Pediatr Nephrol (2017) 32:2063–2070
DOI 10.1007/s00467-017-3716-2

ORIGINAL ARTICLE

The effect of vitamin D and calcium supplementation in pediatric steroid-sensitive nephrotic syndrome

Sushmita Banerjee[1] · **Surupa Basu**[2] · **Ananda Sen**[3] · **Jayati Sengupta**[4]

Received: 26 March 2017 / Revised: 28 May 2017 / Accepted: 30 May 2017 / Published online: 19 July 2017

Abstract
Background Low serum levels of total 25-hydroxy- cholecalciferol (25(OH)D) occur in nephrotic syndrome (NS). We aimed to assess the effects of vitamin D3 and calcium supplementation on 25(OH)D levels, bone mineralization, and NS relapse rate in children with steroid-sensitive NS.
Methods A randomized controlled trial (RCT) was performed in children with steroid-sensitive NS. The treatment group received vitamin D3 (60,000 IU orally, weekly for 4 weeks) and calcium supplements (500 to 1,000 mg/day for 3 months) after achieving NS remission. Blood samples for bone biochemistry were taken during relapse (T0), after 6 weeks (T1) and 6 months (T2) of randomization, whereas a lumbar DXA scan was performed at T0 and T2. Renal ultrasound was performed after study completion in the treatment group and in all patients with hypercalciuria.
Results Of the 48 initial recruits, 43 patients completed the study. Post-intervention, 25(OH)D levels showed significant improvements in the treatment group compared with controls at T1 ($p < 0.001$) and T2 ($p < 0.001$). However, this was not associated with differences in bone mineral content (BMC) ($p = 0.44$) or bone mineral density (BMD) ($p = 0.64$) between the groups. Additionally, there was no reduction in relapse number in treated patients ($p = 0.54$). Documented hypercalciuria occurred in 52% of patients in the treatment group, but was not associated with nephrocalcinosis.
Conclusions Although supplementation with calcium and vitamin D improved 25(OH)D levels significantly, there was no effect on BMC, BMD or relapse rate over a 6-month follow-up. Occurrence of hypercalciuria mandates caution and appropriate monitoring if using such therapy. Appropriate dosage of vitamin D3 remains uncertain and studies examining biologically active vitamin D may provide answers.

Keywords Nephrotic syndrome · Vitamin D · Bone mineral density · Bone mineral content · Hypercalciuria · Children

Study design: randomized controlled open label intervention trial

✉ Sushmita Banerjee
asban@vsnl.com

[1] Department of Paediatric Nephrology, Institute of Child Health and Calcutta Medical Research Institute, Kolkata, India

[2] Department of Biochemistry, Institute of Child Health, Kolkata, India

[3] Department of Family Medicine and Department of Biostatistics, University of Michigan, Ann Arbor, MI, USA

[4] Department of Paediatric Nephrology, Institute of Child Health, Kolkata, India

Introduction

Steroid-sensitive nephrotic syndrome (NS) is the most common chronic relapsing renal disorder diagnosed in children. The occurrence of early osteoporosis in this disease has been reported, and attributed to steroid therapy, which is the main specific treatment used [1–6].

Patients with active NS lose vitamin D binding protein (VDBP) along with albumin in their urine [7, 8]. Low 25-hydroxycholecalciferol (25(OH)D) levels have been described during and after NS relapse, and this may contribute to poor bone health [9–13]. Studies have suggested that 25(OH)D supplementation may protect against osteoporosis [1, 14, 15]. As steroid therapy cannot be avoided in NS, the possibility of vitamin D supplementation providing protection against osteoporosis is attractive, provided that therapy is safe and effective.

Relapses in NS are most commonly precipitated by minor infectious illnesses. Vitamin D deficiency has been associated with numerous infections and inflammatory conditions [16–20]. Vitamin D has complex interactions with the immune system, which include an immunosuppressive effect due to reduction of cytokines such as interleukin-2 and interferon (IFN)-γ, along with an enhancement of T regulatory cells [21]. Such effects may also result in modifying the course of a disease such as NS, in which T cell-mediated immunological abnormalities have been well described [22].

This randomized controlled trial (RCT) performed in children with steroid-sensitive NS, is primarily aimed at assessing if vitamin D and calcium supplementation after NS relapse improves bone health, as measured by bone mineral content (BMC) and bone mineral density (BMD) over a 6-month period. Our secondary aims were:

1. To assess whether the number of NS relapses were reduced by correcting 25(OH)D levels
2. To record longitudinal changes in vitamin D levels and their association with BMC and BMD
3. Monitor for any adverse effects of vitamin D therapy in NS

Materials and methods

A randomized controlled open labeled study was undertaken. Patients with steroid-sensitive NS attending renal outpatient clinics were identified. Patients aged between 2 and 14 years, with NS relapse, i.e., urine protein more than 2+ or urine protein creatinine ratio > 2 mg/mg for 3 consecutive days, were recruited. Patients were excluded if they were steroid-resistant, had secondary forms of NS or were suffering from any other acute or chronic illnesses.

After counseling and informed consent procedures, blood and urine samples were taken during the NS relapse (T0), and a baseline lumbar dual energy X-ray absorptiometry (DXA) scan for BMC and BMD assessment was performed. On achieving NS remission (negative or trace urine protein for 3 consecutive days), the patients were randomized using a computerized program, to either a treatment group or a control group. All children in the treatment group who had 25(OH)D levels below 30 ng/ml received oral cholecalciferol (vitamin D3) 60,000 IU once weekly for four doses along with calcium 250 mg twice daily for patients weighing 20 kg or less, and 500 mg twice daily for children above 20 kg, for 3 months. All patients had blood and urine tests repeated at 6 weeks (T1) and 6 months (T2) post-randomization, and DXA scan was also repeated after 6 months.

Standard treatment with steroids for the NS episode at T0 and any further relapses was prescribed as per the Indian Society of Pediatric Nephrology protocol [23]. Renal ultrasound scans were performed after study completion in all patients in the treatment group and in patients in the control group who demonstrated hypercalciuria (calcium creatinine ratio or CaCR >0.2 mg/mg).

The blood and urine samples were analyzed on automated platforms in the clinical chemistry laboratory of the Institute of Child Health, Kolkata. These investigations are in routine use and regularly evaluated for consistent performance with robust quality control (QC) tools. Serum and urine creatinine (mg/dl) were evaluated using an enzymatic method (coefficient of variation [CV]: 3.5%), serum and urine calcium (mg/dl), using the o-cresolphthalein method (CV: 1.0%), inorganic phosphate (mg/dl), using the ammonium phosphomolybdate method (CV: 2.5%), alkaline phosphatase (ALP; U/L) using the International Federation of Clinical Chemistry method (CV: 4.6%), albumin (g/dl) using the bromocresol green method (CV: 1.9%), and urine protein (mg/dl) using the turbidimetry method (CV: 5.2%). All of the above were measured on the Roche Integra 400 Plus chemistry analyzer. Protein creatinine ratio and calcium creatinine ratio were derived and reported as ratios (mg/mg). The total circulating 25(OH)D (ng/ml) and intact parathyroid hormone (PTH; pg/ml) were measured on a Roche immunoassay platform (Cobas e411) using electrochemiluminescence—25(OH)D (CV: 4.8%) and PTH (CV: 2.2%).

Bone mineral content (g) and bone mineral density (g/cm^2) were assessed in the Calcutta Medical Research Institute's Radiology Department. The GE Medical Systems (Lunar model) based on the DXA method at the lumbar spine levels L1 to L4 was used. This process required about 2 min of immobilization and no sedation or anesthesia was required. The radiation exposure is reported to be about 1–2 μSv. Precision measured by CV is reported to be less than 1%.

Sample size calculations

During the study design, we found only one previous RCT that compared BMD change between a vitamin D + calcium-treated vs control group. This study reported a mean difference in lumbar BMD of 8.4% between groups, after a 2-month period [1]. The reported standard deviations of the changes were roughly 2% and 4% respectively in the treatment and the control groups. Based on a Satterthwaite *t* test for comparing means with unequal variances between groups, to detect such an effect size, a minimal sample size of 8 patients in each group would be required for two-tailed α of 0.01, and 95% power.

We chose to recruit a greater number of patients, as we were assessing different outcome measures, such as longitudinal changes over a 6-month follow-up period after initial vitamin D supplementation, in addition to NS relapse rates. There were no previous RCTs to guide us that included such

parameters. We planned to recruit a total of 48 patients so that even with an estimated maximum of 20% attrition we would have at least 20 patients in each group.

During our study period, a second RCT was published [24], which reported a difference of 20.1% in lumbar BMC, with standard deviations of 16% and 8% in vitamin D-supplemented and control groups respectively, at 12 weeks after treatment of the first episode of NS. Based on the Satterthwaite *t* test, a minimal sample size of 14 patients in each group would be required for α of 0.01, and 95% power to detect such an effect size.

Statistical analysis

All continuous variables were checked for normality using the Shapiro–Wilk test. They are summarized as mean ± standard deviation (sd) when normally distributed and median and interquartile range (IQR) when not normally distributed. For normally distributed variables intergroup comparison was carried out using an unpaired *t* test, whereas over time, comparison was made using a paired *t* test. For variables that were not normally distributed, intergroup comparison was made using the Mann–Whitney *U* test, whereas over time, comparison was made using the Wilcoxon signed rank for two time points and the Friedman test for three time points. Categorical variables are expressed as number and percentages and Fisher's exact test was used to test for intergroup differences. Spearman's correlation analysis was used to test for correlations between variables.

Standard ISKDC terminology was used to define relapse, remission, and NS types [23].

Results

Forty-eight patients with active NS relapse initially consented to participate. However, 5 patients did not complete the study (Fig. 1), and were excluded from the final analysis. A total of 43 patients, therefore, completed the study and were analyzed.

Out of these 43 patients, 33 were males (77%). At study entry, median age was 4.8 years (IQR 3.2–6.9). There were 21 patients in the treatment group and 22 in the control group. The patient characteristics, duration and type of NS, relapse frequency, steroid requirement, and biochemical reports at T0 were not significantly different between groups and are shown in Table 1. At T0, the BMC and BMD of the whole study population correlated strongly with age ($r_s = 0.8$, $p < 0.001$ for both).

At T1, all patients were in NS remission. The 25(OH)D levels (Fig. 2) and urinary CaCR were significantly higher, whereas PTH levels were significantly lower in the treatment compared with the control group (Table 2). There was no difference between groups with regard to the other

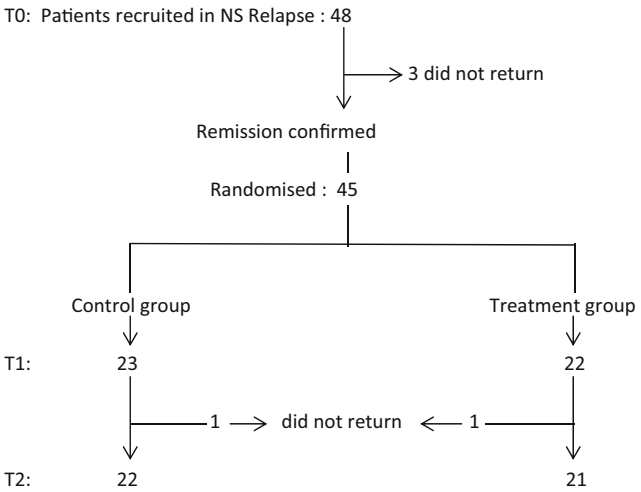

Fig. 1 Consort diagram

biochemical parameters assessed. The increment in 25(OH)D levels in the treatment group did not correlate with the level at study entry ($r_s = -0.07$, $p = 0.77$), nor with age ($r_s = -0.43$, $p = 0.07$), weight ($r_s = -0.37$, $p = 0.12$) or surface area (($r_s = 0.39$, $p = 0.09$) of the patients.

At T2, there remained a significant difference in serum 25(OH)D levels between the treatment and control groups (Fig. 2; Table 2); however, the differences in PTH levels and CaCR ratios were no longer evident. Likewise, there were no differences between groups in any of the other biochemical parameters assessed at this stage.

Thirteen patients in the control group and 10 patients in the treatment group had relapses during this 6-month study period ($p = 0.54$), whereas the average number of relapses were 0.7 in the treatment group and 0.9 in controls ($p = 0.41$). The cumulative prednisolone dose received over the 6-month study period did not vary between groups (110.5; 61.25–115.5 vs 98.0; 61.0–126.0 mg/kg, $p = 0.667$).

At T2, 25(OH)D levels were significantly lower in controls who relapsed than in those who did not (3.21; 1–6.5 vs 13.05; 8.2–15.7 ng/ml, $p = 0.012$), but in the treatment group, the difference was not statistically significant (20.67; 6.4–36.8 vs 25.4, 20.8–37.6 ng/ml, $p = 0.35$).

The median change in BMC over 6 months was +8.7 ± 8.7% in the treatment group vs +6.5 ± 9.5% in the control group, whereas the median change in BMD was +2.8 ± 5.0% for the treated group and +3.6 ± 6.0% for controls. Although the intra-group BMC and BMD in both treated and control patients showed significant increments over 6 months (Table 2), there was no statistical difference between groups ($p = 0.64$; Fig. 3).

The BMC and BMD changes did not vary between relapsers and nonrelapsers in either group. In the whole study group, the changes in BMC and BMD over 6 months showed no correlation with 25(OH)D levels or cumulative steroid dose received over the study period.

Table 1 Data at recruitment (T0)

		Controls, $n = 22$	Treatment, $n = 21$	p value
Sex, male (%)		19 (86)	14 (67)	0.162
Age at NS onset (years)		2.58 (2.0, 3.7)	2.33 (1.8, 2.9)	0.271
Age study entry (years)		4.73 (3.1, 7.4)	5.0 (3.4, 6.9)	0.789
BMI (kg/m^2)		16.8 (15.9, 19.5)	16.9 (14.4, 18.3)	0.45
NS type	FE	2	3	0.664
	IFR	8	8	1.0
	FR	6	4	0.72
	SD	6	6	1.0
Cumulative steroids received in the previous 1 year (mg/kg)		131.25 (52.3, 190.24)	102.5 (55.13, 147)	0.367
NS episodes in the previous year		3 (1, 3)	2 (1, 3)	0.124
Urine	PCR (mg/mg)	7.6 (4.8, 11.9)	7.0 (3.11, 20.35)	0.91
Blood	Creatinine (mg/dl)	0.27 (0.2, 0.34)	0.26 (0.23, 0.36)	0.735
	Albumin (g/dl)	1.8 (1.4, 2.9)	2.1 (1.5, 3.3)	0.305

Continuous variables are reported as median (interquartile range)

NS nephrotic syndrome, *BMI* body mass index, *FE* first episode, *IFR* infrequent relapses, *FR* frequent relapses, *SD* steroid dependence, *PCR* protein creatinine ratio

With regard to adverse events, at T0, 3 patients had hypercalciuria (CaCR 0.37 to 0.98). At T1, CaCR >0.2 was seen in one patient in the control group (0.39) and in 11 patients in the treatment group (range 0.23 to 0.53). The levels of 25(OH)D were above 100 ng/ml in 4 of these latter patients. In the treatment group, CaCR level correlated with serum

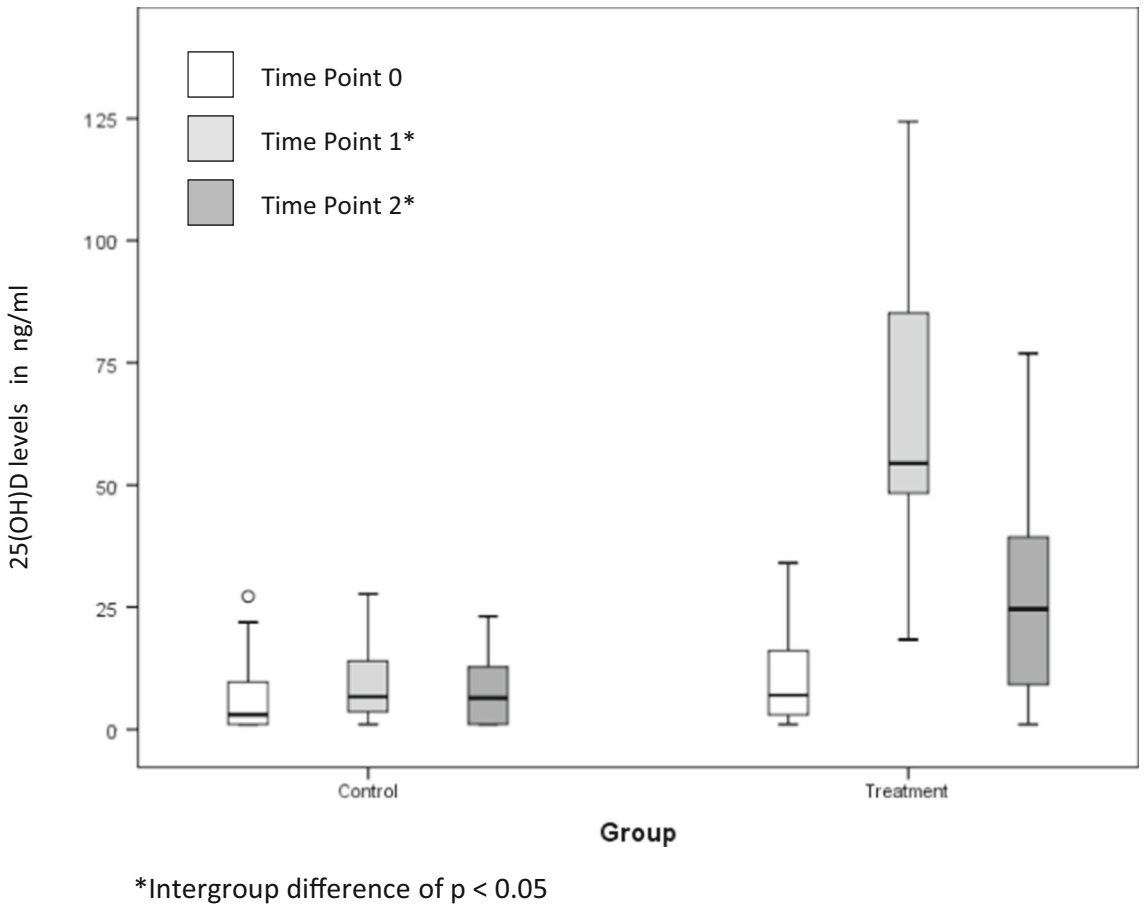

Fig. 2 Serum 25(OH)D Levels

Table 2 Longitudinal changes in parameters over the study period

Groups	Controls				Treatment			
Time point	T0	T1	T2	*****p value	T0	T1	T2	*****p value
Urine CaCR (mg/mg)	0.01 (0.01, 0.06)	0.09 (0.03, 0.14)*	0.04 (0.02, 0.17)	0.001	0.04 (0.01, 0.12)	0.23 (0.11, 0.34)*	0.09 (0.05, 0.2)	0.001
Serum albumin (g/dl)	1.8 (1.4, 2.9)	4.35 (4.1, 4.6)	4.4 (4.1, 4.5)	<0.001	2.1 (1.5, 3.3)	4.4 (3.9, 4.6)	4.5 (3.8, 4.7)	<0.001
Serum Calcium (mg/dl)	8.5 (8.1. 8.9)	10.1 (9.8, 10.2)	10.1 (9.7, 10.3)	<0.001	9.0 (8.4, 9.7)	10.2 (9.4, 10.6)	10.1 (9.5, 10.5)	<0.001
ALP (U/L)	225 (169, 262)	148 (111, 207)	210 (182, 249)	0.076	236 (200, 282)	180 (138, 219)	226 (194, 309)	<0.001
25(OH)D (ng/ml)	3.0 (1, 10.2)	6.62 (3.5, 14.4)**	6.38 (1, 12.9)****	0.107	7.0 (3, 16.7)	54.45 (46.45, 87.75)**	24.61 (8.75, 39.7)****	<0.001
PTH (pg/ml)	35.1 (24.3, 47.37)	41.45 (32.8, 52.0)***	34.7 (33.0, 52.7)	0.097	31.7 (15.5, 34.7)	22.8 (20.1, 28.9)***	27.9 (22.9, 46.8)	0.121
BMC (g)	12.5 (9.37, 18.3)		12.9 (9.4, 20.42)	0.010	12.3 (9.1, 16.15)		13.2 (10.35, 18.35)	0.001
BMD (g/cm^2)	0.577 (0.479, 0.675)		0.586 (0.486, 0.718)	0.015	0.551 (0.484, 0.636)		0.559 (0.500, 0.681)	0.012

Continuous variables are reported as median (interquartile range)

CaCR calcium creatinine ratio, *ALP* alkaline phosphatase, *25(OH)D* 25 hydroxycholecalciferol, *PTH* intact parathyroid hormone, *BMC* bone mineral content, *BMD* bone mineral density

*$p = 0.003$

**$p < 0.001$

***$p = 0.003$

****$p < 0.001$

*****Repeated measure analysis within groups

25(OH)D levels ($r_s = 0.5$, $p = 0.025$). At T2, hypercalciuria was documented in 4 patients in the control group (range 0.22–0.37) and 2 patients in the treatment group (0.22–0.3), and the highest 25(OH)D level was 53 ng/ml (Table 2).

One patient in the control group had hypercalciuria at all three time points (0.37➔0.25➔0.35), whereas the 2 patients who were hypercalciuric in the treatment group at T2 had also been so at T1 (0.53➔0.3 and 0.24➔0.22).

There was no difference in serum calcium levels between groups at any time point. All patients in the treatment group and those with hypercalciuria in the control group had renal ultrasound scans, and none had evidence for nephrocalcinosis.

Discussion

Our study shows that treatment with vitamin D3 and calcium after NS relapse resulted in a significant improvement in serum levels of total 25(OH)D in the treatment group, which persisted over the study period of 6 months. However, this was not associated with any difference in BMC or BMD between groups. Additionally, there was no reduction in relapse rate or number in the treated group. Documented hypercalciuria occurred in 52% of patients (11 out of 21) in the treatment group, compared with 18% of patients (4 out of 22) in the control group.

Glucocorticoid therapy has been extensively associated with reduction in bone mineralization [25, 26]. Several cross-sectional studies have reported low BMD in NS, with the degree of reduction correlating with disease severity and cumulative steroid intake [3–6]. Longitudinal studies by Bak et al. ($n = 40$) and Koşan et al. ($n = 20$) demonstrated BMD reduction occurring *as early as* 2–3 months in patients receiving prednisolone at a dosage of 2 mg/kg/day for 4 weeks, followed by 2 mg/kg/alternate day for 4 weeks [1, 2].

However, there are also a number of studies that have conversely reported no reduction in BMD or BMC, and no correlation with cumulative steroid therapy [27–30]. Polito et al. reported 24 steroid-dependent NS patients on alternate-day steroids for 1 to 6.3 years, who had no significant difference in BMC compared with controls, and no relationship between BMC and dose or duration of therapy [31]. Phan et al. showed that although 65 NS children had low lumbar spinal BMD at baseline and after 3 months of starting steroid therapy, there was a significant increase in BMD-z scores between 3 and 12 months, so that values were normal by 6 and 12 months [32].

The inference from these studies could be that bone mineral loss occurs early after receiving large doses of *daily* steroids. In steroid-sensitive NS, patients in their first episode are treated for 4–6 weeks with daily steroids corresponding to 2 mg/kg/day [20, 33, 34]. Subsequent relapses even when frequent,

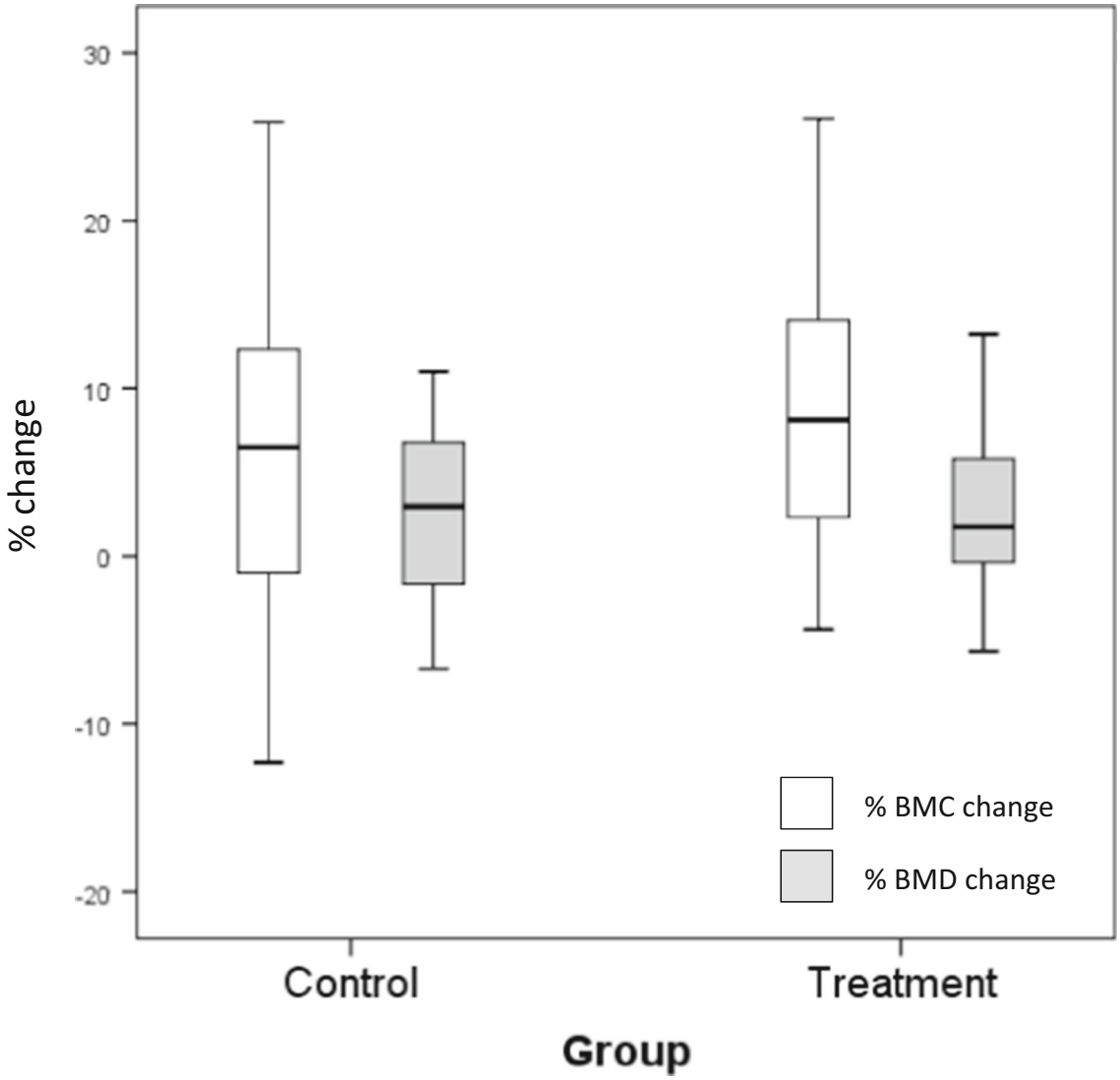

Fig. 3 Percentage change in BMC and BMD over 6 months

are treated with daily prednisolone *only* up to remission, and subsequently with alternate-day therapy, which is rapidly stopped or weaned. Thus, the reduction in bone mineralization possibly occurs maximally after treatment of the first episode and recovers thereafter *if* daily steroid treatment can be limited. In our study, there was no reduction in BMC or BMD in either the control or the treatment group over the 6-month study period. Only 5 of our patients had a first episode of NS, with 0–1 relapses each, during the study period. This is possibly the reason for the lack of reduction of BMD/BMC in our study population.

Low serum levels of total calcium and 25(OH)D during and after NS relapse have been reported by several authors [9–13]. In the RCT reported by Bak et al., supplementation with calcium and 25(OH)D resulted in significantly improved BMD, in comparison to controls [1], over the 2-month study period. A similar RCT by Choudhary et al. reported an increased BMC, but no change in BMD (which actually increased in both) in the supplemented compared with the control group, over 3 months [24]. In a longitudinal study, Gulati et al. reported improved spinal BMD in the supplemented group compared with patients who did not receive such supplements [14]. These studies did not measure vitamin D levels. Pańczyk-Tomaszewska et al. showed that lumbar BMD correlated positively with serum 25(OH)D levels over 1 year [35]. In our study, the treatment group had significant improvements in serum 25(OH)D levels, whereas in the control group levels remained low after 6 months. Despite this, the percentage changes in BMC and BMD were no different in the two groups, and showed no correlation with 25(OH)D levels at any stage.

The previous studies mentioned above [1, 14, 30, 35] used daily doses of vitamin D3 ranging between 200 and 1000 IU per day over 2 months to 1 year. We chose to use an intermittent bolus regime [36], with the expectation of better compliance in our population. In addition, NS is different from other conditions associated with vitamin D deficiency, in that there is actual loss of protein-bound 25(OH)D in the urine during relapse. Our patients were recruited early in relapse, and we assumed that there would be high and continuing loss of urinary 25(OH)D until NS remission, thus warranting the higher dose for rapid correction after remission had been achieved. However, similar to a recent meta-analysis, we found that the increments in 25(OH)D levels in the treatment group were erratic, and not dependent on initial level, age, or anthropometry [37]. Fifty percent of patients had some degree of hypercalciuria 2 weeks after completing vitamin D therapy, which fortunately subsided in most cases and was not associated with hypercalcemia or nephrocalcinosis. Thus, the safe but effective dose of vitamin D therapy in NS remains uncertain, and the drug should be used with caution and with adequate monitoring for adverse effects [37, 38].

Vitamin D is known to have immune modulatory effects. In addition, it may offer protection against pediatric respiratory and gastro-intestinal infections [16, 18, 19], thereby indirectly reducing NS relapses, which are commonly precipitated by such infections. But, our hypothesis that correcting vitamin D levels might reduce NS relapses was not supported by our results, as there was no reduction in the number of relapses in the treatment group. However, we acknowledge that our study is under-powered to exclude the effect of vitamin D on the number of relapses, as to show this difference to be significant at a 5% level with 80% power, about 300 subjects would be required in each group.

Recent data have questioned the validity of using total serum 25(OH)D levels as a marker for body vitamin D stores and for the assessment of effects on osseous and extra-osseous tissues. Aggarwal et al. reported that in adults with NS, BMD correlated positively with bioavailable but not total 25(OH)D levels, whereas bioavailable 25(OH)D showed a strong inverse correlation with PTH [39]. Genetic factors involving vitamin D pathway genes and the *VDBP* gene are likely to be involved in determining bioavailable 25(OH)D levels and dose response [40]. It is possible that Indians, like black Americans, have *VDBP* gene polymorphisms that result in low total but adequate bioavailable levels of 25(OH)D [41]. Thus, the main limiting factor of our study is that we did not measure VDBP levels and are therefore unable to calculate bioavailable 25(OH)D, which is possibly the main determinant of effects on bone and on inflammatory and infectious processes. In addition, increasing the numbers of each of the NS types (first episode/infrequent relapsers/frequent relapsers/steroid-dependent) to enable analysis of each subgroup may have yielded differences in outcome measures. We also did not confirm hypercalciuria by estimation of 24-h excretion during the duration of the study, owing to technical difficulties with timed urine collection; however, all those with persistent abnormalities are under follow-up and further evaluation.

To conclude, in our study, pediatric NS patients supplemented with calcium and vitamin D3 had significantly improved serum levels of total 25(OH)D. However, this was not associated with any other measured benefits, such as improvement in BMD or BMC or number of relapses within the 6-month study period. On the other hand, 50% of patients had documented hypercalciuria. Thus, until further evidence for the beneficial effects of calcium and vitamin D3 supplementation are available, such therapy should be used with caution and with adequate monitoring for possible adverse effects.

Acknowledgements We sincerely thank Dr Ravi Thadani, Chief of Nephrology, Massachusetts General Hospital, for reviewing the manuscript and Dr Subrata Das, H.O.D., Department of Radiology, Calcutta Medical Research Institute, for advice and supervision of the DXA scans.

Authors' contributions Sushmita Banerjee and Jayati Sengupta reviewed and recruited patients, and Surupa Basu supervised biochemical assays. All three were involved in the study design and in manuscript writing. Ananda Sen reviewed and edited the manuscript and guided the statistical analysis.

Compliance with ethical standards The study design was approved by the institutional ethics committees of the Institute of Child Health and Calcutta Medical Research Institute, and adhered to the Declaration of Helsinki. Informed consent was obtained from parents before recruitment.

Conflicts of interest None of the authors involved have any conflicts of interest to declare.

Funding The study was supported by an internal Institute of Child Health Nephrology Fund.

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