Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 171–176
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Diagnostic value of bone remodeling markers in the diagnosis of bone metastases in patients with breast cancer

Dimitrios Pectasides[a,*], Dimitrios Farmakis[b], Maria Nikolaou[b], Ioannis Kanakis[c], Vassiliki Kostopoulou[b], Ioannis Papaconstantinou[d], Nikolaos K. Karamanos[c], Theofanis Economopoulos[a], Sotirios A. Raptis[a]

[a] *Second Department of Internal Medicine-Propaedeutic, Athens University Medical School, Attikon University Hospital, Athens, Greece*
[b] *Second Department of Medical Oncology, Metaxas Memorial Cancer Hospital, Piraeus, Greece*
[c] *Laboratory of Biochemistry, Department of Chemistry, University of Patras, Patras, Greece*
[d] *First Department of Surgery, Athens University Medical School, Laiko Hospital, Athens, Greece*

Received 18 August 2004; received in revised form 3 October 2004; accepted 5 October 2004
Available online 19 November 2004

Abstract

Metastatic spread to bone is common in patients with breast cancer and its early detection is required for the better management of these patients. Several biochemical markers of bone remodeling have been recently developed, in order to assess metastatic bone disease with non radiologic methods. The pyridinolin cross-linked amino-terminal telopeptide of type I collagen (NTx) has been measured in serum and urine as a specific marker of bone collagen breakdown, while the bone-isoform of alkaline phosphatase (BAP) has been used to determine bone formation activity.

Thirty-three consecutive ambulatory patients with metastatic breast cancer and bone metastases and 31 with extraskeletal metastases only, matched for age and menopausal status, were studied. Serum levels of NTx and BAP were measured by enzyme-linked immunosorbent assays. The diagnostic accuracy of both markers was evaluated by receiver operating characteristic (ROC) analysis.

Patients with bone metastases had significantly higher levels of NTx (37.0 ± 36.9 nM BCE versus 23.5 ± 21.0 nM BCE, $P < 0.05$) and BAP (57.8 ± 31.7 U/L versus 36.5 ± 28.5 U/L, $P < 0.01$) compared to those without bone metastases. NTx was positively correlated with BAP ($R = 0.340$, $P < 0.01$). The area under the ROC curve was 0.671 for NTx and 0.755 for BAP. Using a cut-off value of 29.7 nM BCE for NTx, specificity and sensitivity were 87.1% and 45.5%, respectively; in the case of BAP, using a cut-off value of 50.6 U/L, the specificity and sensitivity were 90.3% and 54.5%, respectively. In patients not receiving concomitant hormonal treatment, the area under the ROC curve was 0.724 for NTx and 0.822 for BAP; in this subgroup of patients, using a cut-off value of 30.0 nM BCE for NTx, the specificity and sensitivity were 96.2% and 47.1%, respectively, while using a cut-off value of 50.0 U/L for BAP, the corresponding percentages were 92.3% and 70.6%.

Although serum NTx and BAP are quite specific, they are not sensitive enough to diagnose bone metastases in patients with advanced breast cancer. Their diagnostic accuracy, however, is considerably enhanced in patients not receiving hormonal therapy.

Keywords: Bone remodeling markers; Amino-terminal telopeptide of type I collagen; Alkaline phosphatase; Metastatic breast cancer; Bone metastases; Hormone therapy

1. Introduction

Bone is the second most common site of metastatic spread in breast cancer and accounts for the highest proportion of first site of relapse in these patients [1]. The annual rate of bone metastases development is higher in node-positive and

* Corresponding author. Gravias 5B, Aghia Paraskevi, Athens 15342, Greece. Tel.: +30 210 600 8610; fax: +30 210 600 8610.
E-mail addresses: pectasid@otenet.gr (D. Pectasides), dfarm1@panafonet.gr (D. Farmakis).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.10.007

in estrogen receptor-positive patients [2]. As the disease progresses, almost 70% of patients will develop bone metastases [3]. The median survival from the diagnosis of bone involvement ranges between 18 and 20 months, with major causes of morbidity the skeletal related complications, such as pathologic fractures, hypercalcemia and spinal cord compression [4]. Early detection and treatment is of utmost importance, as skeletal metastases may influence the quality of patients' life.

Plain radiographs remain the standard method for the diagnosis and follow-up of skeletal involvement while bone scintigraphy is also used as a whole-body imaging technique with extreme sensitivity. In addition, several biochemical markers of bone formation and bone resorption have recently been developed, in order to assess metastatic bone disease. Among these markers, bone-specific alkaline phosphatase (BAP) reflects bone formation, while the amino-terminal telopeptide of type I collagen (NTx) represents a degradation product of mature collagen and reflects bone resorption.

The biochemical markers of bone turnover have provided useful data in patients with osteoporosis, Paget's disease, hypercalcemia and bone metastases [5–8]. Bone-specific alkaline phosphatase has been used as a marker of skeletal metastases, although it seems of limited use in assessing the anti-resorptive response [9]. Urinary NTx, on the other hand, has already been shown to correlate with the type and extent of bone metastases and has been used to monitor the anti-resorptive effect of bisphosphonate therapy in patients with hypercalcemia and bone metastases [7,8,10]. Although the standard method of NTx evaluation is the measurement of urine concentrations, normalized to creatinine levels, it seems that serum assays have less sample to sample variability and are more easily available that their urine indices, as serum specimens are also used for numerous other laboratory investigations [6,11,12].

The aim of the present study was to determine the diagnostic validity of serum NTx and BAP in predicting skeletal metastases in patients with breast cancer.

2. Patients and methods

2.1. Patients

All consecutive ambulatory patients with newly diagnosed bone metastases and histologically confirmed breast cancer, with or without extraskeletal involvement, who were referred to our Department between July 2002 and December 2003, were considered for enrollment. Another group of patients with breast cancer bearing only extraskeletal metastases, matched for age and menopausal status, were also studied. Patients with bone metabolic diseases, renal failure, feeding disorders or a second primary malignancy were excluded from the study.

All patients underwent clinical examination, bone survey with Tc-99 bone scan and X-rays and assessment of the extraskeletal disease with chest X-rays, liver ultrasonogram and CT scans when indicated. Bone metastases were classified according to the type and bulk of skeletal lesions. More specifically, lesions were characterized by an independent viewer as lytic, blastic or mixed, whereas the bulk of disease, which concerned the number of segments involved, was characterized as few (<3 independent segments), or multiple (≥3) site involvement.

Post-menopausal status was defined by one of the following criteria: (i) spontaneous amenorrhea for at least 5 years; (ii) spontaneous amenorrhea lasting at least 12 months with gonadotrophin levels within the postmenopausal range (FSH: 20–143 mIU/ml, LH: 16–71 mIU/ml); (iii) secondary amenorrhea due to surgical treatment or radiotherapy.

The trial was approved by the local ethical committee and all patients enrolled gave informed consent.

2.2. Biochemical analysis

Bone markers were measured in serum obtained from fasting morning blood samples and stored at −80 °C until to be assayed. Determination of NTx was performed by an enzyme-linked immunosorbent assay (ELISA, Osteomark™ NTx serum test, Ostex Inernational Inc., Seattle, USA). This assay is a competitive inhibition ELISA format in which the NTx antigen, which has been absorbed to the surface of microtiter plate wells, specifically the a-2 chain of *N*-telopeptide fragment, is recognized by a horseradish peroxidase labeled monoclonal antibody. After adding 100 μl of each calibrator, control and sample into the wells and 100 μl of antibody conjucate diluent (1:101), the plate was incubated for 90 min at room temperature. The next steps included five washes with wash solution, addition of the chromogen reagent (200 μl/well) and incubation for 30 min. Finally, the developed blue color in the wells turned to yellow when added 100 μl 1 M H_2SO_4 as stopping reagent. Optical density was measured at 450 nm with a 630 nm reference filter. Serum NTx levels were expressed in nanomoles of bone collagen equivalents (BCE) per liter (nM BCE). According to the literature, the mean reported value in healthy individuals is 15.9 ± 3.8 nM BCE [13].

The levels of BAP were determined by ELISA, using a commercially available kit (Metra™ BAP Kit, Metra Biosystem, San Diego, CA, USA). ELISA was performed on sterile 96-well round bottomed microplates. Each well contained immobilized monoclonal anti-BAP IgG murine antibody. After the addition of 125 μl of assay buffer, and applying 20 μl of the samples to each well (all runs in triplicate), the mixtures were incubated for 3 h at room temperature. Microplates were washed and the next step was to add 150 μl of substrate comprised of paranitrophenol phosphate (pNPP) in 2-amino-2-methyl-1-propanol and to incubate for 30 min for color development. The reaction was determined with 1 N NaOH and optical density (OD) was measured at 405 nm in Molecular

Devices E-max photometer. All quantitative results for BAP were expressed as units per liter (U/L) where 1 Unit represents 1 μmol of pNPP in the substrate buffer. The reference normal 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).

2.3. 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 S.D. Mean values were compared between groups using Student's *t*-test or Mann–Whitney *U*-test, according to the distribution of the variables tested by the Kolmogorov–Smirnov test. Chi-square test was used to compare categorical variables. Linear regression analysis was used to investigate potential relationships between variables. Receiver operating characteristics (ROC) curves were plotted to evaluate the diagnostic accuracy of bone remodeling markers in predicting bone metastases. A $P < 0.05$ was considered statistically significant.

3. Results

Sixty-four patients were enrolled in the study, 33 with bone and extraskeletal metastases and 31 with extraskeletal metastases only. Patients' baseline characteristics are shown in Table 1. The two patient groups did not differ with respect to age and menopausal status. Patients with bone metastases, however, had a higher percentage of positive hormonal – estrogen or progesterone – receptors. Patients with bone metastases were mainly treated with hormonal therapy, while chemotherapy was more frequently given in patients with extraskeletal metastases only. Concerning the sites of metastases, liver involvement occurred in 27 patients, followed by lung (21 patients), brain (8 patients), skin (8 patients) and lymph nodes (4 patients); 5 patients had locally advanced breast cancer and 6 had other sites of metastatic involvement, including bone marrow, pleura and palate.

Out of 33 patients with skeletal metastases, 10 had only bone metastases and 23 had both skeletal and extraskeletal metastases. The characteristics of this subgroup of patients are demonstrated in Table 2.

The mean NTx and BAP values were significantly higher in patients with bone metastases compared to those without bone disease (37.0 ± 36.9 nM BCE versus 23.5 ± 21.0 nM BCE, $P < 0.05$ and 57.8 ± 31.7 U/L versus 36.5 ± 28.5 U/L, $P < 0.01$, respectively, Fig. 1). Overall, NTx was positively correlated with BAP ($R = 0.340$, $P < 0.01$). However, when the two patient subgroups were studied separately, this correlation was no more significant in patients with no bone disease, while in patients with bone metastases the correlation remained significant and was even stronger ($R = 0.448$, $P < 0.01$).

Table 2
Metastatic status in patients with bone metastases

No. of patients	33
Type of bone metastases	
Lytic	11 (33.3%)
Blastic	10 (30.3%)
Mixed	12 (36.4%)
Bulk of bone metastases	
Few-site involvement (≤3 sites)	10 (30.3%)
Multiple-site involvement (>3 sites)	23 (69.7%)
Extraskeletal metastases	23 (69.7%)

In the group of patients with bone metastases, mean BAP was higher in patients with blastic than lytic bone lesions (56.4 ± 29.8 U/L versus 43.6 ± 21.3 U/L), but the difference was not statistically significant. In contrast, mean BAP was significantly higher in patients with multiple than in those with few bone site involvement (65.7 ± 34.2 U/L versus 39.8 ± 13.8 U/L, $P < 0.01$). On the other hand, mean NTx was significantly higher in patients with blastic skeletal metastases than in those with lytic ones (37.1 ± 25.9 nM BCE versus 18.5 ± 7.3 nM BCE, $P < 0.05$). Mean NTx also showed a trend for being higher in patients with multiple bone lesions compared to those with few lesions (43.3 ± 42.1 nM BCE versus 22.6 ± 14.5 nM BCE, $P = 0.057$).

Regarding the effect of concomitant therapy, NTx and BAP were significantly correlated in patients not receiving hormonal therapy and this correlation was stronger than the one encountered in the overall study group ($R = 0.448$, $P < 0.01$). However, the correlation disappeared in patients treated with concomitant hormonal therapy. Both mean NTx and BAP were higher in patients receiving hormonal therapy (39.8 ± 42.9 nM BCE versus 25.9 ± 22.0 nM BCE for NTx and 50.0 ± 29.8 U/L versus 46.3 ± 33.0 U/L for BAP, respectively), but the differences were not significant. In patients receiving hormonal therapy, mean NTx and BAP did not differ significantly between patients with and without bone metastases (37.3 ± 45.2 nM BCE

Table 1
Patient characteristics

	Total	Bone metastases (−)	Bone metastases (+)	*P*
No. of patients	64	31	33	
Age (years)	61 ± 12	62 ± 12	61 ± 12	NS
Postmenopausal	50 (78.1%)	26 (83.9%)	24 (72.7%)	NS
Hormonal receptor (+)	31 (48.4%)	9 (29.0%)	22 (66.7%)	<0.01
On hormonal therapy	21 (32.8%)	5 (16.1%)	16 (48.5%)	<0.01
On chemotherapy	52 (81.3%)	30 (96.8%)	22 (66.7%)	<0.01

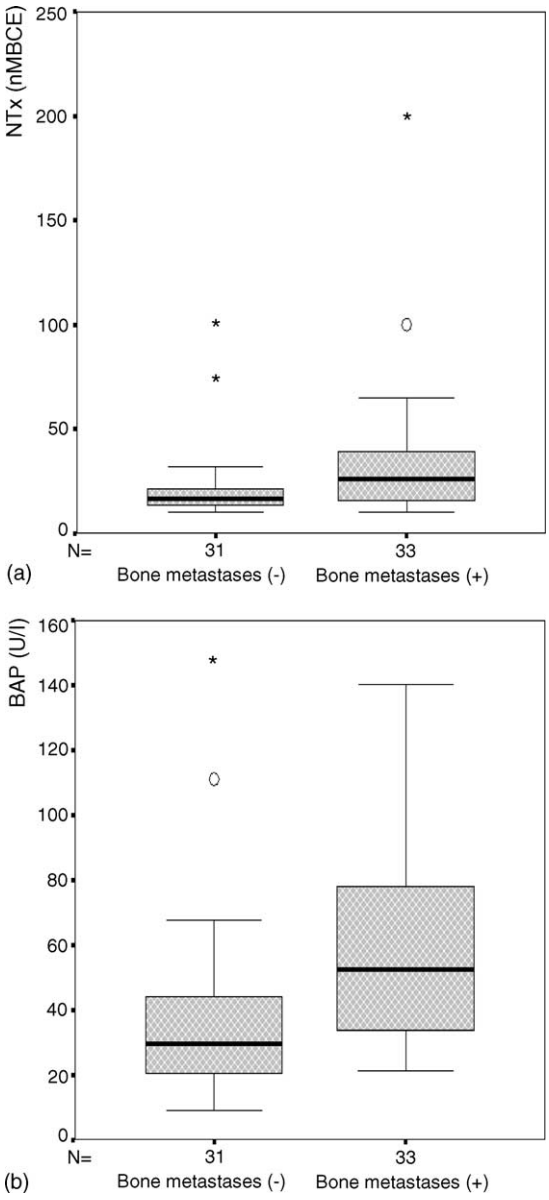

Fig. 1. Box-plot graphs of NTx (a) and bone-specific alkaline phosphatase (b) in metastatic breast cancer patients with and without bone metastases. In each plot, the box represents the interquartile range which contains the 50% of observed values; the bold line across the box indicates the median; the whiskers extend from the box to the highest and lowest values, excluding outliers and extremes; open circles represent the outliers, which are cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box; stars represent the extremes, which are cases with values more than 3 box lengths from the upper or lower edge of the box.

versus 47.8 ± 37.9 nM BCE for NTx and 52.0 ± 33.1 U/L versus 43.6 ± 16.5 U/L for BAP, respectively), while in patients not receiving hormonal therapy, these differences were statistically significant (36.7 ± 28.7 nM BCE versus 18.8 ± 12.5 nM BCE, $P < 0.05$, for NTx and 63.4 ± 30.2 U/L versus 35.2 ± 30.3 U/L, $P < 0.01$, for BAP, respectively).

In what concerns the diagnostic accuracy of NTx and BAP in predicting bone metastases, the corresponding ROC curves for NTx and BAP are shown in Fig. 2. The area under the ROC

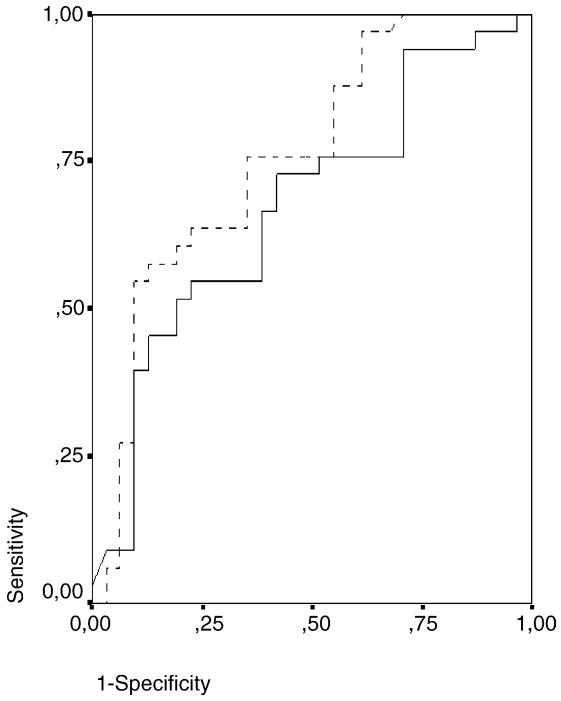

Fig. 2. Receiver operating characteristics curves for NTx (solid line) and bone-specific alkaline phosphatase (dotted line).

curve was 0.671 for NTx (95% confidence intervals (CI), 0.537–0.804, $P < 0.05$ versus the reference area of 0.500) and 0.755 for BAP (95% CI, 0.635–0.875, $P < 0.001$). Using a cut-off value of 29.7 nM BCE, the specificity and sensitivity of NTx were 87.1% and 45.5%, respectively. In the case of BAP, using a cut-off value of 50.6 U/L, the specificity and sensitivity were 90.3% and 54.5%, respectively. The ROC curves were also plotted separately for patients treated with and without concomitant hormonal therapy (Fig. 3). In patients receiving hormonal therapy, the areas under the curve for both NTx and BAP were extremely low (0.400 and 0.513, respectively) and did not differ significantly from the reference area of 0.500. In contrast, in patients not receiving hormonal therapy the corresponding areas were even higher than those of the overall group (0.724, $P < 0.05$ for NTx and 0.822, $P < 0.001$ for BAP). In this subgroup of patients, using a cut-off value of 30.0 nM BCE, the specificity and sensitivity of NTx were 96.2% and 47.1%, respectively; regarding BAP, using a cut-off value of 50.0 U/L, the specificity and sensitivity were 92.3% and 70.6%, respectively.

4. Discussion

Several biochemical markers of bone remodeling have recently been developed as an objective means to assess patients with bone metabolic diseases, such as osteoporosis and Paget's disease, in addition to imaging techniques [6,14]. These biochemical markers are mostly stable-end products released into circulation either during bone formation or dur-

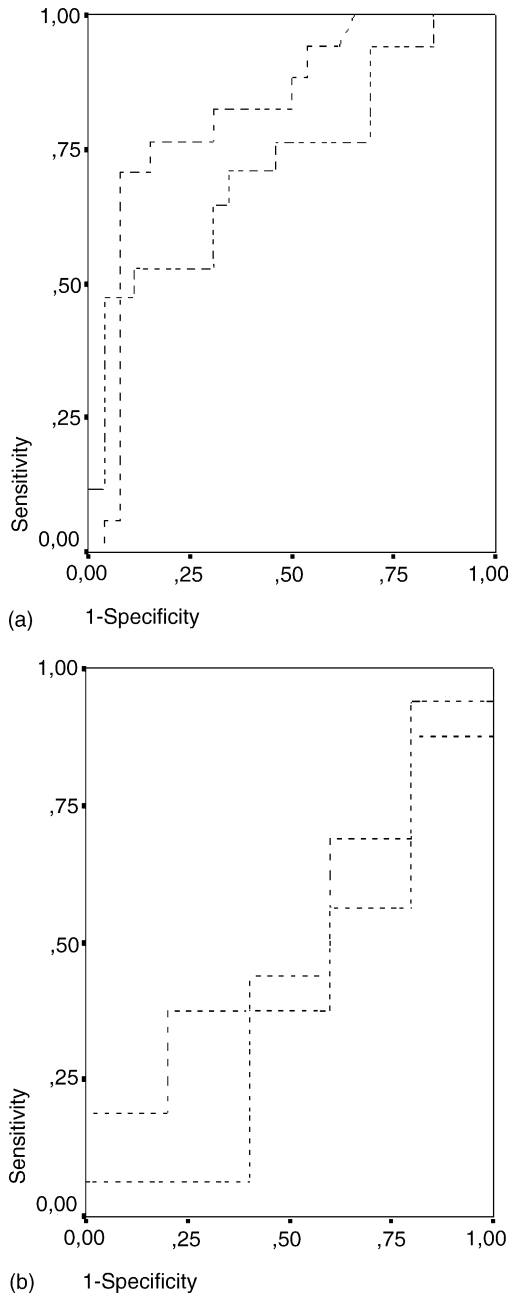

Fig. 3. Receiver operating characteristics curves for NTx (solid line) and bone-specific alkaline phosphatase (dotted line) in patients not receiving hormonal therapy (a) and in those on concurrent hormonal therapy (b).

ing bone resorption and can be detected in serum or urine. Pyridinium cross-links and associated telopeptides – amino-terminal, NTx and carboxy-terminal, CTx – represent fragments of cross-linking amino acid derivatives that stabilize the type I collagen fibrils in bone. Type I collagen telopeptide sequences that contain these cross-linking residues have proven to be more specific markers of bone resorption than the free crosslinks themselves [15]. 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 bone-resorption phase.

Recently, NTx levels have been estimated in patients with bone metastatic disease. Studies have shown that urinary NTx was elevated in patients with untreated bone metastases and correlated with the extent and type of metastases [8,10,16]. Urinary NTx may also assess the anti-resorptive and analgesic effect of bisphosphonate treatment, evaluate the disease progression in bonewhile it may be used to schedule the appropriate dose and efficacy of the bisphosphonate [5,10,11,17]. Among bone formation markers, BAP has been shown to be elevated in patients with untreated skeletal metastases as the overall bone remodeling process is usually imbalanced [10,18]. Nevertheless, it is of limited value in assessing the response to anti-resorptive treatment [10]. Limited and conflicting data are available about the use of these markers in screening bone metastatic disease. Urine NTx had been associated with a high diagnostic validity in patients with bone metastases from a variety of solid tumors, including lung cancer and early stage prostatic cancer [8,19,20].

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 [21]. More specifically, patients with elevated serum NTx levels at baseline had a shorter duration of clinical benefit, time to tumor progression and overall survival compared to those without NTx elevation.

In the present study, serum NTx and BAP concentrations were evaluated in metastatic breast cancer patients with and without bone metastases. The two groups were matched for age and menopausal status, factors that are known to interfere with bone metabolism. Both NTx and BAP were significantly higher in patients with bone metastases. Furthermore, in this subgroup, the correlation between NTx and BAP was stronger than in the entire cohort. Serum NTx and BAP were also correlated, as it was expected, with the bulk of bone disease and the type of lesions.

In the subgroup of patients receiving hormonal therapy, mean NTx and BAP did not differ significantly between patients with and without bone metastases. This is consistent with the well-known correlation between hormonal replacement therapy and osteoporosis in patients with breast cancer [22,23].

According to ROC analysis, BAP seemed to have a better diagnostic accuracy than NTx in predicting bone metastases in patients with advanced breast cancer. Both markers, although quite specific, with overall specificities reaching 90%, were not sensitive enough, as sensitivities were lower than 55%. This lack of sensitivity may be attributed to the concomitant hormone-treatment or to the high incidence of bone micrometastatic disease in patients with metastatic breast cancer. Indeed, it has been shown by autopsy series that over 75% of patients with advanced breast cancer have bone metastases [3]. Regarding the first factor, ROC curves were plotted separately as to the presence or absence of concomitant hormonal therapy. Actually, in patients not receiving hormonal therapy, both NTx and BAP had an enhanced diag-

nostic accuracy for bone metastases compared to the entire group, with NTx specificity reaching 96% and BAP sensitivity reaching 70%. In contrast, in patients on hormonal therapy, the areas under the ROC curves for both NTx and BAP were extremely low and did not differ significantly from the reference area of 0.500.

In conclusion, although serum NTx and BAP are specific markers and their diagnostic accuracy is substantially enhanced in patients not receiving hormonal therapy, they are generally characterized by low sensitivity in predicting skeletal metastases in advanced breast cancer patients. The diagnostic accuracy of these and other markers in detecting the presence of bone micrometastases, before these metastases become clinically apparent by radiologic techniques, may be the aim of future prospective studies.

References

[1] H.K. Brown, J.H. Healey, in: V.T. De Vita Jr., S. Hellman, S.A. Rosenberg (Eds.), Cancer: Principles and Practice of Oncology, 6th ed., Lippincott Williams and Wilkins, Philadelphia, 2001, pp. 2713–2729.
[2] R. Smith, W. Jiping, J. Bryant, A. Kay, A.G. Paterson, N. Wolmark, Proc. Am. Soc. Clin. Oncol. 18 (1999) 120a (abstract 457).
[3] R.E. Coleman, R.D. Rubens, Br. J. Cancer 55 (1987) 61–66.
[4] A.H.G. Paterson, Bone 8 (1987) 17–22.
[5] N.H. Bjarnason, C. Christiansen, Bone 26 (2000) 561–569.
[6] J.D. Clemens, M.V. Herrick, F.R. Singer, D.R. Eyre, Clin. Chem. 43 (1997) 2058–2063.
[7] J. Vinholes, C.Y. Guo, O.P. Purohit, R. Eastell, R.E. Coleman, J. Clin. Oncol. 15 (1997) 131–138.
[8] M. Izumi, Y. Nakanishi, K. Takayama, K. Kimotsuki, K. Inoue, H. Wataya, T. Minami, N. Hara, Cancer 91 (2001) 1487–1493.
[9] L.M. Demers, L. Costa, A. Lipton, Clin. Orthop. Rel. Res. 415 (2003) 138–147.
[10] L. Costa, L.M. Demers, A. Gouveia-Oliveira, J. Schaller, E.B. Costa, M.C. de Moura, A. Lipton, J. Clin. Oncol. 20 (2002) 826–850.
[11] R. Eastell, N. Mallinak, S. Weiss, M. Ettinger, M. Pettinger, D. Cain, K. Fressland, C. Chesnut III, Bone Miner. Res. 15 (2000) 594–598.
[12] H.W. Woitge, M. Pecherstorfer, Y. Li, A.V. Keck, E. Horn, R. Ziegler, M.J. Seibel, J. Bone Miner. Res. 14 (1999) 792–801.
[13] I. Kanakis, M. Nikolaou, D. Pectasides, C. Kiamouris, N.K. Karamanos, J. Pharm. Biomed. Anal. 34 (2004) 827–832.
[14] L.M. Demers, M. Kleerkoper, Clin. Chem. 40 (1994) 1994–1995.
[15] P. Garnero, E. Gineyts, P. Arbault, C. Christiansen, P.D. Delmas, J. Bone Miner. Res. 10 (1995) 641–649.
[16] L.M. Demers, L. Costa, A. Lipton, Cancer 88 (2000) 2919–2926.
[17] J. Vinholes, O.P. Purohit, M.E. Abbey, R. Eastell, R.E. Coleman, Annu. Oncol. 8 (1997) 1243–1250.
[18] P. Magnusson, L. Larsson, G. Englund, B. Larsson, P. Strang, L. Selin-Sjogren, Clin. Chem. 44 (1998) 1621–1628.
[19] H. Miura, I. Yamamoto, M. Takada, Y. Kigami, T. Ohta, I. Yuu, Y. Hamanaka, R. Matsushita, R. Morita, Endocr. J. 44 (1997) 751–757.
[20] C. de la Piedra, N.A. Castro-Errecaborde, M.L. Traba, C. Mendez-Davila, C. Garcia-Moreno, L. Rodriguez de Acuna, J. Rodriguez-Molina, Clin. Chem. Acta 331 (2003) 45–53.
[21] S.M. Ali, L.M. Demers, K. Leitzel, H.A. Harvey, D. Clemens, N. Mallinak, L. Engle, V. Chinchilli, L. Costa, C. Brady, J. Seaman, A. Lipton, Annu. Oncol. 15 (2004) 455–459.
[22] E. Bajetta, A. Martinetti, N. Zilembo, P. Pozzi, I. La Torre, L. Ferrari, E. Seregni, R. Longarini, G. Salvucci, E. Bombardieri, Annu. Oncol. 13 (2002) 1059–1066.
[23] M. Dowsett, Semin. Oncol. 30 (2003) 58–69.