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A Proposal for Standardizing Urine Collections for Bone Resorption Markers Measurement

M. Zaninotto,[1]* D. Bernardi,[1] F. Ujka,[2] P. Bonato,[3] and M. Plebani[1,2]

[1]*Department of Laboratory Medicine, Azienda Ospedaliera di Padova, Italy*
[2]*Center for Biomedical Research, Castelfranco Veneto (TV), Italy*
[3]*Institute of Internal Medicine, University Hospital, Padova, Italy*

Diurnal variations in the excretion of bone resorption markers were assessed in order to identify the type of urine collection which provides the most information on bone resorption rate and its relation to measuring bone dynamics in a postmenopausal population. Sixty women, ages 43–67 and without disease or treatment known to affect bone mineral density, were divided into two groups on the basis of femoral mineral density T-score: <1.5 (Group I), >1.5 (Group II). Bone formation was assessed by measuring bone alkaline phosphatase activity and osteocalcin concentration, bone resorption by urinary hydroxyproline, pyridinoline and deoxypiridinoline, N-telopeptide, galactosyl hydroxylysine, and CrossLaps. To identify the more appropriate collection times, urine samples were collected from 7 am to 3 pm; from 3 pm to 11 pm; from 11 pm to 7 am. Twenty-four hour urine collection and first morning void urine samples were also measured.

The findings suggest that nocturnal collection and first morning void samples provide the most reliable data on the rate of bone degradation, possibly showing bone loss not only in osteopenic patients but also in women with a low T-score. Nocturnal and first morning samples should therefore be recommended in order to standardize sample collection, as they enable an accurate assessment of bone resorption markers and improved comparability to results from different studies, as well as a less cumbersome collection modality. J. Clin. Lab. Anal. 12:145–149, 1998. © 1998 Wiley-Liss, Inc.

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INTRODUCTION

Biochemical markers of bone formation and resorption provide a tool for monitoring changes in bone turnover. Estimation of the rate of bone loss through biochemical assays, combined with an accurate bone mass measurement, may allow an improved assessment of the risk of osteoporosis development in postmenopausal women. Although density measurements may be sensitive enough to measure changes in bone density, biochemical markers are the only diagnostic tool available for the detection of acute changes in skeletal metabolism (1). Biochemical indicators should enable the detection of rapid changes in synthesis and events occurring during bone tissue degradation, which is characterized by a sparse cell distribution with a consequent predominance of the extracellular matrix, consisting of about 90 % of type I collagen. Bone markers may therefore be divided into those reflecting bone cell activities and those primarily derived from events in the extracellular matrix. For an indication of osteoblastic function we can measure bone alkaline phosphatase, an enzyme associated with the osteoblastic plasma membrane, and osteocalcin (also called bone Gla protein, or BGP), a small protein (molecular mass 5800) containing gamma carboxyglutamate residues, presumably binding calcium. In fact, after the phases of osteoblast proliferation, the expression of alkaline phosphatase reaches its maximum during matrix maturation while osteocalcin, a calcium-binding protein, is expressed during the following phase of matrix mineralization (2).

On the other hand, dissolution of minerals and the enzymatic breakdown of bone's organic matrix depend on the action of osteoclasts. The biochemical indicators of bone resorption may therefore be the breakdown products of organic matrix and collagen fibres, resulting in a mixture of peptides and free aminoacids as well as modified aminoacids characteristic of the collagen excreted in urine, such as hydroxyproline and galactosyl hydroxylysine.

In clinical practice, biological variations affecting the mark-

*Correspondence to: Martina Zaninotto, Servizio di Medicina di Laboratorio, Azienda Ospedaliera di Padova, Via N. Giustiniani 2, 35128 Padova, Italy.

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ers themselves, such as the circadian rhythm, should always be considered. This is of particular importance in a disease such as osteoporosis, which is characterized by subtle modifications in bone remodelling activity that, over a long period of time, can lead to a substantial loss of bone mass (3). Careful attention must therefore be paid to the appropriate collection of urine samples if small changes in biochemical variables are to be used as indicators of alterations in bone resorption. It has been observed that some markers have daily cyclical rhythms (4,5); variations over time may consequently be considered fluctuations around a homeostatic set point.

Furthermore, values obtained in samples collected under different physiologic conditions cannot be compared. The different values for excreted molecules reported in different studies may reflect the time at which and the way in which the samples are collected: some authors report findings from early morning urine samples while others use urine collected over a 24-hour period (6,7,8). Also, few data are available on the circadian patterns of urinary markers recently proposed for the evaluation of bone resorption.

The aim of our study was therefore to assess variations in the molecules excreted in different urinary collections in order to identify which samples show a major excretion on bone resorption markers. Once the urine collection providing the most reliable excretion rate of bone degradation had been identified, we evaluated the diagnostic performances of different resorption markers for the measurement of bone dynamics in an osteopenic postmenopausal population.

MATERIALS AND METHODS

We studied 60 postmenopausal women aged 43 to 67 years. The characteristics of the population studied are shown in Table 1. Exclusion criteria included history of hip fracture; vertebral compression fracture at lateral thoracic and lumbar x-rays; malignancy during recent years; treatment with glucocorticoids, estrogen, or anticonvulsivants within the past year; any treatment with bisphosphonates, calcitonin, or oral anticoagulants; and current use of calcium or vitamin-D supplements. Alcohol consumption, cigarette smoking, and physical activity were considered negligible variables in the assessment of patients. Bone-mineral density (BMD) was measured at the lumbar spine (anteroposterior L1-L4 also by lateral scanning), at the hip (neck, trochanter, ward, and total) by dual energy x-ray absorptiometry (QDR 1000, Hologic) and at the distal and ultradistal radius (Osteometer DT 100).

To evaluate the rate of bone turnover in the postmenopausal population, two groups were identified on the basis of their femoral T-scores: Group 1 (controls): T-score <–1.5 (median –0.86; range –1.49 to +1.72), consisting of 30 subjects whose mean duration of menopause was 7.1 years; and Group 2 (osteopenic patients): T-score >–1.5 (median –2.09; range –3.42 to –1.57), consisting of 30 subjects whose mean duration of menopause was 5.7 years. In accordance with the Helsinki II declaration, the study design was throughly explained to the subjects, and informed consent was obtained in all cases.

In both groups the following biochemical markers were measured: bone alkaline phosphatase (B-ALP) activity (lectin precipitation method, Boehringer Mannheim Italia, Milan, Italy) and osteocalcin (BGP) concentration (immunoradiometric assay, Biomed, Padova, Italy) for the evaluation of bone formation. To assess bone resorption we assayed urinary hydroxyproline (HOP) (Beckman Analytical, Milan, Italy), pyridinoline (PYR) and deoxypyridinoline (D-PYR) (Biorad Laboratories, Milan, Italy), galactosyl hydroxylysine (GHYL) (high-performance liquid chromatography Bracco, Milan, Italy); urinary type-I collagen cross-linked N-telopeptide (N-Tx) (Osteomark, Technogenetics, Milan, Italy) and CrossLaps (CrossLapsEIA, CIS DIAGNOSTICI, Vercelli, Italy) measured by immunoenzymatic procedures. The CrossLaps assay, in particular, measures the breakdown products of type-I collagen competing with an immobilized synthetic peptide with an amino acids sequence (Glu-Lys-Ala-His-Asp-Gly-Gly-Arg = CrossLaps peptide) specific for a part of the C-terminal telopeptide of the d1 chain of type I collagen (9). Creatinine was measured in urine samples using the Jaffè procedure so as to normalize the results to the urinary creatinine content.

To evaluate variations in the urinary markers of different urine collections, samples from all subjects were collected through the following periods: 7 am to 3 pm (A); 3 pm to 11 pm (B); and 11 pm to 7 am (C). Moreover, a 24 h urine collection (D) was made, and first morning void urine samples (E) were also obtained. In order to standardize urine collection, subjects were given several volumetric flasks and a supply of urine containers. They were then instructed to collect each urine void separately over a 24 h period. A measured aliquot was saved in a sample vial, on which the total volume and time of collection were recorded. The samples were refrigerated until

TABLE 1. Patients' Characteristics

	No	Age (Years)	Weight (Kg)	Height (Cm)	Postmenopausal years
Osteopenic group T-score: –1.5 to –2.5 femoral neck (DEXA)	30	56.82±5.18	60.43±12.93	160.81±6.03	7.09±5.23
Control group T-score: 0 to –1.49 femoral neck (DEXA)	30	55.59±5.72	69.85±9.55	162.44±5.65	5.74±5.15

[a]Data are means ± 1SD.

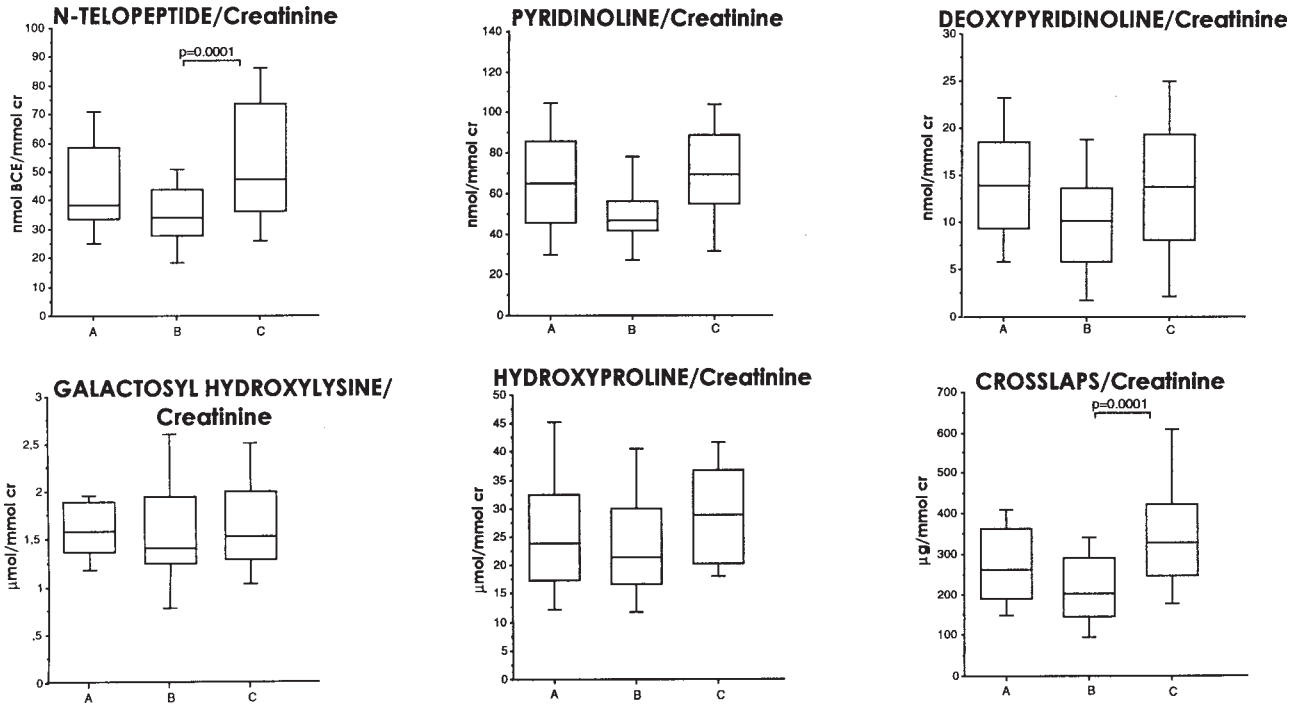

Fig. 1. Excretion rates of the markers studied in the different urine sample collection periods plotted as median values, 25–75% (boxes) and 5–95% (lines) range. **A:** 7 am-3 pm; **B:** 3 pm-11 pm; **C:** 11 pm-7 am.

the end of the 24 h period, transferred to the laboratory, and stored frozen (–20°C) until analysis. On the morning of delivery, a blood sample was drawn from each patient, centrifuged at 3000 rpm, and stored at –80°C until assay; the early morning collection was made after an overnight fast and without any morning exercise.

STATISTICAL ANALYSIS

Trends throughout the day for the urinary data were assessed by ANOVA statistical analysis. A comparison was made between the means values of the two groups using Student's t-test. A value of $P < 0.05$ was considered significant. The statistical significance of differences between medians was calculated using Confidence Interval Analysis (10). The difference was significant when the confidence interval does not include zero.

RESULTS

Figure 1 shows the excretion of urinary markers of bone resorption considered in our study: only N-Tx and CrossLaps showed a marked circadian rhythm with a nocturnal rate (samples C) that was significantly higher than in the second collection samples (B) (median=47.43 versus 33.94 nmol BCE/mmol creatinine and 329.47 versus 201.39 µg/mmol creatinine; P=0.0001). A similar trend was observed for other resorption markers, but no significant difference was found between the values obtained in samples C and B. Significant differences were found within each patients group for CrossLaps and N-Tx values in the same sample, as was also demonstrated by the 95% CI between medians. In fact, in Group 1 the 95% Confidence Interval for the difference between medians ranges from –31.0 to –4.28 for N-Telopeptide

TABLE 2. Comparison Between 24-Hour Urine Collection and First Morning Void Urine Samples

	N-Telopeptide/ creatinine	Pyridinoline/ creatinine	Deoxypyridinoline/ creatinine	Galactosyl-Hydroxylysine/ creatinine	Hydroxyproline/ creatinine	CrossLaps/ creatinine
	Median 5-95% range	Median 5-95% range	Median 5-95% range	Median 5-95% range	Median 5-95% range	Median 5-95% range
24-hour collection	40.58	63.72	13.1	1.47	24.89	209.89
(D)	17.37-71.65	29.93-90.90	3.40-20.74	0.95-2.23	10.56-49.28	98.12-379.01
First morning void	51.99	75.56	15.49	1.58	26.40	348.077
(E)	23.53-97.14	32.88-132.27	3.29-29.16	0.94-3.30	8.10-50.74	177.44-608.91
t-test (two-way)	P=0.004	P=0.32 n.s.	P=0.27 n.s.	P=0.18 n.s.	P=0.66 n.s.	P=0.0001

TABLE 3. Comparison Between Mean Values ($\bar{x}$ ± SD) for Biochemical Bone Markers

	Bone-ALP U/L	Osteocalcin mg/L	Hydroxyproline[a] mmol/mmol cr	N-Telopeptide[a] nmol BCE/mmol cr	Pyridinoline[a] nmol/mmol cr	Deoxypyridinoline[a] nmol/mmol cr	CrossLaps[a] mg/mmol cr
Osteopenic (n=30) group	22.16 ± 17.19	9.46 ± 5.28	30.79 ± 15.50	53.34 ± 23.08	90.62 ± 66.75	15.33 ± 8.86	335.30 ± 142.84
Control (n=30) group	21.12 ± 14.62	8.70 ± 5.94	29.79 ± 9.42	54.91 ± 25.53	60.74 ± 6.90	13.02 ± 7.64	367.01 ± 171.41
t-test (two-way)	*P* = 0.802 n.s.	*P* = 0.596 n.s.	*P* = 0.7839 n.s.	*P* = 0.8239 n.s.	*P* = 0.0387	*P* = 0.3327 n.s.	*P* = 0.4916 n.s.
Premenopausal values	24.2±6.0	10.3±3.4	13.9±6.9	30.4±6.9	33.4±8.1	5.4±1.8	250±110

[a]Nocturnal urine collection.

and from –218 to –40.6 for CrossLaps. In Group 2 the N-Telopeptide values provide a 95% Confidence Interval from –26.2 to –6.02 and CrossLaps from –182 to –56. Although the variability found in the urinary creatinine concentrations may have contributed to the variability found in all the urinary measurements expressed as a ratio, a diurnal variation was also found in the absolute values.

Table 2 shows the results for urinary markers obtained in D, daily, and E, first morning void collection samples. The daytime values (D) for all markers were lower than those found in first morning void samples (E); however, significant differences between collections D and E were found only for N-Telopeptide and CrossLaps concentrations, (P=0.004 and P=0.0001 respectively). Finally, no statistically significant differences (ANOVA test) were found between findings obtained in C (11pm–7am) and E (first morning void) collections.

In order to ascertain the diagnostic usefulness of bone resorption markers assayed in the standardized urine collection found to show the highest excretion rate (nocturnal sample), we compared findings from osteopenic postmenopausal patients with those from age-matched, postmenopausal controls (Table 3). We also evaluated bone ALP and osteocalcin so as to obtain more complete information on bone dynamics.

The findings obtained show a significant overlap between groups without any significant difference between mean values. However, the mean concentrations of bone resorption markers in both groups were higher than those reported elsewhere in premenopausal populations (7,9).

DISCUSSION

Recently developed assays for the measurement of specific biochemical markers of bone turnover may enable a better understanding of the mechanisms underlying bone loss, thus leading to new preventive strategies. However, the potential utility of newly proposed urinary markers will only be fully realized when collections of urine samples have been standardized. We therefore ascertained time trends for urinary data and timely urinary collections were evaluated in order to identify the collection time with the highest excretion rate for urinary markers, thus providing greater information on the bone resorption rate.

The findings obtained suggest that the traditional daily urine collection shows a low excretion rate and therefore does not allow a reliable evaluation of bone resorption, whereas nocturnal and first morning void collections appear to provide more reliable findings for the rate of bone degradation, possibly reflecting increased nocturnal bone turnover. The identification of a urine collection period showing a higher excretion rate of bone resorption markers could provide a more sensitive evaluation of subtle pathophysiological variations in bone dynamics, as occurs in osteopenic patients.

In our study, however, a significant overlap was found between the values of resorption markers in the two patient groups studied. These findings suggest that, in our population, bone mineral density has a greater discriminatory capacity than biochemical markers. Moreover, our finding that the former markers showed a similar trend in both postmenopausal groups studied suggests that these markes are sensitive in evidencing bone loss not only in patients with a high T-score but also in those with a low T-score. However, only when our ongoing longitudinal study has been completed will the markers' clinical usefulness in the assessment of bone resorption be clarified.

In conclusion, our findings indicate that nocturnal or first void urine collections are more suitable for the evaluation of bone turnover. They should, moreover, be recommended in the attempt to standardize the preanalytical phase related to sample collection, because they allow a more accurate assessment of bone resorption markers, enable a more satisfactory comparison between results obtained in different studies, and facilitate the collection process itself by eliciting greater compliance from patients.

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