Calcium-Vitamin D Cosupplementation Influences Circulating Inflammatory Biomarkers and Adipocytokines in Vitamin D-Insufficient Diabetics: A Randomized Controlled Clinical Trial

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Context: To the best of our knowledge, no study has examined the effects of vitamin D-calcium cosupplementation on inflammatory biomarkers and adipocytokines in vitamin D-insufficient type 2 diabetics.

Objective: This study was performed to assess the effects of vitamin D and calcium supplementation on inflammatory biomarkers and adipocytokines in vitamin D-insufficient people with type 2 diabetes.

Methods: Totally, 118 diabetic patients were enrolled in this randomized, placebo-controlled clinical trial. After matching for age, sex, body mass index, type and dose of hypoglycemic agents, and duration of diabetes, subjects were randomly assigned into 4 groups receiving the following: 1) 50000 IU/wk vitamin D + calcium placebo; 2) 1000 mg/d calcium + vitamin D placebo; 3) 50 000 IU/wk vitamin D + 1000 mg/d calcium; or 4) vitamin D placebo + calcium placebo for 8 weeks. Blood sampling was done for the quantification of inflammatory biomarkers and adipocytokines at the study baseline and after 8 weeks of intervention.

Results: Calcium (changes from baseline: -75 ± 19 ng/ml, $P = .01$) and vitamin D alone (-56 ± 19 ng/mL, $P = .01$) and joint calcium-vitamin D supplementation (-92 ± 19 ng/mL, $P = .01$) resulted in a significant reduction in serum leptin levels compared with placebo (-9 ± 18 ng/mL). This was also the case for serum IL-6, such that calcium (-2 ± 1 pg/mL, $P < .001$) and vitamin D alone (-4 ± 1 pg/mL, $P < .001$) and their combination (-4 ± 1 pg/mL, $P < .001$) led to significant reductions compared with placebo (3 ± 1 pg/mL). After adjustment for potential confounders, individuals in the calcium (-3.1 ± 1.3, $P < .05$), vitamin D (-3.1 ± 1.3, $P < .05$), and joint calcium-vitamin D groups (-3.4 ± 1.3, $P < .05$) had greater reductions in serum TNF-α concentrations compared with placebo (0.1 ± 1.2). Individuals who received joint calcium-vitamin D supplements tended to have a decrease in serum high-sensitivity C-reactive protein levels compared with placebo after controlling for baseline levels (-1.14 ± 0.25 vs 0.02 ± 0.24 ng/mL, $P = .09$).

Conclusion: Joint calcium-vitamin D supplementation might improve systemic inflammation through decreasing IL-6 and TNF-α concentrations in vitamin D-insufficient people with type 2 diabetes. (***J Clin Endocrinol Metab*** **99: E2485–E2493, 2014)**

Diabetes is a leading cause of morbidity and mortality worldwide (1). It has been reported that the prevalence of diabetes is approximately 8.5%, worldwide (2). National estimates in Iran revealed that 7.7% of adults were affected by diabetes (3). Systemic inflammation has been linked primarily to insulin resistance, β-cell dysfunction, and type 2 diabetes (4, 5). In addition, increased inflammation is associated with diabetes complications

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Abbreviations: BMI, body mass index; hs-CRP, high-sensitivity C-reactive protein; MET, metabolic equivalent; 25(OH)D, 25-hydroxyvitamin D.

(6). Therefore, finding a strategy to reduce inflammation in diabetic patients is of great importance.

Prior observational studies have demonstrated that vitamin D and calcium intake might influence systemic inflammation (7–11). Few data are also available linking calcium intake to inflammation. An inverse association was reported between the consumption of dairy products and inflammation in healthy adults (12, 13). Calcium intake (≥600 mg/d) was also inversely associated with serum levels of leptin, adiponectin, and inflammatory biomarkers (14). Despite such information from observational studies, few data from clinical trials are available indicating the effects of calcium and vitamin D on inflammation. Vitamin D replenishment of persons with type 2 diabetes has been shown to improve inflammatory biomarkers and glycemic control (15). However, findings in nondiabetic adults are conflicting (16–18).

Calcium and vitamin D has been hypothesized to act jointly rather than independently (19). It seems that their combined effects on inflammation, and consequently on glycemic control in persons with diabetes, might be better than their individual effects. Few clinical trials, mostly in nondiabetic adults, have examined the combined effects of calcium-vitamin D supplementation on inflammatory biomarkers (20–22). Findings from these studies are conflicting. We are aware of no study that examined the effects of calcium-vitamin D cosupplementation on inflammatory biomarkers in vitamin D-insufficient people with diabetes who are on oral hypoglycemic agents. The aim of this study was to assess the effects of vitamin D and calcium supplementation alone and in combination on inflammatory biomarkers in vitamin D-insufficient diabetic patients.

Subjects and Methods

Participants

We recruited participants in this study from Isfahan Endocrine and Metabolism Research Center between March 2012 and September 2012. Based on the formula for parallel-design randomized controlled trials, considering serum high-sensitivity C-reactive protein (hs-CRP) concentrations as a key variable and given the type I error of 5% and the study power of 90%, we needed 104 patients to be enrolled. Nonsmoker individuals aged older than 30 years with type 2 diabetes [fasting blood glucose ≥ 126 mg/dL (≥6.9 mmol/L) or 2 h postglucose load ≥ 200 mg/dL (≥11.1 mmol/L) or both] and insufficient 25-hydroxyvitamin D [25(OH)D] levels [<30 ng/mL (<75 nmol/L)] were included in this study. Patients with a history of renal failure, cancer, liver, or thyroid diseases or any other inflammatory diseases were not included in the study. Individuals with a history of allergy and those taking corticosteroids or injecting insulin were not included as well. We also did not include those taking any kind of vitamin D or calcium supplements as well as those who were pregnant or lactating and those who had a greater than 4 kg weight change during the last 3 months. Participants who were taking any kind of medication except hypoglycemic agents were not included in this study.

Overall, 120 patients who met all the inclusion criteria and expressed their willingness to participate were enrolled in the study. During the intervention, two persons were excluded from the study because of personal reasons (Figure 1). All participants provided informed written consent. The study was approved by the Bioethics Committee of Isfahan University of Medical Science and Isfahan Endocrine and Metabolism Research Center. This study has been registered at Clinicaltrials.gov with the registration number of NCT01662193.

Study design

This is a double-blind, parallel, randomized placebo-controlled clinical trial that was conducted in Isfahan, Iran, between March 2012 and April 2013. Totally, 118 diabetic patients who met the inclusion criteria completed the trial. Before randomization, we stratified participants based on age (±5 y), sex, body mass index (BMI) (±0.5 kg/m^2), type and dosage of hypoglycemic agents use, and duration of diabetes (±6 mo). All investigators and participants as well as laboratory technicians were blinded to the random assignments, except for the study technician who did the randomization. Then patients were allocated randomly into the following four arms using stratified block randomization: 1) individuals in the vitamin D group received 50 000 IU vitamin D3 per week (equivalent to an amount of 1250 μg) plus a daily placebo for calcium; 2) subjects in the calcium group received 1000 mg calcium carbonate per day plus a weekly placebo for vitamin D; 3) participants in the calcium+vitamin D group received 50 000 IU vitamin D3 per week plus 1000 mg calcium carbonate per day; and 4) individuals in the placebo group received separate placebos for calcium daily and for vitamin D weekly. Participants received supplements and placebos for 8 weeks. Calcium supplements and placebos were manufactured by Jalinus Pharmaceutical Co, and vitamin D supplements and placebos were manufactured by Dana Pharmaceutical Co. The use of calcium supplements and placebos throughout the study was checked through asking participants to bring the medication containers. Compliance to the vitamin D supplementation was assessed through the quantification of serum vitamin D levels.

Participants were requested to consume their usual diets throughout the study. They were also asked not to change their routine physical activity levels. To make sure of these recommendations during intervention, 3 days of dietary records (one weekend day and two weekdays) and 3 days of physical activity records (at the same days of dietary records) were obtained. Both dietary and physical activity records were taken at weeks 2, 4, and 6 of intervention. To determine energy and nutrient intakes, all dietary data were converted to a gram scale and then were entered to the Nutritionist 4 software. Physical activity was expressed as metabolic equivalents (METs) in hours per day. To compute the METs for each person, we multiplied the times (in hour per day) reported for each physical activity by its related METs coefficient using standard tables (23). Blood sampling was done at study baseline (wk 0) and after 8 weeks of intervention (wk 8) to quantify serum 25(OH)D, inflammatory biomarkers, and adipocytokines.

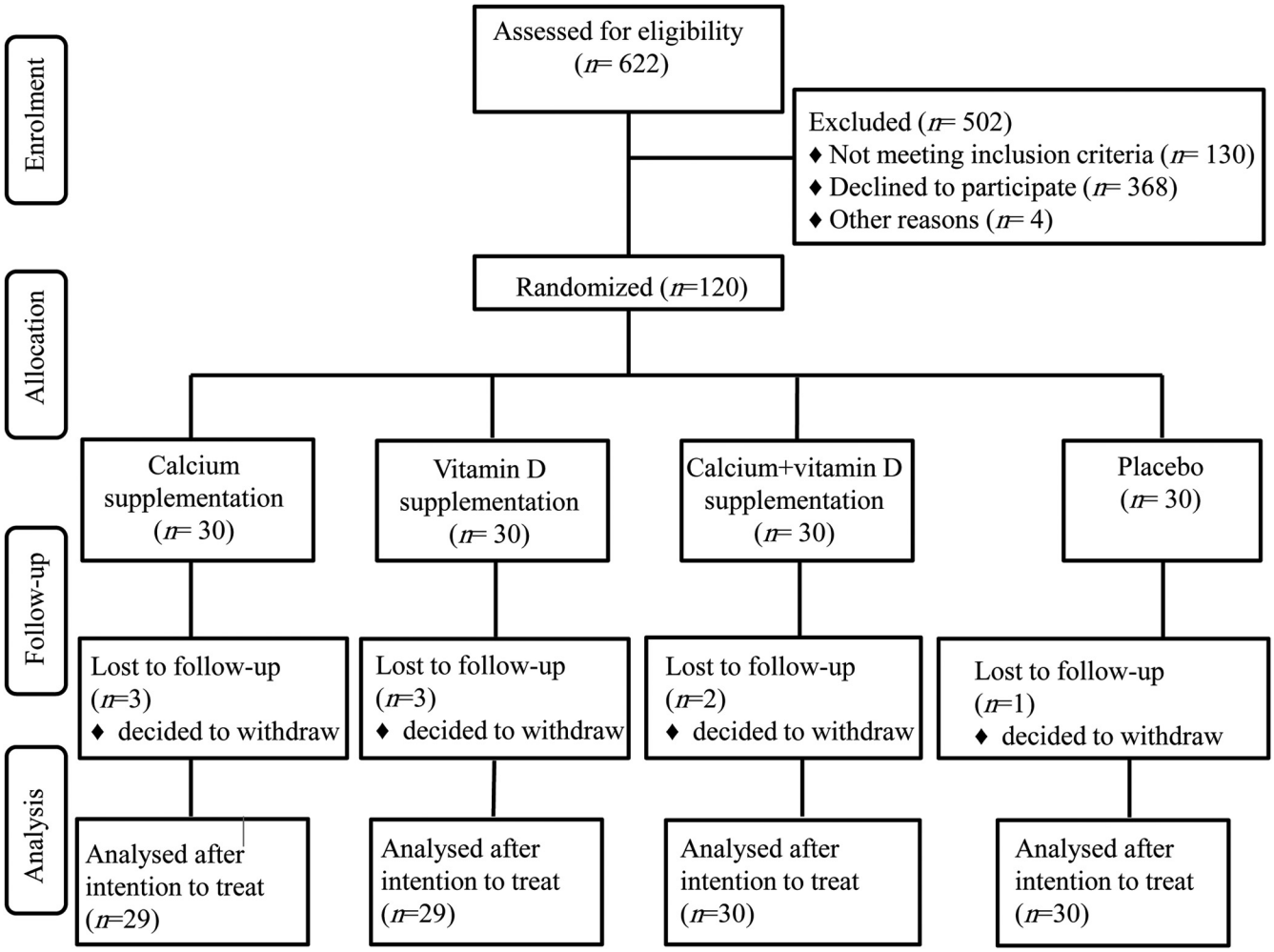

Figure 1. Flow diagram of participants.

Assessment of biomarkers

To examine circulating levels of inflammatory biomarkers and adipocytokines, 10-cc blood samples were taken after 12 hours of overnight fasting at the study baseline and after 8 weeks of intervention. All blood samples were immediately centrifuged for 10 minutes and serum was stored at −70°C until analyses. Serum adiponectin, leptin, TNF-α, and IL-6 levels were quantified by an ELISA method using Booster kits (Orgenium). The intra- and interassay coefficients of variation for adiponectin was 10% or less and 12% or less, for TNF-α 6% or less and 4% or less, and for IL-6 9.4% and 8.6%, respectively. Serum hs-CRP concentrations were assessed using an ELISA through a Hitachi 911 chemistry and immunoassay analyzer (Sentinel). The intra- and interassay coefficients of variation for serum leptin and hs-CRP levels were less than 10%. Serum 25(OH)D concentrations were examined by the RIA method.

Statistical methods

We applied the Kolmogrov-Smirnov test to ensure the normal distribution of variables. Log transformation was applied for nonnormally distributed variables. The analyses were done based on an intention-to-treat approach. Missing values were treated based on the last-observation-carried-forward method. Baseline general characteristics were examined using a one-way ANOVA for continuous variables and χ^2 for categorical variables. Data on dietary intakes and physical activity were compared by a one-way ANOVA. To determine the effects of supplementation on inflammatory biomarkers and adipocytokines, first we computed the changes from baseline by subtracting the baseline values from the end-of-trial values. Then we applied an analysis of covariance. To identify whether the significant differences were influenced by the baseline levels of inflammatory biomarkers, we adjusted for the baseline values of each biomarker. We also controlled for age, gender, and physical activity in these analyses. Additional adjustments were made for BMI to determine whether the changes are mediated through obesity. A linear regression analysis was used to identify the association between changes in serum 25(OH)D levels and changes in the concentration of each biomarker. Values of $P < .05$ were considered significant. All statistical analyses were done using the Statistical Package for Social Science version 17 (SPSS Inc).

Results

Totally, 118 participants were recruited in this study. The number of persons taking only metformin was 22, 23, 22, and 21 in calcium, vitamin D, joint calcium and vitamin D, and placebo groups, respectively. The numbers of patients

who were taking both metformin and glybanclamide were eight, seven, eight, and nine in calcium, vitamin D, joint calcium and vitamin D, and placebo groups, respectively. General characteristics of participants by intervention groups are shown in Table 1. Subjects in the calcium group were older than those in the joint calcium-vitamin D group. Distribution of participants in terms of sex, menopausal status, obesity, and central obesity was not significantly different between the four intervention arms. Mean duration of diabetes in all participants was between 4 and 5 years. Moreover, there were no significant differences among intervention groups in terms of BMI at study baseline. Mean dietary intakes of study participants, obtained from 3 days of dietary records throughout the intervention, are provided in Table 2. No significant differences in dietary energy, and macro- and micronutrient intakes were seen between the 4 intervention groups. Based on 3 days of nonconsecutive physical activity records throughout the intervention, we found no significant differences in physical activity levels among the 4 intervention groups during intervention.

Baseline serum levels of 25(OH)D across the 4 groups was not significantly different ($P = .08$) (Table 3). We found a significant increase in mean serum 25(OH)D concentrations among those in vitamin D group (11.2 ± 5.6 ng/mL at study baseline vs 35.1 ± 14.3 ng/mL after intervention; $P < .001$) as well as those in joint calcium-vitamin D group (12.2 ± 6.6 ng/mL at study baseline vs 35.4 ± 9.6 ng/mL after intervention; $P < .001$). No significant changes in this biomarker were seen in individuals in the calcium (22.3 ± 6.1 ng/mL at study baseline vs 22.2 ± 7.9 ng/mL after intervention; $P = .93$) or placebo groups (18.3 ± 6.6 ng/mL at study baseline vs 19.3 ± 7.7 ng/mL after intervention; $P = .37$). These data indicate good adherence of study participants to vitamin D supplementation. Compliance of participants with calcium supplementation was 88% based on the remaining tablets in the containers. The weight change in all groups was less than 0.5 kg, which was not statistically significant comparing the 4 groups ($P = .31$). Calcium-vitamin D cosupplementation resulted in reduced glycated hemoglobin ($P = .02$) and low-density lipoprotein LDL-cholesterol ($P = .04$) and increased high-density lipoprotein HDL-cholesterol levels ($P = .03$) compared with other groups.

Serum levels of inflammatory biomarkers and adipocytokines at study baseline are shown in Table 3. We observed no significant differences in serum concentrations of adiponectin, leptin, TNF-α, IL-6, and hs-CRP between the 4 intervention groups at study baseline.

The effects of calcium, vitamin D, and joint calcium-vitamin D supplementation on circulating levels of inflammatory biomarkers and adipocytokines are indicated in Table 4. After adjustment for baseline levels of inflammatory biomarkers, we observed that, compared with placebo, supplementation with calcium and vitamin D had no significant effects on serum adiponectin concentrations. However, calcium (changes from baseline: −75 ± 19 ng/mL, $P = .01$) and vitamin D alone (−56 ± 19 ng/mL, $P = .01$) as well as joint calcium-vitamin D supplementation (−92 ± 19 ng/mL, $P = .01$) resulted in a significant re-

Table 1. Baseline General Characteristics of Study Participants[a]

	Calcium (n = 29)[b]	Vitamin D (n = 29)[c]	Ca+D (n = 30)[d]	Placebo (n = 30)	*P* Value[e]
Age, y	53.7 ± 5.7	50.2 ± 6.6	49.8 ± 6.1	51.0 ± 6.1	0.06
Women, n (%)	15 (52)	14 (48)	15 (50)	16 (53)	.98
Postmenopause, n, %	10 (34)	9 (31)	9 (30)	10 (33)	.99
Diabetes duration, mo	53 ± 54	56 ± 38	52 ± 36	57 ± 44	.96
Obesity, n, %[f]	15 (52)	14 (48)	13 (43)	14 (47)	.93
Central obesity, n, %[g]	17 (59)	12 (41)	19 (63)	19 (63)	.27
Serum triglycerides, mg/dL	177 ± 62	168 ± 61	186 ± 89	178 ± 71	.77
Serum HDL-cholesterol, mg/dL	42 ± 7	44 ± 8	47 ± 8	46 ± 7	.11
Serum LDL-cholesterol, mg/dL	93 ± 23	81 ± 23	92 ± 31	85 ± 19	.21
Total cholesterol, mg/dL	170 ± 39[h]	139 ± 20	143 ± 27	147 ± 31	.001
HbA1c, %	6.6 ± 0.8	6.6 ± 0.8	6.7 ± 1.1	6.9 ± 0.9	.61

Abbreviations: Ca, calcium; D, vitamin D; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

[a] All values are means ± SD unless indicated.

[b] Receiving 1000 mg calcium carbonate per day plus a weekly placebo for vitamin D.

[c] Receiving 50 000 IU vitamin D3 per week plus a daily placebo for calcium.

[d] Receiving 1000 mg calcium carbonate per day plus 50000 IU vitamin D3 per week.

[e] Obtained from ANOVA.

[f] Defined as having a BMI of 30 kg/m^2 or greater.

[g] Defined as having a waist circumference of greater than 88 cm for women and greater than 102 cm for men.

[h] $P < 0.05$ compared with other groups, using Tukey's text.

Table 2. Nutrient Intakes of Study Participants Throughout the Intervention[a]

	Calcium (n = 29)[b]	Vitamin D (n = 29)[c]	Ca+D (n = 30)[d]	Placebo (n = 30)	*P* Value[e]
Energy, kcal/d	2052 ± 589	2050 ± 595	2109 ± 666	2147 ± 646	.97
Fat, g/d	89 ± 45	90 ± 46	91 ± 41	99 ± 49	.81
Calcium, mg/d	1018 ± 409	915 ± 355	968 ± 386	951 ± 349	.77
Vitamin D, μg/d	1.7 ± 1.3	1.5 ± 1.1	1.5 ± 1.3	1.5 ± 1.0	.92
Vitamin C, mg/d	144 ± 115	151 ± 110	139 ± 91	121 ± 93	.72
Vitamin E, mg/d	24 ± 25	23 ± 22	26 ± 21	30 ± 27	.73
Magnesium, mg/d	278 ± 87	262 ± 81	278 ± 90	261 ± 98	.79
Cholesterol, mg/d	231 ± 88	237 ± 128	208 ± 153	194 ± 94	.47
Vitamin B2, mg/d	2 ± 0.7	2 ± 0.6	2 ± 1	2 ± 0.6	.71
Iron, mg/d	12 ± 4	12 ± 5	13 ± 4	12 ± 6	.78
Zinc, mg/d	8 ± 2	8 ± 3	8 ± 3	8 ± 3	.70
Selenium, mg/d	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.04 ± 0.01	.84
Omega 3, g/d	0.2 ± 0.3	0.2 ± 0.3	0.2 ± 0.3	0.1 ± 0.1	.38
MUFA, g/d	28 ± 13	29 ± 15	28 ± 12	31 ± 14	.86

Abbreviations: Ca, calcium; D, vitamin D; MUFA, monounsaturated fatty acid.

[a] All values are means ± SD unless indicated.

[b] Receiving 1000 mg calcium carbonate per day plus a weekly placebo for vitamin D.

[c] Receiving 50 000 IU vitamin D3 per week plus a daily placebo for calcium.

[d] Receiving 1000 mg calcium carbonate per day plus 50 000 IU vitamin D3 per week.

[e] Obtained from ANOVA.

duction in serum leptin levels compared with placebo (−9 ± 18 ng/mL). This was also the case for serum IL-6; such that calcium (−2 ± 1 pg/mL, $P < .001$) and vitamin D alone (−4 ± 1 pg/mL, $P < .001$) and their combination (−4 ± 1 pg/mL, $P < .001$) led to significant reductions compared with placebo (3 ± 1 pg/mL). After adjustment for potential confounders, individuals in calcium (−3.1 ± 1.3, $P < .05$), vitamin D (−3.1 ± 1.3, $P < .05$) and joint calcium-vitamin D supplementation groups (−3.4 ± 1.3, $P < .05$) had greater reductions in serum TNF-α concentrations compared with placebo (0.1 ± 1.2). Individuals who received joint calcium-D supplements tended to have a decrease in serum hs-CRP levels compared with placebo after controlling for baseline levels (−1.14 ± 0.25 vs 0.02 ± 0.24 ng/mL, $P = .09$). Further adjustments for BMI did not remarkably alter the above-mentioned findings; however, the significant effect of calcium, vitamin D, and calcium-vitamin D cosupplementation on serum TNF-α concentrations became nonsignificant after adjustment for BMI, indicating that the effect might be mediated through obesity.

Findings from a linear regression analysis revealed that increased serum 25(OH)D concentrations were associated with a significant reduction in serum leptin ($\beta = -.19$, $P < .001$), IL-6 ($\beta = -.11$, $P = .01$), and TNF-α ($\beta = -.10$, $P = .02$).

Table 3. The Baseline 25(OH)D, Inflammatory, and Adipocytokine Levels of Study Participants[a]

	Calcium (n = 29)[b]	Vitamin D (n = 29)[c]	Ca+D (n = 30)[d]	Placebo (n = 30)	*P* Value[e]
25(OH)D, ng/mL	22.3 ± 6.1	11.2 ± 5.6	12.2 ± 6.6	18.3 ± 6.6	.08
Adipocytokines					
Leptin, ng/mL	166 ± 142	209 ± 134	229 ± 163	234 ± 188	.34
Adiponectin, ng/mL	2.5 ± 0.4	2.8 ± 0.5	3.7 ± 0.7	3.0 ± 0.5	.23
Inflammatory biomarkers					
IL-6, pg/mL	4.5 ± 7.1	7.9 ± 6.0	7.5 ± 5.1	6.7 ± 3.0	.10
TNF-α, pg/mL	11.1 ± 10.8	11.5 ± 6.3	13.0 ± 8.6	10.2 ± 3.6	.55
hs-CRP, ng/mL	2.0 ± 1.4	2.3 ± 1.6	1.9 ± 1.1	2.2 ± 1.0	.68

Abbreviations: Ca, calcium; D, vitamin D.

[a] All values are means ± SD unless indicated.

[b] Receiving 1000 mg calcium carbonate per day plus a weekly placebo for vitamin D.

[c] Receiving 50 000 IU vitamin D3 per week plus a daily placebo for calcium.

[d] Receiving 1000 mg calcium carbonate per day plus 50 000 IU vitamin D3 per week.

[e] Obtained from ANOVA.

Table 4. The Effect of Vitamin D and Calcium Supplementation on Inflammation and Adipocytokine Biomarkers[a]

	Calcium (n = 29)[b]	Vitamin D (n = 29)[c]	Ca+D (n = 30)[d]	Placebo (n = 30)	*P* Value[e]
Adiponectin, ng/mL					
Crude	−1.3 ± 0.3	−1.2 ± 0.5	−0.1 ± 1.0	−0.1 ± 0.5	.32
Model 1[f]	−1.1 ± 0.6	−1.3 ± 0.6	0.2 ± 0.6	−0.3 ± 0.6	.29
Model 2[g]	−1.2 ± 0.6	−1.3 ± 0.6	0.1 ± 0.6	−0.3 ± 0.6	.31
Leptin, ng/mL					
Crude	−40 ± 28	−72 ± 31	−88 ± 30	−29 ± 39	.53
Model 1	−75 ± 19	−56 ± 19	−92 ± 19[h]	−9 ± 18	.01
Model 2	−75 ± 20	−56 ± 20	−91 ± 19	−9 ± 19	.01
IL-6, pg/mL					
Crude	−3 ± 1	−5 ± 1	−5 ± 1[h]	3 ± 1	<.001
Model 1	−2 ± 1	−5 ± 1	−4 ± 1	3 ± 1	<.001
Model 2	−2 ± 1	−4 ± 1	−4 ± 1	3 ± 1	<.001
hs-CRP, ng/mL					
Crude	0.01 ± 0.35	−0.48 ± 0.40	−0.75 ± 0.36	−0.06 ± 0.26	.28
Model 1	−0.07 ± 0.25	−0.24 ± 0.24	−1.14 ± 0.25	0.02 ± 0.24	.09
Model 2	−0.09 ± 0.25	−0.25 ± 0.24	−1.19 ± 0.25	0.08 ± 0.24	.09
TNF-α, pg/mL					
Crude	−2.7 ± 1.9	−2.1 ± 2.3	−5 ± 1.6[h]	0.26 ± 1.3	.02
Model 1	−3.1 ± 1.3	−3.1 ± 1.3	−3.4 ± 1.3	0.1 ± 1.2	.04
Model 2	−3.1 ± 1.3	−3.1 ± 1.3	−3.2 ± 1.3	−1.0 ± 1.3	.23

Abbreviations: Ca, calcium; D, vitamin D.

[a] All values are means ± SD unless indicated.

[f] Adjusted for baseline levels, age, sex, and physical activity.

[g] Further adjusted for BMI.

[b] Receiving 1000 mg calcium carbonate per day plus a weekly placebo for vitamin D.

[c] Receiving 50 000 IU vitamin D3 per week plus a daily placebo for calcium.

[d] Receiving 1000 mg calcium carbonate per day plus 50 000 IU vitamin D3 per week.

[e] Obtained from ANOVA.

[h] Compared with others, $P < .05$.

Discussion

In this randomized placebo-controlled clinical trial of vitamin D and calcium supplementation in patients with type 2 diabetes, calcium-vitamin D cosupplementation resulted in a significant reduction in circulating leptin, IL-6, and TNF-α concentrations compared with other groups. Moreover, individuals in the calcium group tended to have greater reductions in serum hs-CRP levels than the other groups. However, supplementation with calcium and vitamin D had no significant effects on serum adiponectin levels after adjustment for baseline levels. To the best of our knowledge, this study is among the first investigations that examined the effects of joint calcium-vitamin D supplementation on inflammatory biomarkers and adipocytokines of type 2 diabetic patients who were on oral hypoglycemic agents. Although metformin might have some antiinflammatory effects, the groups were matched in terms of type and dosage of oral hypoglycemic agent use. Therefore, the effects we found would be independent of oral hypoglycemic agent use.

It is currently recognized that inflammation is involved in the pathophysiology of type 2 diabetes (6, 24). Previous studies have shown that separate vitamin D or calcium supplementations may improve insulin sensitivity and promote β-cell survival (25–27). However, there are very limited data from human studies that examined the effect of vitamin D or calcium supplementation on systemic inflammation in diabetes (15, 28). We found that calcium plus vitamin D supplementation led to decreased levels of serum leptin, IL-6, and TNF-α concentrations. Previously reported trials about the effect of single vitamin D or calcium supplementation on inflammatory biomarkers in nondiabetic subjects have shown conflicting results (20, 21). In a study on British adults of Bangladeshi origin, 54 vitamin D-deficient subjects were randomized and given either a high dose (50 000 IU) or a low dose (500 IU) of a depot (oily) injection of cholecalciferol, every 3 months, during 1 year. The investigators came to the conclusion that reductions in hs-CRP levels were greater in the high than in the low treatment group (8). Others found that a daily supplement of 83.3 μg vitamin D for 12 months among 200 healthy overweight subjects might beneficially influence circulating levels of TNF-α (25). In a 1-year supplementation study with cholecalciferol in 332 persons, reductions in serum IL-6 levels were found (29).

Calcium supplementation has been shown to decrease tumor-promoting proinflammatory markers in sporadic colorectal adenoma patients (20). However, some studies did not find any beneficial effect of vitamin D or calcium supplementation on inflammation (18, 30). Improving vitamin D status through supplementation with different dosages of vitamin D3 in 305 healthy postmenopausal women did not affect inflammatory biomarkers (18). Another trial has shown that supplementation with 50 000 IU/wk vitamin D for 12 weeks cannot decrease proinflammatory cytokines in 90 subjects with coronary artery disease and vitamin D deficiency (30). These studies were short in duration and had included few subjects.

All of the above-mentioned studies used vitamin D or calcium supplementation alone, and we are aware of only a few studies that examined joint calcium-vitamin D supplementation (20, 21). In a study on 90 type 2 diabetic patients with mean 25(OH)D levels of 44 nmol/L, it has been indicated that a daily intake of vitamin D-fortified Doogh (a yogurt based beverage) for 12 weeks improved inflammatory markers, and extra calcium conferred additional benefit only for adiponectin (28). Findings from a clinical trial in colorectal adenoma patients, whose serum 25(OH)D levels were less than 30 ng/mL, revealed that 2 g/d calcium and/or 800 IU/d vitamin D supplementation for 6 months did not significantly change inflammatory biomarkers (20). Moreover, in another randomized controlled trial, daily supplementation with 500 mg calcium citrate and 700 IU vitamin D3 for 3 years had no significant effects on markers of systemic inflammation among those with normal fasting glucose (21). It is worth mentioning that the latter was a post hoc analysis of a randomized controlled trial designed for assessing bone mineral density. This might explain why the investigators could not reach the beneficial effects of joint supplementation on inflammation. Furthermore, participants of that study were apparently healthy individuals who might have near the normal levels of inflammatory biomarkers.

This is in opposite to our study, in which participants were persons with diabetes with elevated levels of inflammation. In addition, the different dosages of calcium and vitamin D can provide further reasons for discrepant findings. It seems that using appropriate doses of calcium and vitamin D supplements in vitamin D insufficient type 2 diabetic patients might help in controlling the elevated levels of inflammatory biomarkers and adipocytokines. Because systemic inflammation is closely involved in the pathogenesis of type 2 diabetes and associated complications such as dyslipidemia and atherosclerosis, lowering inflammatory biomarkers and adipocytokines through the use of calcium-vitamin D supplements might result in better glycemic and metabolic control of diabetic patients.

Several mechanisms may explain the beneficial effects of calcium and vitamin D supplementation on inflammatory biomarkers and adipocytokines in type 2 diabetic patients. Vitamin D interacts with vitamin D response elements in the promoter region of cytokine genes to interfere with nuclear transcription factors implicated in cytokine generation and action. Moreover, vitamin D can down-regulate the activation of nuclear factor-κB, which is an important regulator of genes encoding proinflammatory cytokines implicated in insulin resistance. Also, vitamin D interferes with cytokine generation by up-regulating the expression of calbindin, a cytosolic calcium-binding protein found in many tissues including pancreatic β-cells (31). The mechanism of effects of calcium on inflammation is still unclear. However, it has been claimed that calcitriol regulates the macrophage production of inflammatory factors via calcium-dependent mechanism. Therefore, reducing circulating calcitriol levels via increasing dietary calcium intake or calcium supplementation may regulate the macrophage and thereby attenuate inflammation (32). In addition, it seems that calcium intake might influence inflammation through the suppression of PTH. Circulating levels of IL-6 and TNF-alpha were elevated in primary hyperparathyroidism (33). Therefore, it seems that PTH can regulate circulating levels of IL-6 and TNF-α, which stimulate the production of hs-CRP.

Strengths of this study include the double-blind randomized placebo controlled design, quantification of serum 25(OH)D levels to examine the adherence to the vitamin D supplementation, the relatively good compliance with the supplement use, and taking the variety of confounders including baselines levels of biomarkers into account. Moreover, due to the effect of season on vitamin D status, we confined the intervention into one season. Some limitations must also be considered. This study was conducted during summer. Therefore, the vitamin D status of subjects might not accurately reflect the effect of supplements. However, all patients were vitamin D insufficient at study baseline, even in summer. Furthermore, to assess compliance, we measured serum vitamin D levels at the end, not throughout, the study (eg, wk 4). Because the study has been done among those who were using oral hypoglycemic agents, the findings cannot easily be extrapolated to other diabetic patients (for example, those who are injecting insulin). This study cannot suggest the appropriate dosages for vitamin D and calcium supplements for diabetic patients. To reach this, other studies with different dosages of calcium and vitamin D are required. In addition, a short duration of intervention might prohibit us to observe the effects of supplementation on some biomarkers of inflammation.

In conclusion, joint calcium-vitamin D supplementation resulted in an improved status of inflammation in vitamin D-insufficient type 2 diabetic patients. Further studies to determine the appropriate doses of calcium and vitamin D for these patients are warranted.

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References

1. **Mokdad AH, Ford ES, Bowman BA, et al.** Prevalence of obesity, diabetes, and obesity-related health risk factors. *JAMA*. 2003;289:76–79.
2. **Wild S, Roglic G, Green A, et al.** Global prevalence of diabetes. *Diabetes Care*. 2004;27:1047–1053.
3. **Esteghamati A, Gouya MM, Abbasi M, et al.** Prevalence of diabetes and impaired fasting glucose in the adult population of Iran. *Diabetes Care*. 2008;31:96–98.
4. **Hu FB, Meigs JB, Li TY, et al.** Inflammatory markers and risk of developing type 2 diabetes in women. *Diabetes*. 2004;53:693–700.
5. **Pradhan AD, Manson JE, Rifai N, et al.** C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA*. 2001;286:327–334.
6. **King GL.** The role of inflammatory cytokines in diabetes and its complications. *J Periodontol*. 2008;79:1527–1534.
7. **Pittas AG, Dawson-Hughes B, Li T, et al.** Vitamin D and calcium intake in relation to type 2 diabetes in women. *Diabetes Care*. 2006;29:650–656.
8. **Timms PM, Mannan N, Hitman GA, et al.** Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: mechanisms for inflammatory damage in chronic disorders? *QJM*. 2002;95:787–796.
9. **Chagas CEA, Borges MC, Martini LA, et al.** Focus on Vitamin D, inflammation and type 2 diabetes. *Nutrients*. 2012;4:52–67.
10. **Palomer X, González-Clemente JM, Blanco-Vaca F, et al.** Role of vitamin D in the pathogenesis of type 2 diabetes mellitus. *Diabetes Obes Metab*. 2008;10:185–197.
11. **Danescu LG, Levy S, Levy J.** Vitamin D and diabetes mellitus. *Endocrine*. 2009;35:11–17.
12. **Panagiotakos DB, Pitsavos CH, Zampelas AD, et al.** Dairy products consumption is associated with decreased levels of inflammatory markers related to cardiovascular disease in apparently healthy adults: the ATTICA study. *J Am Coll Nutr*. 2010;29:357–364.
13. **Esmaillzadeh A, Azadbakht L.** Dairy consumption and circulating levels of inflammatory markers among Iranian women. *Public Health Nutr*. 2010;13:1395–1402.
14. **da Silva Ferreira T, Torres MR, Sanjuliani AF.** Dietary calcium intake is associated with adiposity, metabolic profile, inflammatory state and blood pressure, but not with erythrocyte intracellular calcium and endothelial function in healthy pre-menopausal women. *Br J Nutr*. 2013;110:1079–1088.
15. **Shab-Bidar S, Neyestani TR, Djazayery A, et al.** Improvement of vitamin D status resulted in amelioration of biomarkers of systemic inflammation in the subjects with type 2 diabetes. *Diabetes Metab Res Rev*. 2012;28:424–430.
16. **Grossmann RE, Zughaier SM, Liu S, et al.** Impact of vitamin D supplementation on markers of inflammation in adults with cystic fibrosis hospitalized for a pulmonary exacerbation. *Eur J Clin Nutr*. 2012;66:1072–1074.
17. **Wamberg L, Kampmann U, Stødkilde-Jørgensen H, et al.** Effects of vitamin D supplementation on body fat accumulation, inflammation, and metabolic risk factors in obese adults with low vitamin D levels—results from a randomized trial. *Eur J Intern Med*. 2013;24:644–649.
18. **Wood AD, Secombes KR, Thies F, et al.** Vitamin D3 supplementation has no effect on conventional cardiovascular risk factors: a parallel-group, double-blind, placebo-controlled RCT. *J Clin Endocrinol Metab*. 2012;97:3557–3568.
19. **Noda M, Mizoue T.** Relation between dietary calcium and vitamin D and risk of diabetes and cancer: a review and perspective. *J Clin Metab Diabetes*. 2010;1:55–60.
20. **Hopkins MH, Owen J, Ahearn T, et al.** Effects of supplemental vitamin D and calcium on biomarkers of inflammation in colorectal adenoma patients: a randomized, controlled clinical trial. *Cancer Prev Res (Phila)*. 2011;4:1645–1654.
21. **Pittas AG, Harris SS, Stark PC, et al.** The effects of calcium and vitamin D supplementation on blood glucose and markers of inflammation in nondiabetic adults. *Diabetes Care*. 2007;30:980–986.
22. **Gannage-Yared MH, Azoury M, Mansour I, et al.** Effects of a short-term calcium and vitamin D treatment on serum cytokines, bone markers, insulin and lipid concentrations in healthy post-menopausal women. *J Endocrinol Invest*. 2006;26:748–753.
23. **Ainsworth BE, Haskell WL, Whitt MC, et al.** Compendium of physical activities: an update of activity codes and MET intensities. *Med Sci Sports Exerc*. 2000;32:498–504.
24. **Spranger J, Kroke A, Möhlig M, et al.** Inflammatory cytokines and the risk to develop type 2 diabetes: results of the Prospective Population-Based European Prospective Investigation into Cancer and Nutrition (EPIC)-Potsdam Study. *Diabetes*. 2003;52:812–817.
25. **Inomata S, Kadowaki S, Yamatani T, et al.** Effect of 1α25 (OH)-vitamin D3 on insulin secretion in diabetes mellitus. *Bone Miner*. 1986;1:187–192.
26. **Sanchez M, de la Sierra A, Coca A, et al.** Oral calcium supplementation reduces intraplatelet free calcium concentration and insulin resistance in essential hypertensive patients. *Hypertension*. 1997;29:531–536.
27. **Major GC, Alarie F, Dore J, et al.** Supplementation with calcium,

vitamin D enhances the beneficial effect of weight loss on plasma lipid and lipoprotein concentrations. *Am J Clin Nutr*. 2007;85:54–59.

28. **Neyestani TR, Nikooyeh B, Alavi-Majd H, et al.** Improvement of vitamin D status via daily intake of fortified yogurt drink either with or without extra calcium ameliorates systemic inflammatory biomarkers, including adipokines, in the subjects with type 2 diabetes. *J Clin Endocrinol Metab*. 2012;97:2005–2011.
29. **Beilfuss J, Berg V, Sneve M, et al.** Effects of a 1-year supplementation with cholecalciferol on interleukin-6, tumor necrosis factor-α and insulin resistance in overweight and obese subjects. *Cytokine*. 2012;60:870–874.
30. **Sokol SI, Srinivas V, Crandall JP, et al.** The effects of vitamin D repletion on endothelial function and inflammation in patients with coronary artery disease. *Vasc Med*. 2012;17:394–404.
31. **Seshadria KG, Tamilselvana B, Rajendrana A.** Role of vitamin D in diabetes. *J Endocrinol Metab*. 2011;1:47–56.
32. **Sun X, Zemel MB.** Calcitriol and calcium regulate cytokine production and adipocyte—macrophage cross-talk. *J Nutr Biochem*. 2008;19:392–399.
33. **Grey A, Mitnick MA, Shapses S, Ellison A, Gundberg C, Insogna K.** Circulating levels of interleukin-6 and tumor necrosis factor-α are elevated in primary hyperparathyroidism and correlate with markers of bone resorption—a clinical research center study. *J Clin Endocrinol Metab*. 1996;81:3450–3454.