This double-blinded factorial design randomized study investigated extra skeletal manifestation of vitamin-D. The results showed that six months of vitamin-D and calcium supplementation either alone or together did not lead to significant change in skeletal muscle strength of young females. This study changed the prevailing concept on role of vitamin-D on muscle strength. It was accompanied by an Editorial in this prestigious Journal

Publication 1 (a)

Skeletal Muscle Strength in Young Asian Indian Females after Vitamin D and Calcium Supplementation: A Double-Blind Randomized Controlled Clinical Trial

Ravinder Goswami, Manju Vatsa, Vishnubhatla Sreenivas, Upinderpal Singh, Nandita Gupta, Ramakrishnan Lakshmy, Sameer Aggarwal, Arthi Ganapathy, Poonam Joshi, and Himani Bhatia

Departments of Endocrinology and Metabolism (R.G., N.G., S.A., A.G.), College of Nursing (M.V., P.J.), Biostatistics (V.S.), Physical Medicine and Rehabilitation (U.S., H.B.), and Cardiac Biochemistry (R.L.), All India Institute of Medical Sciences, New Delhi 110029, India

Context: Randomized control trials (RCT) of the effect of vitamin D/calcium supplementation on skeletal muscle strength have not shown promising effect in the elderly.

Objective: Our objective was to assess the effect of vitamin D and/or calcium on muscle strength in young adults with vitamin D deficiency.

Design and Setting: We conducted a RCT using a factorial design at a tertiary-care center from September 2010 to April 2011.

Subjects: A total of 173 healthy females with mean age, body mass index, and 25-hydroxyvitamin D [25(OH)D] of 21.7 ± 4.4 yr, 20.8 ± 2.96 kg/m^2, and 9.3 ± 3.37 ng/ml, respectively, were block randomized to 1) double placebo, 2) calcium/placebo, 3) cholecalciferol/placebo, and 4) cholecalciferol/calcium for 6 months. Cholecalciferol was given at 60,000 IU/wk for 8 wk followed by 60,000 IU/fortnight. Elemental calcium was given in doses of 500 mg twice per day for 6 months.

Methods: Assessment included hand grip (primary outcome) and pinch grip strength, distance walked in 6 min, dyspnea score, quality of life by Short Form (36) Health Survey (SP-36), serum 25(OH)D, 1,25-dihydroxyvitamin D, and intact PTH.

Results: The serum 25(OH)D increased significantly to 29.9 ± 8.35 and 27.0 ± 9.54 ng/ml in two groups on cholecalciferol. The mean hand grip strength (19.4 ± 3.92, 21.1 ± 3.31, 20.6 ± 3.92, and 20.1 ± 4.00 kg) and its increase from baseline (0.3 ± 2.25, 0.3 ± 2.64, −0.3 ± 2.41, and 0.6 ± 2.30 kg) were comparable in four groups at 6 months. Quality of life, urinary calcium/creatinine ratio, and adverse effects were also comparable in groups.

Conclusion: Oral cholecalciferol/calcium supplementation in the dose/schedule used is effective and safe in increasing and maintaining serum 25(OH)D. However, this does not lead to improved skeletal muscle strength in young females. **(*J Clin Endocrinol Metab* 97: 4709–4716, 2012)**

There is increased awareness about vitamin D deficiency (VDD) in the general population and its possible extraskeletal effects in malignancy, type 1 and 2 diabetes, multiple sclerosis, and infections (1–4). A recent meta-analysis of 17 randomized control trials (RCT) showed that vitamin D and calcium supplementation improved

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2012-2340 Received May 29, 2012. Accepted July 31, 2012.
First Published Online August 17, 2012

For editorial see page 4366

Abbreviations: iPTH, Intact PTH; 1,25(OH)$_2$D, 1,25-dihydroxyvitamin D; 25(OH)D, 25-hydroxyvitamin D; RCT, randomized control trial; SF-36, Short Form (36) Health Survey; VAS, visual analog scale.

muscle strength and performance only when basal serum 25-hydroxyvitamin D [25(OH)D] was <25 nmol/liter (5). Sixteen of these RCT were in the elderly because the intervention has a bearing on prevention of fall and osteoporotic fractures in this population (5, 6). Only one RCT assessed the effect of vitamin D and calcium supplementation on muscle strength in young people (7). None of the 17 RCT assessed the effects of vitamin D and calcium supplementation on muscle strength in a two by two factorial design.

Here, we report the effect of 6 months of vitamin D and calcium supplementation on skeletal muscle strength, physical performance, and quality of life in apparently healthy young Asian Indian women. The study addresses two questions, namely, what the effect is of vitamin D and calcium supplementation given either alone or in combination on muscle strength in young adults with vitamin D deficiency and whether there is an associated change in quality of life.

Subjects and Methods

Subjects

The study subjects were young female students of the nursing college and other postgraduate courses at the All India Institute of Medical Sciences, Delhi, India. The purpose of the study was explained to all of them, and participation was sought during September to October 2010. Those using drugs affecting bone mineral metabolism such as calcium, vitamin D, or antiepileptic, antituberculous, or antithyroid medications during the previous 6 months were excluded. None of them had muscle aches or weakness. Assessment of daily dietary intake of nutrients, skeletal muscle strength, and biochemical estimations were performed at baseline and after 6 months of supplementation. Blood samples for biochemical estimations were drawn in the fasting state between 0800 and 0930 h.

Randomization, concealment, blinding, and schedule of supplementation

Subjects were assigned to four interventions using block randomization with a block size of four. The four interventions were 1) cholecalciferol sachets and calcium tablets, 2) cholecalciferol sachets and placebo tablets, 3) placebo sachets and calcium tablets, and 4) placebo sachets and placebo tablets. The dose of cholecalciferol (Cadila Pharmaceuticals Ltd., Ahmedabad, India) was 60,000 IU/wk for first 8 wk followed by 60,000 IU twice a month for 4 months. Calcium carbonate (Elder Pharmaceutical Ltd., Mumbai, India) tablets containing 500 mg elemental calcium each was given twice daily for 6 months. The placebos for the calcium tablets and cholecalciferol granules were made of lactose and manufactured by Elder and Cadila Pharmaceuticals, respectively. These were matched with active supplements with respect to color, size, packing and taste. The supplementation schedule followed in this study was based on our previous studies (7, 8).

Packets containing the above interventions were prepared in advance for the whole study duration of 6 months according to the randomization numbers and arranged in serial order. Subjects were enrolled and packets were distributed consecutively to the study participants according to their entry into the trial. The codes of the supplementation packets were retained with an investigator not involved in distribution of the packets. Subjects, caregivers, and assessors were blinded to the intervention assigned to the participants and also to the results of the baseline investigations until the completion of the study.

Assessment of the skeletal muscle strength at baseline and at 6 months

Handgrip and pinch grip strength of the dominant limb were assessed using a computerized dynamometer in isometric mode (Tracker system version 4; JTECH, Salt Lake City, UT) with subjects in sitting position, shoulder adducted and neutrally rotated, elbow flexed at 90°, forearm in midprone neutral position, wrist in 0–30° extension and 0–15° ulnar deviation. For assessing handgrip strength, the dynamometer was presented vertically to the subjects who squeezed it using maximal handgrip force. The strength was measured three times each at two different positions representing increasing resistance levels.

Pinch grip strength between the tips of the thumb and index finger was measured using a pinch gauge. The gauge was presented on its side and at a 45° angle to the forearm, and the average of three measurements was recorded. The coefficients of variation for hand grip dynamometer positions 2 and 3 and pinch grip measurements were 5.4, 4.9, and 3.8% at baseline and 5.0, 5.3, and 3.5% at 6 months, respectively.

The 6-min walk test and assessment of dyspnea on a visual analog scale (VAS) were performed under supervised conditions as described earlier (7, 9). Briefly, brisk walk was demonstrated, and then subjects were asked to perform the same for 6 min on a flat and straight corridor. The distance covered was recorded in meters, and severity of dyspnea experienced after completion of walk was marked on a VAS with 0 indicating no and 100 representing maximal breathlessness.

Quality of life

To assess the change in health-related quality of life, the Short Form (36) Health Survey (SF-36) questionnaire was used after permission (10). The responses made in the physical function, role physical, bodily pain, and general health portion of the SF-36 questionnaire formed a composite score of physical health [Physical Component Score (PCS)-SF-36]. The responses to the role emotional, mental health, social functioning, and vitality portion of the questionnaire were used as an indicator of mental health [Mental Component Score (MCS)-SF-36]. The questionnaire was administered to the study participants before and after 6 months of supplementation.

The daily dietary intake of calories, carbohydrate, protein, fat, calcium, and phytin phosphorus were assessed using an open-ended semiquantitative food frequency *pro forma* questionnaire incorporating information on seven food groups and 40 common Indian food items (11, 12). Nutrient content was estimated using published values of Indian foods (12). Percent body surface area exposed to sunshine was calculated by the rule of nine (13). Serum total T_4 and TSH were measured in all to exclude hypothyroidism at enrollment. Subjects were advised to maintain their usual physical activity and dietary pattern during the study period. The Institutional Ethics Committee approved the study protocol, and written in-

formed consent was obtained from all the study subjects (www.clinicaltrials.gov ID NCT01190683).

Biochemical assessment

Serum total calcium, inorganic phosphorus, and alkaline phosphatase were measured using standard laboratory procedures on an automated analyzer (Beckman Coulter, Hialeah, FL; Syncron clinical system CX4PRO; normal range = 8.4–10.2 mg/dl, 2.5–4.6 mg/dl, and 32–91 IU/liter, respectively. Serum 25(OH)D was measured using chemiluminescence technology [LIAISON 25(OH)D total assay; DiaSorin, Inc., Stillwater, MN]. Serum intact PTH (iPTH) was measured using electrochemiluminescence assay (Elecsys-2010; Roche Diagnostics, Indianapolis, IN); normal range = 15–65 ng/liter. Intra- and interassay coefficients of variation for these assays ranged from 3.5–5.0%. Serum 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] was measured by liquid chromatography and mass spectroscopy (Quest Diagnostics, Nichols Institute, San Juan Capistrano, CA).

Assessment of compliance and safety

Subjects were advised to take the sachet of granules with a glass of milk every week and one tablet in the morning and evening along with a meal. The intake of the first sachet of every month was personally supervised by the physician. A choice of milk or curd was offered to the subjects while supervising the cholecalciferol intake. Only three of the 173 subjects opted for curd for reasons related to taste. Subjects were instructed to bring the sachets and tablets that were not consumed during the previous month when they came to collect the assigned supplements, and compliance was assessed using a pill-counting method. Compliance was also assessed retrospectively by checking the serum 25(OH)D levels in the blood samples drawn at 2 months of enrollment and after the randomization code was broken at 6 months.

To assess the safety of the supplementation, urine calcium/creatinine ratio was measured in the first sample in the morning (representing urine formed overnight), at baseline, and after 6 months. Urinary calcium/creatinine ratio higher than 0.2 mg/mg was defined as hypercalciuria (14), and hypercalcemia was defined as serum total calcium (adjusted for albumin) over 10.6 mg/dl (15). Severe hypercalcemia was defined as higher than 11.0 mg/dl (16). In addition, new symptoms including those related to the gastrointestinal tract were recorded at each visit.

Sample size

Our previous study (7) showed a change of hand grip strength of 3.1 kg in the dual-supplementation group compared with only 0.3 kg in the double-placebo group. Assuming that cholecalciferol alone and calcium alone show a change of 1 kg with a common SD of 3 kg, to detect such a change in hand grip strength in a one-way ANOVA with 5% α-error and 90% power, 33 subjects per group were required. Giving a 20% allowance for attrition, 42 subjects per group (total 168) were needed.

Statistical analysis

Quantitative data are reported as mean and SD and qualitative data as frequencies in percentages. One-way ANOVA or Kruskal-Wallis one-way ANOVA and χ^2 tests were used to analyze the differences in the study parameters among the four groups. Analysis was on the intention-to-treat principle. The average value of a variable in the respective group was used to impute data in subjects who withdrew consent after randomization. The last observation was carried forward for the subjects who were dropped or lost during the intervention period. Differences in the study parameters before and after 6 months of intervention were analyzed by linear regression analysis with adjustment for respective baseline parameters. All *P* values were two tailed and values <0.05 were considered significant. All statistical analyses were implemented on Stata version 11.2.

Results

Figure 1 shows the flow of the study. A total of 300 young women were invited to participate, of which 173 consented and were randomized into four arms. Three subjects withdrew consent after randomization. Four subjects were withdrawn (two for pregnancy, one for hypothyroidism, and one for bronchial asthma). Two subjects were lost to follow-up because they changed their addresses. Eleven subjects failed to complete the follow-up due to undisclosed reasons. Finally, 153 participants completed the study.

Baseline data

Table 1 summarizes the baseline characteristics of the subjects in the four study groups. The mean age, BMI, and body surface area exposed of the 173 study subjects were 21.7 ± 4.4 yr, 20.8 ± 2.96 kg/m^2, and 18 ± 7%, respectively, and were comparable in all the four groups. The average daily dietary calories, protein, fat, carbohydrate and calcium intake of the study subjects were 1634 ± 403 kcal, 51.4 ± 14.86 g, 46.1 ± 14.42 g, 251.1 ± 71.24 g, and 577.8 ± 258.93 mg, respectively, and were comparable in all four groups.

The mean serum 25(OH)D of the 173 subjects was 9.3 ± 3.37 ng/ml at baseline, and none of them had sufficient vitamin D levels (>32.0 ng/ml). Only 34 of the 173 study subjects (19.6%) had serum 25(OH)D values of 12.0 ng/ml or higher, and 69 (39.9%) had serum 25(OH)D of 10.0 ng/ml or higher.

The mean serum iPTH for all subjects was 58.0 ± 22.89 pg/ml with 29.5% of subjects having values in the supranormal range. However, the average serum 1,25$(OH)_2$D values (41.2 ± 11.35 pg/ml) were in the normal range. The mean serum total calcium corrected for albumin, inorganic phosphorus, alkaline phosphatase, 25(OH)D, 1,25$(OH)_2$D, and iPTH values were comparable in the four study groups.

The mean hand grip strength (positions 2 and 3), pinch grip strength, distance covered during 6-min walk test, and the dyspnea experienced in the double-placebo, calcium/placebo, cholecalciferol/placebo, and the double-supplementation groups were 21.1 ± 4.30 kg, 19.1 ± 4.10 kg, 5.4 ± 0.89 kg, 623 ± 41 m, and 24.1 ± 18.06%,

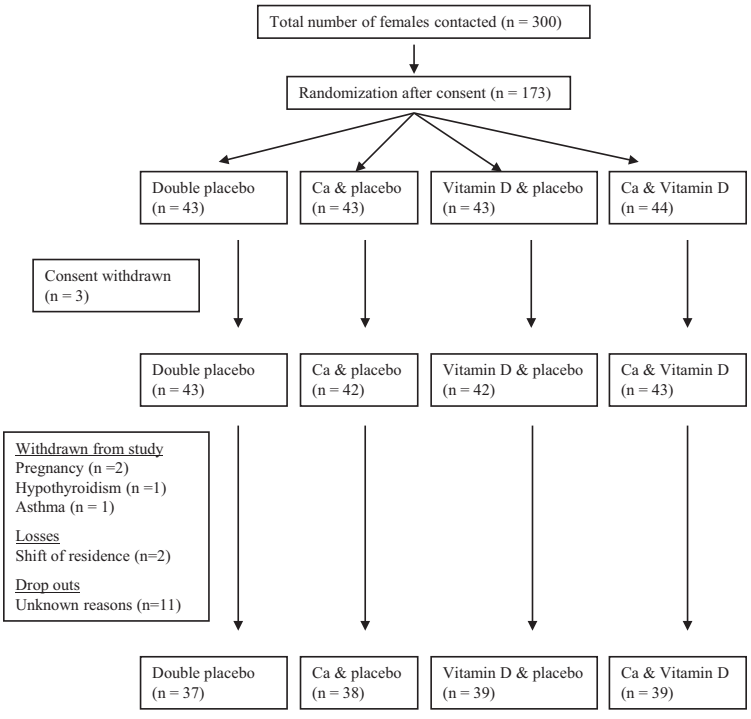

FIG. 1. Flow of the study.

respectively, and were comparable across the groups. Physical and mental health as indicated by PCS-SF-36 and MCS-SF-36 scores were also similar.

The baseline serum 25(OH)D values in all 173 subjects showed an inverse relationship with serum iPTH ($r = -0.39$, $P < 0.001$). Serum $1,25(OH)_2D$ showed no significant correlation with serum 25(OH)D ($r = 0.04$, $P = 0.60$) or with serum iPTH ($r = 0.09$, $P = 0.22$).

Compliance and efficacy of the supplementation

The average compliance for the sachets and tablets as assessed by calculating the percentage consumed of the total allocation varied from 97.6–99.2% and 77.2–81.1%, respectively, and was comparable in the four groups (Table 1). The serum 25(OH)D levels at 2 months were significantly higher in the groups receiving cholecalciferol alone (31.8 ± 13.39 ng/ml) or cholecalciferol with calcium (27.7 ± 11.12 ng/ml) with a mean change of 20.6 ± 8.73 and 17.5 ± 8.61 ng/ml, respectively, from baseline ($P < 0.001$ for both). In contrast, there was no significant increase in the groups receiving calcium plus placebo or double placebo (−0.9 ± 3.13 and −1.8 ± 2.54 ng/ml).

Change in vitamin D status after supplementation at 6 months

The mean serum 25(OH)D values were significantly higher in the two groups receiving cholecalciferol compared with the other two groups. Mean serum iPTH was significantly lower in the cholecalciferol- and calcium-supplemented group compared with the other three groups (Table 2). There were no significant differences in the mean serum total calcium, inorganic phosphorus, and alkaline phosphatase in the four groups. The mean changes in these parameters were also comparable in the four groups. Mean daily dietary calorie intake, its proximate principals, and body surface area exposed were comparable in the pre- and posttreatment phase.

Change in muscle strength and related parameters

Mean hand grip strength at positions 2 and 3 and the average of the two positions were comparable across groups after 6 months of intervention. Average mean grip strength was 19.4 ± 3.92, 21.1 ± 3.31, 20.6 ± 3.92, and 20.1 ± 4.00 kg, respectively ($P = 0.19$). The mean of the differences in hand grip strength between baseline and at 6 months was also comparable in the four groups (0.3 ± 2.25, 0.3 ± 2.64, −0.3 ± 2.41, and 0.6 ± 2.30, $P = 0.34$). Similarly, the mean pinch grip strength, distance covered during the 6-min walk, and dyspnea experienced after 6 months of supplementation and the mean change in these parameters from baseline were comparable in the four groups (Table 2).

Regression analysis adjusting for respective baseline parameters showed no significant difference in any of the study parameters among the four groups.

Effect of supplementation on the quality of life

The mean PCS-SF-36 and MCS-SF-36 scores and the differences in the pre- and post-supplementation scores were comparable in the four study groups at 6 months.

Stratified analysis

Stratified analysis based on preintervention serum 25(OH)D levels (<10.0 and ≥10.0 ng/ml) also showed similar results. The mean change in the hand grip strength in 1) double-placebo, 2) calcium/placebo, 3) cholecalciferol/placebo, and 4) cholecalciferol/calcium supplemented groups in subjects with baseline serum 25(OH)D below 10.0 ng/ml were comparable (−0.2 ± 2.19, 0.0 ± 3.67, −0.9 ± 2.73, and 0.7 ± 1.99 kg, respectively, $P = 0.31$ and adjusted P for respective baseline parameter = 0.43). Similarly, the average hand grip strength in the four intervention arms in subjects with baseline serum

TABLE 1. Baseline parameters (mean and SD) in the four study arms

Parameter	Double placebo (n = 43)	Calcium alone (n = 43)	Cholecalciferol alone (n = 43)	Calcium and cholecalciferol (n = 43)	*P*
Mean age (yr)	22.4 ± 4.91	21.7 ± 4.41	21.3 ± 3.17	21.5 ± 3.50	0.93
BMI (kg/m^2)	20.7 ± 2.98	21.2 ± 3.62	20.7 ± 2.62	20.5 ± 2.53	0.69
Serum total Ca[a] (mg/dl)	9.8 ± 0.48	9.8 ± 0.51	9.8 ± 0.46	9.8 ± 0.51	0.97
Serum PO_4 (mg/dl)	3.9 ± 0.43	4.1 ± 0.37	3.9 ± 0.53	4.0 ± 0.64	0.38
Serum alkaline phosphatase (IU/liter)	65.5 ± 19.66	67.7 ± 21.44	67.0 ± 21.78	63.2 ± 17.81	0.74
Dietary calories (kcal/d)	1572 ± 373	1673 ± 492	1577 ± 343	1716 ± 387	0.26
Dietary Ca intake (mg/d)	567 ± 296	574 ± 265	578 ± 259	591 ± 220	0.98
Dietary phytate (g/d)	936 ± 287	1041 ± 327	933 ± 221	1072 ± 391	0.14
Dietary Ca/phytate ratio	0.6 ± 0.30	0.6 ± 0.23	0.6 ± 0.27	0.6 ± 0.20	0.44
Dietary fat (g/d)	45.2 ± 15.87	45.8 ± 14.3	45.7 ± 13.16	47.6 ± 14.61	0.88
Dietary carbohydrate (g/d)	232.5 ± 75.44	262.9 ± 84.39	241.8 ± 57.29	266.8 ± 61.26	0.11
Dietary protein (g/d)	49.1 ± 13.37	51.7 ± 17.33	49.9 ± 12.08	54.8 ± 15.90	0.28
Serum 25(OH)D (ng/ml)	8.6 ± 3.26	9.9 ± 3.34	9.2 ± 3.40	9.5 ± 3.47	0.38
Serum 1,25$(OH)_2$D (pg/ml)	41.1 ± 9.16	42.5 ± 12.16	40.1 ± 13.23	41.1 ± 10.76	0.80
Serum iPTH (pg/ml)	59.3 ± 23.15	54.3 ± 21.63	62.4 ± 22.06	56.2 ± 24.55	0.38
Urinary Ca/creatinine	0.07 ± 0.05	0.08 ± 0.08	0.06 ± 0.05	0.08 ± 0.07	0.81
Body surface area exposed (%)	19.0 ± 7.79	16.5 ± 5.95	16.9 ± 7.00	18.7 ± 6.30	0.23
Parameters related to muscle strength					
Handgrip strength (kg)					
Position 1	20.1 ± 4.39	21.7 ± 3.09	21.9 ± 4.54	20.8 ± 4.22	0.16
Position 2	18.2 ± 3.65	20.0 ± 4.17	19.9 ± 4.50	18.2 ± 3.83	0.05
Average	19.1 ± 3.86	20.8 ± 3.87	20.9 ± 4.38	19.5 ± 3.94	0.08
Pinch strength (kg)	5.3 ± 0.96	5.5 ± 0.87	5.4 ± 0.90	5.3 ± 0.85	0.69
6-min-walk test (m)	621 ± 47	626 ± 50	621 ± 29	626 ± 36	0.78
Dyspnea-VAS (%)	23.4 ± 16.95	23.1 ± 17.47	19.4 ± 15.09	30.4 ± 20.94	0.10
Health-related quality of life					
PCS-SF-36	49.7 ± 6.49	49.9 ± 5.67	50.2 ± 4.92	49.1 ± 6.10	0.85
MCS-SF-36	44.6 ± 7.19	44.9 ± 6.44	44.7 ± 8.56	42.0 ± 11.35	0.37
Compliance					
Sachet (%)	97.6 ± 4.94	99.2 ± 1.91	98.8 ± 2.35	98.8 ± 3.23	0.39
Tablets (%)	77.2 ± 12.42	78.3 ± 11.09	79.6 ± 8.42	81.1 ± 8.03	0.41

[a] Corrected for serum albumin.

25(OH)D of at least 10.0 ng/ml were not different (0.4 ± 2.65, 0.6 ± 2.01, 0.5 ± 2.69, and 0.2 ± 2.49 kg, respectively, $P = 0.94$ and adjusted $P = 0.73$). There was no significant change in other study outcomes in the four study arms in the two stratified groups of serum 25(OH)D levels.

Adverse events

The mean urinary calcium/creatinine ratio at baseline and the difference between pre- and post-supplementation values were comparable in the four study groups. After 6 months of supplementation, less than 10% of the subjects showed hypercalciuria (4.7, 7.0, 9.3, and 9.0%, respectively, in the four groups, $P = 0.92$). Hypercalcemia was observed in 8.7% of the subjects (2.3, 9.3, 11.6, and 11.4% in double-placebo, calcium/placebo, cholecalciferol/placebo and dual-supplement groups, respectively, $P = 0.38$). The mean serum calcium of the subjects with hypercalcemia was 10.7 ± 0.14 mg/dl, and only 2 of the 15 (one each in calcium/placebo and cholecalciferol/placebo arms) had serum total calcium value over 11.0 mg/dl (11.1 mg/dl for both). Constipation (10.2%), cramps (3.6%), and gastritis (2.3%) were the common complaints reported by the participants, but their frequencies were not significantly different among the study groups.

Discussion

The present study was designed based on the results of our previous study indicating significant effect of cholecalciferol and calcium supplementation on skeletal muscle strength in young individuals. We adopted a two by two factorial design allowing inference regarding the effect of calcium, vitamin D, and their combination, which was not adopted in any earlier study on the effect vitamin D supplementation. In addition, the study subjects were the same sex with comparable age, BMI, dietary practices, and physical activities, which minimized the possible confounding effect on results. All the subjects were recruited within a short span of time, so that seasonal influences would have little influence. In addition, most of the participants in this study stayed within the college campus, near the study site, which

TABLE 2. Mean and SD of various parameters at 6 months and their change from baseline in intention-to-treat analysis

Parameter	Double placebo (n = 43)	Calcium alone (n = 43)	Cholecalciferol alone (n = 43)	Calcium and cholecalciferol (n = 44)	*P*	*P*[a]
Serum total Ca[b] (mg/dl)	9.9 ± 0.55	10.1 ± 0.44	10.1 ± 0.48	10.0 ± 0.48	0.27	
Increase	0.11 ± 0.34	0.23 ± 0.30	0.25 ± 0.31	0.21 ± 0.35	0.17	
Serum 25(OH)D (ng/ml)	7.7 ± 3.64[c]	8.1 ± 2.92[c]	29.9 ± 8.35[d]	27.0 ± 9.54[d]	<0.001	
Increase	−0.9 ± 3.13[c]	−1.8 ± 2.54[c]	20.6 ± 8.73[d]	17.5 ± 8.61[d]	<0.001	
Serum 1,25$(OH)_2$D (pg/ml)	40.1 ± 11.06	39.8 ± 14.16	43.8 ± 11.54	44.0 ± 13.30	0.23	
Increase	−1.0 ± 10.83	−2.8 ± 15.27	3.7 ± 13.07	2.95 ± 13.43	0.13	
Serum iPTH (pg/ml)	68.1 ± 37.51[c,e]	55.3 ± 27.06[d,e]	51.5 ± 15.69[d]	44.2 ± 19.79[d]	<0.001	
Decrease	−8.7 ± 26.04[c,e]	−1.1 ± 22.34[d,e]	10.8 ± 26.16[d]	12.0 ± 22.20[d]	<0.001	
Urine Ca/Creatinine	0.09 ± 0.05	0.10 ± 0.11	0.10 ± 0.06	0.11 ± 0.07	0.35	
Increase	0.02 ± 0.06	0.02 ± 0.11	0.03 ± 0.06	0.03 ± 0.07	0.28	
Parameters related to muscle strength						
Handgrip strength (kg)						
Position 2	20.8 ± 4.02	22.0 ± 3.48	21.9 ± 4.00	21.6 ± 4.06	0.46	
Increase	0.72 ± 2.96	0.28 ± 2.50	0.0 ± 2.52	0.82 ± 2.69	0.45	0.67
Position 3	18.0 ± 3.99	20.3 ± 3.45	19.3 ± 4.12	18.7 ± 4.15	0.06	
Increase	−0.2 ± 2.27	0.3 ± 3.29	−0.6 ± 2.73	0.5 ± 2.44	0.26	0.56
Average	19.4 ± 3.92	21.1 ± 3.31	20.6 ± 3.92	20.1 ± 4.00	0.19	
Increase	0.3 ± 2.25	0.3 ± 2.64	−0.3 ± 2.41	0.6 ± 2.30	0.34	0.74
Pinch strength (kg)	5.3 ± 0.90	5.7 ± 0.90	5.5 ± 0.86	5.4 ± 0.79	0.25	
Increase	−0.03 ± 0.74	0.13 ± 0.56	0.13 ± 0.48	0.09 ± 0.71	0.64	0.44
6-min walk (m)	633 ± 48	634 ± 54	627 ± 47	640 ± 37	0.67	
Increase	12.4 ± 30.31	7.8 ± 28.85	5.73 ± 39.14	13.9 ± 34.61	0.63	0.84
Dyspnea-VAS (%)	18.4 ± 13.83	21.3 ± 14.81	18.9 ± 15.07	21.3 ± 16.06	0.72	
Decrease	5.0 ± 18.09	1.8 ± 20.95	0.4 ± 11.71	9.0 ± 19.41	0.12	0.96
Health-related quality of life						
PCS-SF-36	50.6 ± 6.48	49.3 ± 6.72	51.0 ± 5.83	49.8 ± 4.71	0.55	
Increase	0.9 ± 5.32	−0.6 ± 5.37	0.7 ± 5.22	0.6 ± 5.71	0.56	0.97
MCS-SF-36	44.1 ± 8.56	45.9 ± 7.87	44.8 ± 7.84	43.6 ± 8.07	0.57	
Increase	−0.5 ± 6.82	1.0 ± 7.04	0.1 ± 6.12	1.5 ± 10.04	0.78	0.27

[a] Adjusted for respective baseline values.

[b] Corrected for serum albumin.

[c–e] Data in the same row with *different superscript letters* are significantly different from each other.

allowed an easy contact for motivation and supervised compliance for the supplementation.

Our results show that the cholecalciferol supplementation regimen used can increase and maintain serum 25(OH)D levels close to a vitamin D-sufficient range. This supplementation, however, was not associated with any significant increase in calcium/creatinine ratio, frequency of hypercalciuria, hypercalcemia, or any other complaints. Thus, the schedule of supplementation followed in the study appears to be safe and effective.

Despite significant improvement in the serum 25(OH)D levels, there was no significant change in skeletal muscle strength in any of the supplementation groups. These results are consistent with the overall results reported from a meta-analysis (5). Although, the same meta-analysis showed significant improvement in proximal hip muscle strength in vitamin D-deficient elderly participants, a similar stratified analysis in the present study did not show any improvement in the muscle strength of young females. Such variance could be due to the differences in age and gender compositions.

Vitamin D supplementation is also reported to prevent falls among the elderly, with more prevention among those achieving higher vitamin D levels (17). However, such prevention may not necessarily be due to improvement in muscle strength (18).

The observation of lack of improvement in muscle strength with dual supplementation in the present study is contrary to our earlier findings (7). This is unexpected, especially when we used similar methods for the assessment of skeletal muscle strength and duration of supplementation and closer supervision of the study subjects with minimal confounding effects. It is also unlikely to be due to the differences in the compliance or bioavailability of the supplementation. In fact, the mean serum 25(OH)D levels at 6 months in the subjects of the present study were higher than those in the subjects of the previous study.

Thus, the reasons for variance of results on lack of improvement in muscle strength after cholecalciferol and/or calcium supplementation in the present study are not clear. Possible reasons may include 1) improvement in serum $1,25(OH)_2D$ levels and 2) the gender-specific differences. Bischoff *et al.* (19) reported significant reduction in the frequency of falls after 12 wk of calcium and vitamin D supplementation in elderly women, which was associated with an 8% increase in serum $1,25(OH)_2D$. In the present study, we observed a similar increase in the serum $1,25(OH)_2D$ levels (7.1% in the double-supplementation group and 9.2% in the cholecalciferol/placebo group), but there was no concurrent increase in muscle strength in these groups.

The other factor that might have led to an absence of improvement in skeletal muscle strength in the current study could be the gender-specific effect of vitamin D supplementation. It is known that vitamin D supplementation has a differential effect on the risk of cardiovascular disease, with males showing significant reduction in the risk, whereas the same was not observed among females (20). All the subjects in the present study were females, whereas our earlier study included both males and females. To test the gender-specific differences, the data of our previous study (7) was revisited to assess the effect of cholecalciferol and calcium supplementation on skeletal muscle strength among the males and females separately. Interestingly, sex-stratified analysis revealed improvement in the hand grip strength only in males but not in females. The average increase in hand grip strength among males was 2.8 kg (95% confidence interval = 1.3–4.2, $P = 0.001$) compared with an increase of 0.6 kg (95% confidence interval = −2.6–3.8, $P = 0.71$) among females. This along with the results of the present study indicate that vitamin D and calcium supplementation in the prescribed dose either alone or in combination would not improve skeletal muscle strength as assessed by hand grip strength in young females.

Recently, Wehr *et al.* (21) and Nimptsch *et al.* (22) showed significant positive association between serum 25(OH)D levels and testosterone levels. A RCT in healthy males (23) showed significant increase in serum testosterone levels after cholecalciferol supplementation. Recently, Bhasin *et al.* (24) reported a significant effect of testosterone supplementation on the lean skeletal muscle mass and strength in healthy males aged 18–50 yr. It appears that there is significant association between vitamin D levels and testosterone levels; vitamin D supplementation leads to improvement in testosterone levels, and testosterone supplementation leads to improved muscle mass and strength. Therefore, it is plausible that improved vitamin D levels after supplementation get translated into improved muscle strength, depending on the testosterone levels of the subjects studied. All the subjects in the present study were young females, and their testosterone level is likely to be low. This could be a reason for the absence of any improvement in muscle strength despite significant increased in serum 25(OH)D levels.

Furthermore, the role of female sex hormones in the association between the vitamin D supplementation and improvement in muscle strength is not clear. An average fall of 3% in serum estradiol ($P = 0.04$) and 10% in serum progesterone ($P < 0.001$) per 10-nmol/liter increase in serum 25(OH)D was reported in the luteal phase among young healthy women after cholecalciferol supplementation (25). However, the relevance of these changes in the muscle strength was not assessed. Therefore, studies involving both males and females along with measurement of testosterone and female sex hormones would help understand the vitamin D-mediated improvement in muscle strength better.

Quality of life as assessed by SF-36 scores were similar among the four groups after 6 months of supplementation. This observation is in agreement with earlier reports (26, 18).

Thus, to conclude, oral cholecalciferol supplementation with a loading dose of 60,000 IU/wk for 8 wk followed by 60,000 IU once per fortnight for 4 months is safe and effective in maintaining serum 25(OH)D. However, the improved vitamin D status does not lead to improved skeletal muscle strength in young females.

Acknowledgments

We are thankful to all the students and subjects who participated in this study. The help of Ms. Ankita, dietician, in assessing daily dietary calcium intake is acknowledged.

Address all correspondence and requests for reprints to: Dr. Ravinder Goswami, M.D., D.M. (Additional Professor), Department of Endocrinology and Metabolism, All India Institute of Medical Sciences, New Delhi 110029, India. E-mail: gosravinder@hotmail.com.

This work was supported by the Indian Council of Medical Research for the study (Grant 5/9/77/2009-RHN).

Disclosure Summary: There is no conflict of interest and nothing further to disclose.

References

1. **Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM** 2012. Guidelines for preventing and treating vitamin D deficiency and insufficiency revisited. J Clin Endocrinol Metab 97:1153–1158
2. **Sørensen IM, Joner G, Jenum PA, Eskild A, Torjesen PA, Stene LC** 2012 Maternal serum levels of 25-hydroxy-vitamin D during preg-

nancy and risk of type 1 diabetes in the offspring. Diabetes 61:175–178
3. **Holick MF** 2011 Vitamin D deficiency in 2010: health benefits of vitamin D and sunlight: a D-bate. Nat Rev Endocrinol 7:73–75
4. **Holick MF** 2010 Vitamin D: extraskeletal health. Endocrinol Metab Clin North Am 39:381–400
5. **Stockton KA, Mengersen K, Paratz JD, Kandiah D, Bennell KL** 2011 Effect of vitamin D supplementation on muscle strength: a systematic review and meta-analysis. Osteoporos Int 22:859–871
6. **Murad MH, Elamin KB, Abu Elnour NO, Elamin MB, Alkatib AA, Fatourechi MM, Almandoz JP, Mullan RJ, Lane MA, Liu H, Erwin PJ, Hensrud DD, Montori VM** 2011 Clinical review: The effect of vitamin D on falls: a systematic review and meta-analysis. J Clin Endocrinol Metab 96:2997–3006
7. **Gupta R, Sharma U, Gupta N, Kalaivani M, Singh U, Guleria R, Jagannathan NR, Goswami R** 2010 Effect of cholecalciferol and calcium supplementation on muscle strength and energy metabolism in vitamin D-deficient Asian Indians: a randomized, controlled trial. Clin Endocrinol (Oxf) 73:445–451
8. **Goswami R, Gupta N, Ray D, Singh N, Tomar N** 2008 Pattern of 25(OH)D response at short (2 month) and long interval (one year) after 8 wk of oral supplementation with cholecalciferol in Asian Indians with chronic hypovitaminosis D. Br J Nutr 100:526–529
9. **Goswami R, Guleria R, Gupta AK, Gupta N, Marwaha RK, Pande JN, Kochupillai N** 2002 Prevalence of diaphragmatic muscle weakness and dyspnoea in Graves' disease and their reversibility with carbimazole therapy. Eur J Endocrinol 147:299–303
10. **Contopoulos-Ioannidis DG, Karvouni A, Kouri I, Ioannidis JP** 2009 Reporting and interpretation of SF-36 outcomes in randomised trials: systematic review. BMJ 338:a3006
11. **Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, Hennekens CH, Speizer FE** 1985 Reproducibility and validity of a semiquantitative food frequency questionnaire. Am J Epidemiol 122:51–65
12. **Gopalan C, Sastri BVR, Balasubramanian SC** 1996 Food composition tables. In: Gopalan C, Sastri B, Balasubramanian S, Narasinga B, Deosthale Y, Pant K, eds. Nutritive value of Indian foods. Hyderabad, India: National Institute of Nutrition, Indian Council of Medical Research; 45–95
13. **Mcgrouther DA** 1995 Skin burns. In: Mann CV, Russel RC, Williams NS, eds. Bailey and Love's short practice of surgery. London: Chapman and Hall; 124–148
14. **Sargent JD, Stukel TA, Kresel J, Klein RZ** 1993 Normal values for random urinary calcium to creatinine ratios in infancy. J Pediatr 123:393–397
15. **Gallagher JC, Sai A, Templin T 2nd, Smith L** 2012 Dose response to vitamin D supplementation in postmenopausal women: a randomized trial. Ann Intern Med 156:425–437
16. **Horwitz MJ, Tedesco MB, Garcia-Ocaña A, Sereika SM, Prebehala L, Bisello A, Hollis BW, Gundberg CM, Stewart AF** 2010 Parathyroid hormone-related protein for the treatment of postmenopausal osteoporosis: defining the maximal tolerable dose. J Clin Endocrinol Metab 95:1279–1287
17. **Bischoff-Ferrari HA, Dawson-Hughes B, Staehelin HB, Orav JE, Stuck AE, Theiler R, Wong JB, Egli A, Kiel DP, Henschkowski J** 2009 Fall prevention with supplemental and active forms of vitamin D: a meta-analysis of randomised controlled trials. BMJ 339:b3692
18. **Dhesi JK, Jackson SH, Bearne LM, Moniz C, Hurley MV, Swift CG, Allain TJ** 2004 Vitamin D supplementation improves neuromuscular function in older people who fall. Age Ageing 33:589–595
19. **Bischoff HA, Stähelin HB, Dick W, Akos R, Knecht M, Salis C, Nebiker M, Theiler R, Pfeifer M, Begerow B, Lew RA, Conzelmann M** 2003 Effects of vitamin D and calcium supplementation on falls: a randomized controlled trial. J Bone Miner Res 18:343–351
20. **Sun Q, Shi L, Rimm EB, Giovannucci EL, Hu FB, Manson JE, Rexrode KM** 2011 Vitamin D intake and risk of cardiovascular disease in US men and women. Am J Clin Nutr 94:534–542
21. **Wehr E, Pilz S, Boehm BO, März W, Obermayer-Pietsch B** 2010 Association of vitamin D status with serum androgen levels in men. Clin Endocrinol (Oxf) 73:243–248
22. **Nimptsch K, Platz EA, Willett WC, Giovannucci E** 2012 Association between plasma 25-OH vitamin D and testosterone levels in men. Clin Endocrinol (Oxf) 77:106–112
23. **Pilz S, Frisch S, Koertke H, Kuhn J, Dreier J, Obermayer-Pietsch B, Wehr E, Zittermann A** 2011 Effect of vitamin D supplementation on testosterone levels in men. Horm Metab Res 43:223–225
24. **Bhasin S, Travison TG, Storer TW, Lakshman K, Kaushik M, Mazer NA, Ngyuen AH, Davda MN, Jara H, Aakil A, Anderson S, Knapp PE, Hanka S, Mohammed N, Daou P, Miciek R, Ulloor J, Zhang A, Brooks B, Orwoll K, Hede-Brierley L, Eder R, Elmi A, Bhasin G, Collins L, Singh R, Basaria S** 2012 Effect of testosterone supplementation with and without a dual 5α-reductase inhibitor on fat-free mass in men with suppressed testosterone production: a randomized controlled trial. JAMA 307:931–939
25. **Knight JA, Wong J, Blackmore KM, Raboud JM, Vieth R** 2010 Vitamin D association with estradiol and progesterone in young women. Cancer Causes Control 21:479–483
26. **Latham NK, Anderson CS, Lee A, Bennett DA, Moseley A, Cameron ID; Fitness Collaborative Group** 2003 A randomized, controlled trial of quadriceps resistance exercise and vitamin D in frail older people: the Frailty Interventions Trial in Elderly Subjects (FITNESS). J Am Geriatr Soc 51:291–299

Received: 18 March 2019 | Revised: 15 April 2019 | Accepted: 13 May 2019

DOI: 10.1111/cen.14012

This works shown that problem of vitamin D deficiency in urban area is limited to indoors. Mots outdoors have sufficient vitamin D levels. This work has thus helped physicians in change of clinical practice where vitamin D supplementation is now prescribed mainly to indoors rather than most.

ORIGINAL ARTICLE

WILEY

Absence of vitamin D deficiency among common outdoor workers in Delhi

Pramila Dharmshaktu[1] | Soma Saha[1] | Parmita Kar[1] | Vishnubhatla Sreenivas[2] | Lakshmy Ramakrishnan[3] | Ravinder Goswami[1]

[1]Department of Endocrinology and Metabolism, All India Institute of Medical Sciences, New Delhi, India

[2]Department of Biostatistics, All India Institute of Medical Sciences, New Delhi, India

[3]Department of Cardiac Biochemistry, All India Institute of Medical Sciences, New Delhi, India

Correspondence
Ravinder Goswami, Department of Endocrinology and Metabolism, All India Institute of Medical Sciences, New Delhi 110029, India.
Email: gosravinder@hotmail.com

Funding information
All India Institute of Medical Sciences, Grant/Award Number: No-F.8-496/A-496/2016/RS

Abstract

Background: There is reservation about accepting the notion of widespread vitamin D deficiency (VDD) in sunny countries because information base is largely urban indoors, and the cut-off serum 25(OH)D > 75.0 nmol/L to define sufficiency is perceived as high.

Objective: We assessed the vitamin D status of subjects engaged in six types of outdoor jobs with freedom to seek shade, when needed.

Design: Descriptive observational study.

Subjects and methods: A total of 573 outdoors, (hawkers, n = 144; auto-rickshaw drivers, n = 113; manual rickshaw pullers, n = 49; fuel-station attendants, n = 84; gardeners, n = 96; traffic police personnel, n = 87) were assessed for serum 25(OH)D, iPTH and total calcium during summer and winter. Bank employees were indoor controls (n = 72). Serum 25(OH)D was defined as sufficient if ≥50.0 nmol/L and deficient when <30.0 nmol/L, as per 'Institute of Medicine'.

Results: Mean serum 25(OH)D of 573 outdoors was 44.8 ± 19.6 nmol/L and showed a physiological inverse relation with iPTH (P < 0.001). 77.5% of the outdoors did not have VDD. Hawkers, gardeners, fuel-station attendants and rickshaw pullers had sufficient or near sufficient serum 25(OH)D. The mean serum 25(OH)D (30.6 ± 23.2 nmol/L) of indoors though lower by 12.7 nmol/L than outdoors was above the cut-off of VDD. Proportions with supranormal iPTH were comparable between outdoors and indoors (14.0% vs 20.8%). Despite winter dip, the mean serum 25(OH)D (31.2 ± 14.3 nmol/l) of outdoors was not deficient.

Conclusions: Vitamin D deficiency is not universal. Most urban outdoor workers do not have VDD.

KEYWORDS

25(OH)D, outdoor workers, vitamin D

Pramila Dharmshaktu and Soma Saha contributed equally and are the joint first authors.

1 | INTRODUCTION

Vitamin D deficiency (VDD) is common in countries with cold climate and also in regions with plenty of sunshine as in India, Africa and Middle Eastern countries.[1-3] A systematic study from our centre in the year 2000 revealed a high prevalence of VDD among physicians.[1] Similar VDD was also reported in school children, teachers in Delhi and subjects from other parts of India.[4-7] Dark skin complexion, limited sun exposure and culture of wearing full body covering attire are the major contributory factors for VDD in sunny countries.[1,7,8] With reports suggesting pandemic of VDD, fortification of food with vitamin D is in offing in several countries.[9,10] However, there is a hesitation in accepting the notion of widespread VDD in sunny countries because information base is predominantly from urban indoors with paucity of data from outdoor population.[6,11-13] Besides, serum 25(OH)D level of >75.0 nmol/L to define vitamin D sufficiency is perceived as being rather high leading to an overestimate of prevalence of VDD.[14,15]

Interestingly, our recent study showed normal vitamin D status in outdoor construction workers of Delhi.[16] Absence of VDD in the construction workers was explained by compulsory long hours of sun exposure. However, this study suggested likelihood of good vitamin D status among outdoors working under sun with freedom to seek shade off and on as per their need. It is important to assess the vitamin D status of people working outdoors in order to focus the strategies for tackling VDD in urban cities. If the outdoor workers do not show gross VDD, vitamin D supplementation should be offered to indoor workers only with VDD, and not to all population. The aim of this study was to assess the spectrum of vitamin D status of outdoor workers engaged in different jobs in the urban areas of sunny region and its seasonal variation.

2 | MATERIAL AND METHODS

The study was carried during 2016-18, at the All India Institute of Medical Sciences (AIIMS), Delhi (latitude 28.70°N; longitude 77.11°E). The six groups of outdoor workers were selected based on the variation in their jobs: (a) hawkers or street vendors on pavements around AIIMS, (b) attendants from five fuel stations around AIIMS, (c) drivers of auto rickshaws plying in Delhi, (d) manual rickshaw pullers from a central market near AIIMS, (e) traffic police personnel in Delhi, and (f) gardeners from two plantation nurseries of central Delhi. Only those subjects who had been working for at least past six months in their respective outdoor jobs at Delhi were considered eligible to participate. They were initially investigated during summer, that is in June to September, 2016, when sunshine was available for most of the day with temperatures surging to 40°C. Seasonal variation of vitamin D status was assessed for subjects who had serum 25(OH)D levels ≥50 nmol/L during summer. Re-assessment was carried out during winter, that is January to March 2017. Subjects with use of calcium or vitamin D supplements during past six months were excluded. Those with any chronic illness during this period were also excluded.

Multiple visits were made by the authors to contact hawkers and pavement vendors. They were contacted individually to explain the aim of the study, requested to participate and advised to visit next day after overnight fasting to the Department of Endocrinology at AIIMS for investigations. Auto-rickshaw drivers and manual rickshaw pullers were contacted through their union leaders. The leaders motivated their fellow members to participate in this study. traffic police personnel, gardeners and fuel-station attendants were recruited after permission from their supervisors. These workers were requested to come after overnight fasting for investigations at a prefixed date. To facilitate fasting, camps were arranged at their work place and breakfast was served between 8:00 and 9:00 hours. Blood glucose was also measured using point of care glucometer device.

The controls were employees of a national bank located in the premises of the AIIMS and its nearby branches. These employees worked indoors from 9.30 AM to 5.00 PM and were recruited with the outdoor workers during summer months. All the subjects in the outdoor and indoor groups were interviewed individually about the details of their work schedule, clothes worn and pattern of daily sunlight exposure. Body surface area exposed to sunshine was assessed by the rule of nine.[17] The daily dietary intake of calories, carbohydrate, protein, fat, calcium and phytin phosphorus (phytin-P) was assessed by a trained dietician using a semi-quantitative food frequency questionnaire recording information on food groups and 40 food items commonly consumed by Indians.[18,19]

2.1 | Biochemical estimations

Ten ml of blood was drawn for biochemical investigations, and aliquots of the serum were stored at −20°C.[15] Serum total calcium, inorganic phosphorus, alkaline phosphatase and albumin were measured by Cobas-c111 analyzer (Roche; normal, 2.02-2.60 mmol/L, 0.81-1.45 mmol/L and 40-129 IU/L, 35-52 g/L, respectively). All the assays were performed in batches, and their intra- and inter-assay coefficients of variation were 3.5%-5.0%. Serum 25(OH)D and iPTH were measured by chemiluminescence assay using DiaSorin LIAISON® (DiaSorin, Inc). The measuring range for serum 25(OH)D was 10.0-374.4 nmol/L, with inter- and intra-assay CV of 4.8%-7.7% and 4.2%-7.7%, and 100% cross-reactivity with 25(OH)D_2 and 25(OH)D_3. Serum 25(OH)D and iPTH assays were performed in the Department of Cardiac Biochemistry. The laboratory had participating in the Randox International quality Assessment Scheme (RIQAS, https://www.randox.com/riqas-external-quality-assessment), since 2014 for external quality assessment.[20,21] The laboratory had acceptable performance in the RIQAS with 'Standard Deviation Index' of <0.60 (limit <2.0) during the study tenure. The measuring range for serum iPTH was 3.0-1900 ng/L with normal median (2.5th to 97.5th) of 34.0 (14.5-87.1) ng/L. Vitamin D status was defined as per the report of 'Institute of Medicine committee' with serum 25(OH)D ≥ 50 nmol/L considered as sufficient, <50 nmol/L as insufficient, and values <30 nmol/L as vitamin D deficient.[14]

TABLE 1 Study characteristics of outdoor and indoor workers

Characteristics	Outdoor (n = 573)	Indoor (n = 72)	*P*-value
Age (y)	39.4 ± 12.9	42.9 ± 12.9	0.03
BMI (kg/m^2)	23.5 ± 3.9	24.7 ± 3.8	0.01
Serum total Ca (mmol/L)	2.36 ± 0.12	2.40 ± 0.10	<0.001
Albumin-adjusted total Ca (mmol/L)	2.30 ± 0.10	2.35 ± 0.08	0.003
Serum inorganic phosphorus (mmol/L)	1.08 ± 0.18	1.07 ± 0.17	0.71
Serum albumin (g/L)	43.0 ± 3.7	43.2 ± 2.3	0.6
Serum alkaline phosphatase (IU/L)	88 ± 28	86 ± 27	0.54
Serum 25(OH)D (nmol/L)	44.8 ± 19.6	30.6 ± 23.2	<0.001
≥30.0 nmol/L, n (%)	444 (77.5)	26 (36.1)	<0.001
≥50.0 nmol/L, n (%)	192 (33.5)	13 (18.1)	0.008
Serum iPTH (ng/L)	55.7 ± 30.2	63.6 ± 33.2	0.03
≤87.1 ng/L, n (%)	493 (86.0)	57 (79.2)	
>87.1 ng/L, n (%)	80 (14.0)	15 (20.8)	0.16
Body surface area exposed (%)	12.9 ± 7.2	9.7 ± 3.7	<0.001
Dietary calories (kcal/d)	2085 ± 524	1850 ± 424	<0.001
Dietary calcium (mg/d)	818 ± 513	685 ± 423	0.03
Dietary phosphorus (mg/d)	1572 ± 509	1369 ± 421	0.001
Dietary phytin phosphorus (mg/d)	149 ± 99	136 ± 68	0.29
Dietary carbohydrate (g/d)	310 ± 82	276 ± 62	<0.001
Dietary protein (g/d)	70 ± 25	60 ± 20	<0.001

The study was carried out in accordance with the tenets of the Declaration of Helsinki. The Ethics Committee of AIIMS, Delhi, approved the protocol. Written informed consent was obtained from all the subjects.

2.2 | Sample size and statistical analysis

Sample size was based on the vitamin D status of male construction workers in the previous study.[16] With 14.8% prevalence of serum 25(OH)D < 50.0 nmol/L in construction workers and assuming a similar vitamin D status among the outdoors of the present study, a total of 539 subjects were required to estimate prevalence of VDD at 3% absolute error margin in a two-sided 95% confidence interval.

3 | RESULTS

A total of 605 outdoor workers were contacted (hawkers/pavement vendors, n = 152; auto-rickshaw drivers, n = 113; manual rickshaw pullers, n = 49; fuel-station attendants, n = 104; gardeners, n = 98; traffic police personnel, n = 89). In view of the limited number of females among outdoor workers, results are reported only for males (n = 573). Accordingly, only male indoor subjects were recruited as comparative controls (n = 73). The body surface area exposed was higher in the outdoor than in the indoor group ($P < 0.001$, Table 1).

3.1 | The vitamin D status of outdoor subjects and their comparison with indoor groups

The clinical and biochemical features of the outdoor and indoor workers are listed in Table 1. The mean age and BMI of all the 573 outdoor subjects were 39.4 ± 12.9 years and 23.5 ± 3.9 kg/m^2, respectively. Their daily dietary intake of calorie, protein, carbohydrate and calcium was 2085 ± 524 kcal, 70 ± 25 g, 310 ± 82 g and 818 ± 513 mg, respectively (Table 1). The mean serum 25(OH)D of the outdoors as a group was 44.8 ± 19.6 nmol/L and showed a physiological inverse relation with iPTH ($r = -0.21$, $P < 0.001$). 77.5% of the outdoors did not have VDD.

In comparison with outdoor workers, indoors had significantly lower mean serum 25(OH)D levels with 63.9% having serum 25(OH)D < 30.0 nmol/L ($P < 0.001$). Only 18.1% of the indoors had serum 25(OH)D ≥ 50.0 nmol/L ($P = 0.008$). The mean serum 25(OH)D was lower among indoors by 12.7 nmol/L than the outdoors ($P < 0.001$). The mean age and BMI of outdoors were significantly less than that of the indoors. In view of significant differences in the mean age and BMI between the outdoor and indoor groups, a regression analysis was carried out to assess the significance of difference in the mean serum 25(OH)D level between the two groups. The 25(OH)D levels were significantly higher among the outdoors even after adjustment of age and BMI differences ($P < 0.001$). The mean serum iPTH was higher in indoors than the outdoors and also showed an inverse relationship with serum 25(OH)D ($r = -0.28$, $P = 0.02$).

TABLE 2 Baseline characteristics of six different groups of outdoor workers

Parameter	Hawkers/pavement vendors (n = 144)	Fuel-station attendants (n = 84)	auto-rickshaw drivers (n = 113)	Gardeners (n = 96)	traffic police personnel (n = 87)	Rickshaw pullers (n = 49)	Indoors (n = 72)	*P* value
Age (y)	33.7 ± 12.7[b,c,d,f]	34.0 ± 10.6[g,h,i]	44.4 ± 10.8[n]	46.9 ± 12.1[q]	42.8 ± 12.2[s]	34.3 ± 9.9[u]	42.9 ± 12.9	<0.05
BMI (kg/m^2)	23.2 ± 4.0[b,d,e]	22.4 ± 3.4[g,l,k]	25.3 ± 4.1[l,n]	22.7 ± 3.8[p]	25.1 ± 3.1[s]	21.1 ± 2.7[u]	24.7 ± 3.8	<0.01
Serum total Ca (mmol/L)	2.36 ± 0.11[d]	2.33 ± 0.11[g,i,k]	2.36 ± 0.11[m]	2.33 ± 0.12[p,r]	2.43 ± 0.12[s]	2.33 ± 0.09	2.41 ± 0.10	<0.05
Serum albumin-adjusted Ca (mmol/L)	2.30 ± 0.13[b,d,f]	2.30 ± 0.10[g,i,k]	2.25 ± 0.08[l,m,n]	2.33 ± 0.08[p]	2.38 ± 0.10[s]	2.30 ± 0.08	2.35 ± 0.08	<0.05
Serum phosphate (mmol/L)	1.09 ± 0.18	1.13 ± 0.16	1.06 ± 0.17	1.09 ± 0.17	1.05 ± 0.21	1.06 ± 0.18	1.07 ± 0.17	<0.05
Serum albumin (g/L)	43.5 ± 4.3[a,b,c,d]	41.7 ± 2.4[g,i,k]	45.9 ± 3.3[m]	40.9 ± 2.9[q,r]	42.9 ± 2.7	41.3 ± 1.9	43.2 ± 2.3	<0.05
Serum ALP (IU/L)	87 ± 25	82 ± 23[g]	98 ± 29[m,n,o]	88 ± 31	84 ± 28	80 ± 27	86 ± 27	<0.05
Serum 25(OH)D nmol/L	49.7 ± 20.1[b,d,f]	45.3 ± 18.1[g,k]	36.4 ± 17.9[l]	51.4 ± 23.6[p,r]	39.4 ± 15.2	45.5 ± 13.3[u]	30.6 ± 23.2	<0.05
Median (IQR)	47.2(34.9-61.3)	42.7(30.6-57.4)	34.4(25.0-44.9)	46.2(35.4-63.4)	38.4(30.5-46.9)	43.7(37.7-54.2)	24.2(13.3-40.9)	
≥30.0 nmol/L, n (%)	124 (86.1)	65 (77.4)	69 (61.1)	78 (81.3)	66 (75.9)	42 (85.7)	26 (36.1)	<0.001
≥50.0 nmol/L, n (%)	67 (46.5)	31 (36.9)	18 (15.9)	45 (46.9)	15 (17.2)	16 (32.7)	13 (18.1)	<0.001
Serum iPTH (ng/L)	60.3 ± 33.0[d]	62.7 ± 29.6[i,j]	63.6 ± 34.5[m,n]	53.5 ± 25.7[p]	39.2 ± 19.5[t]	45.7 ± 21.3[u]	63.6 ± 33.2	<0.01
≤87.1 ng/L, n (%)	115 (79.9)	69 (82.1)	92 (81.4)	85 (88.5)	85 (97.7)	47 (95.9)	57 (79.2)	
>87.1 ng/L, n (%)	29 (20.1)	15 (17.9)	21 (18.6)	11 (11.5)	2 (2.3)	2 (4.1)	15 (20.8)	
Body surface exposed (%)	15.6 ± 7.8[a,b,c,d]	12.7 ± 3.8[h,i,j]	10.5 ± 3.7[k]	9.1 ± 3.6[q]	9.2 ± 3.6[s]	23.4 ± 10.8[e,u]	9.7 ± 3.7[f]	<0.05
Dietary Calories (kcal/d)	2016 ± 505[a,c]	2242 ± 598[g,k]	1913 ± 409[l]	2256 ± 612[r]	2088 ± 473[t]	2047 ± 401[u]	1850 ± 424	<0.05
Dietary Ca (mg/d)	665 ± 448[c,d,e]	771 ± 349[h,i,j]	762 ± 428[l,m,n]	1055 ± 570[p,q,r]	1124 ± 588[s,t]	468 ± 3778[u]	685 ± 423	<0.01
Dietary Phosphorus (mg/d)	1462 ± 447[a,c,d]	1639 ± 490[g,j,k]	1438 ± 369[l,m]	1802 ± 584[q,r]	1782 ± 543[s,t]	1245 ± 406	1369 ± 421	<0.05

Note: Inter-group differences are indicated by superscripts: a: hawkers vs fuel-station attendants; b: hawkers vs auto-rickshaw drivers, c: hawkers vs gardeners, d: hawkers vs traffic police personnel; e: hawkers vs rickshaw pullers; f: hawkers vs indoors; g: fuel-station attendants vs auto-rickshaw drivers; h: fuel-station attendants vs gardeners; i: fuel-station attendants vs traffic police personnel; j: fuel-station attendants vs rickshaw pullers; k: fuel-station attendants vs indoors; i: auto-rickshaw drivers vs gardeners; m: auto-rickshaw drivers vs traffic police personnel; n: auto-rickshaw drivers vs rickshaw pullers, o: auto-rickshaw drivers vs indoors; p: gardeners vs traffic police personnel; q: gardeners vs rickshaw pullers; r: gardeners vs indoors; s: traffic police personnel vs rickshaw pullers; t: traffic police personnel vs indoors; u: rickshaw pullers vs indoors.

The mean dietary calcium intake was significantly lower among indoors than the outdoors ($P = 0.03$). Although the mean serum inorganic phosphorus and alkaline phosphatase values were comparable between the outdoor and indoor groups, the mean serum total calcium was higher in indoors (Table 1).

3.2 | Vitamin D status of outdoors according to their different types of jobs

Table 2 lists the demographic characteristics, vitamin D status and related parameters in six types of outdoor jobs. The mean serum 25(OH)D was ≥30.0 nmol/L in all the six types of outdoor jobs with the highest levels in the gardeners (51.4 ± 23.6 nmol/L). The mean serum 25(OH)D was near sufficient range in the hawkers/pavement vendors (49.7 ± 20.1 nmol/L), rickshaw pullers (45.5 ± 13.3 nmol/L) and fuel-station attendants (45.3 ± 18.1 nmol/L). In contrast, the mean 25(OH)D level in traffic police personnel and auto-rickshaw drivers were comparable to that of the indoors. The mean serum iPTH was normal in all the six outdoor groups. Interestingly, auto-rickshaw drivers with the lowest mean 25(OH)D values had the highest mean serum iPTH levels (Table 2). Despite having serum 25(OH)D comparable to that of auto-rickshaw drivers, traffic police personnel had lowest mean PTH levels, highest mean serum total calcium levels and dietary calcium intake among all study groups (Table 2).

3.3 | Seasonal variation

A total of 192 outdoor subjects had serum 25(OH)D values ≥50.0 nmol/L during summer (Table 3). Eighty-four of them could be contacted during winters (hawkers/pavement workers, n = 21; gardeners, n = 29; petrol pump attendants, n = 23; auto-rickshaw drivers, n = 4; and traffic police personnel, n = 7). The mean serum 25(OH)D levels in these outdoors decreased significantly (70.2 ± 18.5 to 31.2 ± 14.3 nmol/L, $P < 0.001$). However, mean serum iPTH levels did not change significantly (49.3 ± 21.7 to 52.3 ± 28.6 ng/L, $P = 0.68$).

TABLE 3 Seasonal variation in 84 outdoor workers

Parameter	Summer	Winter	*P* value
Serum total Ca (mmol/L)	2.34 ± 0.11	2.31 ± 0.07	0.03
Serum albumin-adjusted Ca (mmol/L)	2.30 ± 0.10	2.30 ± 0.05	0.15
Serum inorganic PO4 (mmol/L)	1.11 ± 0.18	1.08 ± 0.17	0.31
Serum albumin (g/L)	41.8 ± 3.4	41.2 ± 2.1	0.08
Serum alkaline phosphatase (IU/L)	79 ± 24	88 ± 26	<0.001
Serum 25(OH)D (nmol/L)	70.2 ± 18.5	31.2 ± 14.3	<0.001
Serum iPTH (ng/L)	49.3 ± 21.7	52.3 ± 28.6	0.68

Though mean serum total calcium showed a fall of 0.03 mmol/L ($P = 0.03$) in winter, the albumin-adjusted serum total calcium did not change significantly in winter (2.30 ± 0.10 to 2.30 ± 0.05 mmol/L, $P = 0.15$).

4 | DISCUSSION

The ability to achieve good vitamin D status under prolonged hours of direct sunshine exposure has been shown by several investigators. The examples include farmers of Nebraska, coastal guards, field workers in Florida, and countries with plenty sunshine as Hawaii, Israel and Australia, where serum 25(OH)D ranged from 75 to 160 nmol/L.[22-27] The vitamin D status of traditional hunters in Tanzanian, Caribbeans and Indian construction workers too ranged from 75 to 115 nmol/L.[16,28,29]

The current study showed that most outdoor workers under indirect and less harsh sun exposure with freedom to seek shade were also free from VDD. The absence of VDD was observed in all the six groups of workers, irrespective of type of their outdoor jobs. In fact, the mean 25(OH)D levels were sufficient or near sufficient in gardeners, pavement vendors/hawkers, rickshaw pullers and fuel-station attendants. The mean serum 25(OH)D of auto-rickshaw drivers and traffic police personnel was also above the cut-off of VDD, but could not reach near sufficiency and can be explained by their pattern of sun exposure. Although auto-rickshaw drivers were plying for several hours, they were mostly seated under roof of their vehicle. Similarly, policemen stood under shade, wore cap and were intermittently out for tackling traffic rules offenders. These observations highlight that though indirect sun exposure can prevent VDD, for vitamin D sufficiency outdoors workers need to have direct sun exposure. The observation of near normal serum 25(OH)D of 50.0 nmol/L is common in southern India that receives more sunlight than north India.[30-32] Present study indicates that it is possible to have normal serum 25(OH)D naturally in outdoor workers of northern Indian region. When the opportunity to expose under the sun was missed as in the auto-rickshaw drivers and traffic police personnel, the serum 25(OH)D could not reach near sufficiency, that is 50 nmol/L, though levels were above the cut-off of VDD.

Not unexpectedly, the mean serum 25(OH)D of indoors was lower by 12.7 nmol/L than the outdoors. However, mean 25(OH)D values were still above 30.0 nmol/L, indicating absence of VDD in noteworthy fraction in summer. The higher mean serum 25(OH)D levels among indoors in the present study in comparison with values of 25.0 nmol/L in the previous studies[13,20] could be explained by several ways. The realization of the importance of sunshine exposure among urban indoors in improving vitamin D status during the last two decades and/or inadvertent use of vitamin D supplements among indoors might explain their improved vitamin D status. Similar improvement in vitamin D status was observed recently by Sowah et al[33] among healthcare professionals.

Present study observed a winter dip in the vitamin D status among outdoor subjects who were vitamin D sufficient during

summer. Multiple factors like lack of available sun exposure, covered body surface area and increasing smog over Delhi due to construction or burning of agricultural crop stubbles in the states near Delhi could be the reasons for decline in serum 25(OH)D levels in winter. Interestingly, its functional effect on serum total calcium was negligible. Though mean serum total calcium showed a fall of 0.03 mmol/L (P = 0.03) during winter, the albumin-adjusted serum total calcium showed no significant change with season (2.30 ± 0.10 to 2.30 ± 0.05 mmol/L, P = 0.15). The mean serum 25(OH)D of 31.2 ± 14.3 nmol/l in winter indicated that several outdoor workers had serum 25(OH)D values below <30 nmol/L. However, despite this low value, they had normal mean serum iPTH and were clinically well and performing physically demanding jobs that required a good skeletal health. This brings the focus on the level of serum 25(OH)D actually needed for good skeletal health and also questions the relevance of term 'vitamin D insufficiency'. Subjects can be simply categorized as 'Vitamin D deficient' or 'non-deficient' using 'IOM' cut-off of 30 nmol/L, doing away with the category 'vitamin D insufficiency'.

Meta-analysis of studies assessing effect of vitamin D supplementation in apparently healthy subjects has not shown any remarkable effect on various functional outcomes.[34,35] In fact, two randomized control trials from our centre showed no significant improvement in muscle strength despite significantly improved vitamin D status after six months of cholecalciferol supplementation.[13,20] In this context, the present study does not show any functional impact of low serum 25(OH)D among indoors other than borderline raised serum PTH. There is possibility that a higher serum PTH among indoors, albeit within the range of normality, might be a fruitful bioadaptive response leading to increased calcium reabsorption in the renal tubules. The higher serum PTH among indoors could theoretically explain the paradox of higher mean serum calcium among indoors despite lower serum 25(OH)D in comparison with outdoors as observed in the present study and also in the previous study on outdoor construction workers in Delhi.[16]

The practice of advising vitamin D supplementation to the urban indoors in presence of the sufficient dietary calcium intake is questionable. The basis of this question and its answer is best exemplified with serum PTH value of traffic policemen group who also had sufficient dietary calcium intake. This group had lowest serum PTH values despite having lowest mean serum 25(OH)D among all outdoor groups.

Currently, vitamin D-fortified milk is being marketed (~250 IU/pack of 500 mL) in Delhi. However, whether this fortification is needed for indoors with sufficient calcium intake or for healthy outdoors needs to be debated in light of the current study. Unnecessary vitamin D supplementation could be associated with risks of hypercalcemia and hypercalciuria and so should be avoided. It would be preferable to depend on sun exposure to achieve vitamin D sufficiency particularly among the subjects with outdoor jobs.

To conclude, the present study indicates that vitamin D status of the majority of the urban outdoors and sizable number of the indoor population was not deficient as per the cut-off suggested by IOM. With complete freedom to move in and out of the shade as per the demand of the work and comfort of the body, the common outdoor workers showed sufficient or near sufficient serum 25(OH)D levels. Thus, contrary to the popular perception, VDD is not universal in urban population and majority of outdoors achieved vitamin D sufficiency by just following their day-to-day schedule. It is important to adopt a conservative view on fortification of food with vitamin D in countries with ample of sunshine due to the possibility of hypercalcemia and renal stone.

ACKNOWLEDGEMENT

The help of Mr. Arun Chandran and Ms. Sunita Sreenivasan in measuring all the biochemical parameters is acknowledged. The help of Ms. Sanghmitra Shakya, research dietician in the study is acknowledged. The help of Mr. Ram Sumer in enrolling subjects is also acknowledged. The study was carried out with the financial support from intramural research grant of All India Institute of Medical Sciences (No-F.8-496/A-496/2016/RS)

CONFLICT OF INTEREST

The authors have nothing to disclose, and there is no conflict of interest.

AUTHORS CONTRIBUTION

Prof Ravinder Goswami designed and guided the study. Dr Soma Saha, Dr Pramila Dharmshaktu*, Parmita Kar and Dr Ravinder Goswami collected the data. Dr Vishnubhatla Sreenivas performed the statistical analysis. Dr Lakshmy Ramakrishnan supervised the vitamin D and PTH assays. All the authors have contributed in writing of the manuscript.

DATA ACCESSIBILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Ravinder Goswami https://orcid.org/0000-0002-3097-5074

REFERENCES

1. Goswami R, Gupta N, Goswami D, Marwaha R, Tandon N, Kochupillai N. Prevalence and significance of low 25-hydroxyvitamin D concentrations in healthy subjects in Delhi. *Am J Clin Nutr*. 2000;72:472-475.
2. Prentice A, Schoenmakers I, Jones KS, Jarjou L, Goldberg GR. Vitamin D deficiency and its health consequences in Africa. *Clin Rev Bone Miner Metab*. 2009;7:94-106.
3. Baroncelli GI, Bereket A, El Kholy M, et al. Rickets in the Middle East: role of environment and genetic predisposition. *J Clin Endocrinol Metab*. 2008;93:1743-1750.

4. Marwaha RK, Tandon N, Reddy D, et al. Vitamin D and bone mineral density status of healthy schoolchildren in northern India. *Am J Clin Nutr*. 2005;82:477-482.
5. Sachan A, Gupta R, Das V, Agarwal A, Awasthi PK, Bhatia V. High prevalence of vitamin D deficiency among pregnant women and their newborns in northern India. *Am J Clin Nutr*. 2005;81:1060-1064.
6. Goswami R, Marwaha RK, Gupta N, et al. Prevalence of vitamin D deficiency and its relationship with thyroid autoimmunity in Asian Indians: a community-based survey. *Br J Nutr*. 2009;102:382-386.
7. Harinarayan CV, Ramalakshmi T, Venkataprasad U. High prevalence of low dietary calcium and low vitamin D status in healthy south Indians. *Asia Pac J Clin Nutr*. 2004;13:359-364.
8. Guzel R, Kozanoglu E, Guler-Uysal F. Vitamin D status and bone mineral density of veiled and unveiled Turkish women. *J Womens Health Gend Based Med*. 2001;10:765-770.
9. Holick MF. The vitamin D deficiency pandemic: approaches for diagnosis, treatment and prevention. *Rev Endocr Metab Disord*. 2017;18:153-165.
10. Manson JE, Brannon PM, Rosen CJ, Taylor CL. Vitamin D deficiency - is there really a pandemic? *N Engl J Med*. 2016;375:1817-1820.
11. Zargar AH, Ahmad S, Masoodi SR, et al. Vitamin D status in apparently healthy adults in Kashmir Valley of Indian subcontinent. *Postgrad Med J*. 2007;83:713-716.
12. Puri S, Marwaha RK, Agarwal N, et al. Vitamin D status of apparently healthy schoolgirls from two different socioeconomic strata in Delhi: relation to nutrition and lifestyle. *Br J Nutr*. 2008;99:876-882.
13. Goswami R, Vatsa M, Sreenivas V, et al. Skeletal muscle strength in young Asian Indian females after vitamin D and calcium supplementation: a double-blind randomized controlled clinical trial. *J Clin Endocrinol Metab*. 2012;97:4709-4716.
14. IOM (Institute of Medicine). *Dietary Reference Intakes for Calcium and Vitamin D*. Washington, DC: The National Academies Press; 2011.
15. Holick MF, Binkley NC, Bischoff-Ferrari HA, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an endocrine society clinical practice guideline. *J Clin Endocrinol Metab*. 2011;96:1911-1930.
16. Goswami R, Saha S, Sreenivas V, Singh N, Lakshmy R. Vitamin D–binding protein, vitamin D status and serum bioavailable 25(OH)D of young Asian Indian males working in outdoor and indoor environments. *J Bone Miner Metab*. 2017;35:177-184.
17. Mcgrouther DA, Mann CV, Russel R, Williams NS. *Bailey and Love's Short Practice of Surgery*. London, UK: Chapman and Hall; 1995:124-148.
18. Willet WC, Sampson L, Stampfer MJ, Hennekens CH, Speizer FE. Reproducibility and validity of a food frequency questionnaire. *Am J Epidemiol*. 1985;122:51-65.
19. Gopalan C, Sastri B, Balasubramanian SC. *Food Composition Tables: Nutritive Value of Indian Foods*. Hyderabad, India: National Institute of Nutrition, Indian Council of Medical Research; 1996:45-95.
20. Saha S, Goswami R, Ramakrishnan L, et al. Vitamin D and calcium supplementation, skeletal muscle strength and serum testosterone in young healthy adult males: randomized control trial. *Clin Endocrinol (Oxf)*. 2018;88:217-226.
21. Jolliffe DA, Greenberg L, Hooper RL, et al. Vitamin D supplementation to prevent asthma exacerbations: a systematic review and meta-analysis of individual participant data. *Lancet Respir Med*. 2017;5:881-890.
22. Barger-Lux MJ, Heaney RP. Effects of above average summer sun exposure on serum 25-hydroxyvitamin D and calcium absorption. *J Clin Endocrinol Metab*. 2002;87:4952-4956.
23. Haddad JG, Chyu KJ. Competitive protein-binding radioassay for 25 hydroxycholecalciferol. *J Clin Endocrinol Metab*. 1971;33:992-995.
24. Levis S, Gomez A, Jimenez C, et al. Vitamin D deficiency and seasonal variation in an adult South Florida population. *J Clin Endocrinol Metab*. 2005;90:1557-1562.
25. Binkley N, Novotny R, Krueger D, et al. Low vitamin D status despite abundant sun exposure. *J Clin Endocrinol Metab*. 2007;92:2130-2135.
26. Azizi E, Pavlotsky F, Kudish A, et al. Serum levels of 25-hydroxy-vitamin D3 among sun-protected outdoor workers in Israel. *Photochem Photobiol*. 2012;88:1507-1512.
27. Nair-Shalliker V, Clements M, Fenech M, Armstrong BK. Personal sun exposure and serum 25-hydroxy Vitamin D concentrations. *Photochem Photobiol*. 2013;89:208-214.
28. Luxwolda MF, Kuipers RS, Kema IP, Janneke Dijck-Brouwer DA, Muskiet FA. Traditionally living populations in East Africa have a mean serum 25-hydroxyvitamin D concentration of 115 nmol/L. *Br J Nutr*. 2012;108:1557-1561.
29. Miljkovic I, Bodnar LM, Cauley JA, et al. Low prevalence of vitamin D deficiency in elderly Afro-Caribbean men. *Ethn Dis*. 2011;21:79-84.
30. Rajasree S, Raman Kutty V, Sreenivasan K, Kartha CC. Elevated serum levels of 25-hydroxyvitamin D_3 in outdoor workers of South India. *Curr Sci*. 1999;77:1136-1138.
31. Harinarayan CV, Ramalakshmi T, Prasad UV, et al. High prevalence of low dietary calcium, high phytate consumption, and vitamin D deficiency in healthy south Indians. *Am J Clin Nutr*. 2007;85:1062-1067.
32. Shetty S, Kapoor N, Naik D, et al. Osteoporosis in healthy South Indian males and the influence of life style factors and vitamin d status on bone mineral density. *J Osteoporos*. 2014;2014:723238.
33. Sowah D, Fan X, Dennett L, Hagtvedt R, Straube S. Vitamin D levels and deficiency with different occupations: a systematic review. *BMC Public Health*. 2017;17(1):519.
34. Theodoratou E, Tzoulaki I, Zgaga L, Ioannidis JP. Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials. *BMJ*. 2014;348:g2035.
35. Zhao JG, Zeng XT, Wang J, Liu L. Association between calcium or vitamin D supplementation and fracture incidence in community-dwelling older adults: systematic review and meta-analysis. *JAMA*. 2017;318:2466-2482.

How to cite this article: Dharmshaktu P, Saha S, Kar P, Sreenivas V, Ramakrishnan L, Goswami R. Absence of vitamin D deficiency among common outdoor workers in Delhi. *Clin Endocrinol (Oxf)*. 2019;91:356–362. https://doi.org/10.1111/cen.14012

This study audited the therapy for the management of hypoparathyroidism and shown that complete efficacy of alfacalcidol in all the Indian patients with normal calcemic control. Further, through a well conducted RCT, there was equal efficacy of both alfacalcidol and calcitriol in the management of hypoparathyroidism (**J Clin Endocrinol Metab, 2021**). This is helping physicians in prescribing oral calcium and vitamin D therapy for the management of hypoparathyroidism with confidence rather than tilt for subcutaneous PTH therapy as a routine

Auditing the Efficacy and Safety of Alfacalcidol and Calcium Therapy in Idiopathic Hypoparathyroidism

Publication 2 (a)

Soma Saha[1] and Ravinder Goswami[1]

[1]Department of Endocrinology and Metabolism, All India Institute of Medical Sciences, New Delhi 110029, India

ORCiD numbers: 0000-0002-3097-5074 (R. Goswami).

Context: Patients with hypoparathyroidism are treated with vitamin D and calcium. PTH is an emerging option because of its physiological action. It is important to assess the efficacy and shortcomings of conventional therapy.

Objective: We assessed the efficacy and safety of alfacalcidol in a large cohort of patients with idiopathic hypoparathyroidism (IH) and identified a subset who could be treated without oral calcium.

Design and Setting: Observational study at tertiary care center.

Subjects and Methods: We assessed 92 patients with IH who were receiving alfacalcidol and oral calcium to maintain an optimal serum total calcium level of 8.0 to 8.5 mg/dL during routine follow-up. Patients with suboptimal control were provided free medicines and followed up frequently. Oral calcium and alfacalcidol doses were titrated sequentially to determine the minimum doses for optimal calcium control. Serum phosphate level, 1,25-dihydroxyvitamin D, fractional excretion of phosphorus (FEPh), and hypercalciuria (urine calcium-to-creatinine ratio, >0.2) were assessed at each step of titration.

Results: Only 38% of patients had optimal calcium control during routine follow-up. With good compliance, all achieved optimal serum calcium and 1,25-dihydroxyvitamin D levels and 43% of patients could stop taking oral calcium. Hyperphosphatemia, hypercalciuria, and low FEPh persisted at all stages of therapy. Serum phosphorus levels normalized when the serum calcium level increased to 9.9 mg/dL, but this level of serum total calcium was associated with hypercalciuria in 90% of patients.

Conclusion: Alfacalcidol is effective in achieving calcemic control in IH. Calcemic control without oral calcium was achieved in 43% of patients receiving alfacalcidol. However, optimal calcium control was associated with hyperphosphatemia and hypercalciuria in most patients. **(*J Clin Endocrinol Metab* 104: 1325–1335, 2019)**

Hypoparathyroidism is a rare disorder with a prevalence of 22 to 37 cases per /100,000 persons (1). Idiopathic hypoparathyroidism (IH) is even rarer. Patients with IH are treated with alfacalcidol (1-α-hydroxycholecalciferol) or calcitriol (1,25-dihydroxycholecalciferol) and oral calcium (2). Recently, various aspects of the conventional therapy in chronic hypoparathyroidism were reviewed in two articles (3, 4), but there is lack of evidence-based information due to rarity of the disease. Areas requiring systematic studies include rationale for maintaining serum total calcium in the low-normal range of 8.0 to 8.5 mg/dL, prevalence of hypercalciuria, efficacy of alfacalcidol therapy in maintaining

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received 15 October 2018. Accepted 28 December 2018.
First Published Online 3 January 2019

Abbreviations: FEPh, fractional excretion of phosphorus; IH, idiopathic hypoparathyroidism.

normal calcium and serum phosphorus levels, and need for oral calcium in all patients with IH.

We are following a large cohort of patients with IH that includes patients enrolled since 1998 (5–15). These patients have been treated with alfacalcidol because of its cost-effectiveness and because PTH deficiency impairs renal 1-α-hydroxylation of vitamin D. The daily dose of alfacalcidol prescribed to the patients varies from 0.25 μg to 3.0 μg. Some patients do not maintain optimal calcemic control. In our earlier study (16), we did not observe increased prevalence of celiac-related autoantibodies (*i.e.*, antitissue transglutaminase) or celiac disease in IH. In addition, patients with IH and coexistent celiac disease maintained normal serum calcium levels while on a gluten-free diet and alfacalcidol therapy given in the usual dosages (16). Thus, the cost of therapy could be a reason for poor compliance and suboptimal calcium control in resource-constrained patients.

The rationale and role of prescribing oral calcium along with alfacalcidol to patients with IH are also not clear if their dietary calcium intake level is normal. There are reports suggesting some patients with IH could be treated without calcium supplements (17–19). It is possible that a subset of patients requires concomitant oral calcium because of inadequate serum 1,25-dihydroxyvitamin D levels with alfacalcidol therapy.

To our knowledge, no systematic study has assessed the efficacy of alfacalcidol and oral calcium in achieving calcium control and the optimum dose requirement in IH. Here, we present an audit of the efficacy and safety of alfacalcidol therapy in our cohort of patients with IH. The primary objective of the study was to assess whether optimal calcium control could be achieved with alfacalcidol therapy.

Subjects and Methods

This study included all patients with IH attending endocrine clinics of All India Institute of Medical Sciences during 2017 and 2018. The diagnosis of hypoparathyroidism was based on clinical features of tetany, cataract, intracranial calcification, hypocalcemia, hyperphosphatemia, normal serum creatinine level, and low or inappropriately normal serum PTH levels (2). All 92 patients included in this study had adult-onset hypoparathyroidism with no known previous family history of hypoparathyroidism.

All patients were assessed for the glial-cell-missing 2 (*GCM2*) gene R110W mutation, the most common genetic mutation in our cohort with IH (20). Only 8.7% of patients had R110W mutation. In a previous study (21), none of these patients had mutation of the calcium-sensing receptor (*CASR*) and *PTH* genes (n = 57 tested). Autoantibodies against CaSR (n = 57 tested) and anti–interferon-α antibodies (n = 74 tested) were present in 10.5% and 1.4% of the cases, respectively (16, 21). Antitissue transglutaminase (n = 73 tested) and thyroid peroxidase antibodies (n = 92 tested) were present in 11.0% and 16.3% of cases, respectively.

Clinically overt autoimmune disorders in IH were primary hypothyroidism (n = 7) and duodenal biopsy–proven celiac disease (n = 3). Four patients had alopecia areata, alopecia totalis, vitiligo, and psoriasis (n = 1 each). Nonautoimmune comorbidities were mental retardation (n = 4), mild hearing loss (n = 2), and type 2 diabetes mellitus, bronchial asthma, coronary artery disease, fibrous dysplasia, history of surgery for acoustic neuroma, acyanotic tetralogy of Fallot, and Takayasu arteritis (n = 1 each). None of the patients in the current study had autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome or postsurgical hypoparathyroidism. Patients with pregnancy or lactation during the previous year, and new patients with <1 year of follow-up were excluded.

At initial presentation, patients were prescribed a daily dose of 0.5 to 1.0 μg of alfacalcidol and 1.0 to 2.0 g of elemental calcium as carbonate salt (each tablet contained 500 mg of elemental calcium and 250 IU of cholecalciferol). The aim was to maintain the serum total calcium concentration between 8.0 and 8.5 mg/dL. The maximum dosage of alfacalcidol prescribed was 3.0 μg/d. No patient received phosphate binder or magnesium supplements. Patients were recommended to take calcium tablets in a divided dose with morning and evening meals. Patients were followed up regularly for monitoring of serum total calcium, phosphorus, and 24-hour urinary calcium and creatinine levels, and fractional excretion of phosphate (FEPh).

The protocol followed to assess the adequacy of serum calcium control is shown in Fig. 1. Briefly, all patients were assessed with a predesigned form to document the duration of follow-up, number of visits since initial presentation in the clinics, education status, income, and compliance with medications. Patients' daily dietary intake of calories, carbohydrate, protein, fat, calcium, and phosphorus was assessed by a trained dietitian using a semiquantitative food frequency questionnaire and information on nutrient content of seven food groups and 40 common Indian food items (22, 23). Patients were advised to follow their usual diet practice in the family.

Efficacy of alfacalcidol in maintaining optimal calcemic control in IH

Patients were categorized as having optimal or suboptimal calcium control on the basis of three serum total calcium values, each measured at intervals of >1 month during their last year of follow-up. Those with at least two serum total calcium values >8.0 mg/dL along with a mean of three measurements of serum total calcium of ≥8.0 mg/dL were considered to have optimal control. Those with serum total calcium concentration ≥10.6 mg/dL were considered hypercalcemic (24, 25). Patients with serum total calcium concentration <8.0 mg/dL on two or more occasions were categorized as having suboptimal control and were assessed further.

Many of the patients in our cohort came from a poor socioeconomic background. Therefore, to minimize noncompliance because of the expense of therapy, patients with suboptimally controlled calcium level were given medicines from the clinic at their follow-up visits every two weeks. The same commercial preparation of alfacalcidol (Panacea Biotech, India) was used throughout the study. Those taking thiazide diuretics were advised to stop that medication at least 1 week before they were enrolled for the prospective analysis. If the level of serum calcium after provision of medicines was >7.0 mg/dL, the ongoing dose of alfacalcidol was continued for another 2 weeks. However, if the level of serum calcium was ≤7.0 mg/dL, the daily dose of alfacalcidol was increased by 0.25 μg. Subsequent 0.25-μg increments were prescribed at 2-week intervals until a serum total

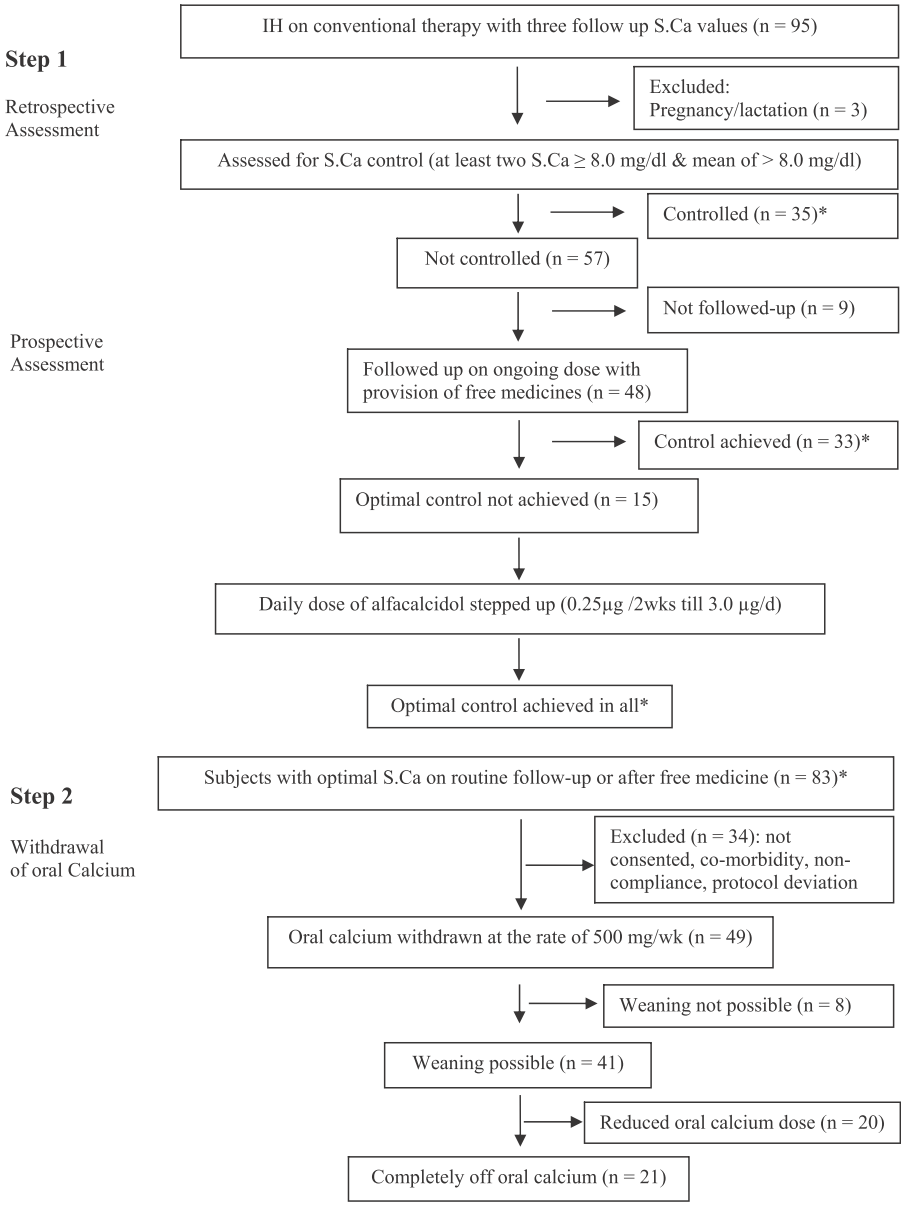

Figure 1. Flow of the study. *Patients eligible for withdrawal of oral calcium and taken for step 2. S.Ca, serum calcium.

calcium concentration 8.0 to 8.5 mg/dL was achieved or the dosage of 3.0 μg/d was reached. Patients who did not attain optimal serum calcium level despite the 3.0-μg dose of alfacalcidol were admitted to the hospital for physician-supervised therapy. The alfacalcidol dose at which patients achieved serum total calcium concentration of ≥8.0 mg/dL was noted. Serum 1,25-dihydroxyvitamin D level was measured before and after optimization to assess the effect of alfacalcidol on calcitriol level (Fig. 1). Patients who did not achieve optimal serum calcium level despite supervised therapy in the hospital were considered resistant to alfacalcidol and planned for calcitriol therapy.

Assessing the need for oral calcium in patients with optimal calcemic control

A stepwise calcium-reduction protocol was used in patients who had optimal control of serum calcium level or achieved it after provision of free medicines. The oral elemental calcium dosage was reduced by 500 mg/d every week. Weaning from oral calcium was continued until the patients' oral calcium supplements were completely withdrawn. If serum total calcium level dropped to <8.0 mg/dL at any step, patients were reverted to their previous dose. The dose at which patients maintained optimal serum calcium level was recorded. After final weaning, patients were followed up at monthly intervals for ≥3 months with continued provision of alfacalcidol therapy from the clinic. Their level of serum 25-hydroxyvitamin D was measured every month and those with values <30.0 ng/mL were recommended to take 60,000 IU of cholecalciferol per month. Patients maintaining a serum total calcium level >8.0 mg/dL for ≥3 months with alfacalcidol therapy alone were considered not to require oral calcium (Fig. 1).

Readjustment of alfacalcidol dose after complete withdrawal of oral calcium

After complete weaning of oral calcium and provision of free alfacalcidol therapy during follow-up, if a patient's serum calcium

level was >9.0 mg/dL, the daily dose of alfacalcidol was reduced by 0.25 μg/d every 2 weeks. Reduction of alfacalcidol was continued until serum calcium concentration was 8.0 to 8.5 mg/dL. Thereafter, patients were followed monthly for ≥3 months to assess the stability of the serum calcium levels.

Biochemical methods and measurements

Serum levels of total calcium, phosphorus, 25-hydroxyvitamin D, and calcitriol, and FEPh and frequency of hypercalciuria were measured at each step of optimization. All the biochemical investigations were carried out after overnight fasting. Serum levels of total calcium, inorganic phosphorus, and alkaline phosphatase (normal ranges, 8.1 to 10.4 mg/dL, 2.5 to 4.5 mg/dL, and 80 to 240 IU/L, respectively) were measured on a Hitachi 917 analyzer (Roche, Mannheim, Germany). Urine calcium was measured in 24-h collection samples at all the follow-up steps; creatinine and total volume were used as estimates of the adequacy of the collection. Urinary calcium-to-creatinine ratio >0.2 mg/mg was defined as hypercalciuria. Percentage of FEPh was calculated using the following equation: [(24-hour urine phosphate in mg × serum creatinine in mg/dL)/(24-hour urine creatinine in mg × serum phosphate in mg/dL)] × 100 (26). The intra- and interassay coefficients of variation for biochemical measurements ranged from 3.5% to 5.0%.

Serum levels of intact PTH (normal range, 13 to 54 pg/mL)were measured by immunoradiometric assay until 2006 (DiaSorin, Stillwater, MN) and subsequently by chemiluminescence (Elecsys-2010; Roche; normal range, 15 to 65 pg/mL). Serum 25-hydroxyvitamin D level was measured by chemiluminescence (LAISON analyzer; DiaSorin) with coefficient of variation ranging from 2.9% to 5.5%. The assays were carried out in the endocrine services laboratory at our institution. However, serum 1,25-dihydroxyvitamin D level (normal range, 18 to 72 pg/mL) was measured at Quest Diagnostics, San Juan Capistrano, CA, by liquid chromatography and mass spectrometry.

The study was carried out in accordance with the tenets of the Declaration of Helsinki. The ethics committee of All India Institute of Medical Sciences, Delhi, approved the protocol (Reference no. IEC-93/03.03.2017, RP-32/2017). Written informed consent was obtained from all patients at the beginning of the study for carrying out the retrospective assessment of their serum calcium control, adjustment of alfacalcidol dose, and stepwise withdrawal of oral calcium.

Statistical analysis

Data are presented as mean and SD, median with interquartile range, and frequencies (reported as percentages). Differences in the age, sex, body mass index, and biochemical parameters were assessed by parametric and nonparametric tests, as appropriate. Regression analysis was used to determine variables associated with optimal calcium control in IH. A two-tailed P value <0.05 was considered significant. All analyses were performed using SPSS, version 20.0 (IBM, Armonk, NY).

Results

A total of 95 patients were available for follow-up during the study. Three were excluded because they were pregnant or lactating. The demographic profile of the 92 patients included in the study was similar to that described in our previous studies (8, 12, 15) and included ratio of male to female patients: 47:45; body mass index, 23.3 ± 4.6 kg/m^2; age at onset of initial hypocalcemic symptoms (25.2 ± 13.4 years); duration of illness (14.9 ± 7.8 years); frequency of cataract (45.7%); intracranial calcification (68.1%); serum total calcium level (5.5 ± 1.0 mg/dL); phosphorus level (6.9 ± 1.4 mg/dL), and median intact PTH level 6.0 (3.2 to 9.8) pg/mL at presentation. Most patients (71%) were vegetarian, 11% consumed eggs along with vegetarian diet, and 18.0% were nonvegetarian. Twelve patients received 12.5 to 25 mg/d thiazides. Their mean 24-hour urine calcium value was not significantly different than that of those who did not receive thiazide (181 ± 105 mg *vs.* 140 ± 102 mg; P = 0.21). The mean duration of their follow-up was 8.2 ± 5.4 years and the mean number of visits made to the endocrine clinic was 29 ±18. The median follow-up period over which serum calcium values were assessed for categorizing 92 patients in the optimal and suboptimal calcium control groups (hereafter referred to as optimal control group and suboptimal control group, respectively) was 13 (6.3 to 19.0) months. Thirty-five patients (38.0%) had optimal calcium control. None had hypercalcemia. The optimal and suboptimal groups had comparable clinical and biochemical features at initial presentation, except serum total calcium level, which was significantly less in the suboptimal group (5.3 ± 0.9 mg/dL *vs.* 5.9 ± 1.0 mg/dL; P = 0.002). Patients in the suboptimal group had significantly less monthly income than those in the optimal group. The daily dietary intake of calcium and phosphorus were comparable between the groups (Table 1).

Follow-up parameters in patients with optimal and suboptimal calcium control

The follow-up period and the total number of visits in endocrine clinic since initial presentation were comparable in the optimal and suboptimal control groups. Patients in the suboptimal group were taking a higher daily dose of alfacalcidol than the optimal group was (Table 1). In fact, 15.8% of patients with IH in the suboptimal group were taking a daily alfacalcidol dose of 3.0 μg, whereas none required such a dose in the optimal group (P = 0.01).

The suboptimal group had higher mean serum phosphorus level but lower mean FEPh than the optimal group (P < 0.001 for both). Despite a similar mean 25-hydroxyvitamin D level of 34.0 ng/mL in the two groups, the mean serum 1,25-dihydroxyvitamin D level was higher in the optimal group by 5.0 pg/mL. The mean 24-hour urine calcium measurement and frequency of calcium-to-creatinine ratio >0.2 were significantly higher in patients with optimal control (Table 1).

To ascertain the factors related to optimal serum calcium control, regression analysis was performed using variables that were significantly different in the univariate analysis (Table 1). Serum total calcium level and cataract

Table 1. Clinical and Biochemical Characteristics of Patients With IH Maintaining Optimal and Suboptimal Calcemic Control During Follow-Up in Endocrine Clinics

Parameter	Mean Serum Total Ca Level During Previous Follow-Up		*P* Value
	<8.0 mg/dL (n = 57)	≥8.0 mg/dL (n = 35)	
M:F, no.	28:29	19:16	0.40
Age at onset of illness, y	23.7 ± 11.4	27.7 ± 16.1	0.20
Duration of illness, y	15.0 ± 8.1	14.8 ± 7.4	0.89
Duration of follow-up in endocrine clinics, y	8.3 ± 5.4	8.1 ± 5.4	0.84
No. of visits	28 ± 18	30 ± 18	0.51
History of seizures, %	56.1	60.0	0.44
Serum Ca at diagnosis, mg/dL	5.3 ± 0.9	5.9 ± 1.0	0.002
Serum phosphate at diagnosis, mg/dL	7.1 ± 1.4	6.6 ± 1.5	0.14
Serum intact PTH at diagnosis, pg/mL, median (IQR)	6.6 (2.7–9.8)	5.3 (3.7–9.5)	0.79
Cataract, %	52.6	34.3	0.07
Intracranial calcification, %	71.4	62.9	0.27
Compliance with therapy, %	15.8	97.1	<0.001
Monthly per-capita income, USD, median (IQR)	28 (20–54)	51 (32–132)	<0.001
Current BMI, kg/m^2	22.7 ± 4.4	24.3 ± 5.0	0.11
Current age, y	38.2 ± 14.2	42.8 ± 16.8	0.16
Period over which three serum Ca values were recorded, mo, median (IQR)	12 (6–19)	15 (8–20)	0.39
Daily alfacalcidol dose, median, μg/d (IQR)	1.5 (1.0–2.25)	1.25 (1.0–1.50)	0.04
Range of daily alfacalcidol dose, %, μg			
0.25-0.75	19.3	20.0	0.03
1.0-1.5	31.6	57.1	
>1.5-3.0	49.1	22.9	
Patients receiving 3.0 μg/d alfacalcidol , %	15.8	nil	0.01
Serum Ca, mg/dL	7.1 ± 0.8	8. 6 ± 0.5	<0.001
Serum phosphate, mg/dL	5.4 ± 1.1	4.6 ± 0.5	<0.001
Ca-phosphate, mg^2/dL^2	38.7 ± 6.2	39.8 ± 4.5	0.34
Serum alkaline phosphatase, IU	215 ± 69	197 ± 49	0.12
Serum albumin, gm/dL	4.6 ± 0.4	4.6 ± 0.4	0.95
Serum 25(OH)D, ng/mL	34. 5 ± 11.5	34.7 ± 20.7	0.95
Calcitriol, pg/mL	31.7 ± 13.0	34.5 ± 14.7	0.35
24-h urine Ca, mg (median; IQR)	113 ± 77 (98; 58–159)	210 ± 114 (187; 120–292)	<0.001
24-h urine creatinine, mg (median; IQR)	1175 ± 561 (1100; 791–1348)	1334 ± 820 (1193; 821–1581)	0.34
Urine Ca-to-creatinine ratio, mg/mg (median; IQR)	0.11 ± 0.07 (0.10; 0.05–0.14)	0.19 ± 0.11 (0.15; 0.10–0.25)	0.001
24-h urine phosphate, mg (median; IQR)	514 ± 393 (430; 320–548)	631 ± 513 (536; 400–651)	0.04
FEPh, % (median; IQR)	6.8 ± 4.4 (6.3; 4.2–8.2)	10.4 ± 5.0(9.2; 7.0–12.9)	<0.001
Urine Ca-to-creatinine ratio > 0.2, %	10.7	34.3	0.006
Daily oral elemental Ca, median (IQR), g	2.0 (2.0–2.0)	2.0 (1.5–2.0)	0.001
Dietary calcium intake, mg/d (median; IQR)	845 ± 500 (715; 558–879)	765 ± 248 (759; 559–903)	0.86
Dietary phosphorus intake, mg/d	1447 ± 490	1340 ± 273	0.28
Total calorie intake, kcal/d	1767 ± 462	1682 ± 283	0.37

Data are reported as mean ± SD unless otherwise indicated.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; Ca, calcium; IQR, interquartile range; SAP, serum alkaline phosphatase.

at presentation, and poor compliance with prescribed therapy during routine follow-up were used as independent variables. Current serum 1,25-dihydroxyvitamin D level was also factored in as additional independent variable because of its biological relevance. This model indicated that poor drug compliance was the only significant factor for suboptimal calcium control during follow-up ($P < 0.001$).

Effect of provision of free therapy in patients with suboptimal calcium control

Of the patients with IH who had suboptimal calcium control during routine follow-up (Fig. 1), 57 were provided alfacalcidol and oral calcium therapy from the clinic and allocated a fixed date for next follow-up. Of these 57 patients, 48 adhered to the scheduled dose and follow-up visits. All 48 achieved target serum calcium levels >8.0 mg/dL (mean, 8.7 ± 0.6 mg/dL) at a median follow-up of 20 (14 to 37) days. Thirty-three achieved calcemic control on the ongoing dose. Two of these 33 patients required admission to the endocrine ward for optimal control. Both these patients were taking 3.0 μg/d alfacalcidol before admission but attained calcium control while taking the same dose with directly supervised therapy in the hospital. Only 15 patients required an increase in the daily alfacalcidol dose; the median increase was 0.25 (0.25 to 0.50) μg/d.

Table 2 lists the changes in biochemical parameters in 48 patients before and after optimal control. Their mean serum levels of calcium increased and of phosphorus decreased significantly ($P < 0.001$ for both). The mean FEPh increased with attainment of optimal control ($P = 0.02$). Serum 1,25-dihydroxyvitamin D level increased significantly (mean, 40.6 ± 14.2 pg/mL; $P = 0.006$) after provision of free alfacalcidol and calcium therapy from the clinic. However, after attaining optimal calcium control, there was a significant increase in hypercalciuria and the frequency of patients with urine calcium-to-creatinine ratio >0.2 increased from 10.4% to 70.8% ($P < 0.001$). Two patients with IH had a serum total calcium level >10.0 mg/dL; however, no patients had serum calcium value in the hypercalcemic range.

Reduction of oral calcium after attainment of target serum calcium level

During the course of the study, 83 patients had optimal calcium control (35 on routine follow-up and 48 after provision of medicines from the clinic). Calcium reduction was attempted in 51 of these 83 patients. Thirty-two patients were excluded from calcium reduction protocol for the following reasons: myocardial infarction (n = 1), past history of head injury (n = 1), recent seizure (n = 1), celiac disease (n = 3), neuropsychiatric disturbances (n = 5), lack of consent (n = 6), difficult in weekly follow-up due to long travel distance (n = 10), and poor compliance with protocols in earlier studies (n = 5). The calcium reduction protocol could not be followed for two patients. A final analysis was carried out in 49 patients who followed all the steps of the calcium reduction protocol. Reduction of daily oral calcium dose was possible in 41 of the 49 patients. Twenty-one (43%) could be completely weaned off oral calcium and maintained stable calcium control as of the writing of this report (*i.e.*, 10.1 ± 4.2 months). The clinical and biochemical characteristics before calcium withdrawal, including daily requirement of alfacalcidol, were similar among the 21 patients who could be weaned off and 28 patients who could not be completely weaned off oral calcium (Table 3).

Biochemical parameters of the 21 patients before and after complete withdrawal of oral calcium supplements are listed in Table 4. The mean serum total calcium level (8.9 ± 0.5 mg/dL to 9.5 ± 0.7 mg/dL; $P < 0.001$) and mean serum 1,25-dihydroxyvitamin D level (38.6 ± 11.8 pg/mL to 47.5 ± 16.6 pg/mL; $P = 0.01$) improved after complete withdrawal of oral calcium. Though there was no significant change in serum phosphorus level, the FEPh increased after withdrawal of oral calcium ($P = 0.01$). Urinary calcium excretion showed no significant decrease after withdrawal of oral calcium (Table 4).

Readjustment of alfacalcidol dose after complete withdrawal of oral calcium

During follow-up of 21 patients receiving alfacalcidol therapy after complete withdrawal of oral calcium, serum total calcium level of 15 patients increased to >9.0 mg/dL. Six of them had a serum calcium level >10.0 mg/dL,

Table 2. Biochemical Characteristics of Patients With IH With Suboptimal Calcemic Control: Before and After Alfacalcidol Therapy

	Alfacalcidol Therapy (n = 48)		
Parameter	**Routine**	**Supervised**	***P* Value**
Serum Ca, mg/dL	7.1 ± 0.8	8.7 ± 0.6	<0.001
Serum phosphate, mg/dL	5.4 ± 1.1	4.9 ± 0.9	<0.001
Ca-phosphate, mg^2/dL^2	38.5 ± 6.7	42.5 ± 8.0	<0.001
SAP, IU	212 ± 71	205 ± 66	0.30
Serum 25(OH)D, ng/mL	33.2 ± 10.0	31.7 ± 7.9	0.14
1,25-Dihydroxyvitamin D, pg/mL	33.5 ± 12.2	40.6 ± 14.2	0.006
24-h urine Ca, mg	119 ± 87	257 ± 177	<0.001
24-h urine creatinine, mg	1238 ± 626	1140 ± 649	0.44
Urine Ca-to-creatinine ratio, mg/mg	0.11 ± 0.06	0.26 ± 0.14	<0.001
24-h urine phosphate, mg	516 ± 254	556 ± 335	0.54
FEPh, % (median; IQR)	7.9 ± 5.1 (6.6; 4.2–8.6)	9.9 ± 5.5 (9.6; 6.0–12.5)	0.02
Urine Ca-to-creatinine ratio >0.2, %	10.4	70.8	<0.001
Range of daily alfacalcidol dose, %, μg			
0.25–0.75	20.8	10.4	0.37
1.0–1.5	29.2	31.2	
>1.5–0.0	50.0	58.3	
Daily alfacalcidol dose, median (IQR), μg/d	1.75 (1.0-2.5)	2.00 (1.0-2.50)	0.001
No. of patients receiving 3.0 μg/d alfacalcidol	9/48	9/48	—

Data are reported as mean ± SD unless otherwise indicated.

Abbreviations: —, no data; 25(OH)D, 25-hydroxyvitamin D; Ca, calcium; IQR, interquartile range; SAP, serum alkaline phosphatase.

Table 3. Clinical Characteristics of Patients With IH Who Did Not Require Oral Calcium Vs Those Who Required Oral Calcium for Optimal Calcemic Control

Parameter	Without Oral Ca (n = 21)	With Oral Ca (n = 28)	*P* Value
M:F, n	11:10	12:16	0.57
Current BMI, kg/m^2	22.1 ± 4.1	24.0 ± 5.4	0.18
Age at onset of illness, y	26.1 ± 12.2	25.8 ± 10.9	0.99
Age at presentation of illness, y	33.5 ± 12.4	34.0 ± 15.2	0.91
Age at present study, y	38.6 ± 14.6	42.7 ± 15.9	0.36
Duration of illness, y	13.5 ± 8.6	16.3 ± 8.4	0.26
History of seizures, %	61.9	46.4	0.39
Duration of follow-up, y	6.1 ± 5.6	9.1 ± 5.1	0.07
Serum Ca at presentation, mg/dL	5.5 ± 1.0	5.8 ± 1.0	0.31
Serum phosphate at presentation, mg/dL	7.0 ± 1.6	6.8 ± 1.7	0.69
Cataract, %	33.3	42.9	0.56
Intracranial calcification, %	65.0	64.3	0.99
Dietary calcium intake, mg/d	1004 ± 660	765 ± 273	0.22
Daily alfacalcidol dose, median (IQR), μg/d	1.5 (1.5-2.5)	1.25 (1.0-2.0)	0.15
Distribution of daily alfacalcidol dose, %, μg			
0.25–0.75	4.8	17.9	
1.0–1.5	47.6	42.9	0.38
>1.5–3.0	47.6	39.3	
Patients receiving 3.0 μg/d alfacalcidol, %	9.5	14.3	0.69
Serum Ca, mg/dL	9.5 ± 0.7	8.9 ± 0.6	0.002
Serum phosphate, mg/dL	4.7 ± 0.8	4.7 ± 0.7	0.89
Ca-phosphate, mg^2/dL^2	44.8 ± 8.1	42.2 ± 6.6	0.21
SAP, IU	234 ± 83	215 ± 63	0.35
Serum albumin, g/dL	4.8 ± 0.42	4.7 ± 0.4	0.30
Serum 25(OH)D, ng/mL	29.6 ± 10.8	30.8 ± 9.4	0.69
Calcitriol, pg/mL	47.5 ± 16.6	40.3 ± 18.6	0.23
24-h urine Ca, mg	296 ± 186	240 ± 199	0.13
24-h urine creatinine, mg	1214 ± 613	1264 ± 838	0.61
FEPh, % (median; IQR)	15.1 ± 7.5 (14.7; 8.8–20.1)	10.2 ± 4.1 (9.9;8.0–11.8)	0.02
Urine Ca-to-creatinine ratio, mg/mg	0.26 ± 0.11	0.21 ± 0.11	0.10
Urine Ca-to-creatinine ratio >0.2, %	66.7	47.8	0.24

Data are reported as mean ± SD unless otherwise indicated.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; Ca, calcium; IQR, interquartile range; SAP, serum alkaline phosphatase.

including two with hypercalcemia (10.6 and 11.2 mg/dL). In 14 of the 15 patients, attempt was made to reduce the daily dose of alfacalcidol to maintain the serum total calcium value between 8.0 and 8.5 mg/dL. Daily alfacalcidol dose could be reduced in 10 patients. They continued to receive free medicines from the clinic and were reassessed after a

Table 4. Biochemical Characteristics in 21 Patients With IH Before and After Calcium Withdrawal

	Oral Calcium		
Parameter	Taking Oral Calcium	No Longer Taking Oral Calcium	*P* Value
Serum Ca, mg/dL	8.9 ± 0.5	9.5 ± 0.7	<0.001
Serum phosphate, mg/dL	4.7 ± 0.7	4.7 ± 0.8	0.85
Ca-phosphate, mg^2/dL^2	41.9 ± 6.5	44.8 ± 8.1	<0.04
SAP, IU	211 ± 70	234 ± 83	0.19
Serum albumin, g/dL	4.7 ± 0.4	4.8 ± 0.4	0.22
Serum 25(OH)D, ng/mL	34.0 ± 11.7	29.6 ± 10.8	0.08
Calcitriol, pg/mL	38.6 ± 11.8	47.5 ± 16.6	0.01
24-h urine Ca, mg	264 ± 126	296 ± 186	0.40
24-h urine phosphate, mg	582 ± 182	810 ± 444	0.01
24-h urine creatinine, mg	1133 ± 322	1214 ± 613	0.54
Urine Ca-to-creatinine ratio, mg/mg	0.24 ± 0.11	0.26 ± 0.11	0.44
Urine Ca-to-creatinine ratio >0.2, %	57.1	66.7	0.75
FEPh, %	11.1 ± 5.7	15. 1 ± 7.5	0.01

Data are reported as mean ± SD unless otherwise indicated.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; Ca, calcium; SAP, serum alkaline phosphatase.

prolonged follow-up period of 8.4 ± 2.6 months. With reduction in dose, their mean calcium level decreased from 9.9 ± 0.6 mg/dL to 8.4 ± 0.2 mg/dL. However, 40% of them had persistently high calcium-to-creatinine ratio. Interestingly, serum phosphorus level was normal at a serum calcium level >9.0 mg/dL, but it significantly increased again (4.3 ± 0.8 mg/dL to 4.8 ± 0.8 mg/dL; $P < 0.01$) when alfacalcidol was reduced to attain a serum calcium level of 8.0 to 8.5 mg/dL (Table 5).

Figure 2 is a histogram of alfacalcidol dosage patterns of the study participants after final adjustment. The daily requirement of alfacalcidol was 1.0 to 2.0 μg in 66% of patients and >1.5 μg in 40% of the patients with IH.

Discussion

Patients with chronic hypoparathyroidism are treated with vitamin D with an aim to maintain their serum total calcium level in the low-normal range for symptomatic relief and to minimize the risk of hypercalciuria and renal stones or nephrocalcinosis. However, retrospective analysis of 120 patients with chronic hypoparathyroidism attending two major hospitals in Boston, Massachusetts, showed a 26% prevalence of hypercalciuria with conventional therapy (27). Similarly, Underbjerg *et al.* (28) observed increased prevalence of renal stones and renal insufficiency in 688 patients with postsurgical hypoparathyroidism identified in the Danish National registry. Serum phosphorus control is usually not the prime focus of conventional therapy. However, our studies showed that progression of intracranial calcification, posterior capsular opacification after cataract surgery, and neuropsychiatric dysfunctions were related to calcium and phosphorus control during long-term follow-up (8, 9, 13). With an ideal therapy, patients with IH should have normal serum calcium and phosphorus levels without any hypercalciuria, as do normal subjects. PTH is being investigated as an alternative therapy for chronic IH (29–31). To prescribe PTH therapy with conviction, it is important to systematically assess the efficacy and shortcomings of conventional vitamin D and calcium therapy, especially persistence of hyperphosphatemia and aggravation of hypercalciuria. The strength of the current study is a large cohort of patients with IH who were closely followed for several years while receiving alfacalcidol therapy at a tertiary care center.

The present study showed that, on routine follow-up, only 38% of patients with IH were maintaining optimal serum total calcium level of 8.0 to 8.5 mg/dL. A similar low rate of calcium control (40%) was observed by Heyburn and Peacock (32) in 28 patients with postsurgical and IH. Of the patients in the current study, 11% also had hypercalciuria despite suboptimal calcium control.

Regression analysis showed poor compliance and inability to afford medicines as the important factors for suboptimal calcium control in IH. To assess the possibility of poor efficacy of alfacalcidol as an additional contributory factor for suboptimal calcium control, medicines were provided free to all the patients. They were also followed up frequently to increase compliance and refilling of medicine stock. Observation of optimal calcium control in all patients within a span of 3 weeks, mostly while receiving their ongoing alfacalcidol dose, indicated good efficacy of conventional therapy in IH. However, this cannot be generalized, because there are reports of patients with primary and postsurgical hypoparathyroidism resistant to high doses of alfacalcidol and calcitriol responding to PTH therapy (33–35).

Table 5. Biochemical Characteristics of Patients With IH Before and After Alfacalcidol Reduction[a]

Parameter	Alfacalcidol Reduction		*P* Value
	Before	After	
Serum Ca, mg/dL	9.9 ± 0.6	8.4 ± 0.2	<0.001
Serum phosphate, mg/dL	4.3 ± 0.8	4.8 ± 0.8	<0.01
Ca-phosphate, mg^2/dL^2	42.5 ± 7.2	40.2 ± 6.8	0.12
SAP, IU	232 ± 68	252 ± 76	0.07
Serum albumin, g/dL	5.0 ± 0.3	4.8 ± 0.4	0.06
Serum 25(OH)D, ng/mL	29.7 ± 7.9	32.7 ± 9.1	0. 24
Calcitriol, pg/mL	42.3 ± 10.7	42.8 ± 12.5	0.91
24-h urine Ca, mg	322 ± 236	261 ± 220	0.17
24-h urine phosphate, mg	728 ± 361	1023 ± 842	0.17
24-h urine creatinine, mg	972 ± 440	1403 ± 1116	0.09
Urine Ca-to-creatinine ratio, mg/mg	0.32 ± 0.09	0.19 ± 0.08	0.004
Urine Ca-to-creatinine ratio >0.2, n	9/10	4/10	0.06
FEPh, %	17.9 ± 8.1	15.0 ± 6.0	0.12

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; Ca, calcium; SAP, serum alkaline phosphatase.

[a]After patients were weaned off oral calcium supplementation and had calcium levels >9.0 mg/dL.

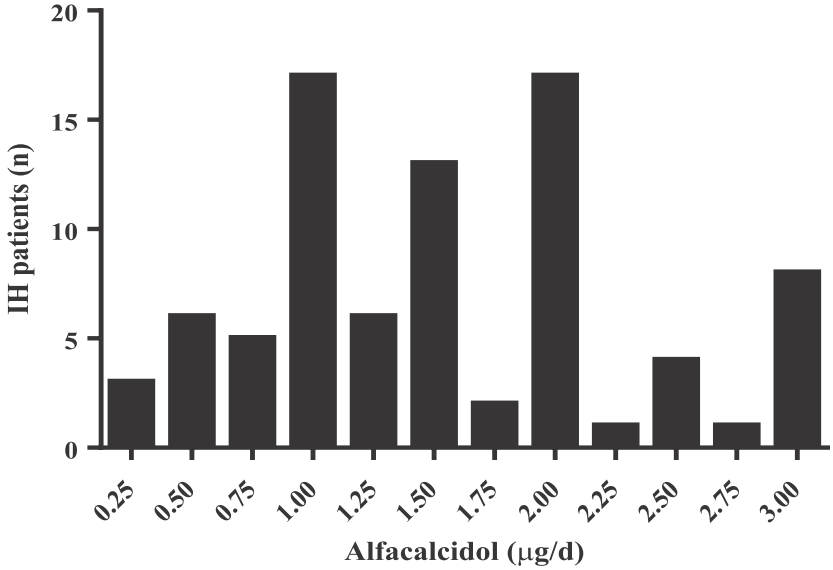

Figure 2. Daily alfacalcidol requirement for optimal calcemic control in patients with IH.

In the current study, at optimal calcium control, alfacalcidol therapy raised the serum 1,25-dihydroxyvitamin D level appropriately to 40 to 47 pg/mL, which is similar to that achieved when patients with hypoparathyroidism were treated with calcitriol or PTH (30, 36) and the levels observed in healthy Asian Indians (24, 25, 37). In fact, in the current study, 21 patients with IH in whom oral calcium could be completely stopped had maximum mean serum 1,25-dihydroxyvitamin D values (*i.e.*, 47.5 pg/mL). Interestingly, despite better compliance, optimum calcium control, and adequate serum 1,25-dihydroxyvitamin D levels, there was only partial correction of hyperphosphatemia and FEPh. Moreover, there was an alarming increase in the frequency of hypercalciuria to 66%. Phosphorus homeostasis was further compromised in patients with suboptimal calcium control with serum phosphorus concentration of 5.4 mg/dL and FEPh of only 7.0%, compared with the normal FEPh of 15% to 20%. Presence of hyperphosphatemia and hypercalciuria in patients with optimal calcium control and those with suboptimally controlled calcium values indicates missing physiological actions of PTH on renal phosphate excretion and calcium reabsorption that could not be rectified with conventional vitamin D therapy in IH. These findings indicate that alfacalcidol and calcium therapy are effective in achieving optimal calcium control but do not normalize serum phosphorus levels. In fact, an increased prevalence of hypercalciuria with optimal calcium control indicates lack of long-term renal safety with conventional therapy.

Withdrawal of oral calcium could have led to worsening of hyperphosphatemia because of loss of its luminal dietary phosphate-binding action; however, no such effect was noticed. On the contrary, serum phosphate homeostasis improved, as indicated by increased FEPh when patients were taking only alfacalcidol. Moderate reduction of serum phosphorus coupled with increased FEPh could be explained by the phosphaturic effect of vitamin D through FGF23 rather than by oral calcium (38). Gupta *et al.* (39) reported increased serum FGF23 values in patients with chronic hypoparathyroidism. Additional studies would help elucidate the interplay and relative importance of various factors that lead to continued improvement in phosphate homeostasis despite calcium withdrawal in IH. In the current study, 43% of the patients could be completely weaned off oral calcium, and the daily oral calcium dose could be reduced in additional 41%. However, none of the study factors, including severity of hypocalcemia at diagnosis, could predict the need for oral calcium. Reduction of oral calcium in the IH would also help reduce the cost of therapy for patients. Thus, oral calcium need not be used as an adjunct of vitamin D therapy on a universal basis in IH. Physicians may consider careful withdrawal or a reduction plan of oral calcium after patients with IH achieve optimal calcium control and maintain normal dietary calcium intake and good compliance with vitamin D analogs.

With continued provision of alfacalcidol and regular follow-up, a subset of patients with IH (mostly those in whom oral calcium could be completely stopped) had a serum total calcium level >9.0 mg/dL. Interestingly, it was observed that their mean serum phosphorus level was lowered to the normal range. However, normalization of serum phosphorus was associated with an alarming increase in hypercalciuria to the extent of 90%. Though, reduction of alfacalcidol could decrease serum calcium values back to 8.0 to 8.5 mg/dL and frequency of hypercalciuria to 40%, it raised their serum phosphorus to the supranormal range.

To summarize, in this study, we showed that during routine follow-up, only 38% of patients with IH maintained the desired serum calcium concentration while receiving conventional therapy. However, all the patients could achieve optimal calcium control and serum 1,25-dihydroxyvitamin D levels provided there was good compliance with therapy. Efficacy of alfacalcidol therapy was also indicated by the fact that 43% of the patients could be treated without any oral calcium. Nevertheless, serum phosphorus levels could not be normalized and hypercalciuria was observed in nearly one-third of

patients taking alfacalcidol even when serum calcium level was maintained between 8.0 and 8.5 mg/dL. More by serendipity than design, serum phosphorus level was normal when the mean serum total calcium level increased to 9.9 mg/dL. However, such serum calcium levels and normalization of serum phosphorus was associated with 90% prevalence of hypercalciuria.

Thus, despite careful optimization of alfacalcidol therapy, ideal control (*i.e.*, normocalcemia, normophosphatemia, and normocalciuria) could not be achieved in patients with IH. Winer (29) summarized the experience with various dosages and modes of administration of PTH 1-34 in chronic hypoparathyroidism and showed its beneficial effect on phosphatemia and hypercalciuria. Mannstadt *et al.* (30) reported a multicentric clinical trial with PTH 1-84 therapy in chronic hypoparathyroidism with its advantages in the control of serum phosphate levels and hypercalciuria in comparison with the conventional therapy. Addition of PTH therapy in patients with IH is likely to help them achieve ideal calcium control. However, in resource-constrained situations where even affording vitamin D analogs is difficult for the patients, there is a need to design cost-effective PTH therapy protocols.

Acknowledgments

We thank Arun Chandran, technician, Endocrinology and Metabolism, for help measuring serum calcium and other biochemical parameters, and Sanghmitra Shakya, dietitian, and Ram Sumer, laboratory assistant, for their help in the study.

Financial Support: This work was carried out using facilities available at our institute and other research projects. S.S. received salary support from the Department of Biotechnology, Government of India (no. BT/PR15041/MED/30/1580/2015).

Correspondence and Reprint Requests: Ravinder Goswami, MD, DM (Endocrinology), Department of Endocrinology and Metabolism, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India. E-mail: gosravinder@hotmail.com.

Disclosure Summary: The authors have nothing to disclose.

References

1. Clarke BL, Brown EM, Collins MT, Jüppner H, Lakatos P, Levine MA, Mannstadt MM, Bilezikian JP, Romanischen AF, Thakker RV. Epidemiology and diagnosis of hypoparathyroidism. *J Clin Endocrinol Metab*. 2016;**101**(6):2284–2299.
2. Shoback D. Hypoparathyroidism. *N Engl J Med*. 2008;**359**(4):391–403.
3. Bollerslev J, Rejnmark L, Marcocci C, Shoback DM, Sitges-Serra A, van Biesen W, Dekkers OM; European Society of Endocrinology. European Society of Endocrinology clinical guideline: treatment of chronic hypoparathyroidism in adults. *Eur J Endocrinol*. 2015;**173**(2):G1–G20.
4. Bilezikian JP, Brandi ML, Cusano NE, Mannstadt M, Rejnmark L, Rizzoli R, Rubin MR, Winer KK, Liberman UA, Potts JT Jr. Management of hypoparathyroidism: present and future. *J Clin Endocrinol Metab*. 2016;**101**(6):2313–2324.
5. Goswami R, Mohapatra T, Gupta N, Rani R, Tomar N, Dikshit A, Sharma RK. Parathyroid hormone gene polymorphism and sporadic idiopathic hypoparathyroidism. *J Clin Endocrinol Metab*. 2004;**89**(10):4840–4845.
6. Goswami R, Bhatia M, Goyal R, Kochupillai N. Reversible peripheral neuropathy in idiopathic hypoparathyroidism. *Acta Neurol Scand*. 2002;**105**(2):128–131.
7. Goswami R, Brown EM, Kochupillai N, Gupta N, Rani R, Kifor O, Chattopadhyay N. Prevalence of calcium sensing receptor autoantibodies in patients with sporadic idiopathic hypoparathyroidism. *Eur J Endocrinol*. 2004;**150**(1):9–18.
8. Goswami R, Sharma R, Sreenivas V, Gupta N, Ganapathy A, Das S. Prevalence and progression of basal ganglia calcification and its pathogenic mechanism in patients with idiopathic hypoparathyroidism. *Clin Endocrinol (Oxf)*. 2012;**77**(2):200–206.
9. Saha S, Gantyala SP, Aggarwal S, Sreenivas V, Tandon R, Goswami R. Long-term outcome of cataract surgery in patients with idiopathic hypoparathyroidism and its relationship with their calcemic status. *J Bone Miner Metab*. 2017;**35**(4):405–411.
10. Saha S, Goswami R. Menstruation associated hypocalcemic symptoms and serum calcium in patients with idiopathic hypoparathyroidism. *BMC Endocr Disord*. 2014;**14**(1):28.
11. Mahtab S, Vaish U, Saha S, Singh A, Goswami R, Rani R. Presence of autoreactive, MHC class I-restricted, calcium-sensing receptor (CaSR)-specific CD8+ T cells in idiopathic hypoparathyroidism. *J Clin Endocrinol Metab*. 2017;**102**(1):167–175.
12. Chawla H, Saha S, Kandasamy D, Sharma R, Sreenivas V, Goswami R. Vertebral fractures and bone mineral density in patients with idiopathic hypoparathyroidism on long term follow-up. *J Clin Endocrinol Metab*. 2017;**102**(1):251–258.
13. Aggarwal S, Kailash S, Sagar R, Tripathi M, Sreenivas V, Sharma R, Gupta N, Goswami R. Neuropsychological dysfunction in idiopathic hypoparathyroidism and its relationship with intracranial calcification and serum total calcium. *Eur J Endocrinol*. 2013;**168**(6):895–903.
14. Goswami R, Ray D, Sharma R, Tomar N, Gupta R, Gupta N, Sreenivas V. Presence of spondyloarthropathy and its clinical profile in patients with hypoparathyroidism. *Clin Endocrinol (Oxf)*. 2008;**68**(2):258–263.
15. Goswami R, Goel S, Tomar N, Gupta N, Lumb V, Sharma YD. Prevalence of clinical remission in patients with sporadic idiopathic hypoparathyroidism. *Clin Endocrinol (Oxf)*. 2010;**72**(3):328–333.
16. Saha S, Saini S, Makharia GK, Datta Gupta S, Goswami R. Prevalence of coeliac disease in idiopathic hypoparathyroidism and effect of gluten-free diet on calcaemic control. *Clin Endocrinol (Oxf)*. 2016;**84**(4):578–586.
17. Davies M, Taylor CM, Hill LF, Stanbury SW. 1,25-dihydroxycholecalciferol in hypoparathyroidism. *Lancet*. 1977;**309**(8002):55–59.
18. Lewin IG, Papapoulus SE, O'Riordan JLH. lalpha-Hydroxyvitamin D in the long-term management of hypoparathyroidism and pseudohypoparathyroidism. *Clin Endocrinol (Oxf)*. 1977;**7**(suppl):203s–207s.
19. Schilling T, Ziegler R. Current therapy of hypoparathyroidism--a survey of German endocrinology centers. *Exp Clin Endocrinol Diabetes*. 1997;**105**(4):237–241.
20. Tomar N, Bora H, Singh R, Gupta N, Kaur P, Chauhan SS, Sharma YD, Goswami R. Presence and significance of a R110W mutation in the DNA-binding domain of GCM2 gene in patients with isolated hypoparathyroidism and their family members. *Eur J Endocrinol*. 2010;**162**(2):407–421.
21. Tomar N, Gupta N, Goswami R. Calcium-sensing receptor autoantibodies and idiopathic hypoparathyroidism. *J Clin Endocrinol Metab*. 2013;**98**(9):3884–3891.
22. Willett WC, Sampson L, Stampfer MJ, Rosner B, Bain C, Witschi J, Hennekens CH, Speizer FE. Reproducibility and validity of a semiquantitative food frequency questionnaire. *Am J Epidemiol*. 1985;**122**(1):51–65.

23. Gopalan C, Sastri BVR, Balasubramanian SC. Food composition tables. In: Gopalan C, Sastri B, Balasubramanian S, Narasinga B, Deosthale Y, and Pant K, eds. *Nutritive Value of Indian foods*. Hyderabad, India: National Institute of Nutrition, Indian Council of Medical Research; 1996:45–95.
24. Saha S, Goswami R, Ramakrishnan L, Vishnubhatla S, Mahtab S, Kar P, Srinivasan S, Singh N, Singh U. Vitamin D and calcium supplementation, skeletal muscle strength and serum testosterone in young healthy adult males: randomized control trial. *Clin Endocrinol (Oxf)*. 2018;**88**(2):217–226.
25. Goswami R, Vatsa M, Sreenivas V, Singh U, Gupta N, Lakshmy R, Aggarwal S, Ganapathy A, Joshi P, Bhatia H. Skeletal muscle strength in young Asian Indian females after vitamin D and calcium supplementation: a double-blind randomized controlled clinical trial. *J Clin Endocrinol Metab*. 2012;**97**(12):4709–4716.
26. Walton RJ, Bijvoet OL. Nomogram for derivation of renal threshold phosphate concentration. *Lancet*. 1975;**2**(7929):309–310.
27. Mitchell DM, Regan S, Cooley MR, Lauter KB, Vrla MC, Becker CB, Burnett-Bowie SA, Mannstadt M. Long-term follow-up of patients with hypoparathyroidism. *J Clin Endocrinol Metab*. 2012; **97**(12):4507–4514.
28. Underbjerg L, Sikjaer T, Mosekilde L, Rejnmark L. Postsurgical hypoparathyroidism--risk of fractures, psychiatric diseases, cancer, cataract, and infections. *J Bone Miner Res*. 2014;**29**(11):2504–2510.
29. Winer KK. Advances in the treatment of hypoparathyroidism with PTH 1-34. *Bone*. 2019;**120**:535–541.
30. Mannstadt M, Clarke BL, Vokes T, Brandi ML, Ranganath L, Fraser WD, Lakatos P, Bajnok L, Garceau R, Mosekilde L, Lagast H, Shoback D, Bilezikian JP. Efficacy and safety of recombinant human parathyroid hormone (1-84) in hypoparathyroidism (REPLACE): a double-blind, placebo-controlled, randomised, phase 3 study [published correction appears in *Lancet Diabetes Endocrinol*. 2014;2(1):e3]. *Lancet Diabetes Endocrinol*. 2013;**1**(4): 275–283.
31. Marcucci G, Della Pepa G, Brandi ML. Drug safety evaluation of parathyroid hormone for hypocalcemia in patients with hypoparathyroidism. *Expert Opin Drug Saf*. 2017;**16**(5):617–625.
32. Heyburn PJ, Peacock M. The management of hypoparathyroidism with 1alpha-hydroxyvitamin D3. *Clin Endocrinol (Oxf)*. 1977; **7**(suppl):209s–214s.
33. Puig-Domingo M, Díaz G, Nicolau J, Fernández C, Rueda S, Halperin I. Successful treatment of vitamin D unresponsive hypoparathyroidism with multipulse subcutaneous infusion of teriparatide. *Eur J Endocrinol*. 2008;**159**(5):653–657.
34. Goussous R, Barakat R, Jarrah N, Abu-Hijleh MO, El-Zaheri M, Ajlouni K. Hypocalcemia resistant to 1,alpha hydroxyvitamin D treated successful with 1,25-dihydroxyvitamin D. *Saudi Med J*. 2003;**24**(5):542–543.
35. Kooh SW, Fraser D, DeLuca HF, Holick MF, Belsey RE, Clark MB, Murray TM. Treatment of hypoparathyroidism and pseudohypoparathyroidism with metabolites of vitamin D: evidence for impaired conversion of 25-hydroxyvitamin D to 1 alpha,25-dihydroxyvitamin D. *N Engl J Med*. 1975;**293**(17):840–844.
36. García-Pascual L, Barahona MJ, Perea V, Simó R. Serum 1,25-dihydroxyvitamin D as a biomarker of the absence of hypercalciuria in postsurgical hypoparathyroidism. *J Clin Endocrinol Metab*. 2017;**102**(1):259–266.
37. Vupputuri MR, Goswami R, Gupta N, Ray D, Tandon N, Kumar N. Prevalence and functional significance of 25-hydroxyvitamin D deficiency and vitamin D receptor gene polymorphisms in Asian Indians. *Am J Clin Nutr*. 2006;**83**(6):1411–1419.
38. Prié D, Friedlander G. Reciprocal control of 1,25-dihydroxyvitamin D and FGF23 formation involving the FGF23/Klotho system. *Clin J Am Soc Nephrol*. 2010;**5**(9):1717–1722.
39. Gupta A, Winer K, Econs MJ, Marx SJ, Collins MT. FGF-23 is elevated by chronic hyperphosphatemia. *J Clin Endocrinol Metab*. 2004;**89**(9):4489–4492.

Basal ganglia calcification is an important clinical feature of hypoparathyroidism. However, little information was available on its pathogenesis. This paper described the molecular basis of its occurrence by a novel approach. The Editors of the highest ranking Journal of Endocrinology i.e. JCEM of the Endocrine Society, USA have acknowledged the significance of this work and included it in `Endocrine focus' on the Journal home page.

ORIGINAL ARTICLE

Endocrine Research

Publication 2 (b)

Expression of Osteogenic Molecules in the Caudate Nucleus and Gray Matter and Their Potential Relevance for Basal Ganglia Calcification in Hypoparathyroidism

Ravinder Goswami, Tabin Millo, Shruti Mishra, Madhuchhanda Das, Mansi Kapoor, Neeraj Tomar, Soma Saha, Tara Shankar Roy, and Vishnubhatla Sreenivas

Departments of Endocrinology and Metabolism (R.G., S.M., M.D., M.K., N.T., S.S.), Forensic Medicine (T.M.), Anatomy (T.S.R.), and Biostatistics (V.S.), All India Institute of Medical Sciences, New Delhi 110029, India

Background: Basal ganglia calcification (BGC) is an interesting example of ectopic calcification in patients with hypoparathyroidism. Its pathogenesis and reasons for predilection of calcification at basal ganglia are not clear.

Objective: To assess the expression of osteogenesis-related molecules in the caudate nucleus and surface gray matter (an area spared from calcification) and discuss potential relevance of the results in context of BGC in idiopathic hypoparathyroidism.

Methods: Caudate nucleus and gray matter were obtained from 14 autopsies performed in accidental deaths. The mRNA expression of bone transcription factors (*RUNX2*/osterix), bone morphogenetic proteins (BMPs) 2 and 4, osteonectin, osteopontin, osteocalcin, vitamin D receptor, calcium sensing-receptor, Na phosphate transporters (PiTs) 1 and 2, N-methyl-D-aspartate receptor 2B (*NMDAR2B*), carbonic anhydrase II (*CA-II*), PTH1 receptor (*PTH1R*), *PTH2R*, and *PTHrP* were assessed by RT-PCR. Western blot, spot densitometry, and immunohistochemistry were performed to assess protein expression of molecules showing differences in mRNA expression between caudate and gray tissues.

Results: The mean mRNA expression of *PiT1* (11.0 ± 10.39 vs 32.9 ± 20.98, $P = .003$) and *PTH2R* (1.6 ± 1.47 vs 13.7 ± 6.11, $P = .001$) were significantly lower in the caudate nucleus than the gray matter. The expression of osteonectin, osteopontin, and CA-II were significantly higher in the caudate nucleus than the gray matter ($P = .01$, .001, and .04, respectively). The mRNA expression of other molecules was comparable in the 2 tissues. The protein expression of both *CA-II* and osteonectin was 24% higher and PiT1 17% lower in caudate than the gray matter. The differences in the PTH2R and osteopontin protein expression were not appreciable.

Conclusions: The presence of several osteogenic molecules in caudate nucleus indicates that BGC would probably be the outcome of an active process. The differences in expression of these molecules in caudate over gray matter could favor BGC at this site in the unique biochemical milieu of hypoparathyroid state. **(*J Clin Endocrinol Metab* 99: 1741–1748, 2014)**

Basal ganglia calcification (BGC) is an interesting example of ectopic calcification in patients with hypoparathyroidism (1, 2). Its pathogenesis and reasons for predilection of calcification at the basal ganglia are not clear. Recently, we reported an association of hyperphosphatemia with the presence and progression of BGC in

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

Received October 22, 2013. Accepted February 3, 2014.
First Published Online February 19, 2014

Abbreviations: BGC, basal ganglia calcification; BMP, bone morphogenetic protein; CA-II, carbonic anhydrase II; CaSR, calcium-sensing receptor; IDV, integrated density value; NMDAR2B, N-methyl-D-aspartate receptor 2B; PiT, phosphate transporter; PTH1R, PTH1 receptor; *RUNX2*, Runt-related transcription factor 2; VDR, vitamin D receptor.

patients with idiopathic hypoparathyroidism (3). Despite a high frequency of calcification in these patients at the basal ganglia (72%), gray-white junction (39.8%), cerebellum (31.2%), and thalamus (29.0%), none of them had calcification within the gray matter at the surface of the brain (3). A similar distribution is reported in several other disorders associated with intracranial calcifications such as osteopetrosis, type 1 diabetes, and Fahr's syndrome (4–6). Autopsies in patients with hypoparathyroidism and Fahr's disease have revealed lamellar deposition of calcium-hydroxyapatite crystals in the region of the basal ganglia (7–9). This observation coupled with association of BGC with hyperphosphatemia suggests an osteogenic mechanism in its pathogenesis. Wang et al (10) reported an inactivating mutation of the *PiT2* gene in patients with familial BGC. The present study assessed the presence of osteogenic molecules in the caudate nucleus and its comparison with other areas of the brain with a lesser predilection for calcification.

In a previous study, we had suggested the possibility of differential expression of several molecular factors facilitating BGC in hypoparathyroidism (3). These factors included overexpression of calcification-promoting and underexpression of mineral-resorbing molecules. Here, we report expression profile of bone transcription factors (Runt-related transcription factor 2 [*RUNX2*] and osterix), bone morphogenetic proteins (BMPs) 2 and 4, osteonectin, osteopontin, osteocalcin, vitamin D receptor (VDR), calcium-sensing receptor (CaSR), phosphate transporters (PiTs) 1 and 2, N-methyl-D-aspartate receptor 2B (*NMDAR2B*), carbonic anhydrase-II (CA-II), PTH1 receptor (PTH1R), PTH2R, and PTHrP in human caudate nucleus. Their expression was compared with that of surface gray matter, an area usually spared from calcification.

Materials and Methods

Autopsy tissues

Caudate nucleus and gray matter of the brain were collected from 14 human autopsies conducted for sudden and accidental deaths at All India Institute of Medical Science, New Delhi. Autopsies from patients dying in the hospital and cases with history of poisoning and or those conducted more than 12 hours after death were excluded. The dissection of the caudate nuclei was carried out by an anatomist and the forensic medicine experts (T.M. and T.S.R.). Gray matter was scraped from the occipital cortex because of the maximum thickness of gray matter in this area. Caudate tissue was obtained from the head and body of the nucleus, which was identified by its location lateral to the third ventricle and caudal to the floor of the anterior horn (Figure 1A). Tissues were immediately transported from the mortuary after dissection on ice to the endocrine laboratory and stored in liquid nitrogen in multiple aliquots. The dissected tissues were examined histologically to confirm the presence of the neuronal tissues. The study protocol was approved by the institutional ethics committee, and all the tissues were collected after written informed consent of the legal heir of the deceased.

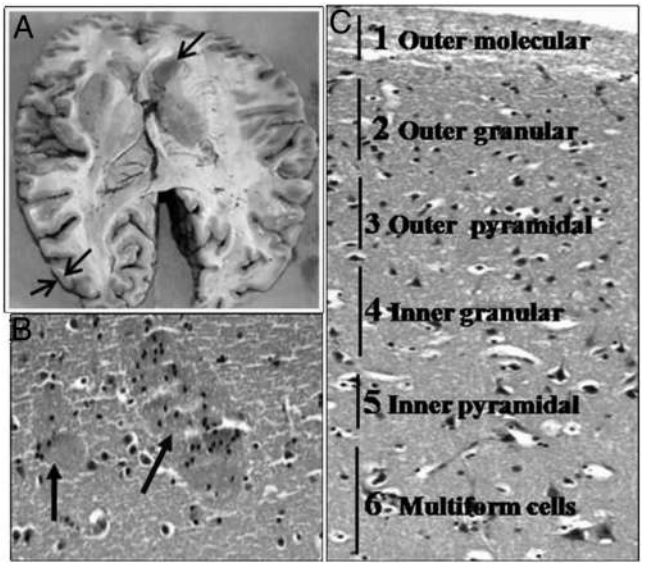

Figure 1. A, Area showing sites of collection of gray and caudate tissue from the brain. B and C, Hematoxylin and eosin-stained photomicrographs from 2 tissues showing mature neurons embedded in a background of glial cells and neurofibrillary material with 2 patches of striatopallidal/pencil fibers (arrows) in the caudate nucleus (B) and organization of neuron into 6 layers (outer molecular, outer granular, outer pyramidal, inner granular, and multiform layer) in the gray matter (C) (magnification, ×100).

RNA isolation, cDNA preparation, and RT-PCR for gene expression analysis

Total RNA was extracted from the tissues using the RNA binding column (Eppendorf AG-22331) as described previously (11, 12). The quality of RNA was checked by presence of 28S and 18S ribosomal bands on agarose gel electrophoresis and quantified using a UV spectrophotometer (GeneQuant; Amersham). The first strand of the cDNA was prepared using random hexamers and Moloney Murine Leukemia Virus Reverse Transcriptase (RevertAidH Minus; Fermentas) and 2.0 μg of total RNA at 42°C.

The mRNA expression of molecules associated with normal bone mineral calcification, ie, *RUNX2*, osterix, *BMP4, BMP2, VDR, PiT1, PiT2* osteonectin, osteopontin, osteocalcin, *CaSR*, and those suggested to be linked with BGC in hypoparathyroidism such as *CA-II, NMDAR2B, PTH1R, PTH2R*, and *PTHrP* were assessed using RT-PCR (11). The expression of dopamine- and cAMP-regulated phosphoprotein (*DARPP-32*) mRNA was also assessed to show its generally higher expression in human caudate nucleus than the gray tissues (13).

Table 1 shows the primers used for specific gene expression, annealing conditions used, and the size of the amplified products and their references (11, 14–25). The conditions for RT-PCR were 20-μL reaction volume with 2 μL cDNA, 3.0mM $MgCl_2$, 0.2mM dNTP, 1.0 U *Taq* DNA polymerase, and 0.1μM each of forward and reverse primers. Initial denaturation was carried out at 94°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds, and extension at 72°C for 30 seconds and fluorescence capture in each cycle (Ta-

Table 1. Forward and Reverse Primers, Annealing Temperatures, and the Product Size of Various Molecules During RT-PCR

Gene	Product, bp	Annealing Temperature (°C)	Forward Primer (5′–3′)	Reverse Primer (5′–3′)	Ref.
GAPDH	381	55	ccaaggtcatccatgacaac tttggt	tgttgaagtcagaggagacca cctg	11
NMDAR2B	465	60	attggtggcagagtggattc	ggcaaaagaatcatggctgt	14
VDR	404	60	gacatcggcatgatgaagg	ctagggtcacagaagggtcatc	11
PiT1	410	55	taccatcctcatctcggtgg	tgacggcttgactgaactgg	15
BMP2	349	55	atggattcgtggtggaagtg	gtggagttcagatgatcagc	16
BMP4	399	55	agcatgtcaggattagccga	tggagatggcactcagttca	16
PTHR2	386	59	aatggagaggttcaggcaga	tctccttggcatccttcagt	17
PTHrP	249	59	ccctctcccaacacaaagaa	ggaggtgtcagacaggtggt	18
RUNX2	288	55	ccccacgacaaccgcaccat	cactccggcccacaaatc	19
Osterix	307	60	cgggactcaacaactct	ccataggggtgtgtcat	20
Osteonectin	291	55	aacgtcctggtcaccctgta	ccaggtcacaggtctcgaa	21
Osteopontin	148	55	tggccgaggtgatagtgtg	cggggatggccttgtatg	22
CA-II	124	55	caatggtcatgctttcaacg	tccatcaagtgaaccccagt	23
CaSR	194	55	attgaggggagcccacctgct	aaagagggtgagtgcgatccca aagg	24
Osteocalcin	297	59	atgagagccctcacactcctc	cgtagaagcgccgataggc	25
PTH1R	361	55	ggaagcccaggaaagataagg	gagtagcccacggtgtaaatc	
PiT2	388	55	gtgttactaggcgccaaagtag	cagcatagaatactgggagtgc	
DARPP-32	385	55	gcaccatctcaagtcgaagag	ctcacttagtgctgggtcttcc	

ble 1). *GAPDH* was amplified in separate tubes along with respective genes using SYBR-I dye (Sigma) fluorescence signals on RT-PCR (CFX98 real-time system; Bio-Rad) as described earlier (11). All the reactions were done in duplicate, and the specificity of molecules amplified was checked by post-PCR melting curve analysis and 1.5% agarose gel electrophoresis. DNA sequencing of all the amplified products was also performed by eluting DNA from the agarose gel as described earlier (26). PCR for assessing mRNA expression of various molecules in the gray and caudate tissues from each of the cases were put in the same PCR plates to minimize differences related to interassay variations. The mRNA expression of each of the molecules studied was measured in relation to that of *GAPDH* transcripts.

Western blot

Western blots were carried out to assess expression of proteins for molecules that showed significant differences in the mRNA expression between caudate and gray matter. The protein for loading in the SDS-PAGE was prepared from a 5×5-mm fraction each from a pair of caudate nuclei and gray tissues. Briefly, the tissues were finely cut with a sterile scalpel blade and homogenized manually in a glass douncer in 1.0 mL lysis buffer (50mM Tris and 1% SDS containing protease inhibitor cocktail). The homogenates were sonicated 3 times under ice (1 minute burst and 1 minute cooling) at 20% amplitude and pulse frequency of 5 seconds (Sonics; Vibra Cell). The protein content was measured by a UV spectrophotometer at 280 nm, and Western blot was performed using 10% SDS-PAGE with 25 μg protein loaded per lane as described earlier (12, 27). After transfer of the protein to the polyvinylidene difluoride, blots were put on incubation manifold for incubating with different primary antibodies on the same blot (Deca-probe; GE, Amersham). Primary antibodies used to detect specific proteins were various rabbit polyclonal antibodies raised against 1) 24 to 84 amino acids in the N-terminal extracellular domain of human PTH2R (SC 30005, 1:500 dilution, H60; Santa Cruz Biotechnology, Inc), 2) 191 to 260 amino acids of human CA-II (SC 25596, 1:1500 dilution; Santa Cruz), 3) middle region of PiT1 protein (catalog item 27570002, 1:500 dilution; Novus Biologicals), and 4) 21 to 145 amino acids of osteonectin protein (NBP1–80971, 1:300 dilution; Novus Biologicals). Monoclonal antibodies were used to detect osteopontin protein expression (NB110–89062, 1:500 dilution; Novus Biologicals). All the blots were developed using alkaline phosphatase-conjugated antirabbit or antimouse IgG (DAKO) and nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-phosphate substrates. The integrated density value (IDV) of the band for proteins was calculated by spot density analysis software using the α-imager (Alpha Innotech Corp) analysis system (27). The ratio of the IDV of the band was used to assess the differences in relative expression of the proteins between caudate and gray matter.

Immunohistochemistry

Caudate and gray tissues obtained from the autopsy were frozen in liquid nitrogen. Immunohistochemistry was performed on 10-μm thin cryostat sections of the tissues taken on gelatin-coated slides. The primary antibodies used for assessing PTH2R, CA-II, PiT1, osteonectin, and osteopontin protein expression were the same as described above. Sections were incubated with primary antibodies in 1:50 dilutions for 60 minutes in humidified conditions, washed with Tris-buffered saline, and incubated again for another 30 minutes with UltraVision one-step horse-

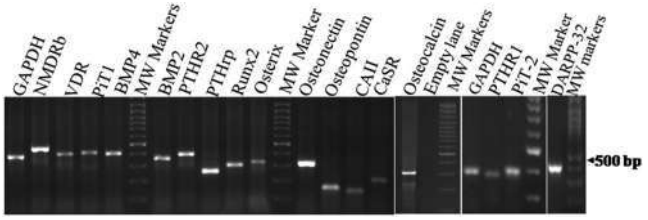

Figure 2. Agarose gel electrophoresis showing specific amplification of various molecules during real-time PCR. MW, molecular weight.

Table 2. Comparison of mRNA Expression of Candidate Molecules Between Caudate Nuclei and Gray Matter

Molecules	mRNA Copies/10^3 GAPDH		P^a	P^b
	Caudate Nuclei	Gray Matter		
NMDAR2B				
Mean ± SD	194.9 ± 190.92	113.9 ± 147.29	.04	.56
Median (IQR)	132 (37–274)	57.8 (18–177)		
VDR				
Mean ± SD	5.9 ± 8.62	2.2 ± 3.06	.35	
Median (IQR)	0.48 (0.02–14.9)	0.44 (0.1–3.0)		
PiT1				
Mean ± SD	11.0 ± 10.39	32.9 ± 20.98	.0002	.003
Median (IQR)	6.71 (1.7–20.4)	31.3 (13.7–59.1)		
BMP4				
Mean ± SD	1.4 ± 1.32	1.4 ± 0.77	.33	
Median (IQR)	0.84 (0.6–2.8)	1.79 (0.7–2.1)		
BMP2				
Mean ± SD	10.1 ± 4.94	9.2 ± 6.61	.34	
Median (IQR)	9.61 (7.0–13.5)	11.6 (2.8–13.8)		
PTH2R				
Mean ± SD	1.6 ± 1.47	13.7 ± 6.12	.0001	.001
Median (IQR)	1.23 (0.6–1.83)	14.8 (7.7–16.3)		
PTHrP				
Mean ± SD	67.8 ± 39.37	71.6 ± 38.24	.53	
Median (IQR)	78.6 (31.03–91.2)	80.2 (33.1–115.4)		
RUNX2				
Mean ± SD	1.4 ± 1.81	1.2 ± 0.79	.72	
Median (IQR)	0.71 (0.5–1.4)	0.76 (0.6–1.8)		
Osteorix				
Mean ± SD	0.5 ± 0.41	0.8 ± 0.58	.08	
Median (IQR)	0.49 (0.3–0.7)	0.62 (0.4–1.0)		
Osteonectin				
Mean ± SD	3920 ± 2734	1097 ± 1597	.0007	.01
Median (IQR)	3162 (2203–5063)	576 (204–939)		
Osteopontin				
Mean ± SD	4083 ± 2197	859 ± 686	.0001	.001
Median (IQR)	3959 (2612–4362)	555 (409–1532)		
CA-II				
Mean ± SD	270.3 ± 185.40	94.6 ± 68.71	.003	.04
Median (IQR)	205 (162–349)	90.9 (38.5–140.6)		
CaSR				
Mean ± SD	5.0 ± 16.06	0.28 ± 0.55	.48	
Median (IQR)	0.15 (0.1–0.3)	0.11 (0.1–0.2)		
Osteocalcin				
Mean ± SD	2.7 ± 4.66	1.9 ± 2.80	.37	
Median (IQR)	0.73 (0.2–2.7)	0.50 (0.1–2.7)		
PTH1R				
Mean ± SD	0.07 ± 0.02	0.2 ± 0.02	.13	
Median (IQR)	0.08 (0.05–0.09)	0.1 (0.07–0.44)		
PiT2				
Mean ± SD	10.9 ± 5.82	15.2 ± 12.86	.40	
Median (IQR)	9.9 (5.6–16.6)	16.1 (2.5–27.3)		

Abbreviation: IQR, interquartile range.

[a] *P* values based on paired *t* test for the log-transformed values.

[b] *P* values adjusted for multiple comparisons.

radish peroxidase polymer system common for detection of both antirabbit and antimouse IgG (Thermo Scientific, Lab Vision Corporation). Color was developed with, 3,3′-diaminobenzidine, and sections were counterstained with hematoxylin. Slides for both gray matter and caudate nucleus for all 5 antigens were stained in one batch along with negative controls (no primary antibody) for both the tissues. Images were taken with a bright-field microscope (Nikon Instruments Inc).

Statistical analysis

Data are shown as mean ± SD, median and interquartile range (25th–75th percentile). The pairwise comparison between the expression of various molecules in the caudate and gray matter was carried out using a 2-sample paired *t* test on log-transformed data. Two-tailed $P < .05$ was considered significant. Statistical analysis was performed using SPSS version 11.0.

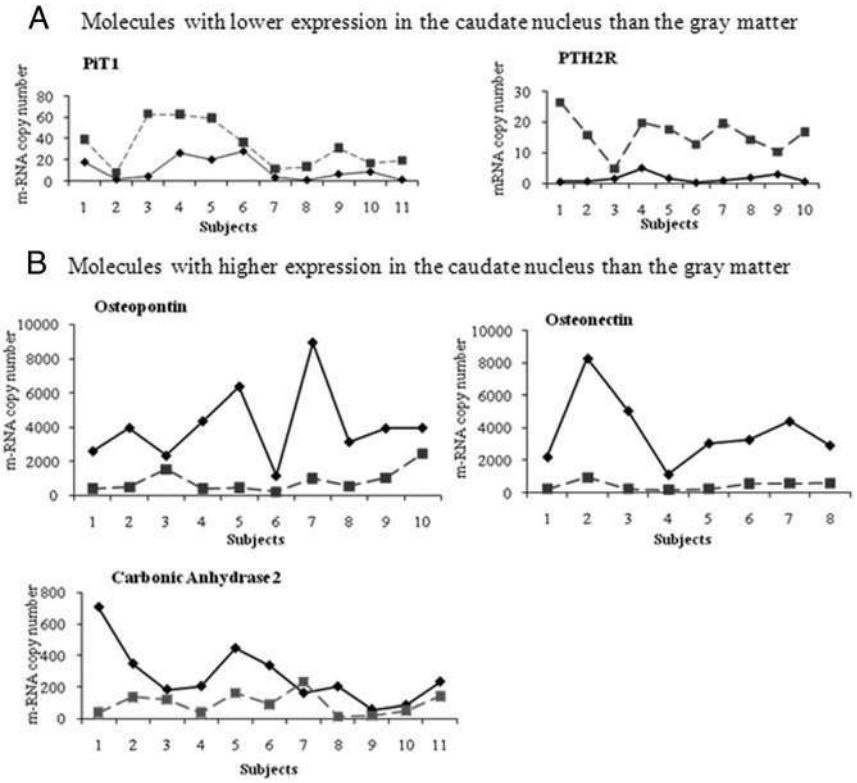

Figure 3. Pairwise comparison of mRNA expression of molecules in the caudate nucleus and gray matter (solid lines represent caudate nuclei; interrupted lines represent gray matter). Molecules with higher expression in the gray matter (A) and caudate (B).

Results

Figure 1 shows the hematoxylin and eosin staining of the caudate and gray matter obtained from a representative case showing the presence of mature neurons and glial cells in both the tissues along with organization into 6 layers in gray matter and the presence of pencil fibers (arrows) typical of the caudate nucleus (28). Expression of *DARPP32* assessed in 2 subjects was higher in the caudate than in the gray matter by 2- and 2–3 fold. Sufficient RNA with intact 28S and 18S bands was obtained in 11 cases from paired caudate nucleus and gray tissues (9 males and 2 females; mean age 36.1 ± 14.5 years), and the results are reported on these cases only. The mRNA expression of *PiT2* and *PTH1R* is reported for 5 subjects in view of the limitation of the cDNA available in paired samples from all the subjects. Figure 2 shows the specific amplification product for various molecules at endpoint RT-PCR. DNA sequencing confirmed the specificity of the molecules amplified (data not shown).

The mean mRNA copy number of various molecules and their difference between caudate and gray tissues are given in Table 2. The mean mRNA expression of *PiT1* (11.0 ± 10.39 vs 32.9 ± 20.98, $P = .003$) and *PTH2R* (1.6 ± 1.47 vs 14.3 ± 6.11, $P = .005$) was significantly lower in the caudate nucleus as compared with the gray matter. The differences in the expression remained significant even after Bonferroni correction for multiple testing (ie, multiplying with n = 14 molecules tested). In contrast, the expression of osteonectin, osteopontin, and *CA-II* were significantly higher in the caudate nucleus as compared with gray matter (corrected *P* values = .01, .001, and .04, respectively; Table 2). The mRNA expression of all other molecules was comparable between caudate nucleus and gray matter.

Figure 3 shows the pairwise comparison of the mRNA transcripts in the caudate nucleus and gray matter. There was consistency in the pattern of lower expression of *PiT1* and *PTH2R* and higher expression of osteonectin, osteopontin, and *CA-II* in the caudate nucleus in all samples as compared with gray tissue except in 1 pair of *CA-II*.

Western blot for protein expression was performed for PTH2R, CA-II, PiT1, osteopontin, and osteonectin because these showed significant differences in the mRNA expression between caudate and gray matter (Figure 4, A and B). All 5 proteins were expressed in both caudate and gray matter. However, the expression of both CA-II and osteonectin proteins as reflected in IDVs was 24% higher in the caudate tissue than the gray matter. The differences in PiT1 expression was 17% lesser in caudate than the gray matter. These differences conformed to the pattern of mRNA expression for these molecules. However, the differences in the PTH2R and osteopontin protein expression could not be appreciated on the IDVs on spot densitometry.

Immunohistochemistry (Figure 4C) showed specific staining for PTH2R, CA-II, PiT1, osteopontin, and osteonectin in the cytoplasm of the neuronal cells. The specific staining also confirmed the mRNA pattern obtained, with higher staining for CA-II, osteonectin, and osteopontin proteins in the caudate nucleus and higher expression of PTH2R and PiT1 protein in the gray matter.

Discussion

BGC is an interesting example of ectopic calcification observed in more than 24 disorders and can also be observed in normal subjects with aging (6). Although there is substantial information on molecular mechanisms of ectopic

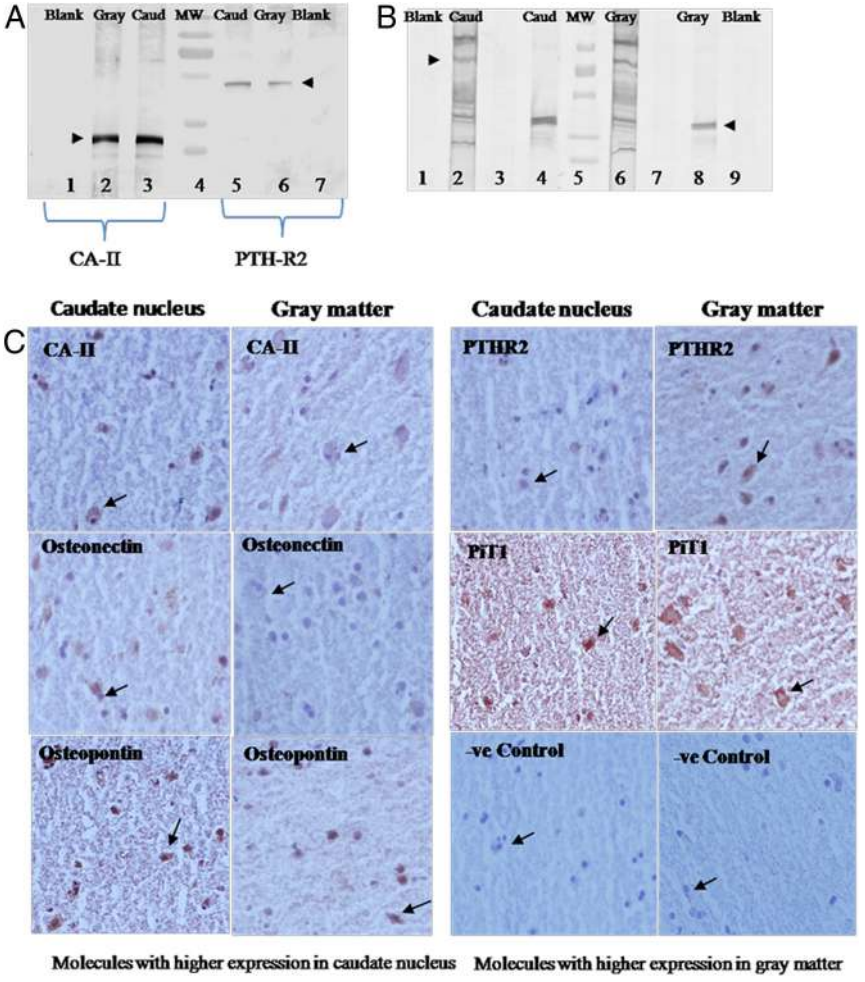

Figure 4. Western blots. A, Expression of CA-II protein (lanes 2 and 3) and PTH2R (lanes 5 and 6) in the caudate and gray matter of the brain; lanes 1 and 7 are blanks for caudate and gray matter, respectively (ie, without primary antibody), and lane 4 is a molecular weight (MW) marker. B, Lanes 2 and 6 showing PiT1 protein in the caudate and gray matter, respectively; lanes 4 and 8 showing osteonectin expression; lanes 1 and 9 are blank for caudate and gray matter, respectively, and lane 5 is a molecular weight (MW) marker. C, Immunohistochemistry showing comparison of specific staining for expression of proteins in the neurons of the caudate nucleus and gray matter (magnification, ×400).

calcification in vessels and the aortic cusp in chronic renal disease, similar information is not available for BGC (29–32). In idiopathic hypoparathyroidism, BGC is observed in up to 70% of cases, and its progression correlates with hyperphosphatemia (3). Although hyperphosphatemia has also been implicated in the ectopic calcification in chronic kidney disease, the molecular biology of BGC is likely to be different because BGC is not common in patients with chronic renal failure (33). The results of the present study provide information on molecules that could be involved in the process of intracranial calcification and predisposition of basal ganglia to such calcifications.

Expression of osteogenesis related molecules in intracranial tissues

The mRNA expression profile of the caudate nucleus and gray matter revealed the presence of several osteogenesis-promoting molecules including BMP2 and RUNX2 molecules. BMP2 and BMP4 in vascular, bone, and aortic cusp tissues promote expression of transcription factor RUNX2 and endochondral ossification (29, 30). Similarly, molecules supporting osteogenesis in bones such as osterix, VDR, CaSR, and type III phosphate transporters (PiT1) were also expressed in caudate and gray matter. In addition, there was a high expression of matrix-associated calcification-promoting osteonectin in both tissues. The profile of mRNA expression suggests the presence of molecules favoring active osteogenesis rather than passive metastatic or dystrophic deposition of minerals. In fact, the active process of calcification is also supported by previous studies showing lamellar deposition of hydroxyapatite crystals in the media and adventitia of vessels in regions of BGC (7–9).

Molecules likely to predispose basal ganglia for calcification

To understand the predisposition of the basal ganglia for calcification over other intracranial regions, surface gray matter was selected for comparison. Calcification at the surface of the gray matter is rare and observed only in a few diseases with underlying susceptibility (34). For example, gyral calcification in Sturge-Weber syndrome and after intrathecal methotrexate/cranial radiation therapy could be explained by leptomeningeal capillary-venous malformations and chemical/physical insult, respectively. Rarity of calcification of surface gray matter is also supported by autopsy studies in patients with extensive intracranial calcification. These studies revealed calcifications at the deeper gray-white junction but not at surface of the gyri (7, 8).

The pairwise comparison of caudate nucleus and surface gray matter in all study subjects revealed interesting differences in the expression of molecules of osteogenesis and resorption. There was a 3-fold reduced mRNA expression of the *PiT1* and similar increased expression of osteonectin in the caudate nucleus as compared with the gray matter. These findings assume significance in view of

a recent report of inactivating mutation of the *PiT2* gene in patients with familial BGC (10, 35). PiT1 and PiT2 are type III Na-phosphate transporters with ubiquitous distribution and are involved in transport of phosphorus in the body tissues, including brain cells (36). It is possible that reduced expression of PiT1 and/or PiT2 would not allow phosphate to enter the neuronal cells of the basal ganglia, pericytes, and medial vascular cells and remain trapped in the interstitium or adventitia of the vessels. Thus, in diseases associated with hyperphosphatemia such as hypoparathyroidism or *PiT2* mutation, a nidus with phosphate in the center is available for calcium hydroxyapatite deposition in the matrix of the vessels around basal ganglia. The mechanism for predisposition of basal ganglia for calcification can be analogous to a 2-hit mechanism. The first hit could be constitutively reduced expression of PiT1 and increased expression of osteonectin in caudate region as seen in the present study. The second hit could either be genetic as with *PiT2* mutation in familial BGC or acquired such as hyperphosphatemia in hypoparathyroidism. A combination of these 2 would synergistically facilitate calcification, especially in the basal ganglia region.

High prevalence of BGC in hypoparathyroidism

Although the above mechanism might explain BGC in idiopathic hypoparathyroidism, it would not explain the lack of predisposition to BGC in other hyperphosphatemic diseases like chronic renal failure and tumoral calcinosis (33). In this regard, an 8-fold reduced expression of *PTH2R* in the caudate nucleus compared with the gray matter provides an important clue. The subnormal circulating PTH in hypoparathyroidism coupled with reduced PTH2R would have a compounding effect on predisposition to BGC. Although tuberoinfundibular peptide of 39 residues (TIP-39) is a natural ligand for PTH2R, PTH can also bind to the same receptor in humans (37). On the contrary, high PTH in chronic kidney disease would have an inhibitory influence on osteogenesis in the vascular tissues (29). The importance of subnormal PTH action in facilitation of BGC is also supported by a report of reduced cAMP response after PTH injection in the cerebrospinal fluid of patients with Fahr's disease (38).

Significantly higher expression of CA-II and osteopontin mRNA and protein in the caudate than in the gray matter in the present study provides another reason for the predisposition of BGC in hypoparathyroidism. The increased prevalence of BGC in osteopetrosis with CA-II mutation indicates an important role of this protein in ossification process (4). A PTH-dependent increase in CA-II action and the resulting hydrogen ion production normally promotes mineral resorption in the bone (4). The high expression of CA-II normally observed in caudate nucleus as seen in the present study could be an example of natural adaptive response to prevent BGC. The subnormal PTH activity and resultant suppression of CA-II expression in idiopathic hypoparathyroidism would theoretically facilitate BGC. The high expression of the osteogenesis-inhibitory molecule osteopontin in the caudate region over gray matter observed in the present study could also be a natural adaptive response to protect against excessive calcification in the basal ganglia region.

In the present study, levels of *NMDAR2b, CaSR, VDR*, and *PTHrP* mRNA expression were comparable in the basal ganglia and gray matter. Thus, these molecules do not seem to be the major determining factor for the predilection of basal ganglia region to calcification. In this study, differences in their protein expression were not studied and are a limitation of the study. The inability to assess the expression of osteogenic molecules in the caudate and gray matter from subjects with BGC and lack of information on the biochemical parameters in the study subjects are other limitations. Although in the present work we have studied several factors possibly related to the process of osteogenesis in BGC, molecules such as receptor activator of nuclear factor κ-B ligand/receptors related to osteoclastogenesis, Wnt-catenin pathways, Msx transcription factor linked with membranous calcification, various calcium binding proteins, pyrophosphate, tissue nonspecific alkaline phosphatase, ectonucleotide pyrophosphatase/phosphodiesterase-1, ecto-5′-nucleotidase/CD73 enzyme, matrix Gla protein, fetuin-A, ligand for PTHR2 (ie, tuberoinfundibular peptide-39), klotho, and fibroblast growth factor-23 and its receptor were not studied. Further studies at cellular and molecular levels are needed to understand the role of these molecules in BGC

Thus, using principles of iterative hypothesis, the present study adds novel information on the presence of various osteogenic molecules in the caudate nucleus. The interplay of these factors in combination with unique biochemical abnormalities of the hypoparathyroid state can theoretically explain the increased prevalence of BGC in patients with hypoparathyroidism.

Acknowledgments

Address all correspondence and requests for reprints to: Dr R. Goswami, Professor, Department of Endocrinology and Metabolism, Room 304, All India Institute of Medical Sciences, New Delhi 110029, India. E-mail: gosravinder@hotmail.com.

This work has been funded by an intramural grant of the All India Institute of Medical Sciences, New Delhi.

Disclosure Summary: The authors have nothing to disclose, and there is no conflict of interest among the authors.

References

1. **Sachs C, Sjöberg HE, Ericson K.** Basal ganglia calcifications on CT: relation to hypoparathyroidism. *Neurology*. 1982;32:779–782.
2. **Rubin MR, Bilezikian JP.** Hypoparathyroidism: clinical features, skeletal microstructure and parathyroid hormone replacement. *Arq Bras Endocrinol Metabol*. 2010;54:220–226.
3. **Goswami R, Sharma R, Sreenivas V, Gupta N, Ganapathy A, Das S.** Prevalence and progression of basal ganglia calcification and its pathogenic mechanism in patients with idiopathic hypoparathyroidism. *Clin Endocrinol (Oxf)*. 2012;77:200–206.
4. **Ohlsson A, Cumming WA, Paul A, Sly WS.** Carbonic anhydrase II deficiency syndrome: recessive osteopetrosis with renal tubular acidosis and cerebral calcification. *Pediatrics*. 1986;77:371–381.
5. **Reske-Nielsen E, Lundbæk K, Rafaelsen OJ.** Pathological changes in the central and peripheral nervous system of young long-term diabetics. I. Diabetic encephalopathy. *Diabetologia*. 1965;1:233–344.
6. **Bonazza S, La Morgia C, Martinelli P, Capellari S.** Strio-pallido-dentate calcinosis: a diagnostic approach in adult patients. *Neurol Sci*. 2011;32:537–545.
7. **Norman RM, Urich H.** The influence of a vascular factor on the distribution of symmetrical cerebral calcifications. *J Neurol Neurosurg Psychiatry*. 1960;23:142–147.
8. **Miklossy J, Mackenzie IR, Dorovini-Zis K, et al.** Severe vascular disturbance in a case of familial brain calcinosis. *Acta Neuropathol*. 2005;109:643–653.
9. **Eaton ML, Camp JD, Love JG.** Symmetric cerebral calcification, particularly of the basal ganglia, demonstrable roentgenographically calcification of the finer cerebral blood vessels. *Arch Neurol Psychiatry*. 1939;41:921–942.
10. **Wang C, Li Y, Shi L, et al.** Mutations in SLC20A2 link familial idiopathic basal ganglia calcification with phosphate homeostasis. *Nat Genet*. 2012;44:254–256.
11. **Goswami R, Mondal AM, Tomar N, et al.** Presence of 25(OH)D deficiency and its effect on vitamin D receptor mRNA expression. *Eur J Clin Nutr*. 2009;63:446–449.
12. **Tomar N, Gupta N, Goswami R.** Calcium-sensing receptor autoantibodies and idiopathic hypoparathyroidism. *J Clin Endocrinol Metab*. 2013;98:3884–3891.
13. **Brené S, Lindefors N, Ehrlich M, et al.** Expression of mRNAs encoding ARPP-16/19, ARPP-21, and DARPP-32 in human brain tissue. *J Neurosci*. 1994;14:985–998.
14. **Lee MC, Ting KK, Adams S, Brew BJ, Chung R, Guillemin GJ.** Characterisation of the expression of NMDA receptors in human astrocytes. *PLoS One*. 2010;5:E14123.
15. **Li X, Yang HY, Giachelli CM.** Role of the sodium-dependent phosphate cotransporter, *Pit-1, in vascular smooth muscle cell calcification. Circ Res*. 2006;98:905–912.
16. **Kochanowska I, Chaberek S, Wojtowicz A, et al.** Expression of genes for bone morphogenetic proteins BMP-2, BMP-4 and BMP-6 in various parts of the human skeleton. *BMC Musculoskelet Disord*. 2007;8:128.
17. **Bagó AG, Palkovits M, Usdin TB, Seress L, Dobolyi A.** Evidence for the expression of parathyroid hormone 2 receptor in the human brainstem. *Ideggyogy Sz*. 2008;61:123–126.
18. **Feeley BT, Liu NQ, Conduah AH, et al.** Mixed metastatic lung cancer lesions in bone are inhibited by noggin overexpression and Rank:Fc administration. *J Bone Miner Res*. 2006;21:1571–1580.
19. **Kanno T, Takahashi T, Ariyoshi W, Tsujisawa T, Haga M, Nishihara T.** Tensile mechanical strain up-regulates Runx2 and osteogenic factor expression in human periosteal cells: implications for distraction osteogenesis. *J Oral Maxillofac Surg*. 2005;63:499–504.
20. **Huang L, Teng XY, Cheng YY, Lee KM, Kumta SM.** Expression of preosteoblast markers and Cbfa-1 and Osterix gene transcripts in stromal tumour cells of giant cell tumour of bone. *Bone*. 2004;34:393–401.
21. **Bonanno G, Perillo A, Rutella S, et al.** Clinical isolation and functional characterization of cord blood $CD133^+$ hematopoietic progenitor cells. *Transfusion*. 2004;44:1087–1097.
22. **Kadkol SS, Lin AY, Barak V, et al.** Osteopontin expression and serum levels in metastatic uveal melanoma: a pilot study. *Invest Ophthalmol Vis Sci*. 2006;47:802–806.
23. **Yoshiura K, Nakaoka T, Nishishita T, et al.** Carbonic anhydrase II is a tumor vessel endothelium-associated antigen targeted by dendritic cell therapy. *Clin Cancer Res*. 2005;22:8201–8207.
24. **Cañadillas S, Canalejo A, Santamaría R.** Calcium-sensing receptor expression and parathyroid hormone secretion in hyperplastic parathyroid glands from humans. *J Am Soc Nephrol*. 2005;16:2190–2197.
25. **Gronthos S, Zannettino AC, Hay SJ, et al.** Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci*. 2003;116(Pt 9):1827–1835.
26. **Tomar N, Bora H, Singh R, et al.** Presence and significance of a R110W mutation in the DNA-binding domain of GCM2 gene in patients with isolated hypoparathyroidism and their family members. *Eur J Endocrinol*. 2010;162:407–421.
27. **Goswami R, Brown EM, Kochupillai N, et al.** Prevalence of calcium sensing receptor autoantibodies in patients with sporadic idiopathic hypoparathyroidism. *Eur J Endocrinol*. 2004;150:9–18.
28. **Hagan CE, Bolon B, Keene CD.** Nervous system. In: Treuting PM, Dintzis SM, Frevert CW, Montine KS, eds. *Comparative Anatomy and Histology: A Mouse and Human Atlas*. 1st ed. San Diego, CA: Academic Press; 2012:339–394.
29. **Vattikuti R, Towler DA.** Osteogenic regulation of vascular calcification: an early perspective. *Am J Physiol Endocrinol Metab*. 2004;286:E686–E696.
30. **Chester AH.** Molecular and cellular mechanisms of valve calcification. Aswan Heart Centre Sci Pract Ser. 2011:4.
31. **Doherty TM, Asotra K, Fitzpatrick LA, et al.** Calcification in atherosclerosis: bone biology and chronic inflammation at the arterial crossroads. *Proc Natl Acad Sci U S A*. 2003;100:11201–11206.
32. **Ketteler M, Floege J.** Calcification and the usual suspect phosphate: still guilty but there are other guys behind the scenes. *Nephrol Dial Transplant*. 2006;21:33–35.
33. **Savazzi GM, Cusmano F, Musini S.** Cerebral imaging changes in patients with chronic renal failure treated conservatively or in hemodialysis. *Nephron*. 2001;89:31–36.
34. **Kırolu Y, Callı C, Karabulut N, Oncel C.** Intracranial calcifications on CT. *Diagn Interv Radiol*. 2010;16:263–269.
35. **Chen WJ, Yao XP, Zhang QJ, et al.** Novel SLC20A2 mutations identified in southern Chinese patients with idiopathic basal ganglia calcification. *Gene*. 2013;529:159–162.
36. **Miyamoto K, Haito-Sugino S, Kuwahara S, et al.** Sodium-dependent phosphate cotransporters: lessons from gene knockout and mutation studies. *J Pharm Sci*. 2011;100:3719–3730.
37. **Usdin TB, Gruber C, Bonner TI.** Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor. *J Biol Chem*. 1995;270:15455–15458.
38. **Smits MG, Gabreels FJ, Froeling PG, de Abreu RA, Thijssen HO, Renier WO.** Calcium-phosphate metabolism in autosomal recessive idiopathic strio-pallido-dentate calcinosis and Cockayne's syndrome. *Clin Neurol Neurosurg*. 1983;85:145–153.