ORIGINAL ARTICLE

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Persistent Effect of Vitamin D Supplementation on Musculoskeletal Parameters in Adolescents One Year After Trial Completion

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ABSTRACT

We showed a beneficial effect of vitamin D supplementation on musculoskeletal parameters in adolescent girls in a 1-year, randomized, double-blinded placebo-controlled trial (RCT). Our objective for this study was to investigate the residual effect of vitamin D supplementation on bone mineral content (BMC), bone mineral density (BMD), at the lumbar spine and hip, lean mass, and height, 1 year after trial completion. We performed post hoc analyses in 167 adolescents, 86 girls and 81 boys, age 13.9 ± 2 years, who received vitamin D or placebo during the trial, and continued into the follow-up trial. Musculoskeletal parameters were measured at baseline, 12 months (intervention), and 24 months (follow-up). ANOVA and *t* tests were used to compare results between the placebo group and the merged vitamin D arms (200 or 2000 IU/day), by gender. Baseline characteristics were comparable between treatment groups at entry into the extension. Girls who had received vitamin D during the trial, had significantly larger hip BMC increments compared to those assigned to placebo, at 24 months compared to study entry, but not 24 compared to 12 months, which persisted in adjusted analyses. There were no significant differences in bone mass changes between treatment groups in boys, at 24 months compared to 12 months or to baseline. The beneficial effect of vitamin D supplementation on hip bone mass, achieved in girls during the trial, persisted 1 year after trial completion. These net cumulative increments, 1 year after discontinuation of supplementation, may have important implications on optimizing peak bone mass accretion in adolescent girls. © 2016 American Society for Bone and Mineral Research.

KEY WORDS: DXA; GENERAL POPULATION STUDIES; NUTRITION

Introduction

By the age of 18 years, most of peak bone mass is attained in humans.[1] Hence, maximizing peak bone mass may reduce age-related bone loss.[2] It is estimated that a 10% increase in peak bone mass reduces the risk of an osteoporotic fracture in adult life by 50%.[3] The predictive ability of decrease in bone mass on fracture risk in adults was roughly similar to that of a 1 standard deviation (SD) increase in blood pressure for stroke, and better than 1 SD increase in serum cholesterol concentration for cardiovascular disease.[4]

The impact of hypovitaminosis D, as opposed to severe deficiency, on musculoskeletal health in children and adolescents is still unclear. The most important function of vitamin D, established nearly a century ago, is to promote skeletal mineralization.[5] Vitamin D, through its action of optimizing intestinal absorption of calcium, plays an essential role in the normal calcification of the growth plate and the mineralization of osteoid in trabecular and cortical bone.[5] Therefore, an adequate intake of vitamin D during childhood and adolescence is necessary to enhance intestinal calcium absorption and ensure normal bone mineralization.[5]

Several trials have investigated means to maximize peak bone mass through lifestyle measures, by increasing physical activity or emphasizing on dairy products consumption or supplementation of calcium and vitamin D.[1,6–9] Two randomized trials using calcium-fortified milk demonstrated increments in bone mass in adolescent Chinese girls and Gambian boys, but could not demonstrate residual effect after trial completion.[10,11]

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Our group had previously reported on the beneficial effects of vitamin D supplementation in adolescent girls,[12] but not boys,[13] on bone mineral content (BMC), bone mineral density (BMD), and lean mass, in a 1-year trial that compared two doses of vitamin D (low dose = 200 IU/day; high dose = 2000 IU/day) to placebo. The increments were comparable at both doses, although there was a trend for larger increments in bone mass at the high dose, in premenarcheal girls.[12]

To our knowledge, no previous studies have investigated the putative residual effects of vitamin D on the musculoskeletal parameters, after trial completion, if any, in adolescents. We hypothesize that vitamin D beneficial effect on muscle mass, BMD, and BMC in adolescents persists between treatment arms 1 year after trial completion (follow-up). We would anticipate that the most substantial increments would be noted in premenarcheal girls, as noted in the main trial.[12]

Subjects and Methods

Subjects

We used data from subjects who participated in the follow-up of the 1-year randomized vitamin D trial.[12] This clinical trial recruited 363 healthy children and adolescents, mean age 12.9 ± 2 years, a critical age for bone mass accretion, from four schools from the greater Beirut area. Of these, 340 subjects completed the intervention trial, and 167 (81 boys and 86 girls), mean age 13.9 ± 2 years, completed the 1-year follow-up. The baseline characteristics of the subjects in the follow-up study ($n = 187$) were similar to the subjects who completed the 1-year intervention trial ($n = 340$) (data not shown).

The study was approved by the institutional review board, and assent or informed consent was obtained from all study subjects and their parents. Full details are provided in the main trial results.[12]

Intervention

Subjects were randomly assigned to receive weekly placebo oil, low-dose vitamin D (1400 IU; equivalent to 200 IU/day) or high-dose vitamin D (14,000 IU, equivalent to 2,000 IU/day) for 1 year, in a double-blind design. Data were collected at baseline, 12 months, and at 24 months (off any supplementation). Our observational follow-up study merged the low-dose and high-dose vitamin D groups, because no significant differences were noted between the two arms, during the intervention.[12]

Data collection

The subjects underwent a baseline physical examination including height, weight, and Tanner stages, with repeat measurements at 12 and 24 months. Although vitamin D dietary intake was not evaluated, we had previously shown the mean total daily vitamin D intake in a similar population to be 145 (95% CI, 125 to 166) IU in children from private schools, and 111 (95% CI, 97 to 124) IU in those from public schools.[14] BMD and BMC of the lumbar spine, hip, and forearm, and subtotal BMD, BMC, and composition were measured at baseline, at 12 months, and at 24 months, using a Hologic 4500A densitometer (Hologic, Bedford, MA, USA). In our center, the mean ± SD precision of the areal BMD (aBMD) measurements, expressed as the coefficient of variation (CV), for 280 same-day duplicate-scans performed during the study duration was less than 1.2% ± 0.9% for the total body spine, total hip, femoral neck, and lean mass. Anthropometric measures and serum alkaline phosphatase, calcium, phosphorus and 25(OH) vitamin D were measured at 0, 6, and 12 months and musculoskeletal parameters: BMD, BMC, height, and lean mass were measured at 0, 6, 12, and 24 months.

Routine chemistries were measured in the Clinical Chemistry laboratory at American University of Beirut Medical Center (AUB-MC), using the Hitachi 912 autoanalyzer (Roche Diagnostics GmBH, Mannheim, Germany). Serum 25(OH) vitamin D was measured at baseline and 12 months by a competitive protein binding radioimmunoassay using the Incstar Kit (Diasorin; Incstar, Saluggia, Italy), with intraassay and interassay CVs less than 13% at a serum concentration of 47 ng/mL. Routine chemistries were run immediately, and serum was stored at −70°C for vitamin D measurements; all vitamin D measurements from an individual subject were assayed together in the same assay, at the end of the study. The Endocrine Core Laboratory and the Clinical Chemistry Laboratory are certified by the quality assurance (QA) programs of Vitamin D External Quality Assurance Surveillance (DEQAS program; http://www.deqas.org/), and of the College of American Pathologists (CAP; http://www.cap.org/), respectively.

Statistical analyses

All analyses conducted in this study are post hoc analyses, and were performed separately for each gender. Outcomes are absolute or percent changes in lean mass, BMD and BMC at the lumbar spine and hip, and percent changes in height.

Continuous variables are reported as mean ± SD or median (25th percentile to 75th percentile) according to normality. Group and subgroup analyses were performed using independent t test or Mann-Whitney tests when applicable. Repeated measures analysis of variance (RM-ANOVA) was done for musculoskeletal parameters to evaluate overall trends across 2 years. Multivariate analyses were conducted to detect the effect of vitamin D treatment at 24 months, off treatment on BMD/BMC as compared to baseline levels, by gender, with and without adjustment for changes in bone area, lean mass, and height. The adjustment for percent lean mass was because of the known effect of vitamin D on muscle and was shown in the original intervention trial.[12] We also adjusted for bone area and height changes to adjust for growth and pubertal maturation. These adjustments were implemented in the repeated measures two-way ANOVA and in the multivariate analyses. A p value <0.05 was used to indicate significance, and were not adjusted for multiple testing in view of the post hoc and exploratory nature of our analyses. Analyses were done using SPSS version 22.0 software (IBM, Armonk, NY, USA).

In view of the lack of any positive effect of vitamin D supplementation in boys in the main trial,[12,13] the post hoc analyses performed in boys are presented in the Supporting Information.

Results

Baseline characteristics

The extension study included 167 adolescents (81 boys and 86 girls) who were randomized into the two treatment arms. At entry into the follow-up, 19 girls were premenarcheal, and 67 were postmenarcheal, whereas in boys, 45 were labeled as early puberty (Tanner I, II, III) and 36 as late puberty (Tanner IV, V). Baseline characteristics were similar among the treatment

Table 1. Baseline Characteristics at Entry Into Trial Extension (12 Months), Stratified by Initial Treatment in Girls

Total sample ($n = 86$)	Placebo ($n = 32$)	Vitamin D ($n = 54$)	p
Age (years)	14.2 ± 2.1	13.7 ± 2.0	0.35
Height (cm)	155.0 ± 9.1	155.2 ± 7.0	0.92
Weight (kg)	49.5 (41.5–59.0)	47.0 (42.0–56.5)	0.65
BMI (kg/m^2)	20.3 (17.4–22.2)	19.7 (17.9–22.6)	0.46
Lumbar spine BMC (g)	42.643 ± 10.998	42.467 ± 10.172	0.94
Total hip BMC (g)	24.882 ± 4.977	25.117 ± 5.784	0.85
Subtotal body BMC (kg)	1.208 ± 0.260	1.214 ± 0.277	0.92
Lumbar spine BMD (g/cm^2)	0.836 ± 0.140	0.819 ± 0.143	0.61
Total hip BMD (g/cm^2)	0.820 ± 0.124	0.815 ± 0.134	0.84
Subtotal body BMD (g/cm^2)	0.847 (0.802–0.904)	0.831 (0.769–0.896)	0.51
Subtotal lean mass (kg)	30.8 ± 5.6	31.5 ± 6.3	0.64
Subtotal body percentage fat mass (%)	29.2 ± 6.5	28.6 ± 7.0	0.68
Serum Calcium (mg/dL)	10.0 ± 0.3	10.0 ± 0.4	0.91
Serum phosphorus (mg/dL)	4.4 (4.2–4.9)	4.6 (4.05–5.1)	0.56
Serum alkaline phosphatase (IU/L)	134.5 (88.3–214.8)	172.0 (107.5–247.0)	0.21
Serum 25 (OH) vitamin D (ng/mL)	12.7 (8.5–19.5)	20.8 (16.3–28.4)	**<0.001**

Values are mean ± SD or medians (Q1–Q3). Values of p represent significance level of independent t tests or Mann-Whitney test as applicable. Bold p values are significant.

groups, with the exception of 25(OH) vitamin D, being higher in the vitamin D groups than placebo at trial completion, in both genders (Table 1, Supporting Table 1).

Effect of treatment on musculoskeletal parameters, at 12 and 24 months

We confirm significant increments in hip BMC and lean mass at 12 months versus baseline, in the sample of girls who had received vitamin D and entered the extension ($n = 86$), as observed in the intervention parent trial. We also detect a new significant change in hip BMD in this subgroup, and a trend was noted for larger increments at the lumbar spine (Table 2).

Importantly, these increments for hip BMD and hip BMC persisted at 24 months compared to baseline (Table 2, Fig. 1*A*), with a trend for larger increments at the lumbar spine BMD and BMC, in the vitamin D group compared to placebo. Multivariate analysis evaluating the effect of vitamin D at 24 months on percent changes BMD/BMC compared to baseline, showed the sustained beneficial effect on hip BMC, persisted on adjusted analyses (Table 3).

Effect of treatment on musculoskeletal parameters, at 24 months versus 12 months

Similarly, there was a consistent trend for larger increments in percent change hip and lumbar spine BMC and BMD, comparing 24 to 12 months posttherapy, in the vitamin D group compared to placebo (Table 4, Fig. 1*B*) that, however, did not achieve significance. Percent change in lean mass and height did not differ between the two treatment groups in girls (Table 4 for lean mass, data not shown for height).

Figure 2 illustrates the sustainable positive effect of vitamin D over the 2 years on BMC (Fig. 2*A*), and percent change in BMC (Fig. 2*B*), at the spine and total hip, in girls, with a significantly larger increase in hip BMC on adjusted analyses (Fig. 2*A*).

Effect of treatment stratified by menarcheal status in girls, at 12 and 24 months

The results in the subset of premenarcheal girls ($n = 19$) who entered follow-up again replicate those of the parent intervention trial, with significant changes in lumbar spine BMD and lean mass and a new significant change in hip BMD and height in this subgroup at 12 months (Supporting Table 2).

The significant changes in hip and lumbar spine BMD also persisted when comparing 24 months to baseline (Supporting Table 2). There was also a nonsignificant trend for larger increments in percent change BMC and BMD (24 months compared to 12 months) at the spine and hip, in the vitamin D group compared to placebo (Supporting Fig. 1).

Table 2. Percent Change in Musculoskeletal Parameters Stratified by Initial Treatment, at 12 and 24 Months Versus Baseline, in Girls

	12 months versus baseline			24 months versus baseline		
Total sample ($n = 86$)	Placebo ($n = 32$)	Vitamin D ($n = 54$)	p	Placebo ($n = 32$)	Vitamin D ($n = 54$)	p
Lumbar spine BMC (% change)	11.7 ± 9.2	15.9 ± 11.7	0.07	20.7 ± 16.4	28.2 ± 22.2	0.08
Lumbar spine BMD (% change)	6.2 ± 5.7	8.8 ± 7.1	0.09	11.3 ± 9.2	16.0 ± 13.4	0.06
Hip BMC (% change)	7.2 ± 7.7	12.9 ± 10.6	**0.01**	13.9 ± 11.3	21.8 ± 18.5	**0.02**
Hip BMD (% change)	4.4 ± 4.3	6.9 ± 5.1	**0.02**	8.4 ± 6.2	11.9 ± 9.5	**0.04**
Lean mass (% change)	6.3 ± 6.8	9.9 ± 8.6	**0.04**	13.5 ± 12.2	16.1 ± 14.6	0.4
Height (% change)	2.2 ± 1.8	3.0 ± 2.5	0.08	3.8 ± 3.6	4.8 ± 4.2	0.25

Values are means ± SD. Bold p values are significant.

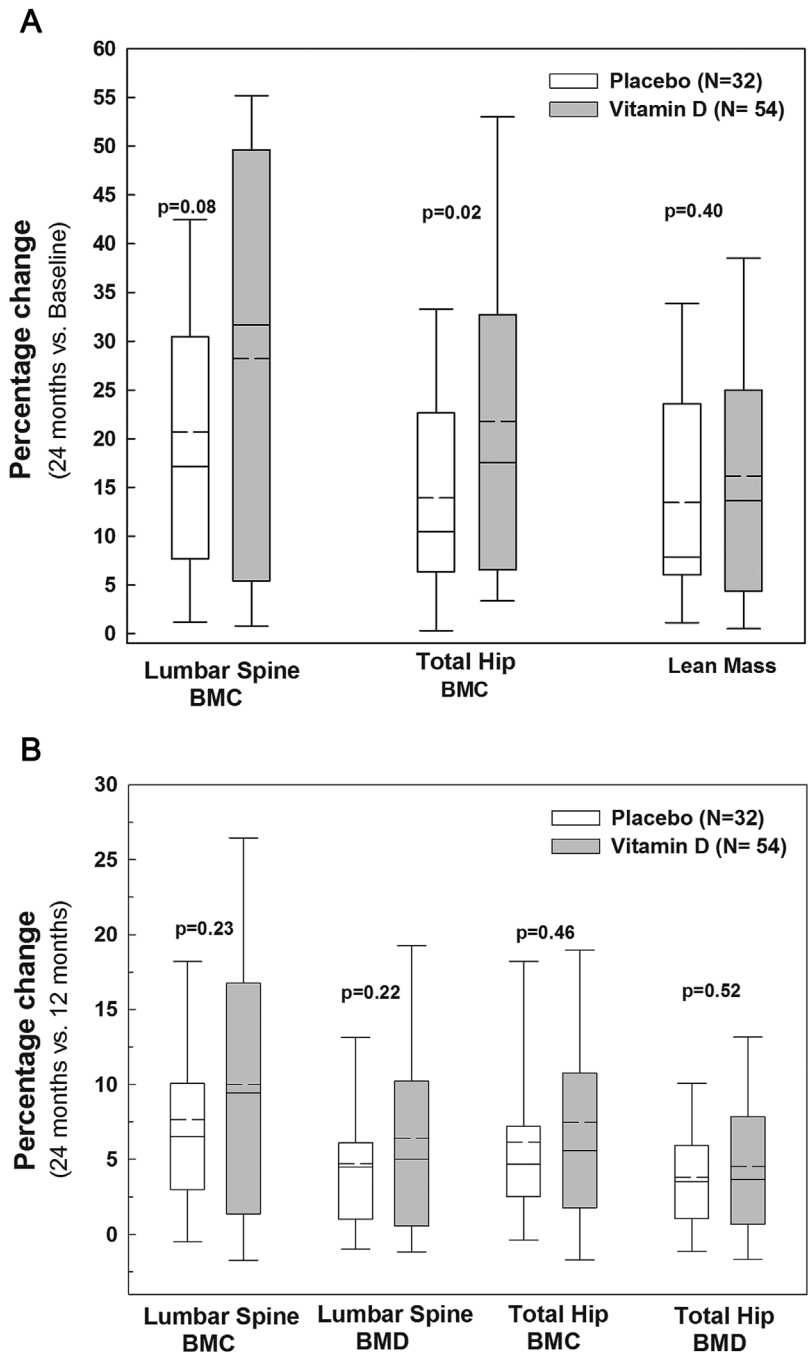

Fig. 1. (*A*) Box plots showing the median and interquartile range of percentage change of lumbar spine BMC, total hip BMC, and lean mass in girls (24 months versus baseline). Medians and means are displayed as solid and dotted lines respectively. Value of *p* depicted represents comparison between the vitamin D group and placebo. (*B*) Box plots showing the median and interquartile range of the percent change in lumbar spine and hip BMCs and BMDs in girls (24 months versus 12 months).

Impact of treatment in boys

Baseline characteristics were similar among the treatment groups, with the exception of 25(OH) vitamin D (Supporting Table 1). No changes were seen in any musculoskeletal parameters in boys at 24 months compared to 12 months, in the overall group of boys and by pubertal status (Supporting Tables 3 and 4).

Discussion

Our study demonstrates that the gains in hip BMC in girls reported in the main intervention trial at 12 months are confirmed in the subgroup who entered the follow-up, but more importantly, maintained at 24 months. This net cumulative significant increase in hip BMC at 24 months in girls who had received vitamin D during the intervention, persisted

Table 3. Multivariate Analysis Relating the Effect of Vitamin D After 2 Years on BMD/BMC as Compared to Baseline Levels, With and Without Adjustment for Percent Bone Area, Lean Mass, or Height, in Girls

All girls	Predictor	*p*
LS BMC (% change)	Vitamin D supplementation	0.098
	Vitamin D supplementation (adjusted for % area)	0.14
	Vitamin D supplementation (adjusted for % lean mass)	0.27
	Vitamin D supplementation (adjusted for % height)	0.22
	Vitamin D supplementation (adjusted for % lean mass and area)	0.16
LS BMD (% change)	Vitamin D supplementation	0.08
	Vitamin D supplementation (adjusted for % area)	0.18
	Vitamin D supplementation (adjusted for % lean mass)	0.2
	Vitamin D supplementation (adjusted for % height)	0.19
	Vitamin D supplementation (adjusted for % lean mass and area)	0.21
Hip BMC (% change)	Vitamin D supplementation	**0.03**
	Vitamin D supplementation (adjusted for % area)	0.19
	Vitamin D supplementation (adjusted for % lean mass)	**0.03**
	Vitamin D supplementation (adjusted for % height)	**0.03**
	Vitamin D supplementation (adjusted for % lean mass and area)	**0.048**
Hip BMD (% change)	Vitamin D supplementation	0.07
	Vitamin D supplementation (adjusted for % area)	0.35
	Vitamin D supplementation (adjusted for % lean mass)	0.13
	Vitamin D supplementation (adjusted for % height)	0.15
	Vitamin D supplementation (adjusted for % lean mass and area)	0.14

Bold *p* values are significant.

after adjusting for height and lean mass. There was no beneficial effect of vitamin D either during the intervention trial or 1 year after its completion (follow-up) in boys.

Studies investigating persistence of a beneficial effect of lifestyle intervention during adolescence on bone mass at later stages, including those that implemented calcium supplementation, through dairy products or tablets, essentially yielded negative results.[10,11,15–19] A 2-year school milk intervention trial showed that 330 mL of a dietary milk supplement (fortified with calcium alone or with both calcium and vitamin D) enhanced the growth and bone mineral accretion in 500 Chinese girls aged 10 years at study entry.[10] However, no persistence of this beneficial effect on change in total BMC and BMD was observed on follow-up 3 years after study completion.[10] Similarly, a prospective 12-year follow-up of 80 prepubertal Gambian boys who had participated in a double-blind, randomized, placebo-controlled trial of calcium supplementation indicated that supplementation in boys with a low calcium diet advanced the adolescent growth spurt, but had no lasting effect on bone mineral or bone size, 12 years later.[11] The improvement in BMD observed in 390 Nigerian toddlers with calcium supplementation also waned at 1 year after discontinuation of therapy.[15] Finally, gains in BMC and BMD attributed to calcium supplementation in 96 girls in the United Kingdom (aged 11 to 12 years) were not evident at 42 months.[16] Lee and colleagues[17] had suggested that the effect of calcium supplementation on bone mineral gain appears to reflect a transient reduction in bone turnover rate. Conversely, the only positive study was one where milk-extracted calcium phosphate taken during the prepubertal period in 116 girls (8 years old) increased BMD at six skeletal sites up to 3.5 years after discontinuation of intervention.[18]

Although several trials emphasized the importance of physical activity, an inexpensive, yet beneficial measure to enhance bone mass in boys and girls, especially during the prepubertal period,[20–23] we could not find any that assessed the delayed or residual benefits of exercise, years after the intervention. Similarly, studies evaluating a sustained effect of vitamin D supplementation after discontinuation of supplementation are scarce. Vitamin D at a dose of 400 IU/day administered during infancy was associated with increased aBMD at the femoral neck, when measured several years later in prepubertal white girls.[24]

The most promising window of intervention seems to be in the premenarcheal period, both for calcium supplementation[18] and vitamin D, in the subset of girls who have low baseline vitamin D levels.[12] The randomized, controlled trial of vitamin D supplementation by Ward and colleagues[9] did not show a beneficial effect on mineral accretion, bone geometry or strength, muscle force, or power, in postmenarcheal females. The lack of an effect of calcium, vitamin D, or exercise after completion of puberty in the above studies,[9,12] strongly suggest that prepuberty and baseline levels are important predictors of bone mass accretion. Finally, the lack of an effect of

Table 4. Percent Change (24 Versus 12 Months) in Musculoskeletal Parameters Stratified by Initial Treatment in Girls

Total sample ($n = 86$)	Placebo ($n = 32$)	Vitamin D ($n = 54$)	*p*
Lumbar spine BMC (% change)	7.7 ± 7.6	10.0 ± 10.3	0.23
Lumbar spine BMD (% change)	4.7 ± 5.3	6.4 ± 7.4	0.22
Hip BMC (% change)	6.2 ± 6.3	7.5 ± 8.6	0.46
Hip BMD (% change)	3.8 ± 4.0	4.5 ± 5.4	0.52
Lean mass (% change)	6.6 ± 7.2	5.7 ± 7.0	0.56

Values are mean ± SD.

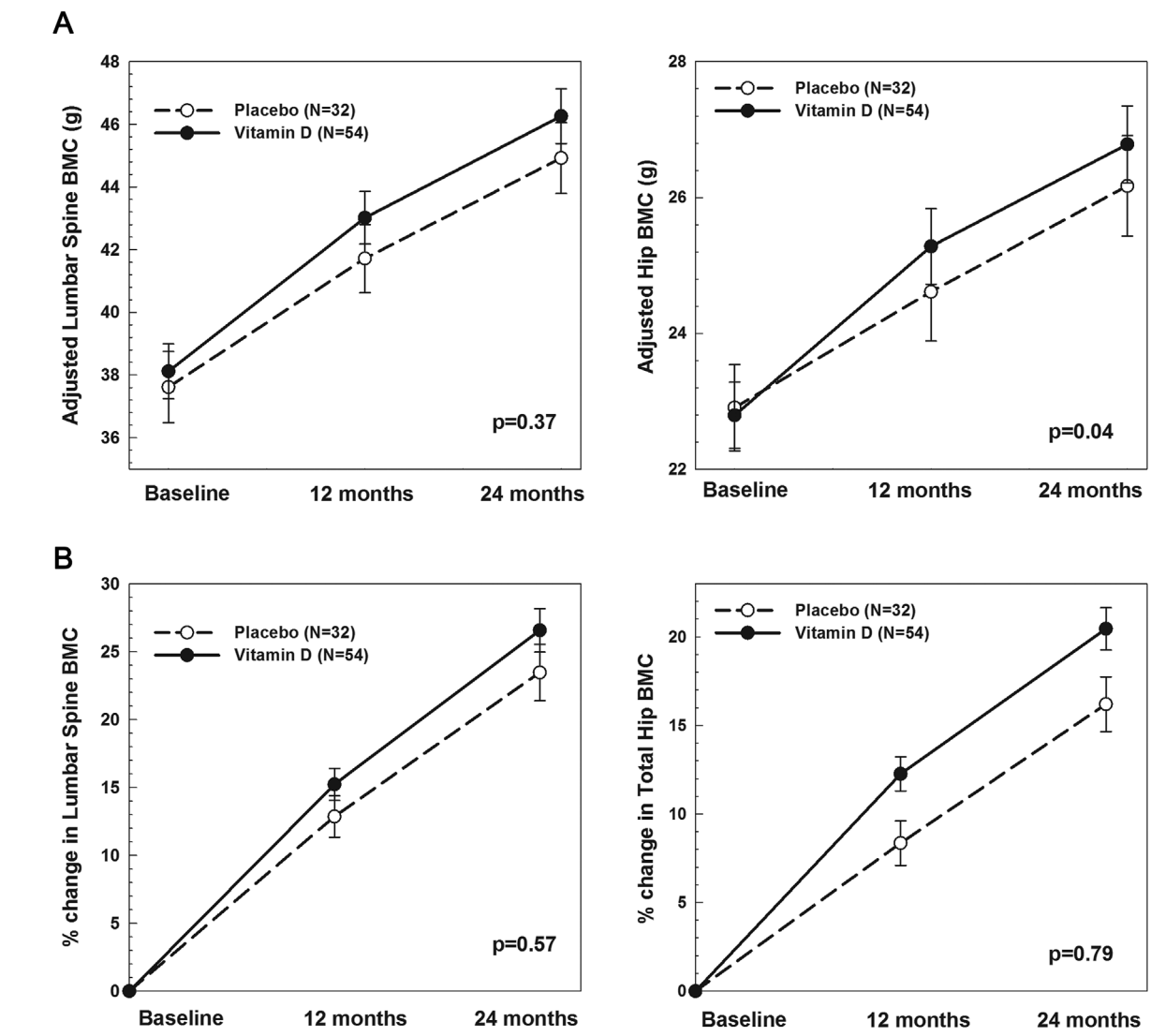

Fig. 2. (*A*) Repeated measures of bone parameters in girls, adjusted for height at baseline and % change in height (24 months versus baseline). Values of *p* represent the significance level for the treatment by time interaction term, for variables of interest between the two groups. Within each treatment group, pairwise comparisons showed significant increase in the variables of interest: 12 months and 24 months as compared to baseline, and 12 months versus 24 months as well, $p < 0.001$. (*B*) Percent change in bone parameters in girls, at 12 and 24 months as compared to baseline. Values of *p* represent significance level for the treatment by time interaction term for variables of interest between vitamin D group and placebo.

vitamin D in boys, be it during the main trial or in the extension, may have several explanations. Boys had a higher calcium intake at baseline, and exercised more.[12,13] There were also gender differences in the severity of hypovitaminosis D at baseline as well as differences in the serum calcitriol levels achieved.[12,13] Furthermore, the different hormonal profiles achieved during puberty might contribute to the observed sexual dimorphism in response to vitamin D supplementation.

Our study had some limitations. Although the original trial was randomized, this follow-up extension in a subset of study participants was not, and selection bias cannot be ruled out. It also may have lacked power to detect persistence of large differences between treatment groups comparing changes at 24 versus 12 months. In fact, assuming that the changes in bone mass at 24 months versus 12 months between treatments are the same as in the main trial (mean $\pm$ SD $=$ 4.2% $\pm$ 10% for total hip BMC), the current sample size in the extension has a 74% power to detect a significant difference between the two arms, 24 months compared to 12 months, with type 1 alpha of 5% between the treatment arm and placebo. Nevertheless, the net cumulative larger increase in bone mass parameters at 24 months after entry into the trial, compared to placebo, despite therapy discontinuation, is quite encouraging. Although the trial is not population-based, it however drew from a large segment of the population in the greater Beirut area and included balanced socioeconomic representation.[12] Lack of

25 OH) vitamin D at 24 months, which was due to a lack of funding, is a limitation to our study. However, we had shared the study results regarding the positive effect of vitamin D on bone, with all participants at study completion, and it would be expected that subjects in the placebo arm may have been encouraged to start taking vitamin D in the follow-up period. This may therefore have resulted in an underestimation of the persistent impact of vitamin D during the follow-up extension at 24 months. Also, although the placebo and vitamin D groups were similar at baseline in terms of calcium intake, exercise, and sun exposure in the intervention trial, such information was not available in the follow-up extension. Several confounding factors could account for our results. Bone mass is not solely a function of vitamin D; it is rather affected by several anatomic, physiologic (sexual maturity, hormonal), nutritional, genetic, and environmental parameters. We did not have Tanner staging during the follow-up, but adjustment for percent lean mass and height in the multivariate analyses took several of these factors into account, and confirmed the robustness of our findings as being independent of such changes. In fact, lean mass was positively affected by vitamin D replacement in girls in the intervention trial. In the subset of girls who continued into the follow-up, we have shown the direct effect of vitamin D supplementation on lean mass at 12 months versus baseline (Table 2). Although this effect was not apparent when we compared 24 months to baseline (Table 2), we nevertheless adjusted for percent lean mass in the multivariate analyses to make sure that the effects of vitamin D on BMC and BMD were independent of lean mass. However, the disappearance of the beneficial effect of vitamin D at 24 months on hip (BMD or BMC, or both) after adjusting for bone area, is consistent with our previous analyses at 12 months for hip BMC[12] and confirms that the effect of vitamin D on bone in adolescents is in part mediated through effects on body size.[12] Finally, no fracture data were available on the study subjects, before, during, and after the intervention trial.

These analyses are to be considered hypothesis-generating and results were therefore not adjusted for multiple testing.

To our knowledge this study is the first to investigate a persistent vitamin D effect 1 year after intervention. In these post hoc analyses, the beneficial effect of vitamin D supplementation on hip BMC that was achieved in girls during the trial, persisted at 24 months; ie, 1 year after trial completion, with net overall increments over 2 years in girls. We had previously shown that vitamin D supplementation improved bone mass and several DXA-derived structural bone parameters at the femoral neck site in adolescent girls[25] and the sustained benefit may indeed carry on into a positive effect on hip fractures in older years. Thus, the results obtained, if validated in a randomized setting, may have important implications with regard to public health and nutritional interventional measures at a critical time for bone mass accretion.

Disclosures

All authors state that they have no conflicts of interest.

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Authors' roles: Study design: GEHF. Study conduct: GEHF, MN, and JM. Data collection: JM. Data analysis: GEHF, LA, and NG. Data interpretation: All authors. Drafting manuscript: GEHF, LA, and NG. Revising manuscript content: All authors. Approving final version of manuscript: GEHF. GEHF takes responsibility for the integrity of the data analysis.

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