

Calcium Supplements Increase the Serum Levels of Crosslinked N-Telopeptides of Bone Collagen and Parathyroid Hormone in Rachitic Nigerian Children

JOHN K. SCARIANO,¹ DOROTHY J. VANDERJAGT,¹ TOM THACHER,² CHRISTIAN O. ISICHEI,³
BRUCE W. HOLLIS,⁴ and ROBERT H. GLEW¹

¹Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, 915 Camino de Salud NE, Albuquerque, NM, 87131, USA, ²Department of General Medical Practice, and ³Department of Chemical Pathology, Jos University Teaching Hospital, Jos, Nigeria, West Africa, and ⁴Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, SC 29425, USA

Objectives: Biochemical markers of bone turnover were measured in the sera of 16 controls and 10 children with calcium-deficiency rickets, during a 12-week course of calcium supplementation (1 g CaCO₃/d) that was effective in healing the bone lesions of the rachitic children.

Design and methods: Serum levels of crosslinked N-telopeptides of bone collagen (NTx), parathyroid hormone (PTH), alkaline phosphatase (ALP), and urinary deoxypyridinoline (LP) were assayed at baseline and during the course of calcium therapy and compared with data of the 16 non-rachitic controls.

Results: Calcium therapy suppressed serum NTx and PTH levels in the rachitic children within 24 h; however, after the first week, PTH and NTx levels increased to the extent that at 12 weeks both were elevated when compared with controls or to baseline levels. Serum levels of NTx and PTH were correlated in the controls and experimental subjects ($r = 0.63$, $p < 0.001$).

Conclusions: The rate of bone resorption, as estimated by serum NTx concentration, is increased during the healing of rachitic lesions. Copyright © 1998 The Canadian Society of Clinical Chemists

KEY WORDS: rickets; calcium-deficiency; serum NTx; parathyroid hormone; alkaline phosphatase; deoxypyridinoline; children; Nigeria; bone collagen; bone markers.

Introduction

In a previous study (1), we showed that levels of the crosslinked carboxyterminal telopeptide of type I-collagen (ICTP) were elevated in the sera of

children in northern Nigeria with calcium-deficiency rickets. These rachitic children had moderate to severe hypocalcemia and significantly elevated circulating levels of 1,25-dihydroxyvitamin D (1,2).

The trivalent lysinylpyridinoline cross-link ICTP is formed in the mature extracellular matrix of bone and other tissues, and because it is released into the circulation as osteoclasts proteolyze collagen, it serves as a non-specific index of bone resorption (3). At least three different mechanisms could account for the increased serum levels of ICTP we observed in the rachitic Nigerian children: (a) an increase in the rate of bone resorption; (b) premature turnover of the inadequately mineralized collagenous matrix of rachitic bone; or (c) an increase in the amount of crosslinking in bone collagen that occurs when mineralization is deficient (4). A study by Sharp and Oginni (5) confirmed that Nigerian children with calcium-deficient rickets have increased turnover of type I and type III collagen, as assessed by their circulating levels of ICTP, the carboxyterminal type I collagen propeptides (PICP), and the aminoterminal propeptides of type I and type III collagen (PINP, PIIINP). However, while ICTP and the extension propeptides of type I and type III procollagen are reliable biochemical indices of collagen turnover, they are not specific for bone collagen.

A method was recently reported which permits measurement of the concentration of crosslinked N-telopeptides (NTx) of bone collagen in serum (6). NTx is a specific product of proteolysis of bone collagen by osteoclasts (7). In a population of elderly females it has been documented that serum concentrations of NTx correlate strongly with bone mineral density measurements made utilizing dual x-ray absorptiometry and to levels of circulating parathyroid hormone and thyroxine (Scariano and Glew,

Correspondence: Robert H. Glew, Ph.D., Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, 915 Camino de Salud NE, Albuquerque, NM 87131-5221. E-mail: rglew@salud.unm.edu

Manuscript received January 5, 1998; revised and accepted April 9, 1998.

This work was supported by a Minority International Research Training (MIRT) grant from the Fogarty International Council of the National Institutes of Health.

submitted for publication). The rate of NTx excretion is widely regarded as a highly specific index of bone resorption (8) and is sensitively suppressed in response to antiresorptive therapies (9,10) and vitamin D and calcium supplementation in vitamin D deficient elderly women (11).

To monitor the rate of bone resorption using a more specific marker, we measured serum levels of NTx, along with alkaline phosphatase, intact parathyroid hormone and urinary deoxypyridinoline (LP) in 10 subjects with radiographically confirmed rickets and 16 non-rachitic age-matched controls. LP is a lysinylpyridinoline crosslink that is abundant in bone collagen and has been widely used as an index of bone turnover (12). These biochemical markers of bone metabolism were monitored in the 10 rachitic subjects before and at least three times during the 12-week course of a therapy that effectively normalized their serum calcium concentrations and, on the basis of radiographic evidence, was effective in healing their bone lesions as well.

Methods and Materials

SELECTION OF THE EXPERIMENTAL AND CONTROL POPULATIONS

Children presenting with the skeletal abnormalities typical of rickets were screened by pediatricians at the Jos University Teaching Hospital in Jos, Nigeria (JUTH) and selected for inclusion in the experimental study group if there was radiographic evidence of active disease. Bone deformities that included genu varum, genu valgum, widening of the distal epiphyseal plates of the wrists and ankles, windswept deformity, beading of the ribs, opening of the anterior fontanelle, cranial bossing, or presence of a rachitic rosary were considered adequate criteria for additional radiologic examination. Subjective criteria of rickets such as leg pain at rest, the inability to walk, frequent falling, or pain upon walking or running were noted. To satisfy the inclusion criteria of the study, the rachitic children must have exhibited radiologic evidence of three of the following five conditions: metaphyseal widening of the wrist or ankle, fraying or cupping of the metaphysis, osteopenia and pathological fracture. Ten of the 16 children we screened met these criteria. They were given 500 mg tablets of calcium carbonate to be taken twice daily for 12 weeks. Sixteen healthy children without evidence of skeletal abnormalities were matched to the rachitic subjects by age, sex, weight and height (Table 1).

Serum and 24-h urine specimens were obtained from the control children at the same time the experimental subjects were enrolled in the study. Serum and 24-h urine specimens were obtained from each subject before treatment, and at intervals of 24 h, and 1, 4, and 12 weeks after calcium supplementation was initiated. The specimens were stored at -40°C , until which time they were transported to Albuquerque on dry ice. At the end of the

TABLE 1
Relevant Anthropometric Parameters of Study Subjects and Controls

	Rachitic Subjects (<i>n</i> = 10)	Controls (<i>n</i> = 16)
Females/Males	7/3	9/7
Age (months)	49 ± 24 ^a	44 ± 22
Height (m)	0.89 ± 0.14 ^a	0.88 ± 0.11
Weight (kg)	13.8 ± 4.8 ^a	11.8 ± 1.8

^aStatistically insignificant ($p > 0.05$). Values given are ± 1 standard deviation.

study period, each of the study subjects underwent a thorough physical examination, their primary caretakers were interviewed, and their recovery was graded as complete, partial, minimal, or unchanged after radiographic examination. All caretakers gave informed consent and the study was conducted in accordance with the Helsinki Declaration and approved by the Human Research Review Committee at the University of New Mexico School of Medicine and the Ethics Review Committee at JUTH.

BIOCHEMICAL ANALYSIS OF SERUM AND URINE

The measurements of calcium, phosphorus, alkaline phosphatase, and creatinine in serum were performed with the aid of a Kodak DT-60 analyzer (Johnson and Johnson Clinical Diagnostics, Rochester, NY, USA). Quality control materials were a gift of the Clinical Chemistry section of the University of New Mexico Hospital Pathology Laboratory. Serum calcium was measured with an Arsenazo III kinetic dye binding assay monitored at 680 nm and pH 5.6, and phosphorus was quantified by reduction of an ammonium phosphomolybdate complex measured at 660 nm, pH 4.2. The method utilized for determining alkaline phosphatase activity was originally described by Bessey, Lowry and Brock as modified by Bowers and McComb (13) and relies on the rate of nitrophenoxide production at pH 10.5 monitored at 400 nm. Prior to assay, aliquots of sera were incubated at 22° C for 18–24 h to ensure full recovery of ALP activity (14). Urinary creatinine was determined utilizing a modified alkaline picrate method (Sigma Chemicals, St. Louis, MO, USA). Creatinine was measured in serum to exclude subjects or controls having disorders in renal function. Calcium levels in urine were quantified by a modification of an ortho-cresolphthalein complexone dye binding assay containing 8-hydroxyquinoline (pH 11) and which was monitored at 575 nm and optimized for maximum sensitivity (Sigma Chemicals). The within-assay coefficient of variation (CV) of the serum and urine measurements was less than 10%. Intact parathyroid hormone was measured utilizing a double antibody sandwich radioimmunoassay (INCSTAR, Stillwater, MN, USA). The within-assay CV for the PTH determination was less than 10%.

BIOCHEMICAL PARAMETERS OF BONE TURNOVER

Urinary deoxypyridinoline

The deoxypyridinoline concentration of an aliquot of a 24-h urine collection was determined utilizing an enzyme-immunoassay kit (Pyrilinks-D®, Metra Biosystems, Mountain View, CA, USA) and read on a 96-well ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Nanomolar LP levels were standardized to millimolar urinary creatinine concentration. The within-assay CV of the LP determination was less than 10%.

Serum NTx

Serum levels of crosslinked N-telopeptides of type I collagen (NTx) were determined using an enzyme-linked competitive inhibition immunosorbent assay (ELISA). The immunoassay utilizes a specific monoclonal antibody (mAb 1H11) that was developed for the measurement of NTx in human urine (8). The antibody mAb 1H11 was conjugated to horseradish peroxidase (HRP) and polystyrene microtiter plates were coated by passive absorption with NTx antigen. Test specimens and assay standards were diluted in buffer and combined with mAb 1H11-HRP in microtiter wells. Following incubation and subsequent washing with detergent solution, a hydrogen peroxide/tetramethylbenzidine buffer was added for color development. The color intensity was measured spectrophotometrically at 450 nm with a 630 nm reference filter. Final assay results are reported as nanomoles of bone collagen equivalents (nmol BCE)/L. Assay precision was <8% CV, with a lower limit of detection of 1.0 nmol BCE/L (6).

STATISTICAL ANALYSIS

All analyses were calculated using the Number Crunching Statistical Software program NCSST version 6.0 (Jerry Hintze, Kayesville, UT, USA). Statistical comparisons were made using paired *t*-tests for observations made within the population of rachitic subjects during the course of calcium therapy. For comparisons made between the rachitic and control children, a two-sample *t*-test was calculated for normally distributed data (calcium, phosphorus, NTx, and LP) and a non-parametric two sample *t*-test (Mann-Whitney test) was utilized for analysis of PTH and ALP data, which were not uniformly distributed. Spearman-Rank correlations were calculated for comparisons of serum NTx and PTH levels using samples obtained from control children and rachitic children after 12 weeks of therapy.

Results

After 12 weeks of calcium supplementation (1 g CaCO₃/d) the lesions of rickets were healing in all 10 children in the experimental group. Radiographic

and clinical criteria of recovery are described in detail elsewhere (Thacher and Glew, in press). The serum calcium concentration of 5 of the 10 children with rickets was below 2.12 mmol/L at the beginning of the study; however, these levels quickly increased and surpassed the lower limit of the method-specific pediatric reference range (2.25 mmol/L) after 1 week of calcium therapy. By the 12th week of supplementation, the mean total serum calcium levels of the rachitic children had increased from 2.17 to 2.37 mmol/L ($p = 0.02$, Table 2). At a median value of 568 U/L, serum alkaline phosphatase activity (ALP) was initially elevated above what has been defined as an age- and method-specific pediatric reference limit of 420 U/L (15) but had decreased after 12 weeks of therapy to 399 U/L ($p = 0.03$). However, by the end of the 12-week study period, the median serum level of ALP in all 10 of the recovering rachitic children was still elevated two-fold relative to the ALP levels of the control subjects ($p = 0.0005$, Table 2, Figures 1 and 2).

SERUM LEVELS OF NTx AND INTACT PARATHYROID HORMONE IN RACHITIC CHILDREN BEFORE AND AFTER CALCIUM THERAPY

As shown in Figure 3, serum levels of NTx in the rachitic children before calcium supplementation was initiated were not significantly different from those of age- and sex-matched controls. After 12 weeks of calcium supplementation, however, the mean serum concentration of NTx in the rachitic group was significantly increased when compared with the NTx levels in either the control group (110.6 vs. 67.8 nmol BCE/L, $p = 0.006$, Table 2, Figure 3) or with baseline values obtained before calcium supplements were provided (69.2 nmol BCE/L, $p = 0.01$, Table 2, Figure 3). Similarly, levels of PTH in rachitic children were not significantly different from control values until the 12th week of calcium supplementation when the median PTH concentration had reached 197.9 pmol/L, a level that was significantly elevated when compared with the baseline (18.9 pmol/L) or the control median of 86.1 pmol/L, ($p = 0.02$, Table 2). As shown in Figure 4, the serum levels of NTx and PTH were both rapidly suppressed in a parallel manner by calcium supplementation; however, NTx and PTH levels began to rise after 1 week of calcium therapy, reaching their highest levels at the end of the study. We calculated a Spearman's-Rank correlation coefficient of 0.63 ($p < 0.001$) for comparisons of serum concentrations of NTx and PTH in the control and experimental populations at the end of the study (Figure 5). As in the case of ICTP (1), we found that serum NTx levels in non-rachitic controls declined steadily from infancy to prepuberty (Figure 6a).

TABLE 2
Relevant Biochemical Parameters of Bone Metabolism in Rachitic Children Before and After 12 Weeks of Calcium Therapy as Compared With Control Population

	RACHITIC SUBJECTS (n = 10)		CONTROLS (n = 16)
	Pre-calcium supplementation Mean ± SD	Post-calcium supplementation Mean ± SD	Mean ± SD
Serum parameters:			
Calcium (mmol/L)	2.17 ± 0.17 ^b	2.37 ± 0.15 ^c	2.42 ± 0.15
Phosphorus (mmol/L)	1.45 ± 0.42 ^b	1.45 ± 0.29 ^b	1.74 ± 2.9
Alkaline phosphatase activity (U/L)	568 (383–1500) ^b	399 (274–1011) ^{bc}	237 (163–320)
Intact parathyroid hormone (pmol/L)	18.9 (3.6–558.9)	197.9 (74.8–402.3) ^c	86.1 (10.7–282.7)
NTx (nmol BCE/L) ^a	69.2 ± 31.5	110.6 ± 36.9 ^{bc}	67.8 ± 34.6
Urinary parameters:			
Deoxypyridinoline (nmol/mmol creatinine)	78.6 ± 19.8 ^b	49.3 ± 15.7 ^c	46.2 ± 15.4
Calcium (mmol/d)	0.63 ± 0.44	1.12 ± 0.68	0.48 ± 0.46

Alkaline phosphatase (ALP) and parathyroid hormone (PTH) concentrations are represented as medians with their respective ranges because these values are not normally distributed as are calcium, NTx, LP, and phosphorus.

NTx, cross-linked N-telopeptides of bone collagen; SD, standard deviation.

^aSerum NTx values are given in nanomoles of bone collagen equivalents per liter.

^bStatistically significant difference as measured by a two-sample *t*-test (parametric or nonparametric) when compared with control value ($p < 0.05$)

^cStatistically significant difference as measured by a paired *t*-test when compared with presupplementation value ($p < 0.05$).

DEOXYPYRIDINOLINE EXCRETION IN HEALTHY AND RACHITIC CHILDREN

The urinary excretion of LP in the rachitic children at the time they entered the study was elevated when compared with age- and sex-matched controls (78.6 nmol/mmol *vs.* 46.2 nmol/mmol, $p < 0.001$, Table 2, Figure 7). All 10 of the rachitic children had urinary levels of LP which were elevated when compared with their respective age- and sex-matched control, and 7 out of 10 rachitic children had urinary LP concentrations that were approximately twice those of their respective controls. As

shown in Figures 2 and 7, during the course of calcium supplementation, the mean level of urinary deoxypyridinoline steadily decreased in the rachitic children from 78.6 nmol/mmol creatinine before treatment to 49.3 nmol/mmol after 12 weeks, a value which was not significantly different from that of the mean of the control group (46.2 nmol/mmol creatinine, Figure 7). Noteworthy is the fact that all 10 of the rachitic children who entered the study manifesting significantly elevated urinary excretion of LP had normalized LP levels by the 12th week of calcium therapy. Urinary excretion of LP in non-rachitic children follows the same expected pattern of age-dependency (Figure 6B) as do serum levels of NTx.

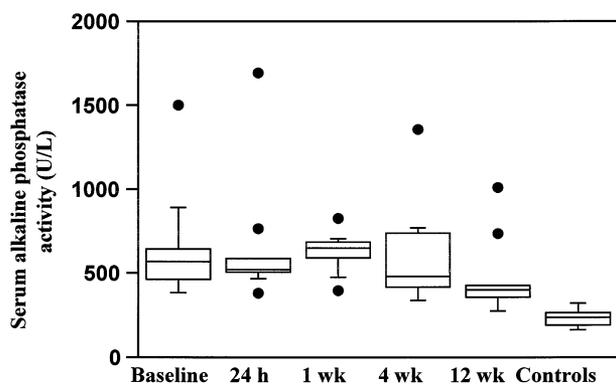


Figure 1 — Box plot showing alkaline phosphatase levels of 16 age-matched controls and the 10 rachitic subjects during 12 weeks of calcium supplementation. Box limits demarcate the 25th and 75th percentile with the median represented as a solid horizontal line. Lines extending from box are maximum and minimum values that fall within the distribution. Circles represent outliers.

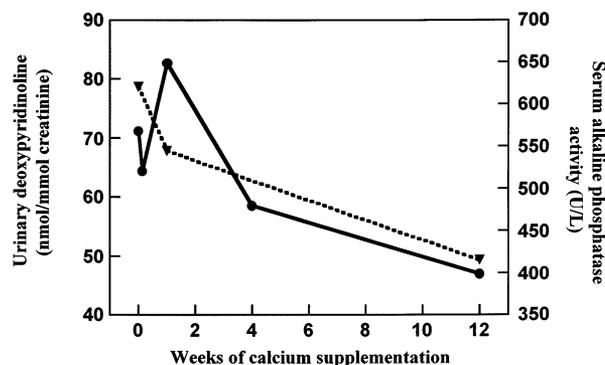


Figure 2 — Median levels of serum alkaline phosphatase (circles, uninterrupted line) and mean levels of urinary deoxypyridinoline (triangles, dashed line) of the 10 rachitic children during the course of 12 weeks of calcium therapy (1 g CaCO₃/d).

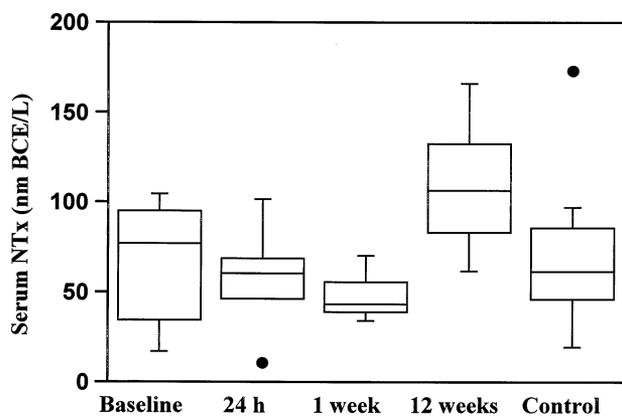


Figure 3 — Box plot showing serum NTx levels of 16 age-matched controls and the 10 rachitic subjects during 12 weeks of calcium supplementation. Box limits demarcate the 25th and 75th percentile with the median represented as a solid horizontal line. Lines extending from box are maximum and minimum values that fall within the distribution. Circles represent outliers.

Discussion

The most significant finding of the present study was the strong association we noted between serum levels of PTH and NTx, a product of osteoclast proteolysis of bone collagen, in the rachitic subjects at baseline and during the course of calcium supplementation. In a study of 202 healthy elderly postmenopausal women (age 60–90 years) we conducted recently in New Mexico (Scariano and Glew, submitted for publication), and in a separate investigation of pre- and postmenopausal Nigerian women (Baca and Scariano, unpublished observations), we found a statistically significant correlation between serum NTx and intact PTH levels. Of additional interest was the significant increase of PTH secretion we observed in each rachitic subject in response to calcium therapy. It appears that the expected feedback inhibition exerted by calcium on parathyroid hormone secretion was blunted in the rachitic children.

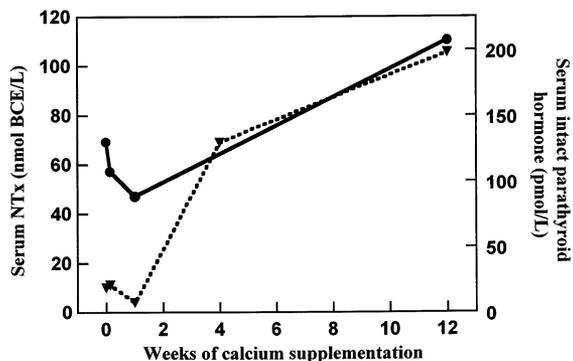


Figure 4 — Mean levels of serum NTx (circles, uninterrupted line) and median levels of intact parathyroid hormone (triangles, dashed line) of the 10 rachitic children during the course of 12 weeks of calcium therapy (1 g CaCO₃/d).

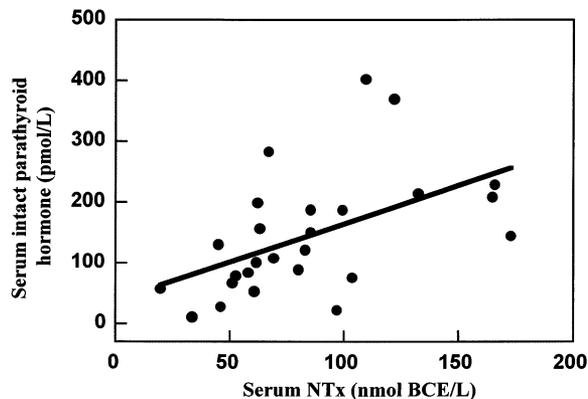


Figure 5 — Scatter plot showing correlation between serum levels of NTx and intact parathyroid hormone (PTH) in 10 rachitic children after 12 weeks of calcium therapy and 16 age-matched controls ($r = 0.63$, $p < 0.001$).

dren. The finding that levels of intact PTH in the sera of most of the rachitic children before treatment were normal conflicts with the conclusions of previous studies which reported that rachitic children

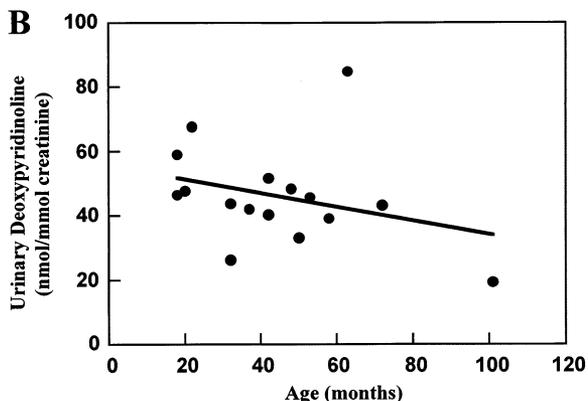
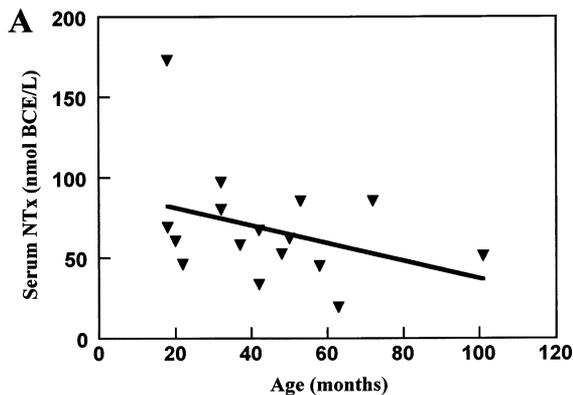


Figure 6 — (A) Scatter plot showing serum NTx as a function of age in 16 non-rachitic Nigerian children who served as controls. (B) Scatter plot showing urinary deoxypyridinoline excretion as a function of age in 16 non-rachitic Nigerian children who served as controls.

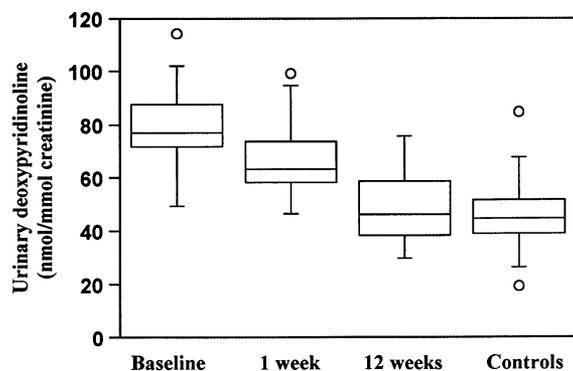


Figure 7 — Box plot showing urinary deoxyypyridinoline excretion of 16 age-matched controls and the 10 rachitic subjects during 12 weeks of calcium supplementation. Box limits demarcate the 25th and 75th percentile with the median represented as a solid horizontal line. Lines extending from box are maximum and minimum values that fall within the distribution. Circles represent outliers.

have secondary hyperparathyroidism associated with calcium deficiency (16,17).

Our results suggest that PTH plays an important role in stimulating bone remodeling and healing in rachitic children. The finding of significantly increased concentrations of NTx and PTH in the sera of the rachitic children after 1 week of therapy also indicates that the calcium supplementation they received had enabled them to overcome an impediment to bone remodeling. It appears that bone resorption, a prerequisite to bone remodeling, then continued at a rate which was greater than that which was occurring in the controls. The present report not only underscores the importance of PTH as a mediator of osteoclast activity (18), but also suggests that PTH is required for remodeling and growth of bone in children who are recovering from rickets.

While serum NTx and PTH levels were both significantly elevated when compared to the controls after 12 weeks of calcium supplementation, the activity of serum ALP and the urinary excretion of deoxyypyridinoline were consistently suppressed by calcium therapy. The significant elevations we observed in the urinary excretion of LP in untreated rachitic subjects in the present study, as well as the increase in serum ICTP levels we reported in our previous study (1), seem paradoxical in the context of the normal baseline NTx levels of these children. Our results are, however, consistent with other studies which have demonstrated different sensitivities in the response of peptide-bound and free pyridinoline collagen crosslinks to estrogen replacement therapy (10,19), and bisphosphonate therapy (11,20,21), and vitamin-D supplementation (11).

One explanation for the apparent discrepancies in the baseline levels and response of LP and NTx to calcium supplementation is that the peptide-bound LP precursor and NTx are metabolized or excreted differently. Clearly, additional studies of the metab-

olism and renal handling of ICTP, NTx, and peptidyl-LP are warranted. Based on the results of the present study, and for the following reason we conclude that a serum based biochemical marker of bone turnover in rachitic children may be superior to a urinary measurement of the same analyte. Administration of large doses of calcium can cause a significant diuresis (22), which in turn results in a dilution of urinary biochemical markers. Given the fact that creatinine may be secreted by renal tubules (23), creatinine concentrations may vary independently in relation to the concentration of urinary bone markers. This effect may render urinary marker values more vulnerable to error when compared to a serum marker of bone turnover such as NTx when assessing the metabolic activity of the skeleton of an individual who is taking calcium supplements.

The fact that serum alkaline phosphatase activity in the rachitic children had declined during the period of supplementation, but remained elevated after 12 weeks of calcium therapy, demonstrates that bone formation, as measured by an index of osteoblast activity, was still occurring at an accelerated rate in the rachitic children at the end of the 12-week study period.

In summary, we have shown that NTx and PTH increase in tandem when rachitic children are administered therapeutically efficacious calcium supplements, thereby providing evidence that markers of endocrine and bone metabolism are tightly coupled. Increases in the serum concentration of NTx and PTH were correlated to the correction of hypocalcemia, decreases in alkaline phosphatase activity and to a favorable clinical response to calcium therapy. Because measurements of collagen metabolites in serum are not influenced by errors introduced by creatinine excretion (23), nor by the pronounced diuretic effect that calcium supplementation has on the renal tubules, we conclude that the measurement of NTx in serum is a reliable indicator of healing bone lesions in calcium-deficient rachitic children.

Acknowledgements

The authors express their gratitude to Dr. J. Daniel Clemens, Joyce Rafferty, Gary Rosenberg, and Rob Mullarkey at Ostex International, Seattle, WA, for providing the reagents and instruments necessary for measuring NTx in serum and Terri Wallace and Dr. Leslie Danielsen of the University of New Mexico School of Medicine for donating quality-control materials.

References

1. Scariano JK, Walter EA, Glew RH, *et al.* Serum levels of the pyridinoline crosslinked carboxyterminal telopeptide of type I collagen (ICTP) and osteocalcin in rachitic children in Nigeria. *Clin Biochem* 1995; 28: 541-5.
2. Walters EA, Scariano JK, Easington CR, *et al.* Rickets and protein-malnutrition in northern Nigeria. *J Trop Ped* 1997; 43: 98-102.

3. Valimaki MJ, Tahtela R, Jones JD, Peterson JM, Riggs BL. Bone-resorption in healthy and osteoporotic postmenopausal women: Comparison markers for serum carboxy-terminal telopeptide of type-I collagen and urinary pyridinium cross-links. *Eur J Endocrinol* 1994; 131: 258–62.
4. Eyre DR, Paz MA, Gallop PM. Cross-linking in collagen and elastin. *Ann Rev Biochem* 1984; 53: 717–48.
5. Sharp CA, Oginni LM, Worsfold M, *et al.* Elevated collagen turnover in Nigerian children with calcium-deficient rickets. *Calcif Tissue Int* 1997; 61: 87–94.
6. Clemens JD, Herrick MV, Singer FR, Eyre DR. Evidence that serum NTx (collagen type I N-telopeptides) can act as an immunochemical marker of bone resorption. *Clin Chem* 1997; 43: 2058–63.
7. Apone S, Lee MY, Eyre DR. Osteoclasts generate crosslinked N-telopeptides (NTx) but not free pyridinolines when cultured on human bone. *Bone* 1997; 21: 129–36.
8. Hanson DA, Weiss MA, Bollen AM, Maslan SL, Singer FR, Eyre DR. A specific immunoassay for monitoring human bone resorption. Quantification of type-I collagen crosslinked N-telopeptides in urine. *J Bone Miner Res* 1992; 7: 125–8.
9. Raisz LG, Wiita B, Artis A, *et al.* Comparison of the effects of estrogen alone and estrogen plus androgen on biochemical markers of bone formation and resorption in post menopausal women. *J Clin Endocrinol Metab* 1996; 121: 31–43.
10. Garnero P, Shih WCJ, Gineyts E, Karpf DB, Delmas PD. Comparison of new biochemical markers of bone turnover in late postmenopausal osteoporotic women in response to alendronate treatment. *J Clin Endocrinol Metab* 1994; 79: 1693–1700.
11. Kamel S, Brazier M, Rogez, JC, *et al.* Different responses of free and peptide-bound cross-links to vitamin-D and calcium supplementation in elderly women with vitamin-D insufficiency. *J Clin Endocrinol Metab* 1996; 81: 3717–21.
12. Daniloff GY, Hesley RP, Ju J, Evans BJ, He P, Seyedin SM. An immunoassay for deoxypyridinoline: a highly specific marker of bone-resorption. *J Bone Miner Res* 1993; 8(S1): S357.
13. Bowers GN Jr, McComb RB. A continuous spectrophotometric method for measuring the activity of alkaline phosphatase. *Clin Chem* 1966; 12: 70–89.
14. Moss DW, Henderson AR. Enzymes. In: Burtis CA, Ashwood ER, eds. *Tietz textbook of clinical chemistry*. 2nd ed. Pp. 831–6. Philadelphia: WB Saunders Co., 1994.
15. Meites S. *Pediatric clinical chemistry reference (normal) values*. Washington: AACC Press, 1989.
16. Okonofua F, Gill DS, Alabi ZO, Thomas M, Bell JL, Dandona P. Rickets in Nigerian children: a consequence of calcium malnutrition. *Metabolism* 1991; 40: 209–13.
17. Pettifor JM, Ross P, Wang J, Moodley G, Couper-Smith J. Rickets in children of rural origin in South Africa: is low dietary calcium a factor? *J Peds* 1978; 92: 320–4.
18. Fitzpatrick LA, Bilezikian JP. Actions of parathyroid hormone. In: Bilezikian JP, Raisz LG, Rodan G, eds. *Principles of bone biology*. Pp. 339–44. San Diego: Academic Press, 1996.
19. Prestwood KM, Pilbeam CC, Burleson JA, *et al.* The short-term effects of conjugated estrogen on bone turnover in older women. *J Clin Endocrinol Metab* 1994; 79: 366–91.
20. Blumsohn A, Naylor KE, Assiri AMA, Eastell R. Different responses of biochemical markers of bone-resorption to bisphosphonate therapy in Paget's disease. *Clin Chem* 1995; 41: 1592–8.
21. Garnero P, Gineyts E, Arbault P, Christiansen C, Delmas PD. Different effects of bisphosphonate and estrogen therapy on free and peptide bound bone cross-link excretion *J Bone Miner Res* 1995; 10: 6491–9.
22. Suki WN, Rouse D. Renal transport of calcium, magnesium and phosphorus. In: Brenner BM, ed. *Brenner and rector's the kidney*. 5th ed. Pp. 482–3. Philadelphia: WB Saunders Co., 1996.
23. Perrone RD, Madias NE, Levey AS. Serum creatinine as an index of renal function: new insights into old concepts. *Clin Chem* 1992; 38: 1933–53.