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Effects of vitamin D₃ and calcium supplementation on serum levels of tocopherols, retinol, and specific vitamin D metabolites

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Abstract

γ -Tocopherol (γ T) protects against DNA-damaging effects of nitrogen oxides, yet its physiologic regulation *in vivo* is unknown. Observational studies indicate inverse associations of 25[OH]-vitamin D with γ T and leptin. To determine whether vitamin D₃ supplementation alters levels of lipid-soluble micronutrients, serum samples (N=85 subjects) from a randomized, double-blind, placebo-controlled clinical trial of vitamin D₃ (800 IU) and calcium (2 g) alone and in combination were analyzed for lipid micronutrients and specific vitamin D metabolites at baseline and after 6-months of supplementation. Serum 25[OH]-vitaminD₃ levels increased 55% ($P < 0.0001$) and 48% ($P = 0.0005$), whereas 25[OH]-vitaminD₂ levels were lower by 48% ($P = 0.26$) and 21% ($P = 0.36$) in the vitamin D₃ and vitamin D₃ plus calcium groups, respectively. At baseline, γ T levels were inversely associated with 25[OH]D ($r = -0.31$, $P = 0.004$). With vitamin D₃ plus calcium treatment, serum α -tocopherol decreased 14% ($P = 0.04$), while similar changes in γ T (19% decrease, $P = 0.14$) were observed. No significant effects were observed for D₃ supplementation on leptin or retinol levels. These results are consistent with the hypothesis that vitamin D₃ +/- calcium affects serum tocopherol and 25[OH]D₂ levels, however, studies utilizing larger populations are warranted.

INTRODUCTION

The tocopherols are important agents in preventing cellular oxidative damage, and are postulated to protect against the development of cancer and other aging-related diseases (1, 2). Although α -tocopherol (α T) has the highest level of vitamin E bioactivity amongst the tocopherols (3) and, consequently, has been the target of most animal and epidemiologic research over the years, more recent findings have pointed to the other tocopherols, particularly γ -tocopherol (γ T), as playing significant roles in inhibiting the development of cancer in animal models (2). Epidemiological evidence, while limited, also suggests an

inverse association of γ T with certain cancers (4, 5) and heart disease (6). γ T has been identified as a key cellular antioxidant that specifically blocks damage to cells resulting from the enzymatic production of nitric oxide (NO), a mediator of inflammation through its oxidation products (7, 8). Conversely, α T appears to be a primary reactant against cellular oxygen-based oxidants, but lacks anti-inflammatory activity (9). Together the two forms of tocopherol protect cells from the major forms of endogenous oxidative assault. The assumption that serum tocopherol levels reflect dietary intake is valid only in cases of severe deficiency or heavy supplement use (10); hence, the physiologic determinants of serum levels of the tocopherols may be key to elucidating their potential functions for either reducing/promoting disease or serving as markers of risk. In particular, serum levels of γ T appear to be unrelated to dietary intake and may reflect to a large extent the level of chronic inflammation as suggested by its positive associations with circulating C-reactive protein and urinary isoprostane (11), thereby complicating the epidemiologic interpretation of its associations with diseases.

Vitamin D is associated with lower risk for cancer, primarily colorectal (12, 13), cardiovascular disease (14), and all-cause mortality (15). Vitamin D deficiency may result in chronic inflammation and decreased immune function (16). It was observed previously that serum 25(OH)-vitamin D (25[OH]D, as the sum of 25[OH]D₂ + 25[OH]D₃), and γ T were significantly inversely associated with one another and in turn associated with all-cause mortality (11). In that same study, a positive association of circulating α T with 25[OH]D was observed. It was hypothesized that deficiency of vitamin D may lead to a state of chronic inflammation resulting in elevated γ T levels which protect against NO-mediated damage, thereby compensating against some of the deleterious consequences of vitamin D deficiency. However, in previous studies (17, 18), it was also observed that both body mass index (BMI) and leptin (an adipocytokine produced predominantly by white adipose tissue) were inversely associated with serum 25[OH]D and positively associated with γ T levels. This suggests the possibility that the inverse association between γ T and 25[OH]D may be incidental to obesity and not directly related to vitamin D status. Also, there is *in vitro* evidence supporting regulation of leptin by vitamin D (19) and regulation of vitamin D by leptin (20), but definitive *in vivo* evidence is lacking.

Circulating retinol levels are reported to rise in winter and decline in summer, inverse to changes predicted for vitamin D (7), suggesting a possible interaction and/or compensation between vitamins D and A. Research evidence in animals (21) and humans (22) suggests that high doses of vitamin A may antagonize vitamin D actions. Support for an interaction between vitamins A and D is also provided in a recent nested case-control study of colorectal cancer by Jenab, et al. (23) where the retinol-colon cancer association was inverse among individuals with low vitamin D but direct among individuals with higher vitamin D levels. To our knowledge, there are no published data on the influence of vitamin D supplementation on circulating vitamin A and tocopherol levels. The primary objective of the current study was, therefore, to examine the effects of vitamin D₃ and/or calcium supplementation on changes of serum levels of α T, γ T, retinol (vitamin A), and leptin levels. In addition, we sought to determine whether supplemental D₃ and calcium, alone or in combination, affect serum levels of specific vitamin D metabolites such as 25[OH]D₂, 25[OH]D₃, and vitamins D₂ and D₃.

METHODS

Study population and protocol

This study utilized stored serum samples from a previous pilot, randomized, double-blind, placebo-controlled, 6-month clinical trial that used a 2 × 2 factorial design to test the effects of calcium and/or vitamin D on biomarkers of risk for colon cancer (24). In brief, 92 adults

aged 30 to 75 years, in general health, capable of informed consent, with a history of at least one pathology-confirmed adenomatous colorectal polyp within the past 36 months, no contraindications to calcium or vitamin D supplementation or rectal biopsy procedures and no medical conditions, habits, or medication usage that would otherwise interfere with the study, and taking 80% of their study pills during 1-month placebo run-in period, were recruited from the patient population attending the Digestive Diseases Clinic at the Emory Clinic, Emory University. The Institutional Review Boards of Emory University and the University of Hawaii approved the current study protocol.

At baseline, eligible participants were randomly assigned to the following four treatment groups: a placebo control group (placebo), a 2.0 g elemental calcium (as calcium carbonate in equal doses twice daily) supplementation group (calcium), an 800 IU [as 400 IU cholecalciferol (D₃) twice daily] vitamin D₃ supplementation group (vitamin D₃), and a calcium plus vitamin D₃ supplementation group (calcium plus vitamin D₃) taking 2.0 g elemental calcium plus 800 IU vitamin D₃ daily. The corresponding supplement and placebo pills were identical in size, appearance, and taste. The placebo was free of calcium, magnesium, vitamin D, and chelating agents. Additional details on the rationale for the doses and forms of calcium and vitamin D₃ supplements were described previously (24).

The treatment period was 6 months, and participants attended follow-up visits at 2 and 6 months after randomization and were contacted by telephone between the second and final follow-up visits. Pill-taking adherence was assessed by questionnaire, interview, and pill count. Participants were instructed to remain on their usual diet and not take any nutritional supplements not in use on entry into the study. Blood was collected at baseline (randomization) and at study completion (6-month follow-up), and diet was assessed with a semi-quantitative food frequency questionnaire (25). A total of 85 subjects (21-22 per group) completing the study with blood samples at baseline and at six months were available for analysis and utilized in the current study.

Sample analyses

Serum levels of vitamins D₂ and D₃, 25[OH]D₂, and 25[OH]D₃ were analyzed using liquid chromatography orbitrap mass spectrometry. An aliquot of 0.3 mL serum was mixed with 0.3 mL methanol, 2 mL hexane, and 15 µL of a methanolic solution containing D₃-d₆ and 25[OH]D₃-d₆ (Medical Isotopes, Pelham, NH). After centrifugation, the hexane layer was removed and subsequently evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.15 mL methanol and 20 µL extract was injected into HPLC (model Accela, ThermoFisher, San Jose, CA). Separation was conducted on an Ascentis Express C18 column (150 × 3.0 mm i.d., 2.7 µm; Supelco, Park Bellefonte, PA) and a pre-filter cartridge (2.1 mm i.d., 0.2 µm; ThermoFisher, San Jose, CA) with a mobile phase gradient consisting of isopropanol/water/methanol/acetonitrile (0/12/ 69.6/18.4 [vol/vol/vol] for 3.5 min, 0/0/60/40 for 4.5 min, 55/0/10/35 for 6 min, and 0/12/69.6/18.4 for 5.1 min, successively). The flow rate was kept at 0.7 mL/min. After electrospray ionization, the orbitrap mass spectrometer (model Exactive, ThermoFisher, San Jose, CA), with exact mass determination to three decimal places, was operated at the following masses for quantitating the respective vitamin D metabolites: m/z 379.336/ 380.339/ 397.346/ 398.349 for D₂, m/z 367.336/ 385.346/ 386.349 for D₃, m/z 377.320/ 378.324/ 395.330/ 396.335/ 413.341/ 414.341 for 25[OH]D₂, m/z 365.320/ 366.323/ 383.330/ 384.333/ 401.341/ 402.341 for 25[OH]D₃, m/z 391.383 for D₃-d₆, and m/z 389.368 for 25[OH]D₃-d₆.

Serum concentrations of total 25[OH]D were also measured according to the manufacturer's directions using an immunoassay kit (Immunodiagnostic Systems, Ltd. Fountain Hills, AZ). Leptin immunoassay kits purchased from R&D System (Minneapolis, MN) were used to analyze serum leptin levels according to the manufacturer's instructions. Serum retinol and

tocopherol levels were analyzed by HPLC with photo-diode array detection, as previously described in detail (11, 26).

Statistical Analysis

Treatment groups (placebo, calcium, vitamin D₃, calcium plus vitamin D₃) were assessed for comparability of characteristics at baseline and at final follow-up by Fisher's exact test for categorical variables and ANOVA for continuous variables.

Primary analyses were based on assigned treatment at the time of randomization regardless of adherence status (intent-to-treat analysis). Means of outcome variables were calculated for each treatment group for the baseline and 6-month follow-up visits. Treatment effects were evaluated by assessing the differences in the outcome variables from baseline to the 6-month follow-up visit between participants in each active treatment group and the placebo group by a repeated-measures linear MIXED effects model. The model included the intercept, follow-up visit effects (baseline and follow-up), and interactions between treatment groups and the follow-up visit effect (the absolute treatment effect). To provide perspective on the magnitude of the treatment effects, we also calculated proportional treatment effects, defined as (absolute treatment effect / treatment group baseline) × 100% (e.g., a proportional effect of 56% would mean a 56% increase in the active treatment group relative to the placebo group). Because the treatment groups were balanced on potentially influential factors (participant characteristics and dietary intakes of certain nutrients) at baseline, no adjustment was made for other covariates in the primary intent-to-treat analyses. Spearman correlation was used to assess the associations between analyte levels. SAS software (SAS Institute, Cary, North Carolina) was used for analyses. All tests were two sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of study participants

The four treatment groups did not differ significantly with respect to participant characteristics measured at baseline (Table 1, based on a total of 92 originally randomized subjects). The mean age of participants was 60.5 ± 7.9 (SD) years, 64% were men, 71% were white, 88% were current non-smokers, and 82% were overweight or obese. The mean values of BMI were 30.1 ± 6.1 kg/m² at baseline and 30.2 ± 6.2 kg/m² at 6-month follow-up ($p = 0.38$). Baseline values of γ T for 30.6% of the 85 subjects who completed the trial were considered hyper-gammatocopherolemic (11) with levels > 2.5 µg/ml. This was comparable to what was observed previously for male control subjects in another study (11), where 28.9% were found to have elevated γ T. After the six-month intervention 22.4% of the 85 subjects in all four treatment groups were observed to be hyper-gammatocopherolemic. Adherence to visit attendance averaged 92% and did not differ significantly among the four treatment groups. On average, at least 80% of pills were taken by 93% of participants at the first follow-up visit and 84% at the final follow up visit. There were no treatment complications.

Effects of supplemental calcium and/or vitamin D₃ on specific serum vitamin D metabolites

Serum specific vitamin D measures are shown in Table 2. At baseline, there were no significant differences between the four treatment groups in 25[OH]D₃, 25[OH]D₂, and vitamins D₃ and D₂ levels. Serum levels of 25[OH]D₃ significantly increased by approximately 50% at the end of 6-month's follow-up in the treatment groups that received supplemental D₃ alone ($P < 0.0001$) or combined with calcium ($P = 0.0005$) relative to placebo. There were no statistically significant treatment effects on serum levels of

25[OH]D₂, vitamin D₂, or vitamin D₃. However, relative to placebo, 25[OH]D₂ levels were 48% lower in the supplemental vitamin D₃ group ($P = 0.26$) and 21% in the vitamin D₃ plus calcium group ($P = 0.36$), and 16% higher ($P = 0.75$) in the calcium group after six months. Serum vitamin D₃ levels were not significantly changed relative to placebo after supplementation, but were observed to be 34% higher ($P = 0.08$) among individuals supplemented with D₃ alone and 17% higher ($P = 0.36$) in those who received D₃ plus calcium, relative to baseline.

Effects of supplemental calcium and/or vitamin D₃ on serum tocopherols, retinol, and leptin

Baseline serum levels of 25[OH]D were significantly inversely correlated with γ T ($r = -0.31$, $P = 0.004$) but were not significantly correlated with α T ($r = 0.12$, $P = 0.29$). Serum α T and γ T levels were not significantly correlated with each other at baseline ($r = -0.10$, $P = 0.38$).

Mean serum levels of α T, γ T, retinol, and leptin for each group at baseline and after six months supplementation are shown in Table 3. While both vitamin D₃ intervention groups had lower γ T levels after six months supplementation with vitamin D₃ relative to baseline, the absolute treatment effect, which also factors in changes in the placebo group, were not statistically significant. Serum α T and γ T levels decreased by 14% ($P = 0.04$) and 19% ($P = 0.14$), respectively, in the calcium plus vitamin D₃ group relative to placebo when analyzed by absolute treatment effect. There also appeared to be a trend for decreasing serum γ T levels as indicated by the relative treatment effects (calcium group, -3%, $P = 0.85$; vitamin D₃ group, -8%, $P = 0.57$; calcium plus vitamin D₃ group, -19%, $P = 0.14$). As shown in Figure 1, when all vitamin D₃-supplemented subjects were combined and stratified by baseline 25[OH]D level as measured by immunoassay, there was a clear trend toward both increased 25[OH]D and lower serum γ T levels after six months of vitamin D₃ treatment across all quartiles, consistent with the inverse association between serum γ T and 25[OH]D observed both at baseline and after treatment.

Although lower retinol levels were observed in supplemental D₃ +/- calcium-treated subjects (Table 3), slightly higher retinol levels were observed in subjects treated with vitamin D₃ alone, indicating no consistent effect of vitamin D₃ supplementation on retinol. In the current study 82% of study participants were overweight or obese and their weight remained constant over the 6-month experimental period (mean BMI: baseline, 30.1 ± 6.1 kg/m²; 6 months, 30.2 ± 6.2 kg/m²). Baseline leptin values were highly correlated with BMI ($r = 0.67$, $P < 0.0001$) and significantly correlated with γ T ($r = 0.24$, $P = 0.03$). No significant changes in leptin levels (Table 3) were observed for any treatment group, consistent with the stable BMI levels observed. The calcium + vitamin D₃ group, relative to the other treatment groups, did have borderline elevated leptin levels at baseline, which remained high at the six-month follow-up.

DISCUSSION

Several studies (27-29) reported that supplemental D₂ or D₃ increases circulating levels of 25[OH]D, an indicator of vitamin D nutritional status in humans, and vitamin D₃ is considered to be the more biologically potent form (30, 31). As expected, this randomized clinical trial of daily supplementation with 800 IU vitamin D₃ demonstrated that increased serum 25[OH]D₃ was responsible for the increase in total 25[OH]D observed, but vitamin D₃ supplementation did not increase 25[OH]D₂ levels. Indeed, lower 25[OH]D₂ levels were observed suggesting that a possible competing effect on 25[OH]D₂ should be considered in larger studies of D₃ supplementation. In contrast, Holick et al. (27) reported that a 1,000 IU dose of supplemental D₂ daily for 11 weeks did not negatively affect serum 25[OH]D₃

levels. In the present study, using a modest dose of D₃ (800 IU per day) in the upper range of current official recommendations (32), serum levels of 25[OH]D₃ increased by approximately 8 ng/ml in individuals who received D₃ +/- calcium, going from an average of approximately 19 ng/ml (inadequate) to approximately 27 ng/ml (near sufficient), whereas mean 25(OH) D₂ levels were lower after supplementation by approximately one ng/ml in those supplemented with D₃ +/- calcium. Vitamin D₃, synthesized in the skin under the influence of solar ultraviolet light, is the primary source of vitamin D (other than dietary supplements) for most people (33). Thus, the clinical significance of reduced circulating 25[OH]D₂ levels due to vitamin D₃ supplementation may not be relevant since 25[OH]D₃ (derived from D₃) is considered the predominant form in the circulation; nevertheless, the physiological significance of 25[OH]D₂ independent of 25[OH]D₃ remains unclear at present.

Results from the present pilot study support, but do not prove, the hypothesis that vitamin D₃ supplementation, alone or in combination with calcium, alters serum αT and γT levels. At baseline, serum 25[OH]D and γT were significantly inversely correlated, whereas αT was positively, but not statistically significantly, associated with 25[OH]D, consistent with previously published findings (11). Lower αT and a trend towards lower γT were observed after D₃ supplementation (Table 3 and Figure 1); however, the sample size was too small to detect the estimated absolute treatment effect on γT. The demonstration of a possible causal relationship between sub-optimal 25(OH) D₃ levels and circulating γT is particularly important, as the means by which γT levels are regulated physiologically are not known. Given the observed associations between 25(OH) and γT in this and a previous study (11), vitamin D would be predicted to account for approximately 5-20% of the variability in circulating γT if a causal relationship existed. The observed borderline significant changes in response to D₃ supplementation (about 12% overall for the combined groups receiving D₃) are consistent with such a postulated effect, where supplementation with D₃ changed vitamin D status from deficiency to near adequate levels. As in a previous study (11), we also observed a significant inverse association between 25[OH]D and γT at baseline and after supplementation (Figure 3). The previous observation that this inverse correlation was strongest in African Americans and Latinos (11) takes on greater significance in light of the current study and suggests that future efforts might be targeted towards a specifically vitamin D-deficient population to fully establish whether there is a causal relationship. The previously reported positive correlation of serum αT and 25[OH]D levels (11) was also observed in the baseline levels of the participants in the present study (although it was not statistically significant); however, vitamin D₃ supplementation in the current study clearly did not increase circulating αT, as αT levels were significantly lower after supplementation in the calcium plus vitamin D₃ group. The positive association for αT in the previous study may have been due to the multi-ethnic population studied (11) and/or the confounding effects of αT supplementation in that population. This effect was not tested in the current study, as there was no significant correlation between αT and γT levels at baseline and no αT was provided as a supplement. Another explanation may be that vitamin D deficiency does indeed cause both tocopherols to rise but αT is depleted to a greater extent because of the increased oxidation associated with an inflammatory state resulting from vitamin D deficiency. Evidence for such a mechanism is provided in cell culture studies where it was observed that inducing oxidative stress causes γT to rise in relation to αT; however, if NO synthesis and a corresponding oxidative assault are inhibited, then levels of both tocopherols would rise in response to the inflammatory trigger (34). In a separate analysis of the subjects used in the current study (35), biomarkers of inflammation were observed to be lower in vitamin D₃-treated subjects, consistent with the hypothesis that tocopherols were lower as a result of decreased inflammation. The potentially interactive effects of calcium with vitamin D₃ seen for tocopherols in the present study are intriguing and warrant further investigation to determine the mechanism of action.

Previous reports indicated that serum 25[OH]D levels were inversely associated with BMI (17, 18), suggesting a possible influence of obesity and its associated effects on leptin on metabolic pathways of vitamin D and /or its precursors. In the current study the majority (82%) of study participants were overweight or obese and their weight remained constant over the 6-month experimental period (mean BMI: baseline, $30.1 \pm 6.1 \text{ kg/m}^2$; 6 months, $30.2 \pm 6.2 \text{ kg/m}^2$), as did serum leptin levels (Table 3). Therefore, our results suggest that: 1) supplementing 800 IU of vitamin D₃ daily for 6 months effectively raised vitamin D status via increased circulating 25[OH]D₃ levels in this middle aged/elderly, overweight study population; 2) supplemental D₃ may depress 25[OH]D₂ levels (46% in the vitamin D₃ group and 21% in the calcium plus vitamin D₃ group); and 3) supplemental 25[OH]D₃ had no significant impact on serum leptin levels. In addition, we found no evidence for increased levels of circulating vitamin D₃ in overweight individuals, indicating that weight and/or leptin did not significantly affect the conversion of circulating vitamin D₃ to 25[OH]D₃.

The strengths of the present study included the randomized, double-blind, placebo-controlled trial design; high protocol adherence by study participants; and that it is the first study to examine both the independent and combined effects of calcium and vitamin D₃ on serum antioxidant micronutrients and specific vitamin D metabolites. Limitations of the current study included the relatively small sample size in each treatment group and the high BMI levels of most subjects. However, the findings from this pilot study are nevertheless novel, consistent with previous observational and cell culture data that point to a possible causal relationship between vitamin D deficiency and circulating tocopherol levels in a general population, and provide important data for the future designs of studies to assess the effects of vitamin D₃ supplementation on biochemical and nutritional parameters.

In summary, in addition to the statistically significant increase in serum 25[OH]D₃ and α T levels and a significant baseline association between 25[OH]D and γ T, the current results suggest that a modest dose of 800 IU of supplemental vitamin D₃, alone or in combination with 2 g of elemental calcium daily for 6 months, may affect circulating 25[OH]D₂ and γ T levels as well, but appears to have no appreciable effects on retinol or leptin levels. The current study cannot rule out the possibility that significantly higher levels of 25[OH]D might impact retinol levels since vitamin D levels were generally low in the study population and the intervention raised circulating levels only moderately. Recommendations for optimal vitamin D levels in nutrition remain controversial and many authorities recommend substantially higher levels clinically for disease prevention (36, 37) which may have additional impacts on lipid and inflammatory biomarkers. No evidence was found for leptin blocking the conversion of D₃ to 25[OH]D₃ *in vivo* or for reductions in leptin levels by vitamin D₃ supplementation. Future larger clinical trials are needed to confirm these observations and to explore the effects of higher doses of vitamin D +/- calcium on physiologically relevant antioxidants and vitamins.

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Figure 1.

Association of serum γ -tocopherol (γ T) with 25(OH)D before and after vitamin D₃ supplementation. All 43 subjects receiving supplemental D₃ +/- calcium were stratified by baseline 25(OH)D level into quartiles. Mean serum γ T level + SE for each quartile was plotted against the median 25(OH)D level as measured by ELISA assay for each quartile. Solid figures represent baseline data and open figures the values for the same subjects after 6 mo of vitamin D₃ supplementation.

Table 1

Selected baseline^a characteristics and dietary intakes of the study participants (n = 92).

	Treatment Group				P ^b
	Placebo (n = 23)	Calcium (n = 23)	Vitamin D ₃ (n = 23)	Calcium +Vitamin D ₃ (n = 23)	
<u>Characteristics</u>					
Age, y	58.5 (8.2)	61.9 (8.2)	60.2 (8.1)	62.1 (7.5)	0.39
Men (%)	70	70	70	70	1.00
White (%)	74	83	65	61	0.39
College graduate (%)	65	61	57	44	0.53
Current smoke (%)	13	9	13	13	0.96
Take multivitamin (%)	30	30	26	39	0.86
Body mass index, kg/m ²	30.6 (7.2)	29.4 (5.5)	28.9 (5.6)	31.6 (6.0)	0.44
Waist to hip ratio	0.93 (0.08)	0.92 (0.09)	0.92 (0.10)	0.98 (0.11)	0.17
<u>Mean dietary intakes</u>					
Energy, kcal/d	1,596 (528)	1,788 (691)	1,848 (821)	1,845 (752)	0.59
Calcium, mg/d ^c	618 (308)	746 (335)	843 (526)	824 (714)	0.41
Vitamin D, IU/d ^c	277 (230)	336 (202)	360 (317)	415 (316)	0.40
Fat, g/d	67 (32)	72 (35)	70 (32)	74 (28)	0.59
Alcohol, g/d	9 (14)	11 (15)	14 (18)	10 (20)	0.84

^aData are given as mean (SD) unless otherwise specified.

^bBy Fisher's exact χ^2 test for categorical variables or ANOVA for continues variables.

^cDiet plus supplements.

Table 2

Serum levels of specific vitamin D measurements at baseline and after 6 months of treatment.

<u>Analyte</u> Treatment Group	Baseline			6-month Follow-up			Absolute Rx Effect ^b		Proportional Rx Effect ^c (%)
	N	Mean (SE) ^a	P ^d	N	Mean (SE) ^a	P ^d	Mean (SE) ^a	P ^d	
<u>25(OH)D₃ (ng/mL)</u>									
Placebo	20	18.8 (1.9)		21	17.2 (1.9)		0		-
Calcium	21	23.4 (1.9)	0.10	21	22.5 (2.0)	0.06	0.8 (2.5)	0.74	3.4
Vitamin D ₃	21	19.4 (1.9)	0.84	21	28.3 (2.0)	0.0001	10.7 (2.4)	<0.0001	55.2
Calcium + Vitamin D ₃	21	18.8 (1.9)	0.98	18	26.0 (2.0)	0.002	9.0 (2.5)	0.0005	47.9
<u>25(OH)D₂ (ng/mL)</u>									
Placebo	20	2.5 (1.3)		21	2.4 (1.3)		0		-
Calcium	21	1.9 (1.3)	0.76	21	2.1 (1.4)	0.89	0.3 (1.0)	0.75	15.8
Vitamin D ₃	21	2.3 (1.3)	0.93	21	1.2 (1.3)	0.52	-1.1 (1.0)	0.26	-47.8
Calcium + Vitamin D ₃	21	4.3 (1.3)	0.35	18	3.3 (1.4)	0.64	-0.9 (1.0)	0.36	-20.9
<u>Vitamin D₃ (ng/mL)</u>									
Placebo	20	13.2 (1.5)		21	12.3 (1.6)		0		-
Calcium	21	12.7 (1.5)	0.78	21	12.7 (1.7)	0.87	0.9 (2.4)	0.70	7.1
Vitamin D ₃	21	12.5 (1.5)	0.81	21	15.8 (1.6)	0.12	4.2 (2.3)	0.08	33.6
Calcium + Vitamin D ₃	21	12.6 (1.5)	0.76	18	13.8 (1.6)	0.50	2.1 (2.3)	0.36	16.7
<u>Vitamin D₂ (ng/mL)</u>									
Placebo	20	0.44 (0.16)		21	0.42 (0.16)		0		-
Calcium	21	0.31 (0.16)	0.56	21	0.34 (0.17)	0.74	0.06 (0.19)	0.77	19.4
Vitamin D ₃	21	0.64 (0.16)	0.39	21	0.54 (0.17)	0.60	-0.08 (0.19)	0.68	-12.5
Calcium + Vitamin D ₃	21	0.49 (0.16)	0.84	18	0.45 (0.17)	0.91	-0.02 (0.19)	0.91	-4.1

^aLeast-squares means are used.^bAbsolute treatment (Rx) effect = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].^cProportional treatment (Rx) effect = (absolute treatment effect / treatment group baseline) × 100% (e.g., a proportional effect of 55% would mean that a 55% increase in the active treatment group relative to the placebo group).^dP value for difference between each active treatment group and the placebo group from repeated measures MIXED model.

Table 3Serum levels of α -tocopherol, γ -tocopherol, retinol, and leptin at baseline and after 6 months of treatment.

Analyte Treatment Group	Baseline			6-month Follow-up			Absolute Rx Effect^b		Proportional Rx Effect^c (%)	
	N	Mean (SE)^a	P^d	N	Mean (SE)^a	P^d	Mean (SE)^a	P^d		
<u>α-Tocopherol (ng/mL)</u>										
Placebo	21	13.4 (1.0)		21	14.3 (1.0)		0		-	
Calcium	21	14.3 (1.0)	0.57	21	14.2 (1.1)	0.93	-1.0 (1.1)	0.36	-7.0	
Vitamin D ₃	22	13.7 (1.0)	0.87	22	13.5 (1.0)	0.59	-1.0 (1.0)	0.32	-7.3	
Calcium + Vitamin D ₃	21	15.5 (1.0)	0.15	21	14.3 (1.0)	0.98	-2.2 (1.0)	0.04	-14.2	
<u>γ-Tocopherol (ng/mL)</u>										
Placebo	21	2.38 (0.25)		21	2.39 (0.25)		0		-	
Calcium	21	1.97 (0.26)	0.24	21	1.93 (0.26)	0.20	-0.05 (0.28)	0.85	-2.9	
Vitamin D ₃	22	2.01 (0.25)	0.28	22	1.87 (0.26)	0.14	-0.15 (0.27)	0.57	-7.5	
Calcium + Vitamin D ₃	21	2.13 (0.26)	0.47	21	1.74 (0.26)	0.07	-0.40 (0.26)	0.14	-18.8	
<u>Trans Retinol (ng/mL)</u>										
Placebo	21	548.4 (33.9)		21	513.5 (34.4)		0		-	
Calcium	21	597.4 (33.9)	0.31	21	583.1 (36.1)	0.17	20.5 (41.3)	0.62	3.4	
Vitamin D ₃	22	573.3 (33.1)	0.60	22	549.7 (34.0)	0.46	11.3 (39.8)	0.78	2.0	
Calcium + Vitamin D ₃	21	567.4 (33.9)	0.69	21	500.0 (33.9)	0.78	-32.4 (39.4)	0.41	-5.7	
<u>Leptin (ng/mL)</u>										
Placebo	21	17.2 (4.5)		21	17.6 (4.5)		0		-	
Calcium	21	18.1 (4.5)	0.89	21	17.3 (4.5)	0.97	-1.1 (2.7)	0.68	-6.0	
Vitamin D ₃	22	14.9 (4.4)	0.72	22	16.8 (4.4)	0.91	1.5 (2.5)	0.54	10.1	
Calcium + Vitamin D ₃	21	26.3 (4.5)	0.15	21	24.1 (4.5)	0.30	-2.5 (2.5)	0.33	-9.5	

^aLeast-squares means are used.^bAbsolute treatment (Rx) effect = [(treatment group follow-up) – (treatment group baseline)] – [(placebo group follow-up) – (placebo group baseline)].^cProportional treatment (Rx) effect = (absolute treatment effect / treatment group baseline) × 100% (e.g., a proportional effect of 55% would mean that a 55% increase in the active treatment group relative to the placebo group).^dP value for difference between each active treatment group and the placebo group from repeated measures MIXED model.