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PINP as Serum Marker of Metastatic Spread to the Bone in Breast Cancer Patients

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Abstract. *Background: Early detection before scintigraphic appearance of osseous metastatic spread might improve the outcome of breast cancer patients. The amino-terminal propeptide (PINP) of type I collagen as an indicator of bone formation is a very promising candidate among all markers of bone metabolism. We investigated the utility of total PINP in breast cancer patients at different stages of the disease. Patients and Methods: Precision tests using controls and serum pools were done for total PINP on the Elecsys®2010 analyzer (electro-chemiluminescence immunoassay – ECLIA). Baseline samples of 51 breast cancer patients with metastatic disease plus 11 patients under neoadjuvant treatment were available. Altogether, 38 patients had been diagnosed with bone metastases while 24 had no evidence of metastatic spread to the bone. Results: For serial precision (intra assay), we found coefficients of variation between 1.2–2%. Total imprecision according to the NCCLS protocol ranged from 1.7–5.4% only. Retrieval in ring trials was between 94% and 103%. ROC analysis of osseous versus non-osseous metastatic disease revealed an area under the curve (AUC) of 0.72. The sensitivity for the detection of bone lesions was 50% at the preliminary normal cut-off of 95 ng/mL. The baseline levels of the patients with bone metastases were significantly higher than those of patients with visceral of soft tissue spread only ($p<0.001$). PINP concentrations correlated with osseous spread in terms of number and size of the bone lesions. Generally, non-osseous metastases did not produce elevated PINP levels in only 2/24 patients without bone metastases showing minimally elevated PINP concentrations (95 and 112 ng/ml). Conclusion: The Elecsys test for total PINP is highly reproducible. PINP concentrations can discriminate patients with bone metastases from those without osseous spread. The moderate sensitivity for the diagnosis of bone lesions may be biologically related to ineffective bone repair in a certain subset of patients. Further studies must focus on the monitoring of patients with elevated baseline levels and on those patients with low PINP levels in the case of otherwise proven bone metastases.*

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More than 50% of patients with breast cancer will develop bone metastases in the long run. It is still unclear whether the course of the disease would be different if the detection of small, clinically asymptomatic bone metastases was possible at an earlier stage than it is at present. The sensitivity of the currently available methods is too small to allow for the detection of clinically still occult bone lesions. The use of specific biochemical markers of bone metabolism might improve the detection of bone metastases leading to early therapeutic interventions (1, 2). However, due to limited sensitivity and specificity, the main use of parameters of bone metabolism is monitoring of treatment outcome of advanced disease with osseous involvement (3, 4).

More than 90% of organic bone matrix consists of type I collagen which is preferentially synthesized in bone (5, 6). 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 degraded to an increased extent, and there is a commensurate rise in the level of type I collagen fragments in blood. On the other hand, reparative mechanisms try to antagonize bone

degradation to keep the net bone mass in a steady-state condition. During this continuous bone remodeling process, mature type I collagen must be formed from precursor molecules by splicing off propeptides at the C-terminal 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) 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 carboxyterminal propeptide PICP is cleared shortly after synthesis, whereas the aminoterminal 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 (mainly breast cancer, prostate carcinoma and multiple myeloma) in both early and advanced disease (7-9). It is also correlated to the loss of bone mass after menopause in breast cancer patients without evidence of relapse (10).

The aim of this study was to evaluate the value of various parameters of bone metabolism, asking the following questions:

- What is the performance of a new, automated PINP assay on the Elecsys®2010 analyzer (electro-chemiluminescence immunoassay – ECLIA)?
- Is PINP determination a reliable tool for the diagnosis of metastatic spread to bone?
- Does the pattern of bone metastases (osteolytic, osteoplastic or mixed) influence the PINP concentrations?
- How well do PINP levels relate to number and possibly size of the bone metastases?
- Do the longitudinal PINP concentrations reflect the course of bone metastatic spread under specific therapy?
- Are PINP determinations valid to monitor an antiresorptive bisphosphonate therapy?
- How do PINP levels compare to other markers of bone turnover?

Patients and Methods

Patient subsets. A total of 62 patients with breast cancer (51 metastatic, 11 under neoadjuvant chemotherapy) were included into this analysis with 729 samples being available for a long-term follow-up. For the patients with osseous spread, samples were drawn before initiation of bisphosphonate therapy (pamidronate 90 mg or zoledronate 4 mg, q3w) and change of specific antineoplastic treatment, which could be hormonal or cytotoxic.

Restaging. Osseous metastatic spread was diagnosed by bone scan with the spotted areas radiologically imaged to asses 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 cases of bone spread). Subgroups were formed of patients with progressive disease (PD, n=5), patients with stable disease (SD, n=35) and patients with disease remission (PR, n=18; CR, n=4). Visceral metastases were monitored using X-ray, CT scan, MRI and ultrasound, as clinically indicated.

Patient sample collection. The serum samples from the 62 patients with breast cancer were collected after the first diagnosis of the primary disease, before initiation of neoadjuvant chemotherapy or at first metastatic spread. Follow-up samples were taken at least every 3-4 weeks depending on the schedule of systemic therapy. The serum samples were protected from light in dark plastic bags and then aliquoted and frozen at -28˚C within 2 h of collection. Serum samples were centrifuged at 2000xg 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 electro-chemiluminescence immunoassay developed by Roche® Diagnostics for use on the Roche Elecsys 1010/2010 and Modular Analytic E170 immunoassay analyzers. Specifically in this kit, there are two incubation phases: 20 μl of serum is 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+)). The three reagents react to form a sandwich complex. During the second incubation, streptavidin-coated microparticles are added and the complex becomes bound to the solid phase *via* interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined *via* a calibration curve.

β-CTX assay. β-Crosslaps were measured in serum samples using an electro-chemiluminescence immunoassay for use on the same analyzer. In this assay, 50 μl of serum and a biotinylated monoclonal anti-β-Crosslaps antibody are incubated together; antigen in the sample is liberated from the serum components. The second incubation begins with the addition of streptavidin-coated microparticles and a monoclonal β-crosslaps-specific antibody labelled with a rubenium complex (Tris(2,2'-bipyridyl)ruthenium(II) complex (Ru(bpy)2/3+)). A sandwich complex is formed which becomes bound to the solid phase *via* biotin-streptavidin interaction. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescence emission which is measured by a photomultiplier. Results are determined *via* a calibration curve.

PINP assay. The free aminoterminal 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 are incubated together. After addition of streptavidin-labelled microparticles and a monoclonal PINP-specific antibody labelled with a rubenium complex (Tris(2,2'-bipyridyl)ruthenium(II) complex (Ru(bpy)2/3+)), a sandwich complex is formed which becomes bound to the solid phase *via* interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound

Table I. *Demographic data of all 62 patients with breast cancer with/without osseous spread to the bone.*

Patients' characteristics		Patients with bone metastases (n=38)	Patients without bone metastases (n=24)
Median age at diagnosis (years)		51.4 (range: 29–76)	47.5 (range: 31-65)
Median age at PINP study (years)		57.2 (range: 29-85)	51.2 (range: 31–70)
Menopausal status at study entry:	pre-	21.1%	45.8%
	post-	78.9%	54.2%
Tumor manifestations	bone	100.0%	---
	breast	28.9%	66.6%
	liver	63.2%	16.2%
	lungs	10.5%	20.8%
	lymph nodes	39.5%	37.5%
	other	21.0%	20.8%
Pretreatment	hormonal	76.3%	20.8%
	cytotoxic	76.3%	45.8%
Therapy during biochemical study	hormonal	13.2%	8.3%
	cytotoxic	84.2%	79.2%
	antibody	28.9%	37.5%
Pattern of bones metastases	osteolytic	36.8%	
	osteoplastic	31.5%	
	mixed	26.3%	
Number of bones metastasis	1-3	21.0%	
	3-7	13.2%	
	> 7	63.2%	
Previous pathological fractures		23.7%	---
Previous bones radiation		42.1%	---
Bones radiation during biochemical study		10.5%	---

substances are then removed with ProCell. Application of a voltage to the electrode then induces chemi-luminescence emission which is measured by a photomultiplier. Results are determined *via* a calibration curve.

Test performance and statistics. The technical evaluation of Elecsys total PINP was performed as part of a multicenter European trial in 5 laboratories (Altötting, Berlin, Brussels, Brno, Essen) on different Elecsys analyzers (E1010, 2xE2010, 2xE170 (E)+(EE)). The precision experiments were performed using 3 levels of PreciControl Bone and 3 different concentrations of human serum pools that were individually prepared by each laboratory themselves. For the intra-assay precision, 21 determinations were analyzed and resulted in coefficients of variation (CV) ranging between 1.1-2.4% for the controls and 1.0-3.7% for the serum pools, respectively.

The total imprecision, which was performed and calculated according to the NCCLS protocol (National Committee for Clinical Laboratory Standards) (n=60), resulted between 2.4-6.5% for the controls and 2.5-6.7% for the serum pools. These excellent results confirm the practicability of the assay for routine application for patients under follow-up investigation. A total of 3 serum samples (concentrations were not disclosed) were distributed to all participants of the study in the form of a study ring trial. The recovery of the individual laboratory compared to the all-laboratory median ranged between 98 and 111% for the controls, and between 101 and 117% for the serum samples.

Statistical analysis was based on pre-programmed macros in Windows Excel (*e.g.* multiple point histogram, min, max, mean, median, SD, CV). The calculation of coefficients of variation (CV) was based on the standard deviation (SD) divided by the mean. *P*-values were calculated using the SAS-program (Statistical Analysis System, version 8.2) from which the following non-parametric tests were applied: the Wilcoxon 2 sample rank test (one- as well as 2-sided with normal approximation and T-approximation) as well as the Kruskall-Wallis test. ROC (receiver operating characteristics) were determined using a C++ program. Sensitivity, specificity, NPV (negative predictive value) as well as PPV (positive predictive value) were calculated *via* this program for every given concentration of total

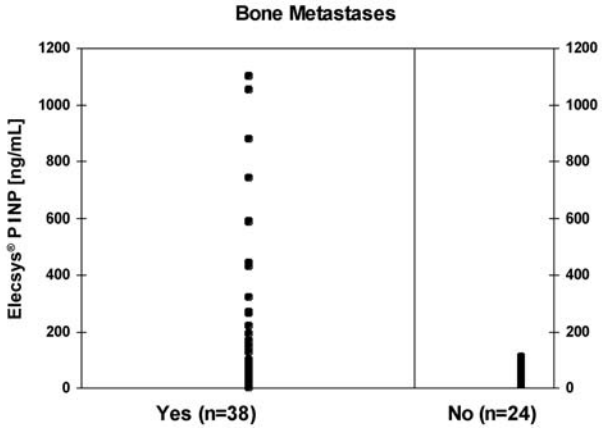


Figure 1. *PINP concentrations of breast cancer patients with bone metastases as compared to the PINP concentrations of women without osseous spread (primary cancer in loco under neoadjuvant chemotherapy or stage IV disease without bone metastases). Statistical comparison gave a p-value <0.001.*

Figure 2. *PINP concentrations in relation to exclusive or combined metastatic spread to bone and other sites like viscera or soft tissue.*

PINP. The Gerhard Plot, another graphic display of the distribution of concentrations together with the sensitivity and specificity, results from the same C++ program.

Results

At the time of this biochemical analysis, 38 patients suffered from bone metastases with 63% having more than 7 bone lesions. Approximately one-third of the patients presented with either purely osteolytic -, with osteoplastic - or with mixed osseous metastases. The majority of patients suffered from liver metastases, too, with 84.2% undergoing palliative chemotherapy alone and/or in combination with hormone or antibody therapy. Among the 24 patients without bone metastasis, 79.2% were under chemotherapy and 37.5 % under antibody therapy, partly in combination. For further demographic data see Table I.

As shown in Figure 1, the baseline PINP levels of the 38 patients with bone metastases were significantly higher than the PINP concentrations of those women with primary disease or metastatic spread with visceral or soft tissue involvement only ($p<0.001$). Patients with bone metastases only reached PINP concentrations of up to 800 ng/ml, while the subset of metastatic breast cancer patients with additional non-osseous visceral spread also reached concentrations of up to 1200 ng/ml (Figure 2). All patients under neo-adjuvant chemotherapy had PINP levels below the preliminary normal cut-off of 95 ng/ml. Patients with soft tissue or visceral spread only generally presented with a normal PINP baseline level, with only 2 patients showing concentrations higher than the preliminary normal cut-off of 95 ng/ml (95.6 and 112 ng/ml).

In terms of statistical evaluation, the baseline levels of PINP showed a sensitivity of 0.50 at a specificity of 0.92.

Table II. *Two-by-two table of the distribution of patients with/without bone metastases and PINP concentrations at baseline higher/lower than the preliminary cut-off of normal of 95 ng/ml.*

	With bone metastases		Without bone metastases		Total
PINP + (> 95 ng/ml)	19	50%	2	8.30%	21
PINP – (≤ 95 ng/ml)	19	50%	22	91.70%	41
Total	38	100%	24	100%	62

The positive predictive value lay at 0.90 with a negative predictive value of 0.54. (for absolute numbers see Table II). Figure 3 displays the sensitivity and specificity for a large range of PINP baseline concentrations with the absolute number of patients who showed this concentration. At the preliminary normal cut-off of 95 ng/mL, we can confirm that the sensitivity of the test is very good. The ROC analysis of osseous *versus* non-osseous metastatic disease revealed an area under the curve (AUC) for PINP of 0.72 (Figure 4). The statistical results for the other markers (β-crosslaps and osteocalcin) are not displayed due to the large data set available in the literature.

Table III gives a general overview of the distribution of the PINP baseline levels and the type and number of metastases. The patients with a supposedly high activity of bone formation (osteoblastic bone metastasis, healing after previous bone fractures) generally had high PINP levels with a median of 148.4 ng/ml for patients with osteoblastic metastases and even a 25%-percentile of 100.4 ng/ml for patients with previous bone fractures. It is interesting to note that only 2 patients with previous bone fractures had PINP concentrations below the normal cut-off, while both patients with the highest PINP

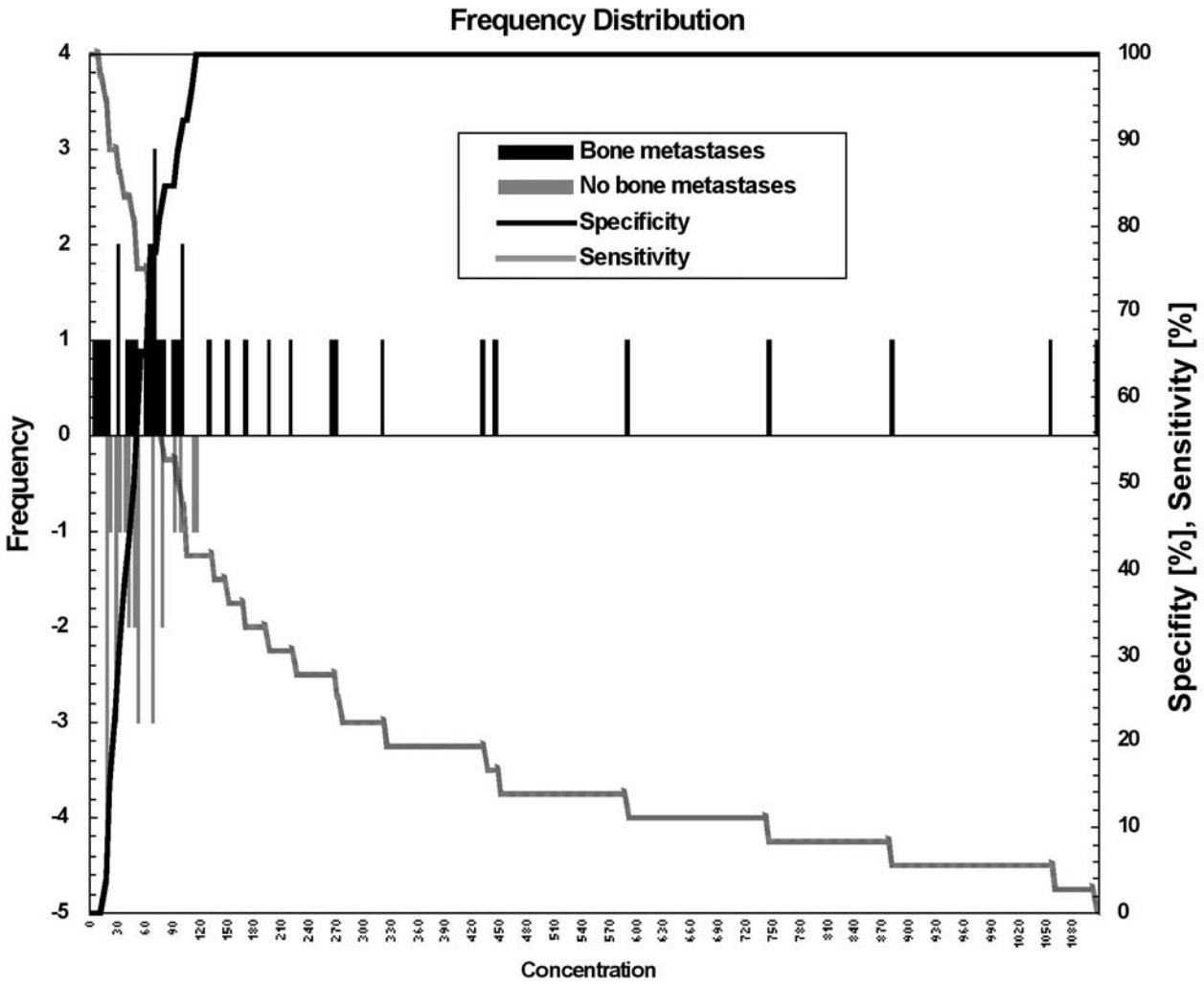


Figure 3. *Frequency of PINP concentrations for patients with/without bone metastases, displayed together with the sensitivity and specificity at this individual concentration (Gerhard plot).*

levels (1104 ng/ml and 1056 ng/ml) had previously suffered from bone fractures.

Figure 5 gives several examples for the biochemical behavior of PINP in relation to β-crosslaps and osteocalcin. The patient in Figure 5A experienced a partial remission after a taxane- and anthracycline-based first-line chemotherapy supplemented with pamidronate as supportive care for osseous spread. The patient had extensive bone metastases with more than 7 bone lesions and liver metastases as visceral involvement. All 3 evaluated markers had decreased at cycle 2 of chemotherapy, with PINP having already reached the 50% level in comparison to baseline. Figure 5B gives the example of a patient without bone metastases, but with a local relapse qualifying as T4 tumor which was only stable under chemotherapy. All the parameters remained within the normal range at any time. Figure 5C displays the example of a patient with initially stable disease under chemotherapy plus trastuzumab antibody treatment. The patient had osteolytic bone metastases which, after a few months, progressed in spite of systemic therapy. Under progression, PINP is the first marker to increase with a lag-time of approximately 3-4 weeks.

Discussion

This analysis on different markers of bone metabolism in metastatic spread from breast cancer shows that PINP determination can discriminate patients without bone metastases from patients with bone lesions. So, our results confirm the data from other investigators, who support the use of PINP or β-CTX as valuable tools to confirm the presence or absence of bone metastases in the first staging of solid tumor patients (11). However, 50% of our patients with bone metastases presented PINP levels below the preliminary normal cut-off of 95 ng/ml, indicating that the search for early

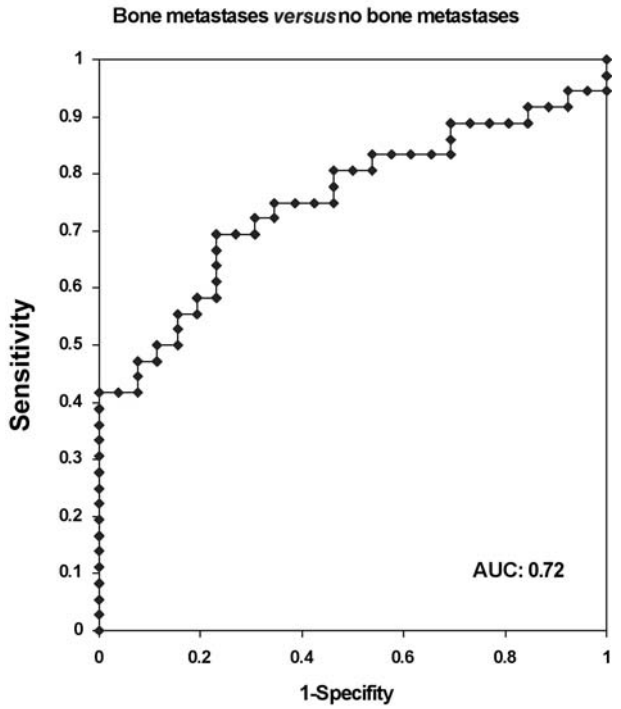


Figure 4. *ROC analysis for the discrimination of patients with bone metastases as compared to patients without bone metastases according to the PINP baseline level.*

bone spread is probably not the most important domain of PINP measurements. It is not surprising that being a bone formation marker, the levels of PINP correlated best with the extent of disease in patients with prostate carcinoma (12). Our results also demonstrated that the levels of PINP were highest in patients with previous bone fractures or an increased bone formation due to predominantly osteoblastic bone metastases.

The question of the best marker of bone turnover to be measured for early detection of bone spread, as well as for monitoring purposes, is still open. Other relevant markers of bone metabolism are the C-terminal telopeptides (CTx). In the C-terminal telopeptides, the α-aspartic acid present converts to the β-form of aspartic acid as the bone ages (β-CTx) (13). These isomerized telopeptides are specific for the degradation of type I collagen dominant in bone. Elevated serum concentrations of isomerized C-terminal telopeptides of type I collagen have been reported for patients with bone metastases (11). Determination of the C-terminal telopeptides of type I collagen in serum has also been recommended for monitoring the efficacy of antiresorptive therapy (14-17).

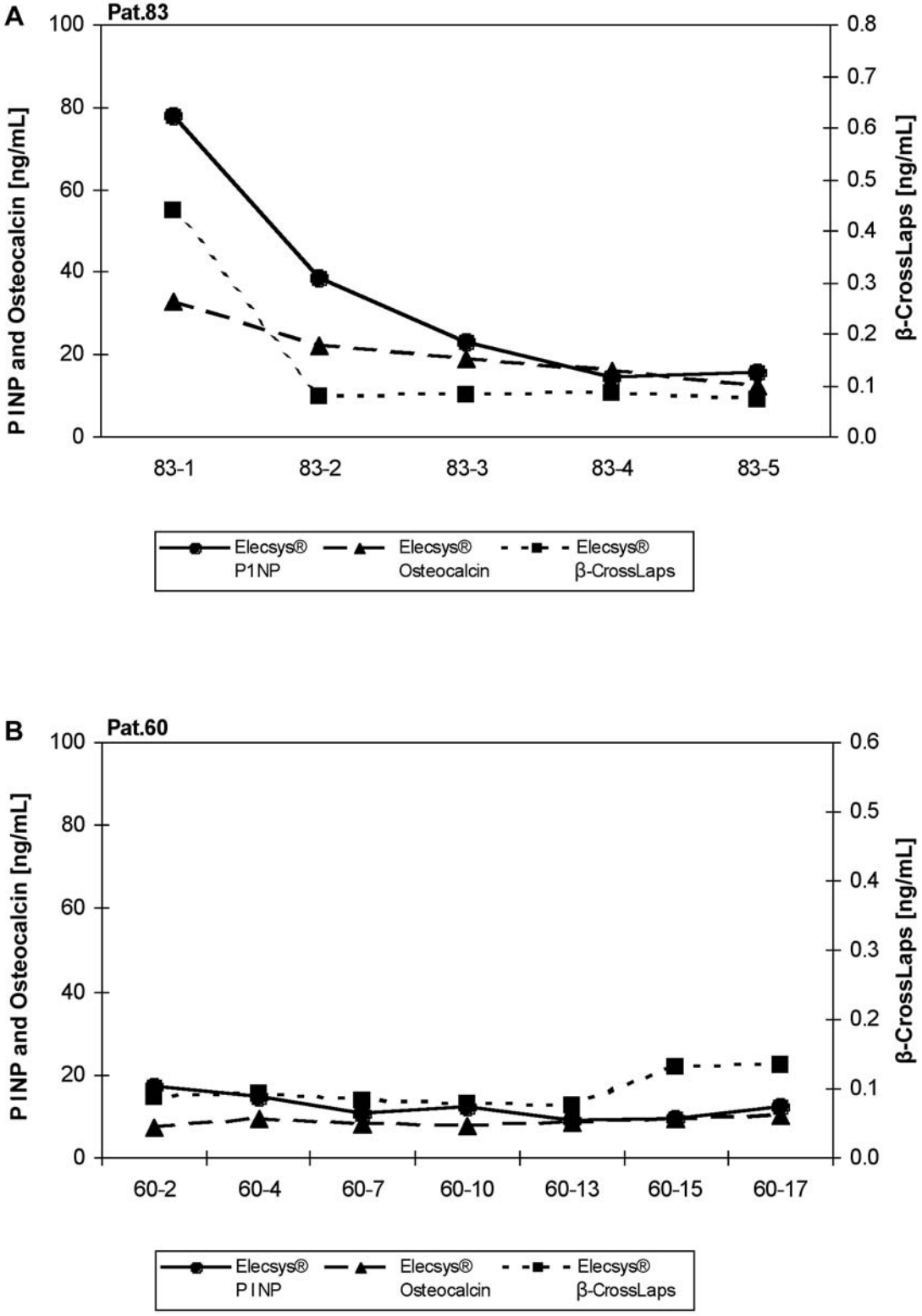
Osteocalcin, the most important non-collagen protein in bone matrix, is a bone-specific, calcium-binding protein which is dependent on vitamin K. During bone synthesis, osteocalcin is produced by the osteoblasts. After release from the osteoblasts, osteocalcin is not only assimilited into the bone matrix, but also secreted into the blood stream. Accordingly, the serum osteocalcin level is related to the rate of bone turnover in various discorders of bone metabolism. Osteocalcin is therefore termed a bone turnover marker and is used for this purpose. By means of osteocalcin measurement, it is possible to monitor the treatment of metastatic bone disease. An increased osteocalcin level 1 month after the start of systemic treatment may be predictive of the treatment efficacy (18).

In metastatic breast cancer patients, the two extension peptides from both ends of the procollagen molecule, carboxy- and aminoterminal propeptides (PICP and PINP, respectively) were investigated in various trials of early detection and monitoring of bone spread. PINP levels were

Table III. *Percentiles of PINP concentration in relation to osteolytic and osteoplastic pattern of bone spread, number of bone metastases, other metastastic sites or previous events of bone fractures.*

PINP concentrations & percentiles			Bone metastases (BM)				Type of bone metastases			Number of bone metastases			Previous bone fracture	
	Yes	No	Only BM	BM and other metastases	No metastases	No BM but other metastases	Osteolytic	Osteoplastic	Mixed	1-3	4-7	>7	Yes	No
0	5.3	13.9	67.6	5.3	13.9	15.2	5.3	10.2	18.8	5.3	30.1	15.2	63.4	5.3
2.5	5.3	13.9	67.6	5.3	13.9	15.2	5.3	10.2	18.8	5.3	30.1	15.2	63.4	10.2
5	10.2	15.2	67.6	10.2	13.9	15.2	5.3	10.2	18.8	5.3	30.1	18.8	63.4	13.9
10	17	17.2	67.6	17	17.2	17.2	15.2	17	27.8	5.3	30.1	27.8	63.4	17
25	49.2	26.2	101.3	45.7	21.3	39.4	45.7	66.5	61.3	10.2	66.5	70.1	100.4	30.1
50	92.8	43.9	131.1	78	33.6	63.2	86.6	148.4	84.4	66.3	67.6	149.7	131.1	61.3
75	266	64.2	445.5	220.3	50.2	75.1	168.3	266	445.5	109.3	100.4	431.5	744.2	95.2
90	744.2	95.6	744.2	588.6	50.5	95.6	879	431.5	1104	445.5	148.4	879	1104	266
95	1056	95.6	744.2	1056	63.9	112.8	1056	588.6	1104	445.5	148.4	1056	1104	445.5
97.5	1104	112.8	744.2	1104	63.9	112.8	1056	588.6	1104	445.5	148.4	1104	1104	588.6
100	1104	112.8	744.2	1104	63.9	112.8	1056	588.6	1104	445.5	148.4	1104	1104	879

Figure 5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Pat.83
P1NP and Osteocalcin [ng/mL]
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Elecsys® P1NP
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P1NP and Osteocalcin [ng/mL]
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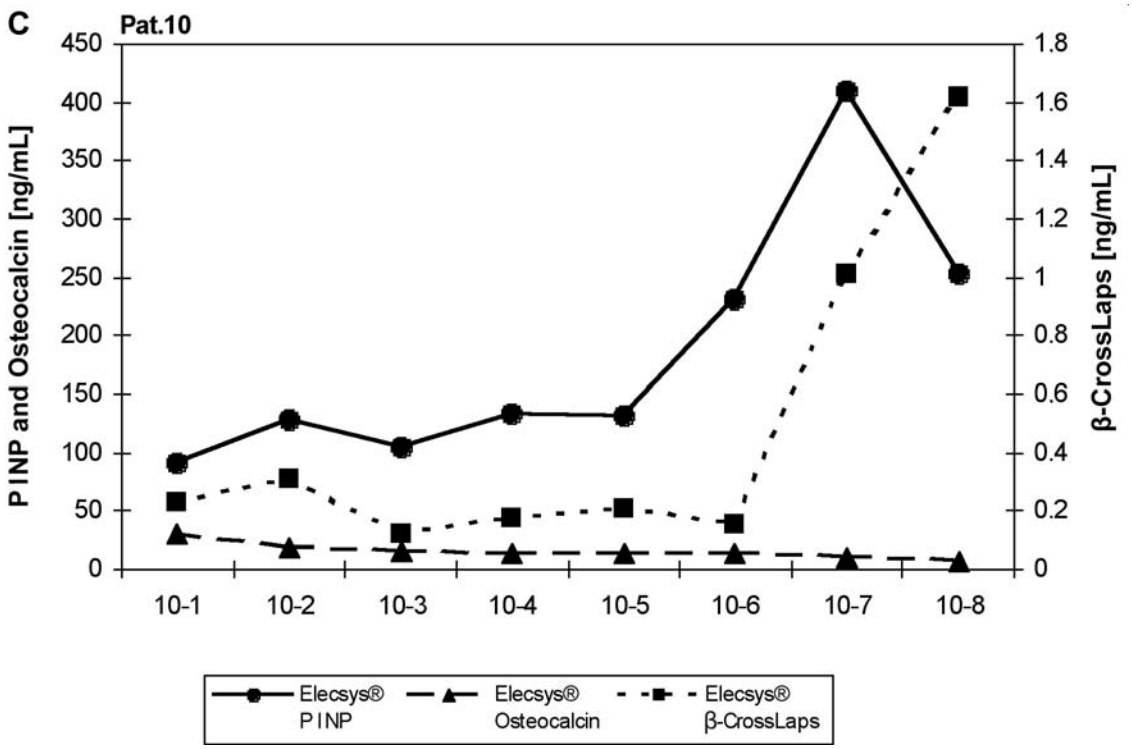

Figure 5. *A: Example for remission: PINP decreases by 50% in comparison to baseline already at the time of the second chemotherapy cycle. B: Example for serum negativity: Having no bone metastases and no distant spread, but a large T4 tumor only, this patient did not show elevated levels of any of the investigated markers. C: Example for stable disease: After a longer time of being stable, the patient suffered from disease progression with PINP being the first marker to show a relevant elevation, having fluctuated around the cut-off of normal before. (The numbers on the X-axis indicate the patient number and the number of the chemotherapy cycle during which the serum sample was taken).*

significantly higher in metastatic patients in comparison to patients without evidence of disease and healthy controls, and the PICP/PINP-ratio was lower (19). PINP was also demonstrated to be valuable in the diagnosis and follow-up of bone metastases in prostate carcinoma (20, 21). It is not astonishing that PINP was also interpreted to be generally reflective of the extracellular matrix homeostasis and aggressiveness of breast cancer (22). Patients with high PINP levels were statistically significantly more sick, 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 node and skin and increasing PINP levels were found if the cancer had spread to the bones and visceral organs. The conclusion from this study was that aggressive breast cancer induced a strong fibroproliferative response with synthesis of type I collagen.

These results fit with another study of 373 node-positive breast cancer patients in whom postoperative PINP levels were measured (23). One hundred and twenty 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 only bone metastases 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, PINP levels may be an important diagnostic and prognostic tool with direct therapeutic implications.

Conclusion

The Roche Elecsys PINP assay shows very good analytical performance. The differentiation of patients with bone metastases as compared to patients without bone metastases is moderate to good. Patients without bone metastasis rarely show just slightly elevated PINP levels higher than the preliminary normal cut-off of 95 ng/ml. The clinical domain of biochemical markers of bone metastases in solid tumors is monitoring rather than early diagnosis of bone spread. Patients with bone metastases and low PINP level require further investigation as they might represent a

subset with insufficient repair mechanisms which could profit from additional treatment options (increase of dose density of bisphosphonate therapy, administration of osteoprotegerin).

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