Growth, bone mass, and vitamin D status of Chinese adolescent girls 3 y after withdrawal of milk supplementation[1–3]

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ABSTRACT

Background: A 2-y 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 of Chinese girls aged 10 y at baseline. Girls who received milk fortified with both calcium and vitamin D also had better vitamin D status than did girls who received nothing or girls who received milk fortified only with calcium.

Objective: The aim was to evaluate whether these effects were sustained 3 y after supplement withdrawal.

Design: Anthropometric measures and dietary intake were reassessed in 501 of the 698 girls whose data had been studied at the end of the intervention. As in the intervention phase, total-body bone mineral content and bone mineral density and serum 25-hydroxyvitamin D concentrations were measured in half of these subjects.

Results: At follow-up, 99% of girls had reached menarche, at a mean (±SD) menarcheal age of 12.1 ± 1.1 y. No significant differences in the timing of menarche were observed between the 3 groups ($P = 0.6$). No significant differences in the changes of total-body bone mineral content and bone mineral density since baseline were observed between the groups. The group receiving calcium-fortified milk had significantly greater gains in sitting height (0.9 ± 0.3%; $P = 0.02$) than did the control group. The group that received calcium- and vitamin D–fortified milk had 17.1 ± 6.7% lower serum 25-hydroxyvitamin D concentrations than did the control group ($P = 0.04$), but the difference was attenuated by additional adjustment for physical activity level (14.2 ± 6.7%; $P = 0.08$).

Conclusion: Milk supplementation during early puberty does not have long-lasting effects on bone mineral accretion. *Am J Clin Nutr* 2006;83:714–21.

KEY WORDS Milk supplementation, growth, bone mineral accretion, vitamin D status, Chinese girls, follow-up study

INTRODUCTION

Bone mass at any time during adult life is determined by peak bone mass achieved at skeletal maturity and the rate of bone loss during aging. An optimal peak bone mass is considered the best protection against age-related bone loss and subsequent fracture risk. Dietary calcium intake is an important nutritional factor that influences the achievement of peak bone mass. Dietary supplementation of calcium has been shown to increase bone mineral accretion in children and adolescents from different ethnic backgrounds (1–9). Enhancement of bone mineral accretion has also been reported in white adolescent girls and Chinese children who received milk or dairy product supplementation (10–13). However, whether the effects of short-term supplementation during childhood or adolescence are maintained after milk or calcium supplement withdrawal is uncertain. Only a few studies have made follow-up measurements, and the results were inconsistent. Three follow-up studies of calcium supplementation (in the form of calcium carbonate or calcium phosphate extract from milk) have found that some of the effects on bone mineral accretion were sustained 1.5–3.5 y after the withdrawal of supplements (14–16). In contrast, other studies have shown that the effects disappeared 1–3 y after the withdrawal of calcium (in the form of calcium carbonate or calcium citrate malate) (17–19) or dairy (12) supplements.

In a school milk intervention study conducted on Beijing girls aged 10 y at baseline, we showed that the subjects who received a 330-mL dietary milk supplement (milk fortified with calcium alone or with both calcium and vitamin D) on school days had greater increases in height (by 0.7–0.8%), sitting height (by 0.7–1.2%), total-body-size–adjusted bone mineral content (BMC; by 1.1–2.5%), and total-body bone mineral density (BMD; by 3.1–5.4%) after 2 y than did the unsupplemented control subjects (20, 21). The group that received milk fortified with both calcium and vitamin D had significantly higher plasma 25-hydroxyvitamin D [25(OH)D] concentrations ($\bar{x}$ ± SD: 47.6 ± 23.4 nmol/L) than both the group that received milk fortified with calcium alone (17.9 ± 9.0 nmol/L) and the control group (19.4 ± 10.2 nmol/L). The purpose of the present study was to evaluate whether the effects of milk supplementation on bone mineral accretion, linear growth, and vitamin D status persisted 3 y after supplement withdrawal.

SUBJECTS AND METHODS

Subjects and study design

The characteristics of the study subjects and the methods used in the intervention study were detailed previously (20). The

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important aspects are described below. Seven hundred fifty-seven girls [$\bar{x}$ (±SD) age at baseline: 10.1 ± 0.4 y] from 9 primary schools in Beijing participated in the school milk intervention study from 1999 to 2001. The 9 schools were randomly assigned to 3 study groups, which were matched for the socioeconomic background of the students. For both ethical and practical reasons, it was not possible to randomize within schools so that different milk supplements or no milk would have been provided to different students in the same class. Two hundred thirty-eight girls were assigned to the calcium milk (Ca milk) group who received 330 mL calcium-fortified ultra-high temperature (UHT) milk per school day, 260 girls to the calcium-vitamin D milk (CaD milk) group who received 330 mL calcium- and vitamin D–fortified UHT milk per school day, and 259 girls to the control group who received no intervention and consumed their habitual diet during the study period. The UHT milk for this project was specially formulated by Murray Goulburn Co-operative Co Ltd (Brunswick, Australia). The milk was fortified with a milk calcium salt (NatraCal) to provide a total 560 mg calcium in 330 mL. NatraCal is a calcium salt derived from fresh milk whey and contains 240 g calcium/kg, 520 g phosphate/kg, 50 g lactose/kg, 50 g protein/kg, 10 g fat/kg, 20 g other inorganic salts/kg, and 110 g water/kg. Each carton of milk (330 mL) also contained 10 g fat and 10 g protein; for milk used in the CaD milk group, 5–8 μg vitamin D (cholecalciferol; Vitamin $D_3$100 CWS/A; Roche Pty Ltd, Sydney, Australia) was added. After correcting for weekends and school holidays when no intervention milk was consumed, the average supplementation over the 2 y was 144 mL milk/d, which contained 254 mg calcium and, for the CaD milk group, 3.33 μg vitamin D.

Of the 698 girls from 9 primary schools who completed the milk intervention study, we were able to contact 587 of them 3 y after the cessation of the intervention, and 504 girls consented to be reexamined. The other 111 girls were unavailable for follow-up measurements because either they had moved from Beijing ($n = 6$) or we were unable to contact them ($n = 105$). The 504 subjects were enrolled in 26 secondary schools, with 1–56 subjects in each school. Thus, 2 types of clusters were involved: the first cluster type was the primary school that the subjects attended, and the second cluster type was the secondary school attended. None of the girls had medical conditions or were taking medications known to influence bone mineral metabolism. The study was approved by the ethics committees of the University of Sydney and the Institute for Nutrition and Food Safety of the Chinese Center for Disease Control and Prevention. Written informed consent was obtained from each subject and their parents.

Anthropometric measurements and pubertal staging

Subjects were weighed to the nearest 0.1 kg while wearing light clothing and no shoes with an electronic digital scale (Thinner, Fairfield, WI). Height and sitting height were measured by the same observer (QZ) with the use of the same stadiometer in the hospital where the bone densitometry scans were made; the subjects were measured in bare feet to the nearest 0.1 cm with a body height–measuring device (TG-III Type, No. 6; Machinery Plant, Beijing, China). Breast development and pubic hair development were ascertained according to Tanner's definitions of the 5 stages of puberty (22) during an interview by the same observer (KZ). Menstrual status was also determined by interview.

Dietary intake and physical activity

Dietary intakes were assessed from two 3-d food records (including 2 weekdays and 1 weekend day). Chinese measures of bowls, plates, and spoons of standard size were used to quantify food items with the assistance of a set of food-measure models. Chinese food-composition tables and data entry programs were used to calculate nutrient intakes, as previously reported (20). Physical activity levels over the previous 12 mo were estimated by a questionnaire, which included 12 categories of school-organized activities, 25 categories of spare-time activities (ie, activities undertaken during class breaks, lunchtime recesses, and outside school hours), and an open question at the end. The subjects were instructed to report the following: *1*) the type, frequency, and duration of school-organized physical activity and spare-time physical activity; and *2*) the extent of any training at sports clubs or in sporting teams over the previous 12 mo. The subjects' school physical activity score, the pattern of spare-time allocation, and their means of traveling to and from school were also recorded.

Bone mineral measurement

Total-body BMC and BMD were assessed in a subset of 277 subjects whose total-body bone measurement data were available both at baseline and after 24 mo of the intervention. Total-body BMC, bone area, and BMD were measured by dual-energy X-ray absorptiometry with the same Norland XR-36 densitometer (Norland, Fort Atkinson, WI) used in the intervention study. Software version 3.94 was used. The densitometer had a variation in precision of $<1.0\%$ for the measured bone site at standard speed. A daily quality assurance test was performed over the study period with the use of a manufacturer-supplied hydroxyapatite phantom, and the accuracy error was $<1.0\%$. Two technicians performed the measurements during the follow-up study under supervision of the technician who performed the measurements during the intervention study.

Biochemical analysis

Samples of blood (drawn after the subjects had fasted overnight, between 0630 and 0900) were obtained during March and April (ie, the end of winter), similar to sampling performed during the intervention baseline and at the end of the intervention. Serum 25(OH)D and parathyroid hormone (PTH) concentrations were assessed in a subset of subjects, similar to the intervention study. Samples from different schools or groups were analyzed in the same batch. Complete baseline and follow-up 25(OH)D and PTH data were obtained from 213 and 98 girls, respectively. Serum 25(OH)D concentrations were measured with ^{125}I radioimmunoassay (DiaSorin, Stillwater, MN), because the competitive protein-binding assay (CPBA) that was used in the intervention study was not available in China. The intraassay CV was 4.2% and the interassay CV was 6.6%. A cross-calibration study with 18 human samples carried out in both CPBA (in the laboratory of Faculty of Veterinary Science, University of Sydney, where the intervention study samples were assayed) and radioimmunoassay (in the laboratory of 304 Hospital in Beijing, where the follow-up samples were assayed) showed that the serum 25(OH)D concentration measured by the radioimmunoassay method was, on average, 23% higher than that measured by CPBA (LHF, unpublished data). Serum PTH concentrations were measured by immunometric assay (Immulite Intact PTH;

Diagnostic Products Corporation, Los Angeles, CA). The intraassay CV was 2.9% and the interassay CV was 5.0%.

Other measurements

The health history of the subjects and family socioeconomic status were obtained by a general information questionnaire. Posteroanterior X-ray radiographs of the nondominant hand and wrist were taken, and bone age was determined to the nearest 0.1 y according to the Chinese standard (23).

Statistical analysis

Descriptive statistics are reported as means ± SDs and differences as means ± SEMs for all variables, unless otherwise indicated. One-factor analysis of variance and 2-factor repeated-measures analysis of variance were used to compare continuous demographical variables at baseline and follow-up. Post hoc analyses were carried out with Tukey's honestly significant difference test. Differences in the frequency data between groups at the same time period were analyzed by a chi-square test.

To allow for clustering by school at both the primary and secondary school levels, a linear mixed model was used to examine the effects of milk supplementation on growth, bone mineral accretion, and serum 25(OH)D and PTH concentrations during follow-up, with schools defined as random effects (24, 25). To investigate any proportional effects of discrete variables (ie, the supplementation group), the continuous variables (ie, bone and anthropometric measures) were natural log-transformed. Because the variable "group" had 3 outcomes (Ca milk, CaD milk, and control groups), 2 dummy variables (Group1 and Group2) were created to represent this variable, where Group1 = 1 for Ca milk and 0 for CaD milk and control, and Group2 = 1 for CaD milk and 0 for Ca milk and control. These 2 dummy variables were treated as a group and were both in each model together. Outcomes were analyzed with adjustment for the baseline value, baseline pubertal stage, and intervention group. Size-adjusted BMC was introduced in a fuller model to study the effects on bone mineral status independently of bone and body size. In this model, BMC was adjusted for bone area, body weight, and height, and we used the mean values and the differences of these variables at baseline and follow-up. Additional modeling was done to adjust for calcium intake, vitamin D intake, and physical activity level. To test whether there were interactions between intervention groups and the baseline value, baseline pubertal stage, calcium intake, vitamin D intake, and physical activity level, the significance of the relevant interaction terms was tested in each model. The regression parameter b_{group} for each intervention group, once multiplied by 100, represents the differences between each of the intervention groups and the control group in percentage change from baseline (26). The differences between the 2 supplemented groups were calculated in a similar way. To show whether the absence of effects was due to reduced statistical power from clustering by school in the linear mixed model, the effects of milk supplementation on growth and bone mineral accretion at follow-up were also evaluated at the person level with a multiple regression model with backward elimination (7, 20). The significance level for test statistics was set at $P < 0.05$. All data were analyzed by SPSS (version 12.0; SPSS Inc, Chicago, IL) and SAS (SAS for WINDOWS version 9.1; SAS Institute Inc, Chicago, IL).

RESULTS

Of the 698 subjects who completed the milk intervention trial, 504 subjects were reexamined 3 y after the end of the intervention (**Figure 1**). No significant difference in the dropout rate was observed between the 3 groups (27.3%, 28.5%, and 28.7% for the Ca milk, CaD milk, and control groups, respectively). In each study group, there were no significant differences in anthropometric and bone measures between the subjects who were reexamined and those who dropped out at baseline and at the end of the intervention. Data for the 501 girls whose complete data were obtained are shown in the present study.

The characteristics of the study and the control groups at presupplementation baseline and 36 mo after the supplement withdrawal are shown in **Table 1**. At presupplementation baseline, there were no significant differences between the 3 groups in age, bone age, pubertal status, and dietary intakes of calcium, phosphorus, vitamin D, and protein, but the control group had significantly higher milk intakes than the CaD milk group and significantly higher energy intake than the Ca milk group. After the supplement withdrawal (at 36 mo), there were no significant differences in bone age, pubertal status, and dietary intake of milk, calcium, phosphorus, energy, and protein between the 3 groups. Compared with the presupplementation baseline, all 3 groups had significantly higher milk and calcium intakes and lower dietary vitamin D intakes 36 mo after the withdrawal of the milk supplement. During the study, the average time spent on spare-time physical activity was higher in the control group than in the supplemented groups. Ninety-nine percent of the girls had reached menarche at follow-up. No significant differences in mean menarcheal age were observed between the 3 groups ($\bar{x}$ ± SD age: 12.2 ± 1.1 y for the Ca milk group, 12.1 ± 1.1 y for the CaD milk group, and 12.2 ± 0.9 y for the control group; $P = 0.6$).

No significant differences in anthropometric and bone measurements were observed between the 3 groups at presupplementation baseline (**Table 2**). At the end of intervention, both supplemented groups had significantly greater gains in height, sitting-height, and total-body BMD than the control group (**Table 3**). The CaD milk group also had significantly greater gains in body weight, BMI, total-body BMC, and size-adjusted BMC than the control group, and significantly greater gains in total-body BMD and size-adjusted BMC than the Ca milk group (Table 3).

Three years after the withdrawal of the milk supplements, the Ca milk group had a significantly greater gain in sitting height than the control group, and this persisted after adjustment for the baseline value, baseline pubertal stage, and clustering by school (Table 3). The Ca milk group showed a significantly greater gain in height than did the control group in analyses performed at the person level ($\bar{x}$ ± SD: 0.5 ± 0.2%, $P = 0.03$), but not after adjustment for clustering by school. No significant differences in the gains of height and sitting height were observed between the CaD milk group and the control group, and there were no significant differences in the gain of weight, BMI, total-body BMC, size-adjusted BMC, bone area, and BMD between the supplemented groups and the control group since presupplementation baseline (Table 3). Additional adjustment for calcium intake, vitamin D intake, and physical activity level had little influence on the differences in the percentage changes of bone and anthropometric variables between the supplemented and control groups

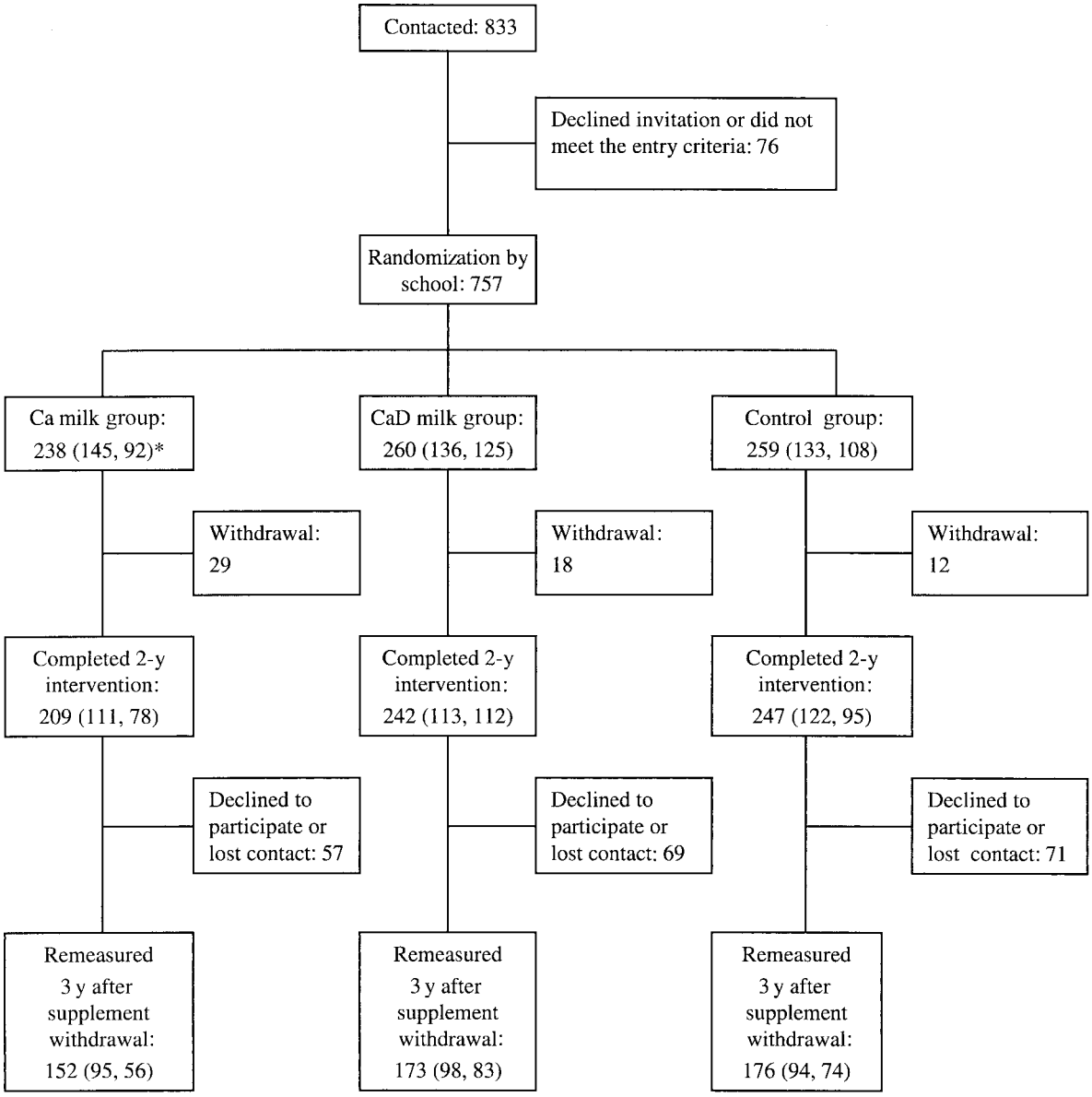

FIGURE 1. . Study profile and number of subjects at each stage of the investigation. *Numbers in parentheses are the number of subjects in whom measurements of total-body bone mineral and plasma 25-hydroxyvitamin D [25(OH)D] concentrations, respectively, were conducted at that stage. Ca milk group, calcium-fortified milk group; CaD milk group, calcium- and vitamin D–fortified milk group.

since presupplementation baseline (data not shown). At follow-up, there were no significant differences in the increase in all anthropometric and bone variables between the Ca and CaD milk groups since presupplementation baseline (Table 3).

The mean (±SD) 25(OH)D concentrations at follow-up were 35.3 ± 16.7, 31.2 ± 13.5, and 37.1 ± 18.5 nmol/L for the Ca milk, CaD milk, and control groups, respectively. Although the CaD milk group had significantly higher serum 25(OH)D concentrations at the end of the 2-y intervention (92.3 ± 12.1%; $P = 0.0003$) than did the control group, the serum 25(OH)D concentration of the CaD milk group was significantly lower than that of the control group at follow-up (17.1 ± 6.7%; $P = 0.04$), and this persisted after adjustment for the baseline value, baseline pubertal stage, and clustering by school. The difference was attenuated, however, by additional adjustment for physical activity level (14.2 ± 6.7%; $P = 0.08$). No significant differences in mean (±SD) serum PTH concentrations were observed between the supplemented and the control groups at follow-up; the values were 36.9 ± 17.7, 42.0 ± 22.7, and 43.3 ± 21.7 pg/mL for the Ca milk, CaD milk, and control groups, respectively. Additional adjustment for calcium and vitamin D intake had little influence on the differences in these 2 biochemical measurements between the supplemented and control groups (data not shown).

DISCUSSION

Our study showed that the effects of milk supplementation on total-body bone mineral accretion had disappeared 3 y after the supplement withdrawal, whereas the effects on sitting height were still evident in the group that received milk fortified with calcium. A lower vitamin D status compared with controls was

TABLE 1
Characteristics and nutrient intakes of the milk-supplemented and control groups at presupplementation baseline and 3 y after supplement withdrawal[1]

	Presupplementation baseline			3-y Follow-up		
	Ca milk group (n = 152)	CaD milk group (n = 173)	Control group (n = 176)	Ca milk group (n = 152)	CaD milk group (n = 173)	Control group (n = 176)
Age (y)	10.1 ± 0.4[2]	10.1 ± 0.4	10.0 ± 0.4	15.1 ± 0.4	15.1 ± 0.4	15.0 ± 0.4
Bone age (y)	9.9 ± 0.9	10.0 ± 1.1	10.1 ± 1.1	15.9 ± 0.9	16.0 ± 0.8	16.0 ± 0.9
Tanner breast stage (%)						
1	42.8	41.0	49.4	0	0	0
2	51.3	47.4	44.9	0	0.6	0
3	5.9	11.6	5.7	2.0	6.4	2.3
4–5	0	0	0	98	93	97.7
Tanner pubic hair stage (%)						
1	96.1	93.6	96.6	0.7	0	0
2	3.9	5.8	2.8	4.7	4.1	2.3
3	0	0.6	0.6	17.3	15.1	10.3
4–5	0	0	0	77.3	80.8	87.4
Postmenarcheal (%)	0.7	1.7	0.6	97.4	99.4	100
Milk intake (g/d)[3]	120 ± 92	106 ± 91[4]	136 ± 97	147 ± 116	134 ± 113	142 ± 106
Calcium intake (mg/d)[3]	415 ± 142	420 ± 183	456 ± 174	457 ± 207	462 ± 202	464 ± 190
Phosphorous intake (mg/d)	782 ± 207	796 ± 211	822 ± 225	805 ± 260	840 ± 229	813 ± 229
Vitamin D intake (μg/d)[5]	0.83 ± 0.56	1.03 ± 0.87	1.03 ± 0.84	0.55 ± 0.53	0.61 ± 0.45	0.68 ± 0.61
Energy intake (kJ/d)	5519 ± 1252[6]	5783 ± 1268	5909 ± 1365	5615 ± 1663	5792 ± 1390	5746 ± 1478
Protein intake (g/d)	52 ± 15	54 ± 15	55 ± 17	53 ± 18	55 ± 15	54 ± 16
Spare-time physical activity (h/wk)[7]	7.3 ± 6.8	7.7 ± 8.0	8.6 ± 7.7	6.2 ± 4.7	6.6 ± 4.6	8.3 ± 5.9

[1] The Ca milk group received 330 mL calcium fortified milk and the CaD milk group received 330 mL calcium– and vitamin D–fortified milk on school days during the 2-y milk intervention trial. There was no significant interaction between group and time for any variables (2-factor repeated-measures ANOVA with interaction).
[2] $\bar{x}$ ± SD (all such values).
[3] Milk and calcium intakes were significantly greater at follow-up than at baseline, $P < 0.004$ (2-factor repeated-measures ANOVA with interaction).
[4,6] Significantly different from the control group at baseline (one-factor ANOVA, post hoc Tukey's test): [4]$P = 0.01$, [6]$P = 0.03$.
[5] Vitamin D intake was lower at follow-up than at baseline, $P < 0.001$ (2-factor repeated-measures ANOVA with interaction).
[7] Significant main effects of intervention group, $P = 0.005$ (2-factor repeated-measures ANOVA with interaction). Post hoc Tukey's test showed a significant difference between the Ca milk and the control groups ($P = 0.005$) and between the CaD milk and the control groups ($P = 0.04$).

also observed in the group that received milk fortified with both calcium and vitamin D.

Our findings on bone mineral accretion are consistent with the results of 4 previous studies, including 2 studies conducted on Chinese prepubertal children that showed that the greater gains in bone mass in children who received calcium or dairy supplementation for 18–36 mo were not sustained when the subjects were followed-up 12–36 mo after the withdrawal of calcium (17–19) or dairy (12) supplementation. However, 3 other studies have found that the some of the benefits of 1-y calcium supplementation on bone mineral accretion were still evident 1.5–3.5 y after the withdrawal of supplements (14–16).

Bonjour et al (14) found that the greater gain in bone mass in prepubertal girls who received 1-y supplementation of calcium phosphate extract from milk was maintained 3.5 y after the cessation of intervention, and the greater gains in bone mass were

TABLE 2
Anthropometric, bone, and biochemical variables of the milk-supplemented and control groups at presupplementation baseline[1]

	Ca milk group	CaD milk group	Control group
Height (cm)	140.0 ± 6.1 [152]	141.1 ± 6.6 [173]	141.2 ± 6.3 [176]
Sitting height (cm)	74.8 ± 3.4 [152]	75.6 ± 3.5 [173]	75.5 ± 3.3 [176]
Weight (kg)	33.8 ± 7.4 [152]	33.6 ± 7.1 [173]	34.0 ± 7.2 [176]
BMI (kg/m^2)	17.1 ± 2.9 [152]	16.8 ± 2.7 [173]	17.0 ± 2.7 [176]
Total-body BMC (g)	1334.0 ± 215.0 [95]	1336.8 ± 199.5 [98]	1374.2 ± 210.4 [94]
Total-body BA (cm^2)	1932.6 ± 177.4 [95]	1944.2 ± 178.5 [98]	1952.1 ± 171.5 [94]
Total-body BMD (g/cm^2)	0.687 ± 0.057 [95]	0.685 ± 0.047 [98]	0.701 ± 0.056 [94]
25(OH)D (nmol/L)	17.8 ± 8.1 [56]	20.2 ± 8.4 [83]	19.3 ± 7.5 [74]
PTH (pg/mL)	37.2 ± 20.8 [34]	36.9 ± 25.4 [33]	41.6 ± 21.2 [31]

[1] All values are $\bar{x}$ ± SD; n in brackets. The Ca milk group received 330 mL calcium-fortified milk and the CaD milk group received 330 mL calcium- and vitamin D–fortified milk on school days during the 2-y milk intervention trial. BMC, bone mineral content; BA, bone area; BMD, bone mineral density; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone. There were no significant differences for any variable between the 3 groups at baseline (one-factor ANOVA with post hoc Tukey's test).

TABLE 3
Effects of milk supplementation on anthropometric and total-body (TB) bone measures 3 y after supplement withdrawal[1]

	Unadjusted percentage change since baseline			Adjusted difference in percentage change since baseline[2]					
				Ca milk group − control group		CaD milk group − control group		CaD milk group − Ca milk group	
	Ca milk group	CaD milk group	Control group	%	*P*	%	*P*	%	*P*
Height									
End of trial	9.5 ± 0.2[3]	9.2 ± 0.2	8.6 ± 0.1	0.8 ± 0.3	0.03	0.7 ± 0.3	0.05	−0.1 ± 0.3	0.6
Follow-up	15.4 ± 0.3	14.5 ± 0.3	14.7 ± 0.3	0.5 ± 0.3	0.1	0.1 ± 0.3	0.6	−0.4 ± 0.3	0.2
Sitting height									
End of trial	9.3 ± 0.2	8.7 ± 0.2	7.9 ± 0.2	1.2 ± 0.3	0.006	0.8 ± 0.3	0.03	−0.4 ± 0.3	0.2
Follow-up	16.1 ± 0.3	14.8 ± 0.3	14.7 ± 0.3	0.9 ± 0.3	0.02	0.3 ± 0.3	0.3	−0.6 ± 0.3	0.09
Weight									
End of trial	34.1 ± 0.8	35.8 ± 0.9	31.1 ± 0.7	2.0 ± 1.2	0.1	3.4 ± 1.2	0.03	1.4 ± 1.2	0.3
Follow-up	65.5 ± 1.6	65.5 ± 1.6	64.3 ± 1.4	0.7 ± 1.1	0.5	0.8 ± 1.0	0.5	0.1 ± 1.1	0.9
BMI									
End of trial	11.7 ± 0.6	13.8 ± 0.7	11.2 ± 0.5	0.3 ± 1.4	0.8	3.3 ± 1.3	0.05	3.0 ± 1.4	0.07
Follow-up	24.1 ± 1.0	25.9 ± 1.0	24.7 ± 0.8	−0.4 ± 1.0	0.7	0.7 ± 1.0	0.5	1.1 ± 1.0	0.3
TBBMC									
End of trial	38.1 ± 1.0	40.7 ± 1.0	36.2 ± 0.9	1.0 ± 1.1	0.4	2.9 ± 1.2	0.04	1.9 ± 1.2	0.2
Follow-up	71.9 ± 1.5	70.8 ± 1.5	70.7 ± 1.5	0.2 ± 0.9	0.9	−0.4 ± 0.9	0.7	−0.5 ± 0.9	0.6
TBBA									
End of trial	29.3 ± 1.0	28.6 ± 1.0	31.2 ± 1.1	−1.4 ± 0.9	0.2	−2.0 ± 0.9	0.07	−0.6 ± 0.9	0.6
Follow-up	32.1 ± 0.8	31.2 ± 1.0	30.8 ± 0.8	1.0 ± 0.6	0.1	0.4 ± 0.6	0.5	−0.6 ± 0.6	0.4
TBBMD									
End of trial	7.0 ± 0.7	9.7 ± 0.7	4.1 ± 0.6	2.3 ± 0.8	0.03	5.0 ± 0.8	0.001	2.8 ± 1.0	0.02
Follow-up	30.1 ± 0.7	30.2 ± 0.6	30.4 ± 0.6	−0.5 ± 0.8	0.5	−0.3 ± 0.8	0.6	0.2 ± 0.8	0.8
Size-adjusted TBBMC									
End of trial	—	—	—	1.5 ± 0.7	0.07	3.8 ± 0.7	0.002	2.4 ± 0.7	0.02
Follow-up	—	—	—	−0.3 ± 0.8	0.7	0.04 ± 0.8	1.0	0.3 ± 0.7	0.7

[1] The Ca milk group received 330 mL calcium-fortified milk and the CaD milk group received 330 mL calcium- and vitamin D–fortified milk on school days during the 2-y milk intervention trial. BMC, bone mineral content; BA, bone area; BMD, bone mineral density. $n = 152$ for the Ca milk group, 173 for the CaD milk group, and 176 for the control group for anthropometric variables. $n = 95$ for the Ca milk group, 88 for the CaD milk group, and 94 for the control group for bone variables.

[2] Analysis with linear mixed model, adjusted for baseline value, baseline pubertal stage, and clustering by schools (*see* Methods for details).

[3] $\bar{x} \pm$ SEM (all such values).

mainly due to a greater increase in bone size, which possibly resulted from the anabolic effects of milk calcium salt on the bones. Although anabolic effects of milk on bone were also shown in our study (as persistent effects on axial growth after supplement withdrawal), we did not observe a sustained effect on bone mass. One possible reason for the different findings of the study by Bonjour et al and our study could be that the average background calcium intake of the subjects in the Bonjour et al study was twice that of our subjects, which was 450 mg calcium/d. Because the calcium intake of our subjects could not meet the amount required for growth, the bone-remodeling rate increased and the additional bone mineral deposited during the supplementation phase was mobilized when supplementation was discontinued, and the benefits on bone mineral status therefore disappeared (27, 28).

The interaction between puberty and dietary intervention may play a role in the long-term effects of short-term supplementation. Additional follow-up (7.5 y after supplement withdrawal) of the subjects in the study by Bonjour et al suggested that calcium supplementation before puberty was associated with an earlier onset of menarche, and sustained effects on BMD were observed only in the girls with earlier menarche (29). In the present study, although there was a trend for more girls who received supplemented milk to have reached menarche at the end of the intervention than those in the control group (48.3% compared with 40.1%; $P = 0.09$), there were no significant differences in age of menarche between the supplemented and control groups at follow-up. Whether the intake of milk calcium salt during prepuberty has a greater effect on menarcheal age or the supplementation amount of the present study of ≈144 mL milk/d (containing 245 mg calcium) is not high enough to affect the age of menarche deserves additional study. In the present study, any residual effects on bone mineral status could also be obscured by the effect of puberty on bone development. Puberty plays a critical role on bone mineral accretion (30), and our subjects progressed from pre-early puberty to mid-late puberty during the 2-y intervention and 3-y follow-up.

A possible long-term effect of milk supplementation during early puberty (from 10- to 12-y-old) on axial growth was observed in the Ca milk group but not in the CaD milk group. A nonsignificant trend toward a lower gain in sitting height was observed for the CaD milk group compared with the Ca milk group ($\bar{x} \pm$ SD: 0.6 ± 0.3; $P = 0.09$). This trend could be related either to the relatively low baseline sitting height of the Ca milk group or to a possible negative effect of vitamin D, which modified a positive effect of the milk supplement itself. In other studies in which dietary supplements of inorganic calcium were supplied (1–4, 6–9), there was no effect on growth rate. Our

results indicated that some components of milk (calcium, protein, other minerals, growth factors, etc), either alone or in combination, were responsible for the increased gain in sitting height during early puberty.

Because dietary vitamin D supplementation is not supposed to have long-term effects on vitamin D status, it is not surprising to find that the mean serum 25(OH)D concentration of the CaD milk group was no longer higher than that of either the control group or the Ca milk group 3 y after supplement withdrawal. However, it was surprising to find that the mean serum 25(OH)D concentration of the CaD milk group was lower than that of the control group at follow-up. Part of this difference could be explained by the relatively higher physical activity level and associated increased sunlight exposure observed in the control group (most of these subjects' activities were outdoors). The long-term effects of dietary vitamin D supplementation on vitamin D status in populations with low vitamin D intake and limited sunlight exposure during the winter deserve additional study.

A recently published 7-y trial conducted in American girls aged 10.9 y at baseline found that calcium supplementation had a significant positive influence on bone accretion during the pubertal growth spurt, but the effects were diminished in late adolescence (31). This finding suggested that BMD could catch up during the bone consolidation phase to compensate for the compromised bone mineral accretion during growth spurts that is caused by inadequate calcium intake. The study conducted on American girls also showed that the subjects with low calcium intakes might not have a complete catch-up (31). The average calcium intake of American girls in that study was 830 mg/d, which approaches the calcium intake of 957 mg/d required for maximum calcium retention in late adolescence (32). We speculate that the catch-up in bone mineral acquisition may not be complete in our Chinese girls, who have habitual calcium intakes of about 450 mg/d, and thus these girls would have a higher possibility of not achieving optimal peak bone mass than would American girls. The average milk intake of 140 g/d at follow-up was higher than both the presupplementation baseline intake of 120 g/d and the reported intake of urban Chinese girls from Beijing aged 12–14 y [$\bar{x}$ intake ($\pm$SD): 83 $\pm$ 74 g/d] (33). This finding indicates that, the milk intake of urban girls increased with economic development in China. However, the 51.1 kg/y per capita milk consumption of our subjects is still lower than the per capita average of $>$100 kg/y in developed countries (34). An additional increase in dairy intake would help to enhance the calcium intake in Chinese girls.

The limitations of the present study included the regression models that were used for data analysis, which did not take into account the subjects who dropped out, the total-body bone measures and biochemistry analysis that were only conducted in one-half of the subjects, and the assessment of bone mass with dual-energy X-ray absorptiometry, which cannot evaluate the changes in bone geometry and volumetric density. Another limitation of the present study was that serum 25(OH)D concentrations were measured by different methods during the intervention (CPBA method) and follow-up (radioimmunoassay method) phases. However, this would not affect comparisons between the groups at the same time point.

In conclusion, the present study showed that the effects of 2-y milk supplementation during early puberty on bone mineral accretion and the effects of vitamin D–fortified milk on vitamin D status largely disappeared 3-y after supplement withdrawal. Additional studies that include bone structure (ie, measured by peripheral quantitative computed tomography) as one of the outcome measurements over a longer term are needed to examine whether increased milk intake during pubertal years influences bone size and peak bone mass attainment in Chinese girls with low habitual calcium intakes. Meanwhile, we conclude that short-term milk supplementation during early puberty does not have long-lasting effects on bone mineral accretion in Chinese girls.

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KZ was involved in the study design, data collection, and data analysis and drafted the manuscript. QZ, LHF, and XH were involved in the data collection. LHF performed the biochemical analyses. AT participated in laboratory analysis consultations. GM and XD were involved in the conception and design of the study. HG, DRF, and CTC were involved in the conception, design, and interpretation of the study. All authors contributed to the writing of the manuscript. None of the authors had any conflicts of interest.

REFERENCES

1. Johnston CC, Miller JZ, Slemenda CW, et al. Calcium supplementation and increases in bone mineral density in children. N Engl J Med 1992; 327:82–7.
2. Lee WTK, Leung SSF, Wang SH, et al. Double-blind, controlled calcium supplementation and bone mineral accretion in children accustomed to a low-calcium diet. Am J Clin Nutr 1994;60:744–50.
3. Lee WTK, Leung SSF, Leung DMY, Tsang HSY, Lau J, Cheng JCY. A randomized double-blind controlled calcium supplementation trial, and bone and height acquisition in children. Br J Nutr 1995;74:125–39.
4. Lloyd T, Martel JK, Rollings N, et al. The effect of calcium supplementation and Tanner stage on bone density, content and area in teenage women. Osteoporosis Int 1996;6:276–83.
5. Bonjour JP, Carrie AL, Ferrari S, et al. Calcium-enriched foods and bone mass growth in prepubertal girls: a randomized, double-blind, placebo-controlled trial. J Clin Invest 1997;99:1287–94.
6. Nowson CA, Green RM, Hopper JL, et al. A co-twin study of the effect of calcium supplementation on bone density during adolescence. Osteoporosis Int 1997;7:219–25.
7. Dibba B, Prentice A, Ceesay M, Stirling DM, Cole TJ, Poskitt EME. Effect of calcium supplementation on bone mineral accretion in Gambian children accustomed to a low-calcium diet. Am J Clin Nutr 2000; 71:544–9.
8. Rozen GS, Rennert G, Dodiuk-Gad RP, et al. Calcium supplementation provides an extended window of opportunity for bone mass accretion after menarche. Am J Clin Nutr 2003;78:993–8.
9. Cameron MA, Paton LM, Nowson CA, Margerison C, Frame M, Wark JD. The effect of calcium supplementation on bone density in premenarcheal females: a co-twin approach. J Clin Endocrinol Metab 2004;89: 4916–22.
10. Chan GM, Hoffman K, McMurry M. Effects of dairy products on bone and body composition in pubertal girls. J Pediatr 1995;126:551–6.
11. Cadogan J, Eastell R, Jones N, Barker ME. Milk intake and bone mineral acquisition in adolescent girls: randomised, controlled intervention trial. BMJ 1997;315:1255–60.
12. Merrilees MJ, Smart EJ, Gilchrist NL, et al. Effects of dairy food supplements on bone mineral density in teenage girls. Eur J Nutr 2000;39: 256–62.

13. Lau EMC, Lynn H, Chan YH, Lau W, Woo J. Benefits of milk powder supplementation on bone accretion in Chinese children. Osteoporosis Int 2004;15:654–8.
14. Bonjour JP, Chevalley T, Ammann P, Slosman D, Rizzoli R. Gain in bone mineral mass in prepubertal girls 3.5 years after discontinuation of calcium supplementation: a follow-up study. Lancet 2001;358:1208–12.
15. Dibba B, Prentice A, Ceesay M, et al. Bone mineral contents and plasma osteocalcin of Gambian children 12 and 24 mo after the withdrawal of a calcium supplement. Am J Clin Nutr 2002;16:681–6.
16. Dodiuk-Gad RP, Rozen GS, Rennert G, Rennert HS, Ish-Shalom S. Sustained effect of short-term calcium supplementation on bone mass in adolescent girls with low calcium intake. Am J Clin Nutr 2005;81:168–74.
17. Lee WTK, Leung SSF, Leung DMY, Cheng JCY. A follow-up study on the effects of calcium-supplement withdrawal and puberty on bone acquisition of children. Am J Clin Nutr 1996;64:71–7.
18. Lee WTK, Leung SSF, Leung DMY, et al. Bone mineral acquisition in low calcium intake children following the withdrawal of calcium supplement. Acta Paediatr 1997;86:570–6.
19. Slemenda CW, Peacock M, Hui S, Zhou L, Johnston CC. Reduced rates of skeletal remodeling are associated with increased bone mineral density during the development of peak skeletal mass. J Bone Miner Res 1997;12:676–82.
20. Du X, Zhu K, Trube A, et al. School-milk intervention trial enhances growth and bone mineral accretion in Chinese girls aged 10–12 years in Beijing. Br J Nutr 2004;92:159–68.
21. Du X, Zhu K, Trube A, et al. Effects of school milk intervention on growth and bone mineral accretion in Chinese girls aged 10–12 years: accounting for cluster randomisation. Br J Nutr 2005;94:1038–9.
22. Tanner JM. Growth at adolescence. 2nd ed. Oxford, United Kingdom: Blackwell Scientific Publications, 1962.
23. National Sports Committee. Assessment of development of metacarpals, phalanges and carpals of Chinese people: national standard of People's Republic of China. Beijing, China: National Sports Committee, 1992.
24. Murray DM. Design and analysis of group-randomized trials: a review of recent developments. Ann Epidemiol 1997;S7:S69–77.
25. Rasbash J, Goldstein H. Efficient analysis of mixed hierarchical and cross classified random structures using a multilevel model. J Educ Behav Stat 1994;19:337–50.
26. Cole TJ. Sympercents: symmetric percentage differences on the 100 $\log_e$ scale simplify the presentation of log transformed data. Stat Med 2000; 19:3109–25.
27. Heaney RP. The bone-remodeling transient: implications for the interpretation of clinical studies of bone mass change. J Bone Miner Res 1994;9:1515–23.
28. Parfitt AM. The two faces of growth: benefits and risks to bone integrity. Osteoporosis Int 1994;4:382–98.
29. Chevalley T, Rizzoli R, Hans D, Ferrari S, Bonjour JP. Interaction between calcium intake and menarcheal age on bone mass gain: an eight-year follow-up study from prepubertal to postmenarche. J Clin Endocrinol Metab 2005;90:44–51.
30. Zhu K, Greenfield H, Du X, Fraser DR. Improvement of bone health in childhood and adolescence. Nutr Res Rev 2001;14:119–51.
31. Matkovic V, Goel PK, Badenhop-Stevens NE, et al. Calcium supplementation and bone density in females from childhood to young adulthood: a randomized controlled trial. Am J Clin Nutr 2005;81:175–88.
32. Matkovic V, Heaney RP. Calcium balance during human growth: evidence for threshold behavior. Am J Clin Nutr 1992;55:992–6.
33. Du XQ, Greenfield H, Fraser DR, Ge KY, Liu ZH, He W. Milk consumption and bone mineral content in Chinese adolescent girls. Bone 2002;30:521–8.
34. Canadian Dairy Information Centre. Consumption for selected countries: dairy consumption 2000 to 2004. Internet: http://www.dairyinfo.gc.ca/cdicwdfcons.htm (accessed 22 June 2005).