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# Utility of Biochemical Markers of Bone Turnover in the Follow-up of Patients Treated with Bisphosphonates

**H. N. Rosen,[1,2,3] A. C. Moses,[2] J. Garber,[2,4] D. S. Ross,[5] S. L. Lee,[6] S. L. Greenspan[1,2,3]**

[1]The Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory of the Beth Israel Deaconess Medical Center, Department of Medicine, Division of Gerontology, Boston, Massachusetts 02215, USA
[2]The Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory of the Beth Israel Deaconess Medical Center, Department of Medicine, Division of Endocrinology, Boston, Massachusetts 02215, USA
[3]The Charles A. Dana Research Institute and the Harvard-Thorndike Laboratory of the Beth Israel Deaconess Medical Center, Department of Medicine, Division of Bone and Mineral Metabolism, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215, USA
[4]Endocrinology section, Health Centers Division, Harvard Pilgrim HealthCare, Boston, Massachusetts 02215, USA
[5]Thyroid Unit, Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts 02215, USA
[6]Endocrinology, Diabetes, Metabolism, and Molecular Medicine Division, Department of Medicine, New England Medical Center, Boston, Massachusetts 02215, USA

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**Abstract.** Biochemical markers of bone turnover are often measured in patients treated with antiresorptive agents to monitor the effects of therapy. In order for a change in these markers to clearly indicate treatment effect, the change in the markers must exceed the amount of spontaneous variation typically seen with no treatment. Based on the measured long-term variability of markers in untreated patients, we defined a minimum significant change (MSC), that is, a change that was sufficiently large that it was unlikely to be due to spontaneous variability. We also examined the changes in markers of bone turnover in subjects treated with pamidronate to see how often observed changes in turnover after treatment exceeded the MSC. We found that urinary markers of bone resorption are best measured on 2-hour fasting samples, because results on random urine showed poor precision and less decline with therapy. We also found that of all the markers, urinary N-telopeptide cross-links (NTX) had the greatest decline after therapy (58%), although it also had the highest long-term variability (29.5%). The marker that most often showed a decline with treatment that exceeded the MSC was serum bone-specific alkaline phosphatase where 74% of observed changes exceeded the MSC. Other markers that often showed a decline with treatment that exceeded the MSC were 2-hour fasting urine NTX and free deoxypyridinoline, where 57% and 48%, respectively, of changes in therapy exceeded the MSC. The ideal marker would combine the large decline after treatment characteristic of NTX (60–70%) with the good precision of bone-specific alkaline phosphatase.

*Correspondence to:* H. N. Rosen, Division of Gerontology, RA414, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston, MA 02215, USA.

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Although millions of patients receive treatment for osteoporosis, it is not clear how these patients should be followed [1, 2]. Longitudinal monitoring of therapy for osteoporosis is important because some patients do not respond well to standard doses of therapy [3–6]. For instance, patients taking anticonvulsants catabolize estrogen at an accelerated rate [7], and these patients are not always optimally protected at standard doses of estrogen. Furthermore, the absorption of bisphosphonates is poor [8], and absorption may be inadequate in patients with small bowel resection or hemigastrectomy. In addition, follow-up is important because patients may not comply with expensive or complex drug regimens for years if there is no feedback regarding a beneficial effect. Therefore there is a need to document that drugs commonly prescribed for osteoporosis are producing the desired effect.

Efficacy of osteoporosis therapy can be documented by serial measurements of bone mineral density (BMD). However, since changes in BMD can be detected only after years, there is a need for a more rapid way to demonstrate the effect of therapy. Since the currently approved therapies for osteoporosis are antiresorptive agents, it seems reasonable to document efficacy by measuring a decrease in bone turnover. Levels of currently available biochemical markers of bone turnover decline within weeks of initiating antiresorptive therapy [9–11], and these markers are reasonable tools for demonstrating promptly that antiresorptive therapy is having the desired effect [1, 2].

Although biochemical markers of bone turnover fall after treatment with antiresorptive therapy, there are some

problems interpreting the changes in these markers during treatment. First, there is spontaneous long-term variability in these markers even when no interventions are made [12–15]. In order to demonstrate efficacy of antiresorptive therapy, observed changes must systematically exceed the changes likely to occur spontaneously. Second, the markers differ in degree of long-term variability and the degree to which they decline with antiresorptive therapy [12]. Therefore, the extent of suppression necessary to prove antiresorptive efficacy for each marker needs to be defined based on *both* its long-term variability and on the anticipated degree of suppression with antiresorptive therapy.

In a study of the efficacy of bisphosphonates in preventing thyroid hormone-induced bone loss [16], we had the opportunity to follow several markers of bone turnover over a 1-year period in patients treated with pamidronate (APD) or placebo every 3 months. In the current study we investigated the spontaneous variability of these markers and the degree of suppression of bone turnover observed after bisphosphonate treatment. We also examined the likelihood that treatment with bisphosphonate would result in a decrease in a marker sufficiently great that it can be clearly attributed to therapy rather than to spontaneous variability.

## Materials and Methods

### *Subjects*

Fifty-five patients who had papillary carcinoma of the thyroid and were taking stable suppressive doses of thyroxine were recruited for a study of bone loss. Subjects were excluded if there was clinical evidence for bony metastases by radioiodine scanning or if they had elevations of serum alkaline phosphatase. Subjects were accepted only if they had been on suppressive doses of thyroxine for at least 6 months, and if they were free of medical conditions that might alter bone metabolism, such as Paget's disease, hyper- and hypoparathyroidism, myeloma, renal or hepatic failure, renal tubular acidosis, malabsorption, or Cushing's syndrome. Subjects were screened for these conditions by a history, physical examination, and measurement of complete blood count and serum chemistries during a screening visit to the General Clinical Research Center (GCRC) at Beth Israel Deaconess Medical Center. Subjects were recruited without regard for racial, social, economic, or other status. This protocol was approved by the Beth Israel Hospital Committee on Clinical Investigations, and written informed consent was obtained from all subjects. Among the 55 subjects, 40 agreed to hospital admission for periodic timed collection of samples, allowing us to collect both random (late afternoon) and 2-hour fasting urine. Since one of the goals of our study was to compare the utility of random and 2-hour fasting urine collections, these 40 subjects form the basis for the current analysis. Additional details concerning these subjects and other aspects of the protocol are described elsewhere [16].

### *Protocol*

Subjects were admitted to the GCRC between 3 p.m. and 8 p.m. at study month 0 and were instructed to void for a collection of random urine. Subjects were NPO (except for water) from 8 p.m. until 6 a.m., when they were instructed to void and then drink 0.5 liter of water. At 8 a.m. on day 1 a blood sample and a 2-hour fasting urine sample were obtained. Blood was collected on ice and allowed to clot for 30 minutes, then centrifuged at +4°C, at 3000 RPM for 5 minutes. The serum was then separated into aliquots and frozen at −70°C. The urine was separated into aliquots and frozen at −20°C. Repeat admissions for collection of blood and urine, as above, were at months 1, 2, 3, and 12 after study entry.

Subjects on suppressive doses of thyroxine were then randomized in a double-blind fashion to receive either APD or placebo (PBO). Subjects treated with active drug received 30 mg of APD in 500 ml of 5% dextrose as an intravenous infusion over 4 hours, and the placebo group received an identical placebo infusion of 5% dextrose. Infusions were repeated at 3, 6, and 9 months after study entry.

### *Measurements*

*Urine.* All specimens were assayed at the end of the study in order to reduce interassay variability. All urine results are reported after correction for creatinine excretion. Peptide-bound N-telopeptide cross-links of type I collagen (NTX) in the urine were measured by ELISA using the Osteomark kit from Ostex International (Seattle, WA) by a method previously described in detail [13]; using this method the intraassay CV is 5–19%. Free pyridinium cross-links (f-PYD) were measured by ELISA with the Pyrilinks kit from Metra Biosystems (Mountain View, CA), using a method previously described in detail [17]; with this method the intrassay CV is 6.6–9.9%. Free deoxypyridinoline (f-DPD) was measured by ELISA with the Pyrilinks-D kit from Metra Biosystems, using a method previously described in detail [18]; this method has an intraassay CV of 4.3–8.4%. Urinary creatinine was measured by standard automated methodology.

*Blood.* All serum specimens were stored at −70°C after collection and were assayed at the end of the study in order to reduce interassay variability. Bone-specific alkaline phosphatase (BSAP) was measured by EIA with the Alkphase-B kit from Metra Biosystems using a method previously described in detail [19]; the intraassay CV is 3.9–5.8% with this method. Serum osteocalcin was measured by immunoradiometric assay (IRMA) using the Immutopics kit from Nichols which measures both intact and the N-terminal midfragment (San Juan Capistrano, CA); the intraassay CV for this method is 3.6–5.3%. PTH assays were performed with the Allegro immunoradiometric assay kit from Nichols (San Juan Capistrano, California); the intraassay CV for this assay is 1.8–3.4%. 25 Hydroxyvitamin D was measured by radioimmunoassay (RIA) using a kit from INCSTAR (Stillwater, MN); the intraassay CV for this assay is 5.6–6.7%.

All assays were performed by a technician who was unaware of the treatment group. Measurements of f-PYD, f-DPD, and BSAP were performed at Metra Biosystems; all other assays were performed in the GCRC core laboratory.

### *Statistical Analysis*

Results are reported as mean ± SEM. The significance of changes in bone turnover over time was computed by paired *t*-test with a Bonferroni correction for multiple comparisons. Coefficient of variation (CV) for a population was computed as SD/mean of all values at baseline for the PBO group. Long-term intraindividual CV was computed for each of the 19 PBO subjects as the SD/mean of measurements from baseline and months 1, 2, and 3. The mean long-term intraindividual CV was computed as the mean of all the individual CVs. The minimum significant change (MSC) of a marker was defined as a change that was sufficiently large that it was unlikely to be due to spontaneous variation. This minimum significant change was defined here as two times (2×) the mean long-term intraindividual CV; any decline in turnover that was greater than 2×CV had a 92% chance of being too great to be due to spontaneous variability. All calculations were performed using the SAS statistical program (SAS Institute; Carey, NC).

## Results

### *Subjects*

Of the 40 subjects, 21 were in the APD group and 19 were in the PBO group. Mean age of subjects in the APD group was 43.2 ± 2.3, and in PBO group was 46.4 ± 1.8 ($P$ = ns

**Table 1.** Coefficients of variation for the biochemical markers of bone turnover in untreated patients

| Marker | Population CV at baseline (%) | Long-term intraindividual CV (%) |
|---|---|---|
| Serum | | |
| BSAP[a] | 28.8 | 7.3 |
| Osteocalcin | 32.1 | 11.6 |
| Random urine | | |
| NTX[b] | 72.6 | 26.2 |
| f-DPD[c] | 36.6 | 13.3 |
| f-PYR[d] | 34.1 | 12.7 |
| 2-Hour fasting | | |
| NTX[b] | 57.2 | 27.0 |
| f-DPD[c] | 26.0 | 10.3 |
| f-PYR[d] | 30.9 | 11.2 |

[a] Bone-specific alkaline phosphatase
[b] N-telopeptide cross-link of type I collagen/creatinine
[c] Free deoxypyridinoline/creatinine
[d] Free pyridinoline deoxypyridinoline/creatinine

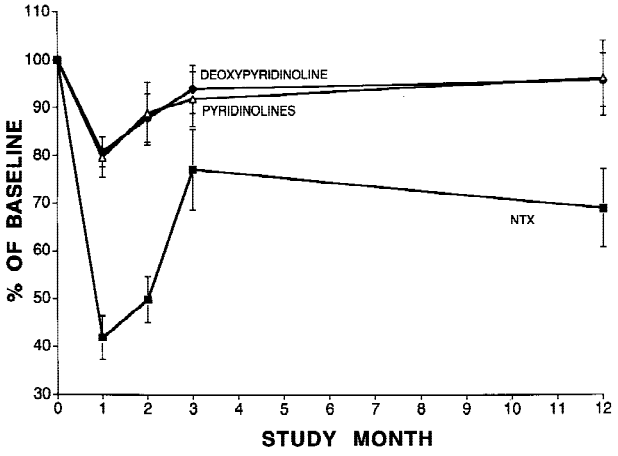


**Fig. 1.** Mean creatinine-corrected 2-hour fasting urine NTX, free deoxypyridinoline (DPD), and free total pyridinolines (PYR) after treatment with intravenous pamidronate every 3 months. Mean NTX and DPD at all timepoints differ significantly from baseline ($P < 0.05$). Mean PYR differs significantly from baseline only at month 1.

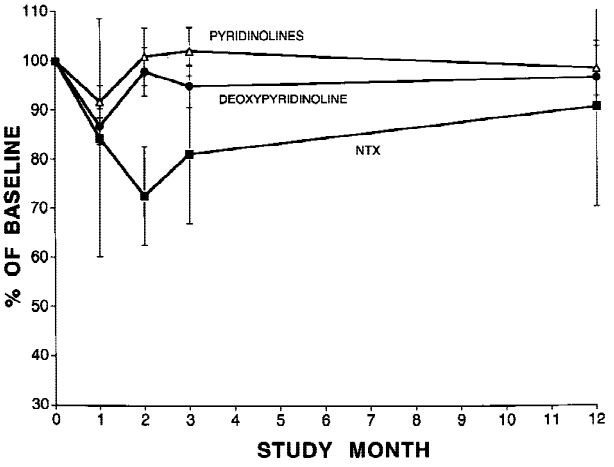


**Fig. 2.** Mean creatinine-corrected random urine NTX, free deoxypyridinoline (DPD), and free total pyridinolines (PYR) after treatment with intravenous pamidronate every 3 months. Mean NTX at all timepoints differs significantly from baseline ($P < 0.05$). Mean DPD and PYR differ significantly from baseline only at month 1.

for the difference). There were 15 women and 6 men in the APD group and 14 women and 5 men in the PBO group ($P$ = ns for the difference). At study entry there were no significant differences between the groups in mean height, weight, calcium intake, levels of PTH, and 25(OH) $D_3$ or any of the measured biochemical markers of bone turnover (data not shown).

*Variability of Assays*

The population CVs for the markers in the PBO group are listed in Table 1. In general, the population CV for random markers was higher than the CV for fasting markers. Among markers of bone formation, BSAP had the lowest population CV (28.8%). Among the resorption markers, 2-hour fasting DPD had the lowest population CV (26.0%).

Mean long-term intraindividual CVs for biochemical markers are listed in Table 1. Not surprisingly, these were much smaller than the CVs for the overall population. Long-term CVs were larger for random than for fasting urine markers, but these differences were not statistically significant. The long-term CVs for NTX were significantly ($P < 0.05$) higher than for DPD for both fasting and random urines. The long-term CV of BSAP (7.3%) was significantly lower than the CV of all other markers ($P < 0.05$).

*Changes in Markers After Treatment With APD*

Changes in markers after treatment with APD are illustrated in Figures 1–3. Markers of bone resorption fell most dramatically at month 1, and gradually rose towards baseline by month 3 (Figs. 1–2). In contrast, markers of bone formation fell more slowly, and reached their nadir at month 3 (Fig. 3).

The decline in 2-hour fasting markers was greater and less variable than that for random markers (Figs. 1–2). Mean 2-hour fasting NTX fell by 58.2% at month 1, whereas mean random NTX fell by only 16%. Similarly, mean 2-hour fasting DPD fell by 19.3% at month 1, whereas random DPD fell by only 13.3%. In addition to mean random urine markers showing less decline, changes in random urine markers were less representative of known changes in bone resorption after treatment with APD. Mean 2-hour fasting NTX and DPD fell maximally at month 1, and gradually rose, but were still significantly below baseline, at month 3; these changes reflect the known effects of an intravenous dose of APD on bone resorption. Mean random urine DPD was no different from baseline at months 2 and 3, so random DPD did not reflect effects of bisphosphonate at months 2 and 3.

Among all the markers, the greatest decline with treatment was in 2-hour fasting NTX; this decline (58.2%) was significantly ($P < 0.05$) greater than the decline in 2-hour fasting DPD (19.3%).

Based on long-term CV for each marker, one can define a minimum change in response to therapy that is greater than the change expected from spontaneous variability. We

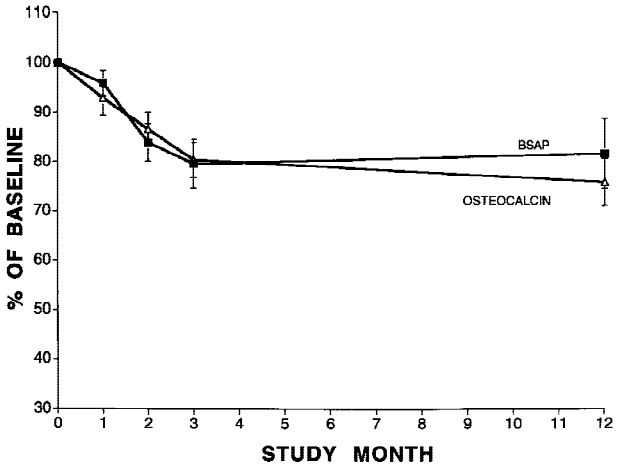


**Fig. 3.** Mean serum BSAP and osteocalcin by Nichols (osteocalcin) after treatment with intravenous pamidronate every 3 months. Mean BSAP and osteocalcin at all time points differ significantly from baseline ($P < 0.05$).

defined this minimum significant change (MSC) as being twice the long-term CV of the marker; a change greater than the MSC would have a 92% likelihood of being due to treatment effect rather than to spontaneous variability. The percent MSC for each marker is listed in Table 2, along with the percent of changes for each marker that actually exceeded the MSC. Since the maximum change for resorption markers after a dose of intravenous APD is after 1 month, the changes in resorption markers that exceeded the MSC at 1 month is given. Since the maximum change for formation markers after a dose of APD is after 3 months, the changes in formation markers that exceeded the MSC at 3 months is given. Although mean DPD fell more modestly than NTX, the low long-term CV for DPD resulted in a small MSC, so the number of patients showing significant changes was just slightly, but not significantly better for NTX than for DPD. So, NTX declines more than DPD after treatment, but a large decline in NTX is needed to be sure the decline is due to treatment and not to spontaneous variability. Therefore, 2-hour fasting NTX and DPD gave useful information about treatment efficacy in a similar percentage of individuals. Despite the modest drop in BSAP with therapy, BSAP yielded the greatest number of significant changes because of the low MSC.

**Table 2.** Changes in bone turnover after treatment with APD that exceeds spontaneous variation

| Marker | Minimum significant change (MSC) (%) | % of results that exceed MSC* |
|---|---|---|
| Serum | | |
| BSAP[a] | 14.6 | 74 |
| Osteocalcin | 23.2 | 35 |
| Random urine | | |
| NTX[b] | 52.4 | 50 |
| f-DPD[c] | 26.6 | 24 |
| f-PYR[d] | 25.4 | 14 |
| 2-Hour fasting | | |
| NTX[b] | 54.0 | 57 |
| f-DPD[c] | 20.6 | 48 |
| f-PYR[d] | 22.4 | 57 |

* After treatment with APD, markers of turnover decline. However, in some individuals the decline could be explained by random variability. Only if the marker declines more than the MSC (see Methods for details) can we be confident that the observed decline is really due to treatment effect. The Table shows the percentage of patients whose markers declined more than the MSC after treatment with APD; i.e., the percentage of patients in whom the decline in markers was too great to be explained by random variability. See text for details

[a,b,c,d] Same as in Table 1

## Discussion

Biochemical markers of bone turnover reflect the effects of antiresorptive therapy [1, 9–11, 20]. There has been a recent proliferation of specific markers of bone turnover, and the clinician must choose the most appropriate to follow in patients treated with antiresorptive agents. In this study we define the advantages associated with some of these markers. We demonstrated that currently available urinary markers of bone resorption measured on random urine samples have substantial variability that greatly limits their utility in following patients on antiresorptive therapy, and 2-hour fasting urine samples should be used to measure these markers. The greatest decline with therapy is seen with urinary NTX, but this advantage is offset somewhat by greater intraindividual variability in NTX than in other markers. The marker that showed the greatest likelihood of a significant change after antiresorptive therapy was BSAP, because of its low long-term variability.

We observed that urinary markers of bone resorption had better precision and greater fall after therapy when measured on 2-hour fasting samples than when measured on random samples (Figs. 1–2). This observation is expected because there is a known diurnal variation in markers of bone turnover [14, 15, 21]. When urine is collected randomly, the diural variation adds to the long-term CV. Obviously it would be most convenient for clinicians and patients for these tests to be ordered without regard to the time of day the patient is seen. Unfortunately, it is less likely that meaningful information will result from random measurements of urinary markers of bone resorption, and therefore measurement of these markers should be ordered on 2-hour fasting samples. However, it is possible that spot fasting samples might be as accurate as the timed 2-hour fasting samples, although this was not directly studied.

In the current study we found that the decline in urinary NTX after antiresorptive therapy was more dramatic than the decline in urinary-free DPD. This finding was expected because we and others have demonstrated that peptide-bound cross-links such as NTX fall abruptly and dramatically after antiresorptive therapy, and that the fall in free cross-links is much more modest [9–11, 22]. Garnero et al. [9] found that 3 days after treatment with pamidronate, peptide-bound crosslinks declined 52–71%, whereas free DPD did not change at all. In keeping with our study [11], most other studies that have examined effects of bisphosphonates after more than 3 days have found that peptide-bound cross-links fall 50–85% whereas free cross-links fall 25–40% [10, 23]. Similarly, previous studies confirm our observation that the intraassay CV and the long-term CV for NTX is higher for NTX than for free DPD [12].

Our results were somewhat surprising and a bit disconcerting in that the best markers clearly show the effects of antiresorptive effect in only 50–75% of individuals on treat-

ment. Either a reduction in long-term CV or a greater decline with treatment would improve the performance of these markers. Since we already have markers that fall dramatically with treatment, most likely future improvements will involve reducing the long-term CV. For instance, if the evolving assay for serum NTX shows changes after treatment similar to those for urinary NTX but with greater precision, we would have a useful marker indeed.

There are some potential criticisms of our study. First, the NTX and osteocalcin assays were run in our GCRC core laboratory using kits from the manufacturer, whereas the DPD and BSAP assays were run by the manufacturer. The expertise of the manufacturer's technicians who developed an assay might have been better than that of our GCRC technician who was not instrumental in developing the assay; this could partially explain the fact that the long-term intraindividual CV for NTX was higher than that of DPD. However, we do not think that the high long-term CV for NTX reported here is due to poor technique because in our technician's hands the analytical intraassay CV for NTX was 5.3%. Furthermore, the intraassay CV for the osteocalcin assay that our technician ran was 5.4–6.7%, close to the CV in the manufacturer's package insert. In addition, the long-term CV results we report here for NTX are similar to results previously reported [12], suggesting that the high CV for NTX excretion is due to substantial variability over time. Second, we comment here only on whether an individual observed change in turnover is real or due to chance, and we do not discuss how great the change in turnover needs to be in order to predict effective treatment. We did not have enough subjects to comment meaningfully on the correlation between change in turnover and change in BMD. Third, the subjects we studied were patients with thyroid cancer, not with osteoporosis. It is possible that the changes in bone turnover after treatment would be greater in subjects with osteoporosis who have a tendency to have high bone turnover [24, 25]. Lastly, though measurement of BSAP by the MetraBiosystems EIA performed favorably in this study, these favorable results may not be applicable to other assays of BSAP. Further study and comparison of the different assays is required.

In summary, we observed that urinary markers of bone resorption must be collected in a timed fashion in the morning for results to be meaningful. Existing markers of bone turnover show limited utility for clearly demonstrating the effects of antiresorptive therapy because many of the changes observed are in the range of change expected with spontaneous long-term variation. The utility would be greatly improved for a marker that combined the large fall with treatment found with NTX and the long-term precision of serum BSAP.

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