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Increased Serum Total and Free 25-Hydroxyvitamin D with Daily Intake of Cholecalciferol-Fortified Skim Milk: A Randomized Controlled Trial in Colombian Adolescents☆

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A B S T R A C T

Background: The efficacy of cholecalciferol (vitamin D3) food fortification in low- and middle-income countries near the Equator is unknown.

Objectives: We examined the effects of providing cholecalciferol-fortified skim milk to adolescents and their mothers on serum total 25(OH)D, free 25(OH)D, and vitamin D–binding protein (DBP) concentrations in a randomized controlled trial.

Methods: We randomly assigned 80 Colombian families each with a child aged 12–14.5 y and their mother 1 L of skim milk daily, either fortified with 2400 IU (60 μg) cholecalciferol or unfortified, for 6 wk. We prescribed 500 mL of milk daily to adolescents; mothers consumed the remainder ad libitum. We estimated intent-to-treat effects as the between-arm difference in the change in serum total and free 25(OH)D and DBP concentrations from baseline to the end of follow-up. Secondary analyses included stratification by baseline characteristics and per-protocol comparisons.

Results: Among adolescents, fortification effects (95% CI) on serum total 25(OH)D, free 25(OH)D, and DBP concentrations were 5.4 nmol/L (2.1, 8.8 nmol/L), 0.6 pmol/L (−0.2, 1.4 pmol/L), and −416 nmol/L (−944, 112 nmol/L), respectively. Effects on total 25(OH)D were stronger in adolescents with lower DBP concentrations, darker skin, less sunlight exposure, and higher compliance than in their respective counterparts. Fortification increased free 25(OH)D concentrations in high compliers. Among mothers, the effects (95% CI) on total 25(OH)D and DBP concentrations were 4.0 nmol/L (0.6, 7.5 nmol/L) and −128 nmol/L (−637, 381 nmol/L), respectively. There were no adverse events.

Conclusions: Provision of cholecalciferol-fortified skim milk increases serum total 25(OH)D concentrations in Colombian adolescents and adult women. *J Nutr 20XX;x:xx–xx.*

Keywords: vitamin D fortification, skim milk, 25-hydroxyvitamin D, free vitamin D, vitamin D–binding protein, Colombia

Introduction

Low vitamin D (LVD) serostatus is highly prevalent worldwide, with variations by geographic location, age, and ethnicity [1]. Although the clinical consequences of suboptimal vitamin D status beyond the musculoskeletal system remain a matter of debate [2, 3], finding effective and safe interventions to enhance vitamin D status at the population level is an important public health priority. Increasing exposure to sunlight may not be safe for everyone because of the skin cancer risk and may not be achievable year-round because of seasonal and climatic variation. Supplementation effectively improves vitamin D concentration but could induce toxicity, and it may not be widely available or within the purchasing reach in all settings. Because few frequently consumed foods naturally carry cholecalciferol (vitamin D3) at high concentrations, fortification of common foodstuffs is recognized as an effective and safe intervention to prevent LVD serostatus [4]. A mean dose of 16.2 μg

Abbreviations used: CLIA, chemiluminescence immunoassay; DBP, vitamin D–binding protein; LVD, low vitamin D; VDD, vitamin D deficiency.

☆ This trial was registered at ClinicalTrials.gov (identifier: NCT01909622).

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cholecalciferol/d from fortified foods results in an estimated 21.2-nmol/L increase in total 25(OH)D concentration [4]. Nevertheless, extremely few countries have implemented mandatory cholecalciferol fortification of common foods [5, 6]. Although its effectiveness has been uneven [7], in some of these settings, fortification has substantially reduced the prevalence of vitamin D deficiency (VDD) [8].

Food fortification with cholecalciferol could prove particularly valuable in low- and middle-income countries where poor vitamin D serostatus can be highly prevalent [9, 10], including tropical regions where sunlight avoidance is common and supplement use is low. In Mexico, for example, a nationally representative survey revealed that 37% of school-aged children [11] and adult women [12] had LVD. In Colombia, one-third of adult women and 20% of adolescents exhibited LVD at the national level [13]. However, evidence of the efficacy of cholecalciferol fortification in these settings is scant. Furthermore, previous studies of cholecalciferol fortification have focused on its effects on total 25(OH)D; nonetheless, approximately 88% of 25(OH)D is bound to vitamin D–binding protein (DBP) and is not bioavailable [14]. It is of substantial interest to learn whether a scalable public health intervention, such as milk fortification, could increase the free, highly bioavailable form of 25(OH)D or could downregulate DBP by increasing vitamin D intake from the diet. Another remaining question on cholecalciferol fortification is whether its effect on total 25(OH)D is heterogeneous with respect to background characteristics of participants related to vitamin D status, including skin pigmentation, sunlight exposure, intake from diet, and DBP concentrations. It is also unknown whether milk fortification can be effective in a family intervention because previous studies have focused on individuals.

We conducted a double-blind, randomized controlled trial to test the effects of providing families with adolescents and their mothers with cholecalciferol-fortified skim milk on serum total 25(OH)D concentrations. The primary targets of the intervention were adolescent children; their mothers were the secondary targets. Secondary aims involved assessing the effect of the intervention on serum free 25(OH)D (in the adolescents) and DBP and examining whether the effect on total 25(OH)D differed according to the background characteristics of the participants.

Methods

Study design and population

The study was conducted in the capital city of Colombia, Bogotá, located at latitude 4° 36′ and at an altitude of 2640 m above sea level. The city's prevalences of VDD [25(OH)D $<$ 30 nmol/L] and LVD [25(OH)D $<$ 50 nmol/L] are among the largest in the country–at 5% and 44% in children and 7% and 46% among nonpregnant women, respectively [13]. Cholecalciferol fortification of dairy products in the country is voluntary; fortified versions of these products are available from some of the manufacturers.

We conducted a parallel, randomized intervention of cholecalciferol-fortified skim milk among 80 families. Families were randomly assigned to receive a 6-wk supply of 1 L of either cholecalciferol-fortified (n = 40) or unfortified skim milk (n = 40) daily. These 80 families were randomly chosen from participants in a longitudinal investigation of nutrition and health among Colombian school-aged children [15, 16]–the Bogotá School Children Cohort (BoSCCo) study. The BoSCCo study consisted of 3202 children aged 5–12 y who were randomly recruited from public primary schools in the city in 2006. Families in this cohort represent low- and middle-income families in Bogotá. Only families with a single child enrolled in the cohort were eligible to participate in the trial. Additional inclusion criteria were as follows: *1*) children living with their mother; *2*) child aged between 144 and 174 mo; *3*) intention of the child and mother to stay in the city for 6 consecutive weeks; and *4*) availability of a refrigerator at home. Exclusion criteria are as follows: *1*) lactose intolerance, allergy to milk or its components, or milk aversion of child or mother; *2*) calcium or bone metabolism disorders such as urinary tract stones and known hypercalcemia in the child or mother; *3*) taking cholecalciferol supplements as treatment of any disease; *4*) receipt of immunosuppressive therapy during the previous year; and *5*) severe clotting disorders. The sample size required was determined using a 2-sided paired t-test to detect minimal difference in serum total 25(OH)D concentration between the 2 treatment arms of 15 nmol/L, per previous food fortification trials [17, 18]. We assumed a SD of 20 nmol/L [19], no effect of the intervention on the SD, $\alpha = 0.05$, and a statistical power of 80%.

Dosage and experimental regimen

We aimed to increase the serum total 25(OH)D in adolescents assigned to the fortification arm by 20 nmol/L. This corresponds to a shift in their distribution from a mean of 68 nmol/L, per preliminary findings in this population [19], to 88 nmol/L, which would decrease the prevalence of LVD from 18% to 3%. This shift would be achievable by increasing the individual daily intake by ≈450 IU (11.3 μg) cholecalciferol, according to fortification trials conducted at the time of the study [17]. Nonetheless, owing to uncertainties in the feasibility of fortifying skim milk with a fat-soluble vitamin and the acceptability of skim milk in this population, we set the target dose for the adolescents at 1200 IU (30 μg) of cholecalciferol daily or twice the recommended dietary allowance [20]. We chose skim milk over whole milk as the fortification vehicle, consistent with dietary guidelines prevalent at the time, which recommended minimizing the intake of whole-fat dairy products to reduce the overall consumption of saturated fat [21].

We aimed to fortify the active treatment arm with 2400 IU (60 μg) of cholecalciferol/L. The largest dairy company in the country manufactured and donated the milk for the trial. Milk was fortified through the direct addition method [22] by first mixing cholecalciferol into a small volume of milk and then adding the mixture to each batch of milk through a triblender speed mixer. The amount of cholecalciferol added was 20% above the target in order to prevent losses through thermal treatment. After a 10-min agitation period, the batches underwent ultrahigh temperature (UHT) processing with a holding temperature of 139°C–142°C for 2–4 s. Finally, the batches underwent 2-stage homogenization under high pressure (150 psi) to ensure emulsification of fat and other ingredients, including cholecalciferol, in the water phase of the milk. Although our goal was to fortify the active treatment arm with 2400 IU (60 μg) of cholecalciferol/L, in practice, the fortification level was lower than the target. Quality control analyses by the manufacturer revealed a mean cholecalciferol concentration of only 896 IU/L in random samples of the fortified experimental milk.

Both arms consumed milk unfortified with other micronutrients, and were identical in appearance, odor, and taste. Milk was packed in plastic bags of 1-L capacity with light-blocking overwrap according to the manufacturer's commercial specifications. Unopened milk bags had a minimal shelf-life of 6 mo. To achieve masking of the participants to the experimental regimens, all bags were identical in appearance and unmarked, except for 1 of the 2 arbitrary codes linked to the identity of each arm. The regimen manufacturer kept this coding from the investigators until the end of primary aim analyses to guarantee double masking. Each family received 1 L/d of UHT-processed skim milk (0.05 g of total fat/100 g milk; Supplemental Table 1) for 6 wk.

Recruitment and randomization procedures

We created a list of identification (ID) numbers from 1 to 80, and randomly assigned each number to either cholecalciferol-fortified or unfortified milk with the use of permuted block randomization in blocks of size = 4. We selected a random sample of 120 participants in the cohort who did not have participating siblings and who fulfilled the age inclusion criterion at the time. Between June and September 2013, a research assistant contacted families in this sample through a phone call to introduce the study, inquire on interest to participate, and verify the eligibility criteria. We contacted 113 families before reaching the required sample size of 80. Primary reasons for exclusion were as follows: the child did not live with their mother ($n = 2$), the family did not intend to stay in the city for the duration of the study ($n = 3$), the child exhibited lactose intolerance ($n = 20$), the mother exhibited lactose intolerance ($n = 2$), the child was allergic to milk ($n = 1$), and the child declined participation ($n = 5$; Supplemental Figure 1). We assigned an ID number to each family that agreed to participate and made an appointment for a home visit within a week. During these visits, the study's team members explained the aims and procedures of the study, answered any queries, confirmed interest in enrolling, and obtained written informed consent from the mothers, as well as written assent from the adolescents to participate. The study protocol and procedures were approved by the Ethics Committee of the Universidad de La Sabana, Colombia, and the Health Sciences and Behavioral Sciences Institutional Review Board of the University of Michigan, United States.

Baseline evaluations

At the baseline visit, research assistants administered questionnaires to collect information on sociodemographic characteristics, sunlight exposure, and milk intake habits. A study dietician administered a 24-h diet recall to the adolescents to inquire the intake of all foods and beverages during the last 24 h, following the United States Department of Agriculture (USDA) 5-step multiple-pass method [23]. Anthropometric measurements from the child and mother were performed with the use of standard techniques. Height was measured to the nearest 1 mm with wall-mounted Seca 202 stadiometers (Seca) and weight to the nearest 0.1 kg with Tanita HS201 electronic scales (Tanita). Objective measures of skin color were obtained using reflectance colorimetry [24] with use of the SmartProbe 400 (IMS, Inc.). A measure of constitutive skin color was taken on the upper gluteal area, which is typically unexposed to the sun. Facultative skin color, representing both constitutive skin color and tanning from sunlight exposure, was measured at the hand's dorsal side. Anthropometry and skin colorimetry were performed in duplicate; the mean of the 2 measures was used in the analysis.

Next, the research assistant confirmed overnight fasting and obtained peripheral blood samples from the adolescents and the mothers through antecubital venipuncture. An aliquot was stored in an anticoagulant-free tube for the separation of serum. Samples were placed in coolers with dry ice and transported while ensuring protection from sunlight on the day of collection to the Universidad de La Sabana in the Bogotá area, where it was cryopreserved at −80°C until transportation to the United States for analyses.

At the end of the visit, research assistants delivered a 3-wk supply of milk to each family, consisting of twenty-one 1-L bags of the milk, which had been randomly assigned to each family. In addition, each family received a 250-mL cup and the adolescents were instructed to drink 2 cups (500 mL) of milk daily to reach the target dose of 1200 IU of cholecalciferol in the fortified group. Mothers were encouraged to use the remaining milk volume for themselves and the rest of the family per their habits. We instructed families to open only 1 bag at a time, to keep it refrigerated, and to store all empty and unopened bags until the next visit. Mothers received a checklist to register the number or fraction of cups the child drank daily, using a semi-quantitative format.

Follow-up visits

We conducted 2 follow-up home visits 3 and 6 wk after the baseline visit. At week-3 interim visit, research assistants inquired the occurrence of hypercalcemia symptoms, collected empty and any unopened milk bags, and provided the families with a new 3-wk supply of 21 milk bags. At the final visit 6-wk postenrollment, research assistants repeated the 24-h diet recall assessment (among adolescents), anthropometry, and blood sample collection. We also inquired on the acceptability of the intervention and retrieved all the remaining milk bags.

Laboratory methods

We quantified serum total and free 25(OH)D at Heartland Assays. Total 25(OH)D was measured using the DiaSorin LIAISON 25-OH Vitamin D Total assay (Diasorin, Inc.) [25, 26], a direct competitive chemiluminescence immunoassay (CLIA), co-specific for 25-hydroxycholecalciferol [25(OH)D3] and 25-hydroxyergocalciferol [25(OH)D2]. The assay's sensitivity is 6.26 nmol/L, and the interassay and intra-assay CVs are 11.2 % and 8.1%, respectively; recovery of endogenous 25(OH)D is 100% [27]. Free 25(OH)D was quantified using the DIASource ELISA, a 2-step immunoassay procedure involving binding to an anti–vitamin D antibody, addition of a chromogenic substrate, and measurement of free 25(OH)D using a plate spectrophotometer; the interassay and intra-assay CVs ranged from 1.9% to 5.5% and 4.0% to 6.1%, respectively. Because of financial constraints, free 25(OH)D was quantified only in the adolescents. DBP was measured with a Quantikine ELISA kit (R&D Systems, Inc.) that uses a monoclonal antibody specific to DBP at the Center for Chemical Genomics, University of Michigan. The mean CV for replicate measures was 13.21%; individual sample CVs ranged from 0.02% to 33.05%.

Other variables

Adolescents' height-for-age and BMI-for-age *z*-scores were calculated according to the WHO growth reference [28]. Caloric and vitamin D intakes of adolescents were estimated from the 24-h diet recalls using each food's nutrient and caloric composition values from the USDA Standard Reference food composition database, supplemented with data from manufacturers and published reports (Food Processor software; ESHA Research), and the Composition Table of Colombian Foods by the Colombian Institute of Family Welfare [29]. Tanning, a proxy for sunlight exposure, was estimated from the colorimetric assessments as the difference between facultative (dorsal hand area) and constitutive (gluteal area) skin color in the International Commission on Illumination scale units, which range from 0 (absolute black) to 100 (absolute white).

Data analyses

All analyses were conducted separately for adolescents and mothers. We first compared the distribution of baseline characteristics between intervention groups using means ± SD and proportions for continuous and dichotomous variables, respectively. We estimated Spearman correlations between vitamin D biomarkers at baseline.

The main analytic strategy followed the intention-to-treat principle. The primary end point was the change in total 25(OH)D concentration from the baseline to the end of the intervention. Secondary endpoints involved changes in free 25(OH)D (among adolescents) and DBP. Treatment effects were the differences in mean change between fortified and unfortified groups. Furthermore, 95% CIs were constructed using mixed-effects linear regression models for repeated measures, with each biomarker as the continuous outcome and treatment assignment (fortified compared with unfortified milk), time (baseline compared with the end of follow-up), and a treatment-by-time interaction term as predictors. All models included a random intercept and an unstructured variance specification. Because there were imbalances in few measured baseline covariates, we conducted supplemental analyses adjusting for these according to the conditionality principle [30].

In exploratory analyses, we examined whether the effect of cholecalciferol fortification on vitamin D status biomarkers differed by the levels of baseline characteristics identified a priori based on their ability to influence vitamin D status or biomarker response to changes in intake. These included sex, BMI, vitamin D status, and sunlight exposure. Continuous characteristics were categorized at conventional cut points or at the median of their distribution. Differences in treatment effects were tested with Wald tests for interaction terms between indicator variables for fortification group assignment, the baseline characteristics, and time (baseline or follow-up). These analyses were exploratory in nature because the study was not designed with sufficient statistical power to allow for subgroup comparisons.

We compared the measures of compliance, acceptability, and safety between treatment groups using Wilcoxon rank-sum and Chi-squared tests for continuous and categorical variables, respectively. Compliance with the intervention was measured primarily using the number of experimental milk bags returned unopened to the research team at the interim and end of follow-up visits. A secondary measure of compliance was the mean reported number of milk cups the child drank every day. Acceptability was assessed as the mean response to questions on tastiness of the milk and likelihood of long-term adoption from Likert-type scales. The primary safety end point to monitor potential cholecalciferol toxicity was the occurrence of hypercalcemia symptoms as reported at the interim visit; these included nausea or vomiting, loss of appetite, excessive thirst, excessive urination, constipation, stomach ache, muscle weakness, muscle or joint pain, confusion, and fatigue. For each symptom, the frequency of occurrence from never to very frequent was registered on a 5-point Likert-like scale. Frequent occurrence of ≥3 symptoms would prompt a calcemia test, but none of the participants reached this threshold. Secondary safety end points were changes in BMI-for-age *z*-score, total energy intake, and saturated fat intake during the intervention period.

In exploratory per-protocol analyses, we estimated the effects of the intervention on vitamin D biomarkers stratified by the degree of compliance according to unopened bag counts. All analyses were performed with Statistical Analysis Software version 9.4 (SAS Institute).

Results

Adolescents

The mean ± SD age of adolescent participants at recruitment was 13.5 ± 0.7 y; 50.0% were girls. The mean ± SD serum total 25(OH)D concentration was 52.1 ± 13.7 nmol/L (range: 27.4–96.8 nmol/L); the proportion in categories <30 nmol/L, 30–50 nmol/L, 50–75 nmol/L, and ≥75 nmol/L was 1.3%, 52.5%, 38.8%, and 7.5%, respectively. The mean ± SD free 25(OH)D and DBP concentrations were 12.8 ± 2.9 pmol/L and 3550 ± 1360 nmol/L, respectively. Baseline correlations (Spearman) of total with free 25(OH)D, total 25(OH)D with DBP, and free 25(OH)D with DBP were 0.85, 0.12, and 0.06, respectively. At baseline, compared with adolescents assigned to the unfortified milk group, those in the fortified milk group were shorter and leaner and had slightly lower total 25(OH)D concentrations, higher DBP concentrations, less food insecurity, and less milk intake in the household (Table 1).

Total 25(OH)D concentrations significantly increased in the fortified milk group and decreased among those assigned to the unfortified group; fortification increased total 25(OH)D by 5.4 nmol/L (95% CI: 2.1, 8.8 nmol/L; $P = 0.002$; Table 2). This effect was not modified by baseline 25(OH)D concentration (Table 3); however, it was significantly stronger in adolescents with low DBP concentrations than in those with high DBP concentrations, in those with darker than with lighter constitutive skin color or with lower than higher tanning, and in those with higher than with lower vitamin D intake (Table 3). Overall, the prevalence of LVD decreased from 58% at baseline to 35% at the end of the intervention in the fortified milk group and increased from 50% to 55% in the unfortified milk group. Fortification resulted in increased free 25(OH)D concentrations, but this effect was not statistically significant overall (Table 2) or within levels of potential modifiers (Supplemental Table 2). Moreover, there was no significant effect on DBP concentrations (Table 2). Adjustment for baseline characteristics that were distributed unevenly between the treatment groups did not change the results (Supplemental Table 3).

TABLE 1
Baseline characteristics of participating adolescents and mothers according to cholecalciferol fortification assignment

Characteristics[1]	Fortified milk ($n = 40$)	Unfortified milk ($n = 40$)
Adolescents		
Female sex	47.5 (19)	52.5 (21)
Age, y	13.6 ± 0.6	13.5 ± 0.7
Height-for-age z^2	−0.72 ± 1.01	−0.34 ± 0.95
Body mass index-for-age z^2	0.09 ± 1.20	0.19 ± 0.91
Serum total 25(OH)D, nmol/L	51.7 ± 14.3	52.5 ± 13.1
Serum 25(OH)D <50 nmol/L	57.5 (23)	50.0 (20)
Serum free 25(OH)D, pmol/L	12.9 ± 3.2	12.7 ± 2.6
Serum DBP, nmol/L	3870 ± 1550	3240 ± 1080
Constitutive skin color[3], L units	56.7 ± 5.7	56.2 ± 4.6
Tanning[4], L units	−4.6 ± 4.0	−3.6 ± 3.2
Vitamin D intake[5], μg/d	2.3 ± 3.3	2.4 ± 3.5
Total energy intake[5], kcal/d	1780 ± 572	1910 ± 520
Saturated fat intake[5], % Energy	12.4 ± 3.6	10.5 ± 2.7
Mothers		
Age, y	41.3 ± 6.6	40.8 ± 7.0
Height, cm	154.5 ± 5.8	154.4 ± 5.0
BMI, kg/m^2	27.1 ± 4.6	27.3 ± 5.7
Education, y	10.1 ± 4.1	9.7 ± 4.4
Parity	2.5 ± 1.0	2.8 ± 1.3
Serum total 25(OH)D[6], nmol/L	45.2 ± 11.1	49.4 ± 10.4
25(OH)D[6] <50 nmol/L	65.0 (26)	61.5 (24)
Serum DBP[6], nmol/L	3580 ± 1460	3330 ± 1090
Constitutive skin color[3], L units	62.3 ± 3.9	61.1 ± 6.2
Tanning[4], L units	−8.6 ± 3.6	−7.5 ± 4.4
Household/environment		
People living in household, *n*	4.5 ± 1.5	4.7 ± 1.9
Household monthly income (minimal wage multiples)	2.8 ± 5.5	2.9 ± 5.3
Socioeconomic status[7]	2.6 ± 0.7	2.5 ± 0.6
Food insecurity in the household	60.0 (24)	70.0 (28)
Milk intake[8], mL/(person d)	206 (110)	235 (145)
Preference low-fat or skim vs. whole milk	10.0 (4)	10.0 (4)
Recruited in June/July vs. August/September	65.0 (26)	67.5 (27)

DBP, vitamin D–binding protein. [1]Values are mean ± SD or % (*n*). [2]According to the WHO growth reference for school-aged children and adolescents (5–19 y) [28]. [3]From colorimetric assessment of typically sun-unexposed skin (gluteal area). Units are in the International Commission on Illumination scale, which ranges from 0 (absolute black) to 100 (absolute white). [4]Proxy for sunlight exposure as the difference between facultative (dorsal hand area) and constitutive (gluteal area) skin color. [5]From a 24-h diet recall administered to the adolescents before randomization. [6]$n = 39$ in the unfortified milk group. [7]Per the local government classification of each household into 1 of the 4 levels in the sample (1 is lowest socioeconomic status). [8]Volume of milk purchased at the household divided by the number of household members.

Compliance with the intervention was generally high and did not differ between the treatment groups (Supplemental Table 4). Of the 42 milk bags dispensed to each family, only a median of 1.8 were returned unopened. Estimated daily experimental milk intake was 1.6 cups. The milk's taste was highly acceptable, although adoptability for long-term use was somewhat low. There were no adverse events, such as hypercalcemia symptoms or changes in BMI or total energy intake, during the intervention (Supplemental Table 4).

In supplemental per-protocol analyses, the effect of fortification was significantly stronger in highly compliant adolescents than in less compliant adolescents (Table 4). Among families who did not return unopened milk bags, fortification increased serum total 25(OH)D by 11.5 nmol/L (95% CI: 7.1, 15.9 nmol/L; $P < 0.0001$) and free 25(OH)D by 1.6 pmol/L (95% CI: 0.4, 2.9 pmol/L; $P = 0.009$).

Mothers

The mean ± SD age of mothers was 41.1 ± 6.8 y. The mean ± SD serum total 25(OH)D concentration was 47.3 ± 10.9 nmol/L (range: 20.9–71.4 nmol/L); the distribution in categories <30 nmol/L, 30–50 nmol/L, and ≥50 nmol/L was 5.1%, 58.2%, and 36.7%, respectively. The mean ± SD DBP concentration was 3450 ± 1290 nmol/L. The correlation (Spearman) between total 25(OH)D and DBP was 0.21. Compared with mothers assigned to the unfortified milk group, those in the fortified milk group reported higher education, lower parity, lower total 25(OH)D status, and higher DBP concentrations (Table 1).

Cholecalciferol fortification increased total 25(OH)D by 4.0 nmol/L (95% CI: 0.6, 7.5 nmol/L; $P = 0.02$; Table 5). This effect did not vary significantly within levels of baseline characteristics (Supplemental Table 5). VDD prevalence decreased from 10% at baseline to 7.5% at the end of the intervention in the fortified milk group and increased from 0% to 7.9% in the unfortified milk group. Corresponding changes in LVD were from 65% to 53% and from 62% to 61% in the fortified and unfortified milk groups, respectively. There was no effect on serum DBP concentrations (Table 5). Results did not change after adjustment for characteristics that differed between the treatment groups (Supplemental Table 6). The effects of treatment were independent of compliance (Supplemental Table 7).

Discussion

In this randomized trial of families living at high altitude in Colombia, provision of cholecalciferol-fortified skim milk for 6 wk increased total 25(OH)D in adolescents, the primary target of the intervention. In addition, a smaller positive effect was observed among their mothers.

The average effect of a target daily dose of 1200 IU (30 μg) cholecalciferol among the adolescents, 5.4 nmol/L or 0.18 nmol/L/μg of fortified cholecalciferol, was substantially smaller than the estimated average dose-response from previous fortification studies, 1.2 nmol/L/μg) [17, 31–33]. There are different possible explanations for the lower effect observed in this trial. First, the prescribed daily dose only reached an average 448 IU (11.2 μg) or 37% of the target owing to problems with the experimental regimen manufacture. Taking this lower effective dose into consideration would yield an estimated effect of 0.48 nmol/L/μg, which is still modest but comparable with a 0.4 nmol/L/μg dose reported in a trial of Canadian children [34]. Second, the lack of compliance with the intervention may have played a role. Although the generally low unopened milk bag count suggested high compliance overall, per-protocol analyses revealed a much stronger effect among adolescents from families who did not return any unopened bags (high compliers), 11.5 nmol/L, compared to those who returned 1 or more unopened bags, 1.8 nmol/L. An 11.5-nmol/L treatment effect from an effective target dose of 448 IU/d would translate into a dose-response estimate of 1.0 nmol/L/μg, much closer to the

TABLE 2
Effect of cholecalciferol fortification of skim milk on serum total 25(OH)D, free 25(OH)D, and DBP concentrations in Colombian adolescents

Vitamin D metabolite[1]	Fortified milk			Unfortified milk			Cholecalciferol fortification effect[2], mean (95% CI)
	Baseline ($n = 40$)	Follow-up ($n = 40$)	Mean change (95% CI)[1]	Baseline ($n = 40$)	Follow-up ($n = 38$)	Mean change (95% CI)	
Total 25(OH)D, nmol/L	51.7 ± 14.3	54.5 ± 13.1	2.8 (0.6, 5.0)	52.5 ± 13.1	49.8 ± 10.6	−2.6 (−5.2, −0.1)	5.4 (2.1, 8.8)
Free 25(OH)D, pmol/L	12.9 ± 3.2	12.3 ± 3.3	−0.7 (−1.3, −0.03)	12.7 ± 2.6	11.4 ± 2.2	−1.3 (−1.8, −0.7)	0.6 (−0.2, 1.4)
DBP, nmol/L	3870 ± 1550	3020 ± 1030	−844 (−1210, −480)	3240 ± 1080	2810 ± 852	−428 (−810, −46)	−416 (−944, 112)

DBP, vitamin D–binding protein.[1]Values are means ± SD unless noted otherwise. [2]From mixed-effects linear regression models for repeated measures. Empirical SEs were specified in all models.

summary 1.2 nmol/L/μg reported in meta-analyses [17, 31–33]. Moreover, fortification resulted in a significant increase in free 25(OH)D among high compliers. The relatively low ratings observed in adoptability of skim milk for long-term use are in line with anecdotal evidence of its limited acceptability over whole milk in this population owing to flavor and texture preferences. Notwithstanding, results of the degree of compliance may lack precision because the trial was not designed to detect effects among subgroups. Third, the CLIA method used to quantify total 25(OH)D has a mean bias that is inversely related to the true value [35], leading to an overestimation of low concentrations. The treatment effect could have been underestimated if low concentrations that remained low postintervention in the unfortified milk group were overestimated pretreatment and posttreatment, whereas in the fortified milk group, only baseline low concentrations, but not higher postintervention concentrations, were overestimated. Finally, the trial duration, 6 wk, was relatively short compared with several other trials, which typically lasted for ≥8 wk. Nonetheless, an effect of duration of the intervention on the magnitude of the effect has not been reported [4].

We conducted exploratory analyses of the effects of the intervention in subgroups defined by baseline characteristics. These results should be cautiously interpreted because the trial was not specifically designed with adequate statistical power for subgroup analyses, and this may affect the precision of the estimates. The effect of cholecalciferol fortification on total 25(OH)D among adolescents did not differ according to baseline 25(OH)D concentrations but was heterogeneous with respect to other baseline characteristics. The effect was stronger among adolescents with a darker compared with lighter constitutive skin and with less compared with more tanning; adolescents with darker skin or less tanning may represent those with low 25(OH)D concentrations from limited sunlight exposure, which could have been overestimated by the CLIA method. The effect was also stronger in adolescents with higher vitamin D intake than in those with lower vitamin D intake from diet. Higher intake could indicate a propensity to increased compliance with the intervention because of enhanced health and nutritional consciousness. Moreover, the effect was stronger among adolescents with lower DBP concentrations compared to those with higher DBP; the nature of this interaction is unclear. DBP concentrations vary genetically according to GC haplotypes [36]; hence, the modifying effect of baseline DBP concentrations on response to fortification could signal a role of specific genetic polymorphisms on hydroxylation efficiency. The 25(OH)D response to weekly vitamin D supplementation among children was related to a combination of "at-risk" GC alleles in another study, although it was independent of individual haplotypes [37]. This finding deserves confirmation and further scrutiny in future investigations.

Fortification did not have an effect on DBP concentrations, consistent with previous supplementation trials in adults [36, 38]. Notwithstanding, there was a significant effect on free 25(OH)D among highly compliant adolescents. Previous investigations had documented increases in free 25(OH)D after supplementation in adults [38, 39] and children [37], but the effect of fortification was not known. This finding is relevant because free 25(OH)D is highly bioavailable [14] in contrast to DBP-bound vitamin D.

Provision of cholecalciferol-fortified skim milk increased total 25(OH)D concentrations in the mothers of the adolescents, even though they were not the primary targets of the intervention. Although the magnitude of the effect was somewhat smaller than that among the adolescents, this result supports the notion that, as opposed to supplementation, fortification can benefit family groups rather than individuals only.

Our study has several strengths. Its randomized, controlled, and masked design allowed estimating the causal effects of vitamin D fortification in a tropical setting with high prevalence of VDD, where no previous fortification trial had been conducted. Follow-up was virtually complete, and compliance with the intervention was high. We had the opportunity to evaluate the effects of cholecalciferol fortification on metabolites that are seldom assessed, including free 25(OH)D and DBP.

However, the study had some limitations. The cholecalciferol dose was substantially lower than that originally planned owing to unanticipated technical difficulties in manufacturing the fortified milk. These problems could have occurred at different stages of the process. For example, although the amount of cholecalciferol added to milk was 20% in excess of the target, vitamin D losses to thermal treatment can reach ≤60% [22]; hence, UHT treatment could have decreased the cholecalciferol concentration in fortified milk. Recirculation of milk through the UHT equipment could compound these losses through redundant heat exposure. Moreover, the dilution of a fat-soluble vitamin, such as vitamin D, in skim milk may be less efficient than that in whole or low-fat milk, rendering the homogenizing step ineffective and leading to the precipitation of the vitamin in the processing equipment or storage tanks. Alternative fortification techniques, including vitamin D encapsulation [22], may be more effective in achieving stability and accuracy of cholecalciferol

TABLE 3
Effect of cholecalciferol fortification of skim milk on serum total 25(OH)D concentration in Colombian adolescents according to baseline characteristics

Baseline characteristics[1] (*n*)	Fortified milk			Unfortified milk			Cholecalciferol fortification effect, mean (95% CI)[2]	*P*-fortification interaction[3]
	Baseline	Follow-up	Mean change (95% CI)[2]	Baseline	Follow-up	Mean change (95% CI)		
Sex								0.74
Female (40)	46.1 ± 12.5	49.6 ± 11.7	3.5 (0.6, 6.5)	47.6 ± 9.1	45.1 ± 7.6	−2.5 (−4.8, −0.2)	6.0 (2.3, 9.8)	
Male (40)	56.8 ± 14.2	59.0 ± 12.9	2.1 (−0.9, 5.2)	57.8 ± 15.0	55.1 ± 11.1	−2.7 (−7.6, 2.2)	4.9 (−0.9, 10.7)	
BMI-for-age *z*								0.71
≤0 (37)	55.2 ± 16.3	57.4 ± 14.9	2.3 (−1.3, 5.8)	56.5 ± 13.7	52.5 ± 11.1	−4.0 (−8.0, 0.0)	6.3 (0.9, 11.6)	
>0 (43)	48.6 ± 11.9	51.8 ± 10.9	3.3 (0.7, 5.8)	49.2 ± 12.0	47.4 ± 9.7	−1.7 (−4.8, 1.3)	5.0 (1.0, 9.0)	
Total 25(OH)D, nmol/L								0.23
≥50 (37)	64.9 ± 11.3	65.8 ± 10.5	0.9 (−3.1, 4.9)	62.3 ± 11.6	55.8 ± 10.4	−6.5 (−10.2, −2.8)	7.4 (2.0, 12.9)	
<50 (43)	42.0 ± 6.2	46.2 ± 7.3	4.2 (2.0, 6.4)	42.6 ± 4.1	43.2 ± 6.0	0.6 (−1.6, 2.8)	3.6 (0.5, 6.7)	
DBP[4], nmol/L								0.04
≥3400 (40)	55.3 ± 15.9	56.4 ± 14.2	1.1 (−1.9, 4.1)	51.4 ± 11.9	50.5 ± 12.6	−1.0 (−4.8, 2.9)	2.1 (−2.8, 6.9)	
<3400 (40)	47.3 ± 11.0	52.2 ± 11.5	4.9 (2.0, 7.7)	53.3 ± 14.3	49.3 ± 9.0	−4.0 (−7.4, −0.6)	8.9 (4.5, 13.2)	
Constitutive skin color[5], L units								0.01
Lighter, ≥57.3 (40)	53.9 ± 17.0	55.5 ± 16.0	1.6 (−1.3, 4.5)	49.9 ± 11.4	50.4 ± 11.4	0.5 (−3.2, 4.3)	1.1 (−3.7, 5.8)	
Darker, <57.3 (40)	49.0 ± 10.0	53.3 ± 8.5	4.3 (1.2, 7.3)	54.6 ± 14.4	49.4 ± 10.2	−5.2 (−8.3, −2.1)	9.5 (5.1, 13.9)	
Tanning[6], L units								0.02
Higher tanning, <−4.6 (40)	55.3 ± 16.2	56.0 ± 15.5	0.7 (−2.4, 3.7)	52.3 ± 10.6	51.2 ± 10.7	−1.1 (−5.4, 3.2)	1.8 (−3.5, 7.1)	
Lower tanning, ≥−4.6 (40)	46.8 ± 9.8	52.5 ± 9.0	5.7 (3.3, 8.0)	52.6 ± 15.0	48.8 ± 10.6	−3.7 (−6.8, −0.6)	9.4 (5.5, 13.3)	
Vitamin D intake[7], μg/d								0.009
Lower, <1.5 (40)	50.3 ± 11.6	52.2 ± 11.7	1.8 (−0.8, 4.5)	48.9 ± 9.5	49.6 ± 10.5	0.7 (−3.0, 4.4)	1.2 (−3.4, 5.7)	
Higher, ≥1.5 (40)	53.1 ± 16.8	56.8 ± 14.2	3.8 (0.4, 7.1)	56.0 ± 15.4	50.0 ± 10.9	−6.0 (−9.1, −2.9)	9.7 (5.1, 14.3)	

DBP, vitamin D–binding protein.
[1] Values are mean ± SD unless noted otherwise.
[2] From mixed-effects linear regression models for repeated measures. Empirical standard errors were specified in all models.
[3] Wald test for an interaction term between indicator variables for fortification group assignment, the baseline characteristic, and time (baseline or follow-up).
[4] The cut point represents the median of the distribution.
[5] From colorimetric assessment of typically sun-unexposed skin (gluteal area). Units are in the International Commission on Illumination scale, which ranges from 0 (absolute black) to 100 (absolute white). The cut point represents the median of the distribution.
[6] Proxy for sunlight exposure as the difference between facultative (dorsal hand area) and constitutive (gluteal area) skin color. The cut point represents the median of the distribution.
[7] From a 24-h recall.

concentrations in food vehicles. Another limitation is that there were imbalances in some measured baseline characteristics between randomly assigned groups, which may have introduced confounding in the intent-to-treat analytic approach. Although adjustment for these characteristics did not change the results, we cannot rule out residual confounding by potential imbalances in other unmeasured variables. Some lack of compliance, likely linked to poor palatability of skim milk, may have attenuated the treatment effect. Implementation of fortification should include milk with any fat content, and future studies need to consider using low-fat dairy instead of skim dairy as food vehicles. Finally, the method used to quantify serum total 25(OH)D concentration may have introduced bias toward the lack of effect because of underestimation of low concentrations. This could hinder the

TABLE 4
Effect of cholecalciferol fortification of skim milk on serum total 25(OH)D, free 25(OH)D, and DBP concentrations in Colombian adolescents according to compliance with the intervention

Compliance[1,2] (*n*)	Fortified milk			Unfortified milk			Cholecalciferol fortification effect, mean (95% CI)[2]	*P*-fortification interaction[d]
	Baseline	Follow-up	Mean change (95% CI)[3]	Baseline	Follow-up	Mean change (95% CI)		
Total 25(OH)D, nmol/L								0.002
Higher compliance (32)	49.4 ± 11.2	55.3 ± 12.5	5.9 (3.5, 8.4)	55.9 ± 18.2	50.3 ± 12.9	−5.6 (−9.2, −1.9)	11.5 (7.1, 15.9)	
Lower compliance (47)	53.6 ± 16.5	53.8 ± 13.8	0.2 (−2.7, 3.2)	51.1 ± 9.2	49.5 ± 9.2	−1.6 (−4.9, 1.7)	1.8 (−2.6, 6.2)	
Free 25(OH)D, pmol/L								0.048
Higher compliance (32)	12.6 ± 3.0	12.3 ± 3.2	−0.2 (−1.2, 0.8)	12.9 ± 3.4	11.1 ± 2.9	−1.9 (−2.6, −1.2)	1.6 (0.4, 2.9)	
Lower compliance (47)	13.2 ± 3.4	12.2 ± 3.4	−1.0 (−1.8, −0.2)	12.6 ± 2.1	11.6 ± 1.7	−1.0 (−1.7, −0.3)	0.0 (−1.0, 1.1)	
DBP, nmol/L								0.93
Higher compliance (32)	3950 ± 1400	3140 ± 1140	−812 (−1240, −385)	3310 ± 1390	2960 ± 958	−353 (−1110, 402)	−459 (−1330, 408)	
Lower compliance (47)	3800 ± 1700	2930 ± 936	−871 (−1430, −308)	3190 ± 908	2730 ± 794	−462 (−892, −32)	−409 (−1120, 299)	

DBP, vitamin D–binding protein.
[1] Values are mean ± SD unless noted otherwise.
[2] Higher compliance was defined as not returning any unopened experimental milk bags to the investigators at the interim and final visits. Lower compliance corresponds to families who returned ≥1 unopened bags.
[3] From mixed-effects linear regression models for repeated measures. Empirical standard errors were specified in all models.
[4] Wald test for an interaction term between indicator variables for fortification group assignment, compliance, and time (baseline or follow-up).

TABLE 5
Effect of cholecalciferol fortification of skim milk on serum total 25(OH)D and DBP concentrations in Colombian women

Vitamin D metabolite[1]	Fortified milk			Unfortified milk			Cholecalciferol fortification effect, mean (95% CI)[22]
	Baseline (*n* = 40)	Follow-up (*n* = 40)	Mean change (95% CI)[1]	Baseline (*n* = 40)	Follow-up (*n* = 38)	Mean change (95% CI)	
Total 25(OH)D, nmol/L	45.2 ± 11.1	47.7 ± 11.7	2.5 (0.3, 4.8)	49.4 ± 10.4	47.9 ± 11.3	−1.5 (−4.1, 1.1)	4.0 (0.6, 7.5)
DBP, nmol/L	3580 ± 1460	3030 ± 1360	−546 (−923, −169)	3330 ± 1090	2910 ± 949	−418 (−759, −77)	−128 (−637, 381)

DBP, vitamin D–binding protein.
[1] Values are mean ± SD unless noted otherwise.
[2] From mixed-effect linear regression models for repeated measures. Empirical standard errors were specified in all models.

generalizability of the findings to other populations; validation and standardization of vitamin D concentrations are warranted in future investigations. External validity may also be limited if other populations show different distributions of the baseline factors that modified the effect of fortification, including sunlight exposure, background dietary intake, and DBP concentrations.

In conclusion, provision of cholecalciferol-fortified skim milk to families over a 6-wk period increased serum total 25(OH)D concentration in adolescents and their mothers. Among highly compliant adolescents, cholecalciferol fortification increased the concentration of serum free 25(OH)D, a bioavailable fraction. Fortification of milk with cholecalciferol is an effective and safe intervention to improve vitamin D serostatus in a setting with high prevalence of VDD. Its effects on clinical outcomes related to poor vitamin D status deserve further investigation.

Author disclosures

The authors report no conflicts of interest.

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The authors' responsibilities were as follows—EV: designed the research, analyzed the data, wrote the article, and had the primary responsibility for final content; HO: contributed to the study design, planned and supervised the study execution, and provided expertise in the interpretation of findings in the local context; CM: coordinated the field activities; CM, SLA, and SA: conducted the research; and all authors: read and approved the final manuscript.

Data Availability

The data used in this report can be made available upon reasonable request to the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2022.11.026.

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Increased total and free 25-hydroxy vitamin D with daily intake of cholecalciferol-fortified skim milk: A randomized controlled trial in Colombian adolescents

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Online Supplementary Material

Supplemental Table 1. Experimental milk nutrient composition[1]

Nutrient	Per 100 g	Per 500 mL[2]
Energy, kcal	28.3	145.8
Carbohydrate, g	4.4	22.9
Protein, g	3.1	16.0
Total fat, g	0.05	0.26
Saturated fat, g	0.03	0.16
Cholesterol, mg	2.0	10.3
Calcium, mg	125	645
Sodium, mg	71	366
Potassium, mg	158	815
Phosphorous, mg	95	490
Iodine, mg	30	155
Magnesium, mg	11	57
Zinc, mg	0.4	2.1
Thiamine, mg	0.04	0.21
Riboflavin, mg	0.17	0.88
Niacin, mg	0.10	0.52
Folic acid, µg	5.0	25.8
Vitamin B-12, µg	0.5	2.6
Vitamin A RAE[3], µg	1.0	5.2

[1] Macronutrient, calcium, and sodium composition and density were provided by the manufacturer. Other micronutrient composition was according to the Colombian Foods Composition Table 2018.

[2] 500 mL (516 g) corresponds to the target daily servings for adolescent participants (2 cups). Nutrient calculations are based on average density of 1.032 g/mL.

[3] Retinol activity equivalents

Supplemental Table 2. Effect of cholecalciferol fortification of skim milk on serum free 25-hydroxy vitamin D in Colombian adolescents according to baseline characteristics

Baseline characteristics[1] (n)	Fortified milk			Unfortified milk			Cholecalciferol fortification effect Mean (95% CI)[2]	*P* fortification interaction[3]
	Baseline	Follow-up	Mean change (95% CI)[2]	Baseline	Follow-up	Mean change (95% CI)		
Sex								0.99
Female (40)	11.6 ± 2.4	10.9 ± 2.3	-0.7 (-1.4, 0.0)	11.5 ± 1.8	10.2 ± 1.7	-1.3 (-1.9, -0.7)	0.6 (-0.4, 1.5)	
Male (40)	14.1 ± 3.4	13.5 ± 3.5	-0.6 (-1.6, 0.4)	13.9 ± 2.8	12.7 ± 2.0	-1.2 (-2.1, -0.3)	0.6 (-0.8, 1.9)	
Body mass index-for-age Z								0.20
≤0 (37)	13.2 ± 3.4	12.6 ± 3.9	-0.6 (-1.5, 0.3)	13.6 ± 3.0	11.9 ± 2.1	-1.8 (-2.5, -1.0)	1.2 (0.0, 2.4)	
>0 (43)	12.6 ± 3.0	11.9 ± 2.6	-0.7 (-1.6, 0.1)	11.9 ± 2.0	11.0 ± 2.2	-0.9 (-1.6, -0.1)	0.1 (-1.0, 1.3)	
Free 25(OH)D, nmol/L								0.40
≥12.3 (40)	15.0 ± 2.7	14.0 ± 3.1	-0.9 (-2.0, 0.1)	15.0 ± 2.1	12.9 ± 1.9	-2.0 (-2.8, -1.2)	1.1 (-0.2, 2.4)	
<12.3 (40)	10.4 ± 1.3	10.1 ± 1.7	-0.3 (-0.9, 0.3)	10.8 ± 0.9	10.0 ± 1.4	-0.7 (-1.3, -0.2)	0.4 (-0.4, 1.3)	
DBP[4], nmol/L								0.12
≥3400 (40)	13.4 ± 3.2	12.7 ± 3.5	-0.7 (-1.3, -0.1)	11.9 ± 2.1	11.4 ± 2.1	-0.6 (-1.2, 0.1)	-0.1 (-1.0, 0.8)	
<3400 (40)	12.3 ± 3.1	11.7 ± 3.0	-0.6 (-1.8, 0.5)	13.3 ± 2.9	11.5 ± 2.3	-1.8 (-2.5, -1.1)	1.2 (-0.2, 2.6)	
Constitutive skin color, L units[5]								0.38
Lighter, ≥57.3 (40)	12.9 ± 3.6	12.4 ± 4.0	-0.5 (-1.4, 0.3)	11.8 ± 2.2	11.1 ± 2.1	-0.7 (-1.4, 0.0)	0.2 (-1.0, 1.3)	
Darker, <57.3 (40)	12.9 ± 2.7	12.1 ± 2.2	-0.8 (-1.7, 0.1)	13.4 ± 2.8	11.7 ± 2.3	-1.7 (-2.4, -1.0)	0.9 (-0.2, 2.0)	
Tanning, L units[6]								0.87
Higher tanning, <-4.6 (40)	13.2 ± 3.2	12.4 ± 3.8	-0.9 (-1.7, 0.0)	12.5 1.9	11.0 ± 2.2	-1.5 (-2.2, -0.7)	0.6 (-0.5, 1.7)	
Lower tanning, ≥-4.6 (40)	12.4 ± 3.2	12.0 ± 2.4	-0.4 (-1.3, 0.6)	12.8 3.1	11.7 ± 2.2	-1.1 (-1.9, -0.4)	0.7 (-0.5, 1.9)	
Vitamin D intake, μg/d[7]								0.05
Lower, <1.5 (40)	12.3 ± 2.1	11.5 ± 2.6	-0.8 (-1.7, 0.0)	11.9 ± 2.0	11.3 ± 2.0	-0.6 (-1.3, 0.1)	-0.2 (-1.3, 0.9)	
Higher, ≥1.5 (40)	13.5 ± 3.9	13.0 ± 3.7	-0.5 (-1.4, 0.5)	13.4 ± 3.0	11.5 ± 2.4	-1.9 (-2.6, -1.2)	1.4 (0.2, 2.6)	

Abbreviations: 25(OH)D, 25-hydroxy vitamin D; DBP, vitamin D binding protein.

[1] Mean ± SD unless noted otherwise.

[2] From mixed effects linear regression models for repeated measures. Empirical standard errors were specified in all models.

[3] Wald test for an interaction term between indicator variables for fortification group assignment, the baseline characteristic, and time (baseline or follow-up).

[4] The cutpoint represents the median of the distribution.

[5] From colorimetric assessment of typically sun-unexposed skin (gluteal area). Units are in the International Commission on Illumination scale, which ranges from 0 (absolute black) to 100 (absolute white). The cutpoint represents the median of the distribution.

[6] Proxy for sun exposure as the difference between facultative (dorsal hand area) and constitutive (gluteal area) skin color. The cutpoint represents the median of the distribution.

[7] From a 24-hour recall.

Supplemental Table 3. Adjusted effect of cholecalciferol fortification of skim milk on serum total 25-hydroxy vitamin D, free 25(OH)D, and vitamin D binding protein in Colombian adolescents

	Mean change (95% CI) from baseline to end of follow-up		Cholecalciferol fortification effect Mean (95% CI)[2]
	Fortified milk	Unfortified milk	
Total 25(OH)D, nmol/L	2.8 (0.6, 5.0)	-3.1 (-5.5, -0.7)	5.9 (2.7, 9.2)
Free 25(OH)D, pmol/L	-0.7 (-1.3, -0.03)	-1.3 (-1.8, -0.8)	0.6 (-0.2, 1.5)
DBP, nmol/L	-844 (-1210, -480)	-441 (-824, -59)	-403 (-931, 125)

Abbreviations: 25(OH)D, 25-hydroxy vitamin D; DBP, vitamin D binding protein.

[1] From mixed effects linear regression models for repeated measures adjusted for baseline characteristics including child's sex, age, height- and body mass index-for-age, total 25(OH)D <50 nmol/L, DBP <3400 nmol/L, constitutive skin color, tanning, dietary vitamin D, maternal education and parity, and household food insecurity, socioeconomic status, and daily milk intake. Empirical standard errors were specified in all models.

Supplemental Table 4. Compliance, acceptability, and safety among adolescents[1]

	Fortified milk n = 40	Unfortified milk n = 39	P^2
Compliance			
Unopened experimental milk bags returned[3], median [IQR]	1.5 [0.0, 6.0]	2.0 [0.0, 5.0]	0.79
Estimated experimental milk intake, (cups[4]/d)	1.5 ± 0.4	1.7 ± 0.5	0.11
Acceptability[5]			
The adolescent liked the experimental milk's taste	4.4 ± 0.7	4.2 ± 0.7	0.24
The adolescent would adopt experimental milk	3.8 ± 0.9	3.7 ± 1.0	0.70
Safety endpoints			
Hypercalcemia symptoms at interim visit, median (IQR)[6]	0.0 (0.0, 1.0)	0.0 (0.0, 1.0)	0.45
Change in adolescent's body mass index-for-age Z (95% CI)	0.04 (-0.02, 0.10)	0.00 (-0.07, 0.06)	0.33
Change in total energy intake, kcal/d (95% CI)	-42 (-242, 158)	-71 (-270, 128)	0.84
Change in saturated fat intake, % Energy (95% CI)	-2.6 (-3.8, -1.4)	-0.2 (-1.4, 1.0)	0.006

[1] Means ± SD unless noted otherwise.
[2] Wilcoxon rank-sum and $\chi 2$ tests for continuous and categorical variables, respectively.
[3] Maximum possible 42 per household.
[4] 1 cup = 516 g = 500 mL.
[5] Scores from Likert-type scales from 1 ='completely disagree' to 5 ='strongly agree'.
[6] Number of symptoms reported as "frequent" or "very frequent" 3 weeks post-enrollment from a list of 10 common hypercalcemia symptoms including nausea or vomiting, loss of appetite, excessive thirst, excessive urination, constipation, stomach ache, muscle weakness, muscle or joint pain, confusion, and fatigue.

Supplemental Table 5. Effect of cholecalciferol fortification of skim milk on serum total 25-hydroxy vitamin D in Colombian women according to baseline characteristics

Baseline characteristics[1] (n)	Fortified milk			Unfortified milk			Cholecalciferol fortification effect Mean (95% CI)[2]	*P* fortification interaction[3]
	Baseline	Follow-up	Mean change (95% CI)[2]	Baseline	Follow-up	Mean change (95% CI)		
Body mass index, kg/m^2								0.12
≤25 (30)	46.3 ± 10.6	50.3 ± 9.5	4.0 (0.7, 7.3)	54.2 ± 9.8	50.9 ± 11.4	-3.3 (-6.5, -0.1)	7.3 (2.7, 11.9)	
>25 (50)	44.3 ± 11.6	45.8 ± 12.9	1.4 (-1.4, 4.3)	47.0 ± 10.0	46.3 ± 11.2	-0.7 (-4.1, 2.8)	2.1 (-2.4, 6.6)	
Total 25(OH)D, nmol/L								0.80
≥50 (29)	57.1 ± 4.9	58.1 ± 5.5	0.9 (-2.0, 3.9)	60.5 ± 6.0	57.1 ± 8.3	-3.3 (-7.0, 0.3)	4.3 (-0.4, 9.0)	
<50 (50)	38.7 ± 7.5	42.1 ± 10.2	3.4 (0.4, 6.4)	42.4 ± 5.2	42.4 ± 8.5	-0.1 (-3.2, 3.0)	3.5 (-0.8, 7.7)	
DBP[4], nmol/L								0.76
≥3170 (39)	47.0 ± 10.4	47.6 ± 9.9	0.6 (-1.8, 3.0)	51.5 ± 12.3	49.2 ± 13.0	-2.3 (-6.1, 1.5)	2.9 (-1.6, 7.4)	
<3170 (40)	43.5 ± 11.7	47.9 ± 13.3	4.3 (0.9, 7.8)	47.2 ± 7.7	47.6 ± 9.1	0.4 (-2.9, 3.7)	3.9 (-0.9, 8.6)	
Constitutive skin color, L units[5]								0.07
Lighter, ≥62.4 (41)	43.9 ± 11.9	48.8 ± 11.3	4.8 (2.4, 7.3)	49.0 ± 10.8	46.8 ± 12.2	-2.2 (-4.9, 0.4)	7.1 (3.5, 10.7)	
Darker, <62.4 (39)	46.4 ± 10.3	46.6 ± 12.2	0.2 (-3.2, 3.6)	49.7 ± 10.3	49.2 ± 10.3	-0.5 (-5.3, 4.2)	0.7 (-5.1, 6.6)	
Tanning, L units[6]								0.76
Higher tanning, <-8.3 (40)	45.5 ± 11.3	49.4 ± 9.9	4.0 (1.5, 6.4)	50.9 ± 12.0	50.5 ± 12.0	-0.5 (-3.7, 2.8)	4.4 (0.4, 8.5)	
Lower tanning, ≥-8.3 (40)	44.8 ± 11.2	45.8 ± 13.4	1.0 (-2.7, 4.6)	47.9 ± 8.7	45.6 ± 10.5	-2.4 (-6.3, 1.6)	3.3 (-2.1, 8.7)	

Abbreviations: 25(OH)D, 25-hydroxy vitamin D; DBP, vitamin D binding protein.

[1] Mean ± SD unless noted otherwise.

[2] From mixed effects linear regression models for repeated measures. Empirical standard errors were specified in all models.

[3] Wald test for an interaction term between indicator variables for fortification group assignment, the baseline characteristic, and time (baseline or follow-up).

[4] The cutpoint represents the median of the distribution.

[5] From colorimetric assessment of typically sun-unexposed skin (gluteal area). Units are in the International Commission on Illumination scale, which ranges from 0 (absolute black) to 100 (absolute white). The cutpoint represents the median of the distribution.

[6] Proxy for sun exposure (tanning) as the difference in color units between typically sun-exposed (dorsal hand area) and sun-unexposed (gluteal area) skin. The cutpoint represents the median of the distribution.

Supplemental Table 6. Adjusted effect of cholecalciferol fortification of skim milk on serum total 25-hydroxy vitamin D and vitamin D binding protein in Colombian women

	Mean change (95% CI) from baseline to end of follow-up		Cholecalciferol fortification effect Mean (95% CI)[2]
	Fortified milk	Unfortified milk	
Total 25(OH)D, nmol/L	2.5 (0.3, 4.8)	-1.4 (-3.8, 1.0)	4.0 (0.7, 7.2)
DBP, nmol/L	-546 (-923, -169)	-344 (-691, 3)	-202 (-717, 314)

Abbreviations: 25(OH)D, 25-hydroxy vitamin D; DBP, vitamin D binding protein.

[1] From mixed effects linear regression models for repeated measures adjusted for baseline characteristics including the woman's age, height, body mass index, education level, parity, total 25(OH)D <50 nmol/L, DBP <3170 nmol/L, constitutive skin color, tanning, household food insecurity, socioeconomic status, and daily milk intake. Empirical standard errors were specified in all models.

Supplemental Table 7. Effect of cholecalciferol fortification of skim milk on serum total 25-hydroxy vitamin D and vitamin D binding protein in Colombian women according to compliance with the intervention

Compliance[1,2] (n)	Fortified milk			Unfortified milk			Cholecalciferol fortification effect Mean (95% CI)[2]	*P* fortification interaction[4]
	Baseline	Follow-up	Mean change (95% CI)[3]	Baseline	Follow-up	Mean change (95% CI)		
Total 25(OH)D, nmol/L								0.53
Higher compliance (32)	46.6 ± 12.5	49.4 ± 11.5	2.7 (-0.2, 5.7)	48.2 ± 12.4	45.2 ± 12.2	-3.0 (-5.8, -0.2)	5.7 (1.7, 9.8)	
Lower compliance (47)	44.0 ± 10.0	46.4 ± 11.9	2.4 (-0.8, 5.6)	50.8 ± 8.8	49.5 ± 10.7	-1.3 (-5.0, 2.4)	3.7 (-1.2, 8.6)	
DBP, nmol/L								0.88
Higher compliance (32)	3240 ± 824	2780 ± 1270	-462 (-1090, 166)	3380 ± 878	3010 ± 886	-370 (-936, 196)	-92 (-938, 753)	
Lower compliance (47)	3850 ± 1800	3230 ± 1420	-615 (-1070, -162)	3300 ± 1240	2860 ± 998	-439 (-877, -2)	-176 (-805, 454)	

Abbreviations: 25(OH)D, 25-hydroxy vitamin D; DBP, vitamin D binding protein.

[1] Mean ± SD unless noted otherwise.

[2] Higher compliance was defined as not returning any unopened experimental milk bags to the investigators at the interim and final visits. Lower compliance corresponds to families who returned ≥1 unopened bags.

[3] From linear models for repeated measures. Empirical standard errors were specified in all models.

[4] Wald test for an interaction term between indicator variables for fortification group assignment, compliance, and time (baseline or follow-up).

Supplemental Figure 1. Trial diagram