

## Supplementation with Three Different Daily Doses of Vitamin D<sub>3</sub> in Healthy Pre-pubertal School Girls: A Cluster Randomized Trial

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**Objective:** To compare the adequacy and efficacy of different doses of vitamin D3 in pre-pubertal girls.

**Design:** Cluster Randomized controlled trial.

**Setting:** Public school in Delhi, India, between August 2015 and February 2016.

**Participants:** 216 healthy pre-pubertal girls, aged 6.1-11.8 years.

**Intervention:** Daily supplementation with 600 IU (*n*=74), 1000 IU (*n*=67) or 2000 IU (*n*=75) of vitamin D3 under supervision for 6 months.

**Outcome measures:** *Primary:* Rise in serum 25 hydroxy Vitamin D (25(OH)D); *Secondary:* Change in bone formation and resorption markers.

**Results:** Following 6 months of supplementation, the mean (SD) rise in serum 25(OH)D was maximum with 2000 IU (24.09 (8.28) ng/mL), followed by with 1000 IU (17.96 (6.55) ng/mL) and 600 IU

(15.48 (7.00) ng/mL). Serum 25(OH)D levels of ≥20 ng/mL were seen in 91% in 600 IU group, 97% in 1000 IU group and 100% in 2000 IU group. The overall mean (SD) rise in urinary calcium creatinine ratio (0.05 (0.28) to 0.13 (0.12) mg/mg), and serum procollagen type I N-terminal propeptide (538.9 (199.78) to 655.5 (218.24) ng/mL), and reduction in serum carboxy-terminal telopeptide (0.745 (0.23) to 0.382 (0.23) ng/mL) was significant (*P*<0.01). The change in the above parameters was comparable among the three groups after adjustment for age.

**Conclusion:** Daily vitamin D supplementation with 600 IU to 2000 IU for 6 months results in Vitamin D sufficiency in >90% of pre-pubertal girls.

**Keywords:** Micronutrient supplementation, Prevention, Vitamin D deficiency.

**Trial registration:** Clinical Trial Registry of India (CTRI): 2017/01/007681

Vitamin D deficiency is a widely recognized public health problem world over, including India. There are limited studies on vitamin D supplementation in Indian children, more so regarding adequate dose of vitamin D3 supplementation in pre-pubertal children [1,2]. Vitamin D deficiency causes secondary hyperparathyroidism with negative consequences on bone mineral density (BMD) resulting in increase in serum bone resorption markers. There are not many studies exploring the impact of vitamin D3 supplementation on serum bone markers in children. In view of the above, we conducted this study to compare the efficacy of daily supplementation of 600 IU, 1000 IU and 2000 IU vitamin D3 in pre-pubertal girls; and to evaluate the effect of vitamin D3 supplementation on serum bone formation and resorption markers.

### METHODS

This was a cluster randomized controlled trial performed

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between August 2015 and February 2016. The study protocol was approved by Institute Ethical Committee. Apparently healthy pre-pubertal school girls (age 6.1-11.8 y), who consented to participate were evaluated from a private school (representing an upper socio-economic strata) in Delhi, India with consent from school authorities, parents/guardians and verbal assent from children. Girls who were either unable to swallow the capsule or were receiving drugs affecting bone mineral metabolism (*e.g.* calcium, vitamin D, glucocorticoids, anti-tubercular or anti-epileptics), or those suffering from any systemic illness were excluded from the study. Eligible and consenting girls were enrolled and randomized into three groups to be supplemented with daily 600 IU (group A), 1000 IU (group B) 2000 IU (group C) of vitamin D3 (capsule form) under supervision for 6 months. There were

4 classes (2nd to 5th) undertaken for supplementation with each class having 3 sections with approximately 40 students per section. Cluster randomization was done within each class considering each section of the class as a cluster. For each class, sections were allocated for interventions using simple random sampling with the help of drawing one chit from three. The randomly allocated interventions were neither shared with class teachers nor the students within each class till the end of the study. The concealment was carried out by removing the labels from the bottles. The different doses of vitamin D<sub>3</sub> were procured as capsules of same shapes but different colours, to avoid mix up and cross-contamination in the allocation arms. Investigators were aware about the intervention allocation to sections; though, the people involved in the laboratory analysis were blinded to the intervention status. The vitamin D<sub>3</sub> capsules were soft gelatine capsules (D rise, USV Pharma Ltd.) manufactured and supplied every month with no overages added.

Baseline height was recorded to the nearest 1 mm using Holtain stadiometer without wearing shoes and weight was recorded to the nearest 0.1 kg by using digital weighing machine. Body mass index (BMI) was calculated as weight (in kg)/ height (m<sup>2</sup>). Blood samples were collected in the fasting state between 0800 Hrs to 0900 Hrs. Serum 25(OH)D was estimated by chemiluminescence (Diasorin, Stillwater, MN, USA) and parathyroid hormone (PTH) was measured using electrochemiluminescence method (Roche Diagnostics). Calcium, phosphates, alkaline phosphatase were estimated by auto analyzer (Roche Diagnostics USA). Serum procollagen type I N-terminal propeptide (PINP) and carboxy-terminal telopeptide (CTX) were measured by Elecsys 2010, based on the principle of electrochemiluminescence immunoassay. Urinary samples were also collected for the spot calcium /creatinine ratio and was performed using Cobas C III (Roche). Repeat collection of fasting blood and urine samples was undertaken one day after the completion of supplementation. Vitamin D deficiency was defined as per Lips criteria; mild (10-20 ng/mL), moderate (5-10 ng/mL), and severe (<5 ng/mL) [3]. Secondary hyperparathyroidism was defined as serum PTH levels >65 pg/mL.

Daily supplementation for 6 days/week was done for a period of 6 months, under supervision of teachers and investigating staff at the study site. Required numbers of vitamin D capsules were provided to the parents/guardians every month along with a record sheet to be maintained by the parents for Sundays and planned holidays as per school calendar. For unplanned holidays, parents were advised to collect their requirement from

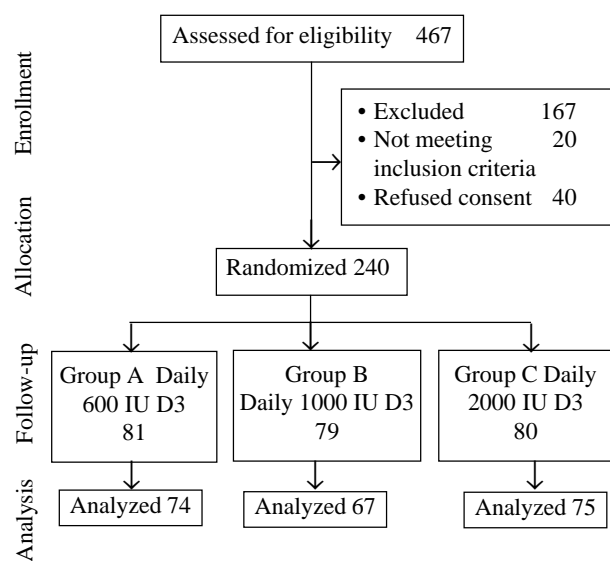
school.

Sample size calculation was based on our earlier study where 70% and 81% of children achieved serum 25(OH)D levels of  $\geq 20$  ng/mL when supplemented with 600 IU and 1000 IU of vitamin D, respectively for 6 months and 90% proportion was expected with 2000 IU [4]. In order to detect a significant difference among the 3 groups in a 2-sided test with a 5%  $\alpha$  error and 80% power, 74 patients per group were required. Considering 10% loss during the follow-up period, a sample size of 82 per group was calculated.

**Statistical analysis:** The proportion of subjects achieving the desirable levels of 20 ng/mL at the end of intervention were compared among the three study groups using chi-square test. Analysis of variance (ANOVA) was used to study the difference in the mean of various parameters, among the three study groups. Multiple linear regression analysis was carried out on change in biochemical and hormonal parameters. A *P* value of <0.05 was considered statistically significant. Analysis was performed using Stata 11.0 (College station Road, TX, USA).

## RESULTS

We approached 467 girls out of which 300 apparently healthy girls who consented were evaluated. All 300 girls had 25 (OH) D below 20 ng/mL. Out of these 300 girls, 240 were found eligible and consented for study; 216 completed the study. Twenty-four were excluded due to lack of proper follow-up, change of school, unavailability of post-treatment laboratory reports, or missed taking supplementation for more than 7 days continuously. Study flow is depicted in **Fig. 1**. Baseline hormonal and



**FIG. 1** Consort flow chart of the trial.

biochemical parameters and the changes following 6 months of vitamin D3 supplementation are shown in **Table I** and **II**, respectively.

All included girls had 25(OH)D levels below 20 ng/mL. Mild, moderate, and severe deficiency was observed in 96 (44.4%), 113 (52.3%) and 7 (3.3%) children, respectively. Post-supplementation mean (SD) serum 25(OH)D levels increased to 29.23 (8.00) ng/mL ( $P<0.01$ ), and a level of 20 ng/mL or more were seen in 67 (91%) girls in group A, 64 (97%) in group B and all (100%) in group C. The difference in the rise of serum 25(OH)D levels between group A and C (7.74 ng/mL,  $P<0.01$ ) and between groups B & C (5.86 ng/mL,  $P<0.01$ ) was significant. The baseline serum PTH was 49.6 (27.2) pg/mL that decreased to 33.7 (14.5) pg/mL following 6 months of vitamin D3 supplementation ( $P<0.01$ ). Secondary hyperparathyroidism was seen in 32 (14.8%) children at baseline, which reduced to 4.5% on follow-up ( $P<0.01$ ). The mean (SD) urinary calcium

creatinine ratio increased from 0.05 (0.28) to 0.13 (0.12) mg/mg following 6 months of supplementation ( $P<0.01$ ) that was not different among the three groups after adjustment for age.

Following supplementation, serum PINP levels increased significantly from 538.9 (199.78) to 655.5 (18.24) ng/mL ( $P<0.01$ ) and serum CTX decreased significantly from 0.745 (0.23) to 0.382 (0.23) ng/mL ( $P<0.01$ ); significant for intra-group comparison but not intergroup comparison. No adverse effects were noted in any of the participants during the study period.

## DISCUSSION

In the current study, a significant dose-dependent increase in serum 25(OH)D with a significant reduction in mean PTH levels was observed following 6 months of vitamin D3 supplementation. Persistent secondary hyperparathyroidism despite achieving serum 25 (OH) D  $\geq 20$  ng/mL following supplementation was noted in few subjects.

**TABLE I** BASELINE HORMONAL AND BIOCHEMICAL PARAMETERS IN PRE-PUBERTAL GIRLS

	600 IU (n=74)	1000 IU (n=67)	2000 IU (n=75)	Total (n=216)
Serum 25(OH)D (ng/mL)	10.13 (3.51)	10.21 (3.71)	9.8 (3.73)	9.99 (3.64)
Serum parathyroid hormone (pg/mL)	43.27 (14.99)	47.90 (23.69)	57.19 (35.85)	49.72 (27.22)
Serum procollagen type-I N propeptide (PINP) levels (mcg/L)	557.44 (211.89)	508.63 (166.52)	560.43 (218.31)	538.9 (199.78)
Serum C-terminal telopeptide of type I collagen (CTX) levels (mcg/L)	0.856 (0.24)	0.649 (0.18)	0.683 (0.16)	0.745 (0.23)
Serum Calcium (mg/dL)	9.43 (0.97)	9.19 (0.46)	9.59 (0.77)	9.38 (0.77)
Serum phosphate (mg/dL)	5.22 (0.61)	5.77 (5.01)	5.34 (0.66)	5.45 (3.08)
Serum alkaline phosphatase (IU/L)	256.33 (69.30)	257.03 (61.72)	276.04 (68.23)	261.95 (66.93)
Urinary calcium creatinine ratio (mg/mg)	0.04 (0.46)	0.03 (0.05)	0.03 (0.03)	0.05 (0.28)

Values in mean (SD).

**TABLE II** CHANGE IN HORMONAL AND BIOCHEMICAL PARAMETERS AFTER VITAMIN D3 SUPPLEMENTATION IN PRE-PUBERTAL GIRLS

Change from baseline	600 IU (n=74)	1000 IU (n=67)	2000 IU (n=75)
Serum 25(OH)D (ng/mL)*	14.93 (10.48, 18.67)	18 (13.1, 21.5)	22.21 (18.38, 28.69)
Serum PTH (pg/mL)*	6.26 (-1.29, 11.33)	14.99 (6.51, 24.35)	17.62 (10.1, 28.58)
Serum PINP (mcg/L)*	54.29 (-59.24, 225.15)	158.55 (40.99, 270.25)	53.10 (-64.20, 216)
Serum CTX (mcg/L)*	0.38 (0.10, 0.61)	0.29 (0.18, 0.47)	0.41 (0.27, 0.62)
Serum Calcium (mg/dL)	0.5 (-0.19, 1.0)*	0.6 (0.29, 1.0)*	0.5 (-0.1, -0.8)
Serum phosphate (mg/dL)	0.14 (-0.39, -0.59)	0.10 (-0.26, 0.73)	0.24 (-0.04, 0.92)*
Serum alkaline phosphatase (IU/L)	-8 (-39, 32)	-20 (-63, 33)	-33 (-72, 52)
Urinary calcium creatinine ratio (mg/mg)	0.07 (0.02, 0.16)	0.05 (0.01, 0.20)	0.02 (0.003, 0.08)

Values represented as median (IQR); \* $P<0.05$ ; PTH: Parathyroid hormone; PNPP: Procollagen type-I N propeptide; CTX: C-terminal telopeptide of type I collagen.

**WHAT IS ALREADY KNOWN?**

- Recommended dietary allowance (RDA) for vitamin D in children (beyond infancy) by Institute of Medicine (US) and Indian Academy of Pediatrics (IAP) is 600 IU/day.

**WHAT THIS STUDY ADDS?**

- Supplementation with 600 IU/d results in adequate 25(OH)D levels in 90% of pre-pubertal girls.
- Higher daily dose requirement of 1000 IU was required to achieve and maintain vitamin D sufficiency in 97% of girls.

Evaluation of bone markers showed a marked increase in serum PINP and significant reduction in CTX levels.

Major limitations of the study were (i) inability to carry out 24-hour urinary calcium excretion, (ii) absence of boys and adolescent girls in the study group, (iii) lack of detailed dietary evaluation of calcium and vitamin D and (iv) lack of intention to treat analysis. We chose daily dose of 600 IU as it is recommended by Indian Academy of Pediatrics (IAP) and Institute of Medicine (IOM), a higher dose of 1000 IU as per our earlier reported prediction equation, and 2000 IU as per one recent study showing that 2098 IU of daily vitamin D supplementation is able to achieve serum 25(OH)D levels of  $\geq 20$  ng/mL in 97.5% of children [4-7]. We did not include a placebo arm as only vitamin D deficient children were included in the current study.

A dose-dependent increase in serum 25 (OH) D levels has been reported in earlier studies evaluating the impact of vitamin D3 supplementation in different doses in children with vitamin D deficiency [8-10]. The response to daily supplementation with 2000 IU of vitamin D3 in the current study was similar to that reported by Dong, *et al.* [8] in American black boys (60 nmol/L) in contrast to Lewis, *et al.* [10] where the increase was only 38 nmol/L. Similarly, the percentage of vitamin D deficient Lebanese children who achieved vitamin D sufficiency following 2000 IU/day of vitamin D3 supplementation for a year [9] was similar to present study. The estimated intake of 2098 IU/day needed to maintain serum 25(OH)D concentration at 20 ng/mL in 97.5% of US children was in sharp contrast to 1000 IU/day required in the present study to achieve sufficiency in 97% subjects [6,11].

The effect of vitamin D3 supplementation on bone markers is less well studied. Few studies in children with vitamin D deficiency or insufficiency have observed higher levels of plasma osteocalcin, CTX and bone-specific alkaline phosphatase (BAP), suggesting the role of vitamin D in maintenance of bone turn-over [12-14]. Rajakumar, *et al.* [15] evaluated the effect of vitamin D3

supplementation on serum bone markers in obese and non-obese children aged 6-10-years with 400 IU of vitamin D3 daily for one month and noted a significant increase in serum 25(OH)D with a decrease in serum osteocalcin, BAP and urine n-telopeptide cross-links of type 1 collagen (urine NTX) in both the groups. We observed a significant decrease in serum CTX and PTH as has also been observed in a previous study [16]. However, the increase in serum PINP without significant decline in ALP following vitamin D3 supplementation as noted in the present study was possibly due to the normal growing phase in this age group. This is in contrast to earlier studies in children and adults which observed decrease in both formation and resorption markers [16-18]. The increase in PINP levels, however, are consistent with the results of a study by Ghazi, *et al.* [17] who observed an increase in osteocalcin and alkaline phosphatase which are bone formation markers after monthly and bimonthly vitamin D supplementation.

The urinary calcium to creatinine ratio shows a wide variation ranging from 0.024 to 0.44 in various geographic areas, including India [20-22]. The ratio in the present study significantly increased post supplementation. The change in this ratio in children following vitamin D3 supplementation has not been studied earlier. Although, urinary calcium concentration is considered to monitor the inadvertent vitamin D toxicity, levels may be affected by improperly timed collections, missed urine voids, and daily variations in calcium intake. Veith, *et al.* [23] had shown good correlation between first-morning urine sample and 24-h urinary calcium excretion, though, others have shown conflicting results [24]. Furthermore, it is highly controversial whether isolated high-normal calcium excretion contributes to stone disease or bone health [25]. Further studies are required to confirm the significance of this finding.

Supplementation with all three daily doses of vitamin D3 resulted in significant increase in the serum 25(OH)D levels. Higher daily dose requirement of 1000 IU to

achieve and maintain vitamin D sufficiency in 97% of girls as against 600 IU/day as recommended by IAP and IOM may be due to several confounding factors such as poor dietary intake of calcium, limited sun exposure and lower serum baseline 25(OH)D values [4,5]. It is therefore important to undertake well-planned studies to ensure whether RDA of 600 IU recommended by IAP and IOM would suffice to achieve and maintain serum 25(OH)D  $\geq$ 20 ng/mL in prepubertal girls.

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**Contributors:** RKM: conceptualized and designed the study, manuscript writing; AM: conceptualized the study; NB: data collection and manuscript writing; GS: data collection and manuscript writing; SG: collection of blood samples and evaluation of bone markers; MS: collection of blood samples and evaluation of bone markers; AN,AC,NG: data collection; VS: statistical analysis; MAG: data collection and analysis.

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**Competing interest:** AM is the founding president of the Endocrine and Diabetic foundation of India.

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