

The Amino-terminal Propeptide (PINP) of Type I Collagen is a Clinically Valid Indicator of Bone Turnover and Extent of Metastatic Spread in Osseous Metastatic Breast Cancer

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Abstract. *Background:* The efficacy control for the treatment of bone metastases in breast cancer is difficult and usually initiated later and with longer time between treatment cycles than the restaging of visceral or soft tissue metastases. The amino-terminal propeptide (PINP) of type I collagen as a biochemical indicator of bone turnover might facilitate early and valid disease surveillance. The utility of total PINP was investigated in metastatic breast cancer patients, with or without bone metastases (for monitoring of therapy). The results were compared to the established markers, osteocalcin and β -carboxyterminal telopeptide (CTX) or crosslaps concentration. *Patients and Methods:* Baseline serum samples of 51 patients with metastatic breast cancer under chemotherapy were investigated. In total, 38 patients had been diagnosed with bone metastases while 13 had no evidence of metastatic spread to the bone. All the patients with bone spread received bisphosphonates in addition to systemic chemotherapy and/or antibody therapy or hormonal treatment. Osteocalcin, CTX and PINP levels were measured on an Elecsys[®]2010 analyzer (electrochemiluminescence immunoassay – ECLIA). The normal cut-off values were: osteocalcin < 41.3 pg/ml, CTX < 1008 pg/ml and PINP < 95 ng/ml. Based on overall treatment outcome, the patients were grouped as responders (CR/PR), with stable disease (SD) or displaying primary progression (PD). *Results:* The baseline levels of PINP were significantly higher in patients with bone metastases (median: 92.8 ng/ml) than in those without (median: 63.2 ng/ml, $p=0.044$). Patients

with more than seven bone metastases had significantly higher PINP levels (median: 149.7 ng/ml) than those with fewer than seven (median: 67.6 ng/ml, $p=0.04$). Significant differences were also found for osteocalcin and CTX, at $p=0.02$ and $p=0.04$, respectively, although the median levels remained under the normal cut-off levels. In terms of response assessment of bone spread, the PINP concentrations decreased in responders from 194.3 ng/ml to 100.4 ng/ml ($p=0.23$). In patients with SD, PINP remained at the same level of approximately 70 ng/ml ($p=0.16$), but increased in patients with PD from 83.4 ng/ml to 176.5 ng/ml ($p=0.14$). These trends rather than statistical difference were probably due to the limited patient cohort. No differences were found for the serum concentrations of PINP, CTX and osteocalcin between post- and pre-menopausal women. *Conclusion:* The PINP levels of the osseous metastatic breast cancer patients were elevated at baseline in comparison to those without bone involvement; the levels correlated to the number of bone metastases but were independent of the menopausal status. Thus, the levels of PINP under therapy might correlate with the response to therapy. Osteocalcin and CTX did not show similar sensitivity for the surveillance of bone metastases.

More than 50% of patients with breast cancer eventually develop bone metastases. However, the efficacy control of treatment for such bone metastases is difficult and usually initiated later than the restaging of visceral, or soft tissue metastases. Specific biochemical markers of bone metabolism might improve the follow-up of bone metastases under therapy and allow for better and earlier monitoring of bone spread.

More than 90% of organic bone matrix consists of type I collagen, which is preferentially synthesized in the bone (1, 2). During normal bone catabolism, mature type I collagen is degraded and small fragments pass into the bloodstream and are excreted via the kidneys. In physiologically or pathologically elevated bone resorption, type I collagen is

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increasingly degraded and there is a commensurate rise in the level of type I collagen fragments in the blood. On the other hand, there are reparative mechanisms against bone degradation to keep the net mass at a steady state. During this continuous bone remodeling process, mature type I collagen must be formed from precursor molecules by splicing off propeptides at the C- and N-terminal ends of the procollagen molecules.

PINP is one of the two propeptides of type I procollagen. These N- or C-terminal propeptides (PINP and PICP, respectively) are cleaved by specific proteinases before the collagen molecules are assembled into fibres. Both peptides can be found in the circulation, with their concentration reflecting the rate of synthesis of type I collagen. The carboxy-terminal propeptide PICP is cleared shortly after synthesis, whereas the amino-terminal propeptide PINP can still be found on the surface of collagen fibres. PINP has been shown to be helpful in the management of bone spread from various malignant disorders (particularly breast cancer, prostate carcinoma and multiple myeloma) at both early and advanced disease stages (3-5). PINP is also correlated to the loss of bone mass after menopause in breast cancer patients without evidence of relapse (6). Consequently, the amino-terminal propeptide (PINP) of type I collagen, as a biochemical indicator of bone turnover, might facilitate early and valid disease surveillance.

The utility of total PINP was investigated in metastatic breast cancer patients with and without bone metastases. The aim of this study was to evaluate various parameters of bone metabolism bearing in mind with the following questions:

- i) Is PINP determination a reliable tool for the diagnosis of metastatic spread to the bone?
- ii) What is the correlation of PINP levels to the number of bone metastases?
- iii) Do the longitudinal PINP concentrations reflect the course of bone metastatic spread under specific therapy?
- iv) Is PINP a valid marker to monitor antiresorptive bisphosphonate therapy?
- v) How do PINP levels compare to other markers of bone turnover?
- vi) Do PINP concentrations change with the menopausal status of the patients?

Patients and Methods

Patient cohort. A total of 51 patients with metastatic breast cancer (38 with bone spread, 13 with visceral or soft tissue metastases only) were included in this analysis with 133 samples being available for long-term follow-up (3 time points per patient: baseline, time of best response to therapy and disease progression).

Restaging. Osseous metastatic spread was diagnosed by bone scintigraphy with subsequent radiological confirmation to assess the size and the osteolytic or osteoblastic pattern, as well as the risk of

fracture. Most patients also had visceral and/or soft tissue involvement. The patients were divided into subsets according to the course of the disease under systemic therapy (plus supportive treatment with bisphosphonates in the case of bone spread), as those with progressive disease (PD, n=5), with stable disease (SD, n=31) and patients with disease remission (PR/CR, n=15). Visceral metastases were monitored using ultrasound, CT scan or MRI, as clinically indicated.

Patient sample collection. The baseline samples from the 51 patients were drawn before the initiation of a new antineoplastic treatment therapy, which could be hormonal, cytotoxic or an antibody. All patients with bone spread received bisphosphonates (pamidronate 90 mg or zoledronate 4 mg, q3w). Follow-up samples were taken at the time of best response and at the time of progression. The serum samples were protected from light in dark plastic bags and then were aliquoted and frozen at -28°C within 2 h of collection. The serum samples were centrifuged at 2000 xg for 10 min. All testing was performed in batches to avoid freeze-and-thaw effects.

Osteocalcin assay. Osteocalcin was measured in serum samples using an electrochemiluminescence immunoassay developed by Roche® Diagnostics for use on the Roche Elecsys 1010/2010 and Modular Analytic E170 immunoassay analyzers. Specifically, there were two incubation phases: 20 μl of serum was mixed with a biotinylated N-MID osteocalcin-specific antibody and with a monoclonal N-MID osteocalcin-specific antibody labelled with a ruthenium complex (Tris(2,2'-bipyridyl)ruthenium(II) complex (Ru(bpy)₂/3+)) to form a sandwich complex. During the second incubation, streptavidin-coated microparticles were added and the complex was bound to the solid phase *via* the interaction between biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically captured onto the surface of an electrode. Unbound substances were then removed with ProCell. Application of a voltage to the electrode induced chemiluminescent emission which was measured by a photomultiplier. The results were determined *via* a calibration curve.

β -CTX assay. β -Crosslaps were measured in the serum samples using an electro-chemiluminescence immunoassay. Fifty ml of serum and a biotinylated monoclonal anti- β -crosslaps antibody were incubated together; the antigen in the sample was liberated from the serum components. The second incubation began with the addition of streptavidin-coated microparticles and a monoclonal β -crosslaps-specific antibody labelled with a ruthenium complex (Tris(2,2'-bipyridyl)ruthenium(II) complex (Ru(bpy)₂/3+)). A sandwich complex was formed which bound to the solid phase *via* the biotin-streptavidin interaction. The reaction mixture was then treated as described above.

PINP assay. The free amino-terminal propeptide of type I procollagen (PINP) concentration was also measured using an electro-chemiluminescence immunoassay. A total of 20 μl of serum and a biotinylated monoclonal PINP-specific antibody were incubated together. After addition of streptavidin-labelled microparticles and a monoclonal PINP-specific antibody labelled with a ruthenium complex (Tris(2,2'-bipyridyl)ruthenium(II) complex (Ru(bpy)₂/3+)), a sandwich complex was formed which was treated as described above.

Table I. Demographic data of all 51 patients with breast cancer with/without osseous spread to the bone.

Patient characteristics	Patients with bone metastases (n=38)	Patients without bone metastases (n=13)
Median age at diagnosis (years)	51.4 (range: 29–76)	50.1 (range: 33–65)
Median age at PINP study (years)	57.2 (range: 29–85)	56.5 (range: 36–69)
Menopausal status at study entry:		
pre-	21.1%	30.8%
post-	78.9%	69.3%
Tumor manifestations		
bone	100%	---
breast	28.9%	7.7%
liver	63.2%	30.8%
lungs	10.5%	38.4%
lymph nodes	39.5%	69.2%
others	21%	46.2%
Pretreatment		
hormonal	76.3%	30.8%
cytotoxic	76.3%	69.3%
Therapy during biochemical study		
hormonal	13.2%	15.5%
cytotoxic	84.2%	61.5%
antibody	28.9%	53.8%
Number of bone metastases		
1–7	34.2%	
>7	63.2%	

Test performance and statistics. Descriptive statistics (mean, median, range, standard deviation) were calculated for each parameter. Diagnostic accuracy was evaluated by ROC curve analysis. For the 3 time points (baseline, best response and progressive disease), all pooled marker concentrations were tested for statistically significant differences between patients with or without bone spread, between patients with more than seven bone metastases in comparison to patients with fewer than seven bone metastases, for patients with bone spread in relation to best response, or for patients with or without bone spread in relation to their menopausal status, using the Kruskal-Wallis and the Mann-Whitney tests. Statistical calculations were performed with SPSS 12.0 for Windows (SPSS, Munich, Germany). A *p*-value of <0.05 was considered statistically significant.

Results

At the time of this biochemical analysis, 38 patients suffered from clinically manifest bone metastases and 63.2% had more than seven bone lesions. A total of 13 patients had visceral or soft tissue metastases. There was no age difference between the two groups of women. The majority

of patients in both groups (70%) suffered from visceral metastases. In the group of patients with bone metastases, 63.2% had liver metastases and 10.5% lung metastases, while among those without bone spread, 30.8% had liver metastases and 38.4% lung metastases. The majority of the patients in both groups received chemotherapy during the study (84.2% in the group with bone spread and 61.5% in the group without bone involvement), which was given alone or in combination with antibody therapy. Only a few patients were on hormonal treatment. For further demographic data see Table I.

ROC analyses were performed to characterize the diagnostic utility of the three markers in order to differentiate between breast cancer patients with or without bone metastasis. The ROC analysis of osseous *versus* non-osseous metastatic disease revealed an area under the curve (AUC) for PINP of 0.75. The AUC results for osteocalcin and β -crosslaps were much worse with AUCs of 0.58 and 0.56, respectively (Figure 1A-C).

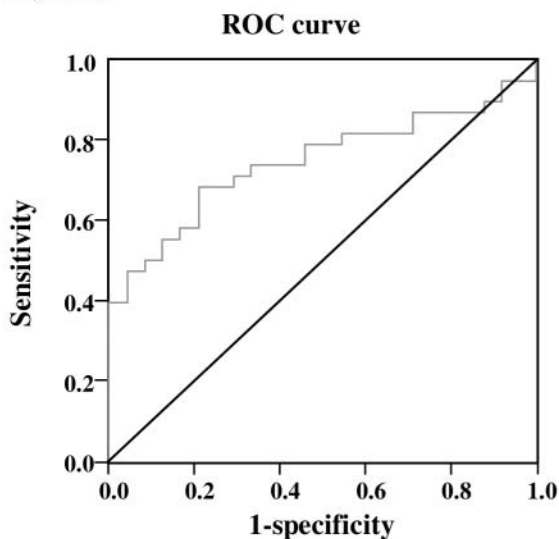
The baseline PINP concentrations of patients with osseous spread (median 92.8 ng/ml) were significantly higher ($p=0.0044$) than the baseline concentrations of patients without bone metastases (median 63.2 ng/ml). Interestingly, these median PINP concentrations remained under the established normal cut-off of 95 ng/ml. For osteocalcin and CTX, no differences were found between either group of patients with or without bone metastases: the median concentrations for CTX and osteocalcin were 314 pg/ml and 19.5 pg/ml, respectively, for patients with bone metastases and 290 pg/ml and 22.3 pg/ml, respectively, for patients without bone spread ($p=0.60$ for CTX and $p=0.92$ for osteocalcin; Figure 2A-C).

Patients with more than seven bone metastases had a significantly higher PINP level (median: 149.7 ng/ml) than those with fewer than seven bone metastases (median: 67.6 ng/ml; $p=0.04$), compared to a normal cut-off of 95 ng/ml. Significant differences were also found for osteocalcin and CTX, $p=0.02$ and $p=0.04$, respectively. However, the median levels (>7 bone lesions: median for CTX 422 pg/ml and median for osteocalcin 26.6 pg/ml; 1–7 bone metastases: median for CTX 167 pg/ml and median for osteocalcin 12.4 pg/ml) remained below the normal cut-off (Figure 3A-C).

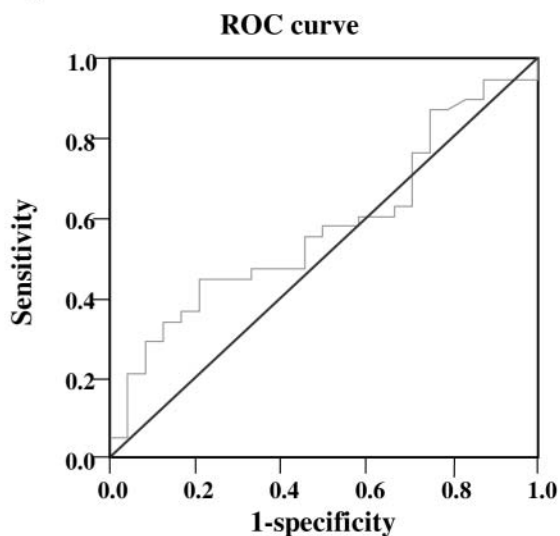
In our study, no significant difference of the serum concentration of PINP, CTX and osteocalcin was found between pre- and postmenopausal women, irrespective of their bone metastatic status (Figure 4). The *p*-values for the comparison of pre- and postmenopausal patients were 0.92, 0.54 and 0.69 for PINP, CTX and osteocalcin, respectively.

For both groups of patients with or without bone metastases, the PINP levels at the time of baseline were compared with those at the time of best response to therapy. Three subgroups of patients were created: responders with complete response (CR) or partial response

A) PINP



C) Osteocalcin



B) CTX

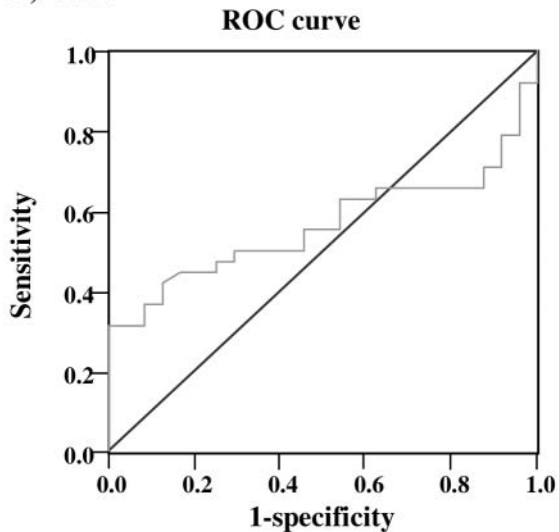


Figure 1. ROC analysis for the discrimination of patients with bone metastases as compared to patients without bone metastases. A) According to the PINP baseline level; B) according to the CTX baseline level; C) according to the osteocalcin baseline level.

Table II. Median concentrations of the three bone markers for patients with/without bone spread at the time of baseline and at the time of best response.

	PINP (ng/ml)	CTX (ng/ml)	Osteocalcin (ng/ml)
	Baseline → Best response	Baseline → Best response	Baseline → Best response
Bone lesions			
CR/PR	194.3 → 100.4	0.44 → 0.28	31.6 → 24.3
SD	71.0 → 65.4	0.28 → 0.21	18.8 → 21.8
PD	83.4 → 176.5	0.25 → 0.17	17.9 → 11.9
No bone lesions			
CR/PR	57.2 → 62.4	0.23 → 0.22	23.5 → 26.3
SD	54.2 → 61.9	0.30 → 0.22	17.7 → 20.6
PD	95.6 → 102.9	0.38 → 0.48	22.3 → 23.4

CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease.

(PR), stable disease (SD) and progressive disease. In patients with bone metastasis, the PINP level decreased significantly in responders (from 194.3 ng/ml to 100.4 ng/ml), remained at the same level in stable disease (ranging from 71 ng/ml to 65.4 ng/ml) and increased in patients with progression (from 83.4 ng/ml to 176.5 ng/ml). However, based on the small number in the response subgroups, these courses did not reach statistical significance ($p=0.23$, $p=0.16$ and $p=0.14$, respectively; Figure 5).

For the patients with bone spread, the CTX and osteocalcin levels decreased in responders (from 439 ng/ml to 279 ng/ml and from 31.6 ng/ml to 24.3 ng/ml, respectively) and, unexpectedly, also in patients with PD (from 245 ng/ml to 174 ng/ml and from 17.9 ng/ml to 11.9

ng/ml, respectively). In patients with SD, the CTX concentrations fell, from 279 ng/ml to 210 ng/ml, while the osteocalcin levels remained constant between 18.8 ng/ml and 21.8 ng/ml. These differences were not significant (Table II and Figure 5). For patients without bone spread, there were no differences between baseline and best response for all markers (see Table II). Only 30 patients suffered from progressive disease during follow-up.

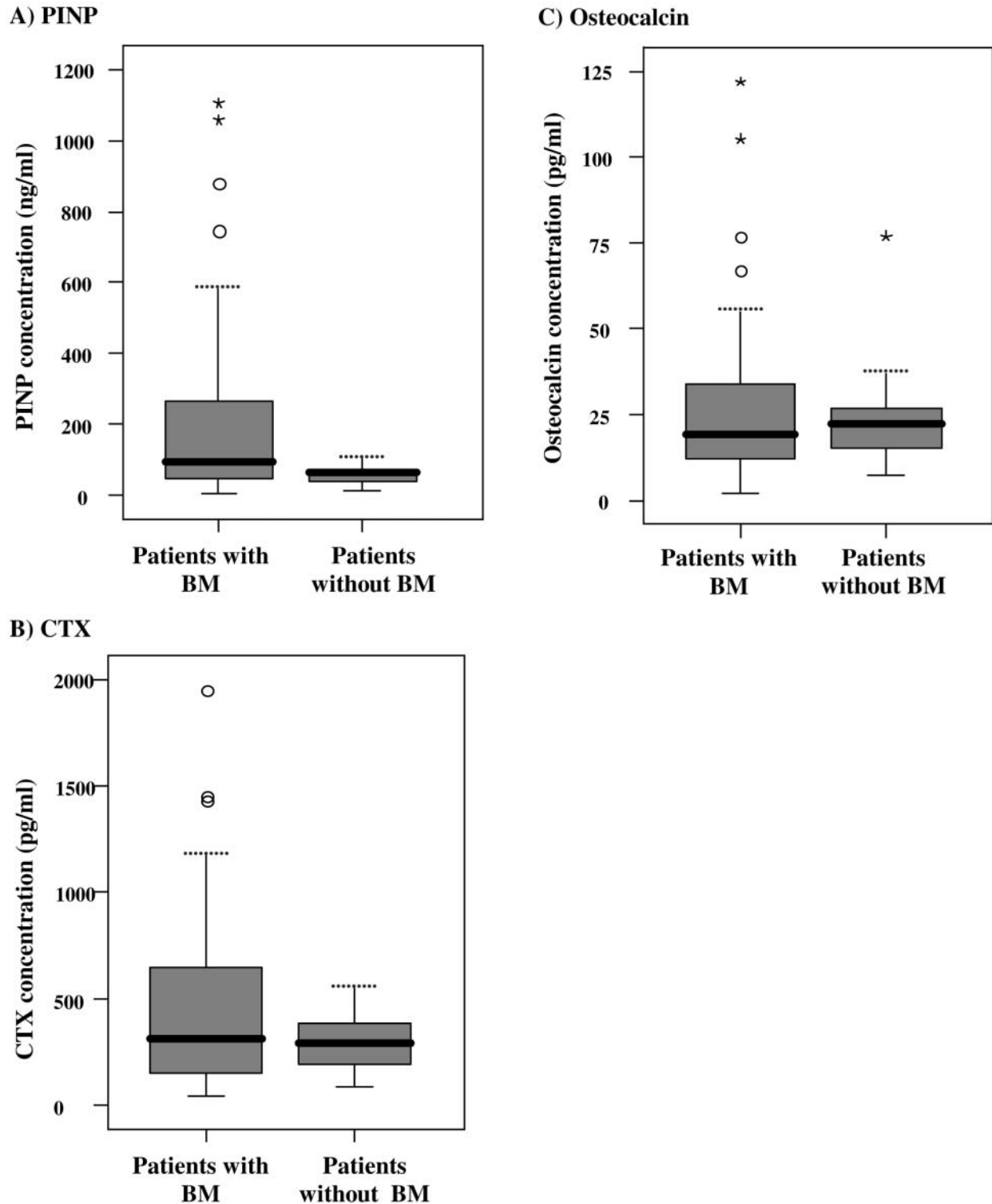


Figure 2. A) Scatter plots of PINP baseline concentrations of breast cancer patients with bone metastases (BM) as compared to the PINP concentrations of women without osseous spread (stage IV disease without bone metastases). Statistical comparison gave a p -value of 0.0044. Median values of the groups are shown as horizontal lines with corresponding figures; dotted lines indicate the upper parametric 95th percentiles of the data, * and ° are outliers. B) Scatter plots of CTX baseline concentrations of breast cancer patients with bone metastases as compared to the PINP concentrations of women without osseous spread. Statistical comparison gave a p -value >0.05 . C) Scatter plots of osteocalcin baseline concentrations of breast cancer patients with bone metastases as compared to the PINP concentrations of women without osseous spread. Statistical comparison gave a p -value >0.05 .

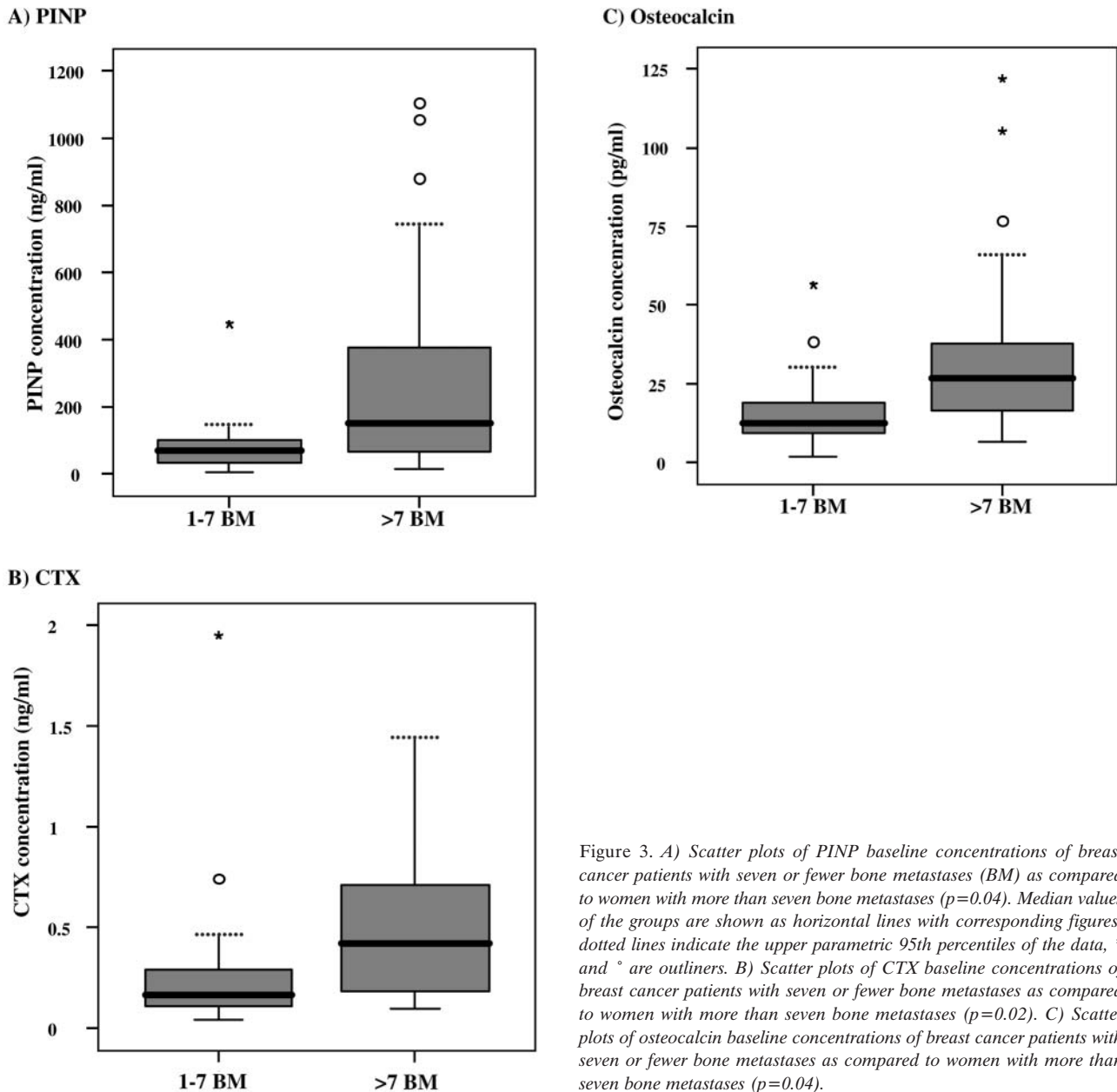


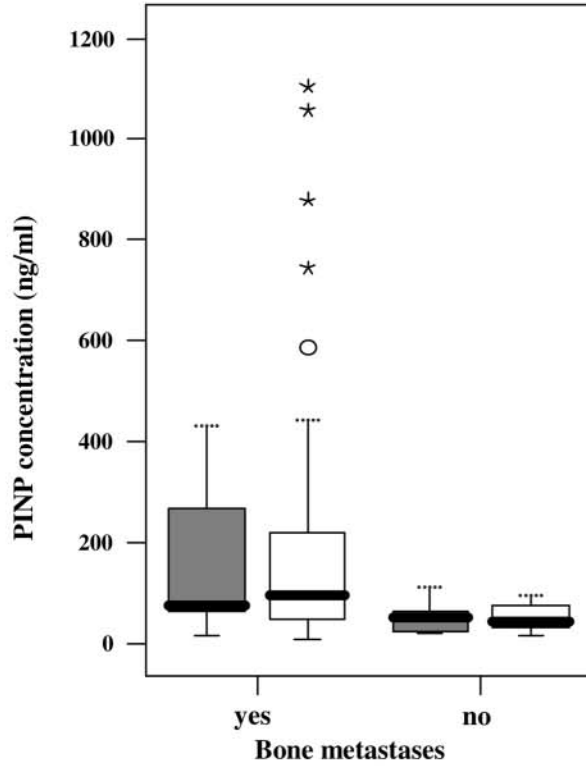
Figure 3. A) Scatter plots of PINP baseline concentrations of breast cancer patients with seven or fewer bone metastases (BM) as compared to women with more than seven bone metastases ($p=0.04$). Median values of the groups are shown as horizontal lines with corresponding figures; dotted lines indicate the upper parametric 95th percentiles of the data, * and ° are outliers. B) Scatter plots of CTX baseline concentrations of breast cancer patients with seven or fewer bone metastases as compared to women with more than seven bone metastases ($p=0.02$). C) Scatter plots of osteocalcin baseline concentrations of breast cancer patients with seven or fewer bone metastases as compared to women with more than seven bone metastases ($p=0.04$).

Discussion

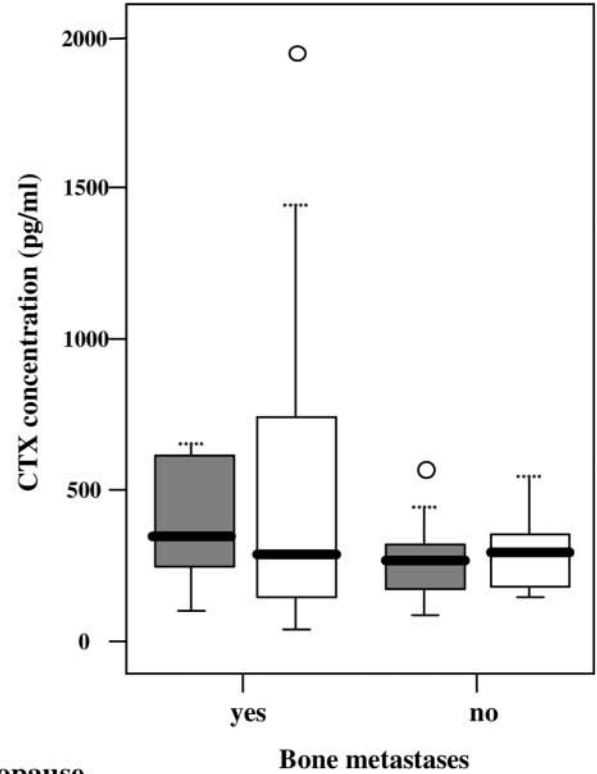
In our analysis, PINP demonstrated its utility for the differentiation of patients with bone metastases from patients without metastatic spread to the bone, while osteocalcin and CTX were not as effective. This result confirms the study of Jung *et al.*, who compared two groups of prostate cancer patients, *i.e.* those with bone metastases and those without. They found similar AUCs for PINP (0.84), CTX (0.59) and osteocalcin (0.56) (7). Several studies showed different results for the sensitivity

and specificity for the bone turnover markers PINP and CTX, such as the investigation by de la PC *et al.* which showed that PINP and β -CTX in serum could both differentiate patients with bone metastases from patients without bone spread in prostate carcinoma (8). In comparison, our results confirmed those of Ebert *et al.*, who also found a better differentiation between metastatic spread to the bone for PINP than for β -CTX in lung cancer (9). Thus, the diagnosis of bone spread by a biochemical marker of bone metabolism is in general possible.

A) PINP



B) CTX



C) Osteocalcin

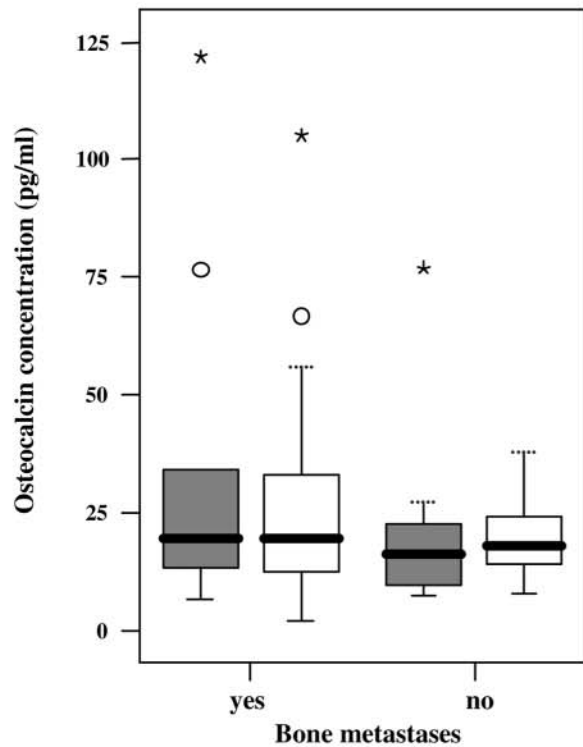
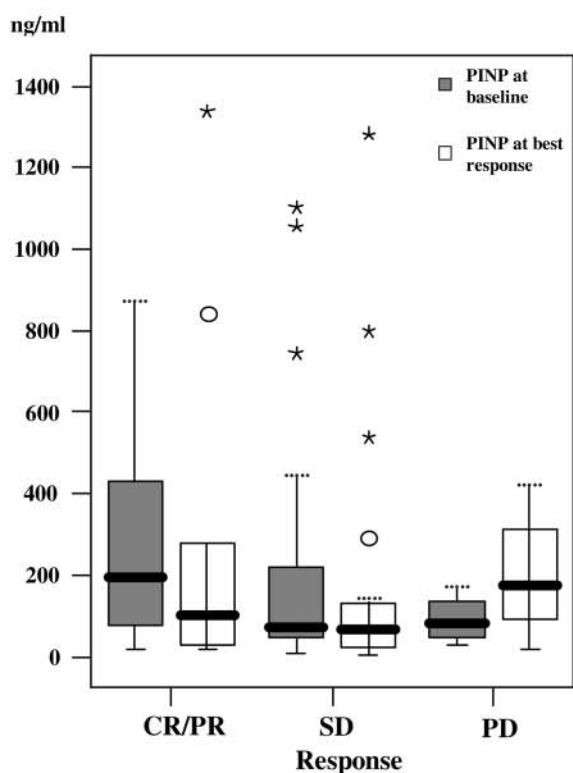
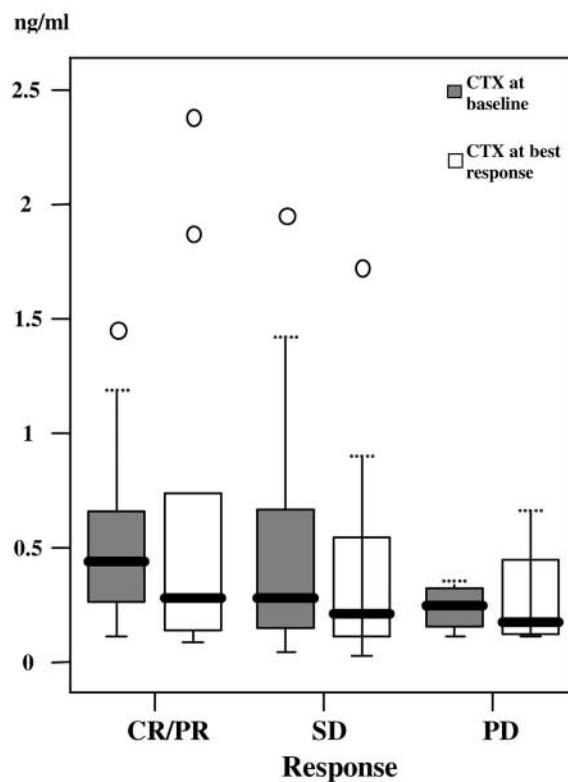


Figure 4. A) Scatter plots of PINP baseline concentrations of women with and without metastatic spread to the bone in relation to their menopausal status. Median values of the groups are shown as horizontal lines with corresponding figures; dotted lines indicate the upper parametric 95th percentiles of the data, * and ° are outliers. B) Scatter plots of CTX baseline concentrations of women with and without metastatic spread to the bone in relation to the menopausal status. C) Scatter plots of osteocalcin baseline concentrations of women with/without metastatic spread to their bone in relation to their menopausal status.

A) PINP



B) CTX



C) Osteocalcin

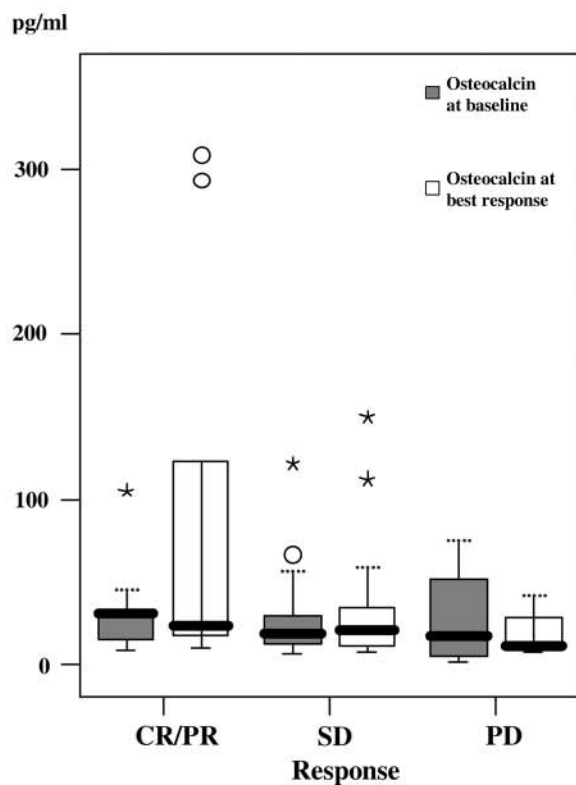


Figure 5. A) Scatter plots of PINP baseline concentrations of women with bone metastases compared to the PINP concentration of the same patients at the time of best response. Median values of the groups are shown as horizontal lines with corresponding figures; dotted lines indicate the upper parametric 95th percentiles of the data, * and ° are outliers. B) Scatter plots of CTX baseline concentrations of women with bone metastases compared to the CTX concentration of the same patients at the time of best response. C) Scatter plots of osteocalcin baseline concentrations of women with bone metastases compared to the osteocalcin concentration of the same patients at the time of best response. CR = complete response, PR = partial response, SD = stable disease, PD = progressive disease.

However, bone scintigraphy is still considered the gold standard for the diagnosis of metastatic bone involvement (9). Many studies have proven the superiority of bone scans for the detection of metastatic spread to the bone for many types of cancer as compared to different bone formation markers (total alkaline phosphatase, bone-specific alkaline phosphatase, PINP, PICP), as well as bone resorption markers (DPD, PYD, ICTP, CTX, tartrate-resistant acid phosphatase 5b) (9, 11). Thus, while the need for an easy non-imaging detection method is still unmet, bone scintigraphy will remain the standard of diagnostic care.

The efficacy of control of any treatment of bone metastases is difficult and usually initiated later than restaging of visceral or soft tissue metastases. The use of specific biochemical metabolism markers might improve the follow-up of bone metastases under therapy and allow for better and earlier monitoring of bone spread. Several studies showed the utility of PINP in the monitoring of patients with bone metastases in breast cancer (12) and prostate cancer (13). However, the results of PINP in both studies are better for the monitoring of bone metastases in prostate cancer, with a significant difference between the group of patients with controlled disease and those with disease progression, than for the monitoring of bone metastases in breast cancer patients where PINP lacked statistical significance. Based on these results, we cannot exclude the fact that the diagnostic and monitoring quality of any marker of bone turnover might be related to the type of solid tumor. Prostate cancer may be a good example for the diagnostic quality of bone markers, as this cancer generally shows the majority of advanced tumor load specifically in bone.

Our analysis is one of the first to show that the levels of PINP reflect the number of bone metastases, with PINP having a median level clearly beyond the normal cut-off. A few studies found a correlation between PINP concentrations and the aggressiveness of the tumor, with PINP generally thought to be reflective of extracellular matrix homeostasis and aggressiveness (14, 15). Breast cancer patients with high PINP levels were statistically significantly more ill, had a higher tumor burden and revealed a lower responsiveness to anthracycline-based therapy as well as an accelerated time-to-disease progression than patients with low PINP levels. The lowest PINP levels were seen when the cancer was restricted to the lymph nodes and skin, while increasing PINP levels were found if the cancer had spread to the bones and visceral organs. The conclusion from this study is that aggressive breast cancer induces a strong fibroproliferative response with the synthesis of type I collagen. These results fit with another study of 373 node-positive breast cancer patients in whom the post-operative PINP levels were measured (10). A total of 120 patients (32%)

developed recurrent disease in the follow-up. The mean PINP level was significantly elevated in the patients who developed metastatic disease as compared to those without metastases. When patients with bone metastases only or patients with bone and soft tissue and/or visceral metastases and patients with only visceral or soft tissue metastases were compared with those not exhibiting metastases, PINP was significantly higher in the group with recurrence in the bone. Thus, the PINP level may be an important diagnostic and prognostic tool with direct therapeutic implications. Patients with metastatic breast cancer and a high PINP level should consult their physician more frequently, have more frequent radiological restaging and receive earlier and more aggressive chemotherapy than those with low PINP levels.

Conclusion

The PINP concentrations of patients with osseous metastatic breast cancer are elevated at baseline in comparison to PINP in patients without bone involvement. The PINP levels are independent of menopausal status and reflect the number of bone metastases. The level of PINP under therapy correlates to therapy response. PINP for monitoring bone metastases in breast cancer requires further investigation. Osteocalcin and CTX did not show a similar biochemical pattern and should be considered less sensitive for the surveillance of bone metastases.

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