fuleihan et al (2006) and Ward et al (2010) studies were 12-month vitamin D intervention trials, in comparison to our study duration of 12 weeks. Although shorter by comparison, our 12-week vitamin D intervention showed significant increases in 25(OH)D, $1,25(OH)_2D$, FFST, handgrip, arm and leg MCSA. Due to the rapid period of growth experienced by adolescents in the early stages of puberty (Rogol et al 2000), it is possible within a 12-week time period to have significant changes in muscle parameters.

Age and maturation are other distinguishing factors between each of the study's populations. Findings from the Ward et al (2010) study were produced in an exclusively

post-pubertal adolescent female population (mean age 14 yrs), study were produced in exclusively post-pubertal adolescent females (mean age 14 yrs), whereas the current study consisted of adolescents in the early stages of puberty. Moreover, the associated increase in total body FFST in the El-Hajj Fuleihan et al (2006) study was only significant in pre-pubertal adolescent females, and not post-pubertal. Although the El-Hajj Fuleihan et al (2006)] study had a similar age distribution in comparison to the current study (both had a mean age of 11 yrs), discrepancies in the findings between the two previous vitamin D intervention trials and the current study could be further explained by the vitamin D status of each the study's population. The population group selected in the El-Hajj Fuleihan et al (2006) and Ward et al (2010) studies had considerably lower baseline 25(OH)D concentrations, of 35 $\pm$ 20 nmol/L and 18 $\pm$ 8 nmol/L, respectively, in comparison to the current study baseline 25(OH)D concentration of 70 $\pm$ 19 nmol/L. Vitamin D deficiency has been shown to be associated with muscle weakness (Valtuena et al 2012; Foo et al 2009; Ward et al 2009) and pain (Van der Heyden et al 2004) in adolescents. By improving the vitamin D status of previously vitamin D deficient populations through supplementation, improvements in muscle parameters are more likely to occur and therefore could differ from the current study's findings. Though results vary, all three studies illustrated that vitamin D supplementation does appear to have an effect on adolescent muscle in both deficient and relatively healthy populations. However, whether 25(OH)D is a sensitive biomarker of the action or effect of vitamin D on muscle in pediatrics is uncertain..

To date, there is a lack of evidence that shows 25(OH)D has an influence or is an ideal biomarker of the action of vitamin D on muscle in pediatrics. While the two previous vitamin D intervention trials conducted in adolescents showed a significant increase in 25(OH)D (El-Hajj Fuleihan et al 2006; Ward et al 2010) and $1,25(OH)_2D$)EL-Hajj Fuleihan et al 2006) following supplementation and that vitamin D supplementation positively affects muscle in comparison to (El-Hajj Fuleihan et al 2006; Ward et al 2010), neither study tested whether associations existed between the individual vitamin D metabolites and their respective muscle outcomes. Whether 25(OH)D is a suitable biomarker in adolescents has also been questioned with respect to calcium absorption. A vitamin D intervention trial in white and black adolescent females (N = 105; aged 11-15 yrs) showed that 25(OH)D was not associated with calcium retention. In fact, blacks with lower 25(OH)D status in comparison to whites had significantly higher calcium retention rates (Weaver et al 2008). Furthermore, in the study by Abrams et al (2005), $1,25(OH)_2D$ and iPTH, but not 25(OH)D, were positively correlated with calcium retention. Outside of being an ideal marker of dietary and cutaneous vitamin D inputs, both studies including the current study question the exact role 25(OH)D plays in early adolescences.

Unlike 25(OH)D, changes in $1,25(OH)_2D$ were positively correlated with changes in arm MCSA. With a short half-life of 4-8 hours (Wootton 2005), $1,25(OH)_2D$ is not traditionally considered a reliable biomarker of vitamin D status (Webb et al 1990; Cranney et al 2007). Therefore, in addition to being a difficult assay to conduct, $1,25(OH)_2D$ is often not assessed in many vitamin D research trials. However the limited

data available still suggest a link between $1,25(OH)_2D$ and muscle (Somjen et al 2005). A hormone, $1,25(OH)_2D$ has been shown to display other functions outside of calcium regulation (Deluca HF 2004; Hewison et al 2000; Lips P 2006), including a potential role in muscle growth and development (Hewison et al 2000; Bischoff-Ferrai et al 2001). In the current study, baseline $1,25(OH)_2D$ concentrations were significantly higher in black males who were also stronger and had larger muscles in comparison white males. Similar associations of a higher $1,25(OH)_2D$ (Harris SS 2006) and muscle parameters (Duey et al 1997; Schuttee et al 1984) have been shown in other cross-sectional studies in non-Hispanic blacks. Due to the rapid period of growth associated with adolescents in the early stages of puberty (Rogol et al 2000), the active form of vitamin D may be needed in much higher amounts in comparison to adults. Although the synthesis of $1,25(OH)_2D$ is tightly regulated and largely dependent upon serum calcium and iPTH concentrations in adults (Deluca HF 2004; Lips P 2006; Murayama et al 1999) perhaps in early adolescents the synthesis of $1,25(OH)_2D$ is less dependent upon calcium and iPTH concentrations and more on the body's overall metabolic demand during growth. Such a theory is further supported by the study's significant increase in $1,25(OH)_2D$ over the 12-week intervention trial in a calcium sufficient population, the study's lack of statistical change in iPTH, and the associated decrease in 25(OH)D with increasing age in early adolescents (Willis et al 2007; Tolppanen et al 2012). However, currently there is no research addressing the conversation rates of 25(OH)D into $1,25(OH)_2D$ in early adolescents. Therefore $1,25(OH)_2D$ should be monitored more closely in future vitamin D research trials in order to understand the exact role of $1,25(OH)_2D$ plays in early adolescence and its potential role in muscle growth and development.

Despite the present study's significant increase in 25(OH)D and $1,25(OH)_2D$, iPTH did not statistically change during the 12-week intervention trial, which could be explained by the study's predominately vitamin D sufficient baseline population (~70 nmol/L). Although a negative correlation between iPTH and 25(OH)D is shown in other pediatric populations (Guillemant et al 1999; El-Hajj Fuleihan et al 2006; Gordon et al 2004; Abrams et al 2005), findings are mixed whether a maximal suppression of iPTH exists in this population. Studies in male adolescents (aged 11-16 yrs) (Gordon et al 2004) and older adults (Hill et al 2010) have shown that a maximal suppression of iPTH is associated with a 25(OH)D concentration of 60 to 80 nmol/L; however, other studies have shown a lack of an inflection point between iPTH and 25(OH)D (Hill et al 2010). Whether there is a 25(OH)D concentration associated with a maximum suppression of iPTH in a pediatric population is still unclear; however, it is evident in cross-sectional studies (Hui et al 2003; Bell et al 1991; Bell et al 1985), intervention trials (Yamaguchi et al 1987; Winer et al 2010; Waller et al 2005), and in the current study that iPTH plays a role in the growth and development of adolescent bone, and potentially muscle.

This study is one of the first studies to report these positive correlations between iPTH and muscle in adolescents. Baseline values of iPTH, adjusted for age, race, sex, and location, were positively correlated to baseline FFST, arm and leg MCSA, and leg IMAT. Although changes in iPTH were not statistically significant following the 12-week vitamin D intervention, changes in iPTH, after adjusting for covariates, were positively correlated with changes in leg MCSA and leg IMAT, and negatively correlated with leg MD; suggesting an overall bigger and fatter muscle with higher iPTH levels. Based upon

adult data, such findings would suggest an impaired muscle function and lower quality (Buford et al 2012; Hilton et al 2008) in the study's population in the study's population. In addition, it has also been documented in adults that increases in IMAT are correlated with decreases in insulin sensitivity and the prevalence of diabetes (Goodpaster et al 2000; Mijovic-Gacic et al 2008). Based solely upon adult data, increases in IMAT, as a result of an increase in serum iPTH, would result in certain negative health outcomes. However, current data supporting this association between IMAT and these metabolic disorders are greatly limited in pediatric populations. To our knowledge, there are no studies that address the effect of IMAT on muscle function and quality in adolescents; whether it elicits a negative effect or is part of the normal growth process. Similarly, it is also important to note that a transient diabetic-like state of insulin resistance occurs during the normal growth process in adolescents (Goran et al 2011; Guzzaloni et al 2002). Therefore it is possible that transient insulin resistant state and this iPTH-induced increase in IMAT coincide with each other to promote muscle growth and development in early adolescences. Such a notion would suggest that increases in iPTH resulting in increase in IMAT are potentially beneficial to adolescent muscle, either acting as a switch for the insulin resistant state seen in early adolescents or potentially as a possible immediate source of dense energy. More research is needed in order to understand this role of iPTH and IMAT in adolescent muscle and their potential interaction during the transient insulin resistant state of the growth process

Overall findings from the current study suggest that an environment of higher serum iPTH is associated with greater changes in muscle in adolescents in the early

stages of puberty. It is important to note that higher concentrations of serum iPTH result in an increase in 25-hydroxyvitamin D-1α-hydroxylase activity (Somjen et al 2005), which subsequently increases serum $1,25(OH)_2D$. Whether an environment of higher iPTH or an increase in $1,25(OH)_2D$ synthesis is responsible for this change in muscle parameters is unclear; however, future vitamin D research in adolescents should include these metabolites in the design.

The current study admittedly had some limitations. There were a significant number of subjects with movement in pQCT-derived muscle outcomes that led to exclusion of their muscle data (arm, n = 46; leg, n = 40). Secondly, the length of the intervention trial may not have been of sufficient duration. Though there were significant changes in serum and muscle outcomes following the 12-week intervention, a longer intervention trial may have generated larger changes in outcome variables.

The strengths of the current study are numerous. This study was the first clinical vitamin D intervention trial that examined muscle outcomes in both black and white male and female adolescents in the early stages of puberty. The 4,000 IU vitamin dose safely generated large changes in 25(OH)D. Furthermore, the study's daily supplementation appears to be the most effective dosing frequency (Saadi et al 2007; Chel et al 2008; Muir et al 2011) and cholecalciferol (vitamin D_3) appears to be superior in raising and maintaining 25(OH)D levels in comparison to ergocalciferol (vitamin D_2) (Klinke et al 1954; Romagnoli et al 2008; Heaney et al 2011). Due to increased nutrient needs (Rogol et al 2000), adolescents in the early stages of puberty were ideal our intervention trial.

The study's two recruitment sites increased generalizability of our findings and the population diversity. In addition, DXA (Elia et al 2000; Bridge et al 2011), pQCT (Sazbo et al 2011; Briggs et al 2010), handgrip dynamometry (Rantanen et al 1999; Wind et al 2010), and RIA (Hollis et al 1993) are all reliable markers of muscle and serum vitamin D status. To our knowledge, this study was the first study to measure IMAT through the use of pQCT in a pediatric population. Though a relatively novel approach in pediatric populations, the assessment of IMAT by way of pQCT has been previously documented in adult populations (Goodpaster et al 2000; Milikovic-Gacic et al 2008; Milikovic-Gacic et al 2009). Validation of this assessment of IMAT was further made by the study's significant correlations between IMAT and muscle outcomes. Future research is warranted in order to further validate this measurement in pediatric populations.

In summary, both iPTH and $1,25(OH)_2D$, but not 25(OH)D, were positively correlated with changes in muscle parameters. If vitamin D supplementation does indeed have an affect on muscle in a relatively healthy adolescent population, it is not reflected by changes in serum 25(OH)D but rather $1,25(OH)_2D$ and iPTH. Based upon the current study and previous adolescent trials (Weaver et al 2008; Abrams et al 2005) 25(OH)D appears to be a suitable biomarker of vitamin D input rather than of effect in adolescents. Although the lack of change in iPTH could be explained by our vitamin D sufficient population, due to the potential interaction of iPTH in adolescent growth (Hui et al 2003; Bell et al 1991; Bell et al 1985; Yamaguchi et al 1987; Winer et al 2010; Waller et al 2005), a minimal concentration of iPTH may be required for optimal growth. However, other muscle metabolites such as IGF-1 (Breen et al 2011), myostatin (Garcia et al 2011),

and other myokines (Hamrick 2011) have also shown to be influenced by vitamin D, suggesting an indirect effect of vitamin D on muscle outcomes. Additional research is needed to clarify the involvement of $1,25(OH)_2D$ and iPTH, in addition to 25(OH)D, on muscle in both male and female adolescent populations, and whether other metabolites are involved in this relationship.

In conclusion, this vitamin D intervention trial in adolescents in the early stages of puberty indicated that a relationship between 25(OH)D and muscle strength, MD, MCSA, IMAT, and FFST did not exist. However this study was the first vitamin D intervention trial to provided important insights into the role of iPTH and $1,25(OH)_2D$ in the development of muscle during this rapid phase of growth. Results from this study encourage future mechanistic studies examining the role of iPTH and $1,25(OH)_2D$ in the growth and development of adolescents.

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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

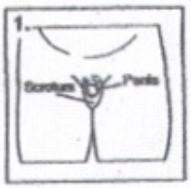
Lewis, Richard D.

SEXUAL MATURATION QUESTIONNAIRE (BOYS)

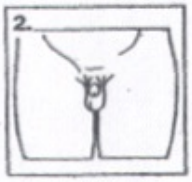
Subject ID#: _____
Date: _____

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.

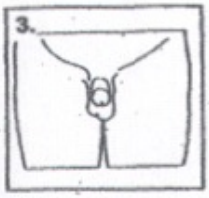


2.



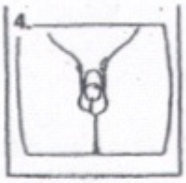
The Scrotum has lowered a bit and the Penis is a little larger.

3.



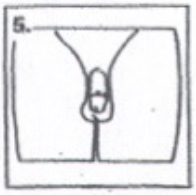
The Penis is longer the Scrotum is larger.

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.

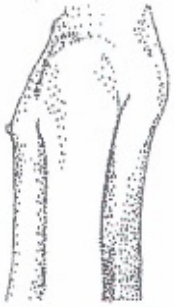
S.J.C. Taylor et al. Pediatric and Perinatal Epidemiology 2001. 15, 88-94.

Lewis, Richard D.

SEXUAL MATURATION QUESTIONNAIRE (GIRLS)

Subject ID#: _____
Date: _____

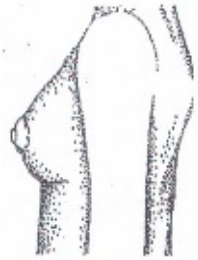
We need to find out what stage of sexual development you are in. Please look at the pictures and circle the one that looks most like you now.



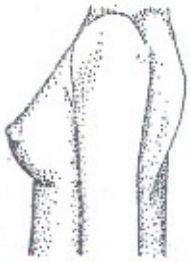
Stage 1: Elevation of papilla only.

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)

APPENDIX E

3-Day physical activity recall

Activities Scale

This purpose of this questionnaire is to estimate the amount of physical activity that you perform. The name of each day (Tuesday, Monday, and Sunday) that you will describe is located in the top right hand corner of each time sheet.

1. For **each** time period, write in the activity number that corresponds to the **main** activity you actually performed during that particular time period.

2. Then rate how physically **hard** each activity was. Place a "✓" in the timetable to indicate one of the following intensity levels for each activity.

• Light - **Slow breathing, little or no movement.**




•Moderate - **Normal breathing and some movement.**




• Hard - **Increased breathing and moderate movement.**




• Very Hard - **Hard breathing and quick movement.**




Activity Numbers

Eating
1.) Eating a meal
2.) Snacking

Work
3.) Working (e.g., part-time job, child care) **(list)**_____
4.) Doing house chores (e.g., vacuuming, dusting, washing dishes, animal care, etc.)
5.) Yard Work (e.g., mowing, raking)

After School/Spare Time/ Hobbies
6.) Church
7.) Hanging around
8.) Homework
9.) Listening to music
10.) Marching band/flag line/drill team
11.) Music lesson/playing instrument
12.) Playing video games/surfing Internet
13.) Reading
14.) Shopping
15.) Talking on phone
16.) Watching TV or movie

Transportation
17.) Riding in a car/bus
18.) Travel by walking
19.) Travel by bicycling

Sleep/Bathing
20.) Getting dressed
21.) Getting ready (hair, make-up, etc.)
22.) Showering/bathing
23.) Sleeping

School
24.) Club, student activity
25.) Lunch/free time/study hall
26.) P. E. Class
27.) ROTC
28.) Sitting in class

Physical Activities and Sports
29.) Aerobics/aerobic dancing
30.) Basketball
31.) Bicycling
32.) Bowling
33.) Calisthenics(i.e., jumping jacks, sit-ups)
34.) Cheerleading
35.) Dancing (social, recreational)
36.) Dancing (ballet, jazz, modern, tap)
37.) Field hockey
38.) Frisbee
39.) Golf
40.) Horseback riding
41.) Ice/roller skating
42.) Jogging/running
43.) Karate/judo/martial arts/ self-defense
44.) Rollerblading
45.) Skateboarding
46.) Soccer
47.) Softball/baseball
48.) Stationary exercise machines (e.g., cycle, ski machine, stair climber, treadmill)
49.) Street hockey
50.) Swimming, water exercise
51.) Tennis
52.) Volleyball
53.) Walking (briskly)
54.) Weight/circuit training
55.) Gymnastics/tumbling
56.) Kickboxing/Tae Bo
57.) Track and field
58.) Trampoline
59.) Other_____

Sample activity time sheet:

The table below shows the correct way to fill out the activity time sheets.
Note that only **one** intensity level is checked for each activity.

	Activity Number	Light	Moderate	Hard	Very Hard
7:00-7:30	22	✓			
7:30-8:00	21	✓			
8:00-8:30	18		✓		
8:30-9:00	28	✓			
9:00-9:30	28	✓			
9:30-10:00	26			✓	
10:00-10:30	26			✓	

Put a "✓" to rate the intensity of each activity.

SUNDAY

Write activity numbers in this column.

		Activity Number	Light	Moderate	Hard	Very Hard
breakfast	7:00-7:30					
	7:30-8:00					
	8:00-8:30					
	8:30-9:00					
	9:00:9:30					
	9:30-10:00					
	10:00-10:30					
	10:30-11:00					
	11:00-11:30					
	11:30-12:00					
lunch time	12:00-12:30					
	12:30-1:00					
	1:00-1:30					
	1:30-2:00					
	2:00-2:30					
	2:30-3:00					
	3:00-3:30					
	3:30-4:00					
	4:00-4:30					
	4:30-5:00					
	5:00-5:30					
supper time	5:30-6:00					
	6:00-6:30					
	6:30-7:00					
	7:00-7:30					
	7:30-8:00					
	8:00-8:30					
	8:30-9:00					
	9:00-9:30					
	9:30-10:00					
	10:00-10:30					
evening	10:30-11:00					
	11:00-11:30					
	11:30-12:00					

Put a "✓" to rate the intensity of each activity.

MONDAY

Write activity numbers in this column.

		Activity Number	Light	Moderate	Hard	Very Hard
before school	7:00-7:30					
	7:30-8:00					
during school	8:00-8:30					
	8:30-9:00					
	9:00:9:30					
	9:30-10:00					
	10:00-10:30					
	10:30-11:00					
	11:00-11:30					
lunch time	11:30-12:00					
	12:00-12:30					
	12:30-1:00					
	1:00-1:30					
	1:30-2:00					
	2:00-2:30					
	2:30-3:00					
	3:00-3:30					
after school	3:30-4:00					
	4:00-4:30					
	4:30-5:00					
	5:00-5:30					
supper time	5:30-6:00					
	6:00-6:30					
	6:30-7:00					
	7:00-7:30					
	7:30-8:00					
	8:00-8:30					
	8:30-9:00					
	9:00-9:30					
	9:30-10:00					
	10:00-10:30					
evening	10:30-11:00					
	11:00-11:30					
	11:30-12:00					

Put a "✓" to rate the intensity of each activity.

TUESDAY

Write activity numbers in this column.

		Activity Number	Light	Moderate	Hard	Very Hard
before school	7:00-7:30					
	7:30-8:00					
during school	8:00-8:30					
	8:30-9:00					
	9:00:9:30					
	9:30-10:00					
	10:00-10:30					
	10:30-11:00					
	11:00-11:30					
lunch time	11:30-12:00					
	12:00-12:30					
	12:30-1:00					
	1:00-1:30					
	1:30-2:00					
	2:00-2:30					
	2:30-3:00					
	3:00-3:30					
after school	3:30-4:00					
	4:00-4:30					
	4:30-5:00					
	5:00-5:30					
supper time	5:30-6:00					
	6:00-6:30					
	6:30-7:00					
	7:00-7:30					
	7:30-8:00					
	8:00-8:30					
	8:30-9:00					
	9:00-9:30					
	9:30-10:00					
	10:00-10:30					
evening	10:30-11:00					
	11:00-11:30					
	11:30-12:00					