THE EFFECTS OF HIGH DOSE VITAMIN D SUPPLEMENTATION ON GLUCOSE METABOLISM AND INFLAMMATION IN OBESE ADOLESCENTS

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

THE EFFECTS OF HIGH DOSE VITAMIN D SUPPLEMENTATION
ON GLUCOSE METABOLISM AND INFLAMMATION
IN OBESE ADOLESCENTS

Presented by Anthony M. Belenchia

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And hereby certify that in their opinion it is worthy of acceptance.

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DEDICATION

There are several other individuals who I feel obliged to thank for helping me achieve this and all of my other life goals. First, my parents, sisters, and nephews—I wouldn't have made it to this point without all of you and the constant love and encouragement that you provided me.

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Introduction

It has been estimated that 40-60% of generally healthy young adults in the United States have some degree of vitamin D deficiency, as measured by circulating 25-hydroxyvitamin D (25(OH)D) concentrations, with an even greater prevalence in minorities, females, elderly, and obese individuals (*1-3*). Compared to healthy controls, obese children and adults have been observed to have significantly lower serum concentrations of 25(OH)D, which is attributed to the sequestration of circulating 25(OH)D by adipose tissue (*4, 5*). The traditional and most widely understood roles of vitamin D involve calcium homeostasis. However, relatively recent research has elucidated several neo-classic roles of vitamin D, which along with correlational data from cross-sectional and epidemiological studies have theoretically linked vitamin D to several health conditions, including obesity and its associated metabolic complications (*6-12*). Among these metabolic complications are insulin resistance and impaired glucose tolerance, with which vitamin D deficiency shares some of the same risk factors, including overweight/obesity, ethnicity, and age.

One of the hallmark characteristics of obesity is the chronic state of subclinical inflammation that it induces (*13, 14*). This inflammation represents what many believe to be the causal link between obesity and the subsequent metabolic conditions that result from obesity (*15*). There are well documented inverse associations between circulating 25(OH)D concentrations and several inflammatory markers that are elevated in obesity (*8, 16*). Moreover, cross-

sectional data linking incidence of diabetes to vitamin D status is plentiful and recent clinical-trials have demonstrated that improving vitamin D status may ameliorate impaired glucose metabolism, insulin resistance, and inflammation (*17-21*). However, the specific mechanisms by which these beneficial effects occur are unclear.

The mounting research suggests great potential for the improvement of vitamin D status on several health outcomes, such as those related to obesity. At the current time, there is a paucity of well-designed, placebo-controlled clinical trials investigating how improving vitamin D status can impact some of these health outcomes. Therefore, the objectives of the current study were (1) to determine the efficacy of 4,000 IU/day of vitamin D_3 in obese adolescents; (2) to determine if the enhancement of circulating 25(OH)D concentration would improve markers of insulin resistance and reduce inflammation in obese adolescents. Furthermore, we sought to determine if the improvements in insulin resistance, if any, would be accompanied by or associated with reductions in biomarkers of inflammation. To our knowledge, the current study is the first to examine the effects of high-dose vitamin D supplementation on metabolic outcomes in severely obese children and adolescents.

EXTENDED REVIEW OF LITERATURE

I. VITAMIN D

Structures and Synthesis

The term vitamin D can refer to one or both of the structural analogues: vitamin D_2 and vitamin D_3. These two fat-soluble compounds, which are classified as secosteroid hormones, are structurally very similar; differing only in their side chains (figure 1). Additionally, these two vitamers are formed from different provitamins through unique reactions/pathways. Vitamin D_2 is formed when the fungal sterol, ergosterol (provitamin D_2), undergoes irradiation upon exposure to sunlight. Vitamin D_3, also known as cholecaciferol, is photochemically produced through two distinct reactions that take place in skin. The first reaction occurs when provitamin D_3 (7-dehydrocholesterol) is exposed to ultraviolet B (UVB) light (290-315 nm) and produces previtamin D_3. Shortly thereafter, a subsequent spontaneous reaction, involving a structural rearrangement of the thermodynamically unstable compound, previtamin D_3, produces vitamin D_3 (figure 2). Cutaneous production of vitamin D_3 begins in the plasma membrane of actively growing layers of the epidermis, where the reaction between ultraviolet B (UVB) radiation (209-315 nm) and provitamin D3 forms up to 90% of all previtamin D_3**.** After the isomerization of previtamin D3 to vitamin D3, the newly formed vitamin is expelled into the extracellular space, where it is rapidly taken up by the dermal capillary bay, and eventually circulation via the vitamin D-binding protein (DBP).

Sources

While cutaneous production of vitamin D_3 represents the primary source of vitamin D for most humans, at higher latitudes individuals are unable to produce the vitamin due to inadequate UVB rays from the sun (*22*). Here, humans and animals acquire the vitamin through dietary intake of vitamin D-rich foods. The availability of vitamin D from naturally occurring dietary sources is limited to fatty fish, oils derived from fatty fish, and irradiated mushrooms. However, in recent years manufacturers have begun to fortify several foods with the vitamin. Among these foods are cereals, pastas, orange juice, milk and other dairy products (table 1). Additionally, dietary supplements containing doses from 400-5,000 IU of vitamin D3 are readily available to the public (*23*). Whereas, D_2 is only available by prescription.

DRI

In 2010, the Institute of Medicine's (IOM) Food and Nutrition Board (FNB) established new dietary reference intakes (DRI) for vitamin D. According to the new guidelines, the recommended dietary allowance (RDA) for individuals from one to seventy years of age is 600 IU per day and 800 IU per day for those above 70 years of age (*24*). Additionally, the tolerable upper intake level (UL) was raised to 4,000 IU per day for all individuals 9 years of age and older. While many of these values are more than 2x greater than pervious amounts, they are not without controversy. The current recommendations are

solely based upon optimizing bone health, which has led several experts in the field to question the appropriateness of the current recommendations (*25-27*). Furthermore, they do not take into account the increased need of specific populations, such as overweight or dark-skinned individuals, who are at an even greater risk for deficiency, and require greater amounts to produce the same rise in serum 25(OH)D concentrations (table 3) (*28*).

Metabolism

The conversion of both forms of vitamin D to the bioactive hormone 1,25-dihydroxy vitamin D (1,25$[OH]_2$D) requires two hydroxylation reactions (figure 2). The first hydroxylation, which occurs in the liver via the 25-hydroxylase enzyme, produces 25-hydroxyvitamin D (25(OH)D). 25(OH)D is the major circulating form of vitamin D and the form by which vitamin D status is assessed. The second hydroxylation, which primarily occurs in the proximal tubule of the kidney in a reaction catalyzed by the 1α-hydroxylase enzyme (CYP27B1), produces 1,25$(OH)_2$D. This second hydroxylation is largely regulated by parathyroid hormone (PTH), which is secreted by the parthyroid gland in response to low calcium concentrations. Following renal activation, 1,25$(OH)_2$D can act in either a endocrine, entering circulation and proceeding to several target organs, or paracrine, acting upon the cell in which it was converted, manner. In addition to the kidneys, several cells that express vitamin D receptors (VDRs) also express 1α-hydroxylase by which they self-

regulate intracellular 1,25$(OH)_2$D concentrations; these include macrophages, epidermal keratinocytes, brain, pancreatic beta-cells, and in some species--skeletal muscle (*29-33*).

Mechanisms of Action

Vitamin D Receptors- The vitamin D receptor (VDR) is a nuclear receptor in the steroid hormone family. As with all nuclear receptors, the VDR contains a ligand-binding domain, an activation domain, a hinge region, and a DNA-binding region (*34*). VDRs are expressed in nearly every type of tissue in the body including but not limited to, the liver, muscle, adipose, pancreatic β-cells, and immune cells, all of which are associated with obesity and its metabolic complications (*32, 35-38*).

Genomic mechanism of action- VDRs, upon activation by the binding of their ligand, 1,25(OH)2D, to the ligand-binding domain, form a heterodimer with retinoid X receptors (RXR). The newly formed dimer then binds to VDR-response elements (VDRE) in the promoter region of a gene, which subsequently induces or represses gene transcription (*38*). Additionally, recent evidence suggests that the VDR is capable of functioning in some capacities even in the absence of its ligand, 1,25$(OH)_2$D (*39*).

Non-genomic mechanisms- In addition to the genomic effects triggered by the binding of the VDR-RXR dimer to a DNA-response element, several non-genomic effects of $1,25(OH)_2D$ and its receptor have been observed. In cultured human adipocytes, $1,25(OH)_2D$ played a role in both lipolysis and lipogenesis through transcaltachia, the rapid channeling of calcium into the cells, (*40*). Additionally, Kajikawa *et al.* demonstrated that through a similar rapid calcium-channeling mechanism, $1,25(OH)_2D$ serves as an insulinotropic agent (*41*).

Roles

While classically implicated in bone health through its regulatory roles on calcium and phosphorous homeostasis, recent evidence suggests that vitamin D has a wide variety of "neo-classic" roles in the body. Additionally, evidence suggests that these roles are both calcium-dependent (Calciotropic) and calcium independent (non-calciotropic). It is well established that the primary function of vitamin D is in calcium homeostasis, which involves both intracellular and extracellular concentrations. Calcium concentrations are maintained through vitamin D's action at three target organs, intestines, bone, and kidneys. In the intestine, the actions of $1,25(OH)_2D$ initiate the synthesis of calbindin, a calcium binding protein necessary for absorption. Furthermore, it is believed that $1,25(OH)_2D$ plays a role interacts with its receptor in a non-genomic way to upregulate calcium and phosphorus absorption, but very little is known about

how this works (*42, 43*). During periods of hypocalcemia, vitamin D promotes the maturation of stem cells into osteoclasts through both genomic and non-genomic pathways, which breakdown bone and in order to increase circulating levels calcium and phosphorus (*43, 44*). Additionally, in the distal tubule, vitamin D promotes reabsorption of calcium when circulating concentrations are low.

Vitamin D Status

Vitamin D status is assessed and defined by the circulating concentration of 25(OH)D. There are several techniques used to determine concentration including but not limited to Radio Immunoassay (RIA), enzyme-linked immunoassay (EIA), and high-performance liquid chromatography (HPLC), which are the most commonly used for research applications (*45*). The recent report released by the Institute of Medicine (IOM) regarding the dietary recommended intakes for vitamin D determined that a circulating 25(OH)D concentration between 12.5 and 20 ng/ml was sufficient for maintaining bone health (*24*). In 2005, Grant and Holick released an updated classification system based upon concentrations that were associated with improvements of several health outcomes (table 2) (*46*). Here, they introduced a new category of "insufficiency" defined as circulating 25(OH)D concentrations of 20-32 ng/ml. Additionally, they proposed that concentrations between 32 and 100 ng/ml were sufficient, those in the 100-150 ng/ml range were excessive, and concentrations

greater than 150 ng/ml was excessive. This new classification was further substantiated when, after conducting two extensive reviews, Bischoff-Ferrari *et al.* concluded that the optimal 25(OH)D status for several health outcomes was between 30-40 ng/ml (*47, 48*).

Factors that affect status- Several factors that affect the absorption, synthesis, metabolism, or mobilization of either vitamin D or its metabolic precursors exist and put certain populations at higher risk of vitamin D deficiency; the majority of which are related to the cutaneous production of vitamin D (*49*). During winter months in temperate climates and year-round at high latitudes, the solar zenith angle is decreased, which translates to increased atmospheric absorption of UV radiation (*50*). In the Northern hemisphere, cutaneous synthesis has been demonstrated to occur between March and October, peaking in mid-summer (*51, 52*). In addition to the changes in solar radiation, colder weather is also associated with more clothing thus, less skin exposure. Another factor that is inversely associated with circulating 25(OH)D is skin pigmentation (*5*). Melanin, which is the primary factor in skin tone, functions as a natural sunblock, thus inhibiting UVB photons from reaching the skin (*53*). Likewise, even low-strength sunblock is capable of inhibiting the majority of cutaneous synthesis (*54*). Some debate exists over the efficacy of oral vitamin D_2 vs. that of D_3 sources, as some studies have shown D_3 to produce greater rises in serum 25(OH)D while others have shown the two to be equivalent (*55, 56*)

Influence of obesity- There is a well-established inverse association between body fat mass and serum 25(OH)D levels in both adults and children (*57, 58*). The central dogma surrounding this inverse relationship is that circulating vitamin D, derived from both cutaneous and dietary sources, is sequestered by adipose tissue prior to the hepatic hydroxylation and therefore, unavailable to be converted to 25(OH)D (*4, 59*) (figure 2). Studies in pigs have demonstrated that upwards of 65% of vitamin D was stored in adipose tissue (*60, 61*). In addition to the lower circulating levels of 25(OH)D seen in cases of excess adiposity, Lee *et al.* noted a similar inverse relationship between BMI and the response in vitamin D status in adults receiving daily vitamin D supplementation (*3*). These results were consistent with the findings of Heaney *et al.,* who reported that obese individuals require approximately twice as much vitamin D to produce the same rise in vitamin D status as lean individuals (*62*).

Prevalence of Deficiency

The prevalence of vitamin D deficiency in the United States has led some experts to classify the problem as an epidemic. In a review of data collected from the 2005-2006 National Health and Nutrition Examination Survey (NHANES), Forrest and Stuhldreher found that overall 41.6% of all adults and 82.1% of African-American adults in the United States had 25(OH)D levels below 20 ng/ml (*1*). Additionally, several recent reports have raised concerns about vitamin D status in healthy children and adolescents. For example, studies of youth living in Cleveland and Boston indicate that up to 54% are of “deficient” or

"insufficient" vitamin D status during winter months (*63, 64*). Another report examining data collected from the NHANES-III determined that approximately 14% of normal weight adolescents 12-19 years of age were vitamin D deficient and 48% fell in the category of insufficient (*5*). By contrast, this same study found the prevalence of deficiency in adolescents who were either overweight or at risk for overweight to be approximately 36%. Furthermore, regression analysis of this data suggested that for every 1% increase in BMI percentile there is a 5% decrease in 25(OH)D concentration.

Vitamin D and Obesity

While there is a well-documented association between vitamin D status and obesity, there is much confusion over whether vitamin D deficiency contributes to, or is a consequence of obesity (*65, 66*). This confusion is perpetuated by the mixed results seen in both human and animal studies investigating the effectiveness of vitamin D supplementation to reduce body weight and adiposity, which have produced mixed results (*67-69*).

Adipogenesis- It is well established that increases in adiposity can occur via hyperplasia or hypertrophy of adipocytes. Hyperplasia (adipogenesis) is the process by which new adipocytes differentiate from precursor cells and involves two primary phases, proliferation of preadipocytes, and differentiation to mature adipocytes. While there are over 100 transcriptional factors involved in

adipogenesis, it is widely believed that the key regulatory factor is peroxisome proliferator-activated receptors-γ (PPAR-γ) (*70-72*). Like VDR, PPAR-γ forms a heterodimer with RXR and binds to specific response elements (PPRE). Calcitriol has been demonstrated to decrease the expression of CCAAT-enhancer binding proteins α and β (C/EBPα & β) (*73, 74*). Previous studies have demonstrated that C/EBPβ plays a role regulating the expression of both PPARγ and C/EBPα, which increases the production of PPARγ ligand and PPAR-γ expression (*74*). *In vitro* studies using 3T3-L1 adipocytes have shown the ability of calcitriol to attenuate pre-adipocyte differentiation by inhibiting PPAR-γ expression. This effect was later attributed to decreases in C/EPBβ levels (*74*). Regardless, the role of calcitriol in adipogenesis is relatively unclear, as *in vitro* studies have shown it to inhibit or stimulate the process (*75, 76*). Furthermore, *in vitro* inhibition of pre-adipocyte differentiation was observed in cell cultures devoid of calcitriol (*77*).

Impaired Glucose Metabolism and Insulin Resistance

Inadequate vitamin D status is frequently implicated in many of the metabolic complications associated with obesity, such as chronic inflammation, insulin resistance, impaired pancreatic β-cell function, and impaired glucose metabolism. Additionally, there is a strong associative link between vitamin D and incidence of type 2 diabetes mellitus, which is characterized by peripheral insulin resistance, impaired glucose tolerance, and β-cell dysfunction. Multiple studies have demonstrated that insulin secretions are greater in vitamin D

sufficient animals and humans than their insufficient counterparts (*78-82*). Furthermore, several studies examining the direct effect of calcitriol administration to vitamin D deficient animal models with impaired insulin secretion capacity have demonstrated increases in insulin secretion following treatment. However, additional studies have been unable to reproduce these responses independent of glucose stimulation, leaving the exact role of vitamin D a mystery (*83, 84*).

Although there are several different parameters and diagnostic criteria used in the diagnosis of prediabetes and type 2 diabetes mellitus (T2D), the estimated numbers of people living with prediabetes or T2D is similar regardless of which diagnostic criteria are used (*85, 86*). The incidence of (T2D) is increasing at an exponential rate with greater than 1.5 million new cases diagnosed every year, up from 1 million annual new cases in 2000 (*87-89*). Once considered a disease almost exclusively seen in adult populations, children and adolescents represented approximately 5-6% of the newly diagnosed cases between 2008-2010 (*87, 90, 91*). T2D is characterized by insulin compensation, which leads to impaired glucose metabolism, and the progressive loss of beta cell function. Several studies have demonstrated that low serum 25(OH)D may be a risk factor for abnormalities in glucose homeostasis. Data from two different large epidemiological studies of adult women indicate that lower intakes of vitamin D and calcium are associated with a higher prevalence of the metabolic syndrome and/or T2D. In addition, the CARDIA study demonstrated a substantial reduction in the incidence of insulin resistance over a 10-year period with

increasing dairy intake in young adults. Although not well-studied in children, an inverse association has been shown between vit D status and elevated fasting glucose and insulin levels in an analysis of NHANES III data; while other studies have established a similar inverse relationship with A1C levels in diabetic and nondiabetic adults. Further, the prevalence of hypovitaminosis (<20 ng/ml) among those with T2D has also been reported to be significantly higher than controls (39 vs 25%).

There is conflicting data showing the effect of vitamin D administration on insulin sensitivity or secretion as well as on plasma glucose levels (table 5). In an intervention study involving subjects that had been previously diagnosed with T2D, treatment with Vitamin D had no effect on glucose metabolism or homeostasis but a significant change in insulin secretion was seen in this study (40). This supports similar findings that showed no change in insulin sensitivity or fasting plasma glucose (FPG) in vitamin D treated individuals (46;47). In one observational study, men and women that had previously been diagnosed with type 2 diabetes were administered a single 300,000 IU injection of ergocalciferol after baseline measurements of 25(OH)D, glycated hemoglobin (HbA_1C), fasting plasma glucose (FPG), fasting insulin (FPI), and insulin resistance were taken. Three months later, significant increases were seen in all five parameters (48). In a case-control study involving 22 British Asians that were classified as at risk for developing type two diabetes, the intramuscular injection of 100,000 IU of vitamin D caused a significant increase in specific insulin (49). Another intervention

study involving postmenopausal non-diabetic women also concluded that supplementation with D_3 had no effect on FPG (50).

Several theoretical explanations have been proposed to explain the potential mechanisms by which vitamin D influences glucose homeostasis. The physiological evidence supporting these theoretical mechanisms were recently summarized by Mitri, Muraru, and Pittas (*92*). Some of the circumstantial evidence presented includes VDR expression on pancreatic β-cells, the expression of 1-α-hydroxylase in pancreatic β-cells, the presence of a vitamin D response element in the human insulin gene promoter, and the presence of VDR in metabolic tissue (*29, 33, 36, 93*). Additionally, 1,25(OH)2D directly activates transcription of the human insulin receptor gene, activates PPAR, stimulates the expression of the glut 4, and enhances insulin mediated glucose transport *in vitro (81, 83, 93, 94).*

Insulin Production- As mentioned, insulin producing pancreatic β-cells express both VDR and 1-α-hydroxylase in high concentrations. The discovery of this intracellular enzyme provided evidence for a method of self-regulation, through which the cell activates 25(OH)D to 1,25$(OH)_2$D as needed. However, evidence supporting a specific mechanism by which calcitriol acts in pancreatic β-cell is inconclusive.

Glucose Uptake- In addition to regulating insulin production and release, there is significant evidence that vitamin D is involved in insulin-stimulated cellular uptake of glucose at various target organs. Among these target organs are skeletal muscle and adipose tissue, where $1,25(OH)_2D$ has been shown to directly activate the transcription of the insulin receptor gene in humans and increase expression of the insulin receptor. Moreover, *in-vitro* studies have provided evidence of calcitriol-dependent insulin-mediated glucose transport involving the GLUT-4 transporter. However, it remains unknown whether Vitamin D deficiency influences insulin sensitivity and glucose uptake through calcium dependent or independent mechanisms (*94, 95*).

Obesity and Inflammation

Obesity is characterized by chronic low-grade inflammation. In the past decade various mechanisms involved in the acute-phase inflammatory response and the activation of the innate immune system have been implicated in the pathological processes underlying insulin resistance, T2D, and other metabolic complications. The central dogma surrounding the immune-linked progression from obesity to disease involves a process that begins with adipocytes. In obesity, adipocytes expand due to increased lipid storage and as a result release several inflammatory cytokines and chemokines (96). The existence of a link between obesity-induced inflammation and insulin resistance

has been clearly established. However, while there are several mechanisms that have been implicated in this association, many are still being uncovered.

Cytokines and Chemokines

Specific pro-inflammatory cytokines and chemokines are believed to have major roles in the progression from obesity to T2D. TNF-α, Interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), and leptin are all produced and secreted in high concentrations with obesity.

Tumor necrosis factor alpha-. Among the most studied of these inflammatory compounds is tumor necrosis factor-alpha (TNF-α), an inflammatory cytokine associated with systemic inflammation that is produced by adipocytes and macrophages. While TNF-α is primarily macrophage-derived, adipocytes have also been shown to produce the cytokine; a process that is upregulated with obesity. TNF-α is often considered a "higher-order" cytokine due to the regulatory role it has over the synthesis and secretion of several other chemokines and cytokines, including several interleukins (*15, 96*). A wide variety of biological roles associated with both innate and adaptive immune responses have been linked to TNF-α, as its release is induced by both pathogens and other cytokines, namely IL-1β (*97, 98*). TNF-α has been shown to inhibit insulin mediated glucose uptake by white adipose tissue (WAT) and muscle in vivo. This decrease in insulin sensitivity has been attributed to

serine phosphorylation of IRS-1 and AS160 through the activation of the serine kinases JNK and AMPK (*99, 100*). Additionally, TNF-α has been implicated in the activation of NF-κB, production of ROS, and suppression of Glut-4 expression(*101, 102*). TNF-α has frequently been identified as a key component in the development of insulin resistance, a process believed to involve several mechanisms (*103*). TNF-α impairs insulin action by interfering with insulin signaling at several points in the insulin signaling cascade (*101*). Additionally, TNF-α has been shown to down-regulate the transcription of GLUT 4 and IRS-1 both *in vivo* and *in vitro* (*102, 104, 105*). *In vitro* studies have shown an inhibitory effect on insulin secretion from pancreatic β-cells and induced apoptosis of β-cells treated with TNF-α. Several studies have demonstrated negative associations between circulating 25(OH)D concentrations and circulating levels of TNF-α in various populations, including the obese (*106-109*). Additionally, several studies have shown that vitamin D supplementation reduces circulating levels of TNF-α, while other studies have shown an increase in the synthesis and release of TNF-α mediated by calcitriol (*108, 110, 111*). It appears that the modulation of TNF-α by calcitriol is highly dependent on the type of cell and current state of inflammation. TNF-α is derived from macrophages, which contain the 1-α-hydroxylase enzyme and self-regulate internal concentrations of calcitriol (*108, 112, 113*).

Interleukin-6- Another key inflammatory cytokine implicated in the pathogenesis of T2D is Interleukin 6 (IL-6). IL-6 is an inflammatory cytokine

that is predominantly released from adipose tissue macrophages, with a greater amount being produced with excess adiposity. In addition other immune cells, skeletal and smooth muscle cells, islet β-cells, hepatocytes, and glial cells have all been shown to produce IL-6 (*114-117*). Also like TNF-α, IL-6 is a component of both the innate and adaptive immune systems. It is believed that IL-6 has a negative effect on signal transduction by hepatic insulin receptors (*118, 119*). IL-6 has also been demonstrated to have both a stimulating and inhibiting effect on peripheral glucose uptake by muscle cells, adipocytes, and several cells in the nervous system (*120-123*). IL-6 has a negative effect on signal transduction by hepatic insulin receptors (*118*). Additionally, IL-6 has is known to stimulate hepatic CRP, another protein implicated in the pathogenesis of T2D, production and secretion. Like TNF-α, multiple studies have shown a negative correlation between vitamin D status and circulating IL-6 and CRP levels (*109, 124*). However, trials involving vitamin D supplementation are inconclusive (*125, 126*).

Adipokines

Leptin- Leptin is an adipocytokine that is predominantly secreted from adipocytes and circulates in concentrations proportional to adiposity. In both children and adults, several cross-sectional studies have reported that plasma leptin levels are positively associated with insulin concentrations and beta-cell secretory capacity. Furthermore, fasting leptin concentration is significantly

correlated with insulin sensitivity in healthy normal-weight adults. The most well studied role of leptin is that of satiety regulation, which occurs through its action in the hypothalamus and arcuate nucleus (*127, 128*). However, leptin is also known to exert direct actions on several other organs and tissue involved in energy metabolism such as skeletal muscle and liver (*129*). In the liver, leptin stimulates glucose uptake and inhibits gluconeogenesis (*130-132*). Additionally, it is believed that leptin is involved in insulin and glucagon regulation, although the role is unclear and controversial (*133-135*). It has been proposed that bidirectional feedback occurs between the endocrine pancreas and adipocytes, where leptin down-regulates insulin production/secretion and insulin stimulates leptin production/secretion (*136, 137*). *In vitro*, cells treated with calcitriol displayed a sharp decrease in leptin secretion from adipose tissue (*138*). Studies have shown that a correlation between leptin concentration and vitamin D status exists that is independent of adiposity (*106, 139*). However, a clear mechanism linking the two has yet to be demonstrated.

Adiponectin- Adiponectin is an adipocytokine that is secreted exclusively from adipose tissue in response to insulin (*140*). However, conversely to leptin, adiponectin secretion has a strong negative correlation with adipose size and number, as well as insulin resistance (*141, 142*). Adiponectin production is also highly regulated by several factors including inflammatory cytokines such as TNF-α (*143*). Adiponectin has been demonstrated to have an insulin-sensitizing effect in peripheral tissues (*144*),

specifically, in muscles where a sharp decrease in adiponectin receptors is seen in humans with Type 2 diabetes (*145*). Additionally, adiponectin has been shown to inhibit gluconeogenesis and stimulate fatty acid (FA) oxidation in the liver. Adiponectin receptors are also expressed in pancreatic β-cells but little is known about their role in these cells (*146*). When bound to its receptors, adiponectin exerts most of its effects via the phosphorylation of AMPK. While studies have shown a correlation between vitamin D status and serum adiponectin, activated PPARγ has a strong stimulatory effect on adiponectin production and, as mentioned earlier, calcitriol is a major inhibitor of PPARγ (*147-149*). This, in addition to the previously mentioned correlation between vitamin D status or calcitriol treatment and many of the factors influencing adiponectin production, suggest a possible link between adiponectin and vitamin D. However, direct mechanisms relating vitamin D to adiponectin have yet to be established.

Summary

There are several studies demonstrating strong independent relationships between vitamin D status and several metabolic complications of obesity. However, it is unknown whether vitamin D deficiency is directly culpable in the pathogenesis of these complications or just a secondary consequence of obesity. Additionally, while several theoretical explanations have been proposed, and some validated, little is known about the

mechanisms by which vitamin D functions in this capacity. For example, there is a growing body of evidence suggesting that through its direct effects on glucose uptake and insulin regulation, vitamin D plays an important role in maintaining glucose homeostasis. However, additional secondary roles, such as decreases in the chronic sub-clinical inflammation seen in obesity, have also been put forward by researchers. The mounting research suggests great potential for the improvement of vitamin D status on several health outcomes, such as those related to obesity. At the current time, there is a paucity of well-designed, placebo-controlled clinical trials investigating how improving vitamin D status can impact some of these health outcomes.

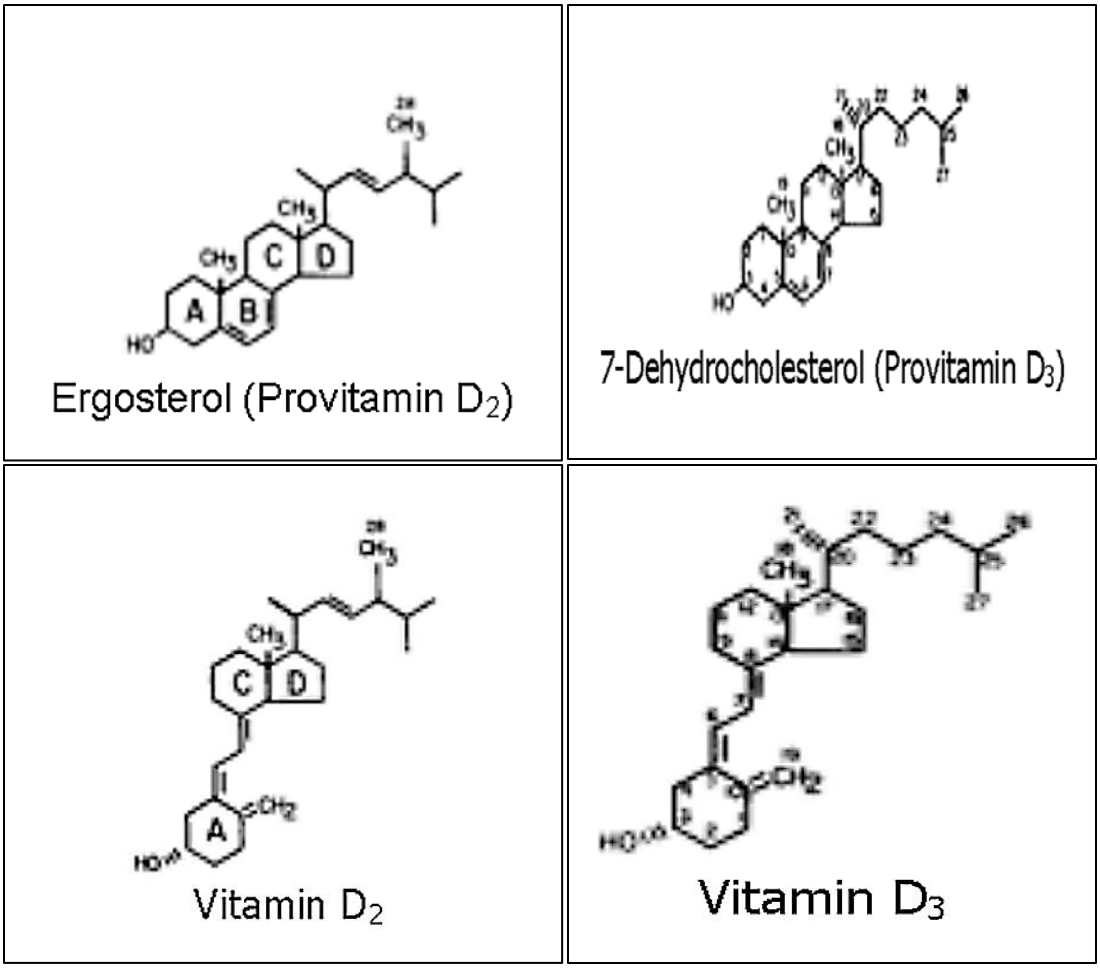


Figure 1- Forms of vitamin D. Adapted from Holick, 2003 (*152*).

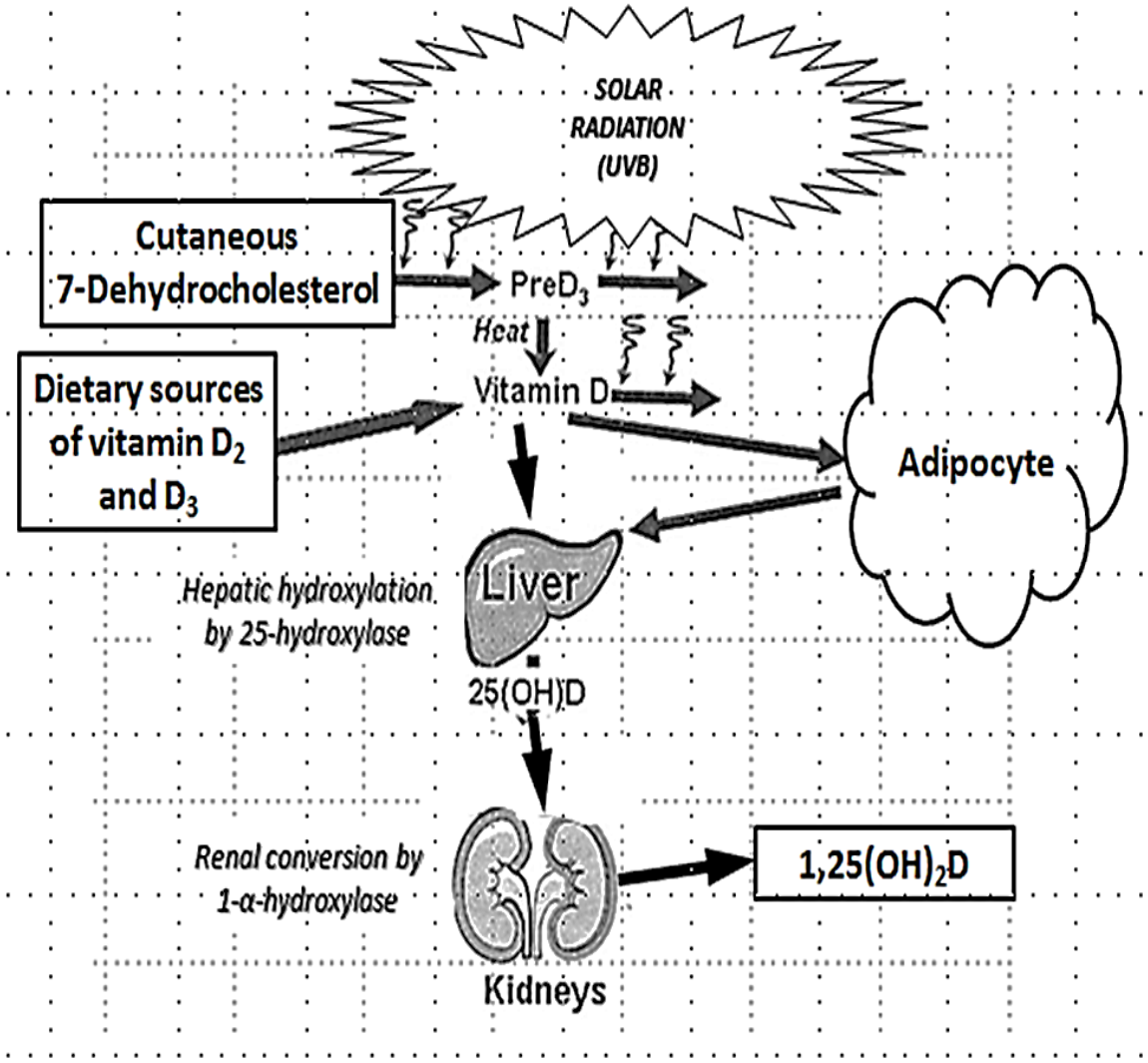


***Figure 2*- Biological pathway of vitamin D.** Adapted from Holick (*59*)

Source	Amount
Cod Liver Oil (1 tablespoon)	1,360 IU
Fortified Orange Juice (8 ounces)	100 IU
Salmon (3.5 ounces)	360 IU
Canned Tuna Fish (3 ounces)	200 IU
Fortified Milk (8 ounces)	100 IU
Fortified Margarine (1 tablespoon)	60 IU
Egg (1 whole)	20 IU

Table 1 *– Common sources of dietary vitamin D. Grant and Holick, 2005; NIH, 2012 (46, 151)*

25(OH)D Concentrations (ng/ml)	Classification
<20	Deficiency
20-32	Insufficiency
32-100	Sufficiency
>100	Excess
>130	Intoxication
***54-90**	Normal in regions near the equator

Table 2 – Classifications of vitamin D status according to Grant and Holick *(46).*

Baseline Concentration (ng/ml)	Intake Required Lean/healthy (IU/day)	Intake Required-Obese (IU/day)
8-12	~2000-2400	4000-4800
12-16	~1800-2000	3200-4000
16-24	~1000-1600	2000-3200
>32 (maintenance)	500-1000	1000-2000

Table 3 – Estimated oral intake required to reach sufficient (>32 ng/ml) 25(OH)D concentrations in lean and obese individuals. Adapted from Wortsman et al.,2000; Grant and Holick, 2005; Holick, 2005; and Bischoff-Ferrari, 2007. (*4, 46, 59, 150*)

Target Organ	Actions
Kidney	↑calcium reabsorption
Bone	↑ growth and development, ↑ remodelling, mineral homeostasis
Gut	↑ calcium and phosphorous absorption
Immune Cells	Promotes monocyte differentiation to macrophages, inhibits antigen-presenting capacity of macrophages, decreases proliferation of activated lymphocytes, reduces activity of NK cells, shifts T-helper cell ratio toward Th2 subset
Pancreatic β-Cells	regulates synthesis and secretion of insulin
Parathyroid Gland	decreases parathyroid hormone secretion
Skeletal Muscle	Stimulates expression of insulin receptor, increases glut-4 transporter concentration

Table 4 – Major target organs of vitamin D_3 and its actions. Adapted from Lee et al., 1994; Lips, 2006; Boucher, 2005; and Borradale, 2009 (*81, 153, 154*).

Author	Subject Characteristics	Sample Size (n=)	Treatment or Intervention	Study Duration	Pre-Post Treatment 25(OH)D	Glycemic Outcome
Inomata et al. (1986) (*82*)	T2D Japanese Adults; mean age 54.3 yrs	14	2 µg alphacalcidiol daily	3 weeks	n/a	Increased insulin secretion and AUC (OGTT)
Boucher et al. (1995) (*155*)	Glucose Intolerant Asians; mean age 44.9 yrs; mean BMI 25.9 kg/m^2	22	100,000 IU vitamin D_3 injection	8-12 weeks	Baseline=3.6 ng/ml Post= 13.5 ng/ml	Increase in post-challenge insulin (OGTT) and C-peptide
Borissova et al. (2003) (*83*)	T2D Women; Mean age (53.8 yrs); Mean BMI 30.9 kg/m^2	10	1332 IU vitamin D_3 daily	1 month	Baseline=14.1 ng/ml Post= 25.3 ng/ml	No change in HOMA-IR; Increase in first-phase insulin secretion (IVGTT)
Pittas et al. (2007) (*156*)	American adults; mean age (71.2 yrs); mean BMI (26.7 kg/m^2)	314	700 IU vitamin D_3 plus 500 mg calcium	3 years	Baseline=28.5 ng/ml Post= 41.0 ng/ml	Improved HOMA-IR (subjects with impaired fasting glucose at baseline)
Tai et al. (2008) (*157*)	Caucasian Adults; Mean age (55 yrs); mean BMI (24.1 kg/m^2)	33	Two 100,000 IU oral doses of vitamin D_3	1 month	Baseline=16 ng/ml Post= 36.1 ng/ml	No change in fasting glucose, insulin, QUICKI, or HOMA-IR

Table 5 – *Select trials examining the effects of vitamin D supplementation on glycemic outcomes.*
AUC= area under curve; OGTT= oral glucose tolerance test; IVGTT=intravenous glucose tolerance test

Author	Subject Characteristics	Sample Size (n=)	Treatment or Intervention	Study Duration	Pre-Post Treatment 25(OH)D	Glycemic Outcome
Jorde et al. (2009) (*158*)	T2D Norwegian Adults; age range (21-70 yrs); mean BMI 32 kg/m^2	32	40,000 IU vitamin D_3 /wk	6 months	Baseline=24 ng/ml Post= 47.3 ng/ml	No change in fasting glucose, insulin, HOMA-IR, or HbA1C vs. placebo
Nagpal et al. (2009) (*159*)	Obese Asian-Indian Men	100	120,000 IU vitamin D_3 every other week	6 weeks	Baseline=14.6 ng/ml Post= 28.6 ng/ml	No change in HOMA-β or HOMA-IR; Increased insulin sensitivity AUC (OGTT)
von Hurst et al. (2009) (*20*)	Insulin-Resistant, South Asian women in New Zealand; mean age (41.8 yrs); mean BMI (27.5 kg/m2)	81	4,000 IU vitamin D3 daily	6 months	Baseline=8.4 ng/ml Post= 32 ng/ml	Decreases in HOMA2%S, HOMA-IR, and fasting insulin vs. placebo
Mitri et al. (2011) (*160*)	American adults; mean age (57 yrs); mean BMI (32 kg/m2)	92	2,000 IU D_3 per day with or without 800 mg calcium	16 weeks	Baseline=26.5 ng/ml Post= 34.2 ng/ml	Vitamin D alone: decrease in HbA1C, FPG, no change in insulin sensitivity indices
Witham et al. (2010) (*161*)	T2D American adults; age range (48-72 yrs); BMI range (28-36)	61	Single oral dose of 100,000 or 200,000 D_3	8 weeks	Baseline=16.4 & 19.2 ng/ml Post= 25.2 & 31.6 ng/ml	100,000 IU group: no change in HOMA IR or HBA1C; 200,000 IU group decrease in HOMA-IR no change in HBA1C

Table 5 continued– *Select trials examining the effects of vitamin D supplementation on glycemic outcomes.*
HOMA2%S= homeostatic model for assessing insulin sensitivity

METHODS

I. SUBJECTS

From November 2009 until January 2011, fourty-four obese children and adolescents, ages 9-19, were recruited from the MU-Adolescent obesity clinic (ADOBE) to take part in a study investigating the effects of vitamin D supplementation on metabolic health factors related to obesity. All subjects were current patients of Dr. Aneesh Tosh at time of enrollment. Following the approval of Dr. Tosh, subjects and their parents were read a copy of the consent form. When necessary, an age appropriate assent form was also read to the subject to ensure that they better understood what the study entailed. After consent was obtained, subjects underwent additional screening for eligibility conducted by the study coordinator. Exclusion criteria included (1) use of vitamin D supplements other than a general multi-vitamin; (2) the use of medication affecting vitamin D metabolism; (3) the use of a tanning bed or undergoing UV therapy; (4) use of oral hypoglycemic agents; (5) previously diagnosed hepatic or renal disorders; (6) pregnancy; (7) the use of tobacco or alcohol. Additionally, subjects who were previously on metformin were to undergo a 2-week washout period prior to beginning the study. However, no such subjects were actually enrolled. Upon enrollment, subjects were randomized to one of two groups by the University of Missouri Hospital Pharmacy- Investigational Drug Service division (UMH-IDS). Treatment group assignment and pill distribution were blinded to both the investigators and subjects. In order to minimize possible age and development-related differences in primary outcomes, randomization was stratified into three

age groups (9-12 years-old, 13-15 years-old, 16-19 years-old). Permuted blocking of group size 4 was used within each age group so that there will be equal numbers assigned to treatment and placebo groups after randomizing each set of four participants.

II. PROTOCOL

The current study was a randomized double-blind, placebo-controlled, vitamin D supplementation trial lasting six months. The study consisted of three visits-- baseline and two subsequent follow up visits at three and six months. Study protocols were approved by the University of Missouri Health Sciences Institutional Review Board (IRB); written consent was provided by participants' legal guardians and written assent was provided by all participants. The study was registered with ClinicalTrials.gov (no.NCT00994396).

Intervention- Adolescents in both the Control and Experimental groups were asked to continue with current standard care, which consisted of quarterly visits with Dr. Tosh and the ADOBE dietitian, and not to use commercial tanning beds for the duration of the study.

Standard diet and behavior counseling- Each session with a dietitian lasted approximately 60 minutes and included an assessment of current dietary intakes and measurement of height and weight. Lifestyle counseling was individually-tailored and included dietary, exercise, and behavioral components.

In addition, adolescents were instructed to gradually increase intentional physical activity to a goal of 60-75 minutes of moderate exercise most days of the week as recommended by the DHHS/USDA 2005 Dietary Guidelines. During the diet counseling sessions, it was also emphasized that subjects consume adequate amounts of calcium to achieve the DRI of 1200 mg/day. Since milk/milk products are the main source of calcium in the US diet, subjects unable to consume milk were instructed to take a calcium supplement (without vit D) along with nonmilk dietary sources to achieve 1200 mg/day.

Placebo and vitamin D pills- Vitamin D_3 and placebo were provided by Reliance Private Label Supplements (Sumerset, NJ). The vitamin D pills contained 2,000 IU of vitamin D_3 and the placebos contained mineral oil but were otherwise indistinguishable. The University Hospital Pharmacy stored and distributed vitamin D_3 and placebo pills. 90 day supplies (180 pills) were dispensed at the baseline and three month visits and subjects were instructed to consume two pills each daily. Additionally, subjects were provided with a calendar to keep track of daily consumption (Appendix E). Subjects were also instructed to return excess pills at the three and six month follow up visits, which were used to monitor compliance.

Data collection time points: The study time table is highlighted in appendix D. At all visits, anthropometrics were measured and blood was drawn

for both standard care and research purposes. Detailed explanations of these procedures can be found in the following sections.

III. MEASUREMENTS

Anthromopetrics

Height, to the nearest 0.5 cm, and weight, to the nearest 0.1 kg, were obtained at the clinic. From these values, body mass index (BMI) was calculated as weight, in kg, divided by height, in meters, squared. Waist circumference was determined at the umbilicus, with a measuring tape parallel to the floor according the standards established by the NIH/NHLBI. BMI-Z scores were calculated based on 2000 normative data, using Epi-Info software (version 3.5.3) from the CDC (www.cdc.gov/epiinfo) (*162*).

Serum Collection and Biochemical Marker Analyses

All blood was collected from the antecubital vein of fasting individuals by a trained phlebotomist at the clinic. Blood collections were conducted as part of the routine treatment; however, additional 10 mL of blood was collected for the purpose of the study. Collection took place in a serum collection tube and sent to the hospital phlebotomy lab, where it was centrifuged according to lab protocol. Upon separation, the supernatant, serum, was collected and transferred to a plastic storage vial, which was picked up by the study staff and stored at -80°C.

Fasting glucose (FPG), Insulin (FPI), serum calcium, and percentage of glycosylated hemoglobin (HbA1C) were determined by the hospital pathology lab

and obtained from the patients' medical charts. From these, homeostasis model assessment-estimated insulin resistance (HOMA-IR) and Quantitative Insulin Sensitivity Check Index (QUICKI), which examine complex, yet normally tightly-controlled relationship between insulin and glucose, were calculated according the following formulae.

$$\textbf{HOMA-IR} = \frac{\left(\boldsymbol{fasting\ insulin\ (\mu U/ml)\ x\ fasting\ glucose(mg/dl)}\right)}{\mathbf{22.5}}$$

$$\textbf{QUICKI} = \frac{\mathbf{1}}{\boldsymbol{log\left(fasting\ insulin(\mu U/ml)\right) + log(fasting\ glucose(mg/dl))}}$$

IL-6, TNF-α, CRP, leptin, total adiponectin, HMW adiponectin, and were measured using enzyme-linked immunosorbent assays (ELISA) (ALPCO Diagnostics, Salem, NH). 25(OH)D was also assessed by ELISA (Immunodiagnostik). Individual protocols and intra-assay CV are found in appendices D-I. Absorbance was measured on a standard micro-plate reader, E-Max Pro, (Molecular Diagnostics).

IV. QUESTIONAIRRE DATA

Questionnaires and Forms

The Sun Exposure Questionnaire (Appendix C) is a one-page questionnaire that assesses exposure to ultra-violet radiation (via sun or artificial sources) and sunscreen use.

Fitzpatrick Method of Skin Typing

The Fitzpatrick method of skin classification is a pigment-based index that was used (Appendix B) (*163*). As previously mentioned, melanin is serves as a natural sunblock, which has been demonstrated to affect cutaneous vitamin D production (*53*).

Dietary and Physical Activity Assessment

Nutrient intake, including vitamin D and calcium, were measured using the Harvard Youth/Adolescent Questionnaire (YAQ), a self-administered semi-quantitative food frequency questionnaire assessing habitual intake. The reproducibility and validity of this measure have been previously demonstrated. The Harvard Youth/Adolescent Activity Questionnaire (YAAQ) is an activity questionnaire designed for children aged 9-18 years old. It is a self-administered tool. It takes approximately 5-10 minutes to complete. The reproducibility and validity of this measure have been previously demonstrated.

V. STATISTICAL ANALYSIS

Power Calculation- It was determined that a sample size of 17 subjects per treatment arm were necessary to detect an increase in the treatment group's 25(OH)D of 8 ng/ml from the anticipated baseline mean of 20 ng/ml with a power of 80%. This calculation was based upon an expected standard deviation of 8 ng/ml.

Hypothesis Testing- Baseline characteristics of all enrolled subjects (n=44) were compared using either independent t-tests (continuous) or chi-square tests (categorical). Only subjects completing at least one follow-up visit were included in change from baseline analyses, according to the intention-to-treat principle. No imputations of missing data were made. Between and within group changes from baseline were analyzed for each outcome variable by repeated measure ANOVA (PROC MIXED) with treatment (between), time (within), and treatment-time interaction serving as independent variables. Due to the equal time intervals, the autoregressive covariance structure was used (AR(1)). The LSMESTIMATE statement followed by the appropriate linear-contrasts were used to make all between and within group comparisons. In the case of non-normally distributed residuals for outcome variables, log transformations were applied prior to statistical testing. Such variables were back transformed, thus means for these variables represent geometric means.

Bivariate relationships were determined using partial correlation coefficients, obtained using either Pearson or Spearman correlation analysis,

after correcting for baseline values of the measured variable, WC, BMI-Z, age, and gender. Additionally, simple and multiple linear regression were used to further examine the nature of the bivariate and multivariate relationships.

Assumptions were tested for all models prior to final analysis. Data represented as mean (95% confidence interval), mean ± SD, except where indicated. $P<0.05$ was considered significant for all tests. All statistical analyses were carried out using SAS 9.22 statistical software (SAS Institute; Cary, NC).

Results

Baseline measures- At baseline, there were no significant differences between those who were assigned to the treatment group versus those in the placebo group for any of the anthropometric or biochemical variables (table 6). There were no significant within group changes from baseline for any of the anthropometric measures BMI, BMI-Z, or WC. Additionally, the two groups were statistically similar in mean age, gender, and Fitzpatrick skin type.

Safety and efficacy- The baseline serum 25(OH)D concentration of individuals whose baseline visit occurred in the winter/spring was significantly less than those who began in the summer/fall (17.5 ± 7.1 vs. 21.8 ± 6.9, respectively; *P=0.046)* (figure3). There were no statistically significant differences in serum 25(OH)D status between Fitzpatrick scale group means as determined by one-way ANOVA (*P=0.486) (*figure 4).

At baseline serum 25(OH)D concentration of all individuals was 19.4 ± 7.3 ng/ml. According to the health risk-based classifications established by Grant and Holick (table 2), only 4.5% of all subjects had sufficient vitamin D status, compared to 53% who were deficient, and 42% who were insufficient (figure 5). After 3 months, 93% of subjects who were received treatment were either sufficient (50%) or insufficient (43%); mean serum 25(OH)D concentration 33.1 ± 9.2 (figure 6). At the end of the study, none of in the vitamin D group were classified as deficient, with 78% being sufficient and 22% insufficient; mean 25(OH)D 38.4 ±9.2. The 25(OH)D concentrations of those in the placebo

group did not increase significantly from baseline to three or six months (*P*=0.389 and 0.401, respectively). These changes were independent of season, for which they were corrected. Serum calcium levels remained unchanged in both groups throughout the study and fell well within the normal limits at all time points.

Insulin resistance and sensitivity- After adjusting for the covariates baseline value of outcome variable, age, gender, WC, and BMI-Z, the within group change from baseline for FPG in the vitamin D group was significant with a decrease of -5.5 mg/dl (-9.8, -1.3; *P=0.016*) in the vitamin D group versus a decrease of -0.2 (-4.6, 4.1; *P*=0.919) in the placebo group (Table 7). However, these between group changes were not significantly different (*P*=0.085). The between group change from baseline in FPI was significant (*P*=0.026), with a decrease of -6.5 μU/mL (-11.7, -1.4; *P=0.014*) in the vitamin D group and an increase of 1.2 μU/mL (-4.1, 6.5; *P*=0.652) in the placebo group. There was a significant difference in the change in HOMA-IR between groups (*P*=0.033), with a significant decrease of -1.63 (-2.84, -0.42; *P=0.009*) in the vitamin D group and an insignificant increase of 0.27 (-0.98, 1.51; *P*=0.670) in the placebo group. There was also significant difference in the between group change from baseline for QUICKI (*P*=0.016), with a significant increase of 0.016 (0.005, 0.027; *P*=0.005) in the vitamin D group and a decrease of 0.004 (-0.014, 0.008; *P*=0.516) in the placebo group. Baseline to month six change in 25(OH)D was

not significantly correlated with the change in FPG, FPI, HOMA-IR, or QUICKI (figure 10).

Inflammatory Markers- After adjusting for the covariates baseline value of outcome variable, age, gender, WC, and BMI-Z, the within group changes from baseline for CRP (*P*=0.209 for vitamin D group and *P*=0.385 for placebo), IL-6 (*P*=0.354 for vitamin D group and *P*=0.651 for placebo), and TNF-α (*P*=0.104 for vitamin D group and *P*=0.128 for placebo) were not significant (Table 8). Likewise, there were no significant between group changes from baseline in any of the inflammatory markers (CRP, *P*=0.137; IL-6, *P*=0.124; TNF-α, *P*=0.986). Simple linear regression revealed a significant inverse relationship between change in 25(OH)D and change in CRP (figure 13) (R^2=0.206; *P*=0.0151).

Adipokines and adipokine ratios- After adjusting for the covariates baseline value of outcome variable, age, gender, WC, and BMI-Z, vitamin D supplementation produced mixed results in adipokine and adipokine ratios (table 9). The between group baseline to six month changes in leptin were not significantly different (*P*=0.087). However, the within group change for the vitamin D group was significant with a decrease of -6.8 ng/ml (-9.7, -3.9; *P*=0.023) in the vitamin D group versus an increase of 0.6 (-4.6, 5.8; *P*=0.993) in the placebo group. Between group changes in total adiponectin were not significantly different (*P*=0.626) nor were the within group changes for vitamin D

or placebo (*P*= 0.447 and *P*=0.958, respectively). Likewise, the between group changes in HMW adiponectin were not significantly different (*P*=0.887) nor were the within group changes for vitamin D or placebo (*P*= 0.382 and *P*=0.290, respectively). There was a significant difference in the change in the leptin to adiponectin ratio between groups (*P*=0.045), with a decrease of -1.41 (-2.13, -0.69; *P=0.009*) in the vitamin D group and an increase of 0.10 (-0.04, 0.24; *P*=0.857) in the placebo group. The change in the ratio of HMW to total adiponectin was not significantly between the two groups (*P*=0.859) nor were the within treatment group changes (*P*=0.298 for vitamin D group and *P*=0.202 for placebo). Simple linear regression determined that changes in leptin and the leptin to adiponectin ratios were inversely related to changes in 25(OH)D (figures 17 and 18). However, none of the other adipokines or adipokine ratios were significantly related to changes in 25(OH)D.

Multivariate linear regression using change in HOMA-IR as a dependent variable was performed to determine which predictors had the greatest influence on changes seen (Table 11). Using the adjusted R^2 selection criteria, the model including change in total adiponectin, change in 25(OH)D, and change in CRP was determined to be the best (adjusted R^2=0.541; *P*=0.0017). This model maintained its significance after being adjusted for baseline WC, baseline BMI-Z, and age (*P*=.0082).

	All (n=44)	Vitamin D (n=21)	Placebo (n=23)	P-Value
Gender (% Male)	50%	52%	48%	0.763
Age (y)	14.2± 2.6	14.6 ± 2.3	13.9 ± 2.4	0.360
Waist Circumf. (cm)	119.3 ± 11.9	122.5 ± 13.4	117.7 ± 13.2	0.268
BMI kg/m^2	39.2 ± 5.9	39.5 ± 5.1	38.9 ± 6.7	0.769
BMI z-score	2.53 ± 0.25	2.54 ± 0.24	2.54 ± 0.28	0.987
Serum 25OHD (ng/mL)	19.4 ± 7.3	19.2 ± 6.3	19.6 ± 7.9	0.865
Fasting Glucose (mg/dl)	89.6± 8.4	90.7± 9.2	88.6 ± 7.6	0.411
Fasting Insulin (µU/L)	24.6 ± 12.5	24.6 ± 12.1	24.5 ± 13.1	0.982
HOMA-IR	5.5 ± 2.9	5.5 ± 2.7	5.5 ± 3.2	0.992
QUICKI	0.306 ± 0.024	0.304 ± 0.020	0.308 ± 0.03	0.631
IL-6	1.90 ± 1.41	2.34 ± 1.56	1.66 ± 1.16	0.129
CRP	2.44 ± 1.37	2.46 ± 1.35	2.43 ± 1.42	0.945
TNF-α	1.94 ± 1.34	2.04 ± 1.19	1.84 ± 1.48	0.637
Leptin	47.7 ± 17.5	49.6 ± 16.8	45.6 ± 18.4	0.503
Total Adiponectin	5.88 ± 1.01	5.81 ± 0.93	5.95 ± 1.11	0.682
HMW Adiponectin	2.41 ± 0.56	2.23 ± 0.45	2.55 ± 0.64	0.141
Leptin:Adiponectin Ratio	8.06 ± 3.01	8.45 ± 3.16	7.69 ± 2.91	0.175
HMW:Total Adiponectin Ratio	0.41 ± 0.07	0.39 ± 0.07	0.43 ± 0.07	0.479

Table 9– Baseline subject characteristics for all enrolled subjects. Data are mean ± SD or proportion.

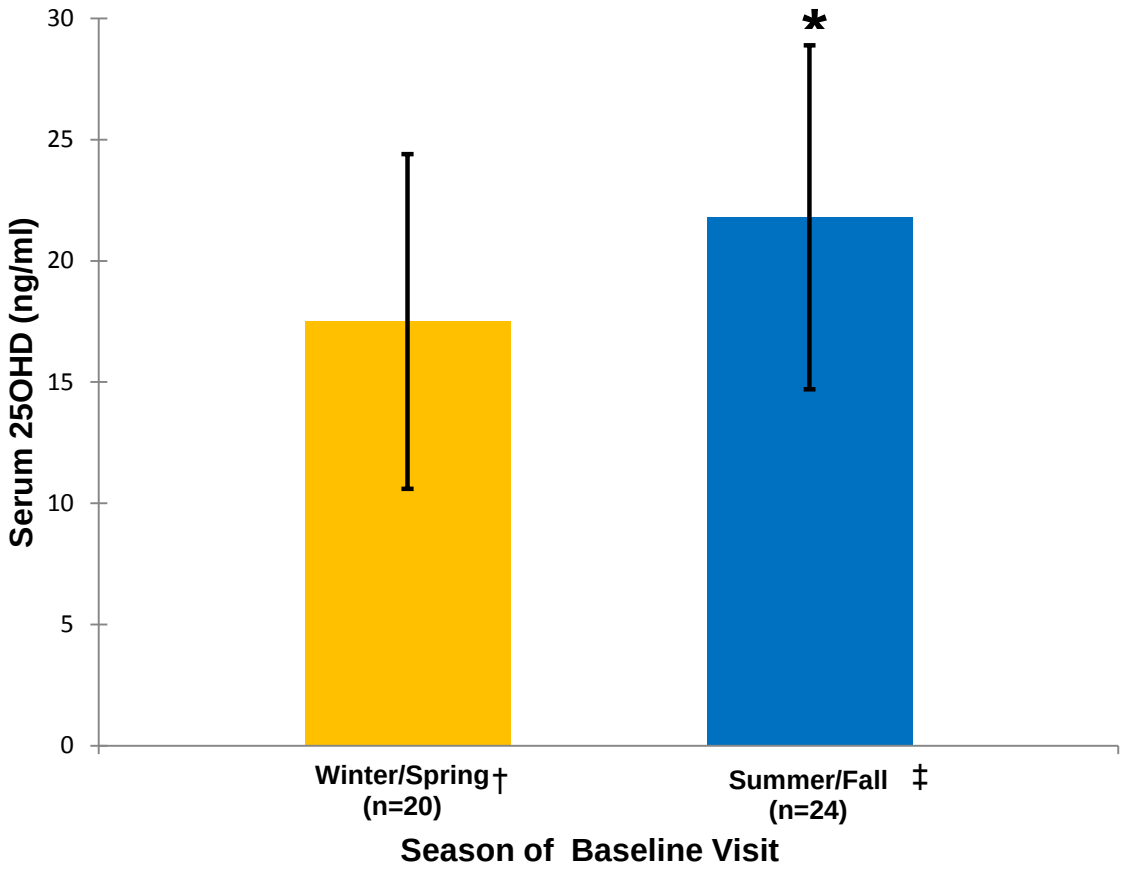


Figure 3– Seasonal difference in baseline serum 25(OH)D concentrations.
†Winter/Spring- November through April
‡Summer/Fall- May through October
Bars represent group means ± SD.
* Means are significantly different from winter/spring, *P*= 0.048

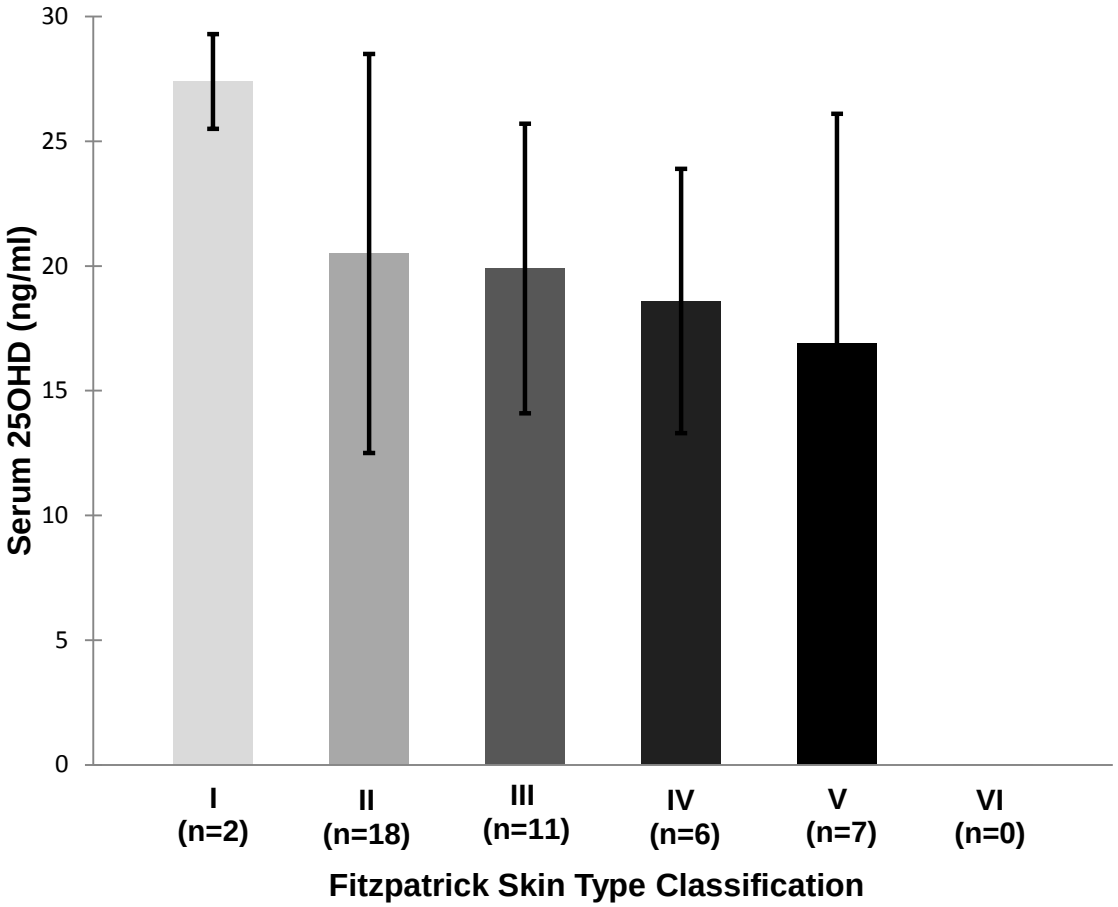


Figure 4– Differences in mean baseline serum 25(OH)D concentrations between Fitzpatrick skin typing classifications.
Bars represent group means ± SD.

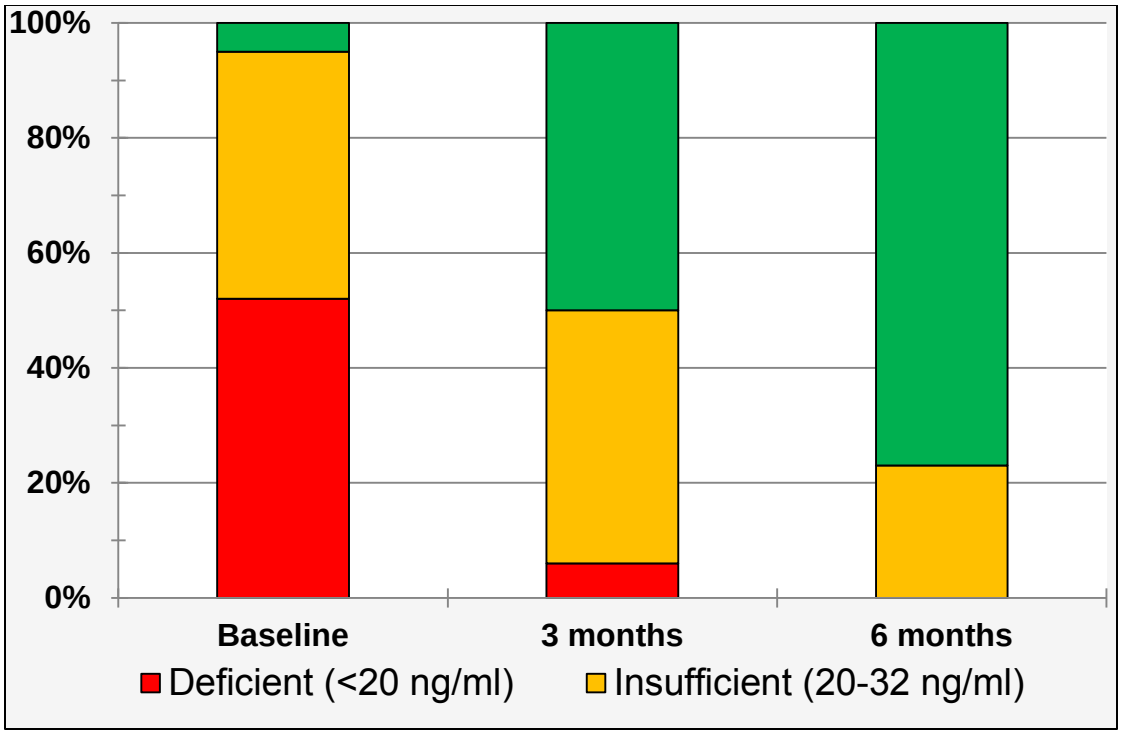


Figure 5- Percent of subjects in the treatment group who fell into each health outcome classification group. Baseline includes all enrolled individuals.

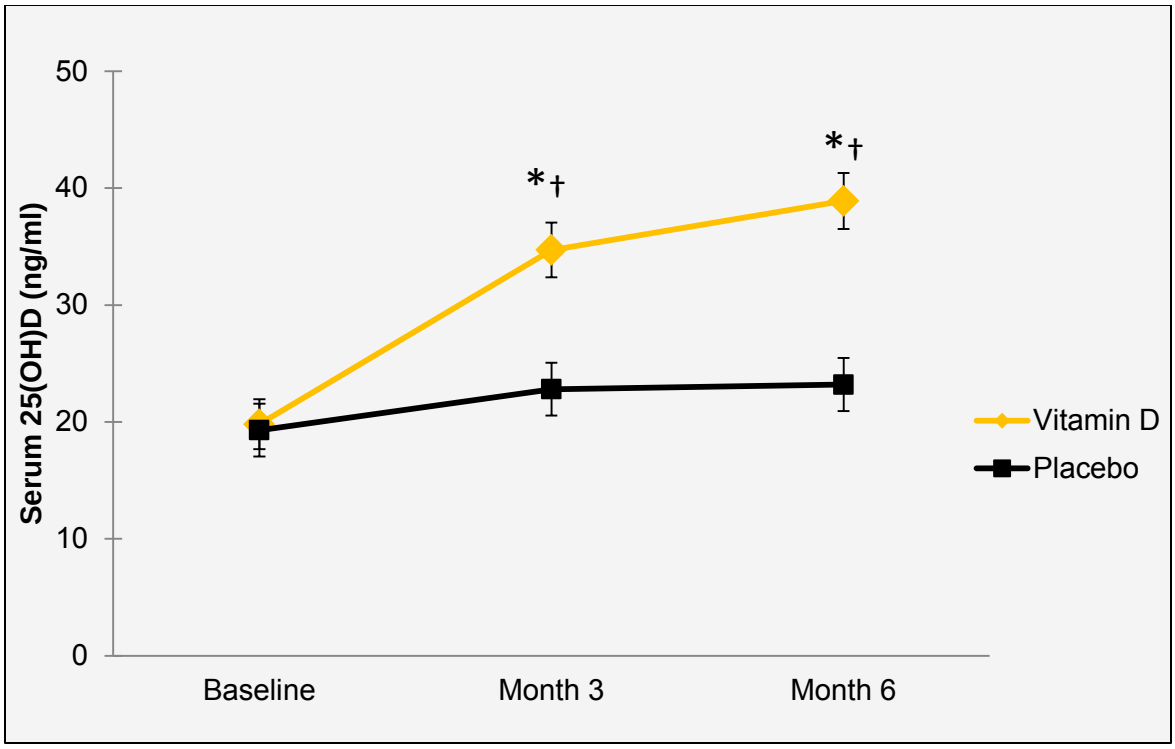


Figure 6- Changes in 25(OH)D concentration across study.
Bar represents 95% confidence interval.
***** *P<0.05 within group change from baseline;*
† P<0.05 between group difference at timepoint

	Vitamin D (n=18)		Placebo (n=17)		
		P-Within		*P*-Within	*P*-Between
FPG (mg/dl)					
Baseline	89.7 ± 1.4		88.9 ± 1.5		*0.607*
End	84.1 ± 1.6		88.7 ± 1.6		*0.141*
Change	-5.5 (-9.8, -1.3)	*0.016*	-0.2 (-4.6, 4.1)	*0.919*	*0.085*
FPI (µU/mL)					
Baseline	23.1 ± 1.7		21.6 ± 1.8		*0.534*
End	16.6 ± 2.0		22.8 ± 1.9		*0.016*
Change	-6.5 (-11.7, -1.4)	*0.014*	1.2 (-4.1, 6.5)	*0.652*	*0.026*
HOMA-IR					
Baseline	5.12 ± 0.40		4.79 ± 0.43		
End	3.49 ± 0.46		5.05 ± 0.46		
Change	-1.63 (-2.84, -0.42)	*0.009*	0.27 (-0.98,1.51)	*0.670*	*0.033*
QUICKI					
Baseline	0.308 ± 0.004		0.311 ± 0.004		*0.419*
End	0.324 ± 0.004		0.308 ± 0.004		*0.010*
Change	0.016 (0.005, 0.027)	*0.005*	-0.004 (-0.014, 0.008)	*0.516*	*0.016*

Table 7 – Covariate adjusted means between and within group changes from baseline in markers of glucose metabolism and insulin resistance/sensitivity. Covariates included age, gender, baseline value of outcome variable, BMI-Z and waist circumference. Data are mean ± SEM or (95% CI).

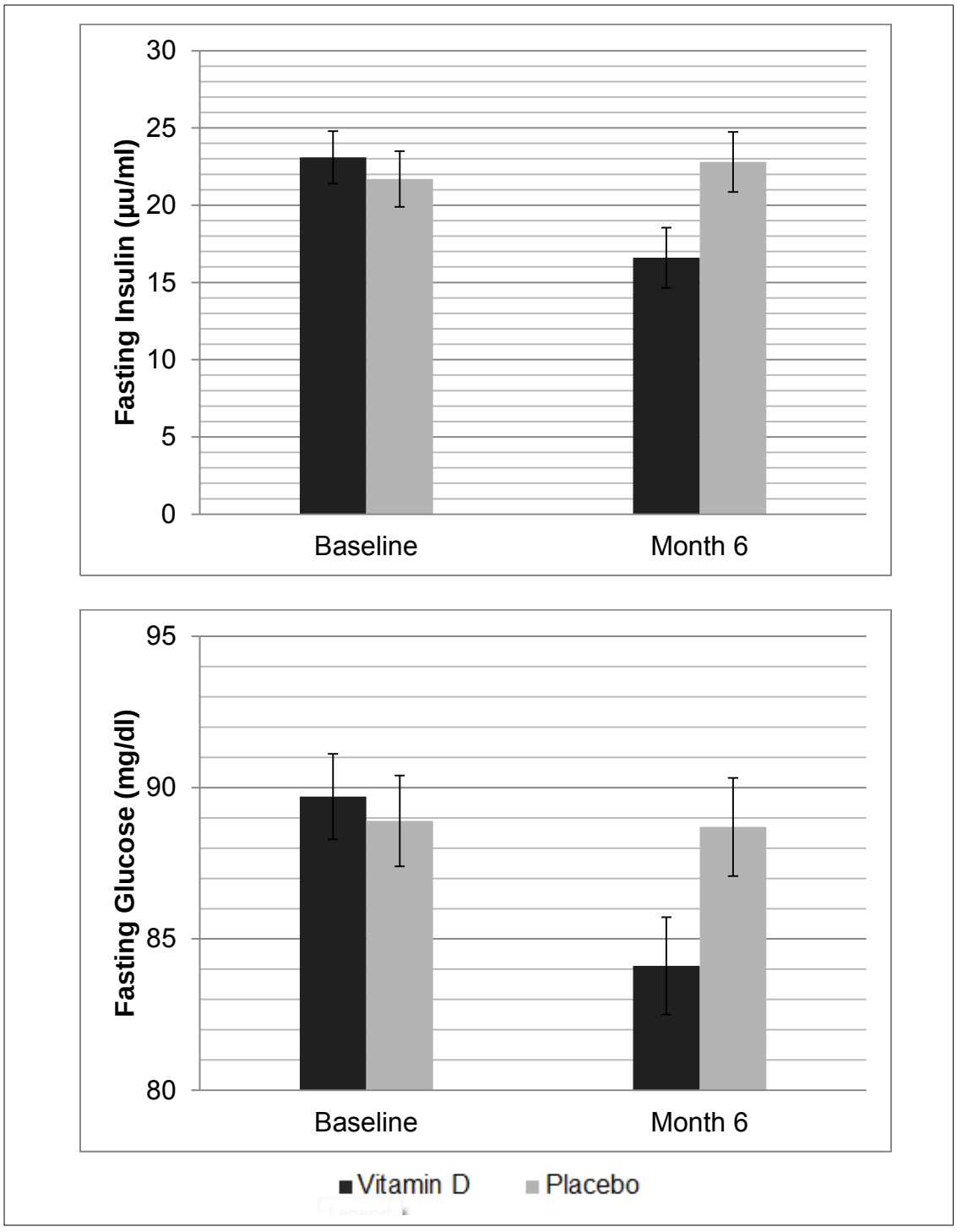


Figure 7- Baseline and 6-month means for fasting glucose and insulin concentrations corrected for baseline concentration, age, waist circumference, and gender. Error bar represents SEM.

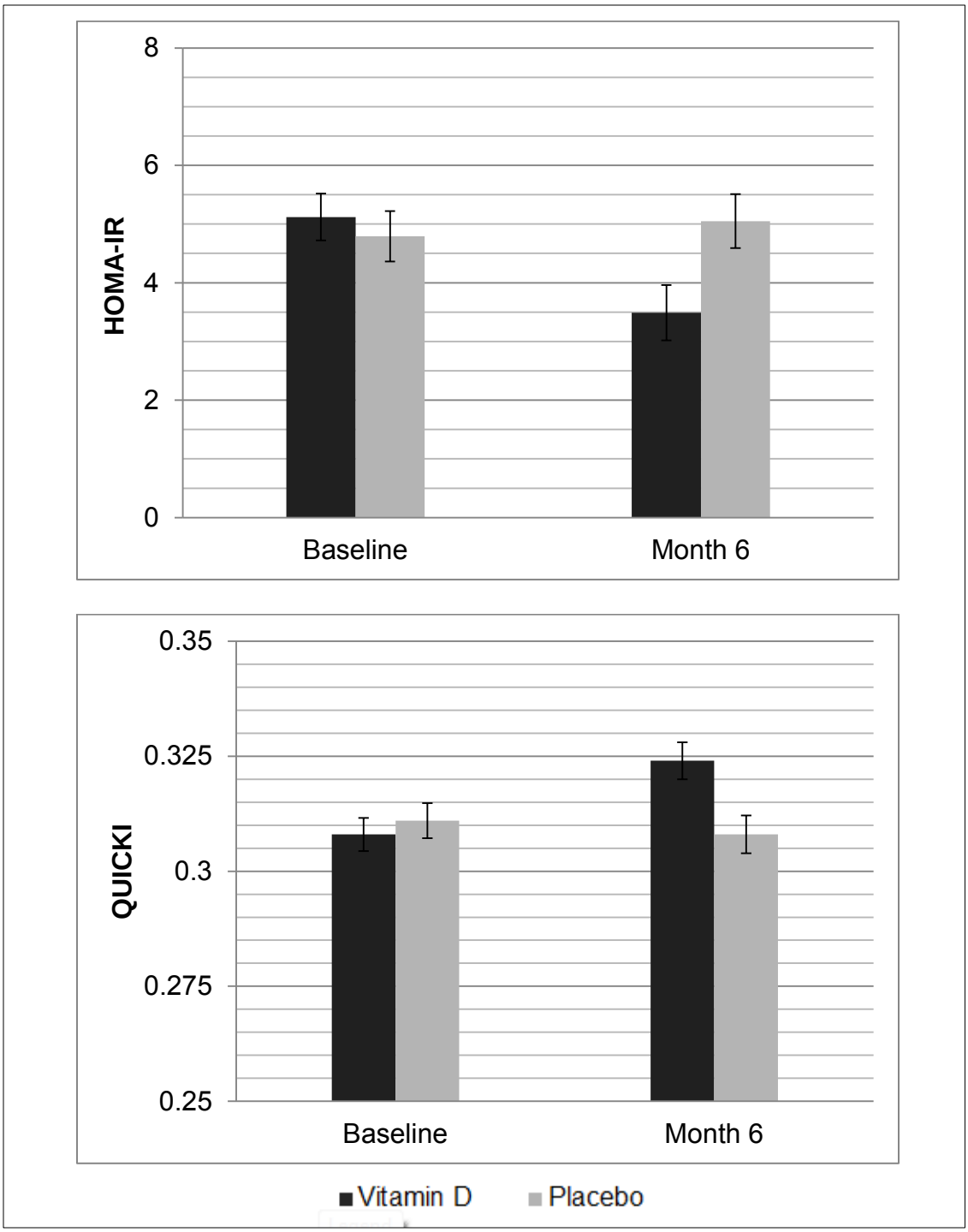


Figure 8- Baseline and 6-month means for indices of insulin resistance and sensitivity corrected for baseline concentration, age, waist circumference, and gender. Error bar represents SEM.

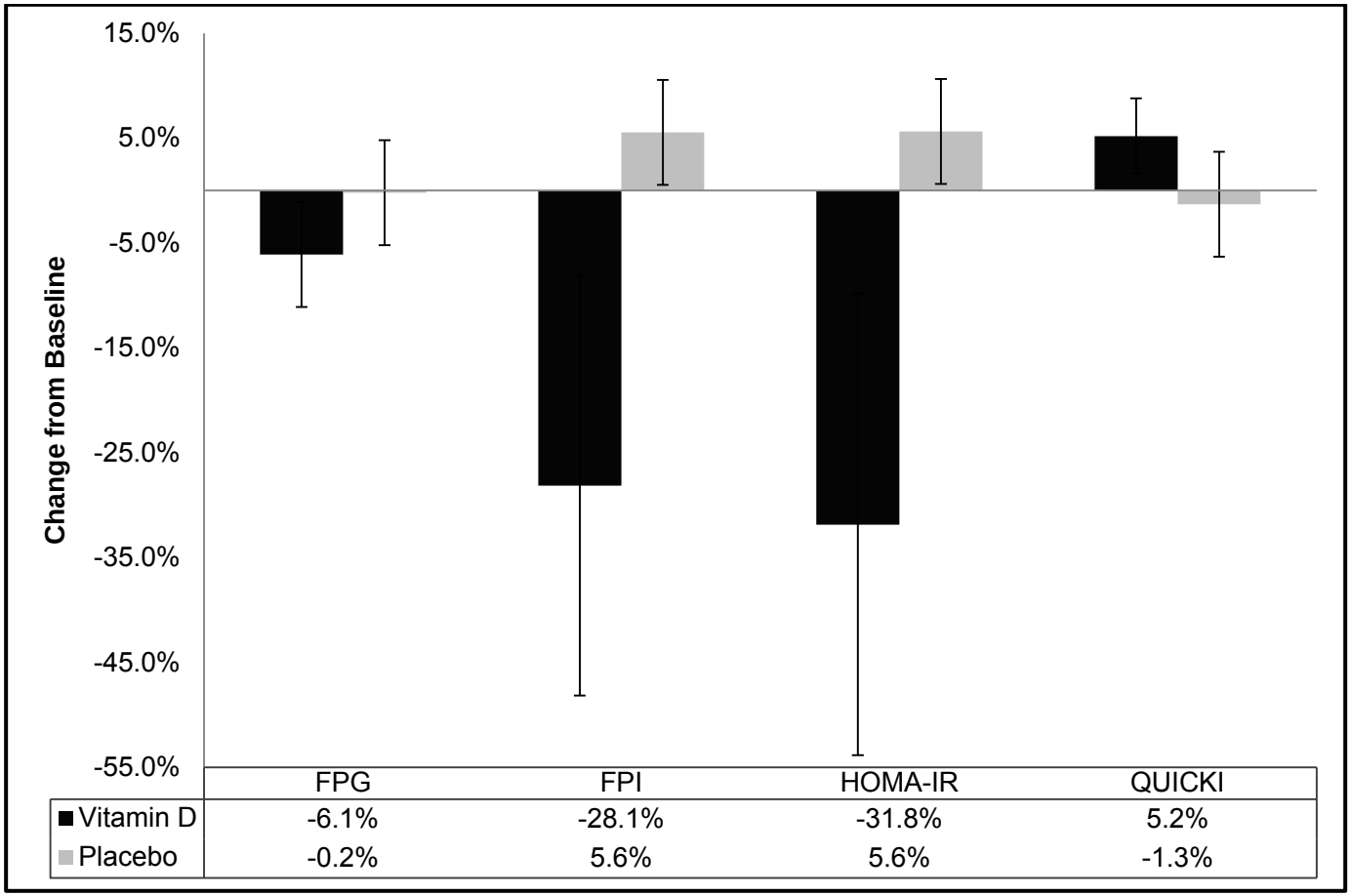
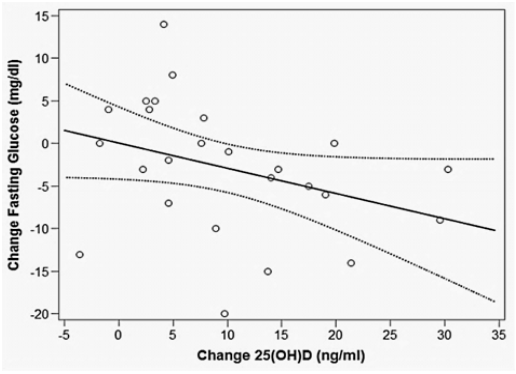
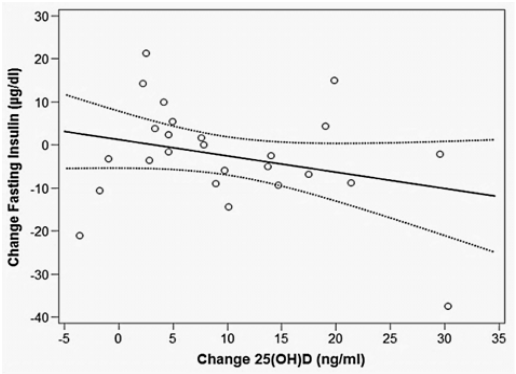


Figure 9- Relative change from baseline for all surrogate markers of glucose metabolism. Percent change from baseline covariate adjusted means. Error bar represents 95% CI.



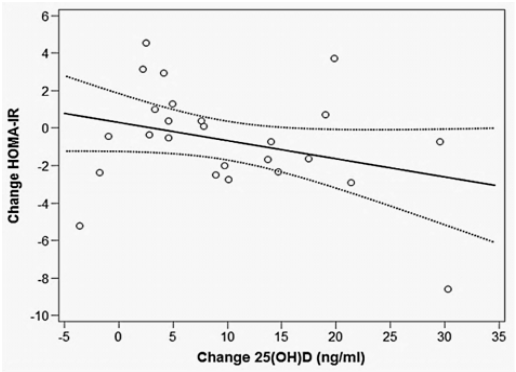
R^2=0.119
P=0.092

y= 0.0376 - 0.2957x



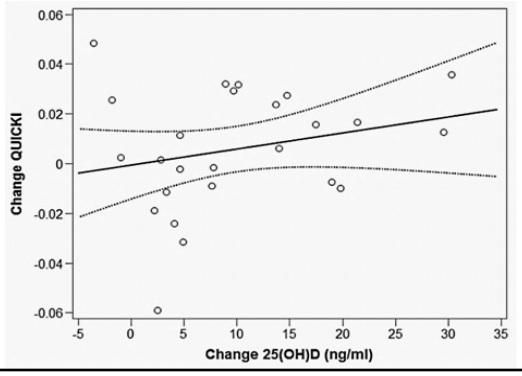
R^2=0.083
P=0.162

y= 1.2299 - 0.3779x



R^2=0.098
P=0.127

y= 0.2987 - 0.0968x



R^2=0.058
P=0.245

y= -0.0005 + 0.0006x

Figure 10- The relationship between the baseline to 6-month change in 25(OH)D and changes in glycemic measures in all subjects.
Linear regression equations for each is presented.
P<0.05 considered significant.

	Vitamin D (n=17)		Placebo (n=16)		
		P-Within		***P-Within***	***P-Between***
HS-CRP (mg/l)					
Baseline	2.51 ± 0.17		2.56 ± 0.18		*0.441*
End	2.20 ± 0.18		2.78 ± 0.19		*0.030*
Change	-0.31 (-0.78, 0.18)	*0.209*	0.22 (-0.28, 0.73)	0.385	*0.137*
IL-6 (pg/mL)					
Baseline	1.64 ± 0.09		1.54 ± 0.10		*0.714*
End	1.44 ± 0.09		1.83 ± 0.10		*0.091*
Change	-0.20 (-0.09, 0.13)	*0.354*	0.29 (-0.20, 0.78)	0.651	*0.124*
TNF-α (pg/ml)					
Baseline	1.88 ± 0.12		1.70 ± 0.13		*0.546*
End	1.58 ± 0.13		1.41 ± 0.14		*0.554*
Change	-0.30 (-0.65, 0.06)	*0.104*	-0.28 (-0.66, 0.08)	0.128	*0.986*

Table 8– Covariate adjusted between and within group changes from baseline in inflammatory cytokines. Covariates included age, gender, baseline value of outcome variable, BMI-Z and waist circumference. Data are mean ± SEM or (95% CI)

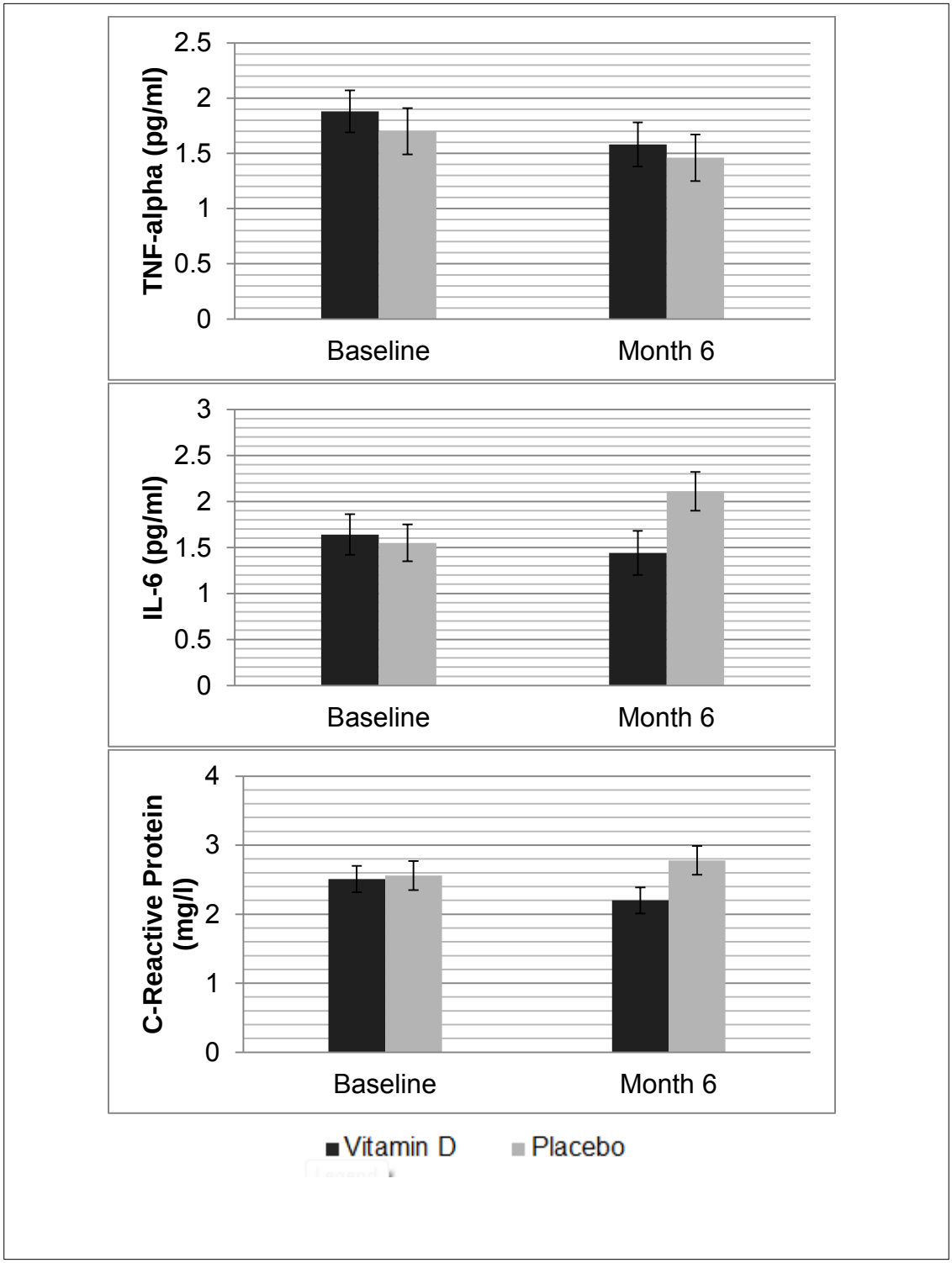


Figure 11- Baseline and 6-month means for inflammatory markers corrected for baseline concentration, age, WC, and gender. Error bar represents SEM.

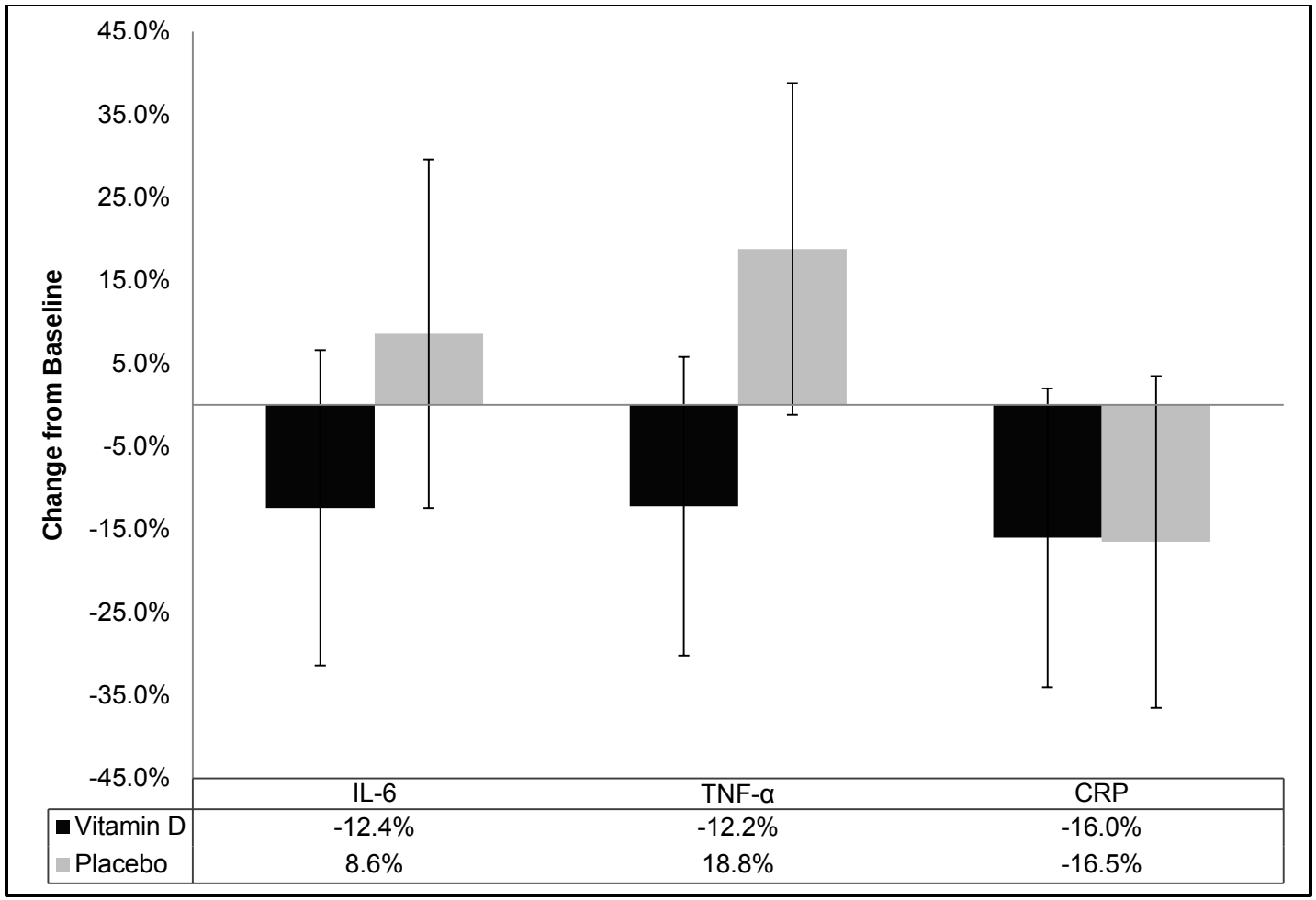
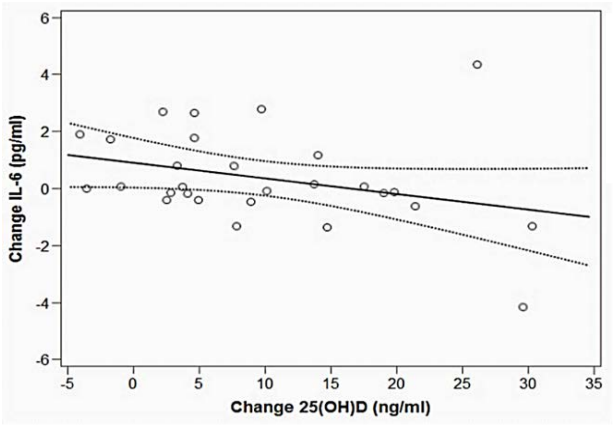
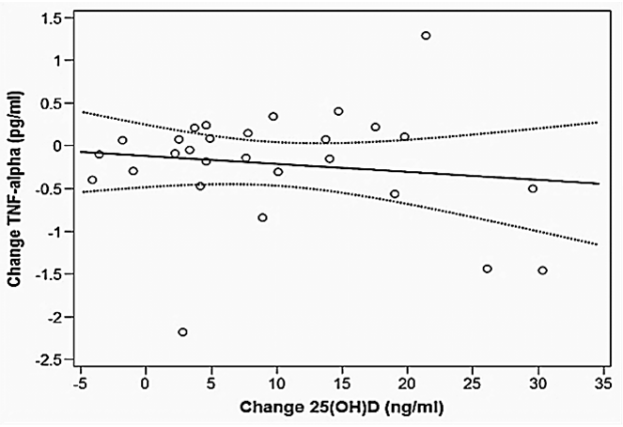


Figure 12- Relative change from baseline for inflammatory cytokines. Percent change from baseline covariate adjusted means. Error bar represents 95% CI.



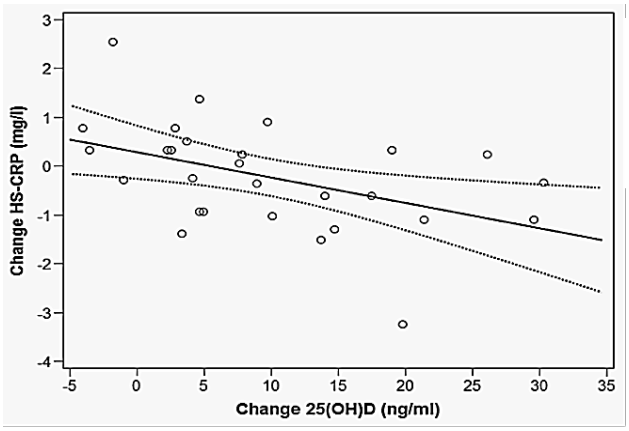
R^2=0.098
P=0.102

y= 0.8983 - 0.0547x



R^2=0.0184
P=0.491

y= -0.119 - 0.0093x



R^2=0.206
P=0.0151

y= 0.2822 - 0.0518x

Figure 13- The relationship between the baseline to 6-month change in 25(OH)D and changes in inflammatory markers in all subjects.
Linear regression equations for each is presented.
P<0.05 considered significant.

	Vitamin D (n=16)		Placebo (n=15)		
		P-Within		*P*-Within	*P*-Between
Leptin (ng/ml)					
Baseline	43.5 ± 1.		43.5 ± 1.9		*0.993*
End	36.7 ± 2.0		44.1 ± 2.1		*0.028*
Change	-6.8 (-9.7, 0.5)	*0.023*	0.6 (-4.7, 5.4)	*0.993*	*0.087*
Total Adiponectin (µg/m)					
Baseline	5.86 ± 0.20		5.88 ± 0.21		*0.985*
End	6.06 ± 0.20		5.90 ± 0.21		*0.499*
Change	0.21 (-0.33, 0.74)	*0.447*	0.01 (-0.55, 0.58)	*0.958*	*0.626*
HMW Adiponectin (µg/m)					
Baseline	2.42 ± 0.09		2.41 ± 0.09		*0.910*
End	2.31 ± 0.09		2.27 ± 0.09		*0.885*
Change	-0.11 (-0.36, 0.14)	*0.382*	-0.14 (-0.39, 0.11)	*0.290*	*0.887*
L/A Ratio					
Baseline	7.45 ± 0.02		7.41 ± 0.005		*0.975*
End	6.04 ± 0.02		7.51 ± 0.005		*0.008*
Change	-1.41 (-2.13, -0.69)	*0.009*	0.10 (-0.04, 0.24)	*0.857*	*0.045*
HMW/Total Adipo. Ratio					
Baseline	0.41 ± 0.01		0.41 ± 0.01		*0.819*
End	0.39 ± 0.01		0.39 ± 0.01		*0.962*
Change	-0.02 (-0.05, 0.02)	*0.298*	-0.02 (-0.06, 0.01)	*0.202*	*0.859*

Table 9– Covariate adjusted between and within group changes in adipokines and adipokine ratios. Covariates included age, gender, baseline value of outcome variable, BMI-Z and waist circumference. Data are mean ± SEM or (95% CI)

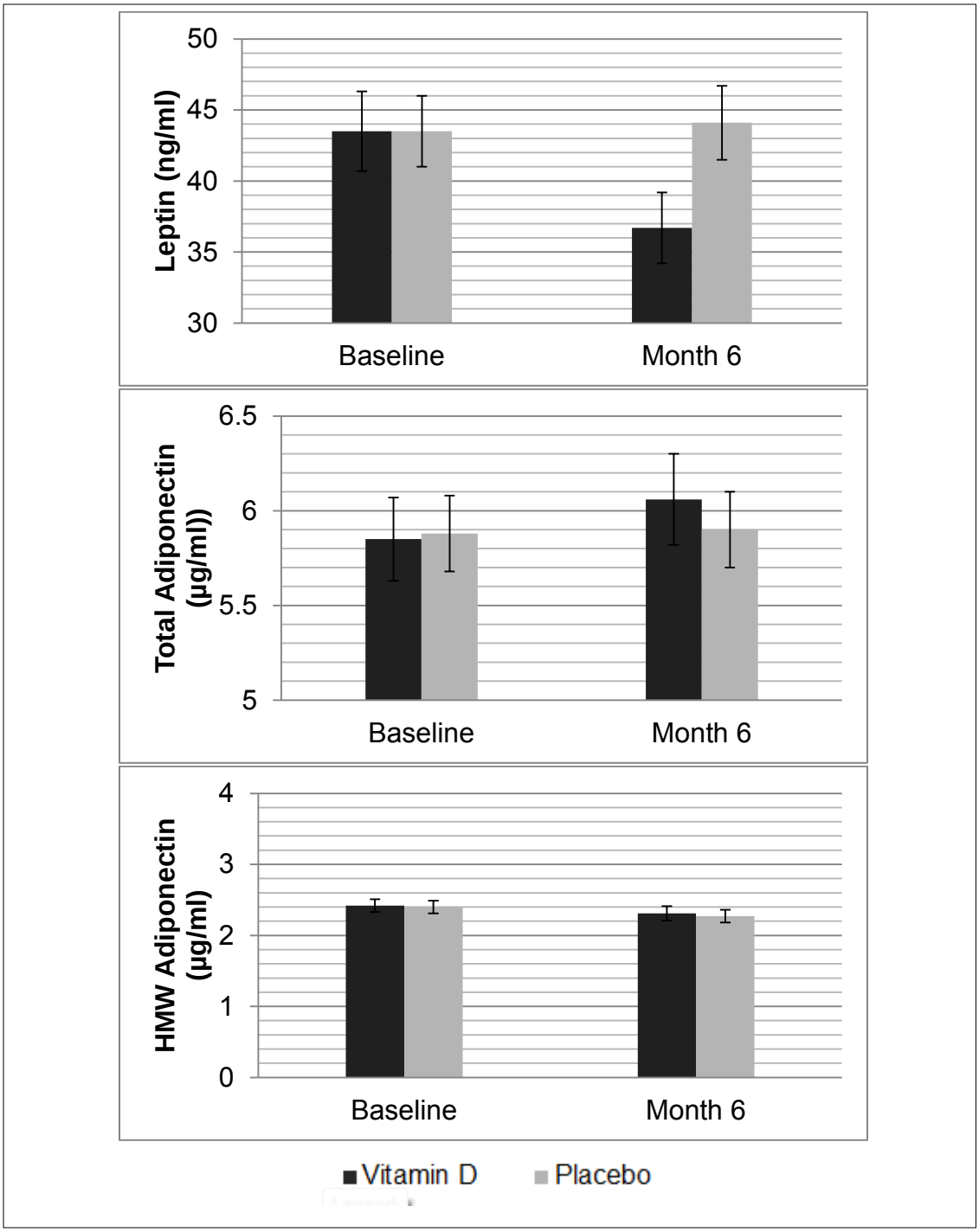


Figure 14- Baseline and 6-month means for adipokines corrected for the covariates baseline concentration, age, waist circumference, and gender. Error bar represents SEM.

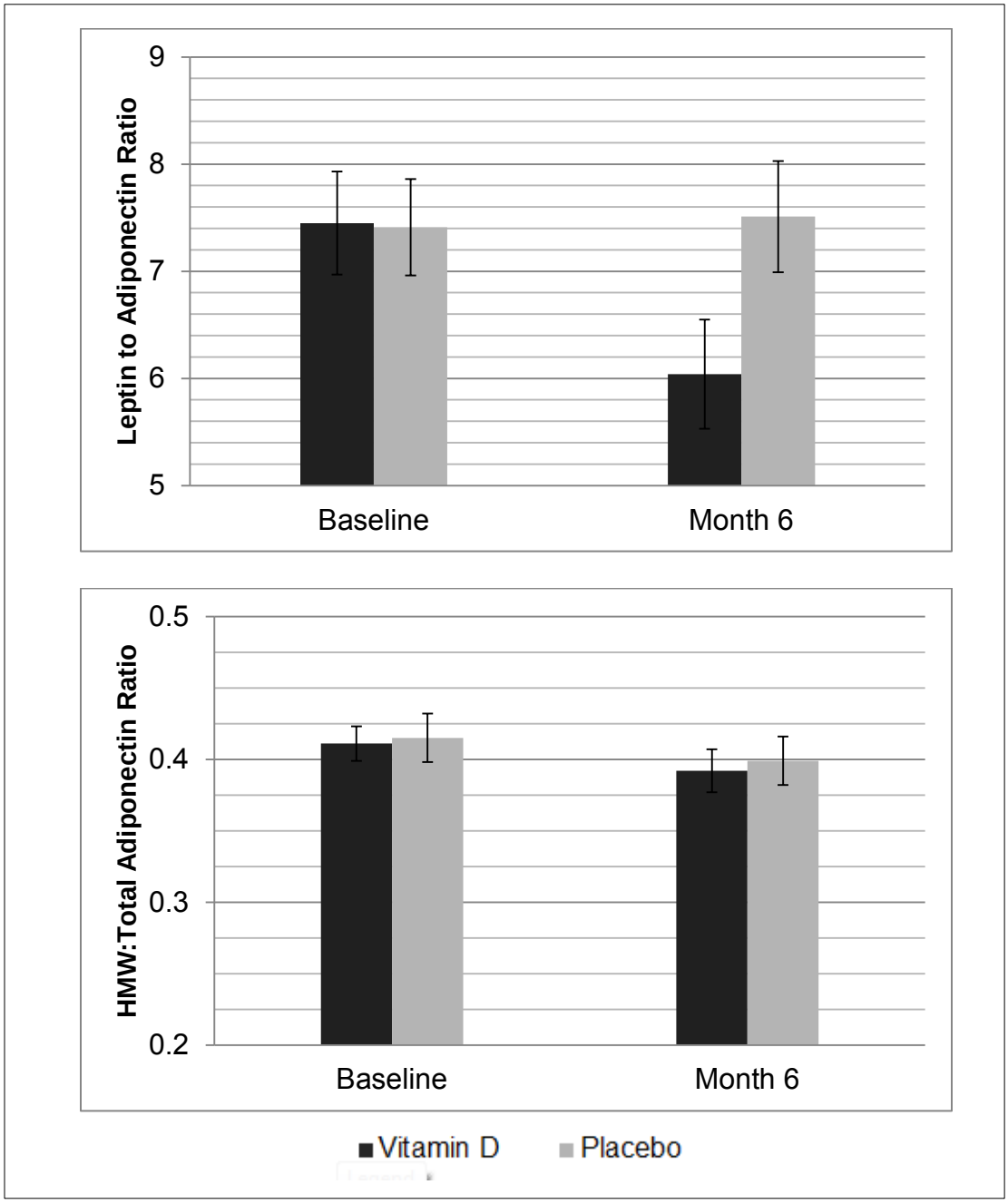
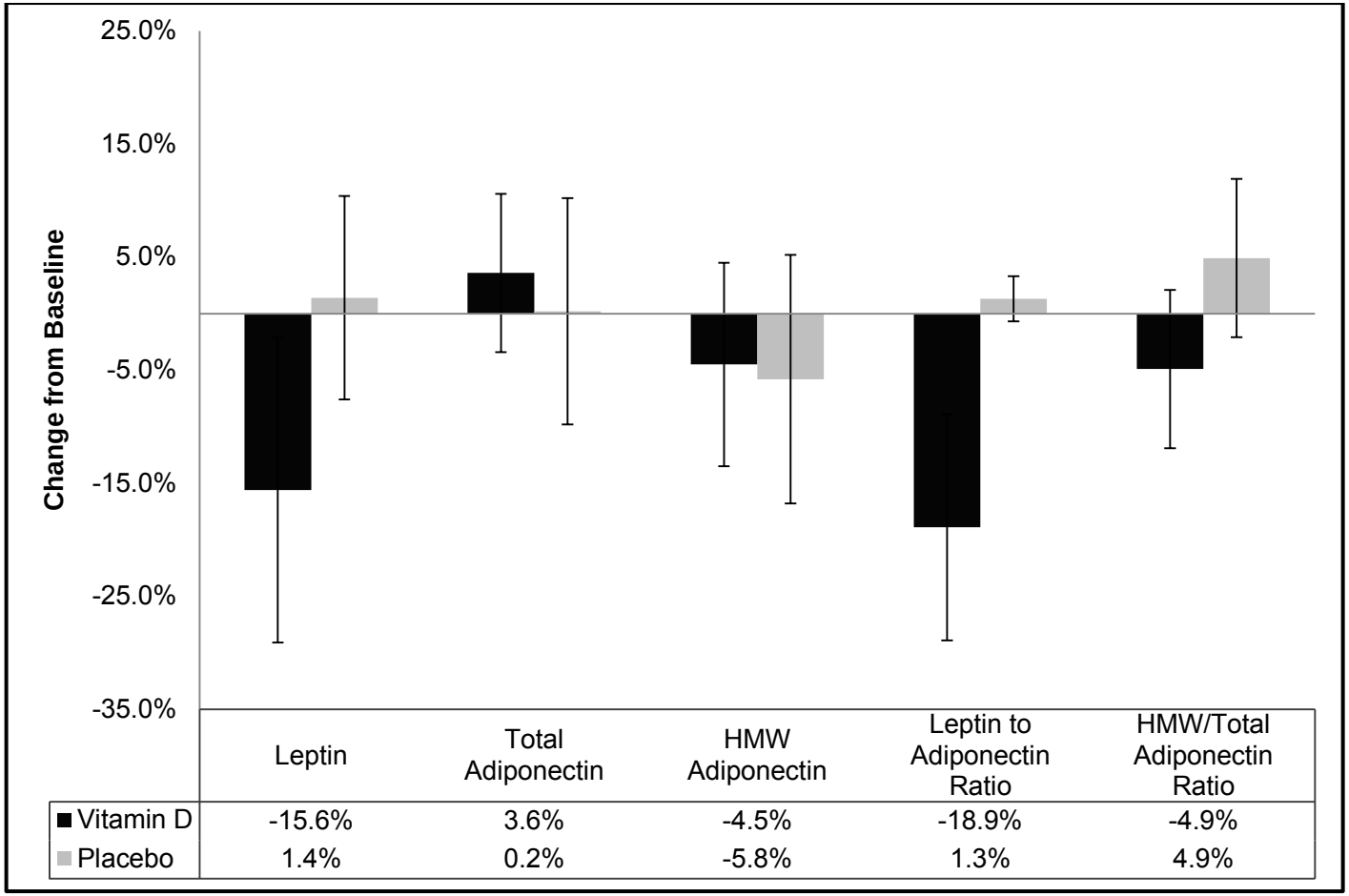
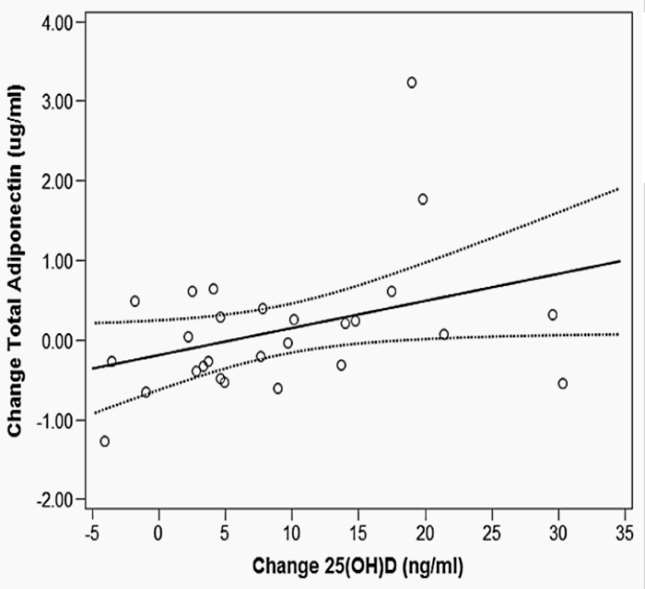


Figure 15- Baseline and 6-month means for fasting adipokine ratios adjusted for baseline concentration, age, waist circumference, and gender. Error bar represents SEM.



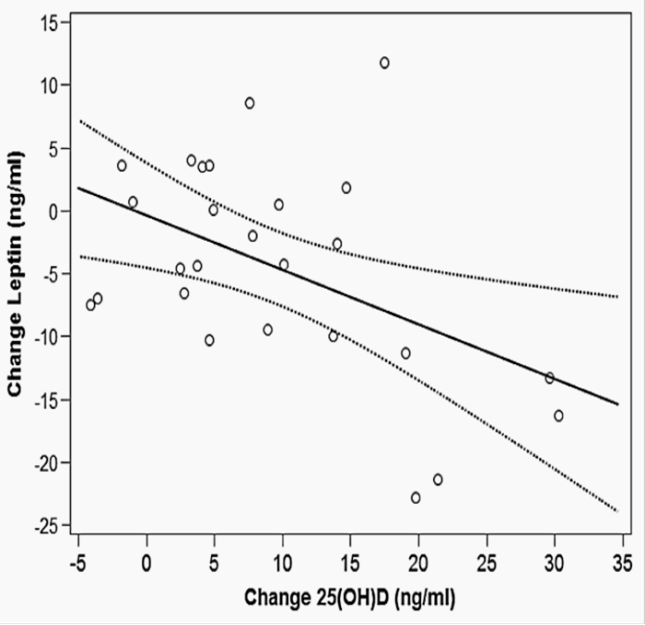
	Leptin	Total Adiponectin	HMW Adiponectin	Leptin to Adiponectin Ratio	HMW/Total Adiponectin Ratio
Vitamin D	-15.6%	3.6%	-4.5%	-18.9%	-4.9%
Placebo	1.4%	0.2%	-5.8%	1.3%	4.9%

Figure 16- Relative change from baseline for all adipokine and adipokine ratios. Percent change from baseline covariate adjusted means. Error bar represents 95% CI.



R^2=0.136
P=0.059

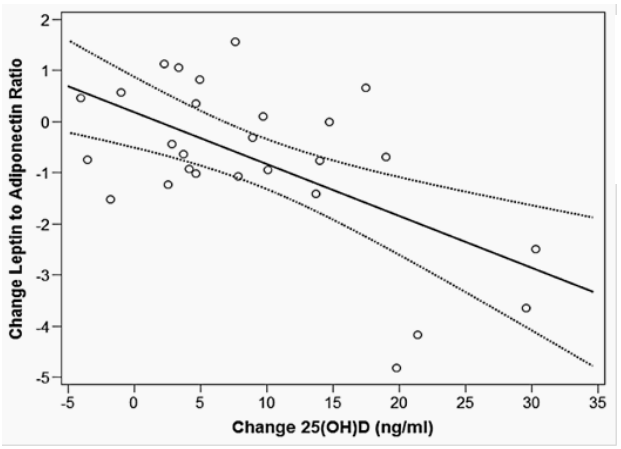
y= -0.186 + 0.034x



R^2=0.225
P=0.0145

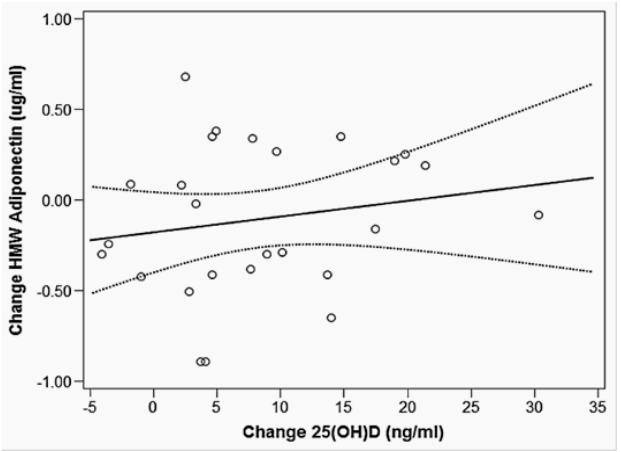
y= -0.3581 - 0.4341x

Figure 17- The relationship between the baseline to 6-month change in 25(OH)D and changes in serum adipokines in all subjects.
Linear regression equations for each is presented.
P<0.05 considered significant.



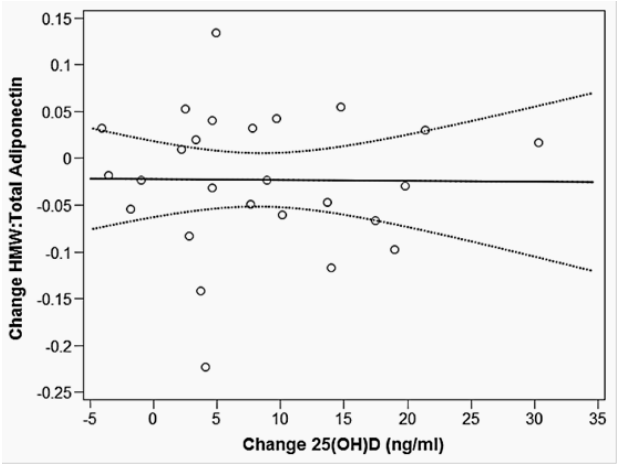
R^2=0.357
P=0.001

y= 0.1799 - 0.1012x



R^2=0.032
P=0.381

y= -0.1779 - 0.0087x



R^2=0.0001
P=0.960

y= -0.0222- 0.0001x

Figure 18- The relationship between the baseline to 6-month change in 25(OH)D and changes in adipokine ratios in all subjects.
Linear regression equations for each is presented.
P<0.05 considered significant.

Partial Correlation Coefficients		
	Baseline HOMA	**Change HOMA**
IL-6	0.09092	-0.34469
P-value	0.6951	0.1260
Leptin	0.01235	0.06680
P-value	0.9576	0.7736
CRP	-0.50372	0.17373
P-value	***0.0199****	0.4514
TNF-α	0.56138	-0.56594
P-value	***0.0081****	**0.0075***
Total Adiponectin	-0.09487	0.05726
P-value	0.6825	0.8053
HMW Adiponectin	-0.38633	0.16529
P-value	0.0837	0.4740
Leptin to Adiponectin Ratio	0.05669	0.04412
P-value	0.8072	0.8494
HMW:Total Adiponectin Ratio	-0.41589	0.22442
P-value	0.0608	0.3281

Table 10– Association between baseline concentrations of cytokines/adipokines and insulin resistance. Adjusted for gender and age.
**P<0.05*

Model	Independent Variable(s)	β	*P-value*	Model R^2
1	Δ Total	1.284	0.062	0.156
2	Δ Total Adiponectin	1.777	0.008	0.371
	Δ 25(OH)D	-0.164	0.017	
3	Δ Total Adiponectin	1.971	0.002	0.542
	Δ 25(OH)D	-0.243	0.001	
	Δ CRP	-1.148	0.015	
4	Δ Total Adiponectin	1.999	0.002	0.569
	Δ 25(OH)D	-0.231	0.002	
	Δ CRP	-0.924	0.069	
	Baseline WC	-0.038	0.308	
5	Δ Total Adiponectin	2.078	0.001	0.588
	Δ 25(OH)D	-0.236	0.002	
	Δ CRP	-0.851	0.101	
	Baseline WC	-0.082	0.199	
	Baseline BMI-Z	2.387	0.385	
6	Δ Total Adiponectin	2.149	0.001	0.623
	Δ 25(OH)D	-0.227	0.003	
	Δ CRP	-0.881	0.087	
	Baseline WC	-0.110	0.110	
	Baseline BMI-Z	3.621	0.219	
	Age (years)	0.244	0.240	

Table 11– Multiple linear regression models for the prediction of the 6-month change in HOMA-IR (dependent variable). β-coefficients, p-values, and determination coefficients (r^2).

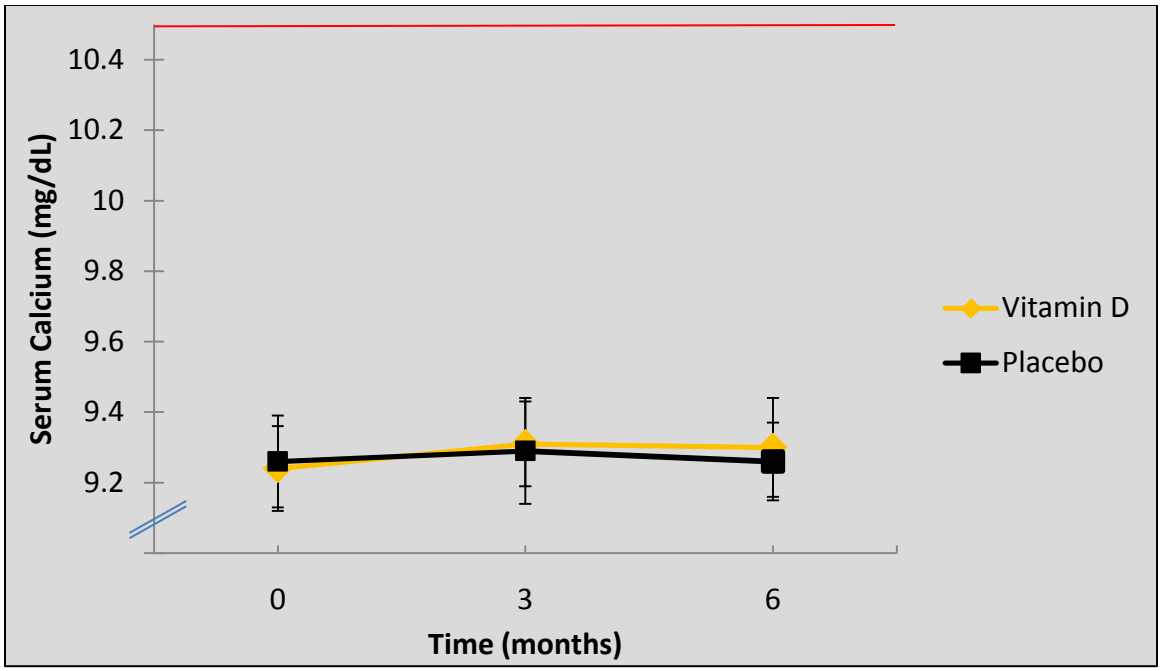


Figure 19- Change in serum calcium across study. Bar represents 95% CI. Red line (at 10.5 mg/dL) indicates clinical hypercalcemia.
****** P<0.05 within group change from baseline;*
† P<0.05 between group difference at timepoint

DISCUSSION

The objectives of the current study were to determine the efficacy of daily supplementation with 4000 IU in raising 25(OH)D concentrations of obese adolescents and to evaluate the effects on glucose metabolism and inflammation that resulted from the enhancement of circulating 25(OH)D concentrations in these individuals. We hypothesized that the dose used would be adequate to raise the serum 25(OH)D concentrations of obese adolescents to levels deemed sufficient (32-100 ng/ml) by multiple experts in field. We also hypothesized that improving the vitamin D status of these individuals would reduce surrogate markers of insulin resistance and the concentrations of three markers of inflammation (IL-6, TNF-α, and CRP). Furthermore, we hypothesized that improving vitamin D status would increase circulating concentrations of total and HMW adiponectin while decreasing leptin concentration, thereby reducing the leptin to adiponectin ratio. Our data back the use of 4000 IU per day as a safe and effective dose to improve the vitamin D status of obese adolescents. Additionally, our data support our hypotheses that improving vitamin D status in obese adolescents will ameliorate impaired glucose metabolism and insulin resistance. Individuals who received vitamin D saw significant reductions in two widely-used surrogate markers of insulin resistance and sensitivity (HOMA-IR and QUICKI, respectively) in addition to a third more recently proposed indicator, the ratio of leptin to adiponectin. However, while there appears to be a possible trend, we did not find adequate evidence to support our hypothesis that improving vitamin D status would reduce markers of inflammation that are

commonly associated with the progression from obesity to impaired glucose metabolism, as none of these markers were significantly reduced.

Baseline vitamin D status- Using 20 ng/ml as the cutoff, it has been estimated, that over 40% of men and 50% of women in the United States suffer from hypovitaminosis D (insufficient vitamin D status) (*1, 164*). Specific populations, such as minorities, the elderly, and overweight/obese individuals are at an even greater risk for vitamin D deficiency (*5, 164, 165*). Using the same criteria (circulating 25(OH)D concentrations less than 20 ng/ml), 53% of our entire sample was vitamin D deficient at baseline. These data are consistent with a large number of epidemiological and cross-sectional studies which have reported widespread vitamin D deficiency and insufficiency across all genders, races, and age groups (*63, 64, 164, 166, 167*).

It is well established that 25(OH)D concentrations are inversely related to skin pigmentation due to the sunscreen like effects of melanin (*53*). To our surprise, there were no significant differences in vitamin D status between the different Fitzpatrick skin categories (figure 4). Examining data from the NHANES III, Saintonge et al. found that the proportion African American adolescents with vitamin D deficiency was more than three times that of Caucasians in the same age group (*5*). The lack of significance seen between skin types was likely due to the limited number of subjects who fell in the extreme categories, I and VI, which had 2 and 0 subjects, respectively. As expected, we found that season of recruitment did have a significant effect on baseline vitamin D status, with those

recruited during the winter/spring (November through April) months having significantly lower circulating concentrations of 25(OH)D than those who enrolled from summer/fall (May to October) (figure 3). 25(OH)D concentrations are positively correlated with UVB exposure, which occurs between the months of March and September, peaking in mid-summer, in the northern hemisphere (50, 51). However, since circulating vitamin D is believed to have a half-life of approximately one month in humans, concentrations following this period of synthesis do not drop off rapidly (*168*).

Safety and efficacy- It has been established that lean individuals supplemented with oral vitamin D_3 experience elevations in serum 25(OH)D of about 1 ng/ml for every 100 IU/d taken (*62*). However, obese individuals have been shown to require as much as twice that amount to see the same rise in serum 25(OH)D (*4*). Our data is consistent with these estimates, as 4,000 IU/day produced a mean increase of 19.5 ng/ml or, 1 ng/ml for every 205 IU/day. Additionally, as anticipated, the increase in serum 25(OH)D during the first three months was of a much larger magnitude than during months three through six. This "plateau effect" is consistent with several other studies that have shown sharp rises in the first 4-6 weeks of supplementation followed by a gradual leveling off of 25(OH)D concentration (*62, 169, 170*). Changes seen were independent of changes in WC and BMI-Z, which did not change over the course of the study, or season of baseline visit and baseline 25(OH)D concentration for which they were covaried.

The hallmark characteristic of vitamin D toxicity is dangerously high concentrations of calcium in the blood, hypercalcemia. Clinically, hypercalcemia is defined as serum calcium concentrations exceeding 10.5 mg/dl (*171*). There was no evidence of toxicity in either group at any point during the study. The mean serum calcium concentrations of our subjects at baseline were approximately 9.2 in both the vitamin D and placebo group. The change from baseline in the group supplemented with vitamin D was negligible, 0.1 mg/dl. It appears that supplementing obese adolescents with 4000 IU of vitamin D_3, the current UL, is both safe and effective.

Changes in Insulin resistance and sensitivity- After adjusting for the covariates (baseline value of outcome variable, age, gender, WC, and BMI-Z), FPI was significantly reduced in the vitamin D group vs. control (*P*=0.026) at six months. However, the reduction in FPG, while trending towards significance, was not (*P*=0.085). While several health agencies use fasting glucose as diagnostic criteria for T2D, HOMA-IR and QUICK provide more physiological insight, as they take into account both fasting glucose and fasting insulin, which are equally important in normal glucose metabolism. HOMA-IR and QUICKI are commonly used clinical indices that provide a quick and easy way to assess insulin resistance and sensitivity from fasting lab values (*172, 173*). In the current population, HOMA-IR values greater than 3.5 are often considered insulin resistant. Likewise, QUICKI values less 0.330 are considered insulin resistant (*174*). At baseline, our study population had values of 5.5± 2.9 and 0.306±

0.020, respectively. The group who received vitamin D had statistically significant decreases from baseline in HOMA-IR and increases in QUICKI vs. placebo (*P*=0.033 and 0.016, respectively). The covariate-adjusted mean decrease in HOMA-IR, -1.63 units, was similar to the mean decrease of approximately 2 units reported in a review of several metformin trials in adolescents (*175*). Relative to baseline values, there was a much greater decrease in FPI (28.1%) than FPG (6.1%). This suggests that the improvements in insulin resistance that resulted from improving vitamin D status were primarily mediated through changes in fasting insulin. The decrease in fasting insulin seems somewhat counterintuitive to the literature suggesting that the actions of $1,25(OH)_2D$ in pancreatic β-cells stimulate the synthesis and secretion of insulin. However, the observed increases in insulin secretion have been seen following glucose challenge, which suggests that vitamin D functions in a regulatory capacity (*82, 83, 155*). Furthermore, the fact that there were not significant change in any of the outcome variables between baseline and three months suggests that the changes seen were either gradual or occurred only after serum 25(OH)D status was elevated. However, studies with more time points are necessary to determine which scenario is true. Simple linear regression analysis revealed that the magnitude of change in 25(OH)D was not significantly associated with any of the outcome variables FPG, FPI, HOMA-IR, or QUICKI. This leaves open the possibility that once sufficient vitamin D status is achieved, little added benefit comes from going beyond it. In other words, more is not necessarily better or a plateau is observed. However, studies aimed at elevating

vitamin D status even more than the current study would be necessary to say this with any degree of certainty.

Changes in Inflammatory markers- We saw no significant changes from baseline within or between treatment groups for IL-6, TNF-α, or CRP (table 8), before or after correcting for the aforementioned covariates. It is not surprising that we did not see a significant decrease in any of the inflammatory markers, as were not adequately powered to detect differences. Strangely, there was a borderline trend towards significance in the reduction of TNF-α seen in both groups. It is possible that this reduction reflects the success of other components of the standard care afforded at the ADOBE clinic. TNF-α drives is a "higher-order" inflammatory cytokine that is believed to be the driving force behind several inflammation-induced metabolic complications. The clinical significance of reducing TNF-α should not be overlooked and longer-term studies should seek to determine if sustained lower levels of circulating TNF-α results in reduced adverse health-outcomes. Simple linear regression revealed that there was not a significant relationship between the 6-month change in 25(OH)D and either TNF-α or IL-6. However, such a relationship did exist between change in 25(OH)D and CRP (P=0.015), where we observed a 0.518 mg/l decrease in HS-CRP for every 10 ng/ml increase in circulating 25(OH)D concentration (figure 13). While several studies exist demonstrating a correlation between serum 25(OH)D and inflammatory markers, as far as we are aware, this is the only study examining the relationship between the magnitude of change in 25(OH)D status

and magnitude of change in these markers (*12, 106, 176*). Although it is an acute-phase protein, C-reactive protein is often implicated as a prime contributor in the development of impaired glucose metabolism in both adults and children (*177, 178*). Additionally, CRP is considered a strong predictor of cardiovascular risk, even in children; and it is believed that reducing concentrations of CRP may decrease risk of future cardiovascular events (*179-181*). The production of CRP is largely driven by IL-6 and TNF-α, which were both highly elevated compared to lean controls found in the literature (*181-185*).

Changes in Adipokine and adipokine ratios- It is well established that as fat mass increases, circulating leptin concentrations increase and adiponectin concentrations decrease (*186-189*). After correcting for gender and age, the ratio of HMW to total adiponectin was inversely associated with waistline (r= -0.36, *P*=0.049) and BMI (r= -0.37, *P*=0.046) at baseline. However, there was not a significant relationship between either of these measures of obesity/adiposity and any of the other adipokines or adipokine ratios. We attributed the lack of correlation with BMI to the restricted range of BMIs that our sample spanned.

Leptin and adiponectin are two adipocyte-derived cytokines that have been independently associated with glucose metabolism. Adiponectin, particularly the HMW isoform, has been shown to have strong insulin sensitizing properties (*190, 191*). Additionally, Lin et al.,demonstrated that increases in serum adiponectin strongly predicted decreases in insulin resistance (*192*).

Likewise, leptin also has several metabolically-beneficial roles such as appetite suppression, improving glucose uptake, and regulating insulin secretion through a negative feedback loop termed the adipoinsular axis(*137, 193*). However, obesity produces a chronic state of hyperleptinemia that leads to leptin resistance. It has recently been suggested that the ratio of leptin to adiponectin could be a useful clinical tool in the assessment of insulin resistance (*194-197*). One study found that this ratio was more strongly correlated with results from a hyperinsulinemic/euglycemic clamp, considered the gold standard of assessing insulin resistance, than were HOMA-IR or QUICKI methods (*198*). In the current study, significant decreases from baseline to six months were seen in the vitamin D group for both leptin and the leptin to adiponectin ratio. However, when compared to the changes seen in the placebo group, only the decrease in leptin to adiponectin ratio was significant. Neither between nor within group changes in total adiponectin, HMW adiponectin, or the ratio of HMW to total adiponectin were significantly different at either three or six months. Simple linear regression revealed that changes from baseline in serum 25(OH)D concentration were inversely related to both leptin and the leptin to adiponectin ratio. In other words, greater improvement in vitamin D status resulted in greater reductions in both leptin and the leptin to adiponectin ratio. Additionally, there was a strong positive correlation between change in 25(OH)D concentration and change in total adiponectin. However, there was no association between change in 25(OH)D and change in HMW adiponectin, which is believed to be the bioactive isoform and therefore, a better indicator of metabolic health (*199*). These data

suggest that vitamin D either directly or indirectly influences circulating levels of adipokines independent of adiposity, as there were no changes in BMI-Z or waist circumference in our subjects. The reduction in leptin accounted for the majority of the change seen in the leptin to adiponectin ratio, which along with the large changes seen in fasting insulin, may indicate modulation of the adipoinsular axis by $1,25(OH)_2D$. However, the exact mechanisms by which it exerts these controls remain unknown. While previous studies have shown that 25(OH)D is inversely correlated with adiponectin and has a positive relationship with leptin, to the best of our knowledge, we are the first to show a significant decrease in the leptin to adiponectin ratio in a clinical trial involving vitamin D supplementation in any population (*106, 200*). While our data did not show a significant relationship between the ratio and either measure of insulin resistance/sensitivity, the ratio has also been associated with other obesity-associated metabolic complications (*201*). Therefore, the potential implications of this finding should not be overlooked.

Predictors of changes in HOMA-IR- We examined the relationship between inflammatory markers, adipokines, and HOMA-IR to determine if there was an association. Partial correlations, corrected for BMI-Z, WC, age, and gender were conducted to determine this relationship (table 10). When looking at baseline values, TNF-α and CRP were both significantly correlated with HOMA-IR; while, HMW adiponectin and the ratio of HMW to total adiponectin both trended towards significance (*0.05<P<0.10)*. Multiple linear regression

determined that change in total adiponectin, 25(OH)D, and CRP had the strongest associations with the change in HOMA-IR. The inverse association observed between change in 25(OH)D and HOMA-IR provides further evidence that improving vitamin D status ameliorated the insulin resistance in our population.

Limitations- The primary limitation of our study was sample size. As discussed in the methodology, the study was powered to detect a biologically relevant change in serum 25(OH)D concentration, as this change was the necessary prerequisite in answering all hypotheses. This limitation was seen in our analysis of the inflammatory markers CRP and IL-6, which despite having seemingly sizable between group differences in baseline to 6-month change, failed to show significant differences. Given the great between subject variances that are seen with many inflammatory markers, large sample sizes are often necessary. Another limitation of our study was the use of fasting measures of insulin resistance and glucose metabolism that we used. While surrogate markers of insulin resistance/sensitivity are a great clinical option due to their low cost and time investment, they are limited in the physiological information that can be ascertained from them. A third limitation to the study was the absence of Tanner stage data for our subjects. While we attempted to control for developmental differences through stratification and blocking based on age, assessing Tanner stage would have been a much more appropriate way to control for these expected differences.

Future Directions- The current study provides immense support for continued research involving the treatment of vitamin D deficiency and the various health effects to which vitamin D deficiency has been associated. Moreover, it substantiates the idea that past studies were using inadequate doses of vitamin D to produce relevant rises in circulating 25(OH)D. The next step in examining the effects of improving vitamin D status on glucose metabolism and insulin resistance should involve more telling measures, such as postprandial load tests. While surrogate measures such as the one we used are useful clinically, the do not provide sufficient mechanistic insight. Furthermore, these studies should look at multidimensional interventions focused on weight loss to examine any additive or synergistic benefits that improving vitamin D status may on metabolic health. Additionally, future studies should recruit across a wider range of body masses, as our group represented an extreme which may have impacted some of the associations and effects seen.

Conclusion

Inadequate vitamin D status is a health concern that has reached epidemic proportions in obese adolescents. Current IOM recommendations for vitamin D intake do not take individuals who are at greater risk for vitamin D deficiency into account and therefore, they are not adequate to raise serum 25(OH)D to desirable levels in many of these individuals. Our findings provide compelling support for routinely monitoring the vitamin D status of obese

adolescents. To the best of our knowledge, we are the first to show that improving vitamin D status in obese adolescents ameliorates surrogate markers of insulin resistance and the leptin to adiponectin ratio. It is evident that vitamin D plays profound roles in metabolic health. Furthermore, our data suggest that correcting poor vitamin D status through dietary supplementation may be an effective addition to the standard treatment of obesity and its associated metabolic complications.

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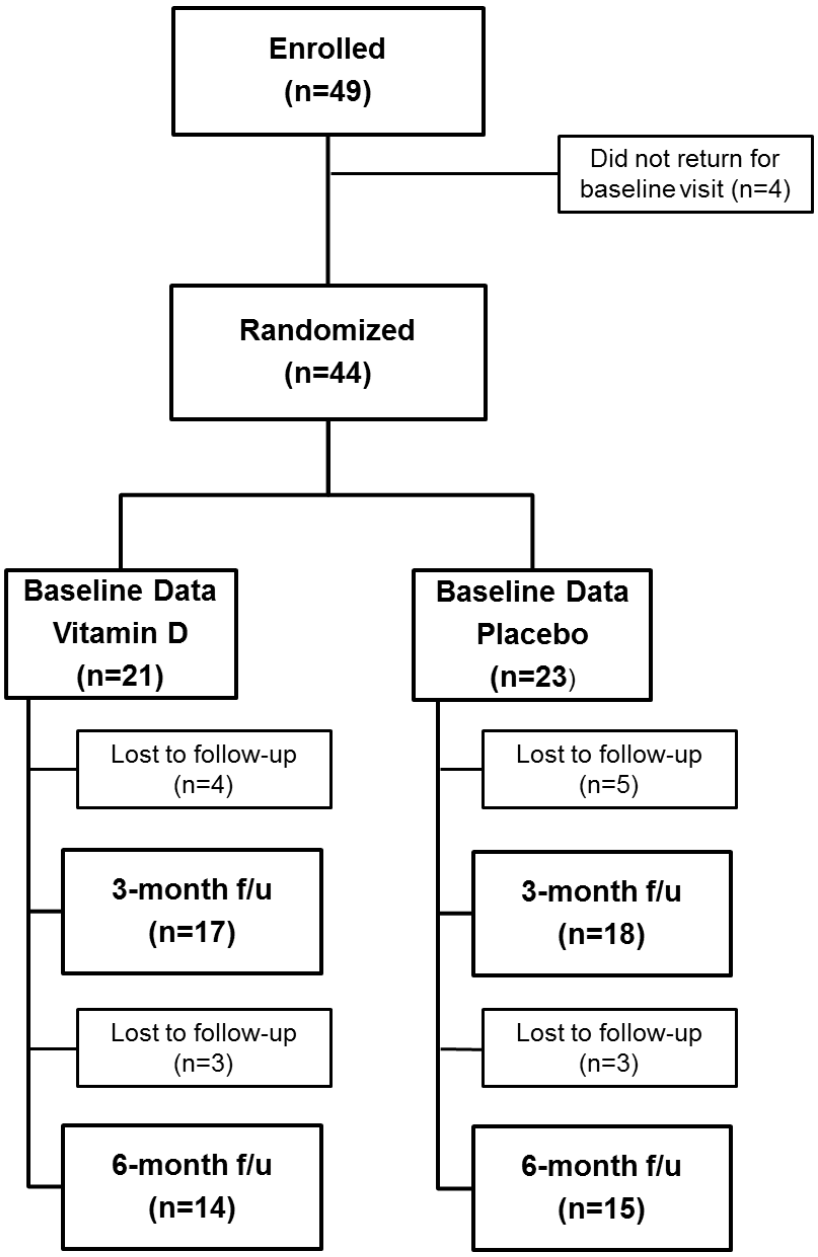
APPENDIX A:
CONSORT Flow Chart



APPENDIX B:
PRE-SCREENING QUESTIONNAIRE

1. UV light exposure:

a. How often do you use a commercial tanning bed? _____
b. For how long have you been following this tanning routine? _____
c. What type of tanning bed do you use? _____
d. Do you ever go out in the sun for the purpose of tanning? **Y / N**
   - **If yes,** how often? _____

2. Supplements/ Medications

a. Do you take any dietary supplements other than a general multivitamin? **Y / N**
   - **If yes,** what kind? _____

b. Do you currently take any of the following:
   - anticonvulsant medications *(ex; Dilantin)*? **Y / N**
   - cholesterol-lowering medications (*ex; cholestyramine, colestipol)*? **Y / N**
   - medications to treat the symptoms of stomach/duodenal ulcers or acid reflux *(ex; Cimetidine, Tagamet, Tagamet HB)*? **Y / N**
   - Hormone Replacement Therapy? **Y / N**
   - corticosteroids *(ex; hydrocortisone, prednisone)*? **Y / N**
   - Heparin to prevent blood clots? **Y / N**

Other medications? List:_____
_____
_____

3. Misc. Conditions

a. Do you smoke? **Y / N**
b. Do you have a history of osteoporosis? **Y / N**
c. Do you have any implanted metal in your body? **Y / N**
d. Are you pregnant or might be pregnant? **Y / N**
e. When was your last menstrual period? _____
f. How many menstrual periods do you have per year? _____
g. Are you on a special diet? **Y / N**
   - If yes, describe:_____

APPENDIX C:
FITZPATRICK SKIN CLASSIFICATION

Skin Type	Sun Exposure Reaction	Pigmentation
I	Always burn, never tan	Pale white, often freckled
II	Usually burn, rarely tan	White complexion; fair
III	Occasionally burn, slow to tan	White to light brown; moderate
IV	Seldom burn, usually tan	Light to moderate brown
V	Rarely burn, always tan	Moderate to dark brown
VI	Never burn	Very dark brown

APPENDIX D:
SUN EXPOSURE QUESTIONNAIRE

Do you use a tanning bed or capsule? **Y / N**

If yes, how often?
times/week **OR** times/month **OR** times/year

If weekly or monthly, for how long have you been tanning using this schedule?
months **OR** years

What kind of bed/capsule do you use?

How much time do you usually spend outdoors per day? (Includes working outside, walking to and from your car, exercising, gardening, purposeful sun exposure for tanning purposes, etc) min

Is this the same on both weekdays and weekends? If no, please explain.

How often do you usually wear sunscreen or sunblock? (Includes SPF in lotions, moisturizers, tanning lotions/oils, and makeup) e.g. *daily, only when out in the sun for long periods of time, while tanning, never, etc.*

What SPF level do you normally use, if any?

APPENDIX E:
Study Timetable

	Month											
	1	2	3	4	5	6	7	8	9	10	11	12
Recruitment of Subjects	X	X	X	X	X							
Vit D/Placebo Intervention		X	X	X	X	X	X	X	X	X	X	
Data Collection	X	X	X	X	X	X	X	X	X	X	X	
Safety/Efficacy Monitoring		X	X	X	X	X	X	X	X	X	X	
Research Data Analysis											X	X

APPENDIX F:
Pill Calendar

Pill Calendar
Start Date: _ Participant ID #:_____
Directions: ***Take 2 gel pills each day.*** *After you take them, mark an "X" on that day of the calendar. If you should happen to forget to take the pills, put a "0" for that day. Share this information with the clinic dietitian or the study coordinator on your next clinic visit.*

SUN	MON	TUE	WED	THU	FRI	SAT

SUN	MON	TUE	WED	THU	FRI	SAT

SUN	MON	TUE	WED	THU	FRI	SAT

APPENDIX H:
25(OH)D ASSAY PROCEDURE
(Immunodiagnostik; Bensheim, Germany)

1. Allow all reagents and samples to reach room temperature (18-26˚C) and mixing gently.
2. Make a protocol sheet with the positions of the standards/samples/controls
3. Reconstitute *Releasing Reagent* (see STEP 4 of reagent preparation)
4. Label V-Tubes (1.5 ml Eppendorf-tubes)
5. Pipette appropriate solution into corresponding tube
6. Add 300 µl of *Releasing Reagent* to each tube and vortex briefly
7. Incubate tubes for 1 hour in a 37˚C water bath (or on a heating block) NO INCUBATORS!
8. Open tubes (WATCH FOR SPLASHING) and add 600 µl of *Sample Dilution Buffer*
9. Close lids and vortex briefly
10. Remove (covered) micro-titer strips from kit
11. Pipette 50 µl of each solution (std/samp/ctrl) [in duplicate] from V-tubes to appropriate well
12. Add 150 µl of *25-(OH)D Antibody* into each well
13. Cover the plate tightly and allow to incubate overnight (min.18 – max. 22 hrs.) at 8-10˚C
14. Aspirate and wash wells 5x with 250 µl of *Diluted Wash Buffer,* using an 8-channel pipette
    a. Be sure to get all *Wash Buffer* out by hitting plate against paper towel
15. Add 200 µl of *Conjugate* into each well
16. Cover plate tightly and incubate for 1 hour at room temperature (18-26˚C) WHILE SHAKING
17. Aspirate and wash wells 5x with 250 µl of *Diluted Wash Buffer,* using an 8-channel pipette
    a. Be sure to get all *Wash Buffer* out by hitting plate against paper towel
18. Add 200 µl of *Substrate* into each well
19. Incubate IN THE DARK for 10-15 minutes at room temperature
20. Add 50 µl of *Stop Solution* into each well
21. Determine absorption with an ELISA reader at 450 nm against a reference (620 or 690 nm)

APPENDIX I:

C-Reactive Protein ASSAY PROCEDURE

(ALPCO Immunoassays; Salem, NH: Cat. # HSCRPHUU-E05)

1. Prepare working solutions of the streptavidin-HRP conjugate and wash buffer.

2. Pipette 20 µL of each calibrator, control, and serum sample into the correspondingly labeled wells in duplicate.

3. Pipette 80 µL of the monoclonal anti-CRP-biotin conjugate into each well.

4. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

5. Wash the wells 3 times with diluted wash buffer (300 µL/well for each wash) and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of an automatic washer is highly recommended.)

6. Pipette0 01 µL of diluted streptavidin-HRP conjugate into each well.

7. Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.

8. Wash the wells again in the same manner as in step 5.

9. Pipette 100 µL of TMB substrate into each well at timed intervals.

10. Incubate on a plate shaker for 10-15 minutes at room temperature.

11. Pipette 50 µL of stop solution into each well at the same timed intervals as in step 9.

12. Read the plate on a micro-well plate reader at 450 nm within 20 minutes after the addition of the stop solution.

APPENDIX J:

HIGH-SENSITIVITY TNF-alpha ASSAY PROCEDURE
(ALPCO Immunoassays; Salem, NH: Cat. #- 45-TNFHUU-E05)

1. *Determine the number of 8-well strips needed for the assay. Insert these in the fra*me(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)

2. Add 50 µL of the Incubation Buffer to all wells. Well(s) reserved for chromogen blank should be left empty.

3. Add 100 µL of the Standard Diluent Buffer to zero wells. Well(s) reserved for chromogen blank should be left empty.

4. Add 100 µL of standards or controls to the appropriate microtiter wells. For all samples (serum, plasma, buffered solution and cell culture medium), add 50 µL of Standard Diluent Buffer to each well followed by 50 µL of sample. Tap gently on the side of the plate to mix. (See REAGENT PREPARATION AND STORAGE, Section B.)

5. Pipette 50 µL of biotinylated anti-TNF-α (Biotin Conjugate) solution into each well except the chromogen blank(s). Tap on the side of the plate to thoroughly mix. Incubate for 2 hours.

6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTIONS FOR WASHING.

7. Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in REAGENT PREPARATION AND STORAGE, Section C.) Incubate for 30 minutes.

8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See DIRECTIONS FOR WASHING.

9. Add 100 µL of Stabilized Chromogen to each well. The liquid in the wells will begin to turn blue.

10. Incubate for 30 minutes at room temperature and in the dark. Please Note: Do not cover the plate with aluminum foil or metalized mylar. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the Stop Solution has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

11. Add 100 µL of Stop Solution to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.

12. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of Stabilized Chromogen and Stop Solution. Read the plate within 2 hours after adding the Stop Solution.

13. Read the Hu TNF-α concentrations for unknown samples and controls from the standard curve plotted in step 15. Multiply value(s) obtained for sample(s) by 2 to correct for the 1:2 dilution in step 4. (Samples producing signals greater than that of the highest standard (32 pg/mL) should be diluted in Standard Diluent Buffer and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

APPENDIX K:

LEPTIN ASSAY PROCEDURE

(ALPCO Immunoassays; Salem, NH: Cat. #-11-LEPHU-E01)

1. Prepare working solutions of the streptavidin-HRP conjugate and wash buffer.

2. Pipette 20 µL of each calibrator, control, and serum sample into the correspondingly labeled wells in duplicate.

3. Pipette 80 µL of the monoclonal anti-leptin-biotin conjugate into each well.

4. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature.

5. Wash the wells 3 times with diluted wash buffer (300 µL/well for each wash) and tap the plate firmly against absorbent paper to ensure that it is dry. (The use of an automatic washer is highly recommended.)

6. Pipette0 01 µL of diluted streptavidin-HRP conjugate into each well.

7. Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.

8. Wash the wells again in the same manner as in step 5.

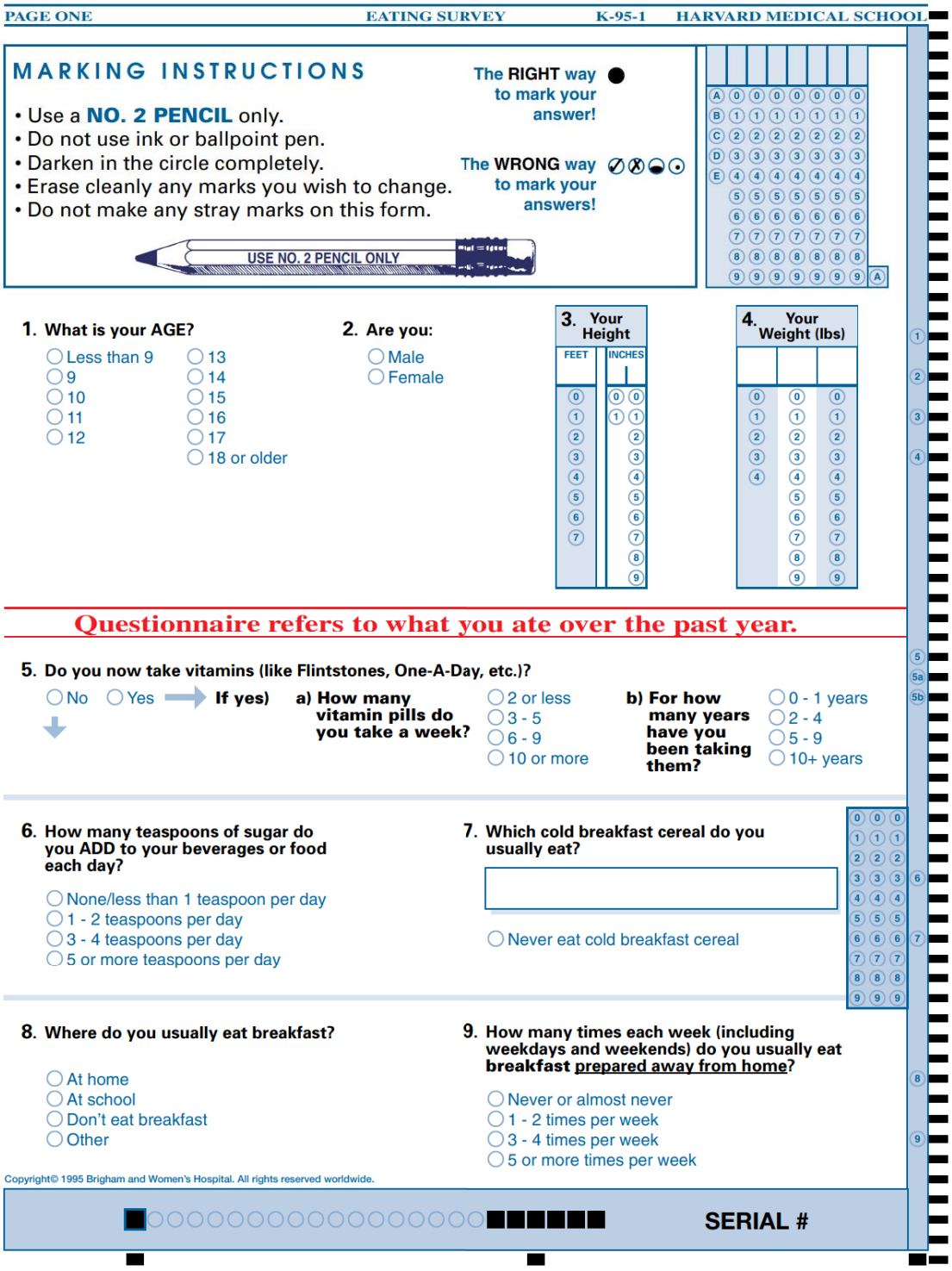
9. Pipette 100 µL of TMB substrate into each well at timed intervals.

10. Incubate on a plate shaker for 10-15 minutes at room temperature.

11. Pipette 50 µL of stop solution into each well at the same timed intervals as in step 9.

12. Read the plate on a micro-well plate reader at 450 nm within 20 minutes after the addition of the stop solution.

APPENDIX L:
Food Frequency Questionnaire (YAQ)



PAGE ONE EATING SURVEY K-95-1 HARVARD MEDICAL SCHOOL

MARKING INSTRUCTIONS

- Use a **NO. 2 PENCIL** only.
- Do not use ink or ballpoint pen.
- Darken in the circle completely.
- Erase cleanly any marks you wish to change.
- Do not make any stray marks on this form.

The **RIGHT** way to mark your answer! ●

The **WRONG** way to mark your answers! ⊘ ⊗ ◒ ⊙

USE NO. 2 PENCIL ONLY

1. What is your AGE?

○ Less than 9 ○ 13
○ 9 ○ 14
○ 10 ○ 15
○ 11 ○ 16
○ 12 ○ 17
○ 18 or older

2. Are you:

○ Male
○ Female

3. Your Height

FEET | INCHES

4. Your Weight (lbs)

Questionnaire refers to what you ate over the past year.

5. Do you now take vitamins (like Flintstones, One-A-Day, etc.)?

○ No ○ Yes → **If yes)**

a) How many vitamin pills do you take a week?

○ 2 or less
○ 3 - 5
○ 6 - 9
○ 10 or more

b) For how many years have you been taking them?

○ 0 - 1 years
○ 2 - 4
○ 5 - 9
○ 10+ years

6. How many teaspoons of sugar do you ADD to your beverages or food each day?

○ None/less than 1 teaspoon per day
○ 1 - 2 teaspoons per day
○ 3 - 4 teaspoons per day
○ 5 or more teaspoons per day

7. Which cold breakfast cereal do you usually eat?

○ Never eat cold breakfast cereal

8. Where do you usually eat breakfast?

○ At home
○ At school
○ Don't eat breakfast
○ Other

9. How many times each week (including weekdays and weekends) do you usually eat breakfast prepared away from home?

○ Never or almost never
○ 1 - 2 times per week
○ 3 - 4 times per week
○ 5 or more times per week

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10. **How many times each week (including weekdays and weekends) do you usually eat lunch prepared away from home?**

- ○ Never or almost never
- ○ 1 - 2 times per week
- ○ 3 - 4 times per week
- ○ 5 or more times per week

11. **How many times each week do you usually eat after-school snacks or foods prepared away from home?**

- ○ Never or almost never
- ○ 1 - 2 times per week
- ○ 3 - 4 times per week
- ○ 5 or more times per week

12. **How many times each week (weekdays and weekends) do you usually eat dinner prepared away from home?**

- ○ Never or almost never
- ○ 1 - 2 times per week
- ○ 3 - 4 times per week
- ○ 5 or more times per week

13. **How many times per week do you prepare dinner for yourself (and/or others in your house)?**

- ○ Never or almost never
- ○ Less than once per week
- ○ 1 - 2 times per week
- ○ 3 - 4 times per week
- ○ 5 or more times per week

14. **How often do you have dinner that is ready made, like frozen dinners, Spaghetti-O's, microwave meals, etc.**

- ○ Never/less than once per month
- ○ 1 - 2 times per week
- ○ 3 - 4 times per week
- ○ 5 or more times per week

15. **How many times each week (including weekdays and weekends) do you eat late night snacks prepared away from home?**

- ○ Never/less than once per month
- ○ 1 - 2 times per week
- ○ 3 - 4 times per week
- ○ 5 or more times per week

16. **How often do you eat food that is fried at home, like fried chicken?**

- ○ Never/less than once per week
- ○ 1 - 3 times per week
- ○ 4 - 6 times per week
- ○ Daily

17. **How often do you eat fried food away from home (like french fries, chicken nuggets)?**

- ○ Never/less than once per week
- ○ 1 - 3 times per week
- ○ 4 - 6 times per week
- ○ Daily

DIETARY INTAKE

How often do you eat the following foods:

Example **If you drink one can of diet soda 2 - 3 times per week, then your answer should look like this:**

E1. **Diet soda (1 can or glass)**

- ○ Never
- ○ 1 - 3 cans per month
- ○ 1 can per week
- ● 2 - 6 cans per week
- ○ 1 can per day
- ○ 2 or more cans per day

BEVERAGES

FILL OUT ONE BUBBLE FOR EACH FOOD ITEM

18. Diet soda (1 can or glass)

- ○ Never/less than 1 per month
- ○ 1 - 3 cans per month
- ○ 1 can per week
- ○ 2 - 6 cans per week
- ○ 1 can per day
- ○ 2 or more cans per day

19. Soda - not diet (1 can or glass)

- ○ Never/less than 1 per month
- ○ 1 - 3 cans per month
- ○ 1 can per week
- ○ 2 - 6 cans per week
- ○ 1 can per day
- ○ 2 or more cans per day

20. Hawaiian Punch, lemonade, Koolaid or other non-carbonated fruit drink (1 glass)

- ○ Never/less than 1 per month
- ○ 1 - 3 glasses per month
- ○ 1 glass per week
- ○ 2 - 4 glasses per week
- ○ 5 - 6 glasses per week
- ○ 1 glass per day
- ○ 2 or more glasses per day

21. Iced Tea - sweetened (1 glass, can or bottle)

- ○ Never/less than 1 per month
- ○ 1 - 3 glasses per month
- ○ 1 - 4 glasses per week
- ○ 5 - 6 glasses per week
- ○ 1 or more glasses per day

22. Tea (1 cup)

- ○ Never/less than 1 per month
- ○ 1 - 3 cups per month
- ○ 1 - 2 cups per week
- ○ 3 - 6 cups per week
- ○ 1 or more cups per day

23. Coffee - not decaf. (1 cup)

- ○ Never/less than 1 per month
- ○ 1 - 3 cups per month
- ○ 1 - 2 cups per week
- ○ 3 - 6 cups per week
- ○ 1 or more cups per day

24. Beer (1 glass, bottle or can)

- ○ Never/less than 1 per month
- ○ 1 - 3 cans per month
- ○ 1 can per week
- ○ 2 or more cans per week

25. Wine or wine coolers (1 glass)

- ○ Never/less than 1 per month
- ○ 1 - 3 glasses per month
- ○ 1 glass per week
- ○ 2 or more glasses per week

26. Liquor, like vodka or rum (1 drink or shot)

- ○ Never/less than 1 per month
- ○ 1 - 3 drinks per month
- ○ 1 drink per week
- ○ 2 or more drinks per week

Example If you eat:

3 pats of margarine on toast
1 - 2 pats of margarine on sandwich
1 pat of margarine on vegetables

5 - 6 pats total all day

then answer this way →

E2. Margarine (1 pat) - not butter

- ○ Never
- ○ 1 - 3 pats per month
- ○ 1 pat per week
- ○ 2 - 6 pats per week
- ○ 1 pat per day
- ○ 2 - 4 pats per day
- ● 5 or more pats per day

DAIRY PRODUCTS

27. What TYPE of milk do you usually drink?

- ○ Whole milk
- ○ 2% milk
- ○ 1% milk
- ○ Skim/nonfat milk
- ○ Don't know
- ○ Don't drink milk

28. Milk (glass or with cereal)

- ○ Never/less than 1 per month
- ○ 1 glass per week or less
- ○ 2 - 6 glasses per week
- ○ 1 glass per day
- ○ 2 - 3 glasses per day
- ○ 4+ glasses per day

29. Chocolate milk (glass)

- ○ Never/less than 1 per month
- ○ 1 - 3 glasses per month
- ○ 1 glass per week
- ○ 2 - 6 glasses per week
- ○ 1 - 2 glasses per day
- ○ 3 or more glasses per day

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30. Instant Breakfast Drink (1 packet)
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

31. Whipped cream
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

32. Yogurt (1 cup) - Not frozen
- ○ Never/less than 1 per month
- ○ 1 - 3 cups per month
- ○ 1 cup per week
- ○ 2 - 6 cups per week
- ○ 1 cup per day
- ○ 2 or more cups per day

33. Cottage or ricotta cheese
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

34. Cheese (1 slice)
- ○ Never/less than 1 per month
- ○ 1 - 3 slices per month
- ○ 1 slice per week
- ○ 2 - 6 slices per week
- ○ 1 slice per day
- ○ 2 or more slices per day

35. Cream cheese
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

36. What TYPE of yogurt, cottage cheese & dairy products (besides milk) do you use mostly?
- ○ Nonfat
- ○ Lowfat
- ○ Regular
- ○ Don't know

37. Butter (1 pat) - NOT margarine
- ○ Never/less than 1 per month
- ○ 1 - 3 pats per month
- ○ 1 pat per week
- ○ 2 - 6 pats per week
- ○ 1 pat per day
- ○ 2 - 4 pats per day
- ○ 5 or more pats per day

38. Margarine (1 pat) - NOT butter
- ○ Never/less than 1 per month
- ○ 1 - 3 pats per month
- ○ 1 pat per week
- ○ 2 - 6 pats per week
- ○ 1 pat per day
- ○ 2 - 4 pats per day
- ○ 5 or more pats per day

39. What FORM and BRAND of margarine does your family usually use?
- ○ None
- ○ Stick
- ○ Tub
- ○ Squeeze (liquid)

WHAT SPECIFIC BRAND AND TYPE (LIKE "PARKAY CORN OIL SPREAD")?

Leave blank if you don't know.

40. What TYPE of oil does your family use at home?
- ○ Canola oil
- ○ Corn oil
- ○ Safflower oil
- ○ Olive oil
- ○ Vegetable oil
- ○ Don't know

MAIN DISHES

41. Cheeseburger (1)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 - 4 per week
- ○ 5 or more per week

42. Hamburger (1)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 - 4 per week
- ○ 5 or more per week

43. Pizza (2 slices)
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

44. Tacos/burritos (1)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 - 4 per week
- ○ 5 or more per week

45. Which taco filling do you usually have:
- ○ Beef & beans
- ○ Beef
- ○ Chicken
- ○ Beans

46. Chicken nuggets (6)
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

47. Hot dogs (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 - 4 per week
- ○ 5 or more per week

48. Peanut butter sandwich (1) (plain or with jelly, fluff, etc.)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 - 4 per week
- ○ 5 or more per week

49. Chicken or turkey sandwich (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 or more per week

50. Roast beef or ham sandwich (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 or more per week

51. Salami, bologna, or other deli meat sandwich (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 or more per week

52. Tuna sandwich (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ One per week
- ○ 2 or more per week

53. Chicken or turkey as main dish (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

54. Fish sticks, fish cakes or fish sandwich (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

55. Fresh fish as main dish (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

56. Beef (steak, roast) or lamb as main dish (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

57. Pork or ham as main dish (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

58. Meatballs or meatloaf (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

59. Lasagna/baked ziti (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

60. Macaroni and cheese (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

61. Spaghetti with tomato sauce (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

62. Eggs (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 eggs per month
- ○ One egg per week
- ○ 2 - 4 eggs per week
- ○ 5 or more eggs per week

63. Liver: beef, calf, chicken or pork (1 serving)

- ○ Never/less than 1 per month
- ○ Less than once per month
- ○ Once per month
- ○ 2 - 3 times per month
- ○ Once per week or more

64. Shrimp, lobster, scallops (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

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65. French toast (2 slices)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

66. Grilled cheese (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

67. Eggrolls (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

MISCELLANEOUS FOODS

68. Brown gravy

- ○ Never/less than 1 per month
- ○ Once per week or less
- ○ 2 - 6 times per week
- ○ Once per day
- ○ 2 or more times per day

69. Ketchup

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

70. Clear soup (with rice, noodles, vegetables) 1 bowl

- ○ Never/less than 1 per month
- ○ 1 - 3 bowls per month
- ○ 1 bowl per week
- ○ 2 or more bowls per week

71. Cream (milk) soups or chowder (1 bowl)

- ○ Never/less than 1 per month
- ○ 1 - 3 bowls per month
- ○ 1 bowl per week
- ○ 2 - 6 bowls per week
- ○ 1 or more bowls per day

72. Mayonnaise

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 6 times per week
- ○ Once per day

73. Low calorie/fat salad dressing

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 6 times per week
- ○ Once or more per day

74. Salad dressing (not low calorie)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 6 times per week
- ○ Once or more per day

75. Salsa

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 6 times per week
- ○ Once or more per day

76. How much fat on your beef, pork, or lamb do you eat?

- ○ Eat all
- ○ Eat some
- ○ Eat none
- ○ Don't eat meat

77. When you have chicken or turkey, do you eat the skin?

- ○ Yes
- ○ No
- ○ Sometimes

BREADS & CEREALS

78. Cold breakfast cereal (1 bowl)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 bowls per month
- ◯ 1 bowl per week
- ◯ 2 - 4 bowls per week
- ◯ 5 - 7 bowls per week
- ◯ 2 or more bowls per day

79. Hot breakfast cereal, like oatmeal, grits (1 bowl)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 bowls per month
- ◯ 1 bowl per week
- ◯ 2 - 4 bowls per week
- ◯ 5 - 7 bowls per week
- ◯ 2 or more bowls per day

80. White bread, pita bread, or toast (1 slice)

- ◯ Never/less than 1 per month
- ◯ 1 slice per week or less
- ◯ 2 - 4 slices per week
- ◯ 5 - 7 slices per week
- ◯ 2 - 3 slices per day
- ◯ 4+ slices per day

81. Dark bread (1 slice)

- ◯ Never/less than 1 per month
- ◯ 1 slice per week or less
- ◯ 2 - 4 slices per week
- ◯ 5 - 7 slices per week
- ◯ 2 - 3 slices per day
- ◯ 4+ slices per day

82. English muffins or bagels (1)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 per month
- ◯ 1 per week
- ◯ 2 - 4 per week
- ◯ 5 or more per week

83. Muffin (1)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 muffins per month
- ◯ 1 muffin per week
- ◯ 2 - 4 muffins per week
- ◯ 5 or more muffins per week

84. Cornbread (1 square)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more per week

85. Biscuit/roll (1)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 per month
- ◯ 1 per week
- ◯ 2 - 4 per week
- ◯ 5 or more per week

86. Rice

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

87. Noodles, pasta

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

88. Tortilla - no filling (1)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 per month
- ◯ 1 per week
- ◯ 2 - 4 per week
- ◯ 5 or more per week

89. Other grains, like kasha, couscous, bulgur

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 or more times per week

90. Pancakes (2) or waffles (1)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 or more times per week

91. French fries (large order)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 orders per month
- ◯ 1 order per week
- ◯ 2 - 4 orders per week
- ◯ 5 or more orders per week

92. Potatoes - baked, boiled, mashed

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

FRUITS & VEGETABLES

93. Raisins (small pack)
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ 1 per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

94. Grapes (bunch)
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

95. Bananas (1)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 4 per week
- ○ 5 or more per week

96. Cantaloupe, melons (1/4 melon)
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ 1 per week
- ○ 2 or more times per week

97. Apples (1) or applesauce
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 6 per week
- ○ 1 or more per day

98. Pears (1)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 6 per week
- ○ 1 or more per day

99. Oranges (1), grapefruit (1/2)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 6 per week
- ○ 1 or more per day

100. Strawberries
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

101. Peaches, plums, apricots (1)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 or more per week

102. Orange juice (1 glass)
- ○ Never/less than 1 per month
- ○ 1 - 3 glasses per month
- ○ 1 glass per week
- ○ 2 - 6 glasses per week
- ○ 1 glass per day
- ○ 2 or more glasses per day

103. Apple juice and other fruit juices (1 glass)
- ○ Never/less than 1 per month
- ○ 1 - 3 glasses per month
- ○ 1 glass per week
- ○ 2 - 6 glasses per week
- ○ 1 glass per day
- ○ 2 or more glasses per day

104. Tomatoes (1)
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 6 per week
- ○ 1 or more per day

105. Tomato/spaghetti sauce
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

106. Tofu
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

107. String beans
- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

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108. Beans/lentils/soybeans

- ◯ Never/less than 1 per month
- ◯ Once per week or less
- ◯ 2 - 6 times per week
- ◯ Once per day

109. Broccoli

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

110. Beets (not greens)

- ◯ Never/less than 1 per month
- ◯ Once per week or less
- ◯ 2 or more times per week

111. Corn

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

112. Peas or lima beans

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

113. Mixed vegetables

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

114. Spinach

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once a week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

115. Greens/kale

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

116. Green/red peppers

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once a week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

117. Yams/sweet potatoes (1)

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once a week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

118. Zucchini, summer squash, eggplant

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

119. Carrots, cooked

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

120. Carrots, raw

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

121. Celery

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 4 times per week
- ◯ 5 or more times per week

122. Lettuce/tossed salad

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 - 6 times per week
- ◯ One or more per day

123. Coleslaw

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 or more times per week

124. Potato salad

- ◯ Never/less than 1 per month
- ◯ 1 - 3 times per month
- ◯ Once per week
- ◯ 2 or more times per week

Think about your usual snacks. How often do you eat each type of snack food.

Example If you eat poptarts rarely (about 6 per year) then your answer should look like this:

E3. Poptarts (1)

- ● Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 - 6 per week
- ○ 1 or more per day

SNACK FOODS/DESSERTS

125. Fill in the number of snacks (food or drinks) eaten on school days and weekends/vacation days.

Snacks	School Days					Vacation/Weekend Days				
	NONE	1	2	3	4 OR MORE	NONE	1	2	3	4 OR MORE
Between breakfast and lunch	○	○	○	○	○	○	○	○	○	○
After lunch, before dinner	○	○	○	○	○	○	○	○	○	○
After dinner	○	○	○	○	○	○	○	○	○	○

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126. Potato chips (1 small bag)

- ○ Never/less than 1 per month
- ○ 1 - 3 small bags per month
- ○ One small bag per week
- ○ 2 - 6 small bags per week
- ○ 1 or more small bags per day

127. Corn chips/Doritos (small bag)

- ○ Never/less than 1 per month
- ○ 1 - 3 small bags per month
- ○ One small bag per week
- ○ 2 - 6 small bags per week
- ○ 1 or more small bags per day

128. Nachos with cheese (1 serving)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 or more times per week

126 127 128

129. Popcorn (1 small bag)

- ○ Never/less than 1 per month
- ○ 1 - 3 small bags per month
- ○ 1 - 4 small bags per week
- ○ 5 or more small bags per week

130. Pretzels (1 small bag)

- ○ Never/less than 1 per month
- ○ 1 - 3 small bags per month
- ○ 1 small bags per week
- ○ 2 or more small bags per week

131. Peanuts, nuts (1 small bag)

- ○ Never/less than 1 per month
- ○ 1 - 3 small bags per month
- ○ 1 - 4 small bags per week
- ○ 5 or more small bags per week

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132. Fun fruit or fruit rollups (1 pack)

- ○ Never/less than 1 per month
- ○ 1 - 3 packs per month
- ○ 1 - 4 packs per week
- ○ 5 or more packs per week

133. Graham crackers

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ 1 - 4 times per week
- ○ 5 or more times per week

134. Crackers, like saltines or wheat thins

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ 1 - 4 times per week
- ○ 5 or more times per week

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135. Poptarts (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 poptarts per month
- ○ 1 - 6 poptarts per week
- ○ 1 or more poptarts per day

136. Cake (1 slice)

- ○ Never/less than 1 per month
- ○ 1 - 3 slices per month
- ○ 1 slice per week
- ○ 2 or more slices per week

137. Snack cakes, Twinkies (1 package)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ Once per week
- ○ 2 - 6 per week
- ○ 1 or more per day

138. Danish, sweetrolls, pastry (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 4 per week
- ○ 5 or more per week

139. Donuts (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 donuts per month
- ○ 1 donut per week
- ○ 2 - 6 donuts per week
- ○ 1 or more donuts per day

140. Cookies (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 cookies per month
- ○ 1 cookie per week
- ○ 2 - 6 cookies per week
- ○ 1 - 3 cookies per day
- ○ 4 or more cookies per day

141. Brownies (1)

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 4 per week
- ○ 5 or more per week

142. Pie (1 slice)

- ○ Never/less than 1 per month
- ○ 1 - 3 slices per month
- ○ 1 slice per week
- ○ 2 or more slices per week

143. Chocolate (1 bar or packet) like Hershey's or M & M's

- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 - 6 per week
- ○ 1 or more per day

144. Other candy bars (Milky Way, Snickers)

- ○ Never/less than 1 per month
- ○ 1 - 3 candy bars per month
- ○ 1 candy bar per week
- ○ 2 - 4 candy bars per week
- ○ 5 or more candy bars per week

145. Other candy without chocolate (Skittles) (1 pack)

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

146. Jello

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

147. Pudding

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

148. Frozen yogurt

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

149. Ice cream

- ○ Never/less than 1 per month
- ○ 1 - 3 times per month
- ○ Once per week
- ○ 2 - 4 times per week
- ○ 5 or more times per week

150. Milkshake or frappe (1)

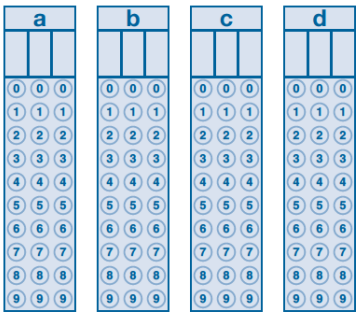
- ○ Never/less than 1 per month
- ○ 1 - 3 per month
- ○ 1 per week
- ○ 2 or more per week

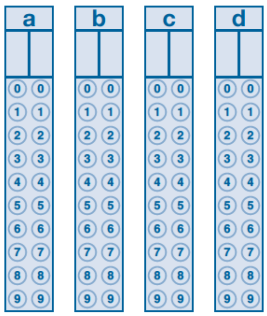
151. Popsicles

- ○ Never/less than 1 per month
- ○ 1 - 3 popsicles per month
- ○ 1 popsicle per week
- ○ 2 - 4 popsicles per week
- ○ 5 or more popsicles per week

152. **Please list any other foods that you usually eat <u>at least once per week</u> that are not listed (for example, coconut, hummus, falafel, chili, plantains, mangoes, etc. . .)**

FOODS	HOW OFTEN?
a) _____	a) _____
b) _____	b) _____
c) _____	c) _____
d) _____	d) _____





THANK YOU FOR COMPLETING THIS SURVEY!

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