Plasma 25-hydroxyvitamin D_3 response to vitamin D supplementation in obese and non-obese men and women

Kelly Choi Ahern

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Mario Kratz

Michael Rosenfeld

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Abstract

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Kelly Choi Ahern

Chair of the Supervisory Committee:
Research Assistant Professor, Mario Kratz, PhD
Department of Epidemiology

Context: Obese adults are more susceptible to poor vitamin D status and response to supplementation than lean adults, but explanatory mechanisms remain unclear. Proposed hypotheses include increased degradation or adipose tissue sequestration of vitamin D.

Objective: The purpose of this study was to investigate how plasma 25-hydroxyvitamin D_3 [$25(OH)D_3$] concentrations respond to vitamin D repletion relative to changes in plasma vitamin D_3 and the major degradation metabolite, 24,25-dihydroxyvitamin D_3 [$24,25(OH)_2D_3$]. We also investigated how obesity and obesity-related adipose tissue inflammation affects vitamin D status.

Methods: This was a randomized intervention pilot study that supplemented daily oral vitamin D_3 doses of either 2,000 IU or 4,000 IU to fifteen vitamin D-deficient, overweight to obese adults for three months. At baseline and 3-month visits, we measured concentrations of vitamin D_3, $25(OH)D_3$, and $24,25(OH)_2D_3$ in plasma and adipose tissue using high performance liquid chromatography-tandem mass spectrometry. We also measured gene expression of the pro-inflammatory cytokine, tumor necrosis factor-alpha (TNFα), in adipose tissue.

Results: Plasma concentration of $25(OH)D_3$ significantly increased after supplementation ($p < 0.001$), and increased more in the 4,000 IU/d group than in the 2,000 IU/d group ($p = 0.04$). Change in plasma $25(OH)D_3$ was positively associated with change in plasma $24,25(OH)_2D_3$ (β-coefficient 3.7, 95% CI 1.9–5.4, $p = 0.001$), but not associated with change in plasma vitamin D_3. We also computed a multivariable model that included change in plasma concentrations of both vitamin D_3 and $24,25(OH)_2D_3$ as independent variables and adjusted for dose. In this analysis, the association between changes in plasma $25(OH)D_3$ and $24,25(OH)_2D_3$ remained significantly positive, but the association between changes in plasma $25(OH)D_3$ and vitamin D_3 became significantly inverse (β-coefficient -0.4, 95% CI -0.8– -0.01, $p = 0.046$). BMI was not significantly associated with the changes in plasma $25(OH)D_3$, $24,25(OH)_2D_3$, or vitamin D_3. Adipose tissue expression of TNFα was not associated with changes in plasma $25(OH)D_3$ or $24,25(OH)_2D_3$.

Conclusion: Our findings were inconclusive about the sequestration hypothesis and did not support the hypothesis that the obese degrade more vitamin D than the lean due to greater adipose tissue inflammation. Future studies should investigate how baseline vitamin D status affects vitamin D response in the obese population.

BACKGROUND

Vitamin D basics

General role of vitamin D

Vitamin D is traditionally known for its role in regulating calcium homeostasis in serum, bone, kidney, and intestinal tissues, which is essential for supporting cellular processes, neuromuscular functions, and bone ossification.[1-3] In the 1980's, the vitamin D receptor (VDR) was found to reside in over 30 tissues, most of which were not involved with calcium homeostasis, such as adipose, lung, cardiac, and pancreatic tissues.[4] This led to further investigation of vitamin D's potential extraskeletal roles, including cell differentiation and proliferation and blood pressure regulation.[5] Another emerging role of vitamin D appears to be its immune system modulating effects that may elicit both pro- and anti-inflammatory processes.[6-8]

Sources and metabolism of vitamin D

Vitamin D is a fat-soluble, steroid-derived vitamin that humans can obtain exogenously from their diet and endogenously from cutaneous synthesis.[9] The richest dietary sources of vitamin D are found in animal products, especially liver, beef, veal, egg yolk, and certain fatty saltwater fish. Some foods, such as milk, yogurt, cheese, orange juice, and cereals, can be artificially fortified with vitamin D. Endogenous vitamin D_3 is synthesized in the skin after a precursor steroid is exposed to sunlight's ultraviolet B (UVB) photons.[9]

With limited dietary sources of vitamin D and decreased UVB exposure due to a growing concern for skin cancer, more people are turning to supplements to fulfill their vitamin D needs. Special analogues, such as calcidiol [$25(OH)D_3$] or calcitriol [$1,25(OH)_2D_3$], are often

administered to patients with liver and/or kidney dysfunction.[10] But most commercial oral supplements are sold as cholecalciferol (vitamin D_3) or ergocalciferol (vitamin D_2). These two forms are generally considered equally effective and interchangeable, though some research has suggested that vitamin D_2 is less "potent" than D_3, due to its lower affinity to the vitamin D-binding protein (DBP) and therefore clears more rapidly.[11, 12]

Dietary vitamin D_3 is transported by chylomicrons and endogenous vitamin D_3 by DBP to the liver. The enzyme 25-hydroxylase then converts vitamin D_3 to 25-hydroxyvitamin D_3 [$25(OH)D_3$], which is the major circulating form of vitamin D. Some $25(OH)D_3$ is further converted to the active hormone, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], by the enzyme 1α-hydroxylase (gene CYP27) in the kidneys.[9] $1,25(OH)_2D_3$ binds to VDR and is responsible for most, if not all, of the biological actions of vitamin D. Excessive amounts of circulating active hormone may have harmful effects, such as hypercalcification, so it must be tightly regulated by a series of feedback mechanisms. Low plasma phosphate and calcium signals parathyroid hormone (PTH) to stimulate 1α-hydroxylase transcription in the kidney, resulting in elevated $1,25(OH)_2D_3$. Elevated $1,25(OH)_2D_3$ in turn suppresses transcription of PTH and 1α-hydroxylase.[13] Additionally, the enzyme 24-hydroxylase (gene CYP24) helps inactivate vitamin D by hydroxylating $25(OH)D_3$ and $1,25(OH)_2D_3$ to the relatively inactive forms, $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$, respectively, for excretion.[9]

Vitamin D receptor

When bound to the active form of vitamin D [$1,25(OH)_2D_3$], VDR forms heterodimers with the retinoid X receptor (RXR), then binds to genomic sites called vitamin D response elements (VDREs) near its target genes.[14] Thus, VDR in the nucleus can elicit genomic actions

that influence gene transcription, but VDR in the plasma membrane can perform non-genomic actions that rapidly activate signal transduction pathways.[4, 9, 15] VDR has been found throughout the body, including adipose tissue (AT) and immune cells.[4, 15-20] The $1,25(OH)_2D_3$-VDR complex likely contributes to both innate and adaptive immunity by regulating expression, differentiation, and activation of macrophages, dendritic cells, and B- and T- lymphocytes.[7, 21] Studies in both rodent and human adipocytes have also shown that this complex can elicit a potent anti-inflammatory cascade, which will be discussed in more detail later.[7, 22-26]

Vitamin D deficiency

Vitamin D deficiency is recognized as a global health issue that affects all ethnicities, ages, and sexes. From 2001 to 2006, the National Health and Nutrition Examination Survey (NHANES) found that 69% of all subjects in the United States were vitamin D insufficient and 29% were deficient.[27] Deficiency is traditionally characterized by an increased risk of rickets in children and infants and osteomalacia in adults.[9, 28] Insufficiency is considered suboptimal vitamin D status that likely has adverse long-term health consequences, but in the absence of rickets or osteomalacia.[29]

Recent research has revealed associations between hypovitaminosis D and increased risk of common cancers, type 1 diabetes, multiple sclerosis, hypertension, depression, and chronic inflammatory conditions, such as rheumatoid arthritis and inflammatory bowel disease.[5, 28, 29] To explain this phenomenon, Ames proposed an evolutionary hypothesis called the triage theory. He suggests that episodes of micronutrient inadequacy forces nature to select micronutrient-dependent functions necessary for short-term survival at the expense of long-term health. In other words, “DNA damage and late onset disease are consequences of a triage allocation

response to micronutrient scarcity".[30] The triage theory hypothesizes that, even in the absence of overt clinical symptoms, insidious changes could still be occurring that lead to an increased risk of diseases associated with aging.[31] Today, micronutrient deficiencies, such as hypovitaminosis D, are still commonplace. Vitamin D's effects on calcium homeostasis are acutely more important for survival than its effects on the immune system. Therefore, according to the triage theory, vitamin D deficient individuals may be suffering from subclinical maladies that can contribute to suboptimal immune function.

Serum concentration of $25(OH)D_3$ is considered the best clinical indicator of vitamin D status, because it reflects the amount of vitamin D from both the diet and endogenous production over the preceding 1-3 weeks.[9, 29] The most commonly used serum $25(OH)D_3$ concentrations for diagnosing levels of hypovitaminosis D are <20 ng/mL (<50 nmol/L, supported by the Institute of Medicine) for deficiency, <30 ng/mL (<75 nmol/L) for insufficiency, and ≥30 ng/mL (≥75 nmol/L) for sufficiency.[9, 27, 29, 32] Nutrition professionals commonly observe a rise in serum $25(OH)D_3$ of 0.24–0.48 ng/mL (0.6–1.2 nmol/L) for every additional daily input of 40 international units (IU) of vitamin D_3 in adults.[33] By that rationale, one would have to consume 2,500-5,000 IU per day (d) in order to achieve a sufficient vitamin D status of serum $25(OH)D_3$ 30 ng/mL.

Interestingly, some researchers argue that serum concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ are so strongly positively correlated that plasma $24,25(OH)_2D_3$ could be just as good of a marker of vitamin D status as plasma $25(OH)D_3$.[34-36] $24,25(OH)_2D_3$ is the major catabolite of $25(OH)D_3$ and accounts for 10-15% of $25(OH)D_3$ serum concentration. New evidence has suggested that it may not be as biologically inactive as once thought and could play a role in embryogenesis, cartilage development, and fracture repair.[34, 35] In a six-week

intervention trial with young, healthy adults (BMI and baseline vitamin D status not reported), Wagner *et al*. found a significant inverse correlation between the change in serum $25(OH)D_3$ concentration and the ratio of initial plasma $24,25(OH)_2D_3$ to $25(OH)D_3$.[35] Therefore, they suggest that the baseline ratio of plasma $24,25(OH)_2D_3$ to $25(OH)D_3$ could serve as an index of vitamin D_3 clearance and predictor of plasma response to supplementation. However, serum $25(OH)D_3$ is still the most well-studied and predominantly-used indicator of vitamin D status.

In November 2010, the Institute of Medicine (IOM) updated the recommended dietary allowance (RDA) for vitamin D intake to 600 IU/d in adults under 70 years old and the upper level (UL) intake to 4,000 IU/d.[37] Vitamin D toxicity [serum $25(OH)D_3$ concentrations exceeding 150 ng/mL or 375 nmol/L] can result in hypercalcemia and subsequent calcification of soft tissues, but is usually only achievable via excessive supplementation of ≥10,000 IU per day for several months.[9] Since vitamin D toxicity is so rare and insufficiency is so rampantly associated with various chronic diseases, many researchers would argue that the IOM's new RDA is still too conservative.[5, 6, 11, 33]

Association between obesity and vitamin D deficiency

Evidence from epidemiological studies

Vitamin D repletion doses likely need to be even higher for certain populations, such as the elderly, ethnic minorities, and obese. Many studies have shown an inverse association between circulating vitamin D concentrations and obesity, using body mass index (BMI) and/or percentage body fat (usually measured by dual energy x-ray absorptiometry).[38-42] NHANES 2005-2006 data showed that the risk of vitamin D deficiency was almost double among obese adults compared to normal weight adults (43.9% vs. 26.8%; $P<0.0001$).[43] However, the nature of

this relationship is less clear. Results from randomized controlled trials (RCTs) show little to no evidence of vitamin D supplementation causing weight or fat mass loss in obese subjects.[44-46] Conversely, a growing body of research suggests that obesity leads to an increased risk of vitamin D deficiency. Some attribute poor vitamin D status to the obese population's tendency to consume nutrient-poor diets and obtain inadequate sunlight exposure due to minimal outdoor activity, wearing clothing that provides more coverage, and/or reduced surface-to-volume ratio.[38]

Evidence from supplementation trials

Not only do the obese seem to suffer from poor intake and exposure to vitamin D, they also have a poor response to vitamin D supplementation. Their vitamin D status does not improve as efficiently or effectively as that of their lean counterparts.[47-50] A recent RCT studied the effect of seven different doses of vitamin D (400–4,800 IU/d for 1 year) on serum 25(OH)D_3 levels in vitamin D-deficient, postmenopausal women. They compared changes in vitamin D status of overweight with normal weight women, categorized by BMIs above or below 25 kg/m^2, respectively.[51, 52] Not surprisingly, the serum 25(OH)D_3 response to the low dose groups (400–800 IU/d) was significantly less than that of the medium dose (1,600–2,400 IU/d) and high dose (3,200–4,800 IU/d) groups in all BMI categories. On average, lean women supplemented with ≥400 IU/d reached sufficient vitamin D status [serum 25(OH)D_3 ≥30 ng/mL] after 1 year, whereas obese women (BMI ≥30 kg/m^2) appeared to require >1,600 IU/d. Interestingly, normal weight women showed a significantly higher response than overweight and obese women (mean difference of 7.1 ng/mL or 17.7 nmol/L, $P < 0.001$) in all dose groups.[51] This study clearly demonstrates that the degree of response to vitamin D supplementation is dependent on body

weight and that obese individuals may require doses of vitamin D that are higher than those recommended for the general population to achieve sufficiency.

Sequestration hypothesis

Along with inadequate intake and sunlight exposure, several other mechanisms have been proposed to explain how obesity might contribute to poor vitamin D status. Some propose that obese individuals have impaired ability to convert pre-vitamin D to vitamin D_3 in their skin or have altered PTH physiology that leads to elevated $1,25(OH)_2D_3$ and subsequently reduced hepatic synthesis of $25(OH)D_3$.[38, 53] In 2000, Wortsman *et al*. proposed, what is now the most commonly cited hypothesis, that obese individuals have more adipose tissue that can sequester this fat-soluble vitamin, resulting in lower serum $25(OH)D_3$.[54] This conclusion was based solely on the observation that obese subjects had significantly lower *serum* vitamin D_2 (ergocalciferol) and $25(OH)D_3$ concentrations than lean controls, 24 hours after one 50,000 IU oral dose of vitamin D_2. But the obese subjects had significantly lower serum vitamin $25(OH)D_3$ levels at baseline. The researchers did not adjust for baseline vitamin D status or measure levels of enzymatic expression, degradation products, or metabolites in adipose tissue. Therefore, their sequestration hypothesis was based on speculation rather than objective measurements.

After developing a liquid chromatography-tandem mass spectrometry (LC/MS) method to measure vitamin D in serum and fat tissue of morbidly obese, vitamin D-insufficient adults, Blum *et al*. found that vitamin D_3 is, in fact, present in adipose tissue.[55] Although this study has since been cited for finding an inverse association between body weight and vitamin D_3 concentrations in both serum *and* AT, many authors fail to mention that these correlations were not statistically significant. Even so, while an inverse association between body weight and

serum vitamin D_3 could support the sequestration hypothesis, an inverse association between body weight and adipose tissue vitamin D_3 does not. Perhaps these subjects simply have low total body vitamin D_3. However, this study is limited by its cross-sectional design, lack of non-obese controls, and failure to measure level of adiposity and serum concentrations of the prominent clinical marker, $25(OH)D_3$. This study confirmed the theory that vitamin D_3 can be stored in AT, but cannot provide any definitive arguments for or against the sequestration hypothesis due to its limitations and statistically weak results.

Lin *et al*. further supported the sequestration hypothesis by tracking serum $25(OH)D_3$ concentrations of obese adults after roux-en Y gastric bypass (RYGB) surgery.[56] They found that that serum $25(OH)D_3$ concentrations increased in the first month along with significant adiposity loss, suggesting that the initial reduction in adiposity led to a release of vitamin D from AT into circulation. This weak assumption lacks the support of adipose tissue data. Additionally, as significant losses of adiposity continued, vitamin D status also gradually decreased for the remaining 23 months of the study period.

Weight loss studies that oppose the sequestration hypothesis

Conversely, Pramyothin *et al*. found no significant initial increase in serum $25(OH)D_3$ immediately following RYGB surgery and subjects remained insufficient throughout the first post-operative year, despite achieving major weight loss and being supplemented with at least 2,500 IU/d vitamin D_2.[57] They also analyzed vitamin D content of discarded abdominal subcutaneous AT taken at the time of RYGB surgery in 11 morbidly obese subjects. No significant correlation was found between baseline AT vitamin D content and change in serum $25(OH)D_3$, suggesting that AT does not release excessive amounts of vitamin D into circulation

during bariatric surgery-induced fat mass loss. Additionally, they found no statistically significant correlation between AT vitamin D content and body weight, BMI, or serum $25(OH)D_3$. While subjects with greater obesity may not necessarily exhibit greater AT vitamin D *concentrations*, they may store a greater amount of overall AT vitamin D due to their greater fat mass. Unfortunately, this study did not account for fat mass. Hence, it remains unclear whether or not these findings support the sequestration hypothesis. This study has several other limitations: (1) samples were taken throughout the year with variable sun exposure, (2) subjects had baseline vitamin D status that ranged from very deficient to sufficient, (3) subjects had varying vitamin D supplementation regimens, and (4) follow-up AT samples were not collected. This study gives us more insight as to how various forms of vitamin D are distributed throughout the body of obese individuals, but a more formal, randomized trial is needed.

If the sequestration hypothesis was valid, then these post-RYGB subjects would be expected to release large amounts of sequestered vitamin D from AT into circulation and progressively improve their vitamin D status as fat mass is lost. However, this is not always the case. Vitamin D deficiency following RYGB surgery is more likely a result of malabsorption related to bypassing the stomach and intestinal absorption sites along with poor mixing of bile salts.[58] Also, restricted dietary intake and food intolerances that commonly follow RYGB could also contribute to vitamin D deficiency.

Moderate non-surgical weight loss also does not appear to affect serum $25(OH)D_3$ concentrations. Despite 12 weeks of energy restriction resulting in 6% total body weight loss and 13% total fat loss, Piccolo *et al*. found that neither AT nor serum $25(OH)D_3$ concentrations significantly changed in overweight adults, most of whom were vitamin D-deficient at baseline.[59] There was a significant positive association between subcutaneous AT $25(OH)D_3$ concentration

and serum 25(OH)D_3 concentration at baseline and post-intervention, suggesting that vitamin D in blood and AT are in equilibrium rather than disproportionately distributed when vitamin D intake remains unchanged. This study provides human evidence that adipose 25(OH)D_3 does not likely contribute to serum 25(OH)D_3 with moderate weight loss alone. However, it would have been beneficial to measure the main storage form, vitamin D_3, in adipose tissue.

Tissue distribution studies that oppose the sequestration hypothesis

Drincic *et al*. further challenged the sequestration hypothesis by showing that volumetric dilution best explains low vitamin D status in obese individuals.[53] When they used a hyperbolic model (which represents the relationship between concentration and volume, or volumetric dilution, more precisely than linear models) to analyze serum 25(OH)D_3 against total body weight, the differences between obese and normal individuals disappeared. They argue that body weight predicts vitamin D status better than BMI, because it more fully captures inter-individual variation in body size, and better than fat mass, because 25(OH)D_3 is widely distributed in non-adipose tissue. They propose that a passive diffusion equilibrium exists between serum cholecalciferol, AT cholecalciferol, and hepatic cholecalciferol destined for 25-hydroxylation. Based on this simple passive diffusion model, a heavier person would exhibit a lower response in total body 25(OH)D_3 and vitamin D_3 than a lighter person, even if they had similar BMIs, when given the same vitamin D dose, simply due to dilution.

Heaney *et al*. investigated the amount, type, and tissue distribution of vitamin D in pigs given a typical adult dose of 2,000 IU/d.[60] They found that 65% of total body vitamin D was present as native vitamin D_3 and 35% as 25(OH)D_3. Of total vitamin D_3, 73% was stored in fat, 16% in muscle, 3% in serum, 2% in liver, and 6% in all other tissues. Conversely, 25(OH)D_3 was

more evenly distributed throughout the body; 34% in fat, 21% in muscle, 30% in serum, 4% in liver, and 11% in all other tissues. This reinforces the notion that, at common vitamin D intake levels, $25(OH)D_3$ is the main circulating form and vitamin D_3 is the main storage form. 2,000 IU/d is a level similar to vitamin D utilization and therefore results in minimal storage. So, two pigs were given an additional single dose of 60,000 IU vitamin D_3 (on top of the 2,000 IU/d) 10-17 days before sacrifice and had substantially higher serum and fat levels of vitamin D than the two control pigs (only given 2,000 IU/d). These superdosed pigs had very different fat concentrations of vitamin D from each other due to their differences in body size, but similar levels of absolute vitamin D.[60] This supports the notion that differences in AT vitamin D concentrations among various body weights can be explained by volumetric dilution rather than sequestration.

In vitamin D-deficient human adults (BMI not reported) supplemented with 4,000–6,400 IU/d vitamin D_3, Heaney *et al*. found that, initially, serum $25(OH)D_3$ concentrations steeply increased while serum vitamin D_3 concentrations remained mostly unchanged.[61] This suggests that at deficient states, any incoming vitamin D is almost immediately 25-hydroxylated and almost none was spared for storage. Increases in serum $25(OH)D_3$ concentrations then slowed down once it reached 40 ng/mL (100 nmol/L). At this time, serum vitamin D_3 concentrations were about 5.8 ng/mL (15 nmol/L) and started increasing more rapidly, suggesting that hepatic 25-hydroxylase became saturated and serum vitamin D_3 began to rise to allow for fat storage.

In summary, the existing literature suggests that circulating $25(OH)D_3$ is a substrate reservoir for conversion into the active hormone that carries out essential metabolic processes. Therefore, plasma $25(OH)D_3$ sufficiency takes precedence over vitamin D storage. Individuals with a poor vitamin D status cannot afford to store the small amount of available vitamin D_3 in

AT. Only when plasma 25(OH)D_3 adequacy is reached should 25-hydroxylation downregulate, allowing some vitamin D_3 to remain in blood and some to be stored in AT. The amount of vitamin D_3 destined for storage may be affected more by plasma 25(OH)D_3 status than level of adiposity. Thus, it is plausible that the obese do not sequester a disproportionately greater amount of vitamin D_3 in adipose tissue than the lean. Measuring both 25(OH)D_3 and vitamin D_3 in blood and adipose tissue in a randomized intervention trial would help elucidate how metabolites of vitamin D are actually distributed in overweight human adults.

Degradation hypothesis

Association between obesity and adipose tissue inflammation

What does differ between the obese and lean is the ability to raise plasma 25(OH)D_3 levels. We are investigating an alternative hypothesis, counter to sequestration, to explain the association between obesity and vitamin D deficiency. We propose that adipose tissue inflammation is the missing link. More specifically, increased adiposity leads to infiltration of activated immune cells that release pro-inflammatory cytokines, which increases degradation and subsequent excretion of vitamin D.[17, 38] Therefore, this degradation hypothesis is based, in part, on the strong association between AT inflammation and obesity.[62-65]

In the past, many considered AT to just passively store energy in the form of triglycerides and other lipids. It is now recognized that AT is an active endocrine organ containing various immune cells that secrete pro-inflammatory cytokines [e.g. tumor necrosis factor-α (TNFα), interleukin (IL)-6, IL-1β, IL-8, interferon-γ (IFNγ), C-reactive protein (CRP)], anti-inflammatory cytokines (e.g. IL-10, adiponectin), and other cytokines that aid in inflammatory processes [e.g. monocyte chemoattractant protein-1 (MCP-1), leptin].[62, 63] Several studies have found that excess

AT promotes infiltration of activated leukocytes, particularly macrophages and T-cells, into adipose tissue that produce pro-inflammatory cytokines.[64-68] As more T- and B-cells become activated, they express higher levels of VDR. Concurrently, most activated immune cells also express 1α-hydroxylase, enabling them to convert circulating $25(OH)D_3$ to the active hormone, $1,25(OH)_2D_3$, that binds to VDR for local use. $1,25(OH)_2D_3$ appears to be a potent regulator of immune cell differentiation, proliferation, and activation that affects both innate and adaptive immunity.[7] Unlike renal 1α-hydroxylase, 1α-hydroxylase produced by macrophages is upregulated by immune stimuli such as IFNγ and is not suppressed by elevated $1,25(OH)_2D_3$.[13] In response to high $1,25(OH)_2D_3$ concentrations, there is some evidence that activated immune cells may upregulate 24-hydroxylase expression[7, 69, 70] in order to inactivate the vitamin D hormone, leading to increased degradation and excretion of vitamin D as $1,24,25(OH)_3D_3$.[7, 13, 21, 71] Although 24-hydroxylase has a higher affinity for $1,25(OH)_2D_3$, it also catabolizes $25(OH)D_3$ to $24,25(OH)_2D_3$, thus, reducing the pool of $25(OH)D_3$ available for 1α-hydroxylation.[13] In summary, we hypothesize that excess adiposity promotes infiltration of activated immune cells that increase AT inflammation and vitamin D degradation, thus, contributing to vitamin D deficiency in obese populations.

Expression of vitamin D hydroxylases in adipose tissue

In 1997, Akeno *et al*. injected mice with $1,25(OH)_2D_3$ and found 24-hydroxylase mRNA not only in the kidneys, but also in the intestines to a great extent and the skin, thymus, bone, lung, testis, spleen, pancreas, and heart to a lesser extent.[71] This study showed that 24-hydroxylase may regulate $1,25(OH)_2D_3$ activity in extra-renal tissues, but it did not investigate adipose tissue. Later, Li *et al*. detected 1α-hydroxylase and 24-hydroxylase in rodent adipocytes

treated with $25(OH)D_3$.[17] Wamberg *et al*. quantified expression of vitamin-D metabolizing enzymes in the adipose tissue of vitamin D-deficient obese and lean women. They found that the obese subjects had significantly lower AT expression of 25-hydroxylase and 1α-hydroxylase than the lean subjects, yet the level of 24-hydroxylase expression did not differ between groups.[72] However, this study is limited by its cross-sectional design. The obese women had significantly lower baseline serum $25(OH)D_3$ than the lean and 24-hydroxylase expression is upregulated only when vitamin D levels are high enough to produce excess $1,25(OH)_2D_3$. An intervention trial that measures the change in vitamin D status and degradation products in response to repletion would better display how enzymatic metabolism of vitamin D may differ in the obese population.

Association between vitamin D and adipose tissue inflammation

Not only does AT inflammation seem to worsen vitamin D status due to degradation, but poor vitamin D status may in turn prevent effectiveness of anti-inflammatory mechanisms. However, some *in vitro* experiments have shown that obese human adipocytes treated with $1,25(OH)_2D_3$ increase expression of pro-inflammatory cytokines and/or inhibit expression of anti-inflammatory cytokines[73-75], whereas others have found that $1,25(OH)_2D_3$ treatment inhibits pro-inflammatory cytokine expression[26, 76] and/or promotes anti-inflammatory cytokine expression.[77] These differential effects of $1,25(OH)_2D_3$ highlight the complexities of studying vitamin D in the context of inflammation. In general, results from *in vitro*, observational, and intervention studies strongly suggest that the vitamin D hormone, $1,25(OH)_2D_3$, plays an important role in inflammatory processes via VDR. This is supported by the findings that (1) VDR and 1α-hydroxylase is expressed in various immune cell types[4, 5], (2) vitamin D deficiency

is associated with risk for chronic inflammatory conditions[28, 29], and (3) there is an inverse association between vitamin D status and systemic inflammation, independent of BMI.[78-80]

Summary

We propose that the association between obesity and vitamin D deficiency can be partially explained by elevated degradation, rather than exceptionally high sequestration in adipose tissue. Our randomized intervention pilot study examines the effect of daily vitamin D_3 supplementation for three months on vitamin D status, storage, and degradation in vitamin D-deficient overweight adults. Few studies have examined the relationship between the vitamin D contents of both serum and fat in humans. Additionally, even fewer studies have examined the effect of AT inflammation on the degradation of plasma $25(OH)D_3$ into $24,25(OH)_2D_3$. Other limitations of previous studies include supplementing vitamin D in addition to the confounding effect of calcium, having a cross-sectional design, using *in vitro* or animal models, and enrolling participants with any baseline vitamin D status. We will improve upon these common limitations by directly measuring vitamin D metabolites in both the blood and AT of human adults at two time-points, measuring AT inflammation, supplementing only with vitamin D_3, and enrolling subjects with baseline vitamin D deficiency.

Implications of research

Chronic, subclinical vitamin D deficiency is highly prevalent and associated with detrimental health consequences. Obese individuals appear to be at greater risk of developing vitamin D deficiency than lean individuals. By measuring the amount of vitamin D degradation product [$24,25(OH)_2D_3$] and storage form (vitamin D_3) in blood, we can determine the

differential contribution of degradation or sequestration to low circulating vitamin D levels in obese populations. Results from this pilot study will help researchers design larger randomized controlled trials that can further elucidate biological pathways affecting the clinical marker of vitamin D status, serum $25(OH)D_3$. These studies can help determine why the obese population is disproportionately affected by vitamin D deficiency.

Aims and hypotheses

Objective: To investigate how plasma levels of $25(OH)D_3$, the main clinical marker of vitamin D status, respond to three months of daily vitamin D_3 supplementation. Our overall hypothesis is that a less substantial increase in $25(OH)D_3$ is due to a greater increase in vitamin D_3 and $24,25(OH)_2D_3$, reflecting that a greater portion of the supplemented vitamin D_3 enters pathways related to storage and degradation of vitamin D, respectively.

- Primary Specific Aim 1: To investigate the relationship between the change in plasma levels of $25(OH)D_3$ and the change in plasma levels of vitamin D_3 in response to oral supplementation. We hypothesize that the change in plasma $25(OH)D_3$ is inversely associated with the change in plasma vitamin D_3.
- Primary Specific Aim 2: To investigate the relationship between the change in plasma levels of $25(OH)D_3$ and the change in plasma levels of the major degradation metabolite, $24,25(OH)_2D_3$, in response to oral supplementation. We hypothesize that the change in plasma $25(OH)D_3$ is inversely associated with the change in plasma $24,25(OH)_2D_3$.
- Primary Specific Aim 3: To investigate the relationship between the change in plasma levels of $25(OH)D_3$ and the change in plasma levels of vitamin D_3 and $24,25(OH)_2D_3$ in response to oral supplementation. We hypothesize that the change in plasma $25(OH)D_3$ is

inversely associated with both the change in plasma vitamin D_3 and plasma $24,25(OH)_2D_3$.

We are also interested in exploring how obesity and obesity-related adipose tissue inflammation affect vitamin D status.

- Secondary Specific Aim 1: To investigate the relationship between BMI and changes in plasma levels of $25(OH)D_3$ and vitamin D_3. We hypothesize that BMI is inversely associated with both the change in plasma levels of $25(OH)D_3$ and vitamin D_3.
- Secondary Specific Aim 2: To investigate the relationship between adipose tissue expression of the key pro-inflammatory cytokine, TNFα, and changes in plasma levels of $25(OH)D_3$ and $24,25(OH)_2D_3$. We hypothesize that expression of TNFα is positively associated with the change in plasma levels of $24,25(OH)_2D_3$ and inversely associated with the change in plasma levels of $25(OH)D_3$.

METHODS

Subjects

Potential subjects were recruited via flyer postings, the Craigslist web site, newspaper advertisements, and grocery store newsletters throughout the greater Puget Sound area. Interested participants then underwent a telephone screening interview to assess eligibility. **Table 1** lists the inclusion and exclusion criteria.

Table 1. Inclusion and exclusion criteria of the study population.[81]

Inclusion criteria:	**Exclusion criteria:**
• Age: 18-65 years; • BMI ≥25 kg/m^2; • Plasma 25(OH)D_3 between 7 and 20 ng/mL • Weight stable to within 10 pounds for 6 months prior to entering the study, and within 30 pounds of their lifetime maximum weight (excluding pregnancy); • Ability to be admitted for 6.5 hours on three occasions to the FHCRC Prevention Center, • Ability to provide informed written consent; • Willingness to take vitamin D_3 capsules daily for 6 months • Willingness to have blood drawn and undergo adipose tissue biopsies on three occasions	• Chronic disease such as thyroid disease, liver disease, or kidney disease; • Diabetes mellitus, or fasting glucose >125 mg/dL; • Chronic inflammatory condition such as autoimmune disease or inflammatory bowel disease; • Malabsorption syndromes (untreated celiac disease; condition after stomach or intestinal resection); • Current or recent (within one month) chronic intake of medications likely to interfere with study endpoints [(insulin, antidiabetics, anabolic steroids, glucocorticosteroids, statins, blood thinners (warfarin, aspirin), non-steroidal anti-inflammatory drugs (if daily)]; • Current or recent (within 3 months) intake of vitamin D in excess of 600 IU/day; • Anemia, recent history (within 3 months) of anemia; recent (within 3 months) blood donation; recent (within 3 months) participation in another study that involved blood draws; or plans to participate in other research that involved blood draws during the study period; • Pregnancy in the last 6 months, plans to become pregnant during the study period, or current breastfeeding. • Allergy to local anesthetics

Screening Visit

Potentially eligible subjects were then invited to the Prevention Center at Fred Hutchinson Cancer Research Center (FHCRC) for a screening visit in the morning after having fasted for at least 12 hours. The study coordinator reviewed consent forms, tested for pregnancy (if applicable), measured anthropometrics (weight, height, waist and hip circumference), and administered a standardized medical and medication history questionnaire. Clinicians then took

resting blood pressure and pulse measures, and collected 10 mL of fasting blood to measure serum 25(OH)D_3 and glucose levels, which must have been 7–20 ng/mL and <125 mg/dL, respectively, to enroll in the study.

Study Design

Eligible subjects were then invited back to the Prevention Center for their baseline clinic visit, and randomized to receive either 2,000 IU or 4,000 IU (double-blinded) of vitamin D_3 per day for three months. They were admitted for a total of two clinic visits, at baseline and three months. We attempted to have subjects complete the study over the winter months (October-May) to prevent confounding by sun exposure. In the winter months, solar exposure in the Pacific Northwest is insufficient to contribute meaningfully to vitamin D status. Further, subjects were asked not to take any additional vitamin D or multivitamin supplements, and not to visit sun-tanning salons.

Vitamin D supplement

Subjects were asked to take capsules providing either 2,000 IU or 4,000 IU vitamin D_3 per day for three months. Subjects received a monthly supply of capsules in the original manufacturer's packaging. Any language on the container providing information about the vitamin D dose was removed or covered. They were asked to keep a daily log of their intake and to return all bottles for counting of capsules that were not taken. Capsules were purchased from J.R. Carlson Laboratories (Arlington Heights, IL).

Our choice for a daily dose of 2,000–4,000 IU of vitamin D_3 per day is based on published findings on the efficacy and safety in obese and non-obese populations. Vieth *et al*.

used a dose of 4,000 IU of vitamin D_3 for 5 months, and showed that this dose was effective in raising plasma 25(OH)D_3 concentrations in almost all subjects to >30 ng/mL within 3 months, without any subject developing hypercalcemia over the 5-month study period.[82] In this study, plasma 25(OH)D_3 concentrations reached a plateau at 3 months, which provides our rationale for conducting the second clinic visit at that time point.[82] Another consideration in choosing this particular dose was that Heaney *et al*. recently showed that 25-hydroxylation of newly ingested/formed vitamin D_3 is almost quantitative up to a plasma vitamin D_3 concentration of ~5.8 ng/mL, which is equivalent to a daily intake of ~2,000 IU.[61] Only an intake in excess of 2,000 IU can therefore be expected to raise plasma vitamin D_3 concentrations appreciably, which is likely needed to increased adipose tissue storage of both vitamin D_3 and 25(OH)D_3. This has been hypothesized based on experiments done in rodents and pigs[60], but has never been shown to be the case in humans.

Using 2,000 IU vs. 4,000 IU per day in our study that includes adipose tissue measurements of both vitamin D_3 and 25(OH)D_3 in obese individuals will allow us to address the question whether vitamin D is indeed stored in appreciable amounts in human adipose tissue, whether vitamin D_3 or 25(OH)D_3 is the predominant storage form, and which daily dose is needed to induce appreciable adipose tissue storage.

Clinic Visits

Subjects completed two clinic visits: one at baseline and one after three months. At each clinic visit, subjects were asked to come to the FHCRC Prevention Center at 7:15 AM after a 12-hour fast. Study coordinators took body measurements, asked about any medical or medication

changes, and tested for pregnancy (if applicable). Clinicians recorded vitals, drew fasting blood, and performed an adipose tissue biopsy.

Fasting blood draw

A nurse or phlebotomist drew 53 mL of fasting blood using an intravenous catheter from the forearm vein into chilled EDTA tubes. The EDTA-plasma was immediately spun in a chilled centrifuge, so the plasma layer could be aliquoted and stored at -70°C.

Adipose tissue biopsy

After the fasting blood draw, the physician assistant extracted 1 g of subcutaneous adipose tissue, approximately two inches lateral of the umbilicus, by needle aspiration biopsy. Approximately 200 mg of this sample is snap frozen in an -80°C freezer for gene expression analysis and 250 mg for measurement of adipose tissue concentrations of vitamin D_3 and $25(OH)D_3$.

Laboratory Methods

Measurements of vitamin D and metabolites in plasma and adipose tissue

Fasting plasma concentrations of vitamin D_3 (cholecalciferol), $25(OH)D_3$, and $24,25(OH)_2D_3$ were determined by plasma high performance liquid chromatography-tandem mass spectrometry (LC/MS/MS) on a Waters Xevo mass spectrometer (Waters Corp., Milford, MA). Plasma $25(OH)D_3$ concentration was measured for clinical care and standardized using National Institute of Standards and Technology control material SRM-972.[83] This assay has an inter-assay coefficient of variation of 5.4% for $25(OH)D_3$ and 10.9% for $24,25(OH)_2D_3$,

substantially lower than other published methods.[84, 85] We added analysis of vitamin D_3 to the assay for this study by including this analyte in the calibrators and using a tri-deuterated analog as an internal standard to reduce the variability due to ion suppression and extraction efficiency.[81]

To quantify vitamin D_3 and 25(OH)D_3 in tissues, 150-250 mg of frozen tissue was pulverized using a mortar and pestle on a bed of dry ice. The tissue was scraped into a glass tube and extracted overnight with 2 ml of chloroform/methanol (2:1) on a rotary mixer. The tubes were then centrifuged at 3000 x g and the aqueous layer was removed and re-extracted (2 h). The organic layer was removed and pooled with the first extract, and dried under nitrogen. The residue was reconstituted in 200 μL acetonitrile, derivatized, and analyzed as for plasma.[86]

Gene expression analysis of inflammatory cytokines in adipose tissue

A research associate extracted total RNA from whole AT using RNeasy Lipid Tissue kit (Qiagen, Hilden, Germany) and quantified using RiboGreen (Invitrogen Corp., Carlsbad, CA). Then, cDNA synthesis was carried out on ~1 mg of total RNA using the RETROscript® Kit (Ambion/Applied Biosystems, Austin, TX) and PCR performed using pre-designed TaqMan® Gene Expression Assays (Applied Biosystems, Austin, TX) on an ABI Prism® 7900HT Sequence Detection System. The gene targets were TNFα, IL-6, and IL-1β. We measured β-glucuronidase and 18s rRNA as potential housekeeping genes as both of these are relatively stably expressed in adipose tissue across different conditions.[87] Using the method proposed by Vandesompele et al.[88], we used Genorm to assess the variability of these genes in our samples, and used one or both to calculate a normalization factor that was applied to all target genes.

Calculations and Statistics

All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, version 22.0) in consultation with the University of Washington's Biostatistics Department. We assessed the distribution of all variables of interest by conducting Shapiro-Wilk tests and by plotting normal plots and histograms. Variables that did not appear consistent with a normal distribution was log-transferred prior to statistical analyses. For repeated measures analysis of variance (RM-ANOVA) and multiple linear regression analyses, we assessed the distribution of the residuals, and log-transformed the variable if the residuals were not consistent with a normal distribution.

For the primary specific analyses, we conducted multiple linear regression analyses, with the change in $25(OH)D_3$ between baseline and the 3-month time point as the dependent variable and the change in vitamin D_3 between baseline and the 3-month visit (primary specific aim 1), the change in $24,25(OH)_2D_3$ between baseline and the 3-month visit (primary specific aim 2), or both (primary specific aim 3) as independent variables. We included the vitamin D_3 dose administered as a covariate in all models.

Similarly, we conducted multiple linear regression analyses to address the secondary specific aims. Including the change in $25(OH)D_3$ or vitamin D_3 as the dependent variable and baseline BMI and vitamin D_3 dose as independent variables, we assessed whether BMI is a determinant of the changes in $25(OH)D_3$ and vitamin D_3, when adjusting for the vitamin D_3 dose administered (secondary specific aim 1). Including the change in $25(OH)D_3$ or $24,25(OH)_2D_3$ as the dependent variable and the expression of tumor necrosis factor alpha (TNFα) in subcutaneous adipose tissue at baseline and vitamin D_3 dose as independent variables, we assessed whether adipose tissue TNFα as a measure of adipose tissue inflammation is a determinant of the changes

in $25(OH)D_3$ and $24,25(OH)_2D_3$, when adjusting for the vitamin D_3 dose administered (secondary specific aim 2).

We conducted descriptive analyses by computing means and standard deviations for all variables at baseline and the 3-month visit for normally distributed variables. For non-normally distributed variables, we computed medians and the range. Plasma concentrations of vitamin D_3, $25(OH)D_3$, $1,25(OH)_2D_3$, $24,25(OH)_2D_3$ and changes in adipose tissue concentrations of vitamin D_3 and $25(OH)D_3$ at baseline and the 3-month visit were compared using RM-ANOVA, which included the dose of vitamin D_3 administered as a covariate. P-values less than 0.05 were considered significant.

RESULTS

Baseline characteristics

A total of 18 adults enrolled in our study between October 2011–June 2012 and October 2012–June 2013. Of these enrolled subjects, one dropped out before the baseline visit and two dropped out before the 3-month visit. Therefore, 15 adults completed both the baseline and 3-month visits (n=8 given 2,000 IU/d, n=7 given 4,000 IU/d). The 2,000 IU/d group consisted of six Caucasians and two African-Americans. The 4,000 IU/d group consisted of three Caucasians, two African-Americans, one Asian, and one Hispanic.

Table 2. Baseline characteristics of the study population*.

	All (n=15)	2,000 IU/d group (n=8)	4,000 IU/d group (n=7)	P-value
Age (years)	34.9 ± 10.1	30.3 ± 9.5	40.3 ± 8.3	**0.050**
Gender (male / female)	4 / 11	2 / 6	2 / 5	0.880
Body weight (kg)	95.3 ± 18.6	91.4 ± 19.3	99.7 ± 18.3	0.410
Body mass index (kg/m^2)	34.1 ± 6.7	31.9 ± 5.3	36.6 ± 7.6	0.178
Plasma fasting 25(OH)D_3 (ng/mL)	12.7 ± 4.0	12.5 ± 3.7	12.9 ± 4.6	0.852
Plasma fasting vitamin D_3 (ng/mL)	0.1 (0.1–1.1)	0.1 (0.1–0.4)	0.1 (0.1–1.1)	0.457
Plasma fasting 24,25$(OH)_2D_3$ (ng/mL)	1.0 ± 0.6	1.0 ± 0.4	1.0 ± 0.7	0.959
Adipose tissue 25(OH)D_3 (pg/mg)	1.1 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	0.979
Adipose tissue vitamin D_3 (pg/mg)	10.0 ± 6.1	10.8 ± 4.6	9.1 ± 7.8	0.620
Adipose tissue 24,25$(OH)_2D_3$ (pg/mg)	0.09 (0.05–0.11)	0.09 (0.05–0.11)	0.09 (0.09–0.11)	0.183

* Data are means ± standard deviations or medians (range). P-values for normally distributed variables are based on independent samples t-tests, comparing the two dose groups (2,000 IU/d and 4,000 IU/d). P-values for non-normally distributed variables [gender, plasma fasting vitamin D_3, adipose tissue 24,25$(OH)_2D_3$] are based on Mann Whitney U-tests.
Abbreviations: 25$(OH)D_3$ = 25-hydroxyvitamin D_3, 24,25$(OH)_2D_3$ = 24,25-dihydroxyvitamin D_3

When comparing baseline characteristics between the two dose groups (**Table 2**), the only significant difference was age, by 10 years ($p = 0.05$). There were no statistically significant differences in gender distribution, body weight, or body mass index (BMI). Although, it is notable that baseline BMI was about 5 kg/m^2 higher in the 4,000 IU/d group than in the 2,000 IU/d group ($p = 0.18$). The average BMI of all subjects was 34.1 ± 6.7 kg/m^2. Plasma

concentrations of $25(OH)D_3$, vitamin D_3, and $24,25(OH)_2D_3$ were very similar between groups (not statistically different). The mean baseline plasma $25(OH)D_3$ concentration for the whole study population was 12.7 ± 4.0 ng/mL. Adipose tissue (AT) concentrations of $25(OH)D_3$, vitamin D_3, and $24,25(OH)_2D_3$ were also very similar between groups and not statistically different.

Post-intervention changes

Plasma vitamin D metabolites

After three months of daily vitamin D_3 supplementation of either 2,000 IU/d or 4,000 IU/d, body weight remained relatively stable with no significant difference over time or between groups (**Table 3**). Plasma $25(OH)D_3$ concentrations significantly increased over time ($p < 0.001$), and increased significantly more in the 4,000 IU/d group than in the 2,000 IU/d group ($p = 0.04$). All 15 study subjects achieved plasma $25(OH)D_3$ concentrations of at least 20 ng/mL after three months of supplementation. Furthermore, all seven subjects given 4,000 IU/d achieved vitamin D sufficiency [plasma $25(OH)D_3$ concentration ≥30 ng/mL], whereas only four of the eight subjects given 2,000 IU/d reached this level. Plasma $25(OH)D_3$ concentrations rose by two- to three-fold from baseline. Vitamin D_3 plasma concentration increased significantly over time ($p < 0.001$), and increased significantly more in the 4,000 IU/d group than in the 2,000 IU/d group ($p = 0.006$). To further explore the relationship between plasma $25(OH)D_3$ and vitamin D_3, we plotted all of the subjects' plasma $25(OH)D_3$ status against their plasma vitamin D_3 status at both visits (**Figure 1)**. Plasma concentrations of $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ significantly increased over time, but did not differ between groups, although we observed a trend for a greater increase in $24,25(OH)_2D_3$ in the 4,000 IU/d group ($p = 0.09$).

Table 3. Clinical and biochemical characteristics of subjects at baseline and 3 months post-intervention*.

			RM-ANOVA p-value	
	Baseline	**3-month visit**	**Time**	**Time-by-dose**
Body weight (kg)				
2,000 IU/d (n=8)	91.4 ± 19.3	91.4 ± 19.3	0.711	0.752
4,000 IU/d (n=7)	99.7 ± 18.3	100.1 ± 16.2		
Plasma fasting $25(OH)D_3$ (ng/mL)				
2,000 IU/d (n=8)	12.5 ± 3.7	30.4 ± 6.5	<0.001	0.042
4,000 IU/d (n=7)	12.9 ± 4.6	37.9 ± 5.6		
Plasma fasting vitamin D_3 (ng/mL)				
2,000 IU/d (n=8)	0.1 (0.1–0.4)	8.1 (2.5–13.8)	<0.001	0.006
4,000 IU/d (n=7)	0.1 (0.1–1.1)	19.0 (5.5–24.0)		
Plasma fasting $24,25(OH)_2D_3$ (ng/mL)				
2,000 IU/d (n=8)	1.0 ± 0.4	3.8 ± 1.5	<0.001	0.090
4,000 IU/d (n=7)	1.0 ± 0.7	5.1 ± 1.5		
Plasma fasting $1,25(OH)_2D_3$ (pg/mL)				
2,000 IU/d (n=8)	32.1 ± 5.8	40.8 ± 7.3	0.034	0.193
4,000 IU/d (n=7)	35.0 ± 6.1	37.3 ± 8.8		
Adipose tissue $25(OH)D_3$ (pg/mg)				
2,000 IU/d (n=8)	1.1 ± 0.3	2.5 ± 0.8	<0.001	0.013
4,000 IU/d (n=7)	1.1 ± 0.3	3.5 ± 0.3		
Adipose tissue vitamin D_3 (pg/mg)				
2,000 IU/d (n=8)	10.8 ± 4.6	21.3 ± 9.3	<0.001	0.012
4,000 IU/d (n=7)	9.1 ± 7.8	31.8 ± 14.3		
Adipose tissue $24,25(OH)_2D_3$ (pg/mg)				
2,000 IU/d (n=8)	0.09 (0.05–0.11)	0.11 (0.05–0.11)	0.481	0.214
4,000 IU/d (n=7)	0.09 (0.09–0.11)	0.10 (0.09–0.11)		
Adipose tissue TNFα (mRNA copy number/ng total RNA)				
2,000 IU/d (n=8)	1.5 ± 0.5	1.7 ± 0.8	0.409	0.679
4,000 IU/d (n=7)	1.7 ± 0.7	1.7 ± 0.5		
Adipose tissue IL-6 (mRNA copy number/ng total RNA)**				
2,000 IU/d (n=8)	1.4 (0.9–3.6)	1.4 (1.0–2.7)	0.775	0.863
4,000 IU/d (n=7)	1.1 (0.8–6.1)	1.3 (0.8–3.0)		
Adipose tissue IL-1β (mRNA copy number/ng total RNA)**				
2,000 IU/d (n=8)	1.3 (0.4–5.1)	0.6 (0.4–17.5)	0.505	0.964
4,000 IU/d (n=7)	1.3 (0.4–3.5)	1.2 (0.6–1.5)		

* Baseline and 3-month visit data are means ± standard deviations or medians (range). P-values based on RM-ANOVA (repeated measure analysis of variance) tests of within-subjects effect that compare the overall change over time (between the baseline and 3-month visit) and compare the change over time between the two dose groups (2,000 IU/d and 4,000 IU/d).
** Used log-transformed data for RM-ANOVA.
Abbreviations: $25(OH)D_3$ = 25-hydroxyvitamin D_3, $24,25(OH)_2D_3$ = 24,25-dihydroxyvitamin D_3, $1,25(OH)_2D_3$ = 1,25-dihydroxyvitamin D_3, TNFα = tumor necrosis factor-alpha, IL = interleukin.

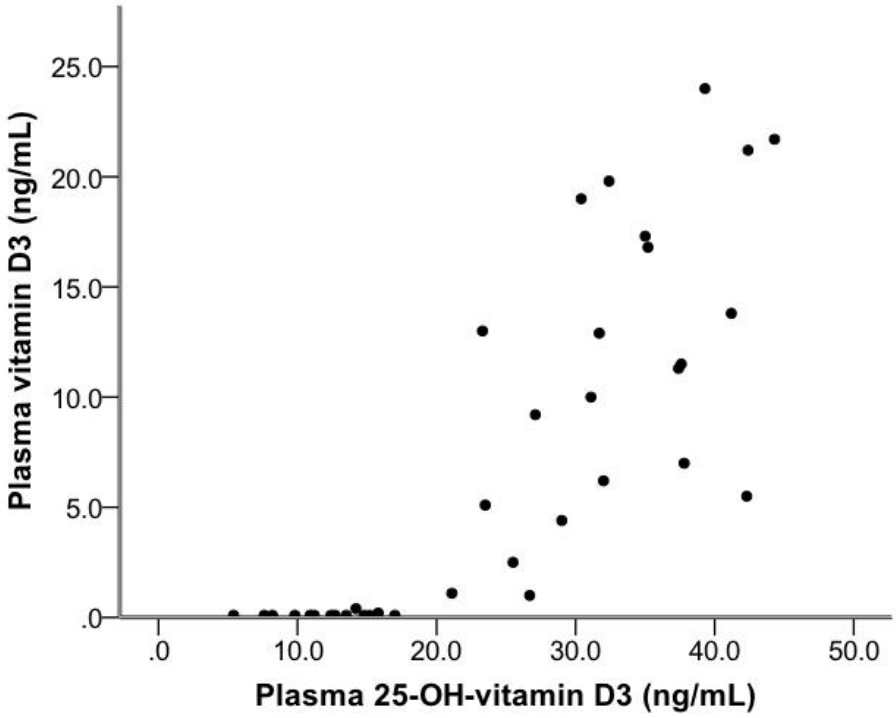

Figure 1. Scatter plot displaying all fifteen study subjects' plasma concentration of 25-hydroxyvitamin D_3 with their plasma concentration of vitamin D_3 at the baseline and three-month visits.

Adipose tissue vitamin D metabolites and inflammatory cytokine expression

Adipose tissue concentrations of 25(OH)D_3 and vitamin D_3 increased significantly over time, and increased significantly more in the 4,000 IU/d group than in the 2,000 IU/d group (Table 3). Both AT 25(OH)D_3 and vitamin D_3 levels rose by about two- to three-fold from baseline. AT concentration of vitamin D_3 was almost 10-fold greater than that of 25(OH)D_3 at baseline and post-intervention. Concentration of AT 24,25$(OH)_2D_3$ was very low, and did not significantly change over time or differ between groups. We also measured gene expression levels of three pro-inflammatory cytokines, TNFα, IL-6, and IL-1β, that are released by immune cells in adipose tissue. None of these inflammatory markers significantly changed over time or differed between groups.

Associations and interrelationships

Change in plasma concentration of 25(OH)D_3

Linear regression analysis showed that the higher vitamin D dose of 4,000 IU/d did increase plasma 25(OH)D_3 concentrations significantly more than the lower dose of 2,000 IU/d (β-coefficient 7.1, 95% CI 0.3–13.9, p = 0.04) (**Table 4**). Therefore, we reported regression data as both unadjusted and adjusted for dosage. Change in plasma 25(OH)D_3 showed no significant association with change in plasma 1,25$(OH)_2D_3$, whether adjusted for dose or not. Change in plasma 25(OH)D_3 concentrations showed a significant positive association with change in plasma 24,25$(OH)_2D_3$ but no association with change in plasma vitamin D_3. However, we also computed another regression model that included change in plasma concentrations of both vitamin D_3 and 24,25$(OH)_2D_3$ as independent variables and was adjusted for dose. In this analysis, the association between changes in plasma 25(OH)D_3 and 24,25$(OH)_2D_3$ remained significantly positive, but the association between changes in plasma 25(OH)D_3 and vitamin D_3 became significantly inverse.

Although all subjects had deficient baseline vitamin D status, their plasma 25(OH)D_3 levels varied greatly, ranging from 5.4–21.1 ng/mL. Therefore, we also investigated the association between baseline plasma 25(OH)D_3 concentration and change in plasma 25(OH)D_3, but no significant association was found, both unadjusted and adjusted for dose.

We also assessed whether measures of obesity (BMI and body weight) or measures of AT inflammation (pro-inflammatory cytokines) were inversely associated with the level of plasma 25(OH)D_3 response. Since there was no significant change in BMI, body weight, or AT expression of TNFα, IL-6, or IL-1β over time or between groups (Table 3), we used their baseline values in our analyses. No significant associations were found between change in

plasma 25(OH)D_3 and baseline values of BMI, body weight, or AT expression of TNFα, IL-6, or IL-1β, both unadjusted and adjusted for dose (Table 4). While not significant, the associations between change in plasma 25(OH)D_3 and baseline AT levels of TNFα, AT IL-6, and AT IL-1β were all positive.

Table 4. Linear regression analyses – Associations between change in plasma 25-hydroxyvitamin D_3 concentration after 3 months of daily vitamin D_3 supplementation and vitamin D dose, changes in plasma vitamin D metabolites, and baseline measures of plasma 25-hyrdoxyvitamin D_3, body weight, and adipose tissue inflammation.

	Unadjusted		Adjusted for Dose	
Independent Variable	**Co-efficient (95% CI)**	**Significance**	**Co-efficient (95% CI)**	**Significance**
Vitamin D_3 dose	7.1 (0.3, 13.9)	**0.042**	-	-
Δ plasma 1,25(OH)$_2$$D_3$ (ng/mL)	-0.2 (-0.6, 0.2)	0.316	-0.1 (-0.5, 0.4)	0.704
Δ plasma 24,25(OH)$_2$$D_3$ (ng/mL)	4.1 (2.5, 5.7)	**<0.001**	3.7 (1.9, 5.4)	**0.001**
Δ plasma vitamin D_3 (ng/mL)	0.3 (-0.2, 0.9)	0.214	-0.04 (-0.8, 0.7)	0.919
Multiple regression				
Δ plasma vitamin D_3 (ng/mL)	-0.2 (-0.6, 0.2)	0.377	-0.4 (-0.8, -0.01)	**0.046**
Δ plasma 24,25(OH)$_2$$D_3$ (ng/mL)	4.5 (2.6, 6.4)	**<0.001**	4.3 (2.6, 5.9)	**<0.001**
Baseline plasma 25(OH)D_3 (ng/mL)	-0.4 (-1.4, 0.5)	0.351	-0.5 (-1.4, 0.4)	0.235
Baseline BMI (kg/m^2)	0.2 (-0.4, 0.8)	0.231	0.04 (-0.6, 0.6)	0.879
Baseline body weight (kg)	0.03 (-0.2, 0.3)	0.783	-0.02 (-0.2, 0.2)	0.856
Baseline adipose tissue TNFα (mRNA copy number/ng RNA)	2.0 (-5.3, 9.4)	0.559	0.9 (-5.8, 7.6)	0.765
Baseline adipose tissue IL-6** (mRNA copy number/ng RNA)	7.8 (-8.8, 24.4)	0.328	9.1 (-5.1, 23,4)	0.188
Baseline adipose tissue IL-1β** (mRNA copy number/ng RNA)	1.6 (-12.3, 15.4)	0.811	2.2 (-10.1, 14.4)	0.709

Dependent variable for all analyses: Change in plasma 25-hydroxyvitamin D_3 (ng/mL)
Coefficients are unstandardized.
**Used log-transformed data.
Abbreviations: 25(OH)D_3 = 25-hydroxyvitamin D_3, 24,25(OH)$_2$$D_3$ = 24,25-dihydroxyvitamin D_3, 1,25(OH)$_2$$D_3$ = 1,25-dihydroxyvitamin D_3, BMI = body mass index, TNFα = tumor necrosis factor-alpha, IL = interleukin, Δ = Change in value from baseline to 3-month visit.

Changes in plasma concentrations of vitamin D_3 and 24,25$(OH)_2D_3$

After three months, the 4,000 IU/d dose increased plasma vitamin D_3 concentrations significantly more than the 2,000 IU/d dose (β-coefficient 8.9, 95% CI 3.1–14.8, p = 0.006) (**Table 5**). However, the higher vitamin D dose did not increase plasma 24,25$(OH)_2D_3$ concentrations significantly more than the lower dose. Changes in plasma concentrations of vitamin D_3 and 24,25$(OH)_2D_3$ were not significantly associated with baseline BMI, body weight, or plasma 25$(OH)D_3$. This lack of associations is preserved even after adjusting for dosage.

There were no significant associations between change in plasma 24,25$(OH)_2D_3$ and baseline AT expression levels of TNFα or IL-1β (Table 5). However, there was a significant positive association between change in plasma 24,25$(OH)_2D_3$ and baseline AT IL-6 after adjusting for dose (β-coefficient 3.0, 95% CI 0.3–5.8, p = 0.03). We attempted to quantify the level of CYP24 gene expression in adipose tissue, but there were no detectable amounts in any subject at any time point.

Association between adipose tissue vitamin D and obesity

Since so few studies have evaluated vitamin D metabolites in the adipose tissue of overweight humans, we also investigated the relationship of BMI or body weight with AT 25$(OH)D_3$ and AT vitamin D_3 (**Table 6**). We found no significant association between change in AT 25$(OH)D_3$ and baseline BMI or body weight. However, change in AT vitamin D_3 showed a significant inverse association with baseline BMI after adjusting for dose (β-coefficient -0.9, 95% CI -1.4 – -0.4, p = 0.003). In other words, subjects with higher BMIs showed a less substantial increase in AT vitamin D_3 concentration than subjects with lower BMIs. But the more overweight subjects had significantly lower baseline plasma 25$(OH)D_3$ concentrations.

Therefore, we used a multiple linear regression model with change in AT vitamin D_3 as the dependent variable, baseline BMI and baseline plasma 25(OH)D_3 as independent variables, and dose-adjusted. The association between change in AT vitamin D_3 and baseline BMI was attenuated, but remained inverse (β-coefficient -0.8, 95% CI -1.7–0.03, p = 0.058). This inverse association was further attenuated upon replacing baseline BMI with baseline body weight (p = 0.08).

Baseline BMI and body weight were significantly inversely associated with baseline AT 25(OH)D_3 concentration (Table 6). However, baseline BMI and body weight were not significantly associated with baseline vitamin D_3 in plasma or adipose tissue.

Table 5. Linear regression analyses – Associations between changes in plasma concentrations of vitamin D metabolites after 3 months of daily vitamin D_3 supplementation and vitamin D dose, baseline body mass index, body weight, plasma 25-hydroxyvitamin D_3, and measures of adipose tissue inflammation.

		Unadjusted		Adjusted for Dose	
Dependent Variable	**Independent Variable**	**Co-efficient (95% CI)**	**Significance**	**Co-efficient (95% CI)**	**Significance**
Δ plasma vitamin D_3 (ng/mL)	Vitamin D_3 dose	8.9 (3.1, 14.8)	**0.006**	-	-
Δ plasma $24,25(OH)_2D_3$ (ng/mL)	Vitamin D_3 dose	1.2 (-0.2, 2.7)	0.090	-	-
Δ plasma vitamin D_3 (ng/mL)	Baseline BMI (kg/m^2)	0.02 (-0.6, 0.6)	0.942	-0.3 (-0.8, 0.2)	0.246
Δ plasma vitamin D_3 (ng/mL)	Baseline body weight (kg)	0.003 (-0.2, 0.2)	0.978	-0.06 (-0.2, 1.1)	0.480
Δ plasma vitamin D_3 (ng/mL)	Baseline plasma $25(OH)D_3$ (ng/mL)	0.6 (-0.3, 1.6)	0.172	0.6 (-0.1, 1.3)	0.100
Δ plasma $24,25(OH)_2D_3$ (ng/mL)	Baseline BMI (kg/m^2)	-0.02 (-0.2, 0.1)	0.736	-0.06 (-0.19, 0.06)	0.273
Δ plasma $24,25(OH)_2D_3$ (ng/mL)	Baseline body weight (kg)	-0.02 (-0.06, 0.03)	0.373	-0.03 (-0.07, 0.01)	0.150
Δ plasma $24,25(OH)_2D_3$ (ng/mL)	Baseline plasma $25(OH)D_3$ (ng/mL)	0.02 (-0.2, 0.2)	0.844	0.01 (-0.2, 0.2)	0.904
Δ plasma $24,25(OH)_2D_3$ (ng/mL)	Baseline adipose tissue TNFα (mRNA copy number/ng total RNA)	0.5 (-1.0, 2.0)	0.475	0.3 (-1.1, 1.8)	0.635
Δ plasma $24,25(OH)_2D_3$ (ng/mL)	Baseline adipose tissue IL-6** (mRNA copy number/ng total RNA)	2.8 (-0.3, 5.9)	0.076	3.0 (0.3, 5.8)	**0.033**
Δ plasma $24,25(OH)_2D_3$ (ng/mL)	Baseline adipose tissue IL-1β** (mRNA copy number/ng total RNA)	0.8 (-2.1, 3.6)	0.574	0.9 (-1.8, 3.5)	0.489

Coefficients are unstandardized.
**Used log-transformed data.
Abbreviations: $25(OH)D_3$ = 25-hydroxyvitamin D_3, $24,25(OH)_2D_3$ = 24,25-dihydroxyvitamin D_3, BMI = body mass index, TNFα = tumor necrosis factor-alpha, IL = interleukin, Δ = Change in value from baseline to 3-month visit.

Table 6. Linear regression analyses – Associations between baseline body mass index or body weight and vitamin D metabolites in adipose tissue and plasma.

		Unadjusted		Adjusted for Dose	
Dependent Variable	**Independent Variable**	**Co-efficient (95% CI)**	**Significance**	**Co-efficient (95% CI)**	**Significance**
Δ adipose tissue 25(OH)D_3 (pg/mg)	Baseline BMI (kg/m^2)	0.03 (-0.05, 0.10)	0.463	-0.003 (-0.07, 0.06)	0.914
Δ adipose tissue 25(OH)D_3 (pg/mg)	Baseline body weight (kg)	0.002 (-0.02, 0.03)	0.842	-0.004 (-0.03, 0.02)	0.699
Δ adipose tissue vitamin D_3 (pg/mg)	Baseline BMI (kg/m^2)	-0.4 (-1.3, 0.4)	0.285	-0.9 (-1.4, -0.4)	**0.003**
Δ adipose tissue vitamin D_3 (pg/mg)	Baseline body weight (kg)	-0.2 (-0.5, 0.1)	0.191	-0.3 (-0.5, -0.1)	**0.010**
Baseline plasma 25(OH)D_3 (ng/mL)	Baseline BMI (kg/m^2)	-0.4 (-0.7, -0.2)	**0.005**	-	-
Baseline plasma 25(OH)D_3 (ng/mL)	Baseline body weight (kg)	-0.1 (-0.2, -0.003)	**0.044**	-	-
Δ adipose tissue vitamin D_3 (pg/mg)	Baseline BMI (kg/m^2)	0.1 (-1.0, 1.2)	0.831	-0.8 (-1.7, 0.03)	0.058
	Baseline plasma 25(OH)D_3 (ng/mL)	1.3 (-0.5, 3.2)	0.144	0.1 (-1.2, 1.5)	0.812
Δ adipose tissue vitamin D_3 (pg/mg)	Baseline body weight (kg)	-0.07 (-0.4, 0.3)	0.645	-0.2 (-0.4, 0.03)	0.080
	Baseline plasma 25(OH)D_3 (ng/mL)	1.0 (-0.6, 2.6)	0.182	0.6 (-0.5, 1.7)	0.238
Baseline adipose tissue 25(OH)D_3 (pg/mg)	Baseline BMI (kg/m^2)	-0.03 (-0.05, -0.008)	**0.009**	-	-
Baseline adipose tissue 25(OH)D_3 (pg/mg)	Baseline body weight (kg)	-0.01 (-0.02, -0.002)	**0.015**	-	-
Baseline plasma vitamin D_3 (ng/mL)	Baseline BMI (kg/m^2)	-0.01 (-0.04, 0.008)	0.187	-	-
Baseline plasma vitamin D_3 (ng/mL)	Baseline body weight (kg)	-0.003 (-0.011, 0.005)	0.459	-	-
Baseline adipose tissue vitamin D_3 (pg/mg)	Baseline BMI (kg/m^2)	-0.4 (-0.9, 0.08)	0.091	-	-
Baseline adipose tissue vitamin D_3 (pg/mg)	Baseline body weight (kg)	-0.09 (-0.3, 0.1)	0.347	-	-

Coefficients are unstandardized.
Abbreviations: 25(OH)D_3 = 25-hydroxyvitamin D_3, BMI = body mass index, Δ = Change in value from baseline to 3-month visit.

DISCUSSION

Summary of Main Findings

Association of change in plasma 25(OH)D_3 with changes in vitamin D_3 and 24,25(OH)$_2D_3$

The primary purpose of this study was to investigate how levels of the main clinical marker of vitamin D status, plasma 25(OH)D_3, respond to three months of daily vitamin D_3 supplementation in overweight to obese adults. We hypothesized that change in plasma 25(OH)D_3 would be inversely associated with change in plasma vitamin D_3. As expected, baseline plasma 25(OH)D_3 was inversely associated with change in plasma 25(OH)D_3 (Table 4) and positively associated with change in plasma vitamin D_3 (Table 5), but neither association was significant. We did not find a significant association between changes in plasma 25(OH)D_3 and vitamin D_3, even after adjusting for dose and baseline plasma 25(OH)D_3 (Appendix Table A). Besides baseline vitamin D status, plasma 25(OH)D_3 response is potentially affected by other factors, such as obesity and adiposity, chronic inflammation, degradation, or vitamin D dosage and form. Without a larger sample size or control group, we lack the statistical power to account for all confounding factors and clearly determine if plasma 25(OH)D_3 and vitamin D_3 responses are inversely related.

Although no significant associations were found between changes in plasma 25(OH)D_3 and vitamin D_3, we wanted to further investigate their relationship with each other in plasma and adipose tissue. In plasma, 25(OH)D_3 concentrations were always greater than vitamin D_3 concentrations, but to considerably different degrees in each group at both visits. In adipose tissue, the concentration of vitamin D_3 was always about 9-fold greater than that of 25(OH)D_3 in both groups at baseline (when all subjects were vitamin D-deficient) and post-intervention [after plasma concentrations of 25(OH)D_3 and vitamin D_3 greatly improved]. Additionally, plasma

vitamin D_3 and AT vitamin D_3 were strongly positively associated with each other (Appendix Table A). Therefore, vitamin D_3 appears to be the predominate form of vitamin D used for AT storage, independent of the plasma concentrations of either $25(OH)D_3$ or vitamin D_3.

Furthermore, when all subjects were vitamin D-deficient at baseline, very little vitamin D_3 was detected in plasma (Table 3). Only after plasma concentrations of $25(OH)D_3$ reached at least 20 ng/mL did the plasma concentrations of vitamin D_3 substantially increase (Figure 1). While our results showed that baseline vitamin D status may not be strongly associated with plasma vitamin D response as a continuous variable, categorizing plasma $25(OH)D_3$ concentrations as above or below 20 ng/mL may be a more physiologically-relevant strategy. In the setting of vitamin D deficiency, achieving adequate plasma $25(OH)D_3$ levels for 1α-hydroxylation may take precedence over vitamin D storage. Once plasma $25(OH)D_3$ reaches a sufficient threshold concentration (>20 ng/mL), some plasma vitamin D_3 can remain unhydroxylated and become available for storage in adipose tissue.

We also proposed that a small increase in plasma $25(OH)D_3$ is due to high levels of degradation. Although one study has quantified the level of CYP24 expression in murine adipocytes[17], we could not detect a measureable amount in any subject. Therefore, we used plasma concentrations of the major vitamin D catabolite, $24,25(OH)_2D_3$, as an indicator of degradation activity. We hypothesized that change in plasma $25(OH)D_3$ would be inversely associated with change in plasma $24,25(OH)_2D_3$. However, we actually found a significant positive association, both unadjusted and adjusted for dose and even after further adjustment for the change in vitamin D_3. This suggests that degradation may be less a regulated process than driven by $25(OH)D_3$ status. We found a significant positive association between plasma

$24,25(OH)_2D_3$ and $25(OH)D_3$ at baseline ($p < 0.001$) and post-intervention ($p < 0.001$) (Appendix Table A), further supporting this notion. The strong association between plasma $25(OH)D_3$ and $24,25(OH)_2D_3$ was consistent with findings from the literature.[35] However, unlike Wagner *et al*., we did not find a significant association between change in plasma $25(OH)D_3$ and the ratio of baseline plasma $24,25(OH)_2D_3$ to $25(OH)D_3$.[35] Therefore, our results do not support the use of this ratio as a predictor of plasma response to supplementation. This may be due to our small sample size and inability to adjust for as many factors as Wagner *et al*. did, such as baseline plasma $25(OH)D_3$, $24,25(OH)_2D_3$, BMI, age, gender, PTH, calcium, phosphate, and creatinine. In summary, catabolism of $25(OH)D_3$ into $24,25(OH)_2D_3$ appears to simply rise with increasing $25(OH)D_3$ concentrations, independent of the amount of vitamin D supplementation or vitamin D status.

Only after including both changes in plasma vitamin D_3 and $24,25(OH)_2D_3$ in a multiple regression model and adjusting for dose did we find a significant inverse association between changes in plasma $25(OH)D_3$ and vitamin D_3. This multiple regression model also resulted in an even stronger positive association between change in plasma $25(OH)D_3$ and change in plasma $24,25(OH)_2D_3$. The relationship between plasma concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ continues to appear simply balanced. Whereas, the relationship between plasma concentrations of $25(OH)D_3$ and vitamin D_3 may be affected by the threshold effect. 25-hydroxylase could become saturated when plasma $25(OH)D_3$ concentration reaches ~20 ng/mL, resulting in more unhydroxylated vitamin D_3 in plasma. However, with a sample size of only 15 subjects, we were unable to also adjust for baseline plasma $25(OH)D_3$ concentration in this multiple regression model. We, therefore, could not test whether the inverse association between the changes in vitamin D_3 and $25(OH)D_3$ was at least partly due to the fact that the plasma vitamin D_3

concentration increased only after a plasma 25(OH)D_3 concentration of ~20ng/mL had been reached. Overall, our results do not support the hypothesis that disproportionately high levels of degradation explain obese individuals' poor response to vitamin D supplementation.

Association between obesity and plasma vitamin D response

The secondary purpose of this study was to investigate how obesity is related to vitamin D metabolites in blood and adipose tissue in response to vitamin D repletion. Contrary to the popular sequestration hypothesis, we proposed that greater vitamin D degradation causes the obese to suffer from a less substantial plasma 25(OH)D_3 response than the lean. We also proposed that vitamin D_3 would be quickly 25-hydroxylated in order to replenish the diminished 25(OH)D_3 levels. Therefore, we hypothesized that BMI would be inversely associated with changes in plasma 25(OH)D_3 and vitamin D_3, but we found no such associations. Although baseline BMI did not statistically differ between groups, the average BMI in the 4,000 IU/d group was 5 kg/m^2 greater than in the 2,000 IU/d group. The higher dose group also exhibited a significantly greater improvement in plasma 25(OH)D_3 and vitamin D_3 than the lower dose group. If BMI is, in fact, inversely associated with vitamin D response, then having a higher average BMI in the higher dose group could mask this association. To better account for volumetric dilution[53], we replaced baseline BMI with baseline body weight in our analyses and still did not find any association with plasma vitamin D response. Other factors, such as degradation and sequestration, may be affecting the relationship between BMI and plasma vitamin D response.

Degradation of vitamin D may be playing a role in the strength of vitamin D response, but not in the way we originally predicted. Baseline BMI was inversely associated with plasma $25(OH)D_3$ at baseline and post-intervention, which is consistent with the literature.[38-42] Baseline BMI was also inversely associated with plasma $24,25(OH)_2D_3$ at baseline and post-intervention (Appendix Table A). In other words, a greater BMI was associated with lower levels of plasma $25(OH)D_3$ and lower levels of the primary vitamin D degradation product. As previously discussed, low levels of plasma $24,25(OH)_2D_3$ could simply be a result of low levels of $25(OH)D_3$. Therefore, we wanted to account for degradation when investigating the relationship between obesity and plasma $25(OH)D_3$ response. After adjusting for change in plasma $24,25(OH)_2D_3$, baseline BMI showed a significant positive association with change in plasma $25(OH)D_3$ ($p = 0.03$), which was slightly attenuated after adjusting for dose ($p = 0.07$) (Appendix Table A). Thus, after adjusting for degradation, a greater BMI was associated with a greater improvement in plasma $25(OH)D_3$. A potential limitation of this approach is that it was over-adjusted, because change in plasma $25(OH)D_3$ is so strongly associated with change in plasma $24,25(OH)_2D_3$.

In summary, our results were consistent with the literature that obesity is associated with low baseline vitamin D status. However, we did not find any association between BMI and plasma $25(OH)D_3$ or vitamin D_3 response. These findings are in contrast to many larger human intervention trials that have found a significant inverse association between obesity and plasma $25(OH)D_3$ response.[47-50] In fact, after adjusting for change in plasma $24,25(OH)_2D_3$, BMI became positively associated with change in plasma $25(OH)D_3$. Perhaps past studies failed to adjust for degradation. Perhaps we over-adjusted or our statistical power was too weak due to the small sample size and lack of normal weight controls. When analyzing vitamin D response,

larger future studies may benefit from analyzing both $25(OH)D_3$ and $24,25(OH)_2D_3$. None of our results, however, have supported the degradation hypothesis.

We also investigated the hypothesized contribution of disproportionately high levels of vitamin D AT **sequestration** to obese individuals' poor response to supplementation. While baseline BMI and change in AT $25(OH)D_3$ concentration were not associated, there was a significant inverse association between baseline BMI and change in AT vitamin D_3, adjusted for dose (Appendix Table A). This inverse association remained significant even when we used baseline body weight instead of BMI. AT vitamin D_3 concentrations increased in all subjects, but increased less substantially in those with a greater BMI. Perhaps the incoming vitamin D is diluted in subjects with greater overall mass or volume. Although some would argue that these findings oppose sequestration, we only measured concentrations (not absolute amounts) and, therefore, cannot quantify the proportion of supplemented vitamin D that was distributed in blood versus adipose tissue.

As previously discussed, deficient plasma $25(OH)D_3$ levels were associated with low plasma vitamin D_3 levels and, thus, little vitamin D_3 would be available for storage. Additionally, baseline plasma $25(OH)D_3$ concentration was inversely associated with baseline BMI. Therefore, we accounted for the possible effect of baseline plasma $25(OH)D_3$ by adding it to the model. The association between BMI and change in AT vitamin D_3 was slightly attenuated but remained inverse. BMI or body weight appears to play a role in AT storage of vitamin D_3, independent of baseline plasma $25(OH)D_3$ status. Whether this is more likely due to sequestration or volumetric dilution is unclear, especially since we could only measure vitamin D concentrations and not factor in whole body fat tissue storage due to a lack of data on fat mass.

Literature has shown that the obese suffer from poor plasma vitamin D response. We have found that subjects with higher BMIs suffered from poor vitamin D response in adipose tissue but not in blood. The findings from this study did not support our degradation hypothesis and did not support or oppose the sequestration hypothesis. An alternative explanation is that obese individuals tend to have lower baseline plasma $25(OH)D_3$ levels that are further from the sufficiency threshold. Along with inadequate sunlight exposure and nutrient-poor diets, another potential explanation for low baseline plasma $25(OH)D_3$ levels is volumetric dilution. This is based on the notion that vitamin D passively diffuses throughout the body and, thus, vitamin D is more diluted in larger bodies. By this rationale, a larger person needs a much larger vitamin D dose to overcome deficiency than a smaller person, even if the two people have similar BMIs and/or different body compositions. Perhaps vitamin D dosages should be based on body weight as well as vitamin D status. In the setting of severe vitamin D deficiency, it is plausible that more of the supplemented vitamin D would be immediately metabolized to carry out essential functions and/or diluted than sequestered in adipose tissue.

Association of adipose tissue inflammation with vitamin D degradation and response

Lastly, we investigated adipose tissue inflammation as a possible mechanism to explain high levels of vitamin D degradation in the obese. Compared to lean individuals, we proposed that obese individuals exhibit weaker responses to vitamin D repletion because their adipose tissue is more highly infiltrated with activated immune cells that release pro-inflammatory cytokines (e.g. TNFα, IL-6, IL-1β) and the vitamin D-degrading enzyme, 24-hydroxylase. In other words, greater AT inflammation may be indicative of greater vitamin D use and degradation, leading to a less substantial plasma $25(OH)D_3$ response. Therefore, we

hypothesized that baseline levels of the major pro-inflammatory cytokine in AT, TNFα, would be positively associated with change in plasma $24,25(OH)_2D_3$ and inversely associated with change in plasma $25(OH)D_3$. However, none of these associations were statistically significant. Since immune cells also express other pro-inflammatory cytokines in AT, we also used AT levels of IL-6 and IL-1β in this model. Although not significant, the associations between change in plasma $25(OH)D_3$ and baseline AT TNFα, IL-6, and IL-1β all suggest a positive, rather than the hypothesized inverse relationship. The associations between change in plasma $24,25(OH)_2D_3$ and baseline AT levels of the three pro-inflammatory cytokines were also all positive, but only IL-6 was significantly associated with change in plasma $24,25(OH)_2D_3$ after adjusting for dose. This finding could indeed suggest that AT inflammation promotes increased degradation. However, it may also simply be a false positive finding, particularly in the absence of any similar significant trend for TNFα and IL-1β. Furthermore, baseline BMI was not associated with baseline AT concentrations of TNFα, IL-6, or IL-1β (Appendix Table A). This contradicts our theory that greater obesity leads to greater expression of pro-inflammatory cytokines in AT. But most importantly, none of the pro-inflammatory cytokines under investigation were associated with poor plasma $25(OH)D_3$ response. Therefore, it appears unlikely that AT inflammation is a major determinant of how plasma $25(OH)D_3$ concentration responds to supplementation in the obese.

Summary of Other Findings

We successfully treated hypovitaminosis D in overweight adults with supplementation of 2,000 IU/d or 4,000 IU/d for three months. The 4,000 IU/d dose was more effective than the 2,000 IU/d dose at achieving vitamin D sufficiency. Depending on how we define vitamin D

sufficiency (>20 ng/mL vs. >30 ng/mL), the current IOM vitamin D intake recommendation for adults (600 IU/d) may be inadequate for vitamin D-deficient overweight individuals. Improvements in plasma $25(OH)D_3$ reached a plateau by three months in all groups. Therefore, three months may be an adequate intervention length for studies that are investigating plasma $25(OH)D_3$ response to vitamin D repletion. Based on relative concentrations at baseline and post-intervention, our results support the notions that $25(OH)D_3$ is the main circulating form of vitamin D and vitamin D_3 is the main storage form in adipose tissue. Baseline BMI and body weight were significantly inversely associated with baseline plasma $25(OH)D_3$ and baseline AT $25(OH)D_3$.

Strengths and Limitations

To our knowledge, this pilot study was one of the first randomized intervention trials to measure various vitamin D metabolites in both blood and adipose tissue in vitamin D-deficient overweight human adults. Very few studies have quantified more than two metabolites [usually $25(OH)D_3$ and/or vitamin D_3], taken AT samples from the same subject before and after vitamin D repletion, or measured AT expression of pro-inflammatory cytokines *in vivo*. Several studies have mentioned the degradation hypothesis, but few have measured vitamin D degradation products directly. We supplemented subjects with two different doses of pure vitamin D_3 without the confounding effect of added calcium as in many previous studies. Our small pilot study helped determine which variables to measure and which dose to supplement in a larger randomized control trial.

This study also has several limitations. Our greatest limitation is the small sample size. With only 15 study subjects, our statistical power was limited. Our analysis was especially

sensitive to outliers, which prevented normal distribution of certain variables. Also, we lacked lean and/or placebo control groups. A lean control group would have allowed us to analyze a broader spectrum of BMI categories, rather than just the overweight to obese. Without a placebo group, we cannot completely account for hidden time effects, such as cutaneous sources of vitamin D from sun exposure. We assume that by performing the study during the winter and spring months, when sunlight in Seattle is minimal, the endogenous contribution to overall vitamin D levels was negligible. Due to this assumption, we did not adjust for race or skin type. To better understand the relationship between adiposity and vitamin D storage, this study would have benefited from measuring fat mass or body composition rather than just body weight and BMI. Without measuring body composition, we could only assume that subjects with greater BMIs had greater fat mass. We recruited subjects with BMIs ≥25 kg/m^2, but some "overweight" subjects could actually have very little fat mass. Requiring a higher BMI, such as ≥30 kg/m^2, would better capture subjects with excess adiposity and more clearly demonstrate vitamin D response in just the obese. One study found that 23,25-dihydroxyvitamin D_3 and $24,25(OH)_2D_3$ were constantly excreted in the urine of six healthy adults.[89] If a great proportion of $24,25(OH)_2D_3$ is found in the urine, then measuring just the plasma concentration would result in an underestimation of degradation product. Measuring the urinary clearance of $24,25(OH)_2D_3$, in addition to the plasma concentration, would more accurately represent the true amount of vitamin D degradation and more completely test the degradation hypothesis.

Conclusion

We conducted this study in order to investigate how vitamin D-deficient obese adults respond to vitamin D supplementation. Consistent with the literature, we found that BMI was

inversely associated with plasma 25(OH)D_3 status. However, we could not replicate the results from previous studies that have found an inverse association between obesity and plasma 25(OH)D_3 response. This could be due to our small sample size or lack of controls. Within its limitations, findings from our small pilot study were inconclusive about the sequestration hypothesis and opposed the hypothesis that obese individuals degrade more vitamin D than lean individuals due to greater adipose tissue inflammation. Future studies should further investigate how sequestration, baseline vitamin D status (or the "threshold hypothesis"), and volumetric dilution affect vitamin D response in the obese population.

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Appendix Table A. Linear regression analyses – Associations between changes in vitamin D metabolites, at baseline, or at 3-months.

		Unadjusted		Adjusted for Dose	
Dependent Variable	**Independent Variable**	**Co-efficient (95% CI)**	**Significance**	**Co-efficient (95% CI)**	**Significance**
Baseline plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline plasma 25$(OH)D_3$ (ng/mL)	0.1 (0.09, 0.2)	**<0.001**	-	-
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	3-month plasma 25$(OH)D_3$ (ng/mL)	0.2 (0.1, 0.3)	**<0.001**	0.2 (0.1, 0.3)	**<0.001**
Baseline plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline BMI (kg/m^2)	-0.06 (-0.1, -0.02)	**0.004**	-	-
Baseline plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline body weight (kg)	-0.02 (-0.03, -0.003)	**0.023**	-	-
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline BMI (kg/m^2)	-0.08 (-0.2, 0.06)	0.232	-0.1 (-0.3, -0.009)	**0.038**
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline body weight (kg)	-0.04 (-0.08, 0.01)	0.118	-0.05 (-0.09, -0.005)	**0.031**
Δ plasma 25$(OH)D_3$ (ng/mL)	Ratio baseline plasma 24,25$(OH)_2D_3$ to 25$(OH)D_3$	-56.1 (-236.4, 124.2)	0.513	-41.2 (-202.9, 120.6)	0.589
Δ plasma 25$(OH)D_3$ (ng/mL)	Δ plasma vitamin D_3 (ng/mL)	0.5 (-0.07, 1.1)	0.077	0.2 (-0.6, 1.0)	0.625
	Baseline plasma 25$(OH)D_3$ (ng/mL)	-0.8 (-1.8, 0.2)	0.113	-0.6 (-1.6, 0.4)	0.218
Δ plasma 25$(OH)D_3$ (ng/mL)	Baseline BMI (kg/m^2)	0.3 (0.04, 0.6)	**0.030**	0.3 (-0.03, 0.7)	0.072
	Δ plasma 24,25$(OH)_2D_3$ (ng/mL)	4.2 (2.9, 5.6)	**<0.001**	4.1 (2.5, 5.8)	**<0.001**
Baseline adipose tissue vitamin D_3 (pg/mg)	Baseline plasma vitamin D_3 (ng/mL)	11.0 (-1.3, 23.2)	0.075	-	-
3-month adipose tissue vitamin D_3 (pg/mg)	3-month plasma vitamin D_3 (ng/mL)	1.0 (0.04, 1.9)	**0.042**	0.8 (-0.5, 2.1)	0.197

Coefficients are unstandardized.
Abbreviations: 25$(OH)D_3$ = 25-hydroxyvitamin D_3, 24,25$(OH)_2D_3$ = 24,25-dihydroxyvitamin D_3, BMI = body mass index, Δ = Change in value from baseline to 3-month visit.

Appendix Table A (continued). Linear regression analyses – Associations between changes in vitamin D metabolites, at baseline, or at 3-months.

		Unadjusted		Adjusted for Dose	
Dependent Variable	**Independent Variable**	**Co-efficient (95% CI)**	**Significance**	**Co-efficient (95% CI)**	**Significance**
Δ adipose tissue vitamin D_3 (pg/mg)	Δ plasma vitamin D_3 (ng/mL)	0.8 (0.09, 1.5)	**0.031**	0.4 (-0.6, 1.3)	0.426
Baseline plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline adipose tissue TNFα (mRNA copy number/ng RNA)	-0.3 (-0.9, 0.3)	0.328	-	-
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline adipose tissue TNFα (mRNA copy number/ng RNA)	0.2 (-1.5, 2.0)	0.775	0.04 (-1.7, 1.7)	0.964
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	3-month adipose tissue TNFα (mRNA copy number/ng RNA)	-0.6 (-2.0, 0.9)	0.419	-0.6 (-2.0, 0.8)	0.366
Baseline plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline adipose tissue IL-6** (mRNA copy number/ng RNA)	-0.2 (-1.6, 1.2)	0.781	-	-
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline adipose tissue IL-6** (mRNA copy number/ng RNA)	2.6 (-1.1, 6.3)	0.155	2.8 (-0.6, 6.3)	0.098
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	3-month adipose tissue IL-6** (mRNA copy number/ng RNA)	2.4 (-3.4, 8.2)	0.392	2.6 (-3.0, 8.1)	0.332
Baseline plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline adipose tissue IL-1β** (mRNA copy number/ng RNA)	-0.6 (-1.6, 0.5)	0.282	-	-
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	Baseline adipose tissue IL-1β** (mRNA copy number/ng RNA)	0.2 (-3.1, 3.4)	0.902	0.3 (-2.8, 3.4)	0.841
3-month plasma 24,25$(OH)_2D_3$ (ng/mL)	3-month adipose tissue IL-1β** (mRNA copy number/ng RNA)	-1.1 (-3.5, 1.2)	0.328	-1.2 (-3.0, 0.5)	0.343
Baseline adipose tissue TNFα (mRNA copy number/ng RNA)	Baseline BMI (kg/m^2)	0.01 (-0.04, 0.06)	0.602	-	-
Baseline adipose tissue IL-6** (mRNA copy number/ng RNA)	Baseline BMI (kg/m^2)	-0.003 (-0.02, 0.02)	0.787	-	-
Baseline adipose tissue IL-1β** (mRNA copy number/ng RNA)	Baseline BMI (kg/m^2)	0.002 (-0.03, 0.03)	0.895	-	-

Coefficients are unstandardized.
Abbreviations: 25$(OH)D_3$ = 25-hydroxyvitamin D_3, 24,25$(OH)_2D_3$ = 24,25-dihydroxyvitamin D_3, BMI = body mass index, TNFα = tumor necrosis factor-alpha, IL = interleukin, Δ = Change in value from baseline to 3-month visit.
**Used log-transformed data.

Appendix Table B. Commonly used abbreviations and vitamin D nomenclature.

Nomenclature	Abbreviation	Synonyms
Vitamin D_2		Ergocalciferol
Vitamin D_3		Cholecalciferol
25-hydroxyvitamin D_3	$25(OH)D_3$	Calcidiol
1,25-dihydroxyvitamin D_3	$1,25(OH)_2D_3$	Calcitriol
24,25-dihydroxyvitamin D_3	$24,25(OH)_2D_3$	
1,24,25-trihydroxyvitamin D_3	$1,24,25(OH)_3D_3$	
1-alpha-hydroxylase	$1\alpha(OH)$ase	CYP27 (gene)
24-hydroxylase	24(OH)ase	CYP24 (gene)
25-hydroxylase	25(OH)ase	
Vitamin D receptor	VDR	
Body mass index	BMI	
International unit	IU	
Adipose tissue	AT	
Tumor necrosis factor-alpha	TNFα	
Interleukin	IL	
High sensitivity C-reactive protein	hs-CRP	
Monocyte chemoattractant protein-1	MCP-1	
Interferon-gamma	IFNγ	
Parathyroid hormone	PTH	
Repeated measures analysis of variance	RM-ANOVA	
Institute of Medicine	IOM	
Roux-en Y gastric bypass	RYGB	
Randomized controlled trial	RCT	