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Clinical Value of Bone Remodelling Markers in Patients with Bone Metastases Treated with Zoledronic Acid

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Abstract. *Background: Bisphosphonates have an established role in the treatment of bone metastases from a variety of solid tumours. The objective response to anti-resorptive treatment cannot be evaluated by imaging techniques. A number of bone remodelling markers have been associated with bone metastases status; among them, urine and serum levels of N-terminal telopeptide of collagen type I (NTx) seem to have the best diagnostic accuracy. However, serum NTx has not yet been properly evaluated. Patients and Methods: Seventy-one consecutive patients with newly diagnosed skeletal metastases were enrolled in this prospective study. All of them were treated with zoledronic acid at 4 mg, every 3 or 4 weeks. Serum NTx and bone-isoform of alkaline phosphatase (BAP) were measured by enzyme-linked immunosorbent assays at baseline and every 2 months thereafter. Results: At baseline, serum NTx and BAP levels were significantly higher in patients with blastic than lytic bone lesions and in those with multiple rather than few bone site involvement. Forty-seven patients were followed for a median period of 139 days. Zoledronic acid resulted in a significant NTx reduction at first and second post-treatment evaluations (mean reduction of 43% at first evaluation); thereafter, mean NTx levels remained suppressed. In contrast, BAP levels did not show any significant changes. Bone disease progression resulted in a significant NTx elevation by an average of 69%. The initial response of NTx to zoledronic acid was correlated with the long-term clinical outcome of bone disease: patients with an initial NTx elevation had a significantly higher rate of bone disease progression compared to those with an initial NTx decline (66.7% versus 18.8%, p=0.001). Extraskeletal disease or bone irradiation did not influence NTx response. Conclusion: Serum NTx appears to be a useful marker in monitoring patients with skeletal metastases, as it is correlated with the type and bulk of bone disease and reflects bone disease progression. It is also useful in monitoring bisphosphonate therapy, while the initial response to this therapy seems to bear a prognostic significance for bone disease outcome.*

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Bone constitutes the third most common site of distant metastases, following lungs and liver, in patients with solid tumours and is often the only metastatic site, particularly in breast and prostate cancer (1). The resulting skeletal events, including bone pain, pathological fractures, hypercalcemia and spinal cord compression, affect the quality of the patients' life.

Bisphosphonates have an established role as palliative therapy in patients with skeletal metastases (2, 3). At the cellular level, bisphosphonates bind with high affinity to hydroxyapatite crystals, hence inhibiting osteoclast activity and macrophage proliferation, while promoting osteoclast apoptosis and stimulating osteoblast differentiation (4-8). In addition, bisphosphonates have been shown to inhibit cancer cell invasion and adhesion to bone matrix and to induce apoptosis of human cancer cell lines (9-11).

Plain radiographs remain the standard method for the diagnosis and characterization of bone metastases. Bone scintigraphy is extremely sensitive, but any abnormal scintigraphic findings should always be verified by radiographic ones. However, the objective response to anti-resorptive treatment is difficult to evaluate by imaging techniques, mostly because radiographic changes

generally develop slowly. Thus, the assessment is basically clinical, including performance status, different pain score scales and record of analgesic treatment. The need for a more objective method for the monitoring of patients with bone metastases receiving anti-resorptive treatment is, therefore, clear.

Recently, the development of several biochemical markers of bone remodelling has provided useful clinical data in patients with osteoporosis, hypercalcemia and bone metastases (12-14). These biochemical markers are mostly stable end-products released into the circulation either during bone formation or during bone resorption and can be measured in serum or urine. Several clinical studies have already shown that the urinary concentration of amino-terminal telopeptide of type I collagen (NTx) represents a specific marker of bone resorption and correlates strongly with the presence and extent of skeletal metastases (14-16). Assays to determine serum NTx have also become available, but this marker has not yet been properly evaluated. Bone formation markers, such as the bone-specific isoform of alkaline-phosphatase (BAP), have also been used to evaluate skeletal metastases (17, 18).

The aims of this prospective study were to assess the clinical value of serum NTx along with BAP in patients with bone metastases from a variety of solid tumours and determine whether these markers can be used in evaluating the response to treatment with the new-generation bisphosphonate, zoledronic acid.

Patients and Methods

Patients' selection. All consecutive ambulatory patients with radiological evidence of newly diagnosed bone metastases, admitted to our Department between July 2002 and December 2003, were initially considered for enrolment. Exclusion criteria were the presence of metabolic bone disease, renal failure, cachexia or feeding disorders and second primary malignancy. The study protocol was approved by the local ethics committee and all patients enrolled gave informed consent.

Patients' evaluation. Baseline evaluation included clinical assessment, bone survey, evaluation for extraskeletal disease and serum NTx and BAP determination. During follow-up, clinical assessment was performed at monthly intervals; bone survey was repeated every 4 months or on bone disease progression; evaluation of extraskeletal disease, if any, was performed every 2 months; blood samples for NTx and BAP determination were obtained approximately every 2 months or on bone disease progression.

Clinical evaluation included assessment of performance status according to the World Health Organization (WHO) criteria, bone pain evaluation according to the Radiation Therapy Oncology Group (RTOG) pain score scale and recording of concomitant treatments (analgesics, bisphosphonate, anti-cancer therapy). Skeletal-related events, including pathological fractures, hypercalcemia, neurologic abnormalities due to spinal cord compression and need for bone irradiation, were also recorded. Bone survey included X-rays and bone scintigraphy with Tc-99, as well as computed tomography scans when necessary.

Table I. *Patients' characteristics at baseline.*

	No.	%
No. of patients	71	
Age, years	62.1±15.1	
Gender, male	32	45.1
Primary site		
Breast	31	43.7
Prostate	10	14.1
Lung	17	23.9
Head & neck	6	8.5
Other	7	9.9
Metastatic status		
Bones only	22	31.0
Bones plus extraskeletal	49	69.0
Type of bone disease		
Lytic	29	40.9
Blastic	18	25.4
Mixed	24	33.8
Bulk of bone disease		
Few site involvement (≤ 3 sites)	30	42.3
Multiple site involvement	41	57.8

Patients were initially classified according to the type and bulk of bone disease, based on the findings of the bone survey. Bone disease type was characterised as lytic, blastic or mixed. The bulk of bone disease concerned the number of sites involved and was classified as few (<3 sites) or multiple (≥3 sites) site involvement.

Bone disease progression was defined as either the patient's clinical deterioration, expressed as increase of pain score, increased need for analgesics, bone disease-induced deterioration of PS or need for bone irradiation or the occurrence of new findings in imaging techniques (X-rays, bone scintigraphy, computed tomography scans).

All patients enrolled in the study received treatment with zoledronic acid (Zometa™, Novartis Pharma AG, Basel, Switzerland) at 4 mg as a 15-minute intravenous infusion every 3 or 4 weeks, along with radiotherapy, chemotherapy or hormonotherapy if needed.

Biochemical analysis. Bone markers were measured in serum obtained from fasting morning blood samples, stored at -80˚C until assessment. Determination of NTx was performed by an enzyme-linked immunosorbent assay (ELISA, Osteomark™ NTx serum test, Ostex Inernational, Inc., Seattle, USA). Serum NTx levels were expressed in nanomoles of bone collagen equivalents (BCE) per litre (nM BCE). According to the literature, the mean reported value in healthy individuals is 15.9±3.8 nM BCE (19). Levels of BAP were also determined by ELISA, using a commercially available kit (Metra™ BAP kit, Metra Biosystem, San Diego, CA, USA) and the results were expressed as units per litre (U/L). The normal reference range for BAP is 14.2-47.7 U/L. Calibration and validation of results were performed using the Softmax PRO software (version 2.4.1).

Table II. *Pre-treatment and first 3 post-treatment NTx and BAP evaluations.*

	Pre-treatment evaluation	Post-treatment evaluations							
		1st evaluation		2nd evaluation			3rd evaluation		
			p^*		p^*	$p^\dagger$		p^*	$p^\dagger$
No. of patients	71	47		30			17		
Time, days	0	55		115			148		
NTx, nM BCE	31.6±30.0	18.0±10.7	<0.001	17.4±16.9	0.001	<0.05	21.0±22.2	<0.05	NS
BAP, U/L	52.8±41.5	52.8±50.6	NS	50.8±42.1	NS	NS	74.8±68.4	NS	NS

NTx indicates N-terminal telopeptide of collagen type I; BAP, bone-isoform of alkaline phosphatase; time, the median number of days from the first evaluation; NS, not significant.

* *versus* pre-treatment evaluation

† *versus* previous evaluation

Statistical analysis. Statistical analysis was performed using the SPSS 10.0 statistical software package (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± 1 standard deviation. Mean values were compared between patient subgroups using the Student's *t*-test or Mann-Whitney *U*-test, according to the distribution of variables tested by the Kolmogorov-Smirnov test. Similarly, the paired *t*-test or Wilcoxon paired test were used to evaluate NTx and BAP level changes. Categorical variables were compared using the Chi-square test. Linear regression analysis was used to investigate potential relationships between variables. Kaplan-Meier curves for bone disease progression were plotted and compared between patient subgroups using the log-rank test. A p value <0.05 was considered statistically significant.

Results

Baseline evaluation. Seventy-one patients, aged 62.1±15.1 years, were enrolled in the study. The patients' characteristics at baseline are reviewed in Table I. Pre-treatment serum NTx levels were positively correlated with those of BAP ($p<0.001$). Neither NTx nor BAP differed significantly between patients with bone plus extraskeletal metastases and those with bone metastases only (34.1±33.3 nM BCE *versus* 26.1±20.6 nM BCE, $p>0.05$, for NTX and 51.5±41.3 U/L *versus* 55.6±43.0 U/L, $p>0.05$, for BAP, respectively). In contrast, both markers correlated with the extent and type of bone disease. More specifically, NTx was significantly higher in patients with multiple bone site involvement *versus* those with few bone site involvement (38.9±36.3 nM BCE *versus* 21.7±13.3 nM BCE, $p<0.05$); BAP also showed a trend for being higher in patients with multiple site involvement (63.1±49.3 U/L *versus* 38.6±21.5 U/L, $p=0.053$). Both markers were significantly higher in patients with blastic than in those with lytic bone lesions (41.8±30.7 nM BCE *versus* 21.4±11.2 nM BCE, $p<0.001$, for NTx and 82.7±58.2 U/L *versus* 34.8±20.6 U/L, $p<0.05$, for BAP, respectively).

Follow-up. Forty-seven out of 71 patients had at least one post-treatment evaluation and were followed for a median period of 139 days (range, 31 to 396 days) after the initiation of zoledronic acid therapy. The main reasons for censoring were progression of the main disease and death. During the study period, 17 out of 47 patients (36.2%) required bone irradiation, 32 patients (68.1%) received chemotherapy and 17 (36.2%) were on hormonal therapy. Seventeen of the 47 patients (36.2%) died during follow-up.

Baseline and post-treatment with zoledronic acid NTx and BAP levels are shown in Table II. At the first post-treatment evaluation, mean NTx levels were significantly reduced, with an overall average reduction of 43%. More specifically, NTx was decreased in 32 patients (68.1%, average decrease 54.7%) and elevated in 15 patients (31.9%, average increase 26.2%). At the second re-evaluation, a further significant reduction of mean NTx was observed, while at the third post-treatment evaluation NTx levels showed a tendency to form a plateau within the reported normal range. Thereafter, the sample of evaluated patients was small and the observed changes were not statistically significant (Figure 1). The mean BAP concentrations, in contrast, did not show any stastically significant changes in the repetitive post-treatment evaluations (Table II).

Subgroup analysis. A subgroup analysis was performed to assess the effect on NTx response of factors that are known to interfere with bone metabolism, namely bone irradiation and hormonal therapy.

In the subgroup of patients not having received bone irradiation, baseline NTx and BAP levels were significantly higher than in patients who had been treated with radiotherapy (36.6±37.8 nM BCE *versus* 20.7±7.9 nM BCE, $p<0.05$, for NTx and 64.1±51.5 U/L *versus* 40.1±17.1 U/L, $p<0.05$, for BAP, respectively). In this subgroup, the pattern

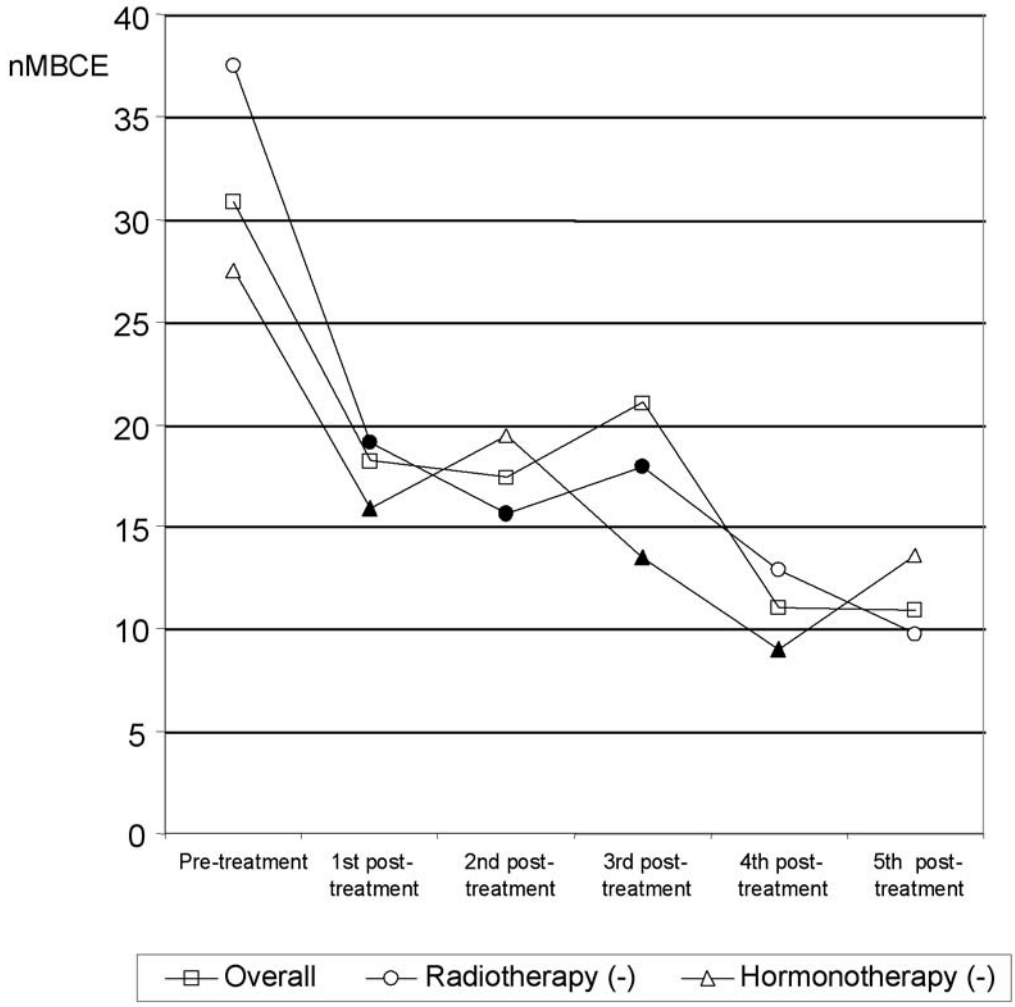

Figure 1. *Pre- and post-treatment mean NTx values in the whole follow-up group, in the subgroup of patients not receiving concurrent bone irradiation and in those not receiving concurrent hormonal therapy (solid marks indicate significantly different values [$p<0.05$] versus the corresponding pre-treatment values).*

of NTx response to zoledronic acid was similar to that of the overall group (Figure 1).

From the 17 patients who received hormone treatment, 14 had breast cancer and 3 had prostate cancer. Hormonal therapy included aromatase inhibitors (12 patients), luteinizing hormone-releasing hormone analogues (4 patients) and tamoxifen (1 patient). The mean pre-treatment NTx and BAP values, although higher in the subgroup of patients on hormonal therapy, were not significantly different from those of patients not receiving hormonal therapy (36.9±45.4 nM BCE *versus* 27.5±19.6 nM BCE for NTx and 67.0±58.2 U/L *versus* 48.9±32.3 U/L for BAP, respectively). In this latter subgroup, the pattern of NTx response to zoledronic acid was also similar to that of the overall group (Figure 1).

Bone disease outcome. During the follow-up period, bone disease showed a partial response or remained stable in 31 of the 47 patients (65.9%), while 16 patients (34.1%) experienced bone disease progression. The median time to bone disease progression was 45.5 days (range, 20-211 days). Progression included clinical deterioration (8 patients), need for bone irradiation (4 patients), appearance of new bone lesions (3 patients) and pathological fracture (one patient). At the time of progression, the mean NTx increased significantly with respect to the mean value before progression (27.1±20.4 nM BCE *versus* 20.0±16.0 nM BCE, $p<0.01$). More specifically, NTx was elevated in 15 out of 16 patients (93.7%) with bone disease progression, by an average increase of 69%. The one patient who did not show NTx elevation during bone disease progression had blastic metastases from prostate cancer.

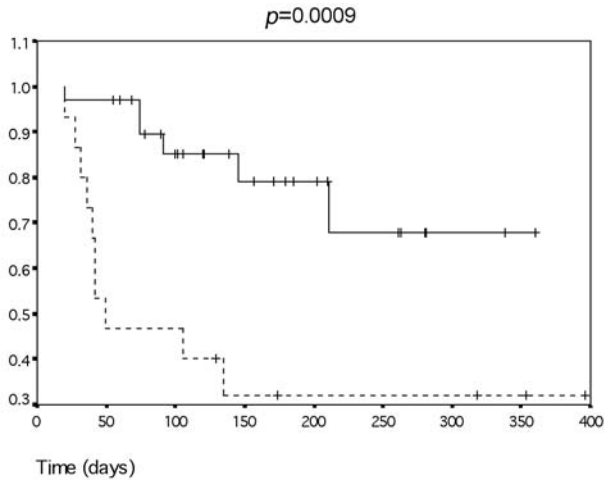

Figure 2. *Kaplan-Meier curves for bone disease progression in patients with initial NTx decline (solid line) and in those with initial NTx increase (dotted line).*

The rate of bone disease progression was significantly higher in patients with NTx elevation compared to those with NTx decline at first post-treatment evaluation. More specifically, 10 out of 15 patients (66.7%) with an initial NTx elevation experienced bone disease progression *versus* 6 out of 32 patients (18.8%) with an initial NTx decline ($p=0.001$). Moreover, the median time to bone disease progression was shorter in patients with an initial NTx elevation (41 days) compared to those with an initial NTx decline (82.5 days); the corresponding Kaplan-Meier curves for bone disease progression are shown in Figure 2 (log rank test $p<0.001$).

Discussion

Bone metabolism involves a continuous and dynamic process, known as the bone remodelling cycle. The presence of tumour cells in the bone microenvironment alters the remodelling balance in many circumstances in favour of bone resorption. Many factors have been implicated in this tumour-induced osteolysis. These factors are either normal growth and signalling molecules that are activated by the presence of tumour cells, including interleukin-1, interleukin-6, tumour growth factor-β, platelet-derived growth factor (PDGF), receptor activator of NF-Kappa-ligand (RANK-L), prostaglandin E_2, matrix metallo-proteinases and osteoprotegerin or tumour-released factors, such as parathyroid hormone-related peptide (14, 20). However, most of these molecules remain within the bone microenvironment and cannot be measured in the circulation (14). Factors that are released into the circulation are enzymes, such as BAP, osteocalcin, produced by osteoblasts, tartate-resistant acid phosphatase, produced by osteoclast, or products of collagen synthesis or degradation.

Among bone formation markers, BAP has been shown to be elevated in patients with untreated skeletal metastases (21), while type I procollagen C- and N- propeptides have also been evaluated with similar results to that of BAP (22).

Pyridinium cross-links and associated telopeptides – amino-terminal, NTx and carboxy-terminal, CTx – represent fragments of cross-linking amino acid derivatives that stabilize the collagen type I fibrils in bone. Type I collagen telopeptide sequences that contain these cross-linking residues have proven to be reliable and more specific markers of bone resorption than the free cross-links themselves (23). The specificity of NTx results from the fact that it originates solely from type I collagen and is produced as a neo-epitope by osteoclast activity during the bone-resorption phase.

Recently, NTx levels have been estimated in patients with bone metastatic disease. Studies have shown that NTx was elevated in patients with untreated bone metastases and correlated with the extent and type of metastases (16-18). The established method of evaluating NTx is in urine specimens, normalized to creatinine concentration. Urinary NTx may assess the anti-resorptive effect of bisphosphonate treatment, evaluate the disease progression in bone (17) and the analgesic effect (24), while it may be used to schedule the appropriate dose and efficacy of the bisphosphonate (12). The reduction of NTx in urine has also been correlated with reduced fracture incidence in patients treated with pamidronate (25).

Urine samples are easier to collect and reflect the release of these telopeptides over a few hours, hence allowing for a more dynamic picture of telopeptide secretory rates (26). Serum assays, on the other hand, have less sample to sample variability, greater availability of blood specimens, which are used for other measurements as well and are also available in renal failure patients, in whom urine may not be an option (27-29).

In a very recent study, serum NTx levels were found to bear a prognostic significance in a cohort of 250 breast cancer patients with bone metastases, who participated in two randomized studies of second-line hormone-therapy (30). More specifically, patients with elevated serum NTx levels at baseline had a shorter duration of clinical benefit, time to tumour progression and overall survival, compared to those without NTx elevation.

In the present study, serum NTx and BAP levels were correlated with the extent and type of bone metastatic disease, as they were significantly elevated in patients with large bone disease burden and in those with blastic lesions. This is in accordance with the knowledge that bone

metabolism is strongly activated when blastic lesions are apparent in X-rays. Moreover, the presence of extraskeletal metastatic disease did not seem to influence the NTx or BAP concentrations. Patients on hormonal therapy had higher values of NTx; this difference, although not statistically significant, may be attributed to the osteoporosis caused by the long-term effects of anti-estrogens on bones (31-33). During follow-up, serum NTx levels declined significantly following the administration of zoledronic acid. The pre-treatment NTx levels were higher in the group of patients who did not receive bone irradiation, probably because of the dominance of blastic lessions in this subgroup of patients. Furthermore, it seems that the NTx reduction following zoledronic acid therapy was not an effect of the concurrent application of radiotherapy for bone disease, as the pattern of NTx response in the subgroup of patients not receiving radiotherapy was similar to that of the overall group.

Following the initial decline, serum NTx levels remained suppressed as long as patients responded to the anti-resorptive treatment. When bone disease progressed, in contrast, an average NTx increase of 87% was observed, indicating that the serum NTx concentration closely follows the evolution of bone disease. Interestingly, the initial response of NTx to zoledronic acid was found to bear a prognostic significance for the long-term outcome of bone disease. More specifically, the increase of NTx after the initial bisphosphonate administration was significantly correlated with bone disease progression that happened sometime later in the course of the disease. As shown by the corresponding Kaplan-Meier curves, the majority of patients not responding directly to zoledronic acid with NTx decline at first post-treatment evaluation experienced a rapid progression of bone disease within the first 50 days.

In contrast, BAP levels did not show any significant change following the administration of zoledronic acid. An explanation for this observation could be that zoledronic acid targets the osteoclasts and inhibits bone resorption, while bone formation is a longer process and, as a result, changes in bone formation markers happen at longer intervals.

In conclusion, serum NTx appears to be a useful marker in assessing bone metastatic status in patients with solid tumours and could be applied as an adjunct method for the monitoring of patients on bisphosphonate therapy. More specifically, it correlates with the type and extent of bone disease burden, reflects both the response to anti-resorptive treatment and the progression of bone disease, while it may also bear a prognostic significance with respect to bone disease outcome. In view of these findings, prospective studies to investigate whether NTx can detect early micrometastatic bone lesions in patients with a malignancy prone to bone metastases, before these lesions become apparent by imaging techniques, seems warranted.

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