Pergamon

European Journal of Cancer, Vol. 34, No. 13, pp. 2021–2026, 1998

Printed in Great Britain
0959-8049/98/$—see front matter

PII: S0959-8049(98)00277-9

Original Paper

Markers of Bone Resorption in Patients Treated with Pamidronate

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Pyridinoline (PYD), deoxypyridinoline (DPD), and *N*-telopeptide (NTX) are markers of bone resorption. In cancer patients with bone metastases, NTX is more often elevated than either of the pyridinolines. Bisphosphonates inhibit osteoclasts and their treatment decreases skeletal complications of malignancy. The aim of this study was to correlate urinary PYD, DPD, and NTX levels with clinical events in patients receiving pamidronate. 25 cancer patients with lytic bone disease were treated with monthly pamidronate combined with endocrine or chemotherapy; 27 others were on placebo. Twenty-four hour urines were collected at baseline, 1, 3 and 6 months. NTX values were determined by enzyme-linked immunosorbent assay (ELISA); PYD and DPD values were determined by reverse phase high performance liquid chromatography (HPLC). Two hour urines were also collected weekly for 21 patients. The greatest difference as a result of pamidronate treatment was observed in NTX values. Maximum suppression was achieved 2 weeks after treatment. Of the 25 patients who received pamidronate, 21 had initially elevated NTX values. 12 of the 21 finished with normal NTX values, whilst 9/21 had NTX values which remained abnormally elevated. The proportions of patients with fractures between these two subgroups approached statistical significance ($P = 0.07$) while the proportions with bony disease progression were significant ($P = 0.03$, Fisher's exact test). Measuring NTX levels appears useful in monitoring bisphosphonate therapy of bone metastases. The goal of treatment should be to normalise NTX excretion. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: bone resorption, bisphosphonates, *N*-telopeptide
Eur J Cancer, Vol. 34, No. 13, pp. 2021–2026, 1998

INTRODUCTION

THE SPREAD of cancer to bone occurs in approximately 25% of cancer patients who develop metastatic disease [1]. Bone metastases can be lytic, blastic or a combination of these [2]. Increased osteoclast activity has been associated with lytic bone metastases [3]. Various urine tests have been used to reflect osteolytic activity. These include calcium/creatinine and hydroxyproline/creatinine ratios. The collagen crosslinks pyridinoline (PYD), deoxypyridinoline (DPD), and more recently the crosslinked *N*-telopeptides of type-I collagen (NTX) have been proposed as more specific biochemical markers than the aforementioned traditional markers of bone resorption [4–15].

Bisphosphonates are inhibitors of osteoclast activity and their use results in decreased crosslink values in patients with increased osteoclastic activity. Bisphosphonates act by preventing the maturation of precursor cells into osteoclasts, are directly toxic to osteoclasts, and prevent osteoclasts from attaching to the bone surface. The purpose of this study was to determine the level of these newer urinary markers of bone resorption in patients treated with either the bisphosphonate pamidronate (Aredia; APD) or placebo.

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Received 28 Oct. 1997; revised and accepted 13 Jul. 1998.

PATIENTS AND METHODS

Patients

In the initial phase of this study, 52 postmenopausal breast cancer patients with bone metastases (median age 61 years; range 46–78 years) were treated with either the bisphosphonate pamidronate administered at a dose of 90 mg intravenously (i.v.) every month or placebo in addition to standard endocrine therapy or chemotherapy as appropriate for their malignancy. A 24 h urine specimen was collected prior to initiation of therapy and at each monthly visit for 6 months.

In the second phase of this study, 2 h urine specimens after the first morning void were collected weekly for 4 weeks from 21 patients: 13 patients (8 postmenopausal breast cancer; 5 myeloma—4 male, 1 female) were treated with pamidronate 90 mg i.v. every month and 8 patients (5 breast cancer; 3 myeloma–2 male, 1 female) received placebo in addition to standard endocrine therapy or chemotherapy. These specimens were obtained during the first 1–3 months after initiation of treatment.

Crosslink assay

For PYD and DPD, equal volumes (250 µl) of urine and 12 N HCl were hydrolysed overnight, concentrated using CF-1 cellulose columns, and eluted with deionised water. Lyophilisation followed. After reconstitution, the samples were analysed by reverse phase high performance liquid chromatography (HPLC) using an acetonitrile linear gradient and purified standards from Metra Biosystems (Palo Alto, California, U.S.A.). The results were quantitated using the crosslinks' natural fluorescence and normalised to creatinine excretion. The results were expressed in µmol/mol creatinine [13, 14]. The assay detection limit was 1 pmol, with an interassay imprecision averaging 12% at PYD and DPD concentrations of 26 and 9.2 µmol/mol creatinine, respectively. The reference ranges for PYD and DPD for a normal premenopausal female population were 18–40 and 4–10 µmol/mol creatinine, respectively.

NTX assay

The NTX values were determined by enzyme-linked immunosorbent assay (ELISA; Ostex International, Inc., Seattle, Washington, U.S.A.). Briefly, a small amount of urine was added to a 96 well microtitre plate coated with antigen. A purified mouse monoclonal antibody, conjugated to horseradish peroxidase (HRP), was added to each well. During an initial incubation period, antigen in the samples competed with the solid phase antigen for binding to the antibody. The wells were washed to remove unbound material and tetramethyl benzidine/hydrogen peroxide (TMB/H_2O_2) was added as the chromogenic substrate. The colour intensity indicated the amount of conjugated antibody bound to the solid phase antigen and was indirectly proportional to the amount of antigen in the sample. The reaction was stopped using 1N sulphuric acid and the plates were read at 450 nm with a microtitre plate reader. In this immunoassay the low end of sensitivity of NTX was 20 nmol of NTX expressed in bone collagen equivalents (BCE) with an interassay coefficient of variation (CV) of 4–6%. All values were corrected for creatinine and expressed as nmol BCE/mmol creatinine. The normal values for NTX were obtained from Ostex, Inc., on 258 normal premenopausal women. The upper limit for 95% of normal premenopausal women is 65 BCE.

Statistical methods

Because repeated measurements of PYD, DPD, and NTX were collected over time, a longitudinal data analysis was applied (PROC MIXED of SAS^R) . For each of the placebo

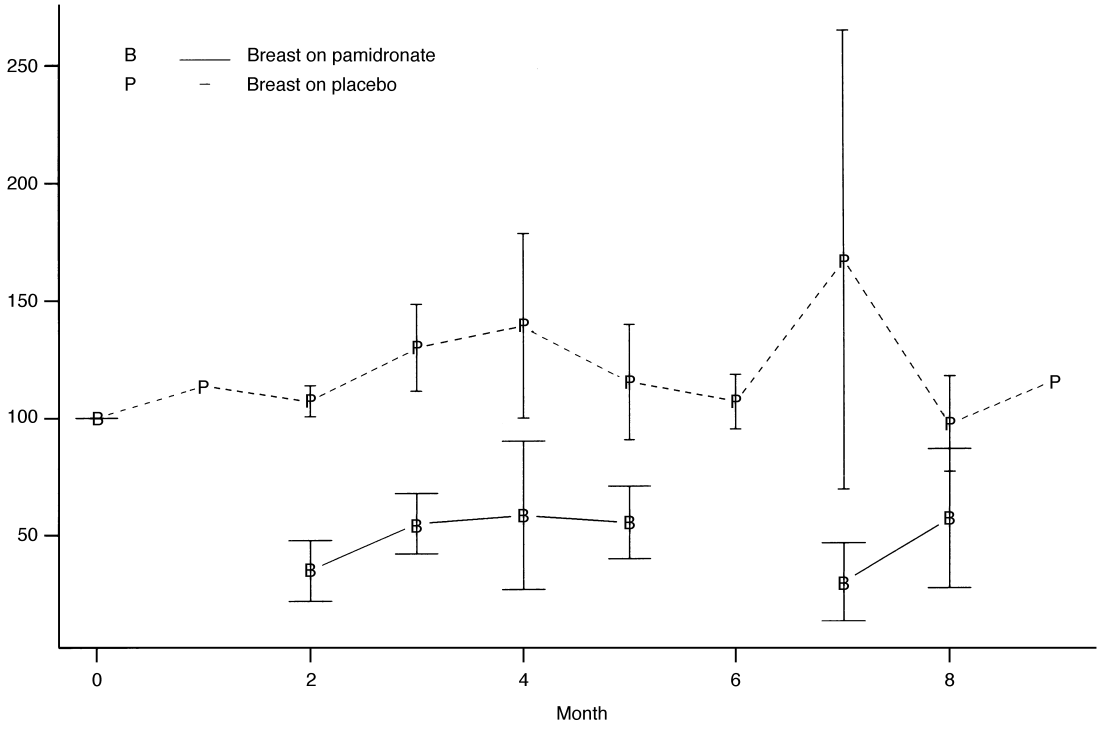

Figure 1. Percentage change in *N*-telopeptide (NTX) values in 24 h urines from 25 breast cancer patients treated with pamidronate versus 27 patients on placebo at baseline, 1, 3 and 6 months.

and pamidronate groups, an intercept-slope model was fitted over time. A significant slope estimate ($P < 0.05$) was indicative of a change within a group. A significant difference between the slope estimates ($P < 0.05$) was indicative that the placebo and pamidronate groups differed with respect to their changes over time. For the weekly data, additional exploratory analyses were performed whereby the changes from baseline were examined via t tests to determine when significant changes occurred.

RESULTS

Monthly urines

Twenty-four hour urine specimens were collected from 52 patients with bone metastases. Over the period of 6 months of the study, there was no significant difference in PYD values between the patients who received pamidronate or placebo (data not shown, $P = 0.51$). There was a significant decrease in DPD over time in the patients treated with pamidronate (data not shown, $P = 0.04$), but when compared

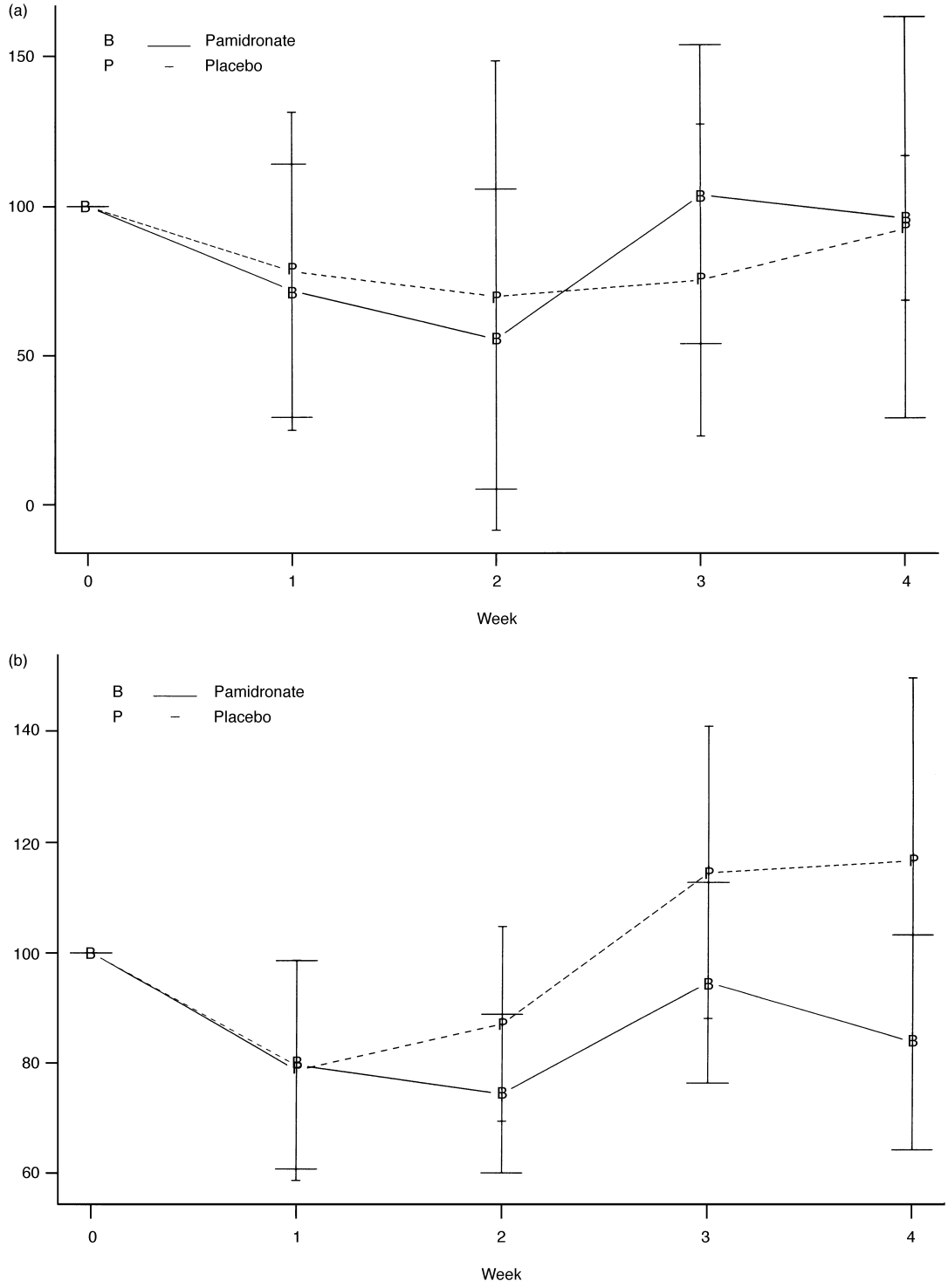

Figure 2. Percentage change in (a) deoxypyridinoline (DPD) values; and (b) *N*-telopeptide (NTX) values in 2 h urines after the first morning void from 13 patients treated with pamidronate versus 8 patients on placebo. Urines were collected weekly for 4 weeks.

with the placebo group, this was not significant (data not shown, $P = 0.35$). The greatest difference was observed with the NTX values for patients treated with pamidronate versus placebo ($P = 0.002$) (Figure 1).

Weekly urines

In this phase of the study, 2 h urines after the first morning void were collected weekly for 4 weeks from 21 patients. 13 patients had metastatic breast cancer and 8 patients had multiple myeloma. There were no significant changes in PYD, DPD, or NTX over the 4-week period for the patients who received placebo. In the patients who received pamidronate, there was no significant change from baseline in the PYD values over the 4-week study period following pamidronate administration (data not shown). In contrast, the levels of DPD and NTX were significantly suppressed in the pamidronate group over baseline values. The maximum suppressive effect was achieved 2 weeks after pamidronate treatment (Figure 2a and b). The decrease observed was significant for both DPD (mean = − 5.1, standard error (S.E.) = 1.9, $P = 0.02$) and NTX (mean = − 19.4, S.E. = 9.3, $P = 0.06$).

Comparison between breast cancer and multiple myeloma

Destruction of bone in multiple myeloma is due to osteoclast hyperactivity and is not accompanied by increased osteoblast activity—an uncoupling of the bone remodelling unit. Most often in metastatic breast cancer, there is some osteoblastic activity associated with osteoclast mediated osteolysis. Thus, to determine whether there was a differential response when each of these diseases was treated with an osteoclast inhibitor, 8 breast cancer patients and 5 multiple myeloma patients who were treated with pamidronate were then assayed at weekly intervals and the results compared. There were no significant differences observed in PYD, DPD, or NTX levels between the breast cancer or multiple myeloma patients receiving pamidronate (Figure 3). The maximum effect was again observed at week 2. Within each group it appeared that there were patients whose cross-link values decreased as well as those whose levels increased.

Correlation of NTX, DPD values and clinical events

With respect to the 27 placebo patients, only 14 experienced a decrease in NTX values (mean change of 18%, standard deviation (S.D.) = 86%. 20 of the 27 placebo patients had initial values of NTX in the abnormal range. Only 6 of the 20 placebo patients had final NTX values in the normal range ($P = 0.12$ compared with the pamidronate group, Fisher's exact test). Of the 25 patients randomised to pamidronate, 23 experienced a decrease in NTX value (mean decrease of 44%, S.D. = 56%). Of these 25 patients, 21 had initial values of NTX in the abnormal range (> 65 BCE, i.e. greater than 95% of premenopausal women). 12 of the 21 patients finished with an NTX value in the normal range. Therefore, two subgroups of patients were defined, namely, Group A, the 12 patients whose NTX value went from abnormal to normal and group B, the 9 patients whose NTX value stayed abnormal (Figure 4a and b). The observed proportions of patients with fractures, 5/12 (42%) versus 8/9 (89%), were close to statistical significance ($P = 0.07$, Fisher's exact test). The observed proportions of patients with disease progression in bone, 3/12 (25% group A) versus 7/9 (78%, group B), were statistically significant ($P = 0.03$, Fisher's exact test).

Similarly, we examined DPD levels in patients treated with pamidronate. There were 21 patients who had initial values of DPD in the elevated range (> 10 μmol/mol creatinine). At the final study visit, 7/21 patients had values in the normal

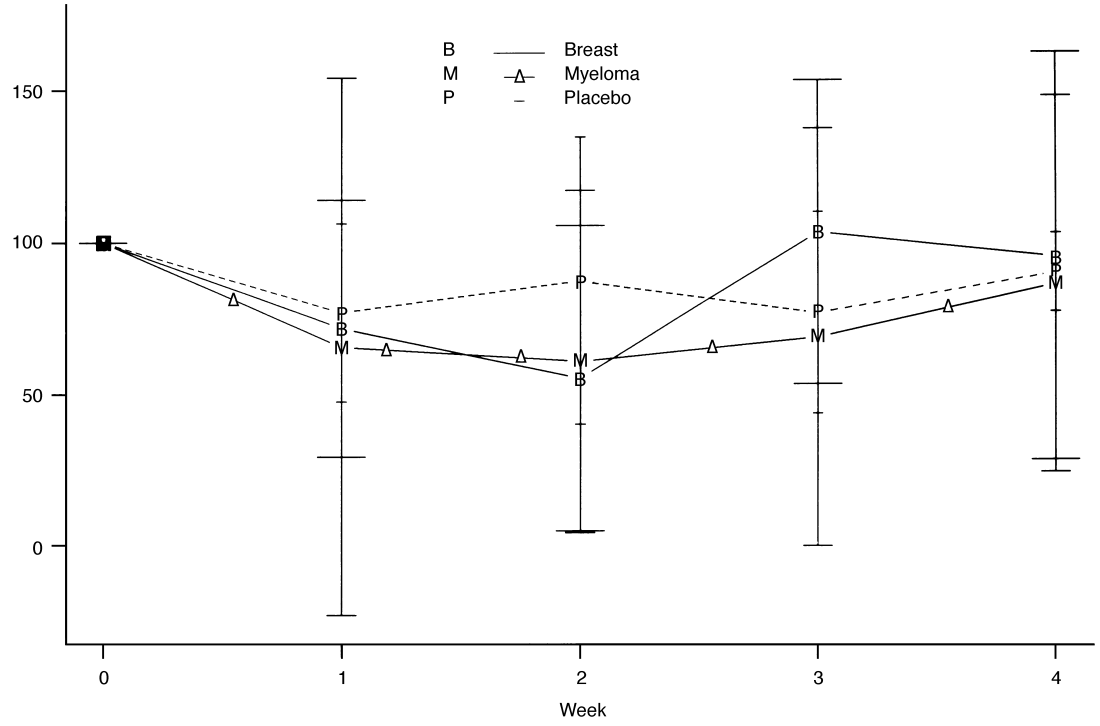

Figure 3. Percentage change in deoxypyridinoline (DPD) values in 2 h urines after the first morning void in 8 breast cancer patients on pamidronate, 5 multiple myeloma patients on pamidronate, and 8 patients on placebo. Urines were collected weekly for 4 weeks.

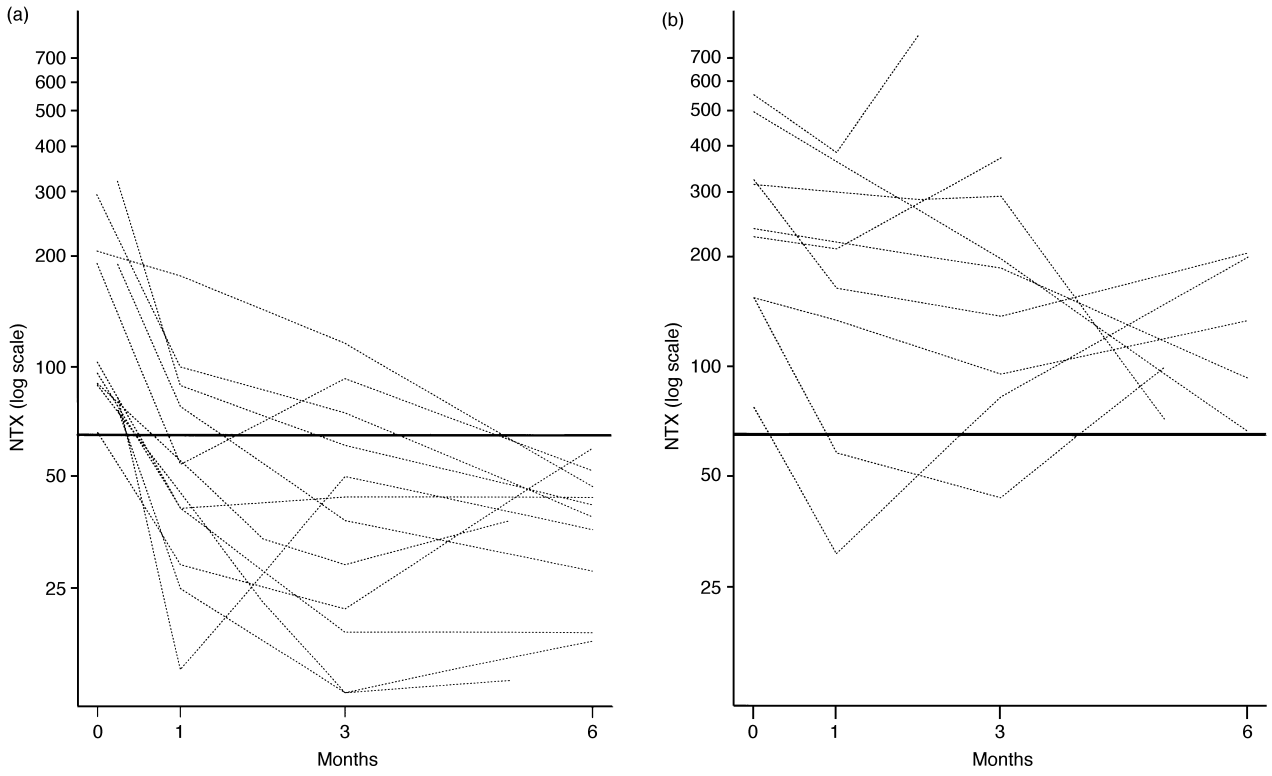

Figure 4. (a) Profile plot for patients on pamidronate with *N*-telopeptide (NTX) > 65 bone collagen equivalents (BCE) at the first visit but not at the last visit. A value > 65 BCE is elevated, i.e. greater than 95% of premenopausal women (b) Profile plot for patients on pamidronate with NTX > 65 BCE at the first and last visit. All values are expressed in BCE.

range. There was no statistical significance between the 7 patients whose values decreased into the normal range and the 14 patients whose values remain elevated with respect to incidence of fractures ($P = 0.58$) and disease progression in bone ($P = 0.61$).

DISCUSSION

Bisphosphonates have recently been employed to treat a variety of bone diseases. Positive results, as defined by reduced osteoclast activity, decreased fracture incidence, and decreased pain, have been obtained with second generation bisphosphonates in Paget's disease, osteoporosis, multiple myeloma, and recently in metastatic breast cancer [15–28]. It would be quite useful to be able to monitor the dose of bisphosphonate required to achieve a clinical antiresorptive benefit prior to significant changes in bone density at X-ray or the development of bone related clinical events.

Several newer biochemical tests have recently been developed to monitor osteoclast activity. Which test is optimal for this purpose is still a question. It is important for clinicians to know which test has the greatest utility to monitor response to therapy. We have previously shown in a group of cancer patients with metastatic disease to bone that NTX is more often abnormally elevated than either PYD or DPD [13, 14]. Similar results were again observed in this study. Of the three biochemical markers evaluated, the urine NTX level appears to best reflect decreased bone resorption in patients receiving antiresorptive therapy.

The optimal dose and schedule of bisphosphonate treatment for patients with osteolytic bone metastases is still unknown. So far, clinicians have employed a monthly treatment schedule with pamidronate. We found that maximal suppression of the markers of osteoclast activity occurred 2 weeks after the start of bisphosphonate treatment. Future schedules employing bisphosphonate therapy at 2 or 3 week intervals would appear to be indicated.

It is not known if all cancers metastatic to bone respond similarly to bisphosphonate therapy. Current thinking suggests that all cancers cause lytic destruction of bone by increasing osteoclast activity. Results presented here suggest that the bisphosphonates can have a beneficial effect in this regard and suppress the levels of resorption markers in most cancers causing osteolysis. There was no difference in the decrease of crosslink levels between a cohort of breast cancer patients and those with multiple myeloma, although patient numbers were limited. In addition, there were myeloma and breast cancer patients in whom a decline of crosslink levels was not observed despite therapy with monthly infusions of 90 mg of pamidronate. This situation may be analogous to the requirement of higher doses of pamidronate in patients with severe cancer associated hypercalcaemia (corrected serum calcium level ≥ 13.5 mg/dl) to achieve normocalcaemia than that required by patients with a moderate degree of hypercalcaemia to achieve the desired effect of suppressing bone resorption.

Most patients with lytic bone metastases (21/25) had an initial value of NTX in the abnormal range (> 65 BCE, i.e. greater than 95% of normal premenopausal women). 12 of these 21 patients after 6 months of treatment had a normal NTX value. Therefore, two subgroups of patients were defined: group A, the 12 patients whose NTX value went from abnormal to normal and group B, the 9 patients whose

NTX value stayed abnormal. The observed proportions of patients with fractures, 5/12 (42%) versus 8/9 (89%), were close to statistical significance ($P = 0.07$). The observed proportions of patients with progression of disease in bone, 3/12 (25%) versus 7/9 (78%), were statistically significant ($P = 0.03$). This result is similar to the observation that a decrease in NTX values after pamidronate therapy correlates with a decrease in pain (Vinholes and Coleman, Weston Park Hospital, Sheffield, U.K.). It would thus appear that measurement of NTX could be used to monitor results of bisphosphonate therapy of bone metastases. The goal of treatment should be to normalise excretion of NTX. In refractory patients, this might be obtained by using more frequent bisphosphonate administration, or a higher dose of pamidronate, or newer more potent bisphosphonates.

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Acknowledgements—The authors wish to thank Mrs Pierrette Brydle for her excellent help in the preparation of this manuscript.