VITAMIN D, INSULIN SENSITIVITY, AND CARDIOMETABOLIC RISK IN CHILDREN

by

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(Under the Direction of RICHARD D. LEWIS)

ABSTRACT

Background: The childhood obesity epidemic has increased the prevalence of cardiometabolic disease and pre-diabetes prevalence. Vitamin D's role in diabetes and cardiometabolic disease is an emerging, extraskeletal function that has been explored via mechanistic research, as well as observational and intervention studies, predominantly in adults.

Objective: To review the relevant literature, report the effects of vitamin D supplementation on glycemia and insulin sensitivity in apparently healthy children, and examine the cross-sectional relationship between serum vitamin D and cardiometabolic risk in overweight children.

Methods: The intervention trial includes black and white early pubertal (N = 320) males (aged 10 to 13 years) and females (aged 9 to 12 years) who participated in a 12-week randomized, triple masked, multi-site, five-dose (0, 400, 1,000, 2000, and 4,000 IU vitamin D_3) placebo-controlled trial. The cross-sectional study includes 222 sedentary, overweight children (aged 7-11 years, 58% female, 58% black, 86% obese).

Anthropometry, body composition, and biochemical indices related to serum vitamin D status, glucose-insulin homeostasis, and cardiometabolic risk were examined in both studies.

Results: The vitamin D intervention did not significantly alter glycemia or insulin sensitivity over 12-weeks. Glucose, insulin, and insulin resistance increased, while insulin sensitivity decreased, over the 12-weeks, likely attributable to organic pubertal maturation phenomena. Lower serum vitamin D concentrations were associated with multiple cardiometabolic risk factors, insulin resistance, and adipokines and associated with an increase in cardiometabolic disease risk, however, model adjustment with visceral adipose tissue attenuated all of these relationships.

Conclusions: We report novel findings from the first pediatric vitamin D intervention to examine effects on glycemia and insulin sensitivity. In this trial, the apparently healthy, vitamin D sufficient status of our children, are key facets that may have contributed to our null finding. Furthermore, we may have avoided the glucose-insulin homeostatic alterations induced by pubertal growth if we had examined a pre- or post-pubertal population. Our cross-sectional findings highlight the important mediating role of visceral adiposity in the vitamin D-cardiometabolic disease relationship. Future, rigorously designed trials are warranted to elucidate the causal effect of vitamin D on cardiovascular disease and diabetes, with particular attention paid to pediatric populations with higher cardiometabolic risk and vitamin D insufficiency.

INDEX WORDS: 25-hydroxyvitamin D, vitamin D, insulin sensitivity, diabetes, cardiometabolic, obesity, children, pediatric

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Soli Deo Gloria

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CHAPTER 1

INTRODUCTION

The prevalence of obesity in the US has significantly increased over the past three decades in children and adolescents,[1] contributing to a rise in the incidence of diseases previously associated with adulthood, such as metabolic syndrome, pre-diabetes, and type 2 diabetes mellitus (T2DM).[2, 3] In conjunction, hypovitaminosis D exists in a significant percentage of the US pediatric population,[4] and overweight status and abdominal adiposity are associated with lower circulating 25-hydroxyvitamin D [25(OH)D] levels.[5] One of the emerging extraskeletal roles of vitamin D currently under investigation is the role of vitamin D in heart disease and diabetes. Lower circulating vitamin D levels may increase risk for glucose and insulin homeostatic abnormalities and cardiometabolic disease.[6, 7] Biologically plausible mechanisms for the role of vitamin D in diabetes and cardiovascular disease include vitamin D's involvement in insulin secretion[8] and action,[9] inflammatory processes,[10] heart proteomics, structure,[11] and function,[12] the renin-angiotensin-aldosterone system,[13] and atherosclerotic progression.[14] Observational studies in children[15] and adults[16, 17] demonstrate inverse associations between 25(OH)D concentrations and adiposity, insulin resistance, and cardiometabolic risk, and prospective studies in adults present vitamin D as an important modifier of diabetes[18] and cardiovascular disease risk.[19] With the rise in obesity and associated comorbidities and healthcare costs, cost-effective public health prevention measures are needed. Vitamin D supplementation is a cheap, safe way to achieve 25(OH)D sufficiency. In the latest

Dietary Reference Intakes report for calcium and vitamin D, the Institute of Medicine dubbed the roles of vitamin D in diabetes and cardiovascular disease to be "hypotheses of emerging interest" and pointed to the paucity of rigorous intervention trials to establish causality and dose-response relationships.[20] Vitamin D supplementation trials to date, conducted almost exclusively in adults, have utilized heterogeneous study designs and provide mixed but convincing evidence for the role of vitamin D in cardiometabolic disease and insulin sensitivity.[21] One trial in adolescents[22] has shown improvements in cardiovascular outcomes, but no supplementation trials have examined the effect of vitamin D on cardiometabolic risk or insulin sensitivity in children. Thus, future research is needed to elucidate the role of vitamin D in cardiovascular disease and diabetes, particularly utilizing well-designed supplementation trials in understudied populations like children.

In Chapter 2 of this document is a comprehensive review of the pertinent literature to date on vitamin D, insulin sensitivity, and cardiometabolic disease. Chapter 3 presents the first manuscript, which reports the results from the first pediatric vitamin D supplementation trial to examine insulin sensitivity outcomes, analyzing data from the NIH-funded, multi-site, dose-response Georgia, Purdue, and Indiana University School of Medicine (GAPI) vitamin D trial. The second manuscript in Chapter 4 examines the cross-sectional relationship between serum vitamin D and cardiometabolic risk in overweight children, focusing on the role of robust measures of adiposity. Finally, chapter 5 includes a summary of the findings from the aforementioned clinical trial and cross-sectional data in light of the cumulative evidence from the literature and provides direction for future research.

CHAPTER 2

LITERATURE REVIEW

Overview

The following review of the literature provides background information on and a delineation of the roles of vitamin D in diabetes and cardiometabolic disease during pediatric years. The current pediatric obesity prevalence rates and trend over time are described, as well as the concurrent increase in associated comorbid conditions, such as pre-diabetes, type 2 diabetes mellitus (T2DM), and metabolic syndrome. Next, since the extraskeletal role of vitamin D in diabetes and cardiometabolic disease is the focus of this dissertation, background information on the sources of vitamin D, most recent dietary recommendations for vitamin D, and the challenges regarding measurement methodologies and ascertaining estimates of hypovitaminosis D are covered.

The next section delves into the basic science and human data regarding vitamin D, diabetes, and cardiometabolic outcomes, but first, the dynamic and static measures of insulin sensitivity are detailed, since these are the outcomes reported in the animal and human research. The proposed mechanisms from animal and cellular data will be explored before covering the current observational research available in children and adults; cross-sectional, case-control, and prospective cohort studies in the area of vitamin D, diabetes, and heart disease are reviewed. The current vitamin D supplementation trials that examine diabetes and cardiometabolic outcomes are thoroughly detailed, before providing the cumulative evidence of all human studies from recent systematic reviews

and meta-analyses. Finally, a summary of the literature review is included, along with a description of current gaps in the literature and recommendations for future research.

Pediatric Obesity

Healthy People 2010 called for a reduction in the percentage of children and adolescents who are obese[23], however, minimal progress has been made to achieve this goal, so it has been carried over as a Healthy People 2020 goal.[24] Nationally representative data from the most recent National Health and Nutrition Examination Survey (NHANES) survey 2007-2008 reveals that 17 percent of children and adolescents aged two through 19 years are obese (defined as a BMI-for-age $\geq 95^{th}$ percentile), while 32 percent are overweight (defined as a BMI-for-age $\geq 85^{th}$ percentile) or obese.[25] NHANES data also demonstrate the drastic increase over the past three decades in pediatric overweight and obesity, with obesity prevalence increasing from five to 10 percent in children aged two to five, from seven to 20 percent in children aged six to 11, and from five to 18 percent among adolescents aged 12 to 19 years.[1] Furthermore, since 1999-2000, a significant increase has been observed in boys ages six to 19 for the highest BMI cut point (BMI-for-age $\geq 97^{th}$ percentile), demonstrating that the severity of obesity is worsening.[25] Overweight and obesity during pediatric years increase the risk for obesity during adulthood.[26]

Cardiometabolic Disease and Diabetes in Children

Atherosclerotic cardiovascular disease and diabetes are the first and seventh leading causes of death in US adults, respectively, however, the pathology and risk factors responsible for the development of these diseases commence during childhood.[27] Pediatric obesity can lead to myriad comorbid conditions, including dyslipidemia, insulin

resistance, hyperinsulinemia, hypertension, obstructive sleep apnea syndrome, nonalcoholic fatty liver disease, polycystic ovary syndrome, and musculoskeletal disorders.[28] The community-based Bogalusa Heart Study found that approximately 40 percent of obese children aged five to 17 years had at least two cardiovascular disease risk factors (e.g. dyslipidemia, hyperinsulinemia, hypertension),[29] and The National Heart, Lung, and Blood Institute Growth and Health Study observed that overweight status in girls was significantly associated with increased blood pressure, high-density lipoprotein cholesterol (HDL-C), and triglyceride levels.[30] A prospective cohort study of American Indian children reports sobering findings that obesity, glucose intolerance, and hypertension during childhood are strongly associated with increased rates of premature death (death before 55 years of age).[31]

In 2010, 25.8 million and 79 million US adults suffered from diabetes or pre-diabetes, respectively.[32] Globally, the total number of people with diabetes for all age-groups was 171 million in the year 2000; this figure is expected to rise to 366 million people by 2030.[33] Overall, sixteen percent of US adolescents from NHANES 2005-2006 met pre-diabetes criteria; overweight and obese teenagers had higher pre-diabetes prevalence values of 18 and 30 percent, respectively.[2] Estimates of T2DM during pediatric years are lacking, however, silent T2DM was identified in four percent of obese adolescents in one multiethnic cohort study.[34] In obese youth, severe obesity, pre-diabetes, and the African American race are predictors of T2DM development.[35] When conducting research in pre- and peri-pubertal youth, it is important to note that several studies confirm the phenomenon of transient insulin resistance during the pubertal transition in both sexes regardless of adiposity status.[36, 37]

Although a standard pediatric definition has not been developed,[38] metabolic syndrome represents a clustering of five cardiometabolic factors (elevated triglyceride concentrations, low HDL-C, hypertension, hyperglycemia, and abdominal adiposity) that increase the risk of developing T2DM and cardiovascular disease. Prevalence of metabolic syndrome has increased concurrently with the childhood obesity epidemic. NHANES 1988-1994 data found the prevalence of metabolic syndrome in adolescents to be four percent overall, and 29 percent in obese adolescents,[3] while more recent NHANES 2001-2006 data reports an overall prevalence of nine percent, with approximately half of all participants having at least one risk factor.[39] Obese children with insulin resistance but without metabolic syndrome demonstrate 10 times higher high-sensitivity C-reactive protein than controls, as well as higher fibrinogen, interleukin-6, and plasminogen activator inhibitor-1; these findings demonstrate that childhood obesity is associated with a systemic pro-inflammatory and pro-thrombotic state even before metabolic syndrome comorbidities are clinically detectable.[40]

Prevention of cardiometabolic disease and diabetes is vital since a plethora of complications, such as heart disease, myocardial infarction, stroke, blindness, kidney disease, limb amputations, and pregnancy complications, can ultimately result.[32] The best prevention method is achieving and maintaining an active lifestyle and healthy weight by consuming a variety of nutrient-dense foods in moderation. The Diabetes Prevention Program, a multi-site clinical trial utilizing lifestyle intervention methods in over 3,000 adults with pre-diabetes, resulted in a five to seven percent weight loss, which significantly reduced the incidence of T2DM by 58 percent.[41] Specific dietary factors, may also play a key role in the prevention and treatment of chronic disease. Although its

efficacy as a preventative or treatment method requires further research, vitamin D has been repeatedly associated with and presented as an important modifier of risk for cardiometabolic disease and diabetes in the research literature.[6, 42]

Vitamin D: Background, Sources, and Recommendations

Vitamin D is a fat-soluble vitamin and pro-hormone which is converted to the circulating 25(OH)D form in the liver and subsequently converted 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] in the kidney; the latter compound is the active metabolite, which acts as a hormone in a variety of body tissues. Through the endocrine actions of 1,25$(OH)_2$D, vitamin D promotes calcium absorption in the gut and enables normal mineralization of bone by maintaining adequate serum calcium and phosphate levels, preventing rickets in children, osteomalacia in adults, and together with calcium, protecting older adults from osteoporotic fracture.[20] Through autocrine mechanisms, which account for more than 80 percent of the metabolic utilization of daily vitamin D, 1,25$(OH)_2$D alters gene expression through stimulatory and inhibitory actions.[43] Aside from renal cells, many cells types in the body possess the vitamin D receptor (VDR), and some cells are able to convert 25(OH)D to 1,25$(OH)_2$D.[20]

Vitamin D can be cutaneously synthesized from ultraviolet B radiation, which converts 7-dehydrocholesterol in the skin to pre-vitamin D_3, which in turn, becomes vitamin D_3. Vitamin D is found in few natural (e.g. fatty fish) and fortified food sources (e.g. milk, cereal), in the form of vitamin D_2 and vitamin D_3, the latter of which is derived from natural animal sources. Vitamin D_3 is considered superior to D_2 because the former is more bioavailable, more efficient in raising 25(OH)D concentrations, and possesses a higher affinity for plasma vitamin D-binding proteins.[43, 44] Serum 25(OH)D is

recognized as the best functional indicator of vitamin D status because its concentration captures cutaneous, dietary, and supplemental sources of vitamin D and has a relatively long circulating half-life of 15 days, unlike the biologically active metabolite $1,25(OH)_2D$, which has a short half-life of 15 hours and whose levels are tightly regulated by parathyroid hormone (PTH), calcium, and phosphate.[45]

After numerous scholarly articles requested an update of the 1997 vitamin D dietary intake recommendations,[46, 47] the Institute of Medicine (IOM) was charged by the US and Canadian governments to review the pertinent research data and identify evidence-based Dietary Reference Intakes (DRIs) for calcium and vitamin D. Although a variety of potential indicators of health outcomes, including cardiovascular diseases, hypertension, T2DM, and metabolic syndrome, were examined for nutrient adequacy for vitamin D by the IOM, the available research evidence was judged equivocal and inadequate in terms of causality or a dose-response relationship. The resulting IOM 2010 recommendations were based upon skeletal health indicators and based on the assumption of minimal sun exposure and calcium adequacy.[20] **Table 2.1** outlines the new vitamin D DRIs. Although not specifically within the charge to the IOM committee, the Food and Nutrition Board also identified 25(OH)D cut-points relative to bone health. They concluded that persons are at risk of deficiency at a serum 25(OH)D level of less than 30 nmol/L and identified serum 25(OH)D levels corresponding to the Estimated Average Requirement (EAR) and Recommended Dietary Allowance (RDA) of 40 nmol/L and 50 nmol/L, respectively.[20]

Since their release, these cumulative recommendations by the IOM have received significant criticism. Many well-respected scholars in the field of vitamin D believe that

skeletal research alone, particularly regarding fracture incidence reduction, clearly points to a 25(OH)D cut-point of 75 nmol/L for sufficiency.[48]

Table 2.1 Vitamin D 2010 Dietary Reference Intakes by Age[20]

Age	AI	EAR	RDA	UL
0-6 months	400 IU	---	---	1,000 IU
6-12 months	400 IU	---	---	1,500 IU
1-3 years	---	400 IU	600 IU	2,500 IU
4-8 years	---	400 IU	600 IU	3,000 IU
9-70 years	---	400 IU	600 IU	4,000 IU
> 70 years	---	400 IU	800 IU	4,000 IU

AI = Adequate Intake; EAR = Estimated Average Requirement; RDA = Recommended Dietary Allowance; UL = Tolerable Upper Intake Level; IU = International Units

Furthermore, the 600 IU RDA is criticized due to its inability, without appreciable solar input, to increase 25(OH)D levels to 25 nmol/L, much less to the 50 nmol/L committee recommendation. This is because several studies indicate a response increment of 1.75-2.5 nmol/L/100 IU of vitamin D.[49] This is further exacerbated by the fact that 600 IU/day barely produces detectable changes in 25(OH)D concentrations in those who are overweight or obese, which describes the majority of US adults and one third of children and adolescents.[48] Finally, the UL of 4,000 IU/day has been criticized as overly conservative since the report acknowledges that intakes up to 10,000 IU/day are likely safe based on work by Hathcock et al.[50]

Vitamin D: Measurement and Status

Ascertaining prevalence estimates of vitamin D insufficiency, using 25(OH)D concentrations as the marker of status, is challenging due to diverse assay methodologies. The major assay methodologies currently in use include radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), high-performance liquid chromatography (HPLC), liquid chromatography tandem mass spectrometry (LC-MS), and random access

automated assays based on chemiluminescence (RAAA). Each method has its strengths and weaknesses, including the ability to detect 25(OH)D_2 and D_3 metabolites equally and to differentiate between other, impertinent, metabolites (e.g. 24,25$(OH)_2$D) and inactive epimers (e.g. 3-epi-25(OH)D_3). Direct detection methodology, including HPLC followed by UV detection and LC-MS, are considered the candidate reference methods by many, however, HPLC is cumbersome and sample throughput is slow, while LC-MS equipment and maintenance are expensive, and the use and interpretation are complex.[51] The best commercial assay appears to be the DiaSorin RIA, since it detects D_2 and D_3 metabolites equally and received the highest accuracy score via the Vitamin D Quality Assessment Scheme (DEQAS);[52] furthermore, high correlations have been noted between Diasorin RIA and LC-MS.[53, 54]

Based on NHANES 2003-2006 data, the average vitamin D intake tends to be less than the RDA in adults and children even though 53 percent of the US population report use of any dietary supplement, and 37 percent reporting use of a vitamin D supplement.[55] Use of vitamin D supplements has increased over time in adults and remained stable in the pediatric population,[55] although only four percent of one to 21 year olds had taken 400 IU of vitamin D via supplementation in the previous 30 days.[56] The average vitamin D intake from the diet and all sources in youth aged one to 18 years is 328 IU and 290 IU in boys and girls, respectively.[55] Although average vitamin D intake falls below the RDA, mean serum 25(OH)D concentrations are above 50 nmol/L; this discrepancy suggests that the majority of Americans are attempting to meet their vitamin D needs via sun exposure instead of through foods and supplements.[57]

Overall, approximately one-quarter of the US population has 25(OH)D levels less than 50 nmol/L, placing them at risk for vitamin D inadequacy based on the 2010 IOM cut-points.[58] Ascertaining prevalence values for hypovitaminosis D is challenging due to diverse definitions of vitamin D deficiency. In the pediatric literature, values corresponding to vitamin D deficiency range from less than 12.5 to 30 nmol/L, while those for insufficiency range from less than 25 to 80 nmol/L; hence, hypovitaminosis D estimates yielded from these studies range from one to 78 percent.[59] Although the recent 2010 IOM DRI report for vitamin D concluded that the majority of Americans are receiving adequate amounts of vitamin D, this is based on 25(OH)D cutoffs that may not be applicable to pediatric populations or extraskeletal roles of vitamin D. Studies, such as the NIH-funded multi-site dose-response Georgia, Purdue, and Indiana University School of Medicine vitamin D trial (GAPI Trial), are currently underway to help define vitamin D sufficiency in pediatric populations. Until such definitions are available, extrapolation from the aforementioned adult 25(OH)D cutoffs has been used for pediatric studies, demonstrating that hypovitaminosis D exists in a significant number of children and adolescents. Recent studies utilizing nationally representative NHANES data, demonstrate that nine percent and 61 percent of children and adolescents have 25(OH)D levels less than 37 nmol/L and 72 nmol/L, respectively, representing 50.8 million US youth.[56] In children aged one to 11 years, 18 percent have serum vitamin D levels less than 50 nmol/L.[4] Vitamin D insufficiency has been observed in both the northern[60-62] and southern[63-66] US pediatric samples.

The following factors are associated with lower 25(OH)D levels in the pediatric literature: older age, female gender, winter season, northern latitude, black or Mexican-

American race/ethnicity, not using vitamin D supplementation, drinking milk less than once per week, spending greater than four hours per day using the television, video, or computer, elevated parathyroid hormone concentrations, overweight status, and abdominal adiposity.[4, 56, 59, 67] Adiposity is highlighted in the IOM 2010 DRI vitamin D report as a condition of interest because serum vitamin D concentrations are inversely associated with BMI percentile, waist circumference, total fat mass, body fat percentage, visceral adipose tissue, and subcutaneous abdominal adipose tissue in youth[52] and adults. Hypovitaminosis D prevalence is significantly increased in obese pediatric populations, particularly in minority populations, compared to their normal-weight counterparts. Anywhere from 32 to 78 percent of obese adolescents, depending on the ethnicity studied, and 57 percent of obese African-American children have 25(OH)D levels below 50 nmol/L.[5, 68-70]

Intervention trials in children demonstrate the ability of vitamin D supplementation to significantly increase 25(OH)D concentrations.[70, 71] This increase is potentiated by a greater degree of vitamin D insufficiency at baseline, which in research is often achieved by testing during wintertime or in high-risk groups, in which 25(OH)D values are at their nadir. In contrast, obesity blunts the serum vitamin D response following supplementation, with decreased bioavailability being shown from both cutaneous and dietary sources, possibly resulting from the deposition of vitamin D in body fat compartments.[70, 72, 73]

Insulin Sensitivity Measures

Different research methods exist to quantify insulin sensitivity in animals and humans. The euglycemic hyperinsulinemic clamp (EHC), considered the gold standard

for assessing insulin sensitivity, and insulin suppression tests use intravenous (IV) infusion methods to directly assess insulin-mediated glucose utilization and reflect a stimulated state; however, these methods are labor and time intensive and expensive.[74] The EHC measures the amount of glucose necessary to compensate for an increased insulin level. It requires an IV infusion of insulin and glucose over a two-hour period, and is therefore too invasive for most research settings and for pediatric participants.[74] A less complex indirect assessment of insulin-mediated glucose utilization can be achieved through the minimal model analysis of a frequently sampled intravenous glucose tolerance test (FSIVGTT).

Finally there are numerous simple surrogate measures of insulin sensitivity and insulin resistance assessed under fasting or dynamic, after an oral glucose load, conditions.[74] An oral glucose tolerance test (OGTT) is an example of a surrogate measure in a dynamic state, and its results reflect the ability of the body to dispose of glucose after a standard oral glucose load.[74] While the OGTT does not require IV access, it does employ multiple blood draws required over the two- or three-hour period following oral glucose administration. Several insulin sensitivity indexes (ISI) surrogate measures are derived from OGTT data, including ISI-Matsuda, ISI-Stumvoll, Gutt $ISI_{0,120}$, Avignon's SiM, and Belfiore area.[75] Furthermore, many surrogate measures exist to test fasting, or basal, conditions: fasting glucose, fasting insulin, 1/fasting insulin, fasting glucose-to-insulin ratio (FGIR), fasting insulin-to-glucose ratio (FIGR), homeostasis model assessment for insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI), Raynaud, Bennet's S_I, Belfiore basal, FIRI, and McAuley index.[75] These measures are less labor and time intensive and cheaper, because

they simply require a fasting blood serum sample. Of the measures listed above, only three, the OGTT, fasting glucose, and fasting insulin, are used clinically.

The three most widely used and validated surrogate measures are fasting insulin, HOMA-IR, and QUICKI.[76] The latter two measure insulin resistance and sensitivity, respectively. HOMA-IR = {[fasting insulin (uU/ml)] x [fasting glucose (mg/dL)] / 405}. Developed by Matthew et al. in 1985, HOMA assesses pancreatic beta-cell function and insulin resistance and is designed to be used in cohort and epidemiological studies.[77, 78] QUICKI = 1 / {log [fasting insulin (uU/ml)] + log [fasting glucose (mg/dl)]}. Fasting insulin, HOMA-IR, and QUICKI correlate well (r = 0.6 – 0.8) with the gold standard EHC technique[78, 79] and FSIVGTT, across race/ethnicity groups.[75] QUICKI has a better linear correlation with EHC than HOMA-IR, however, utilizing the logarithmic transformation (logHOMA-IR) achieves a stronger linear association with the EHC method, similar to that seen with QUICKI.[74]

Many insulin sensitivity measures have been assessed in pediatric samples. Keskin et al. evaluated the reliability of HOMA-IR, FGIR, and QUICKI for assessing insulin resistance in obese children and adolescents and found HOMA-IR to be the most reliable, demonstrating the greatest sensitivity and specificity for measuring insulin resistance.[80] Additionally, their analyses revealed a HOMA-IR cutoff point for diagnosis of insulin resistance to be > 3.16 for adolescents, which is different than the cutoff point of > 2.5 for adults.[80] In non-diabetic children and adolescents, the EHC method is strongly correlated with fasting insulin, FGIR, HOMA-IR, and QUICKI.[79] Across pubertal maturation stages (as described by Tanner) stages one to five in obese children, the FIGR, HOMA-IR, and QUICKI indexes prove to be good measures of insulin

resistance and sensitivity,[37] as well as the FGIR.[81] In contrast, the OGTT has been found to have poor reproducibility in obese youth with higher insulin resistance.[82] Depending on the study design and population being studied, the aforementioned measures are useful in understanding the insulin sensitivity status of a child or effect of an intervention on their insulin sensitivity.

Basic Science: Mechanisms behind Vitamin D's Role in Heart Disease and Diabetes

Cellular and animal studies reveal the underlying mechanisms of vitamin D's role in cardiovascular disease and diabetes. The VDR is found in vascular smooth muscle, endothelium, and cardiomyocytes,[83, 84] and vitamin D plays a role in heart proteomics, structure, and function.[11, 12] Independent of calcium status, vitamin D deficiency results in increased systolic blood pressure (SBP), cardiac contractility, left ventricular hypertrophy, and fibrotic lesions, and VDR knockout mice develop heart hypertrophy.[11, 12, 85] Vitamin D plays a key role in the renin-angiotension-aldolesterone-system (RAAS) independent of calcium metabolism. Mice lacking the VDR have elevated renin and angiotensin II levels that lead to hypertension and cardiac hypertrophy; injection of $1,25(OH)_2D_3$ reduces renin synthesis in mice, and in cell cultures, $1,25(OH)_2D_3$ has been shown to directly suppress renin gene transcription by a VDR-dependent mechanism.[13] Detrimental cardiovascular effects are also mediated by secondary hyperparathyroidism, which is caused by vitamin D deficiency.[86]

Vitamin D is involved in diabetes, with functions in both insulin secretion and action. The VDR[87] and 1-alpha-hydroxylase enzyme[88, 89] required to activate vitamin D, have been discovered in pancreatic beta cells. Vitamin D deficiency, independent of calcium status, decreases insulin secretion;[90] mice lacking functional VDR on their beta

cells demonstrate impaired insulin secretion,[8, 91, 92] but vitamin D supplementation restores insulin secretion.[8] In terms of insulin action, the VDR is found on skeletal muscle cells,[93] albeit on a very limited basis according to recent research,[94] and a vitamin D response element has been identified in the promoter region of the human insulin receptor gene.[95] Additionally, $1,25(OH)_2D_3$ stimulates insulin receptor expression, PI3-kinase activity, and insulin responsiveness.[9, 96]

A recent study offers an explanation for the acceleration of cardiovascular disease seen in diabetic individuals. Oh et al. studied human macrophages obtained from obese, diabetic, hypertensive patients with vitamin D deficiency; these macrophages were cultured in vitamin D deficient or $1,25(OH)_2D_3$ supplemented media. The $1,25(OH)_2D_3$ suppressed foam cell formation by reducing oxidized low-density lipoprotein cholesterol (LDL-C) uptake.[14] Furthermore, deletion of the VDR in macrophages accelerated foam cell formation induced by oxidized LDL-C.[14]

Vitamin D, through its effects on cytokines, plays a role in improving systemic inflammation, affecting risk for both diabetes and cardiovascular disease. By interacting with vitamin D response elements in the promoter region of cytokine genes, vitamin D interferes with nuclear transcription factors implicated in cytokine generation and action.[10, 97, 98]

Observational Studies: Vitamin D, Cardiometabolic Disease, and Insulin Sensitivity

Cross-Sectional Studies

A few cross-sectional studies in pediatric samples exist that examine vitamin D status, cardiometabolic risk factors, and insulin sensitivity (see **Table 2.2**). NHANES 2001-2006 data in adolescents demonstrates a positive association between serum

vitamin D and HDL-C levels, as well as inverse associations between serum vitamin D and waist circumference, SBP, HOMA-IR, and likelihood of having metabolic syndrome.[15] Reis et al. also examined NHANES data during adolescence and found inverse relationships between 25(OH)D concentrations and SBP, plasma glucose levels, abdominal obesity, odds of having hypertension, and odds of having metabolic syndrome.[67] In a broader age range of one to 21, 25(OH)D deficiency (< 37 nmol/L) was correlated with higher SBP and lower HDL-C in another NHANES data analysis.[56]

In a study of obese children and adolescents living in Wisconsin, serum vitamin D levels were positivity correlated with QUICKI and inversely associated with BMI, fat mass, and hemoglobin A1C (HbA_{1C}).[69] In obese African-American adolescent females living in Alabama, OGTT-derived insulin sensitivity was positively associated with 25(OH)D levels.[68] In a recent, small study of African-American adolescents aged ten to 20 years, dietary vitamin D intake was correlated positively with adiponectin levels, which were significantly lower in those who were overweight.[99] Finally, two retrospective analyses in pediatric samples demonstrate positive associations between serum vitamin D concentrations and HDL-C and negative correlations with fasting glucose and SBP.[100, 101]

Many of the aforementioned studies adjusted for potential confounders (e.g. age, gender, race/ethnicity, BMI, socioeconomic status, physical activity) in their analyses. However, as a recent study by Rajakumar et al. elegantly demonstrates, the relationship between adiposity and serum vitamin D levels is robust, and therefore, any investigation of the associations between 25(OH)D status and cardiometabolic outcomes or insulin

Table 2.2 Pediatric Cross-Sectional Studies of Serum Vitamin D, Cardiometabolic Outcomes, and Insulin Sensitivity

Authors & Year	Sample size (N)	Age of subjects	Race of subjects & location	Disease state or healthy	25D positively associated with:	25D inversely associated with:	Adiposity covariate:
Rajakumar et al., 2011[102]	237	8-18	C, AA	Apparently healthy (47% obese)	HDL-C	BMI, BMI percentile, total body fat %, VAT, SAT	Not specified
Ganji et al., 2011[15]	5867	12-19	National (NHANES 2001-2006)	Apparently healthy	HDL-C	Waist circumference; SBP; HOMA-IR; MetS	BMI
Kumar et al., 2009[56]	6275	1-21	National (NHANES 2001-2004)	Apparently healthy	HDL-C	PTH, SBP	BMI
Reis et al., 2009[67]	3577	12-19	National (NHANES 2001-2004)	Apparently healthy	None	Obesity, waist circumference, SBP, fasting glucose, HT, MetS	BMI
Ashraf et al., 2009[68]	51	12-16	AA in SE (Alabama)	Obese	Insulin sensitivity (Matsuda index)	None	BMI
Johnson et al., 2009[39] *	302	2-18	70% C; Minnesota	Pediatric outpatients	HDL-C,	Fasting glucose	BMI
Alemzadeh et al., 2008[69]	127	6-17	C, AA, & H; Wisconsin	Obese	QUICKI	BMI, fat mass, HbA_{1c}	Fat mass
Smotkin-Tangorra et al., 2007[101] *	217	7-18	Not specified; New York	Obese	HDL-C	BMI, SBP	Not specified

C = Caucasian; AA = African American; H = Hispanic; HDL-C = high-density lipoprotein cholesterol; BMI = body mass index; VAT = visceral adipose tissue; SAT = subcutaneous adipose tissue; SBP = systolic blood pressure; HOMA-IR = homeostasis model assessment of insulin resistance PTH = parathyroid hormone; HT = hypertension; MetS = metabolic syndrome; HbA_{1c} = glycated hemoglobin

* Retrospective study design

sensitivity requires adequate correction of adiposity status. In eight to 18 years old black and white children, circulating vitamin D levels were inversely associated with BMI, BMI percentile, total body fat percentage, visceral adipose tissue (VAT), and subcutaneous adipose tissue (SAT), and VAT was found to independently predict 25(OH)D status.[102] As Table 2.2 demonstrates, the most common adiposity covariate used in these pediatric studies is BMI, which lacks sensitivity.

Cross-sectional relationships in adults that examine vitamin D and cardiometabolic relationships have also been investigated. Studies of insulin sensitivity have been explored in healthy, glucose tolerant individuals, as well as in those with pre-diabetes. Significant positive relationships are demonstrated between serum vitamin D concentrations and the following outcomes: OGTT-derived insulin sensitivity,[16, 103] OGTT-derived glucose,[104, 105] OGTT-derived beta cell function,[16] and adiponectin.[106] Significant inverse associations have been noted between 25(OH)D concentrations and the following outcomes: fasting glucose,[105, 107-109] fasting insulin,[105, 107] HOMA-IR,[105-107] odds of having metabolic syndrome,[109, 110] odds of having T2DM,[105] and HbA_{1C}.[111]

Studies of cardiometabolic disease have been conducted in healthy individuals, as well as those with T2DM or high risk of coronary artery disease. Significant inverse associations are reported between 25(OH)D levels and the following outcomes: vascular calcification,[112] heart rate,[17] SBP,[17] tissue plasminogen activator antigen,[113] fibrinogen,[114] prevalent cardiovascular disease,[114] N-terminal pro-atrial natriuretic peptide,[115] cell adhesion markers,[19] triglycerides, and triglyceride/HDL-C ratio.[110] Serum vitamin D levels have also been inversely associated with markers of systemic inflammation,

including high sensitivity C-reactive protein,[19, 114, 116] interleukin-6,[116] and TNF-α[116] in adults.

However, not all investigations report significant associations between vitamin D status and cardiometabolic or insulin sensitivity outcomes. Cheng et al. studied non-diabetic adult participants from the "Third Generation Study", which includes grown grandchildren of the original Framingham Heart Study patients; the measures of adiposity used were VAT and SAT, determined by computed tomography. The prevalence of vitamin D deficiency (defined as 25(OH)D < 50 nmol/L) was three-fold higher in subjects with high SAT and VAT volumes than those with low SAT and VAT ($p < 0.0001$), and 25(OH)D was inversely related to SAT (-2.7 nmol/L per standard deviation increment in SAT) and VAT (-5.7 nmol/L per standard deviation increment in VAT).[117] In multivariate adjusted regression models, the association of 25(OH)D with insulin sensitivity outcomes no longer achieved statistical significance after adjusting for VAT. This may be because VAT is closely tied to insulin resistance and metabolic risk, particularly through the actions of adipokines.[118] For example, adiponectin levels have been found to be negatively correlated with obesity and insulin resistance, and case-control studies present low adiponectin as an independent risk factor for future T2DM development.[118]

In the aforementioned studies, a variety of covariates were adjusted for, including age, gender, race, sociodemographics, physical and leisure activity, supplement use, parathyroid hormone, smoking status, alcohol intake, and sampling month. The most common adiposity covariates used were BMI, waist circumference, and fat mass, however, the well-designed Cheng et al. study sheds light on the importance of

adequately controlling for adiposity, an important confounding variable, using robust, sensitive measures.

Case-Control Studies

Several case-control studies in adults have compared vitamin D status in patients with diabetes or heart disease with matched controls. When compared to controls, serum vitamin D concentrations are significantly lower in T2DM patients,[111, 114, 119-125] myocardial infarction patients,[126] acute stroke patients,[127] and congestive heart failure patients.[115] However, other studies demonstrate no significant differences between diabetics and controls.[128-130]

The confounding issue of adiposity carries over into these case-control study designs. In an Italian study examining postmenopausal women with T2DM, cases had significantly higher BMIs and lower 25(OH)D concentrations than controls; although age and time since menopause were adjusted for, BMI was not adjusted for, so cases may have had lower 25(OH)D levels because they were more obese.[125] Scragg et al. attempted to correct for adiposity differences using BMI and related metabolic disturbances (cholesterol levels and BP) in their investigation of Polynesian and Caucasian subjects with newly diagnosed pre-diabetes and T2DM living in New Zealand, but even with these adjustments, cases still had significantly lower serum vitamin D levels than controls.[123]

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The majority of these observational investigations across different life stages and disease states demonstrate significant cross-sectional associations between vitamin D status and myriad measures of cardiometabolic risk and insulin sensitivity and lower
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25(OH)D levels in cases vs. controls. However, observational studies have limitations. Case-control studies are flawed by the measurement of 25(OH)D status after the heart disease or diabetes diagnosis, while cross-sectional studies simultaneously measure 25(OH)D status and cardiometabolic status, thereby introducing an ambiguous temporal relationship between the two variables. Both types of studies preclude causal inferences. There is considerable variability in samples that have been studied, particularly regarding diabetes status (non-diabetic, pre-diabetic, diabetic, newly diagnosed T2DM, established T2DM, insulin-treated T2DM vs. other medications), ethnicity, and latitude. Also, control groups in case-control studies were matched on different factors per study (e.g. age, sex, ethnicity, season), or matching was not mentioned at all. In fact, in all three case-control studies that demonstrated no difference in 25(OH)D between cases and controls, no matching was mentioned, and furthermore, the subjects in these particular studies spanned the widest age ranges of all the case-control studies: 18-75 years;[128] 19-80 years;[129] and 14-63 years.[130] Age matching is critical, since insulin resistance, blood pressure, and risk of T2DM and heart disease increase with age.

Addressing adiposity in observational analyses is crucial. Being overweight or obese increases one's risk for developing pre-diabetes, T2DM, and heart disease. Furthermore, BMI, waist circumference, and fat mass are inversely related to 25(OH)D concentrations in the majority of studies on vitamin D status. Obese people may consume fewer foods and beverages fortified with vitamin D (e.g. dairy products) or that naturally contain vitamin D (e.g. salmon), and overweight and obese people report lower use of multivitamin and vitamin-D containing supplements.[131] Obese individuals may participate in less outdoor activities or belong to an ethnic group with darker skin

pigmentation. All of these facets would decrease their vitamin D status through reduced intake or cutaneous synthesis. Additionally, due to its lipophilic nature, vitamin D sequestration in adipose tissue is a plausible mechanism to explain lower vitamin D status in overweight and obese individuals.[72] Animal studies in rats[132, 133] and pigs[134] confirm storage of cholecalciferol and 25(OH)D in fat tissue, especially at high vitamin D doses. Although total carcass data is lacking for humans, one human study used subcutaneous fat samples from obese patients at the time of bariatric surgery and found cholecalciferol and 25(OH)D present in the fat tissue.[135] Although vitamin D is found in the fat, muscle, liver, serum, and remaining tissues, storage in fat predominates for cholecalciferol, while 25(OH)D is found in highest concentrations in the serum.[134] Additional research, particularly in humans, is needed in this area.

It is apparent that obesity modifies both cardiometabolic risk and vitamin D status, and is therefore, an important confounding variable. Controlling for anthropometric measures like BMI and waist circumference may not adequately adjust for the effect of adiposity. In the one study that used a detailed, reliable characterization of adiposity through VAT and SAT volume measurements, the relationship between 25(OH)D and insulin sensitivity lacked statistical significance after adjusting for VAT.[117] Most of the cross-sectional studies attempt to control for adiposity with BMI or waist circumference, but only one of the case-control studies did so using BMI.[123]

Other important confounders include age, physical activity, and calcium status. Calcium intake is rarely controlled for, even though the homeostatic mechanisms between calcium and vitamin D are intertwined. Furthermore, both calcium and vitamin D, independently and in combination, have been shown to modify one's risk for

T2DM.[136] Only three of thirteen case-control studies examining vitamin D status in diabetic or heart disease patients controlled for potential confounders.[119, 123, 125] Other considerations that confound the inverse association observed between vitamin D status and cardiometabolic outcomes include: 1) vitamin D status is positively associated with younger age, healthy body weight, and healthy lifestyle practices, and inversely associated with smoking, alcohol intake, and parental history of myocardial infarction; therefore, vitamin D status is a marker of good health; 2) the inverse associations observed in cross-sectional studies may be due to reverse causation, since lower vitamin D status may reflect chronic nonspecific illness; 3) we consume whole foods, and dietary sources of vitamin D may affect diabetes risk; also, foods rich in vitamin D are typically healthy foods, and they may replace less healthy foods, which increase one's risk for diabetes (e.g. drinking fortified milk instead of sweetened beverages); 4) single 25(OH)D measurements used in observational analyses may not reflect long-term vitamin D status.[21]

Prospective Cohort Studies

Several prospective studies to date have examined vitamin D intake or 25(OH)D status in relation to future glycemic status and insulin resistance,[137] T2DM incidence,[138] cardiovascular disease risk factors and incidence,[139] and mortality[19] exclusively in adult cohorts. Two prospective studies assessed vitamin D intakes in relation to metabolic syndrome and T2DM risk.[140, 141] Vitamin D intake was assessed in over 10,000 women from the Women's Health Study, and neither total nor supplemental vitamin D were significantly associated with metabolic syndrome; however, a greater incidence of T2DM was seen for women who consumed less than 159 IU vitamin D/day versus greater than

511 IU vitamin D/day, respectively.[140] In the Nurses' Health Study, over 83,000 women were followed for 20 years, and 4,843 incident T2DM cases were ascertained. After adjustment for confounders, no association was seen between total vitamin D intake and T2DM incidence, but when comparing the highest (400 IU/day) versus lowest (≤ 100 IU/day) vitamin D supplement intake levels, the relative risk for T2DM was 0.87 [95%CI: 0.75-1.00], p = 0.04), which barely missed statistical significance based on the confidence interval.[141] Furthermore, a combined daily intake of great than 1200 mg calcium and greater than 800 IU vitamin D was associated with a 33 percent lower risk of T2DM incidence (RR = 0.67 [95%CI: 0.49-0.90] compared to an intake of less than 600 mg calcium and less than 400 IU vitamin D.[141]

Several prospective studies have examined vitamin D status and subsequent diabetes incidence using 25(OH)D concentrations. The Australian Diabetes, Obesity, and Lifestyle Study found a lower five-year incidence of diabetes in those with a higher 25(OH)D concentration (65 nmol/L versus 58 nmol/L), for those who developed diabetes), and each 25 nmol/L increment in serum vitamin D was associated with a 24 percent reduced risk of diabetes.[142] Over a seven-year follow-up period in the Framingham Offspring Study, subjects in the highest 25(OH)D tertile had a 40 percent lower incidence of T2DM compared to those in the lowest tertile, after adjusting for a plethora of variables.[143] One study pooled the data from two Finnish nested case-control studies at the 22-year follow-up time point and found that men had a reduced risk of T2DM between the highest and lowest 25(OH)D quartiles (OR= 0.28 [95%CI: 0.10-0.81]).[18] Forouhi et al. followed a British cohort in the Ely Prospective Study for ten years and found that baseline 25(OH)D concentrations were inversely associated with ten

year risk of hyperglycemia, insulin resistance, and metabolic syndrome, after adjustments.[137] Interestingly, this study endured a 50 percent loss to follow-up over the ten years, and the remaining participants were healthier at baseline than those lost to attrition, which could have produced a more conservative estimate of the association between vitamin D and risk of hyperglycemia, insulin resistance, and metabolic syndrome.[137] Mattila et al. examined a cohort from the Mini-Finland Health Survey over 17 years of follow-up and observed a significant inverse association between 25(OH)D and risk of T2DM between highest (> 70.9 nmol/L) and lowest (< 22.4 nmol/L) 25(OH)D quartiles (RR = 0.60 [95%CI: 0.36-0.98], p = 0.01), however, this relationship was attenuated and failed to reach statistical significance in multivariate analyses after further adjustments that included BMI, leisure exercise, smoking, and education. [138] Finally, a nested case-control study in a prospective cohort of over 900 American pregnant women identified gestational diabetes mellitus (GDM) cases and frequency matched them to controls.[144] Women who developed GDM had significantly lower 25(OH)D levels (60 nmol/L) than controls (75 nmol/L) at 16 weeks gestation, after adjusting for maternal age, race, family DM history, and pre-gravid BMI. Furthermore, vitamin D deficiency (< 50 nmol/L) was associated with a 2.66-fold increase in GDM risk.[144]

The relationship between serum vitamin D status and heart disease incidence has also been explored in prospective cohorts. In a ten-year follow-up analysis of men from the Health Professionals' Follow-Up Study, men with 25(OH)D levels less than 37.5 nmol/L were at increased risk for myocardial infarction compared with their vitamin D sufficient counterparts (> 75 nmol/L); even men with intermediate vitamin D status (37.5-75 nmol/L) were at elevated risk relative to the vitamin D sufficient group.[145] In a

study combining men and women from the Health Professionals' Follow-Up Study and the Nurses' Health Study, respectively, the pooled relative risk for hypertension was 3.18 (95%CI: 1.39-7.29) for those with 25(OH)D levels less than 37.5 nmol/L compared with those whose levels were greater than 75 nmol/L.[146] Framingham Offspring Study participants were followed for an average of 5.4 years, and those with a 25(OH)D level less than 37.5 nmol/L experienced an increased incidence of cardiovascular events compared to those with levels greater than 37.5 nmol/L, but this relationship was only statistically significant in the hypertensive participants.[139]

Finally, cardiovascular, diabetes, and all-cause mortality have been related to vitamin D status in prospective studies. Older adults from the NHANES III (1988-1994) dataset were followed through the year 2000, and serum 25(OH)D levels were independently, inversely associated with cardiovascular disease mortality and all-cause mortality.[147] In T2DM patients followed for a median of 15 years, all-cause mortality was two times higher in patients with severe vitamin D deficiency (< 13.9 nmol/L) compared to those with higher levels.[148] The Ludwigshafen Risk and Cardiovascular Health Study found significantly higher hazard ratios for cardiovascular mortality and all-cause mortality for patients in the lower two 25(OH)D quartiles (medians of 18 and 33 nmol/L) compared to patients in the highest serum vitamin D quartile (median of 71 nmol/L).[19]

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Described above, the prospective cohort studies and nested case-control studies are varied in methodology. They were conducted for varying durations during different life stages (but exclusively in adults), utilized different measures and cut-points for
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vitamin D status, estimated the risk of different outcomes, and were conducted in predominantly Caucasian cohorts. Assessing vitamin D status using self-report responses to food frequency questionnaires and other such tools to estimate vitamin D intake is not ideal, as they rely on retrospective data and recall bias from participants. For example, the Nurses' Health Study[141] assessed vitamin D and calcium intakes from diet and supplemental use every two to four years during the 20-year follow-up. Neither of the vitamin D intake studies quantified sun exposure, a major source of vitamin D. In addition, adjustments in the two vitamin D intake studies[140, 141] demonstrate the importance of adjusting for calcium intake in vitamin D studies, because these micronutrients may act together to modify the risk of T2DM, and may therefore not be independent of one another. Vitamin D intake is an inferior estimate of vitamin D status when compared to 25(OH)D concentrations, which reflect one's dietary intake (foods and supplements) and cutaneous synthesis via sun exposure.

Still exclusively in adults, cohort studies utilizing 25(OH)D concentrations demonstrate clearer inverse relationships in heart disease and diabetes risk factors, incidences, and mortality. Prospective cohort studies are the best of all observational study design types, because the temporal ambiguity of vitamin D status and disease is removed.[149] Taken together, these studies provide evidence that low circulating vitamin D levels may increase one's risk for hyperglycemia, insulin resistance, T2DM, metabolic syndrome, cardiovascular disease risk factors and mortality, myocardial infarction, and all-cause mortality. All five of these studies adjusted for adiposity using either BMI or waist circumference, which is a strength, although a more direct assessment of obesity (e.g. using computed tomography or dual energy X-ray absorptiometry) would have

strengthened these studies. Most of the studies relied on self-report for T2DM diagnosis, but two studies confirmed the diabetes diagnosis via national registry-based data.[18, 138]

Although a 25(OH)D concentration > 50 nmol/L has been proposed by the IOM to define sufficiency based on skeletal outcomes, the desirable concentration based on cardiometabolic and diabetes outcomes may be higher. The prospective cohort studies reviewed here provide evidence for this possibility in adults, since the 25(OH)D concentrations reported to lower cardiovascular or diabetes disease risk consistently hover around 70 nmol/L (> 70 nmol/L;[138] 65 nmol/L;[142] median of 71 nmol/L;[19] > 75 nmol/L[144-146]).

Supplementation Trials: Vitamin D, Cardiometabolic Disease, & Insulin Sensitivity

Vitamin D intervention studies are necessary to further understand the relationships revealed in observational studies. Pertinent vitamin D supplementation trials (see **Table 2.3**) to date have been conducted almost exclusively in adults and have evaluated facets of cardiometabolic disease, glycemia, and insulin sensitivity. Only two pediatric vitamin D supplementation RCTs were found that examine cardiometabolic and insulin sensitivity outcomes. One was conducted in 11 adolescent end-stage renal disease patients undergoing regular hemodialysis and 11 healthy volunteers; subjects received an intravenous dose of $1,25(OH)_2D_3$, which resulted in a 38 percent increase in glucose uptake, a 48 percent increase in the early component of insulin secretion, and a 32 percent increase in the late component of insulin secretion in the renal disease patients.[150] The other study involved 49 African-American adolescents, who were given 2,000 IU vitamin D or a placebo daily over four months; the supplemented group experienced a

significant improvement in carotid-femoral pulse wave velocity, a measure of arterial stiffness.[22]

Overall, the supplementation trials to date provide hopeful but mixed results, likely due to the heterogeneity in study designs. The diversity of study design makes it challenging to draw overarching conclusions concerning the effect of vitamin D supplementation on cardiometabolic disease and diabetes. The following trial components varied across studies: trial duration (four days – seven years), sample size (N = 14 – 33,000+), race/ethnicity (Caucasians from the US and Europe, African-American, South Asian, Indian, Iranian, Japanese, Turkish, and Lebanese), age (14 – 80 years old), gender, disease status (healthy, overweight or obese, pre-diabetic, T2DM of short duration, established T2DM with multiple medications, GDM, hypertensive, end-stage renal disease), supplement form (D_3, $1\alpha(OH)D_3$, $1,25(OH)_2D_3$, in combination with calcium, fortified yogurt drink, and UV irradiation), dosage (for D_3: 400 IU to 200,000 IU), frequency of supplementation (single dose, daily, weekly, fortnightly) and outcome measures (cardiovascular function markers, EHC, IVGTT, and OGTT-derived measures, fasting surrogate measures, T2DM incidence, and inflammatory markers). Although the majority of the supplementation trials were randomized controlled trials (RCT), a few were not. The GDM trial did not include a control group.[151] Three trials did include a control group but lacked blinding, a placebo-control, and randomization.[150, 152, 153]

The majority of studies used cholecalciferol (vitamin D_3), which is a strength in study design since this form has been shown to increase 25(OH)D more potently than vitamin D_2[44] and because some 25(OH)D assays have < 100% cross-reactivity between D_3 and D_2, causing the latter to be underestimated.[154] Other forms of vitamin D used

Table 2.3: Vitamin D Supplementation Trials and Outcomes of Cardiometabolic Disease and Diabetes

Authors & sample size	Age	Sex	Race/ Ethnicity	Disease State	Baseline 25(OH)D (nmol/L)	Vitamin D form & dose	Duration	Pertinent outcomes	Significant effects ($p < 0.05$)
Harris et al. 2011[159] (N=57)	avg=30	M/F	African-American	OW or obese (BMI≥25)	~ 36	D_3: 60,000 IU monthly	4 mos	Flow-mediated dilation (FMD)	↑FMD, ↑diameter, ↑FDM/shear
Mitri et al. 2011[160] (N=92)	≥40 (avg=57)	M/F	Mostly Caucasian (US); 1% Hispanic; 2%Other	OW or obese (BMI≥25)	~ 25	D_3: 2,000 IU/day	4 mos	Disposition index (insulin sensitivity), HbA_{1c}, Fasting glucose, 2-h glucose	↑Disposition index
Nikooyeh et al. 2011[161] (N=90)	30-60 (avg=51)	M/F	Iranian	T2DM	~ 44	D_3: D-fortified yogurt drink (500 IU + 150 mg Ca/250ml) 2x/day	3 mos	Fasting glucose, HbA_{1c}, HOMA-IR; waist circ, BMI, lipids	↓Fasting glucose, ↓Hb_{A1c}, ↓HOMA-IR, ↓waist circ, ↓BMI
Dong et al. 2010[22] (N=49)	14-18 (avg=16)	M/F	African-American	Apparently healthy (BMI-for-age<95th%)	~ 33	D_3: 2,000 IU/d	4 mos	SBP, DBP, carotid-femoral pulse wave velocity (measure of arterial stiffness)	↓PWV
Witham et al. 2010[162] (N=61)	≥18 (avg=65)	M/F	Caucasian (Britain)	T2DM	~ 45	D_3: single dose of 100,000 IU or 200,000 IU	4 mos	SBP, BNP, HOMA-IR, HbA_{1c}	↓SBP in both trt arms; ↓BNP at 16wks w/ 200,000 IU; trend for ↓HOMA-IR at 8wks w/ 200,000 IU (p=0.05)
von Hurst et al. 2010[163] (N=81)	23-68 (avg=41)	F	South Asian	Insulin resistant	~ 20	D_3: 4,000 IU/day	6 mos	HOMA-IR, HOMA2%S, HOMA2%B	↑HOMA2%S, ↓HOMA-IR, ↓fasting insulin
Nagpal et al. 2009[164] (N=100)	≥ 35 (avg~40)	M	Indian	Non-diabetic, but central obesity	~ 33	D_3: 3 doses of 120,000 IU every 14 days	6 wks	HOMA-IR, QUICKI, OGIS, HOMA%B	↑OGIS in per protocol analysis (p=0.055 intent-to-treat analysis)

Avenell et al. 2009[165] (n=5,292) §	≥ 70 (avg=77)	M/F	Caucasian (Britain)	8% w/ T2DM (self-report) at BL	Not provided	D_3: 800 IU/day alone & combined w/ 1,000mg Ca carbonate	2-5 yrs	Development of T2DM, usage of meds for T2DM (both self-report)	None
Jorde & Figenschau 2009[166] (N=36)	21-75 (avg~56)	M/F	Caucasian (Norway)	T2DM, treated w/ metformin & bed-time insulin	~ 59	D_3 : 40,000 IU per week	6 mos	Fasting insulin & glucose, C-peptide, fructosamine, Hb_{A1c}	None
Zittermann et al. 2009[167] (N=200)	18-79 (avg~48)	M/F	Caucasian (Germany)	OW or obese (BMI>27)	30	D_3: 3,332 IU per day	1 yr	TNFα	↓TNFα
de Boer et al. 2008[168] (N=33,951)	50-79 (avg=62)	F	84% Cauc; 8% AA; 4% Hispanic; 2% Asian/Pacif. Islander; <1% Am.Ind.	None, all post-menopause	89% <80 61% <50	D_3: 400 IU/day + 1,000mg Ca	7 yrs (median follow-up)	Incident T2DM w/ med usage	None
Tai et al. 2008[169] (N=33)	19-75 (avg=55)	M/F	Caucasian (Australia)	None	40	D_3: 2 doses of 100,000 every 2wks	1 mo	Avignon's SiM, HOMA, QUICKI	None
Pittas et al. 2007[170] (N=314) §	≥65 (avg=71)	M/F	Caucasian (US)	29% w/ IFG at Baseline	76	D_3: 700 IU/day + 500mg Ca citrate	3 yrs	Fasting plasma glucose, HOMA-IR, CRP, IL-6	In IFG suppl. group, sig. smaller ↑FPG & HOMA-IR
Gannage-Yared et al. 2003[171] (N=47)	Adults (age?)	F	Lebanese	None, all post-menopause	?	D_3: 800 IU/day + 1000mg Ca	3 mos	TNFα, CRP, fasting insulin	None
Borissova et al. 2003[172] (N=10)	Adults (age?)	F	Caucasian (Bulgaria)	T2DM, treated w/ oral hypoglycemia agents	~ 35	D_3: 1,332 IU/d	1 mo	First (FPIS) and second (SPIS) phases of insulin secretion	↑FPIS

Krause et al. 1998[173] (N=18)	26-66 (avg=48)	M/F	Caucasian (Germany)	Untreated mild essential HT	~ 48	UV: Full-body UVB vs. UVA 3x/wk w/ exposure of 6min at 0.7 MED	6 wks	24-h ambulatory SBP and DBP	↓SBP & ↓DBP in only UVB group
Rudnicki & Molsted-Pedersen 1997[151] (N=12)	Not provided	F	Caucasian (Denmark)	GDM	Not provided	$1,25(OH)_2D_3$: 2ug/m^2 IV at day 2 & oral 0.25ug/d for next 14 days	16 days	Glucose and insulin means from OGTT	↓Glucose & insulin after IV treatment, but not oral
Fliser et al. 1997[157] (N=18)	Adults (age?)	M	Caucasian (Germany)	None	?	$1,25(OH)_2D_3$: 1.5ug/day orally	7 days	Glucose disposal rate, mean plasma glucose and insulin	None
Orwoll et al. 1994[157] (N=35)	40-70 (avg=61)	M/F	Caucasian (US)	T2DM treated w/ diet and/or meds	35	$1,25(OH)_2D_3$: 1ug/day	4 days	Glucose, insulin, C-peptide, and glucagon responses to Sustacal meal	Shorter T2DM duration assoc. w/ improvement in insulin & C-peptide
Mak 1992[150] (N=22)*	16-23	M/F	Caucasian (US)	Uremic end-stage renal disease patients undergoing hemodialysis	Not provided	$1,25(OH)_2D_3$:2ug/m^2	Single dose then tested	Glucose mean from IVGTT, insulin secretion from hyperglycemic clamp	↑Glucose uptake, ↑insulin secretion
Ljunghall et al. 1987[155] (N=65)	Adults ("middle -aged")	M	Caucasian (Sweden)	IGT	?	$1\alpha(OH)D_3$: 0.75ug/day	3 mos	Hb_{A1c}, fasting glucose, IVGTT insulin and glucose	None
Gedik & Akalin 1986[152] (N=14)**	17-54 (avg=33)	F	Turkish	Clinical vitamin D deficiency	Not provided	D_3: 2000 IU/day	6 mos	Insulin and glucose AUC from OGTT, IGI	↑Insulin AUC, ↑IGI
Inomata et al. 1986[156] (N=14)***	Adults (age ?)	M/F	Asian (Japan)	T2DM	?	$1\alpha(OH)D_3$: 2ug/day	3 wks	Insulin AUC from OGTT	↑Insulin AUC

Nilas & Christiansen 1984[153] (N=238) §	Adults (age ?)	F	?	?, all post-menopause	?	2000 IU D_3/day or 0.25ug/day $1\alpha(OH)D_3$ or 0.25-0.50ug/day $1,25(OH)_2D_3$	2 yrs : D_3, $1\alpha(OH)_3$ 1 yr : $1,25(OH)_2D_3$	Fasting glucose	None

? denotes inability to retrieve full-text of article, so information lacking

***** N = 22 (n = 11 end-stage renal disease patients; n = 11 healthy volunteers of same age). Design lacks blinding, randomization, and placebo-control.

****** N = 14 (n = 4 cases of vitamin D deficiency, assessed at BL and post-repletion; n = 10 healthy controls [not vitamin D deficient] who received no supplementation). Design lacks blinding, randomization, and placebo-control.

******* N = 14 (n = 7 T2DM subjects given daily $1\alpha(OH)D_3$ for 3wks; n = 7 control subjects instructed to follow balanced diet). Design lacks blinding, randomization, and placebo-control.

§ Post-hoc analysis of osteoporosis RCT

include 1α(OH)D_3[153, 155, 156] and the biologically active metabolite hormone, 1,25$(OH)_2D_3$. [150, 151, 153, 157, 158] One study delivered vitamin D cutaneously via full-body UVB irradiation.[173] This variety, coupled with the diverse dosages, makes study comparison and compilation challenging. Some studies used daily doses, while others delivered single or periodic doses, which may not be as efficacious at sustaining increases in 25(OH)D concentrations. This may have partially contributed to the borderline significant results seen in the Witham et al.[162] and Nagpal et al.[164] trials and the null effects reported by Jorde & Figenschau[166] and Tai et al.[169]

Trials conducted in apparently healthy, non-diabetic adults fail to show significant improvements in cardiometabolic and diabetic outcomes.[153, 157, 165, 169, 171] Also, once an individual has established T2DM, particularly of longer duration and requiring medication usage, vitamin D supplementation may not provide a substantial benefit,[166] although one small trial found an improvement in insulin secretion in diabetics treated with oral hypoglycemic agents after receiving 1,332 IU vitamin D per day over one month.[172] Conversely, favorable evidence from supplementation trials appears to occur in high-risk individuals (i.e. glucose intolerant, insulin resistant, hypertensive, overweight, or obese). Vitamin D supplementation has positive effects on DM-related outcomes in subjects with T2DM of shorter duration, pre-diabetes, and overweight and obese individuals.[158-162, 164, 170] A critical window may exist for the efficacy of vitamin D supplementation, namely at an early stage of diabetes or perhaps during pre-diabetes. This assertion is supported by the results of Pittas et al. in which supplementation was found to protect those with pre-diabetes but not those with normal glucose tolerance.[170] This is an important finding, as it may reflect the ability of vitamin D supplementation to

delay the progression to clinical T2DM. Vitamin D supplementation has positive effects on cardiometabolic-related outcomes in subjects with hypertension, overweight, obesity, and T2DM.[159, 162, 173] Finally, vitamin D supplementation improves inflammatory markers in overweight and obese individuals.[167]

Vitamin D insufficient populations also appear to benefit more from supplementation than their vitamin D-sufficient counterparts. This phenomenon likely contributed to the significant results demonstrated in interventions in which subjects were vitamin D-deficient at baseline, as well as the null results seen in vitamin D-sufficient subjects.[155] It is possible that Pittas et al. may have found more pronounced improvement in fasting glucose and HOMA-IR if the subjects had not been vitamin D-sufficient (76 nmol/L) at baseline.[170] However, "deficiency", "insufficiency", and "sufficiency" terminology should be used cautiously when discussing extraskeletal indicators, since the current IOM recommendations stem from skeletal outcomes. Although a 25(OH)D concentration greater than 50 nmol/L has been proposed by the IOM to define sufficiency based on skeletal outcomes, the desirable concentration based on cardiometabolic outcomes may be higher. Evidence to support this assertion comes from the von Hurst et al. trial in which a 25(OH)D between 80 – 119 nmol/L was found to optimally increase insulin sensitivity in vitamin D-deficient, insulin resistant South Asian women.[163]

Lack of adherence to RCT supplementation regimens can dilute the treatment effect. In the Women's Health Initiative Calcium/Vitamin D Trial, participants were allowed to continue personal use of calcium, vitamin D, and multivitamin supplementation. At year three of follow-up, 44 percent of participants reported using multivitamins, and 4 and 16 percent used vitamin D and calcium supplements,

respectively.[168] It is feasible that the treatment and placebo group were consuming relatively similar levels of these nutrients (especially since the vitamin D supplement level was so low [400 IU D_3 daily]), and this may have diluted the results. In fact, on the basis of dose and difficulty with adherence over the seven-year trial, Lappe et al. estimated the supplementation effect on 25(OH)D concentrations to be only 5 nmol/L, an increment unlikely to affect metabolic outcomes.[174]

Finally, several of these trials,[153, 165, 168, 170] were designed to answer bone-related research questions, which is evidenced by their excessively long follow-up durations. These *post hoc* analyses are not ideal. In order to sufficiently answer the research question of whether vitamin D supplementation affects diabetes- and cardiovascular-related outcomes, appropriately-powered trials designed for that explicit purpose are needed.

Aside from Mak's study in adolescents with end-stage renal disease[150] and the cardiovascular outcome trial by Dong et al. in adolescents,[22] to date, no published vitamin D intervention studies exist that investigate the glucose-insulin responses to vitamin D supplementation in children or adolescents. Future research of vitamin D's role in cardiovascular disease and diabetes should utilize rigorously designed, adequately powered RCT designs with multiple doses, examining high-risk populations, such as obese individuals with pre-diabetes, particularly during the pediatric years and including minority populations.

Cumulative Evidence from Systematic Reviews and Meta-analyses

Several systematic reviews and meta-analyses have compiled the findings from observational and intervention studies of vitamin D related to cardiometabolic and

diabetes outcomes. In terms of glycemia, insulin sensitivity, and diabetes outcomes, a systematic review and meta-analysis by Pittas et al. found a relatively consistent association from observational research between low vitamin D status and T2DM prevalence and incidence.[136] Vitamin D intake greater than 500 IU/day decreases the risk of T2DM by 13 percent when compared with an intake less than 200 IU/day, and those with the higher vitamin D status (> 62 nmol/L) versus lower vitamin D status (< 35 nmol/L) have a 43 percent reduced risk of developing T2DM.[6] Looking at the totality of vitamin D supplementation trials, Mitri et al. and Pittas et al. conclude that there is no clinically significant effect of vitamin D supplementation at the dosages given, however in patients with baseline pre-diabetes, supplementation with vitamin D improves insulin resistance and may retard the progression to DM.[6, 136, 175]

Reviews of the role of vitamin D in cardiometabolic disorders found that the highest level of serum vitamin D is associated with a 43 percent reduction in cardiometabolic disorders, and this reduction is particularly substantial in middle-age and elderly populations.[42] Lower 25(OH)D levels are associated with an increased risk of incident hypertension in longitudinal cohort studies, and a compilation of supplementation trials reveals that vitamin D non-significantly reduces SBP.[21] Vitamin D supplementation at moderate to high doses (approximately 1,000 IU/day) may reduce cardiovascular disease risk.[7] As with diabetes outcomes, clinically significant effects from cardiometabolic trials are lacking.

Observational studies are criticized for failing to adjust for important confounders, and the diversity of the intervention trials (e.g. vitamin D formulations and populations studied) and design flaws (e.g. short duration of follow-up, small sample

size, lack of control groups, and *post hoc* analyses) make the available evidence somewhat limited.[136] Although significant heterogeneity exists between studies, no evidence of publication bias exists.[42]

Summary and Gaps in the Literature

In summary, the pediatric obesity epidemic and associated rise in comorbid metabolic disturbances have occurred concurrently. In conjunction, hypovitaminosis D is present in a significant percentage of children and adolescents, particularly in those with excess adiposity and those in minority groups. The body of literature that supports the extraskeletal role of vitamin D in diabetes and cardiometabolic disease is growing, providing biologically plausible mechanistic evidence via animal and cellular models and producing reproducible cross-sectional and longitudinal relationships for disease risk factors in humans. Vitamin D supplementation trials to date are extremely diverse in design and taken together, demonstrate equivocal findings, although a reduction in cardiovascular disease risk and a slowed progression to T2DM have been demonstrated.

There are several areas that represent gaps in the current literature. Underlying mechanisms behind the observed, lower serum vitamin D levels in overweight and obese people compared to their normal weight counterparts should be explored. Total human body composition data is needed to determine tissue storage sites and concentrations for vitamin D sequestration. There is a need for continued cellular research to discover the precise alterations in gene expression responsible for vitamin D's role in heart disease and diabetes, and VDR genetic polymorphisms should continue to be explored, as they may mediate risk for cardiometabolic disease and/or response to supplementation.

Future cross-sectional investigations must adequately adjust for pertinent confounders, particularly adiposity. No longitudinal studies exist that commence during childhood and adolescence and extend into adulthood to examine the risk of heart disease and diabetes incidence based on prior vitamin D intake and 25(OH)D status. These types of studies are worth exploring, since some of the strongest evidence for vitamin D's role in heart disease and diabetes originates from adult prospective cohort studies. Future supplementation trials should be rigorously-designed, dose-response studies in adult and pediatric populations, with a particular focus on high-risk groups (e.g. obese, pre-diabetic, mild hypertension). Clinically relevant and significant outcomes should be assessed using rigorous methodology. Finally, although the vitamin D recommendations were recently updated by the IOM and based on skeletal outcomes, significant gaps remain in the literature regarding recommendations specific to pediatric years, disease states such as heart disease and diabetes, particular racial groups, and those who are overweight or obese, which represents a large proportion of the US population.

The current evidence is not strong enough to support vitamin D supplementation on a population-based scale as the panacea for diabetes or heart disease. Additional findings from well-controlled trials with diabetes and heart disease as the primary outcomes will help ascertain the public health significance of vitamin D for the prevention of these diseases. Ultimately vitamin D supplementation is cheap and safe, and this simple intervention may have significant implications for those at risk for developing diabetes and cardiovascular disease, potentially devastating, chronic, and costly conditions.

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CHAPTER 3

VITAMIN D SUPPLEMENTATION AND INSULIN SENSITIVITY IN EARLY PUBERTAL CHILDREN: RESULTS FROM THE RANDOMIZED CONTROLLED GAPI TRIAL[1]

[1] Ferira AJ, Laing EM, Hausman DB, Hall DB, Weaver CM, McCabe GP, Martin BM, Peacock M, Warden SJ, Hill KM, Lewis RD. To be submitted to *The American Journal of Clinical Nutrition*.

Abstract

Background: The increase in pediatric obesity prevalence has contributed to a rise in the incidence of many comorbid cardiometabolic conditions, including pre-diabetes. The role of vitamin D in pre-diabetes and type 2 diabetes mellitus (T2DM) is an emerging area of research, supported by mechanistic findings, as well as cross-sectional and longitudinal studies. Vitamin D supplementation trials that examine diabetes-related outcomes have been conducted exclusively in adults and cumulatively provide equivocal findings, largely due to the heterogeneity in study designs. Rigorously designed intervention trials are needed in pediatric populations.

Objective: Our primary objective was to determine the dose-response effect of vitamin D supplementation over 12 weeks on fasting and surrogate measures of glycemia and insulin sensitivity in 320 early pubertal children who participated in the multi-site Georgia, Purdue, and Indiana University School of Medicine (GAPI) vitamin D trial.

Design: Black and white early pubertal (N = 320) males (aged 10 to 13 years) and females (aged 9 to 12 years) participated in the GAPI vitamin D supplementation trial, a 12-week randomized, triple masked, multi-site, dose-response, placebo-controlled trial. Participants were equally randomized to receive placebo or vitamin D_3 (400, 1,000, 2,000, or 4,000 IU/day). Anthropometry, pubertal maturation, maturity offset, and body composition were assessed at baseline and 12 weeks. Serum 25-hydroxyvitamin D ([25(OH)D], glucose, and insulin concentrations were assessed at baseline, 6-, and 12-weeks. Homeostasis model assessment of insulin resistance (HOMA-IR) and quantitative insulin sensitivity check index (QUICKI) were used as surrogate measures of insulin resistance and sensitivity, respectively. Dietary intake was estimated using 3-day diet

records at baseline and 12 weeks. Statistical analyses were conducted using a mixed effects model and intent-to-treat analysis with alpha = 0.05 to denote significance.

Results: Baseline 25(OH)D was significantly, inversely associated with fasting insulin ($r = -0.140$, $p = 0.017$) and HOMA-IR ($r = -0.146$, $p = 0.012$), as well as positively associated with QUICKI ($r = 0.142$, $p = 0.015$) after controlling for race, sex, age, pubertal maturation, fat mass, and body mass index. Main effects for time were significant for all four outcomes. Glucose ($F = 5.79$, $p = 0.003$), insulin ($F = 11.59$, $p < 0.001$), and HOMA-IR ($F = 12.41$, $p < 0.001$) increased over time, while QUICKI ($F = 12.13$, $p < 0.001$) decreased over time. Even though serum 25(OH)D concentrations significantly increased in a dose-dependent manner, the effect of vitamin D supplementation over time was non-significant for the four dependent variables across all treatment groups. Treatment*time interactions are as follows: glucose ($F = 0.60$, $p = 0.780$), insulin ($F = 0.41$, $p = 0.918$), HOMA-IR ($F = 0.46$, $p = 0.883$), and QUICKI ($F = 0.43$, $p = 0.905$). Important baseline covariates (gender, race, pubertal maturation, and adiposity) did not interact significantly with the treatment*time effect.

Conclusions: We report novel findings from the first pediatric vitamin D intervention to examine effects on glycemia and insulin sensitivity. Although 12 weeks of vitamin D supplementation did not improve glycemia and insulin sensitivity, the heterogeneity introduced into our glucose-insulin outcomes due to the pubertal maturation of our subjects, as well as the apparently healthy, vitamin D sufficient status of our children, are key facets that may have contributed to our null finding. Future research on the role of vitamin D in diabetes should utilize rigorously designed trial designs in high-risk, homogeneous populations, particularly during the post-pubertal pediatric years.

Introduction

The prevalence of pediatric obesity in the US has significantly increased over the past three decades.[1] Data from the most recent National Health and Nutrition Examination Survey (NHANES 2007-2008) indicated that in the United States, 17 percent of children and adolescents aged two through 19 years are obese, and 32 percent are overweight or obese.[2] Concurrently, the prevalence of many comorbid cardiometabolic conditions, including pre-diabetes, has increased. In 1999-2000 the pre-diabetes estimate for adolescents was seven percent.[3] The most current estimate, based on 2005-2006 data, has more than doubled, with 16 percent of US adolescents meeting pre-diabetes criteria, with overweight and obese teenagers having even higher prevalence values of 18 and 30 percent, respectively.[4] In obese youth, pre-diabetes is a significant predictor of type 2 diabetes mellitus (T2DM) development.[5] Estimates of T2DM during pediatric years are lacking; however, silent T2DM was identified in four percent of obese adolescents in one multiethnic cohort study.[6]

Cost-effective public health prevention measures for pre-diabetes and T2DM are crucial since myriad sequelae, including heart disease, blindness, kidney disease, limb amputations, and pregnancy complications, can ultimately result.[7] The role of vitamin D in pre-diabetes and T2DM is an emerging extraskeletal area of research, supported by animal and cellular mechanistic findings, as well as cross-sectional and longitudinal studies in humans. This secosteroid hormone has been shown to be involved in both insulin secretion[8, 9] and action[10, 11] at the autocrine level of gene expression. In pediatric cross-sectional studies, vitamin D status has been positively associated with insulin sensitivity[12] and inversely related to fasting glucose[13] and homeostasis model assessment

of insulin resistance.[14] Longitudinal investigations of adult prospective cohorts present vitamin D status as an important modifier of diabetes risk.[15]

In the latest Dietary Reference Intake (DRI) report for calcium and vitamin D, the Institute of Medicine (IOM) dubbed the role of vitamin D in diabetes to be a "hypothesis of emerging interest" and pointed to the paucity of randomized controlled trial (RCT) data to establish causality and dose-response relationships.[16] Rigorously designed vitamin D intervention trials are needed to improve our knowledge generated from the observational studies. To date, randomized vitamin D supplementation trials examining diabetes-related outcomes have been conducted almost exclusively in adults.[17-35] Taken cumulatively, these trials provide equivocal results thus far, in large part due to the heterogeneity in study designs. Trial durations range from four days to seven years, while sample sizes vary from ten to over 33,000. Although Caucasians have predominantly been the race studied, South Asians, Indians, Iranians, Japanese, Turkish, and Lebanese populations have been examined as well. The ages studied range from 17 to elderly individuals. The disease status has widely varied (apparently healthy, overweight or obese, pre-diabetes, T2DM of short duration vs. established, gestational diabetes mellitus, and end-stage renal disease), as has the supplement form (D_3, $1\alpha(OH)D_3$, $1,25(OH)_2D_3$, in combination with calcium, and fortified yogurt drink), dosage (for D_3: 400 IU to 200,000 IU), and frequency of supplementation (single dose, daily, weekly, fortnightly). Outcome measures used include euglycemic hyperinsulinemic clamp-, intravenous glucose tolerance test-, and oral glucose tolerance test-derived measures of glucose uptake and insulin sensitivity, fasting surrogate measures in glycemia and insulin sensitivity, T2DM incidence, and inflammatory markers. Nonetheless, vitamin D trials to

date demonstrate improvements in hyperglycemia,[18] glucose control,[18] hyperinsulinemia,[20, 30] insulin sensitivity or resistance,[17-20, 25, 34] and insulin secretion.[20, 27, 31, 33] Interventions that have studied high-risk (e.g. pre-diabetes, overweight/obesity) samples[20, 25] with poor baseline vitamin D status[20] appear to be more efficacious than those conducted in healthy,[29] vitamin D replete individuals,[32] or those with established T2DM.[22] To our knowledge, only one vitamin D trial has assessed diabetes-related endpoints in a pediatric sample and that was using the active metabolite, $1,25(OH)_2D_3$, in 11 end-stage renal disease patients undergoing hemodialysis. Significant increases in glucose uptake, as well as in early and late components of insulin secretion, were observed.[31]

Although the recent 2010 IOM DRI report for vitamin D concluded that the majority of Americans are receiving adequate amounts of vitamin D, this is based on 25(OH)D cut-points that may not be applicable to pediatric populations or extraskeletal roles of vitamin D. Studies are currently underway to help define vitamin D sufficiency in pediatric populations. For now, the loosely-defined "hypovitaminosis D" exists in a significant number of children and adolescents. Recent studies utilizing nationally representative data, demonstrate that nine percent and 61 percent of children and adolescents have 25(OH)D levels less than 37 nmol/L and 72 nmol/L, respectively, representing 50.8 million US youth.[36] In children aged one to 11 years, 18 percent have serum vitamin D levels less than 50 nmol/L.[37]

Based on the aforementioned mechanistic, observational, and limited RCT data, suboptimal vitamin D status in children and adolescents may contribute to an increased risk of pre-diabetes and progression to T2DM. The purpose of this study was to conduct a

multi-site, randomized, dose-response, placebo-controlled trial over 12 weeks to determine the effects of vitamin D supplementation on fasting and surrogate measures of glycemia and insulin sensitivity in 320 early pubertal black and white, male and female children.

Subjects and Methods

Study Participants

Early pubertal (N = 320) males (aged 10 to 13 years) and females (aged 9 to 12 years) participated in the GAPI vitamin D supplementation trial, a 12-week randomized, triple-masked, multi-site, dose-response, placebo-controlled trial. Within each of four strata defined by race (black and white) and location (n = 160 at The University of Georgia [UGA] in Athens, GA; n = 160 at Purdue University [PU] in West Lafayette, IN and Indiana University School of Medicine [IUSM] in Indianapolis, IN), participants were equally randomized to receive oral placebo or vitamin D_3 (400, 1,000 2,000, 4,000 IU/day). Randomization was blocked by sex, race, and latitude, resulting in n = 8 per dose/sex/race/latitude group. Subjects were tested in two cohorts during the winters (October – March) of 2009-2010 and 2010-1011, when 25(OH)D levels were at their nadir. Recruitment targeted pediatricians' offices, elementary schools, and media, utilizing flyers, newspapers, postcards, and radio in the University and local communities.

Screening and Testing Protocol

All procedures were approved by the Institutional Review Boards for Human Subjects at UGA, PU, and IUSM. Each participant and their guardian completed informed assent and consent forms, respectively. Screening proceeded in two phases.

First, potential participants who met the age requirement were screened over the telephone using a medical questionnaire (**see Appendix A**) to assess race/ethnicity, medication and supplement use, and medical history. Potential subjects were mailed a sexual maturation self-assessment form, completed the form at home, and mailed it back to the laboratory. Males and females classified as early pubertal (i.e. stages 2 or 3 for genitalia and breast development as described by Tanner[38]), were scheduled for the first testing session and randomly assigned to one of the five doses. Exclusionary criteria included a self-rating of pubertal maturation other than stages 2 or 3, commencement of menarche, race/ethnicity other than non-Hispanic black or white, and medication use known to affect vitamin D or glucose/insulin metabolism. If participants were taking dietary supplements prior to enrollment, they were required to complete a one-month wash-out period before their first testing session. Subjects were asked to refrain from taking any herbal or dietary supplements during the duration of the study. There were five testing sessions at baseline, 3, 6, 9, and 12 weeks.

Supplement Formulation

Vitamin D_3 was manufactured by Douglas Laboratories, who packaged the supplements in tablet form and labeled them with product code numbers unknown to the research staff and study participants. A computer-generated randomization scheme was provided by the statistician (Hall) to the PI (Lewis) only. Subjects were instructed to consume 1 tablet/day of their assigned treatment. To facilitate compliance, the bottles were returned to the researchers at each testing session, and pill counts were performed. Potential adverse events of supplemental vitamin D were assessed using a questionnaire to document

any clinical symptoms, as well as serum and urinary calcium concentrations and 25(OH)D status to detect hypercalcemia, hypercalciuria, and hypervitaminosis D, respectively.

Anthropometry, Pubertal Maturation, and Maturity Offset

Measurements were conducted at baseline and 12 weeks according to the Anthropometric Standardization Reference protocol.[39] Weight was measured within 0.1 kg using an electronic scale (Seca Bella 840). Standing height was measured using a wall-mounted stadiometer within 0.1 cm (Novel Products Inc., Rockton, IL). Participants were measured in light outdoor clothing after the removal of shoes. Height and weight were assessed for calculation of sex- and age-specific BMI percentiles using CDC growth charts[40] (**see Appendix B**). Pubertal maturation was self-assessed for screening purposes and again at 12 weeks using a form for criteria for stages of genitalia and breast development as described by Tanner.[38] Maturity offset was employed to provide information regarding the timing of maturational growth to capture -1.5 to -0.3 years preceding peak height velocity[41] using sitting height (50 cm box) and leg (subischial) length using a wall-mounted stadiometer (**see Appendix C** for maturity offset sex-specific equations). One-way random effects model, single measure intraclass correlation coefficients (ICC) were computed for anthropometric procedures in females, 6 to 10 years of age (n = 10), measured by the same individual twice in a 2-week period. The ICC (R-value) and test-retest CVs (%) were as follows: standing height (0.99 and 0.4%), body weight (0.99 and 1.4%) and sitting height (0.97 and 0.9%), respectively.

Body Composition Measurement

Percentage body fat, total body fat mass (g), and fat free soft tissue (g) were determined by dual-energy X-ray absorptiometry (DXA; Delphi A [at UGA] and

Discovery W [at IUSM], Hologic Inc., Bedford MA; GE Healthcare Lunar iDXA; GE Medical Instruments, Madison, WI [at PU]) at baseline and 12 weeks by the same technician within each site using Pediatric Whole Body Analysis software versions. Quality assurance for DXA was performed by daily calibration against the standard phantoms provided by the manufacturer. At UGA, one-way random effects model, single measure ICCs were calculated in young females 5 to 8 years of age (n = 10) scanned twice at the laboratory during a 7-day period for measures of total body composition (all $R \geq 0.98$). Study-specific conversion equations (**see Appendix D**) were developed to standardize the instruments across sites.

For secondary analyses, subjects were divided into normal- and high-fat categories. Percent body fat cutoffs of ≥ 25 % for males and ≥ 32 % for females were used to delineate high-fat categories, while percent body fat values < 25% for males and < 32% for females were used as normal-fat categories. These cut-points align with the Cooper Institute's Fitnessgram Health Fitness Zone standards for body composition,[42] which are based on longitudinal results from the Bogalusa Heart Study, which examined the relationship between total body adiposity and cardiovascular disease risk in a large bi-racial sample of boys and girls.[43]

Biochemical Indices: Serum 25(OH)D, Glucose, and Insulin

At baseline, 6 weeks, and 12 weeks, blood samples were collected following an overnight fast for analysis of serum 25(OH)D, glucose, and insulin. Blood was placed on ice until centrifugation, and serum samples were stored at –80 °C until analysis. Reference controls (from each kit) and internal controls (in-house pooled samples) were included with each assay run for quality control. All biochemical analyses were assayed

using a block design, such that baseline, 6-week, and 12-week samples from the same subject were assayed at one time by the same investigator using the same kit.

Serum 25(OH)D samples were assayed using a 2-step radioimmunoassay (RIA; DiaSorin Laboratories, Stillwater, MN) and run in duplicate. The interassay coefficient of variations (CVs) ranged from 5.2 – 8.7 % and the intra-assay CVs ranged from 5.5 – 8.2%. The detection limits of the assay are 5 – 100 ng/mL. Analytical reliability of 25(OH)D assay was further monitored through participation in DEQAS (Vitamin D External Quality Assessment Scheme). Serum glucose concentrations were determined in triplicate using a microtiter modification of the enzymatic Autokit Glucose method (Wako Chemicals USA, Richmond, VA). The detection limit for this assay is 0 – 500 mg/dL. The mean intra- and interassay CVs were 1.8% and 2.2%, respectively. Serum insulin was analyzed, in duplicate, by Human Insulin Specific RIA (HI-14K; St. Charles, MO). The detection limit is 3.125 – 100 uU/mL. The mean intra- and interassay CVs were 3.5% and 5.3%, respectively.

Surrogate Measures of Insulin Sensitivity

Fasting Glucose and insulin values were incorporated into the homeostasis model assessment of insulin resistance (HOMA-IR) as our surrogate for insulin resistance. HOMA-IR = [(fasting glucose [mg/dL] x fasting insulin [uU/mL]) / 405].[44] Our surrogate measure for insulin sensitivity was the quantitative insulin sensitivity check index (QUICKI), which equals 1/[log(fasting glucose{mg/dL}) + log(fasting insulin{uU/mL})]. HOMA-IR and QUICKI correlate strongly (r = 0.6 – 0.8) with the gold standard euglycemic hyperinsulinemic clamp technique.[45, 46]

Dietary Intake

Three-day food records (**see Appendix E**), a valid and reliable method for estimating energy and nutrient intakes in children,[47-49] were completed by participants and their parents at baseline and 12 weeks. The form specifically inquired about the consumption of calcium-fortified foods and nutritional supplements. Each record included two weekdays and one weekend day and included time of eating, type of food and amount, as well as preparation method. Diet records were analyzed using Food Processor SQL version 9.7.3 (ESHA Research, Salem, OR, USA) to assess energy (kcal), vitamin D (IU), and calcium (mg). One-way random effects model, average measure (i.e. 3-days) ICCs were conducted in female children 6 to 10 years of age (n = 10), whose 3-day diet records were completed twice in a 2-week period and were calculated for vitamin D (R = 0.98), calcium (R = 0.93), and energy (R = 0.86).

Statistical Analyses and Power Analysis

Descriptive statistics were generated, as well as range and normality checks. Analysis of variance (ANOVA) was used to compare means for baseline variables across the five treatment groups. If the test of between-subjects effect was significant for treatment, Tukey post hoc tests for all pair-wise comparisons were used to determine which groups were significantly different. Two-tailed independent samples *t*-tests were used to compare means between race, gender, sites, and adiposity groups for all baseline variables.

Linear mixed model analyses using repeated measures analysis of variance (RM-ANOVA) were used to examine the effect of vitamin D supplementation on the four dependent variables: glucose, insulin, HOMA-IR, and QUICKI. These models assumed a

general variance-covariance structure to capture within-subject correlation and heteroscedasticity, rather than the compound symmetry structure of classical RM-ANOVA. Models included main effects of treatment and time, and interactions between these factors (treatment x time). Secondarily, repeated measures analysis of covariance (RM-ANCOVA) models were considered to control for possible confounding or effect-modifying effects of important baseline covariates. In particular, five covariates of interest (sex, race, pubertal maturation stage, maturity offset, and percent body fat) were considered for inclusion in the models and retained based upon a backward selection procedure, using an alpha = 0.1 significance level for retention in the model following rules of hierarchy (e.g. drop 3-way interaction before a 2-way interaction or main effect). The adequacy of the fitted models was checked using residual plots and other standard diagnostics. Analyses were conducted on an intent-to-treat basis.

Secondary analyses were conducted using Pearson correlations to compute associations between baseline and percent change values of biochemical variable and age, anthropometry, maturation, body composition, and dietary intake. Partial correlations were utilized to relate baseline 25(OH)D with insulin sensitivity dependent variables, controlling for covariates of interest (race, gender, age, pubertal maturation, fat mass, BMI). For all analyses, insulin and HOMA-IR were analyzed on the natural log scale to correct for non-constant variance observed on the original scale, and an alpha = 0.05 was used to denote statistical significance. The mixed effects model analyses were computed using Statistical Analysis Software (SAS, Cary, NC, Version 9.2). All other analyses were performed using SPSS version 18.03 (PASW Statistics, Chicago, IL).

Sample size was computed to detect statistical differences in 12-week changes in the primary outcome variables (serum glucose, insulin, HOMA-IR, and QUICKI) after supplementation with vitamin D or placebo. For power calculations, we assumed that the variance distribution of these outcome variables would be uniform within the range of 0-20 and a root mean square error of 0.38 (based on preliminary data). Under these conditions n = 64/group will provide > 90% power using an alpha level of 0.05. Allowing for an attrition rate of ≥ 20% (based on our prospective childhood studies) and equal randomization to the five treatment arms, n = 64/group were recruited (N = 320).

Results

Participant Characteristics

In the sample of 320 early pubertal children, 319 were tested at baseline, and 299 completed the 12-week intervention, for a 94% retention rate. Based on CDC gender-specific BMI-for-age percentiles, 2% of participants were classified as underweight, 55% normal weight, 21% overweight, and 21% obese. Dose-response changes in mean serum vitamin D concentrations from baseline to 12-weeks were: 71.3 to 63.4 nmol/L for placebo group, 71.2 to 76.6 nmol/L for 400 IU group, 71.0 to 90.2 nmol/L for 1,000 IU group, 65.7 to 101.8 nmol/L for 2,000 IU group, and 69.9 to 147.3 nmol/L for 4,000 IU group. Therefore, at baseline only 1% of the sample had serum 25(OH)D levels < 30 nmol/L, 15% < 50 nmol/L, and 66% < 80 nmol/L, but by 12 weeks, 0.7%, 9%, and 41% had serum vitamin D concentrations < 30 nmol/L, < 50 nmol/L, and < 80 nmol/L, respectively. In the overall cohort at baseline, the 25(OH)D levels ranged from 25.3 to 114.7 nmol/L; after 12 weeks of placebo or vitamin D supplementation, serum vitamin D

levels ranged from 24.2 to 237.4 nmol/L. Based on fasting glucose measurements, only 5% of the children met fasting pre-diabetic criteria during at least one measurement.

Baseline descriptive characteristics of the participants overall and by treatment group are presented in **Table 3.1**. Significant group differences were observed for weight and lean mass, as the 2,000 IU group was heavier than the 1,000 IU, 400 IU, and placebo groups. The 2,000 IU group also had greater FFM than the 1,000 IU treatment arm. There were no significant differences in the other baseline variables. Baseline characteristics divided by gender and race are also displayed in Table 3.1. Males had more lean mass and were older and taller than the females. The males were more mature (based on secondary sexual characteristics described by Tanner) yet farther from their PHV (based on maturity offset). Females had higher fat mass, percent body fat, BMI percentile, insulin, and HOMA-IR but lower QUICKI. Racial differences included higher BMI percentile, insulin, and HOMA-IR for blacks, as well as lower 25(OH)D concentrations, QUICKI, and calcium intake. Their white counterparts were older and closer to PHV.

Although not shown in the table, children in the high-fat category were younger and less insulin sensitive but more insulin resistant than their normal-fat peers. The high-fat children had lower 25(OH)D levels than their leaner counterparts. Site differences were also noted. When comparing latitudes (southern latitude: UGA [n = 80 blacks, n = 80 whites] vs. northern latitude: PU and IUSM [n = 80 blacks, n = 80 whites]), serum vitamin D, pubertal stage, and lean mass were higher in the southern cohort, while percent body fat and dietary vitamin D intake were higher in the northern children. These differences were driven by the blacks, since no differences were observed between whites in the southern vs. northern cohorts. In contrast, blacks in the south had higher serum

vitamin D (64.2 vs. 56.1 nmol/L) and lean mass than their northern counterparts, and northern blacks had higher fat mass, percent body fat (35.1% vs. 28.5%), and dietary vitamin D intake (179.5 IU/day vs. 148.0 IU/day) than their southern black peers.

Vitamin D Supplementation Effects on Glycemia and Insulin Sensitivity

Profile plots of estimated joint treatment means for glucose, insulin, HOMA-IR, and QUICKI across the 12-week trial are displayed in **Figure 3.1**. The effect of vitamin D supplementation over time was non-significant for all treatment groups. Treatment*time interactions are as follows: glucose ($F = 0.60$, $p = 0.780$), insulin ($F = 0.41$, $p = 0.918$), HOMA-IR ($F = 0.46$, $p = 0.883$), and QUICKI ($F = 0.43$, $p = 0.905$). However, main effects for time were significant for all four outcomes. Glucose ($F = 5.79$, $p = 0.003$), insulin ($F = 11.59$, $p < 0.001$), and HOMA-IR ($F = 12.41$, $p < 0.001$) increased over time, while QUICKI ($F = 12.13$, $p < 0.001$) decreased over time.

Interaction Effects of Race, Gender, Adiposity, and Maturation

Important baseline covariates (gender, race, pubertal maturation, and percent body fat) were investigated, but after employing our backward selection procedure, none of the 3-way interactions (treatment*time*covariate of interest) remained in the model, using an alpha = 0.1.

Pearson and Partial Correlations

Consistent with the high- vs. normal-fat group differences noted for biochemical indices, Pearson correlations in **Table 3.2** relate baseline anthropometry and body composition measures with baseline serum vitamin D, glycemia, and insulin sensitivity data. Serum 25(OH)D and QUICKI were significantly and negatively associated with

weight, height, BMI percentile, fat mass, percent body fat, and lean mass, while insulin and HOMA-IR were positively related with the same parameters.

Significant Pearson correlations were also seen for maturation indices and biochemical parameters. Pubertal maturation was positively associated with insulin ($r = 0.159$, $p = 0.005$) and HOMA-IR ($r = 0.148$, $p = 0.008$) and negatively related to QUICKI ($r = -0.154$, $p = 0.006$). Similar but stronger associations were noted for maturity offset and insulin ($r = 0.231$, $p < 0.001$), HOMA-IR (0.197, $p < 0.001$), and QUICKI ($r = -0.203$, $p < 0.001$). Neither maturation measures were significantly associated with serum glucose.

Figure 3.2 presents the partial correlations relating serum vitamin D to the four dependent variables of interest. These revealed that baseline serum 25(OH)D was significantly and inversely associated with fasting insulin ($r = -0.140$, $p = 0.017$) and HOMA-IR ($r = -0.146$, $p = 0.012$), as well as positively associated with QUICKI ($r = 0.142$, $p = 0.015$) after controlling for race, gender, age, pubertal maturation, fat mass, and BMI. Pearson correlations relating percent change values between biochemical parameters, anthropometry, and body composition lacked statistical significance. Lastly, using 3-day diet record estimates, dietary vitamin D and calcium intake were positively associated ($r = 0.689$, $p < 0.001$), and a trend between serum vitamin D and dietary calcium was noted ($r = 0.108$, $p = 0.059$).

Discussion

This is the first pediatric supplementation trial to examine glycemia and insulin sensitivity outcomes in response to varying doses of vitamin D. The main finding of this prospective intervention is that although glycemia and insulin sensitivity outcomes

changed over the course of the trial, vitamin D supplementation did not significantly alter these parameters over 12 weeks in early pubertal children.

There are several factors that likely contributed to our null treatment*time findings for vitamin D supplementation on insulin sensitivity. Despite the fact that a high percentage of the children in the current study were overweight or obese, the study sample was apparently healthy, as well as normo-glycemic and –insulinemic. Previous research in adult vitamin D trials suggests that a critical window may exist for the efficacy of vitamin D supplementation, namely at an early stage of diabetes or perhaps during pre-diabetes.[15] Trials conducted in apparently healthy, non-diabetic adults have not succeeded in showing significant improvements in cardiometabolic and diabetic outcomes.[24, 26, 29, 35, 50] Also, once an individual has established T2DM, particularly of longer duration and requiring medication usage, vitamin D supplementation may not provide a substantial benefit,[22] although one small trial found an improvement in insulin secretion in diabetics treated with oral hypoglycemic agents after receiving 1,332 IU vitamin D daily over one month.[27] Conversely, favorable evidence from supplementation trials appears to occur especially in high-risk individuals (i.e. glucose intolerant, insulin resistant, overweight, or obese). Vitamin D supplementation has positive effects on DM-related outcomes in subjects with T2DM of shorter duration, pre-diabetes, and overweight and obese individuals.[17-19, 21, 25, 30] Pittas et al. found vitamin D supplementation protective for those with pre-diabetes, but not those with normal glucose tolerance,[25] while Orwoll et al.[30] reported improvements in insulin response to a glucose load in those subjects with shorter T2DM duration. These are important findings, as they

may reflect a critical period for vitamin D efficacy and the ability of supplementation to delay the progression to clinical T2DM.

Another possible contributing factor to our null finding is baseline vitamin D status. Although hypovitaminosis D is currently loosely-defined in pediatric populations, on average, our children were considered vitamin D "sufficient" (70 nmol/L) based on IOM adult standards for skeletal outcomes. Vitamin D insufficient populations appear to benefit more from supplementation than their vitamin D-sufficient counterparts. This phenomenon likely contributed to the significant results demonstrated in interventions in which subjects were vitamin D-deficient at baseline,[17-21, 27, 30] as well as the null results seen in vitamin D-sufficient subjects.[32] However, "deficiency", "insufficiency", and "sufficiency" terminology should be used cautiously when discussing pediatric vitamin D status and when exploring extraskeletal indicators, since the current IOM recommendations stem from skeletal outcomes. Although a 25(OH)D concentration greater than 50 nmol/L has been proposed by the IOM to define sufficiency, the desirable concentration based on cardiometabolic outcomes may be higher. Support for this assertion comes from the von Hurst et al.[20] trial in which a 25(OH)D between 80 – 119 nmol/L was found to optimally increase insulin sensitivity in vitamin D-deficient, insulin resistant South Asian women. Because lower 25(OH)D values are found in populations living at higher latitudes, one half our participants were recruited from the northern latitude of 40° N (PU and IUSM). Also, participants were only tested in the wintertime to take advantage of the seasonal declines in serum 25(OH)D observed during the winter months. Both of these strategies proved insufficient in generating a population with low baseline circulating 25(OH)D concentrations.

The effect of pubertal maturation on glucose and insulin concentrations may have introduced significant heterogeneity or noise into our data set, leading to non-significant outcomes. When conducting research in pre-, early-, and peri-pubertal youth, it is important to consider the established phenomenon of transient insulin resistance that occurs during the pubertal transition in both sexes regardless of adiposity status.[51, 52] In fact, our significant main effects for time for all four outcomes confirm that this phenomenon was occurring in our children over the course of the trial. Glucose, insulin, and HOMA-IR increased significantly over the 12 weeks, while QUICKI significantly decreased. In this study, females, black children, those in pubertal maturation stage 3, and high-fat children were more insulin resistant than their respective counterparts. We addressed these possible confounding variables; however, none remained significant in the final models. Future trials should be aimed at a more homogenous sample such as post-pubertal adolescents, to minimize the confounding effect of maturation on insulin sensitivity.

The baseline differences we observed related to 25(OH)D status, glycemia, and insulin sensitivity, based on race, gender, maturation, and adiposity categories served as an impetus for their inclusion as covariates in our model. However, none of these possible confounders appeared to interact with the effect of vitamin D supplementation over the course of our trial.

We observed significant associations between baseline 25(OH)D and fasting insulin, insulin resistance, and insulin sensitivity after adjusting for all pertinent covariates. Similar cross-sectional relationships have been described for HOMA-IR in adolescents[14] and for QUICKI in children and adolescents.[53] Cellular and animal studies

help reveal the underlying mechanisms of vitamin D's role in insulin secretion and action. The VDR[54] and 1-alpha-hydroxylase enzyme[55, 56] are present in pancreatic beta cells. Vitamin D deficiency and VDR-knockout models, independent of calcium status, reduce insulin secretion,[8, 9, 57, 58] but vitamin D supplementation restores insulin secretion.[9] In terms of insulin action, the VDR is found on a limited basis on skeletal muscle cells,[59, 60] and a vitamin D response element has been identified in the promoter region of the human insulin receptor gene.[61] Additionally, $1,25(OH)_2D_3$ has been shown to stimulate insulin receptor expression, phosphoinositide 3-kinase activity, and insulin responsiveness.[10, 11]

The overweight (21%) and obesity (21%) prevalence in our children is higher than national estimates. In the US approximately 15% and 17% of youth aged two through 19 are overweight and obese, respectively.[2] More specifically, obesity prevalence is 20% in children aged six to 11 and 18% in adolescents aged 12 to 19 years.[1] This discrepancy is important since excess adiposity is associated with myriad comorbidities, including glucose-insulin abnormalities. Even though our baseline pre-diabetes prevalence of five percent is well below NHANES pre-diabetes estimate for adolescents (16%),[4] our children did display relatively high HOMA-IR values (overall mean = 4.4). An estimated cutoff point for HOMA-IR to identify metabolic syndrome in healthy and obese children is > 3 in non-obese and obese Hispanic children,[62] and a HOMA-IR > 4 was estimated for insulin resistance diagnosis in German adolescents.[63]

In terms of dietary intake, 3-day diet records captured that our participants consumed approximately 169 IU of vitamin D per day, which is less than the national average total vitamin D intake (304 IU daily) for children ages nine to 13 years.[64]

Assuming minimal sun exposure, our subjects would require an additional 431 IU of vitamin D per day in order to meet the new Recommended Dietary Allowance of 600 IU per day for those ages 1 to 70 years.[16] This would be challenging without a significant increase in fortified dairy products, fatty fish, or supplementation. We also observed an average calcium intake 400 mg lower than the current RDA (1,300 mg/day for 9-13 year olds).

Our study possesses several strengths, including the rigorous study design, which included randomization, placebo control, triple masking, four treatment arms of sufficient dose range, multiple latitudes, large sample size, both genders, and two distinct races. Using a vitamin D_3 formulation ingested daily is also a strength, since this form has been shown to increase 25(OH)D more potently than vitamin D_2.[65] Additionally, we aimed to understand the effect of pertinent confounders, age, race, gender, maturation, and adiposity, on the supplementation results over time. Our study also has some limitations. We did not utilize oral glucose tolerance test-derived or clamp-derived measures of insulin sensitivity, although in non-diabetes children, like our study sample, fasting insulin, HOMA-IR, and QUICKI correlate strongly ($r = 0.6 – 0.8$) with the gold standard euglycemic hyperinsulinemic clamp (EHC) technique[45, 46] and the frequently sampled intravenous glucose tolerance test, across race/ethnicity groups.[66] HOMA-IR and QUICKI have proved to be good measures of insulin resistance and sensitivity, respectively, across the five stages of sexual maturation as described by Tanner.[52]

With the rise in obesity and associated comorbidities and healthcare costs, cost-effective public health prevention measures are needed. Vitamin D supplementation is a affordable, safe way to achieve 25(OH)D sufficiency. Future research on the role of

vitamin D in diabetes should utilize rigorous study designs like the current project. High-risk populations, such as obese individuals with pre-diabetes and hypovitaminosis D, particularly during the pediatric years and including minority populations, should be of particular interest. Clinically relevant outcomes should be assessed using rigorous methodology. Trials conducted in pediatric populations should aim to examine homogeneous samples, especially with regard to pubertal maturation, as this affects glucose and insulin profiles. Such research will help elucidate the question of whether vitamin D sufficiency improves insulin sensitivity during pediatric years.

In summary, we report novel findings from the first pediatric vitamin D intervention to examine effects on glycemia and insulin sensitivity. Although 12 weeks of vitamin D supplementation failed to improve glucose, insulin, HOMA-IR, and QUICKI, the heterogeneity introduced into our glucose-insulin outcomes due to the pubertal maturation of our subjects, as well as the apparently healthy, vitamin D sufficient status of our children, are key facets that may have contributed to our null finding.

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Table 3.1 Baseline Participant Characteristics Overall, by Treatment, and Divided by Gender and Race Categories[1,2]

	Overall Characteristics					Characteristics by Treatment					
	Overall (N = 320)	**Male (n = 160)**	**Female (n = 160)**	**White (n = 160)**	**Black (n = 160)**	**Placebo (n = 64)**	**400 IU (n = 64)**	**1,000 IU (n = 64)**	**2,000 IU (n = 64)**	**4,000 IU (n = 64)**	***P*-value for Treatment[3]**
Age (yrs)	11.3 ± .07	12.0 ± .10	10.7 ± .10[§]	11.6 ± 0.1	11.2 ± 0.1*	11.6 ± .20	11.2 ± .20	11.1 ± .20	11.4 ± .20	11.5 ± .20	0.183
Anthropometry											
Weight (kg)	47.4 ± .70	48.6 ± 1.0	46.1 ± .80	46.1 ± 1.0	48.6 ± 1.0	46.5 ± 1.6[b]	46.4 ± 1.5[b]	46.6 ± 1.6[b]	52.0 ± 1.5[a]	46.9 ± 1.6	.042
Height (cm)	150.7 ± .50	152.7 ± .80	148.7 ± .70[§]	151.3 ± .80	150.1 ±.70	150.6 ± 1.2	150.6 ± 1.2	149.3 ± 1.2	153.5 ± 1.2	150.0 ± 1.2	.120
BMI-for-age (%)	68.0 ± 1.6	64.4 ± 2.4	71.6 ± 2.2*	61.3 ± 2.4	74.4 ± 2.1[§]	65.6 ± 3.8	67.0 ± 3.7	70.9 ± 3.7	71.5 ± 3.7	68.6 ± 3.7	.766
Maturation											
Pubertal Maturation[4]	2.4 ± .03	2.4 ± .04	2.3 ± .04*	2.3 ± .04	2.4 ± .04	2.5 ± .10	2.3 ± .10	2.3 ± .10	2.5 ± .10	2.2 ± .10	.027
Maturity Offset (yrs)	-1.1 ± .07	-1.5 ± .10	-0.6 ± 0.1[§]	-0.9 ± .10	-1.3 ± .10*	-1.1 ± .10	-1.3 ± 0.1	-1.4 ± 0.1	-1.0 ± 0.1	-1.2 ± .10	.212
Body Composition[5]											
Fat mass (kg)	14.9 ± .40	13.7 ± .60	16.1 ± 0.6*	14.2 ± .60	15.5 ± .60	14.0 ± 1.0	14.2 ± 1.0	14.9 ± 1.0	16.4 ± 0.9	15.0 ± 1.0	.424
Percent body fat	31.2 ± .50	28.2 ± .70	34.4 ± 0.7[§]	30.5 ± .80	31.8 ± .80	29.8 ± 1.2	30.6 ± 1.2	31.9 ± 1.2	31.6 ± 1.2	32.1 ± 1.2	.660
Lean mass (kg)	30.1± .40	32.3 ± .60	28.5 ± 0.4[§]	30.4 ± .60	30.6 ± .50	30.4 ± .90	30.0 ± .90	29.3 ± .90[b]	33.0± .90[a]	29.7 ± .90	.027
Biochemical											
25(OH)D (nmol/L)	70.0 ± 1.0	70.6 ± 1.3	69.1 ± 1.6	80.0 ± 1.2	60.1 ± 1.3[§]	71.5 ± 2.3	71.2 ± 2.3	71.0 ± 2.3	66.3 ± 2.4	69.9 ± 2.3	.494
Serum Glucose (mg/dL)	89.0 ± .40	89.3 ± .60	88.7 ± 0.6	88.5 ± .60	89.4 ± .60	88.6 ± .90	90.6 ± .90	88.2 ± .90	88.9 ± .90	88.9 ± .90	.408
Serum Insulin (uU/mL)	20.0 ± .60	18.5 ± .80	21.4 ± 0.8*	18.1 ± .80	21.8 ± .80[§]	20.6 ± 1.3	19.3 ± 1.3	19.5 ± 1.3	21.1 ± 1.3	19.0 ± 1.3	.759
HOMA-IR	4.4 ± .10	4.1 ± .2.0	4.7 ± 0.2*	4.0 ± .20	4.8 ± .20*	4.6 ± .30	4.4 ± .30	4.3 ± .30	4.6 ± .30	4.2 ± .30	.809
QUICKI	.31± .001	.32 ± .002	.31 ± .002*	.32 ± .002	.31 ± .002[§]	.31 ± .003	.31 ± .003	.31 ± .003	.31 ± .003	.32 ± .003	.537
Dietary Intake											
Energy Intake (kcal/d)	2001 ± 31	2085 ± 46	1914 ± 43*	2061 ± 44	1939 ± 45	1978 ± 69	1996 ± 72	1986 ± 70	2000 ± 72	2048 ± 74	.968
Vitamin D Intake (IU/d)	169.2 ± 7.1	178.8 ± 10.8	159.5 ± 9.2	173.0 ± 11.6	165.4 ± 8.1	150.7 ± 15.2	197.8 ± 15.9	143.1 ± 15.4	183.7 ± 15.9	174.8 ± 16.4	.078
Calcium Intake (mg/d)	901 ± 23	907 ± 33	894 ± 31	964 ± 34	837 ± 29*	837 ± 48	1000 ± 51	823 ± 49	914 ± 51	945 ± 52	.065

[1] Values are presented as means ± SE.

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

[3] Test of significance based on ANOVA. Tukey post hoc tests for all pair-wise comparisons were used to determine which groups were significantly different. Means in a row with superscripts without a common letter differ, $P < 0.05$

[4] Stages based upon secondary sexual characteristics as described by Tanner.

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

* Group means for gender or race are significantly different, $p < 0.05$. Test of significance between groups was based on independent samples *t*-tests.

§ Group means for gender or race are significantly different, $P < 0.001$. Test of significance between groups was based on independent samples *t*-tests.

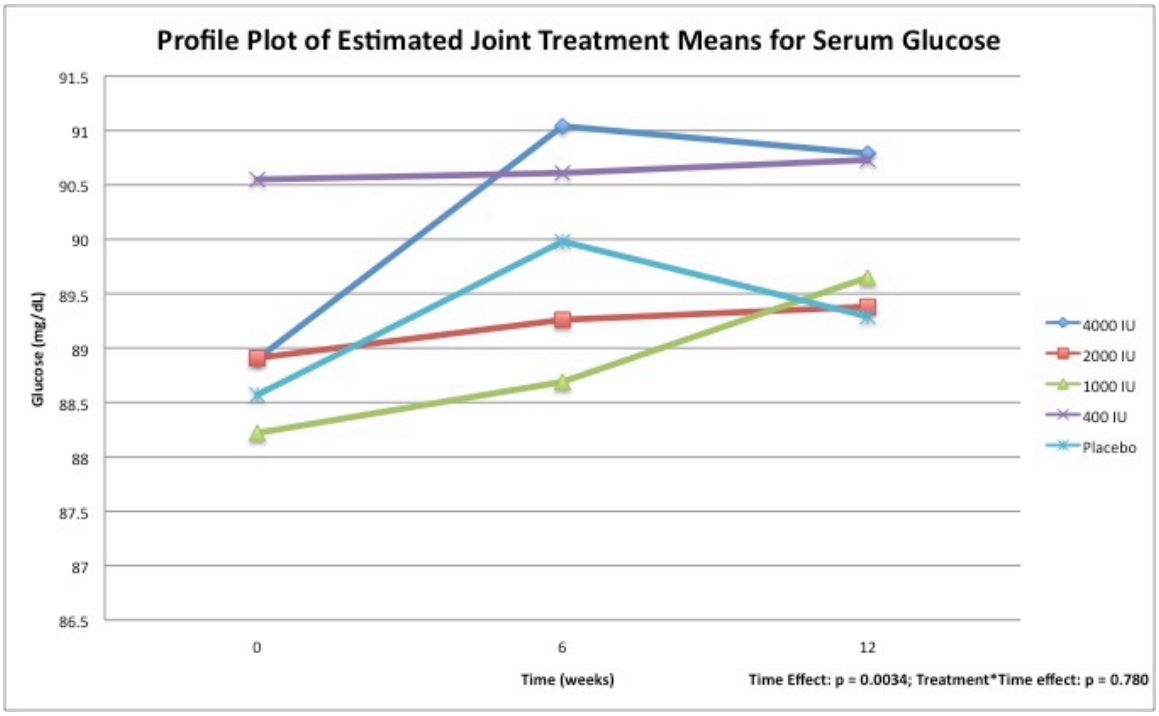
Table 3.2 Relationships between Biochemical Parameters, Anthropometry, and Body Composition[1]

	Weight		Height		BMI Percentile		Fat Mass		Percent body fat		Lean Mass	
	r	***p*-value**	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**	**r**	***p*-value**
25(OH)D	-0.177*	0.002	- 0.81	0.152	-0.163*	0.003	-0.139*	0.016	-0.085	0.143	-0.111	0.054
Serum Glucose	0.027	0.630	0.001	0.983	0.071	0.211	0.032	0.581	0.040	0.491	0.007	0.909
Serum Insulin	0.498*	< 0.001	0.195*	< 0.001	0.473*	< 0.001	0.511*	< 0.001	0.413*	< 0.001	0.290*	< 0.001
HOMA-IR	0.472*	< 0.001	0.183*	0.001	0.461*	< 0.001	0.486*	< 0.001	0.395*	< 0.001	0.273*	< 0.001
QUICKI	-0.467*	< 0.001	-0.192*	0.001	-0.462*	< 0.001	-0.473*	< 0.001	-0.383*	< 0.001	-0.278*	< 0.001

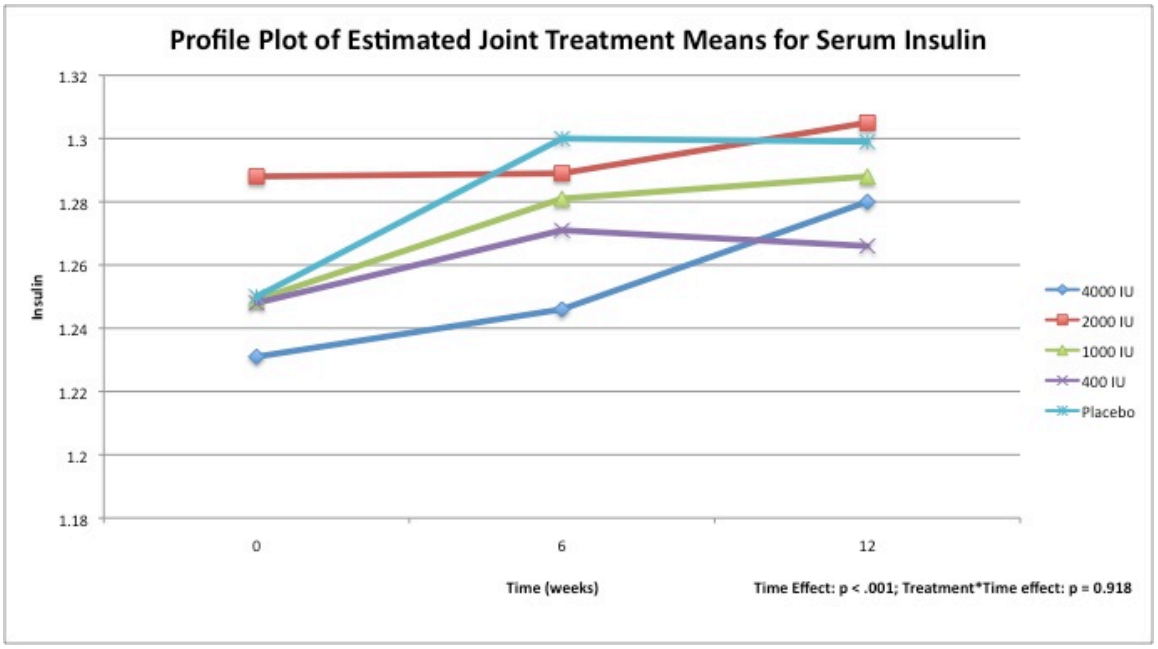
[1] Data presented as unadjusted Pearson correlations and accompanying *p*-values. Insulin and HOMA-IR were analyzed on the natural log scale to correct for non-constant variance observed on the original scale

* *P*-value < 0.05

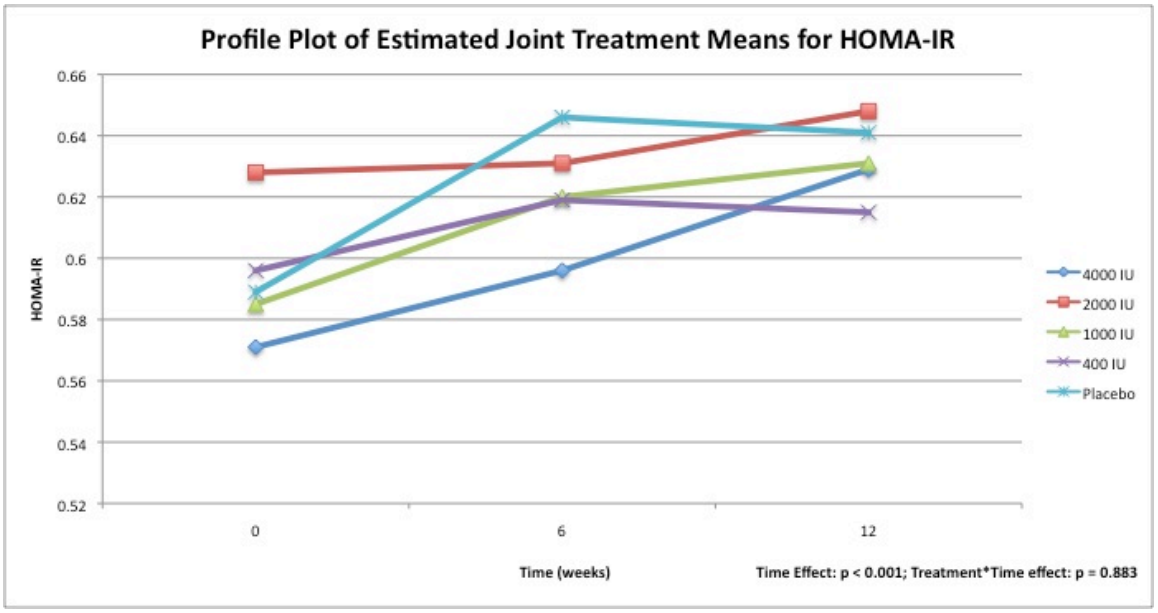
(A) Glucose



(B) Insulin



(C) HOMA-IR



(D) QUICKI

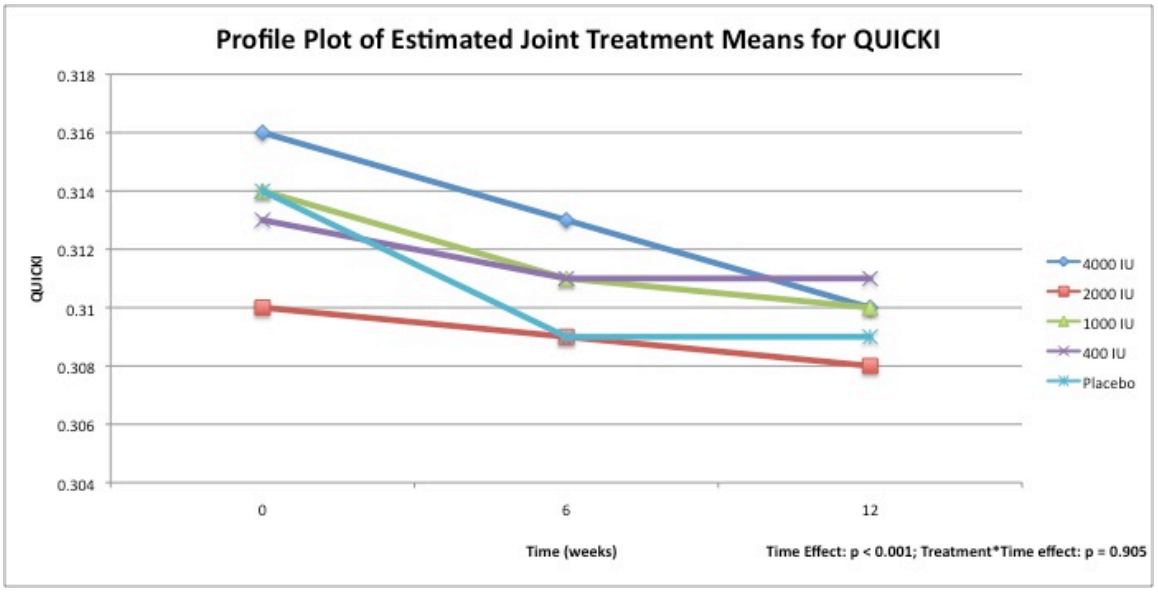
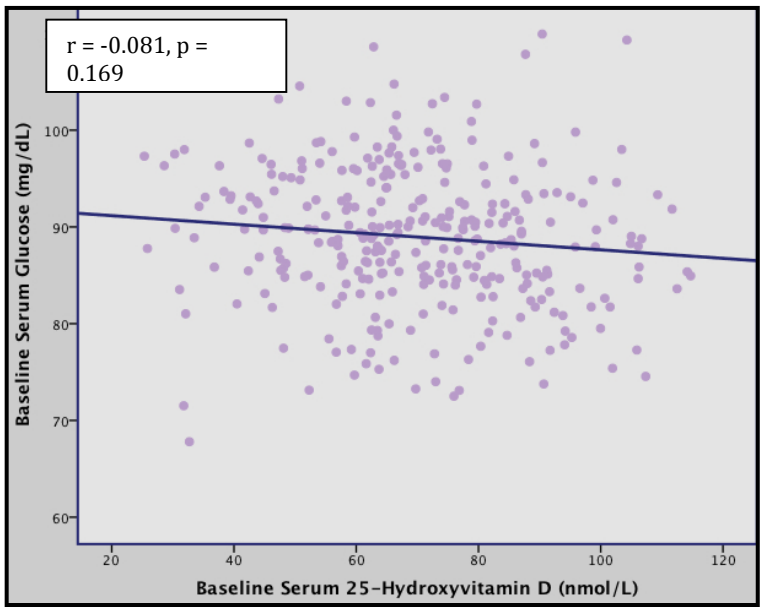


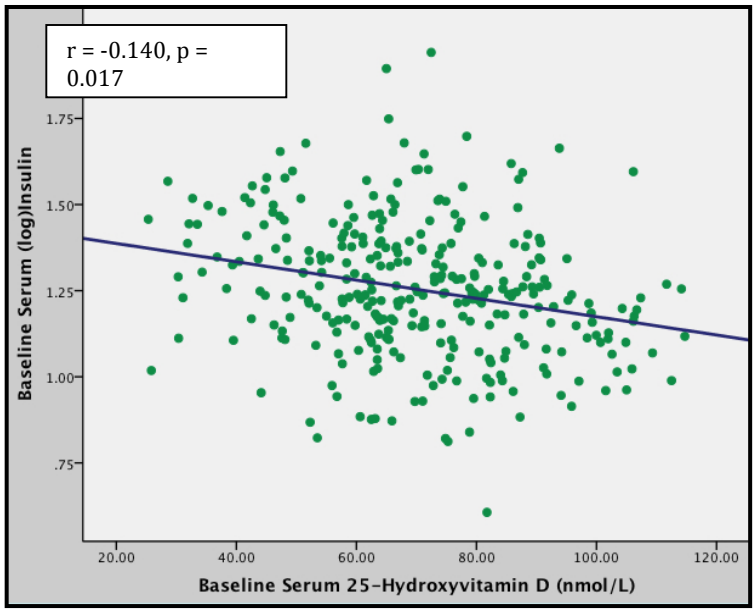
Figure 3.1 Profile Plots of Estimated Joint Treatment Means for Glucose, Insulin, HOMA-IR, and QUICKI over 12-Weeks of Supplementation[1]

[1] Linear mixed model analyses using repeated measures analysis of variance (RM-ANOVA) were used to examine the effect of vitamin D supplementation on the four dependent variables: glucose, insulin, HOMA-IR, and QUICKI. Profile plots represent the treatment*time interaction. Insulin and HOMA-IR were analyzed on the natural log scale to correct for non-constant variance observed on the original scale.

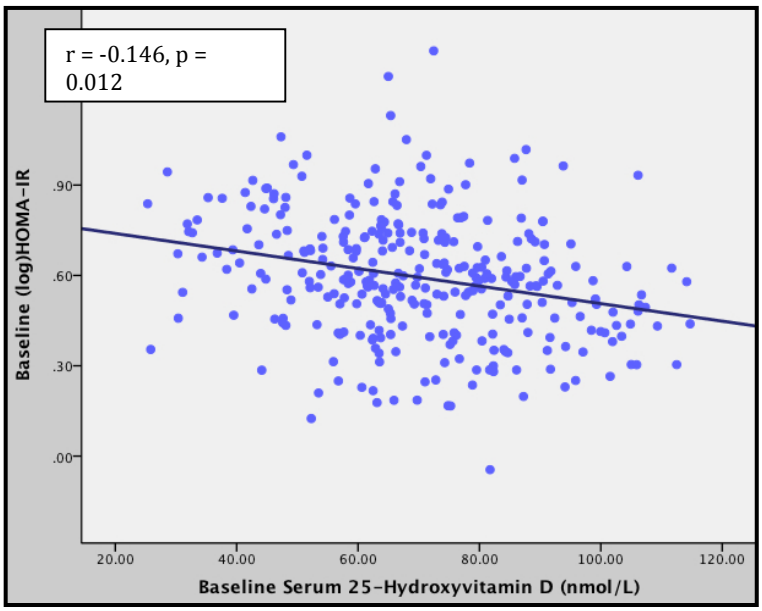
(A) Glucose and 25(OH)D



(B) Insulin and 25(OH)D



(C) HOMA-IR and 25(OH)D



(D) QUICKI and 25(OH)D

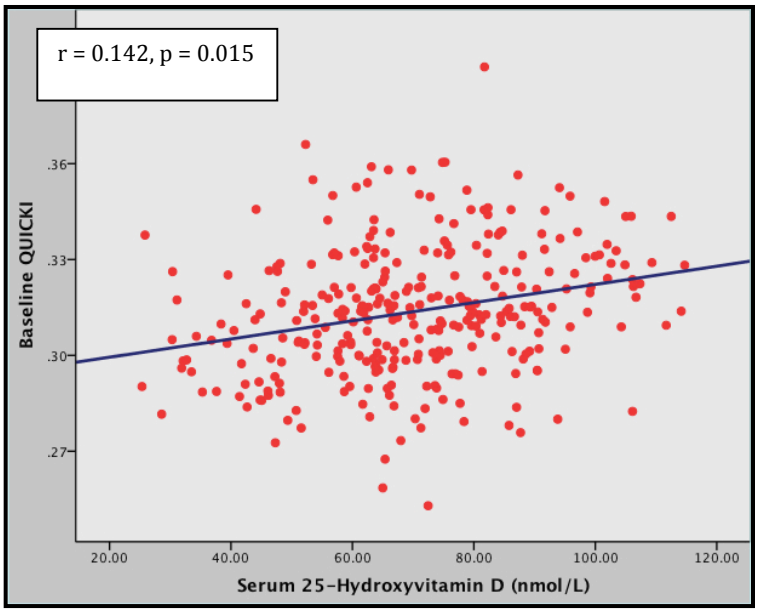


Figure 3.2 Relationships between Baseline 25-Hydroxyvitamin D, Glycemia, and Insulin Sensitivity[1]

[1] Partial correlations were adjusted for race, sex, age, pubertal maturation, fat mass, and BMI. Insulin and HOMA-IR were analyzed on the natural log scale to correct for non-constant variance observed on the original scale.

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CHAPTER 4

SERUM VITAMIN D CONCENTRATION AND ITS ASSOCIATION WITH MARKERS OF DIABETES AND CARDIOVASCULAR DISEASE RISK IN OVERWEIGHT CHILDREN[1]

[1] Ferira AJ, Pollock NK, Laing EM, Hausman DB, Gower BA, Allison JD, Zhu H, Dong Y, Lewis RD, David CL. To be submitted to *Diabetes Care*.

Abstract

Objective: To determine the relationship between serum 25-hydroxyvitamin D [25(OH)D] and the presence of individual cardiometabolic risk factors (CMR), two or more CMR, insulin, insulin sensitivity (Matsuda index), beta-cell function (insulinogenic index), inflammatory-related markers, and total and visceral fat in 222 sedentary, overweight children (aged 7-11 years, 58% female, 58% black, 86% obese).

Research, Design, and Methods: Fasting serum glucose, insulin, Matsuda index, insulinogenic index, lipids, C-reactive protein (CRP), leptin, adiponectin, and 25(OH)D concentrations were derived from an oral glucose tolerance test. CMR were measured with standard methods and defined according to pediatric definitions of metabolic syndrome. Percentage body fat (%fat) and visceral adipose tissue (VAT) were measured by dual-energy X-ray absorptiometry (DXA) and magnetic resonance imaging (MRI), respectively.

Results: The odds for having ≥ 2 CMR was higher in the first tertile (< 52.7 nmol/l) of 25(OH)D than in the third tertile (≥ 69.4 nmol/l) of 25(OH)D (OR: 3.52; 95% CI: 1.39, 8.98; $P < 0.01$), after adjusting for sex, race, sexual maturation stage, and season. Waist circumference, insulin, HOMA-IR, triglycerides, leptin, and VAT were inversely related, and Matsuda index, HDL-cholesterol, and adiponectin were directly related with 25(OH)D (all $P < 0.05$), after adjusting for the same covariates; further adjustment with VAT attenuated the significance of these associations and the OR (all $P > 0.05$). No significant relations were found between 25(OH)D and blood pressure, fasting glucose, insulinogenic index, C-reactive protein, or %fat.

Conclusions: In overweight and obese preadolescent children, lower serum 25(OH)D concentrations are associated with multiple measures known to increase risk for diabetes and cardiovascular disease. In addition, our findings suggest that visceral, rather than general, adiposity may be more relevant to vitamin D metabolism.

Introduction

Over the past three decades, the prevalence of pediatric obesity has significantly increased, rising from seven to 20 percent in children aged six to 11 years.[1] This obesity epidemic has contributed to a rise in the incidence of comorbid conditions previously associated with adulthood, such as dyslipidemia, insulin resistance, hyperinsulinemia, and hypertension.[2] Metabolic syndrome represents a clustering of five cardiometabolic factors (elevated triglyceride concentrations, low high-density lipoprotein cholesterol, hypertension, hyperglycemia, and abdominal adiposity)[3] that increase the risk of developing cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM). A concurrent increase in pediatric obesity and metabolic syndrome prevalence has been observed, with the overall prevalence in adolescents increasing from four to nine percent from 1988-1994 to 2001-2006 NHANES cycles.[4, 5]

There has been emerging interest in the non-skeletal role of vitamin D, specifically related to CVD and diabetes. Furthermore, cardiometabolic disorders have been linked to poor vitamin D status.[6] Biologically plausible mechanisms for the role of vitamin D in CVD and diabetes include its involvement in heart proteomics, structure, and function,[7-9] the renin-angiotensin-aldosterone system,[10] atherosclerotic progression,[11] insulin secretion and action,[12-15] and inflammatory processes.[16-18]

In adults, cross-sectional analyses have linked 25(OH)D to cardiometabolic risk factors,[19-21] and longitudinal investigations of prospective cohorts' disease incidence reveal an increase in cardiometabolic risk with lower vitamin D status.[6, 22] Supplementation trials have shown that vitamin D may reduce cardiovascular risk[23] and retard the progression of pre-diabetes to T2DM,[24] although clinically significant effects are lacking, and the heterogeneity and weakness of study designs limit the available evidence.

In pediatric populations, evidence for the extraskeletal role of vitamin D in cardiometabolic disease is limited to cross-sectional investigations. The findings from these studies[25-30] are consistent with adult cross-sectional findings, having related 25(OH)D to cardiometabolic risk factors, such as abdominal adiposity, hyperglycemia, and blood pressure; however, there are no published data on 25(OH)D and cardiometabolic disease risk exclusively in pre-adolescent children, particularly in those who are overweight and obese.

Determining the relationships between vitamin D status and increased cardiometabolic risk is important since a significant number of children have hypovitaminosis D. Nationally representative data demonstrate that 18 percent of children aged one to 11 years are vitamin D insufficient [25(OH)D < 50 nmol/L].[31] Furthermore, serum vitamin D concentrations are inversely associated with a wide range of adiposity measures in youth.[25, 32] Visceral adipose tissue (VAT) has recently been shown to independently predict 25(OH)D status during pediatric years,[25] and VAT is closely tied to insulin resistance and metabolic risk.[33] Therefore, adiposity is an important confounding variable that must be measured using robust, sensitive measures and

subsequently adjusted for in cross-sectional analyses. Studies to date examining the relationship between vitamin D and cardiometabolic outcomes in pediatric samples have adjusted for adiposity using BMI[26, 28-30, 34] or total body fat mass,[27] which lack sensitivity and specificity.

Previous studies in pediatric samples have not utilized robust measures of total and central adiposity, nor have they examined 25(OH)D in relation to a wide range of cardiometabolic risk factors, inflammatory markers, and adipokines. The purpose of the present study was to determine relations between serum 25(OH)D and the presence of individual cardiometabolic risk factors (CMR), two or more CMR, fasting insulin, insulin sensitivity, beta-cell function, an inflammatory marker C-reactive protein (CRP), adipokines implicated in inflammation, and robust measures of total and central adiposity in overweight and obese preadolescent children.

Research Design and Methods

Participants

Participants were 222 sedentary, overweight and obese children residing in the Augusta, Georgia area (latitude: 33°N) who were enrolled in an investigation to determine the effects of aerobic exercise on metabolism.[35] Inclusion criteria for the trial were the following: white or black race, aged 7-11 years, overweight (BMI ≥ 85th percentile for age and sex[36]), and sedentary (no regular participation in an exercise program more than 1 h/wk). Children were excluded if they had a medical condition that would affect study results or limit physical activity. Participants underwent anthropometric measurements, an oral glucose tolerance test (OGTT), body composition scans and interviews that assessed physical activity and diet. Data were collected in six

different cohorts (due to time and space constraints) in either summer (June through August) or winter (December through February) between December 2003 and June 2006. Informed consent and assent were obtained from all parents and children, respectively. All procedures were approved by the human assurance committee (Institutional Review Board) at the Georgia Health Sciences University.

Anthropometry, blood pressure, and pubertal stage

Height (m), body weight (kg), and waist circumference (cm) measurements were collected by a trained laboratory technician. Participants were measured in light, indoor clothing after the removal of shoes. Height and body weight were assessed for calculation of sex- and age-specific BMI percentiles using CDC growth charts.[37] Waist circumference was then obtained at the midpoint between the lowest rib and the iliac crest. Seated blood pressure was measured 5 times at 1 min intervals after a 10-minute rest using the Dinamap Pro 100 (Critikon Corporation, Tampa, FL), and the last 3 measures were averaged. Pubertal maturation stage was determined by a trained pediatrician based on secondary characteristics of sexual maturation as described by Tanner.[38] When an individual was observed to have discordant stages of pubic hair and breast or genital development, the higher of the two stages was used.

Oral glucose tolerance test

After an overnight fast, a 2-h OGTT was conducted in which the participants ingested a standard oral glucose solution (1.75 g/kg of body weight up to a maximum of 75 g) at time 0. Blood was sampled for glucose and insulin at - 15, - 10, - 5, 0, 30, 60, 90, and 120 min relative to the oral glucose ingestion. Fasting glucose and insulin levels were determined from averaging the - 15, - 10, and - 5 min blood collections. From the

measures of glucose and insulin, the HOMA-IR was calculated: fasting insulin (μU/ml) x fasting glucose (mg/dl)/405.[39]

Assay of glucose, insulin, lipids, C-reactive protein, leptin, adiponectin, and 25(OH)D

Glucose was measured in 10 μl sera using an Ektachem DT system (Johnson and Johnson Clinical Diagnostics, Rochester, NY). The mean intra-assay CV for this analysis was 0.61%, and the mean interassay CV was 1.45%. Specific insulin was assayed in duplicate 100 μl with reagents obtained from Linco (St. Charles, MO). The mean intra- and interassay CVs for the insulin assay were 5% and 5.6%, respectively. Commercial quality control sera of low, medium, and high insulin concentration are included in every assay to monitor variation over time.

Between the -15 and 0 min time points, an additional 10 ml was drawn for determination of lipids, CRP, leptin, adiponectin, and 25(OH)D concentrations. Triglycerides, total cholesterol, and HDL-cholesterol were measured with the Ektachem DT II system. With this system, HDL-cholesterol is analyzed using a two-reagent system involving stabilization of low-density lipoprotein, very-low-density lipoprotein, and chylomicrons using cyclodextrin and dextrin sulfate, and subsequent enzymatic-colorimetric detection of HDL-cholesterol. For total cholesterol, the intra- and interassay CVs were 2.5 and 3.5%, respectively. For triglycerides, the interassay CV was 5.3%. The intra- and interassay CVs were 2.0 and 2.5%, respectively, for HDL-cholesterol. LDL-cholesterol was determined using the Friedewald formula.[40] Serum concentrations of CRP were measured using high-sensitivity enzyme-linked immunosorbent assay (ALPCO Diagnostics, Windham, NH), with intra- and interassay CVs of 10% and 10.2%, respectively. Leptin and adiponectin were both measured in serum and assayed in

duplicate using RIA (Millipore, Billerica, MA). Intra- and interassay CVs were 3.1% and 6.2%, respectively, for leptin and 4.1% and 7.1%, respectively, for adiponectin. Serum 25(OH)D concentrations were assayed using RIA (DiaSorin Laboratories, Stillwater, MN) and run in duplicate, with intra- and interassay CVs of 5.6% and 7.1%, respectively. Analytical reliability of the 25(OH)D assay was monitored via participation in the Vitamin D External Quality Assessment Scheme (DEQAS).

Dynamic measurements of insulin sensitivity and beta-cell function

The Matsuda index, an insulin sensitivity index that reflects a composite estimate of hepatic and muscle insulin sensitivity, was calculated using the following formula: *Matsuda index* = 10,000/√(fasting glucose × fasting insulin) × (mean glucose × mean insulin), where fasting glucose and insulin data are taken from the OGTT and mean data represent the average glucose and insulin values obtained during the OGTT (i.e., fasting, 30, 60, 90, and 120 min).[41] The Matsuda index has been validated using the euglycemic clamp method.[41] The insulinogenic index was used as our surrogate of beta-cell function, which has been shown to correlate well with insulin secretion measured by euglycemic clamp, and each predict future development of T2DM.[42, 43] The insulinogenic index was calculated by dividing the increment in insulin at 30 min by the increment in glucose at 30 min of the OGTT (i.e., $\Delta Insulin_{0\text{-}30}/\Delta Glucose_{0\text{-}30}$).

Adiposity, physical activity and dietary intake measurements

Percentage body fat was measured by dual-energy X-ray absorptiometry (DXA) (QDR-4500W, Hologic Inc., Waltham, MA). VAT and subcutaneous abdominal adipose tissue (SAAT) were quantified via magnetic resonance imaging (MRI) (1.5T, General Electric Medical Systems, Milwaukee, WI) from five 1 cm transverse slices around the

L4-L5 disk of the lumbar spine. Assessments of VAT and SAAT are described in detail elsewhere.[44] Using a one-way random effects model, single-measure intraclass correlation coefficients were calculated in 219 adolescents aged 13 to 18 years. Each subject was scanned twice within seven days for bone mineral content, bone area, area bone mineral density, fat-free soft tissue mass, fat mass, and percentage body fat (all $R \geq 0.97$).

Information on moderate and vigorous physical activity was collected using questions 80 and 81 from the 2001 CDC Youth Risk Behavior Survey[45] (**see Appendix F**). Moderate PA (d/wk) was determined by the question, 'On how many of the past 7 days did you participate in PA for at least 30 min that did not make you sweat or breathe hard, such as fast walking, slow bicycling, skating, pushing a lawn mower or mopping floors?' Vigorous PA (d/wk) was determined by the question, 'On how many of the past 7 days did you exercise or participate in PA for at least 20 min that made you sweat or breathe hard, such as bicycling, fast dancing or similar aerobic activities?' These moderate and vigorous PA variables have shown modest reliability and validity in this age group.[46]

To assess mean daily intakes of energy and dietary calcium and vitamin D, the participants completed three 24-hour diet recalls (**see Appendix G**) with a registered dietitian or trained research assistant on the day of blood collection (Nutrition Data System for Research, software version 2006, Minneapolis, MN). During the recall, participants were able to use their diet record for assistance. Prior to the recall, a registered dietitian instructed the child and parent on how to maintain a diet record using food models, portion booklets, and serving containers for estimating serving size.

Definitions of cardiometabolic risk factors (CMR)

For this analysis, the majority of CMR were defined according to the National Cholesterol Education Program Adult Treatment Panel III definition modified for age.[47] These risk factors were defined accordingly: waist circumference ≥ 90th percentile for age and sex;[48] systolic or diastolic blood pressure ≥ 90th percentile for age, gender, and height;[49] fasting glucose ≥ 100 mg/dl;[50] triglycerides ≥ 110 mg/dl; and HDL cholesterol ≤ 40 mg/dl.

Statistical analyses

To assess differences in potential confounders and to determine the relations between serum 25(OH)D and CMR, fasting insulin, Matsuda index, insulinogenic index, total cholesterol, LDL-cholesterol, CRP, leptin, and adiponectin, participants were equally divided into tertiles of serum 25(OH)D. For the preliminary analyses, group differences in potential confounding variables (i.e., age, sex, race, pubertal stage, season of study visit, percentage body fat, SAAT, VAT, physical activity, and dietary intake of energy, calcium and vitamin D) were determined using either ANOVA (continuous variables) or chi-square test of independence (categorical variables). To assess the post-test for linear trend, *P*-trend values were determined for continuous variables using linear orthogonal polynomial analyses. To further explore the association between serum 25(OH)D and the robust measures of total and central adiposity, we calculated partial correlations between serum 25(OH)D and percentage body fat, SAAT and VAT, after adjustment for sex, race, pubertal stage and season of study visit.

For comparison of the primary dependent variables (i.e., CMR, insulin, HOMA-IR, Matsuda index, insulinogenic index, total cholesterol, LDL-cholesterol, CRP, leptin,

and adiponectin), an *F* test was performed to test the assumption of homogeneity of regression slopes by assessing the interactions between the independent variable (i.e., 25(OH)D tertile groups) and the covariates (sex, race, sexual maturation stage, and season). Because there were no significant interactions, ANCOVA was used to compare the primary dependent variables across serum 25(OH)D tertiles after adjusting for sex, race, Tanner stage, and season. Linear orthogonal polynomial analyses were used to assess the post-test for linear trend. If a trend for difference in the dependent variable of interest across a tertile of serum 25(OH)D was significant ($P < 0.05$), multiple comparisons with Bonferroni correction were performed. In obese children, visceral adiposity, more so than subcutaneous abdominal fat and total body fat, has a greater influence on markers known to increase the risk for T2DM and CVD.[51] Therefore, associations between 25(OH)D and the primary dependent variables were explored taking VAT into account. Therefore, we subsequently tested whether any association observed with serum 25(OH)D was dependent on the same covariates plus VAT. By using this approach, if an observed association with 25(OH)D was dependent on VAT, there would be less association between 25(OH)D and the primary dependent variable of interest when controlled for VAT.[52] We also treated 25(OH)D as a continuous variable and investigated the relationship with the aforementioned primary dependent variables using partial correlations, controlling for sex, race, sexual maturation, and VAT. However, we elected to report the 25(OH)D tertile results since the our findings treating serum vitamin D as a continuous variable were similar, to reduce redundancy, and because the tertiles approximate clinically relevant cutoffs.

Adjusted means are reported as mean ± SE. Prior to analyses, insulin, triglycerides, CRP, leptin, and adiponectin were log-transformed so that each of these variables followed an approximate normal distribution. However, given that the results in the log-transformed models and untransformed models were similar, the untransformed data were reported for clarity.

In addition, we calculated partial odds ratios (ORs) and 95% CI using multinomial logistic regression to determine the presence of ≥ 2 CMR for each serum 25(OH)D tertile category after the analysis was adjusted for sex, race, sexual maturation stage, and season. Additional partial ORs were calculated using the same covariates plus VAT. ORs for tertiles 1 and 2 in relation to tertile 3 (reference category) were compared. All statistical analyses were conducted using SPSS version 18.03 (PASW Statistics, Chicago, IL) and statistical significance was set at P-value < 0.05.

Results

The sample was composed of 222 overweight black and white children of which 58% were female and 58% were black. The majority of participants (85%) were in stages I and II for sexual maturation; however, 26 subjects were in stage III and eight in stage IV. The percentages of overweight and obese participants were 14% and 86%, respectively. Of the 222 participants, 42% were identified as having at least one CMR and 39% with ≥ 2 CMR. The CMR distributions among the participants were: 67% (n = 149) with waist circumference ≥ 90th percentile for age and gender; 3% (n = 7) with blood pressure ≥ 90th percentile for age, gender, and height; 19% (n = 42) with fasting glucose ≥ 100 mg/dl; 18% (n = 40) with triglycerides ≥ 110 mg/dl; and 25% (n = 56) with HDL-cholesterol ≤ 40 mg/dl.

Descriptive characteristics of the participants by tertiles of serum 25(OH)D concentrations are shown in **Table 4.1**. The proportion of participants in the lowest 25(OH)D tertile category (< 52.7 nmol/l) was significantly higher for blacks than for whites and for those examined in the winter than for those examined in the summer (both $P < 0.03$). Significant linear downward trends in sexual maturation stage and VAT were observed across tertiles of serum 25(OH)D (both P-trend < 0.04). There were no significant differences in age, sex, BMI percentile, percentage body fat, SAAT, physical activity, and intakes of energy, calcium or vitamin D across tertiles of serum 25(OH)D. Consistent with the ANOVA data, partial correlations revealed that serum 25(OH)D was significantly, inversely associated with VAT (r = -0.190, $P = 0.01$), but not percentage body fat (r = -0.082, $P = 0.25$) or SAAT (r = -0.134, $P = 0.07$) (**Figure 4.1**).

Table 4.2 displays markers known to increase risk for diabetes and cardiovascular disease across tertiles of serum 25(OH)D concentrations, adjusting for sex, race, sexual maturation stage, and season of study visit (model 1). Significant linear downward trends were revealed for waist circumference, fasting insulin, HOMA-IR, triglycerides, and leptin across tertiles of serum 25(OH)D (all P-trend < 0.05). Conversely, significant linear upward trends across tertiles of serum 25(OH)D were observed for Matsuda index, HDL-cholesterol, and adiponectin (all P-trend < 0.03). When VAT was included as a covariate, it attenuated these significant differences (all P-trend > 0.05) (model 2). There were no statistically significant differences in blood pressure, fasting glucose, insulinogenic index, total or LDL-cholesterol, or CRP, across tertiles of serum 25(OH)D (all P-trend >0.05).

The association between serum 25(OH)D levels and the prevalence of ≥ 2 CMR is presented in **Table 4.3**. Participants in the lowest tertile of serum 25(OH)D concentrations (< 52.7 nmol/l) were more likely to have ≥ 2 CMR compared with those in the highest tertile (≥ 69.4 nmol/l) (44% vs. 26%, respectively; $P = 0.03$). After adjustment for sex, race, sexual maturation stage and season of study visit, the multivariate-adjusted odds ratio for ≥ 2 CMR for the lowest (< 52.7 nmol/l) compared with the highest (≥ 69.4 nmol/l) tertile of serum 25(OH)D concentrations was 3.52 (95% CI: 1.39, 8.98; $P < 0.01$) (model 1). After adding VAT as a covariate, this significant association was attenuated (model 2).

Discussion

This is the first pediatric study to investigate the relationships between 25(OH)D and cardiometabolic risks in a sample composed exclusively of overweight and obese, preadolescent children. Furthermore, to our knowledge, this is the first pediatric study to control for adiposity in the vitamin D/cardiometabolic relationship using MRI-derived measures of adiposity. Our findings suggest that lower vitamin D status is associated with multiple measures known to increase risk for diabetes and CVD in overweight and obese, preadolescent children and that VAT, instead of general adiposity, is a key confounder in these associations.

We examined the relationship between vitamin D status and a wide range of cardiometabolic risk factors, inflammatory markers, and adipokines. Significant linear downward trends were observed between 25(OH)D tertile levels and fasting insulin, HOMA-IR, triglycerides, and leptin. Children in the lowest 25(OH)D tertile were estimated to be 3.5 times more likely in terms of odds to have ≥ 2 CMR when compared

to the children in the highest tertile. Fasting insulin[20, 53] and triglycerides[54] have been inversely related to serum vitamin D in adult studies, but the present study is the first to report these relationships in children. HOMA-IR, a surrogate measure of insulin resistance highly correlated with fasting insulin in children,[55] has been previously negatively related to 25(OH)D levels in adolescents[28] and adults.[20, 53, 56] Adipokines have not been explored in pediatric studies examining the vitamin D-cardiometabolic relationship, so this is the first study to report the inverse association with leptin. Significant linear upward trends were seen in the children between 25(OH)D tertiles and Matsuda index, HDL-cholesterol, and adiponectin. Similar to our findings, previous positive associations have been shown between vitamin D status and HDL-cholesterol,[25, 26, 28, 34, 57] OGTT-derived insulin sensitivity via the Matsuda index,[30] and adiponectin[58] in youth. Nunlee-Bland et al.[58] only used dietary vitamin D information, so we are the first study in children to relate serum vitamin D levels to the adipokine, adiponectin.

In the present study, no significant differences across 25(OH)D tertiles were noted for blood pressure, fasting glucose, insulinogenic index, total or LDL-cholesterol, or CRP. Our null findings contrast with previous studies in pediatric samples, in which inverse associations have been seen between serum vitamin D and systolic blood pressure[28, 29, 57] and fasting glucose,[29, 34] However, these cohorts were predominantly adolescents, which may contribute to the discrepancy, particularly for glycemia, which is dependent on pubertal maturation.[59] In agreement with our non-significant findings, 25(OH)D has not been previously related to insulinogenic index, total or LDL-cholesterol, or CRP in pediatric studies; however, two of these indices, OGTT-derived

beta cell function[60] and CRP,[61-63] have been negatively related to serum vitamin D levels in adults.

The inverse associations observed between 25(OH)D and fasting insulin and HOMA-IR, as well as a positive association with the Matsuda index, may be attributed to the role of vitamin D in insulin secretion[12, 13] and action[14, 15] at the cellular level. Triglyceride levels and HDL-cholesterol were the two CVD-related outcomes related to vitamin D status. Mechanistic research currently provides convincing evidence for vitamin D's involvement in CVD, particularly through the autocrine actions of the active $1,25(OH)_2D_3$ metabolite, but most of the studies have focused on atherosclerotic progression,[11] heart structure and function,[9] and the renin-angiotensin-aldosterone-system.[10] Future mechanistic research should focus on isolating the effects of VAT and secreted adipocytokines on vitamin D metabolism and cardiometabolic risk.

The most important finding in the present study is that when VAT was entered as a covariate in the statistical model, all aforementioned downward linear trends with serum vitamin D tertiles and insulin, HOMA-IR, triglycerides, and leptin and upward linear trends for Matsuda index, HDL-cholesterol, and adiponectin, were attenuated. Moreover, the increased odds of having CVD risk factors in children in the lowest tertile for serum 25(OH)D were also attenuated. Previous cross-sectional investigations in children, adolescents, and adults have described the relationships between vitamin D and CMR to be independent of adiposity. Most cross-sectional investigations of vitamin D and CMR have controlled for anthropometric measures such as BMI and waist circumference or DXA-derived measures such as fat mass, but these methods may not adequately adjust for the effect of adiposity. In contrast, our findings suggest that this

relationship is in fact fat-dependent, with VAT playing a confounding role. Accordingly, one adult cross-sectional study that used a detailed, reliable characterization of adiposity through VAT and SAT volume measurements determined that the relationship between 25(OH)D and insulin sensitivity lacked statistical significance after adjusting for VAT.[64]

Adiposity is highlighted in the Institute of Medicine's 2010 Dietary Reference Intake vitamin D report as a condition of interest.[65] Hypovitaminosis D prevalence is significantly increased in obese pediatric populations, particularly in minority populations. Anywhere from 32 to 78 percent of obese adolescents, depending on the ethnicity studied, and 57 percent of obese African-American children have 25(OH)D levels below 50 nmol/L.[27, 30, 66] Due to its lipophilic nature, vitamin D sequestration in adipose tissue is a possible mechanism to explain lower vitamin D status in overweight and obese individuals.[67] Animal studies in rats[68, 69] and pigs[70] confirm storage of cholecalciferol and 25(OH)D in fat tissue, especially at high vitamin D doses. One human study used subcutaneous fat samples from obese patients at the time of bariatric surgery and found cholecalciferol and 25(OH)D present in the fat tissue.[71] Although vitamin D is found in the fat, muscle, liver, serum, and remaining tissues, storage in fat predominates for cholecalciferol, while 25(OH)D is found in highest concentrations in the serum.[70] Additional research, particularly in humans, is needed in this area.

In our predominantly obese sample of children, serum vitamin D concentrations were not significantly associated with total body fat or SAAT but were inversely related to VAT. The role of ectopic lipid deposition in the visceral abdominal depot and insulin-sensitive tissues in the vitamin D/cardiometabolic relationship goes beyond the issue of vitamin D sequestration in fat tissue. If this were simply a case of fat storing this

lipophilic vitamin, then one would expect 25(OH)D to be inversely related to SAAT and total body fat percentage in our sample as well. Instead, the observed association was strongest with VAT, and when VAT was adjusted for, the significant trends between serum vitamin D tertiles and markers known to increase risk for diabetes and CVD were attenuated, becoming non-significant. This suggests that the observed associations are dependent on VAT. Fat tissue is no longer viewed as an energy storage organ, but instead possesses endocrine and immune functions. VAT is more prone to lipolysis[72] and is located close to the hepatic portal vasculature, which leads to the delivery of free fatty acids to the liver, leading to increased circulating triglycerides, reduced HDL-cholesterol, and decreased insulin response; this explains VAT's positive association with dyslipidemia, hyperinsulinemia, and glucose intolerance.[73] In pre-pubertal children, VAT is independently related to triglyceride levels and fasting insulin.[74] VAT's role in metabolic dysfunction is also likely mediated through the actions of adipocytokines (e.g. leptin, adiponectin, resistin, visfatin) and classical cytokines (e.g. TNF-α, IL-6, IL-1).[33, 75] In fact, the removal of VAT in aging rats restores peripheral and hepatic insulin action to the levels of young rats, mediated through the reduction in expression of TNF- and leptin.[76] Perhaps these adipokines and cytokines produced by the metabolically active VAT also act on 25(OH)D, stimulating its conversion to inactive metabolites such as $24,25(OH)_2D$. This would ultimately cause less 25(OH)D to be available for conversion to the active $1,25(OH)_2D$ metabolite, which could then act on cardiovascular and insulin-sensitive tissues and improve inflammation. This is a hypothesis that future mechanistic research should investigate.

In our pediatric sample, the majority (67%) had waist circumference ≥ 90th percentile for age and gender, and waist circumference was negatively associated with vitamin D status. Serum vitamin D concentrations have been inversely related to various indices of overweight or obesity, including BMI, BMI percentile, waist circumference, total fat mass, and total body fat percentage,[27-29, 32] in children and adolescents. Additionally, racial differences may exist, with the 25(OH)D-total body fat inverse association perhaps being stronger in whites compared to blacks.[77] Regional adiposity has also been inversely related to serum vitamin D status. Dong et al. reported negative associations for both VAT and SAAT and vitamin D status in their adolescent sample.[32] Similar to our findings, Rajakumar et al. demonstrated that VAT independently predicts 25(OH)D concentrations in children.[25]

Our study confirms previous findings regarding racial, seasonal, and pubertal differences in vitamin D status. We found the proportion of children in the lowest 25(OH)D tertile category (< 52.7 nmol/L) to be significantly higher for blacks than their white counterparts. In the pediatric literature, lower 25(OH)D levels in the pediatric literature are consistently seen in the black race.[25-27, 31] Nationally, in children ages six to 11 years, over 50% of black children had serum vitamin D levels < 50 nmol/L, compared to a prevalence of approximately 11% and 20% for white children and overall, respectively.[31] Not only does the higher melanin content of black children reduce their ability to cutaneously synthesize vitamin D, but their prevalence of high BMI for sex and age is higher than white children,[78] which may contribute to greater sequestration of vitamin D in their adiposity depots.[67] In the present study, there were a greater number of vitamin D insufficient children during the winter season of testing. Cutaneous synthesis is

significantly reduced during the winter months due to increased clothing, reduced outdoor activity, and altered angle of ultraviolet B irradiation.[79, 80] Season has been shown to independently predict vitamin D status in black and white children.[25] Pubertal status has also been demonstrated to independently predict 25(OH)D levels, and in the current study, there was a significant linear downward trend in sexual maturation stage across 25(OH)D tertiles. The underlying mechanisms are unknown, but hypotheses involve increased sequestration of vitamin D in fat and lean tissues,[67] an increase in blood volume and subsequent dilution effect, or an increased rate of conversion to the active $1,25(OH)_2D$ metabolite during this life stage. Future research is needed to confirm the exact cause.

The prevalence of pre-diabetes was 19% in our study. Although this is higher than the overall national pre-diabetes estimate (16%) for adolescents, this value is in agreement with the pre-diabetes prevalence for overweight and obese teenagers, of 18 to 30%, respectively.[81] In terms of dietary intake, 24-hour recalls captured that our participants, collapsing across tertiles, consumed approximately 145 IU of vitamin D per day, which is less than the national average of total vitamin D intake (324 IU daily) for children ages four to 13 years.[82] Assuming minimal sun exposure, our subjects would require an additional 455 IU of vitamin D per day in order to meet the new Recommended Dietary Allowance of 600 IU per day for those ages 1 to 70 years.[65] This would be challenging without a significant increase in dairy products, fatty fish, or supplementation.

Although our cross-sectional study design limits causal inference, since vitamin D status and measures of cardiometabolic risk were simultaneously measured, our study

possesses several strengths. This is the first pediatric study to investigate the relationship between 25(OH)D and cardiometabolic outcomes in a homogeneous sample of overweight and obese preadolescent children, a high-risk population. Additionally, the homogeneous level of pubertal maturation in our participants reduced the possibility for confounding effects of maturation on glucose and insulin metabolism, since research confirms that transient hyperglycemia, hyperinsulinemia, and insulin resistance occur during the pubertal transition in both genders, regardless of race or obesity.[59] Our sample is diverse, including both genders and multiple races, which increases the generalizability of our findings. Utilizing MRI measures of adipose tissue is another strength, as this allowed us to accurately assess the relationship between adiposity, serum vitamin D levels, and cardiometabolic risk using robust measures of fatness. We examined a wide range of cardiometabolic risk factors, inflammatory markers, and adipokines. Furthermore, we assessed insulin sensitivity and resistance under fasting and dynamic conditions; the OGTT provides valuable information about the ability of the body to dispose of glucose.[83] To our knowledge, we are the first pediatric study to associate serum vitamin D concentrations with fasting insulin, triglycerides, leptin, and adiponectin.

Although its efficacy as a preventative or treatment measure requires further research, vitamin D has been associated with and presented as an important modifier of risk for cardiometabolic disease and diabetes in the pediatric and adult research literature.[6, 22] Future observational investigations in adult and pediatric populations should adequately adjust for pertinent confounders, particularly adiposity. Well-designed vitamin D supplementation trials in children and adolescents are warranted to elucidate

the causal effect of vitamin D and cardiometabolic outcomes. Supplementation trials, almost exclusively in adults, have thus far yielded equivocal findings.[84] To our knowledge, no pediatric vitamin D supplementation trials have assessed cardiometabolic disease precursors or insulin sensitivity during preadolescence.

In summary, we report in overweight and obese sedentary preadolescents that lower circulating vitamin D levels are associated with multiple measures known to increase risk for diabetes and cardiovascular disease. Our findings between serum 25(OH)D and VAT suggest that visceral, rather than general, adiposity may be more pertinent to vitamin D metabolism.

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Table 4.1. Participant characteristics by tertiles of serum 25-hydroxyvitamin D concentrations in overweight children aged 7-11 years[1]

	Serum 25-hydroxyvitamin D[2]			
	Tertile 1	Tertile 2	Tertile 3	
	43.8 nmol/l (21.6 – 52.7 nmol/l)	61.0 nmol/l (52.8 – 69.2 nmol/l)	79.4 nmol/l (69.4 – 119.4 nmol/l)	*P*-trend[3]
n	74	74	74	
Age (y)	9.4 ± 1.0	9.3 ± 1.0	9.3 ± 1.2	0.327
Sex (%)[4]				0.145
Males	32	50	45	
Females	68	50	55	
Race (%)[4]				0.029
White	12	47	68	
Black	88	53	32	
Sexual maturation stage, 1-5	1.8 ± 0.9[a]	1.6 ± 0.8	1.3 ± 0.5[b]	0.002
Season of study visit (%)[4]				0.003
Winter	63.5	44.6	31.1	
Summer	36.5	55.4	68.9	
BMI percentile	97.8 ± 2.8	97.5 ± 2.5	96.2 ± 3.2	0.439
BMI percentile category (%)[4]				0.065
Overweight	9.5	10.8	21.6	
Obese	90.5	89.2	78.4	
Percentage body fat (%)	41.2 ± 6.3	41.0 ± 5.6	39.4 ± 6.3	0.120
SAAT, cm^3	287 ± 14.1	287 ± 12.7	243 ± 14.1	0.109
VAT, cm^3	36.7 ± 11.4[a]	34.4 ± 16.8	29.9 ± 13.9[b]	0.035
Physical activity and diet parameters				
Moderate physical activity (d/wk)	2.0 ± 2.2	2.1 ± 2.1	2.1 ± 2.1	0.763

Vigorous physical activity (d/wk)	3.3 ± 2.3	3.6 ± 2.3	3.0 ± 2.5	0.545
Energy intake (kcal/d)	1634 ± 653	1798 ± 736	1722 ± 614	0.524
Calcium intake (mg/d)	711 ± 280	700 ± 261	720 ± 302	0.809
Vitamin D intake (IU/d)	144 ± 100	144 ± 88	148 ± 112	0.650

[1] Values are means ± SD or %.

[2] Values are median (range) serum 25-hydroxyvitamin D levels in a given tertile.

[3] *P*-values comparing differences between tertile groups of serum 25-hydroxyvitamin D levels were calculated using ANOVA. Means in a row with superscripts without a common letter differ, $P < 0.05$ (Bonferroni correction test for multiple comparisons). *P*-trend values were determined using linear orthogonal polynomial analysis.

[4] *P*-value test of significance between groups were based on chi-square test.

Table 4.2. Markers known to increase risk for diabetes and cardiovascular disease across tertiles of serum 25-hydroxyvitamin D concentrations in overweight children aged 7-11 years[1]

	Serum 25-hydroxyvitamin D			
	Tertile 1 (<52.8 nmol/l)	Tertile 2 (52.8 – 69.2 nmol/l)	Tertile 3 (≥69.4 nmol/l)	*P*-trend[2]
Median (nmol/l)	43.8	61.0	79.4	
Waist circumference (cm)				
Model 1	79.0 ± 1.3[a]	77.7 ± 1.1	74.5 ± 1.2[b]	0.027
Model 2	79.2 ± 1.0	77.5 ± 0.9	76.4 ± 0.9	0.080
Systolic BP (mm Hg)				
Model 1	97.0 ± 1.3	98.1 ± 1.1	96.5 ± 1.2	0.791
Model 2	96.9 ± 1.4	97.4 ± 1.1	96.4 ± 1.3	0.780
Diastolic BP (mm Hg)				
Model 1	52.5 ± 0.8	53.2 ± 0.7	53.9 ± 0.8	0.205
Model 2	53.1 ± 0.9	53.2 ± 0.7	53.9 ± 0.8	0.554
Fasting glucose (mg/dl)				
Model 1	92.8 ± 1.0	94.1 ± 0.9	92.4 ± 1.1	0.786
Model 2	91.8 ± 1.2	94.3 ± 1.0	92.3 ± 1.1	0.760
Fasting insulin (μU/ml)				
Model 1	24.1 ± 1.6[a]	21.9 ± 1.4	18.9 ± 1.6[a]	0.045
Model 2	23.8 ± 1.7	21.5 ± 1.4	20.6 ± 1.5	0.197
HOMA-IR				
Model 1	5.5 ± 0.4[a]	5.1 ± 0.3	4.3 ± 0.4[b]	0.048
Model 2	5.3 ± 0.4	5.0 ± 0.3	4.8 ± 0.4	0.296
Matsuda Index				
Model 1	2.1 ± 0.2[b]	2.4 ± 0.2	2.6 ± 0.2[a]	0.035
Model 2	2.4 ± 0.2	2.4 ± 0.2	2.5 ± 0.2	0.266

Insulinogenic index				
Model 1	2.6 ± 0.2	2.8 ± 0.2	2.7 ± 0.2	0.783
Model 2	2.5 ± 0.2	2.8 ± 0.2	2.7 ± 0.3	0.703
Triglycerides (mg/dl)				
Model 1	81.9 ± 5.2[a]	78.4 ± 4.6	65.7 ± 5.3[b]	0.004
Model 2	82.7 ± 5.9	80.5 ± 4.7	70.1 ± 5.4	0.098
Total cholesterol (mg/dl)				
Model 1	166.1 ± 4.3	164.4 ± 3.8	161.0 ± 4.3	0.360
Model 2	166.8 ± 4.7	163.2 ± 3.7	162.4 ± 4.3	0.424
HDL cholesterol (mg/dl)				
Model 1	44.3 ± 1.5[b]	48.7 ± 1.4[a]	52.4 ± 1.6[a]	0.001
Model 2	45.9 ± 1.5[b]	46.3 ± 1.2[a]	49.9 ± 1.4[a]	0.088
LDL cholesterol (mg/dl)				
Model 1	101.9 ± 3.4	95.9 ± 3.0	94.3 ± 3.4[a]	0.222
Model 2	103.3 ± 3.8	94.5 ± 3.0	96.7 ± 3.4	0.248
C-reactive protein (mg/dl)				
Model 1	3.2 ± 0.3	3.1 ± 0.3	2.6 ± 0.3	0.251
Model 2	3.1 ± 0.4	3.2 ± 0.3	2.7 ± 0.4	0.502
Leptin (ng/ml)				
Model 1	29.3 ± 1.7[a]	28.8 ± 1.5	23.7 ± 1.7[b]	0.037
Model 2	28.4 ± 1.7	28.9 ± 1.4	25.5 ± 1.5	0.258
Adiponectin (μg/ml)				
Model 1	8.8 ± 0.6[b]	9.0 ± 0.6[b]	11.1 ± 0.6[a]	0.022
Model 2	9.0 ± 0.7	9.1 ± 0.6	10.9 ± 0.7	0.084

[1] n = 222; values are least-square (LS) means ± SE. LS means are the mean values in the outcomes when covariates are held constant: Model 1 was adjusted for sex, race, sexual maturation stage, and season of study visit; model 2 was further adjusted for visceral adipose tissue.

[2] *P*-values comparing differences between tertile groups of serum 25-hydroxyvitamin D levels were calculated using ANOVA. Means in a row with superscripts without a common letter differ, $P < 0.05$ (Bonferroni correction test for multiple comparisons). *P*-trend values were determined using linear orthogonal polynomial analysis.

Table 4.3. Partial odds ratios (95% CI) for having two or more cardiometabolic risk factors (CMR) across tertiles of serum 25-hydroxyvitamin D concentrations in overweight children aged 7-11 years[1]

	Serum 25-hydroxyvitamin D			
	Tertile 1 (<52.8 nmol/l)	Tertile 2 (52.8 – 69.2 nmol/l)	Tertile 3 (≥69.4 nmol/l)	*P*-trend
Median (nmol/l)	43.8	61.0	79.4	
Unadjusted prevalence of ≥2 CMR (%)	43.7	38.9	25.8	0.030
Model 1	3.52 (1.39, 8.98)[2]	1.91 (0.89, 4.16)	1.0	0.025
Model 2	2.92 (0.95, 7.62)	1.52 (0.61, 3.86)	1.0	0.165

[1] *n* = 222; CMR were defined according to pediatric definitions of metabolic syndrome: waist circumference ≥90th percentile for age and gender; HDL cholesterol ≤40 mg/dl; triglycerides ≥110 mg/dl; systolic or diastolic blood pressure ≥90th percentile for age, gender, and height; and fasting glucose ≥100 mg/dl. Model 1 adjusted for sex, race, sexual maturation stage, and season of study visit. Model 2 adjusted for the same covariates in model 1 and further adjusted for visceral adipose tissue.

[2] Significantly different from the reference group, tertile 3.

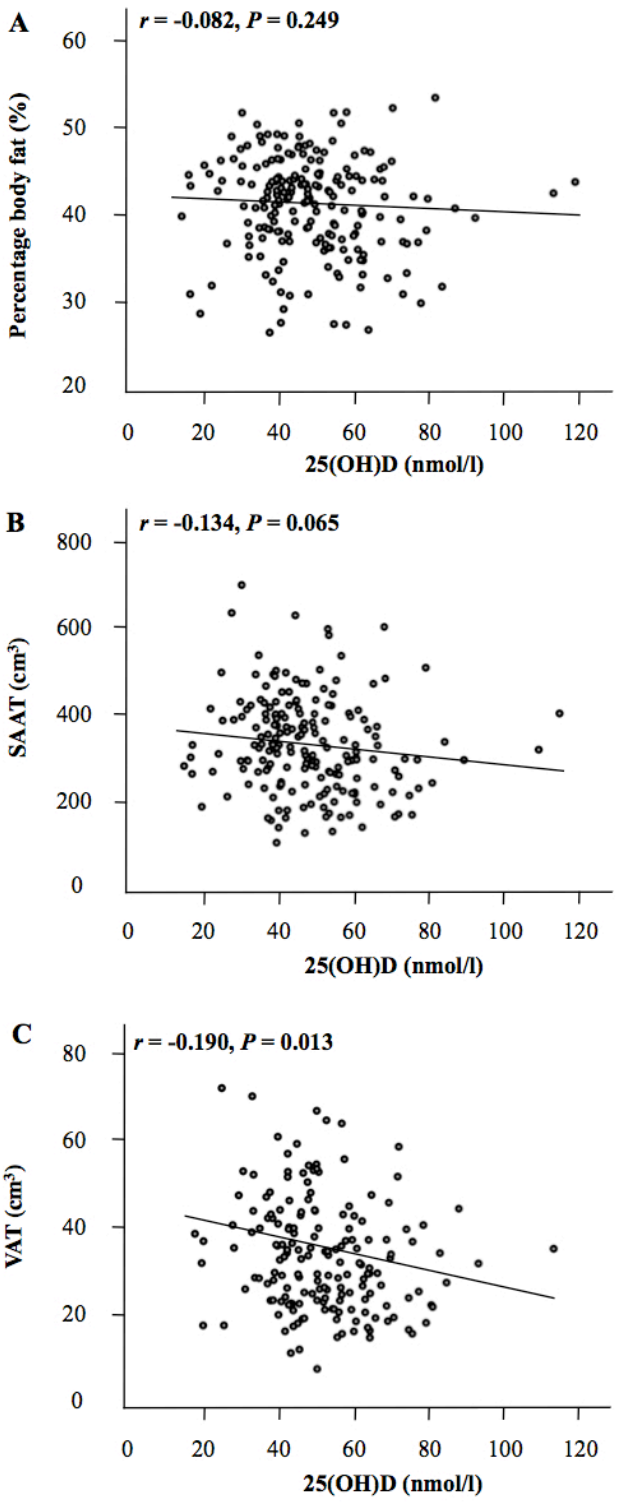

Figure 4.1. Relationships between plasma 25-hydroxyvitamin D concentrations and total and central adiposity in 222 overweight children aged 7 – 11 years.
(A) Percentage body fat vs. 25(OH)D, (B) Subcutaneous abdominal adipose tissue (SAAT) vs. 25(OH)D, and (C) Visceral adipose tissue (VAT) vs. 25(OH)D. Relationships are adjusted for sex, race, sexual maturation stage, and season.

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CHAPTER 5

SUMMARY

Public health prevention measures to reduce cardiometabolic risk factors and pre-diabetes are crucial since a myriad of sequelae, including type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), myocardial infarction, stroke, kidney disease, and blindness, are likely to result. This is particularly important in children and adolescents, as the high prevalence of obesity in youth is contributing to increases in pre-diabetes[1, 2] and cardiometabolic disturbances[3, 4] at younger ages. Increasing evidence from animal and cell culture studies, as well as cross-sectional, longitudinal, and intervention studies in humans, suggests that vitamin D has a protective role in diabetes and cardiometabolic disease.[5-7] Because hypovitaminosis D is present in a significant percentage of the United States pediatric population,[8, 9] especially in youth with excess adiposity,[10] the potential public health ramifications of vitamin D supplementation are enormous. Biologically plausible mechanisms linking vitamin D deficiency to diabetes and CVD involve vitamin D as a direct and indirect modifier of insulin secretion and action, inflammation, heart structure and function, atherosclerotic progression, and the renin-angiotensin-aldosterone-system.

Chapter 3 presented novel findings from the first pediatric vitamin D supplementation trial to address glycemia and insulin sensitivity outcomes. Our intervention examined the dose-response effect of five difference doses of vitamin D_3 supplementation over 12 weeks on fasting and surrogate measures of glycemia and

insulin sensitivity in 320 early pubertal children who participated in the randomized, placebo-controlled, triple masked, multi-site Georgia, Purdue, and Indiana University School of Medicine (GAPI) vitamin D trial. The main finding of this intervention is that although glycemia and insulin sensitivity outcomes changed over the course of the trial, vitamin D supplementation did not alter these parameters over 12-weeks and none of the possible confounders (gender, race, maturation, adiposity) significantly interacted with the effect of vitamin D supplementation.

There are several factors that likely contributed to our null treatment*time findings for vitamin D supplementation on insulin sensitivity. First, despite the fact that a high percentage of participants were overweight or obese, the study sample was relatively healthy normo-glycemic and –insulinemic children. Previous research in adult vitamin D trials suggests that a critical window may exist for the efficacy of vitamin D supplementation, namely at an early stage of diabetes or perhaps during pre-diabetes.[6] Another possible contributing factor to our null finding was the "sufficient" baseline vitamin D status of our children. Vitamin D insufficient populations[11] appear to benefit more from supplementation than their vitamin D-sufficient counterparts.[12] Because lower 25(OH)D values are found in populations living at higher northern latitudes, one half the participants were recruited from a northern latitude of 40° N. Also, participants were only tested in the wintertime to take advantage of the seasonal declines in serum 25(OH)D observed during the winter months. Both of these strategies were insufficient in generating a population with low baseline circulating 25(OH)D levels. Finally, the organic effect of pubertal maturation on glucose and insulin concentrations may have introduced significant heterogeneity or noise into our data set, leading to non-significant

outcomes. A phenomenon of transient insulin resistance occurs during the pubertal transition in both genders regardless of adiposity status.[13, 14] Our significant main effects for time for all four outcomes confirm that this phenomenon was occurring in our children over the course of the trial; glucose, insulin, and HOMA-IR increased significantly over the 12-weeks, while quantitative insulin sensitivity check index (QUICKI) significantly decreased.

In the latest Dietary Reference Intakes (DRI) report for calcium and vitamin D, the Institute of Medicine (IOM) committee dubbed the role of vitamin D in cardiovascular disease and diabetes to be "hypotheses of emerging interest" and pointed to the paucity of randomized controlled trial data to establish causality and dose-response relationships.[15] Future supplementation trials should focus on high-risk groups (e.g. obese, pre-diabetic, hypertensive), and clinically relevant outcomes should be assessed utilizing rigorous methodology. Moreover, younger, pre-pubertal children, should be targeted in an effort to avoid the pubertal changes in serum insulin and insulin sensitivity.

Chapter 4 presented our cross-sectional study findings on the relationships between serum vitamin D levels and cardiometabolic disease risk factors in 222 overweight and obese, preadolescent children. This is the first pediatric study to investigate the relationships between serum vitamin D status and cardiometabolic risk in a sample composed exclusively of overweight and obese, preadolescent children. Furthermore, to our knowledge, this is the first pediatric study to control for adiposity in the vitamin D/cardiometabolic relationship using MRI-derived measures of adiposity. Our findings suggest that lower vitamin D status is associated with multiple measures known to increase risk for diabetes and CVD in overweight and obese, preadolescent

children and that visceral adipose tissue (VAT), instead of general adiposity, is instrumental in mediating these associations.

We examined the relationships between vitamin D status and a wide range of cardiometabolic risk factors, inflammatory markers, and adipokines. Significant linear downward trends were observed between 25-hydroxyvitamin D [25(OH)D] tertile levels and fasting insulin, homeostasis model assessment of insulin resistance (HOMA-IR), triglycerides, and leptin, while significant linear upward trends were seen between 25(OH)D tertiles and Matsuda index, HDL-cholesterol, and adiponectin. Furthermore, children in the lowest 25(OH)D tertile were 3.5 times more likely to have ≥ two cardiometabolic risk factors (CMR) when compared to children in the highest tertile. The most important finding was that when VAT was entered as a covariate in the statistical model, all of the aforementioned trends and increased odds were attenuated.

Previous cross-sectional investigations in children, adolescents, and adults have described the relationships between vitamin D and cardiometabolic risk factors to be independent of adiposity. These studies have addressed adiposity by controlling for anthropometric measures like BMI and waist circumference or DXA-derived measures like fat mass, but these methods may not adequately adjust for the effect of adiposity. In contrast, our findings suggest that this relationship is in fact fat-dependent, but dependent on the location of the fat. Visceral adipose tissue has been shown to independently predict vitamin D status in children,[16] and in the one adult cross-sectional study that used a detailed, reliable characterization of adiposity through VAT and subcutaneous adipose tissue (SAT) volume measurements, the relationship between 25(OH)D and insulin sensitivity lacked statistical significance after adjusting for VAT.[17]

Mechanistic research is needed to elucidate the role of metabolically-active VAT in the relationship between vitamin D status and cardiometabolic disease risk. Along with Cheng et al.,[17] our findings demonstrate the instrumental, mediating role that visceral adiposity plays in vitamin D status and cardiometabolic risk, which should shift the paradigm and improve the future designs of observational studies in the area of vitamin D, CVD, and diabetes.

There are several areas that represent gaps in the current literature. Underlying mechanisms behind the observed, lower serum vitamin D levels in overweight and obese individuals compared to their normal weight counterparts should be explored. Human data is needed to determine tissue storage sites and concentrations for vitamin D sequestration. There is also a need for continued cellular research to discover the precise alterations in gene expression responsible for vitamin D's role in heart disease and diabetes, and vitamin D receptor genetic polymorphisms should continue to be explored, as they likely mediate risk for cardiometabolic disease and/or response to supplementation. Future cross-sectional investigations must adequately adjust for pertinent mediating variables, particularly adiposity. No longitudinal studies exist that commence during childhood and adolescence and extend into adulthood to examine the risk of heart disease and diabetes incidence based on prior vitamin D intake and 25(OH)D status. These types of studies are worth exploring, since some of the strongest evidence for vitamin D's role in heart disease and diabetes originates from adult prospective cohort studies.

Finally, although the vitamin D recommendations were recently updated by the IOM and based on skeletal outcomes, significant gaps remain in the literature regarding

recommendations specific to pediatric years, disease states such as diabetes and CVD, particular racial groups, and those who are overweight or obese, which represents a large proportion of the United States population. With the rise in obesity and associated sequelae, cost-effective public health prevention and treatment measures are needed. Total healthcare costs for diabetes alone were estimated to be 174 billion dollars in 2007.[18] Vitamin D supplementation is a cheap, safe way to achieve 25(OH)D sufficiency. Future research may point towards the importance of vitamin D sufficiency in ameliorating or preventing insulin resistance and cardiometabolic dysfunction, ultimately informing future IOM DRI recommendations issued for this fascinating micronutrient and improving the health status of millions of Americans.

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APPENDIX A

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

1. B. 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 Non-Hispanic or Latino	Hispanic or Latino Non-Hispanic or Latino
Race:	American Indian or Alaska Native Asian Black or African American Native Hawaiian/ Pacific Islander White any combination of the above	American Indian or Alaska Native Asian Black or African American Native Hawaiian/other Islander White 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 Non-Hispanic or Latino	Hispanic or Latino Non-Hispanic or Latino
Race:	American Indian or Alaska Native Asian Black or African American Native Hawaiian/ Pacific Islander White any combination of the above	American Indian or Alaska Native Asian Black or African American Native Hawaiian/other Islander White 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? _____

5. How tall is your child? ____ft____ in How much does he/she weigh? _____ lbs.

Calculate BMI and plot on CDC growth chart – must be between 5th and 95th percentile BMI for age and sex to qualify.

6. Has your child lost or gained weight in the past 3 months? YES _____ NO _____

If yes, how much? _____lbs

7. 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 _____
Zinc Malabsorption (e.g. acrodermatitis enteropathica)	YES _____ NO _____
Psychological Illness	YES _____ NO _____

8. Is your child currently taking any medications? YES _____ NO _____

If yes, what medication(s)? _____
(*check approved and non-approved medication list: check specifically for Adderall, Ritalin, and steroid medications*)

9. 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*)

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 sexual 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 sexual 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_____ S ____

"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

CDC BMI Percentile Charts for Age and Sex

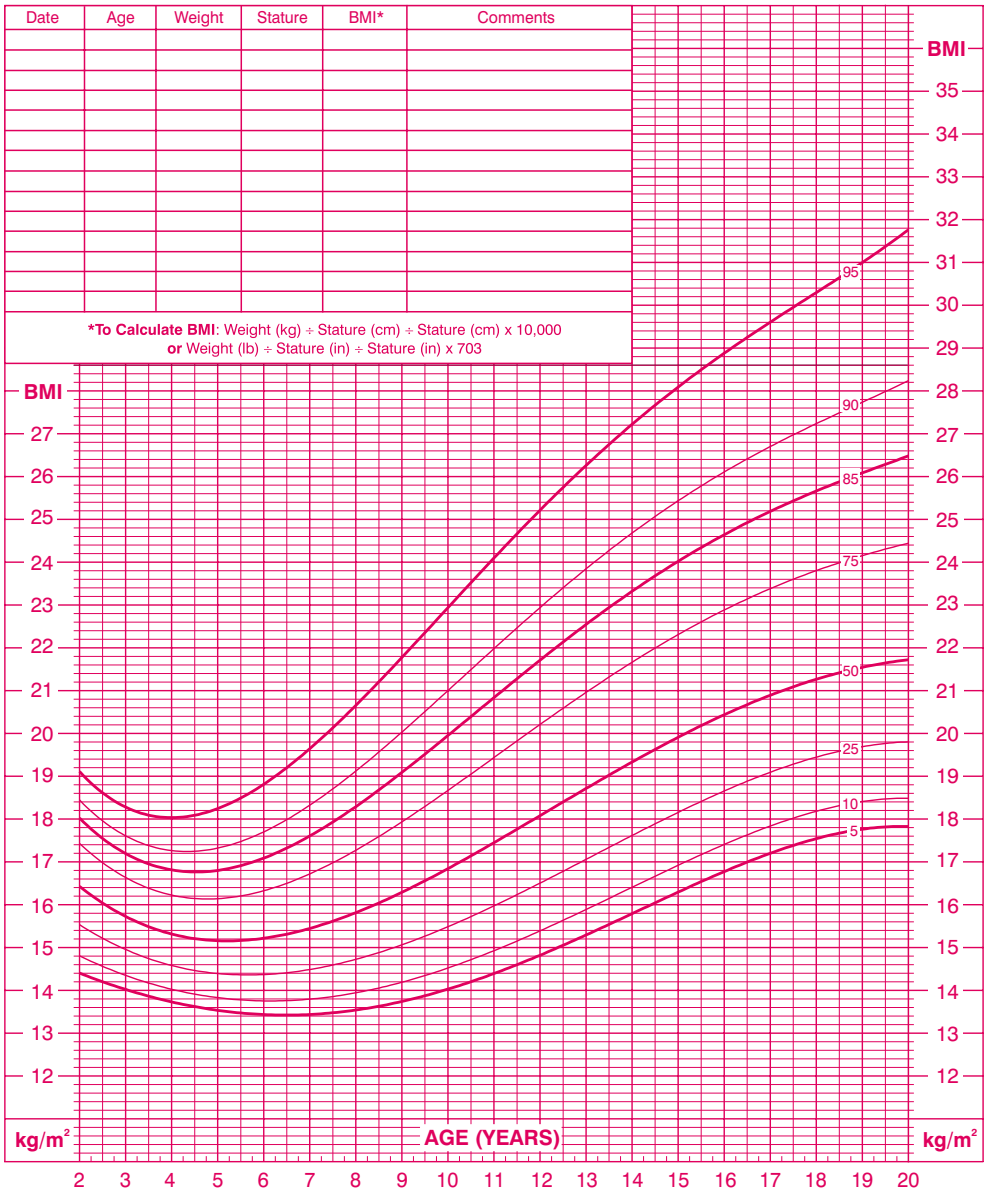
2 to 20 years: Girls
Body mass index-for-age percentiles

NAME _____

RECORD # _____

Date	Age	Weight	Stature	BMI*	Comments

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



Published May 30, 2000 (modified 10/16/00).
SOURCE: Developed by the National Center for Health Statistics in collaboration with the National Center for Chronic Disease Prevention and Health Promotion (2000).
http://www.cdc.gov/growthcharts

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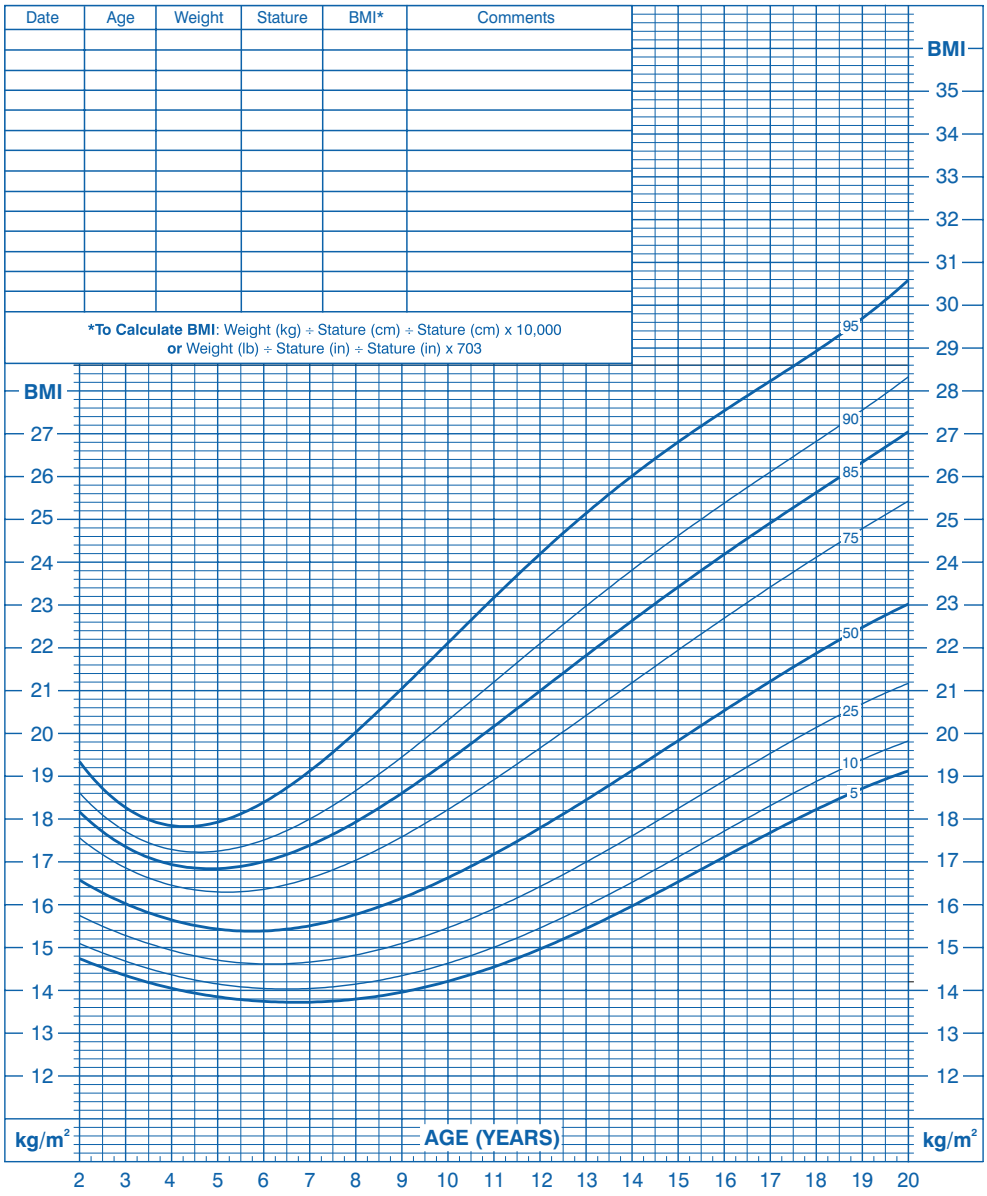
2 to 20 years: Boys
Body mass index-for-age percentiles

NAME _____

RECORD # _____

Date	Age	Weight	Stature	BMI*	Comments

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



Published May 30, 2000 (modified 10/16/00).

SOURCE: Developed by the National Center for Health Statistics in collaboration with the National Center for Chronic Disease Prevention and Health Promotion (2000).
http://www.cdc.gov/growthcharts

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APPENDIX C

Maturity Offset Calculations

Maturity Offset Prediction Equations

Variables:

- Age (yrs)
- Height (cm)
- Weight (kg)
- Leg Length (cm)
- Sitting Height (cm)

Maturity Offset (Males) =
-9.236 + [0.0002708*(Leg Length*Sitting Height)] + [-0.001663*(Age*Leg Length)] + [0.007216*(Age*Sitting Height)] + [0.02292*(Weight/Height*100)]

Maturity Offset (Females) =
-9.376 + [0.0001882*(Leg Length*Sitting Height)] + [0.0022*(Age*Leg Length)] + [0.005841*(Age*Sitting Height)] + [-0.002658*(Age*Weight)] + [0.07693* (Weight/Height*100)]

Mirwald, R. L., A. D. Baxter-Jones, et al. (2002). "An assessment of maturity from anthropometric measurements." Med Sci Sports Exerc **34**(4): 689-94.

APPENDIX D

GAPI Body Composition Conversion Equations

Dual Energy X-ray Absorptiometry Body Composition Conversion Equations

Total Fat Mass
To convert UGA to PU:
= 0.891816 x UGA total fat content + 700.831037

To convert IUSM to PU
= 1.0572 x IUSM total fat content + 1848.1

Lean Mass
To convert UGA to PU:
= 1.027785 x UGA lean mass – 980.601068

To convert IUSM to PU
= 0.9113 x IUSM lean mass + 299.43

Percent Body Fat
To convert UGA to PU:
= 0.927841 x UGA %fat + 1.989372

To convert IUSM to PU
= 1.0502 x IUSM %fat + 3.7823

UGA = The University of Georgia

PU = Purdue University

IUSM = Indiana University School of Medicine

APPENDIX E

GAPI Three-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.

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

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

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

   Each food that you ate.

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

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

3. **It is important to describe each food you eat in detail.**
   For example:

   Write down brand names for each food you ate if you know them.

   Write down the type of milk (whole, 2%, or skim) and bread (white, wheat, etc).

   Write down if the food was fresh, frozen, or canned.

   If you ate a casserole or a salad, write down the foods there were in it and amounts.

   If you add things like butter, jelly, sugar, honey, or cream to foods or beverages, please write them down with the amounts used.

4. **Do you drink whole _____, 2% _____, 1% _____, or skim ____ milk?**

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

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

   _____

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

DAY 1 OF THE DIET DIARY

ID: _____ **CHECKED BY:** _____

DATE: _____ **DAY OF THE WEEK:** _____

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

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

_____

_____

_____

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

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

APPENDIX F

Physical Activity Questionnaire

YOUTH RISK BEHAVIOR SURVEY 2001

This survey is about health behavior. It has been developed so you can tell us what you do that may affect your health. The information you give will be used to develop better health education for young people like yourself.

DO NOT write your name on this survey. The answers you give will be kept private. No one will know what you write. Answer the questions based on what you really do.

Completing the survey is voluntary. Whether or not you answer the questions will not affect your grade in this class. If you are not comfortable answering a question, just leave it blank.

The questions that ask about your background will be used only to describe the types of students completing this survey. The information will not be used to find out your name. No names will ever be reported.

Make sure to read every question. Fill in the circles completely. When you are finished, follow the instructions of the person giving you the survey.

Thank You Very Much For Your Help.

The next 7 questions ask about physical activity.

80. On how many of the past 7 days did you exercise or participate in physical activity for <u>at least 20 minutes that made you sweat and breathe hard</u>, such as basketball, soccer, running, swimming laps, fast bicycling, fast dancing, or similar aerobic activities?

a. 0 days
b. 1 day
c. 2 days
d. 3 days
e. 4 days
f. 5 days
g. 6 days
h. 7 days

81. On how many of the past 7 days did you participate in physical activity for <u>at least 30 minutes</u> that did <u>not</u> make you sweat or breathe hard, such as fast walking, slow bicycling, skating, pushing a lawn mower, or mopping floors?

a. 0 days
b. 1 day
c. 2 days
d. 3 days
e. 4 days
f. 5 days
g. 6 days
h. 7 days

82. On how many of the past 7 days did you do exercises to <u>strengthen or tone your muscles</u>, such as pushups, sit-ups, or weight lifting?

a. 0 days
b. 1 day
c. 2 days
d. 3 days
e. 4 days
f. 5 days
g. 6 days
h. 7 days

83. On an average school day, how many hours do you watch TV?

a. I do not watch TV on an average school day
b. Less than 1 hour per day
c. 1 hour per day
d. 2 hours per day
e. 3 hours per day
f. 4 hours per day
g. 5 or more hours per day

84. In an average week when you are in school, on how many days do you go to physical education (PE) classes?

a. 0 days
b. 1 day
c. 2 days
d. 3 days
e. 4 days
f. 5 days

85. During an average physical education (PE) class, how many minutes do you spend actually exercising or playing sports?

a. I do not take PE
b. Less than 10 minutes
c. 10 to 20 minutes
d. 21 to 30 minutes
e. 31 to 40 minutes
f. 41 to 50 minutes
g. 51 to 60 minutes
h. More than 60 minutes

86. During the past 12 months, on how many sports teams did you play? (Include any teams run by your school or community groups.)

a. 0 teams
b. 1 team
c. 2 teams
d. 3 or more teams

The next question asks about AIDS/HIV education.

87. Have you ever been taught about AIDS or HIV infection in school?

a. Yes
b. No
c. Not sure

APPENDIX G

GPI 24-Hour Dietary Recall

Georgia Prevention Institute
Department of Pediatrics
Medical College of Georgia
Building HS-1640
1120, 15th Street
Augusta, Georgia 30912

24-HOUR DIETARY RECALL

Participant ID #:

Date: Check one: *Weekday____ Weekend____*

Visit #:

Interviewer:

Telephone #:

Time of day	Meal/Snack/Drink (Choose one)	What did you eat/drink? (Include cooking method & brand name)	Amount