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der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Plasma osteopontin in comparison with bone markers as an indicator of distant metastases and a predictor of survival outcome in prostate cancer and renal cell carcinoma patients

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von

Azizbek Ramankulov

aus Bischkek, Kirgisien

Gutachter: 1. Prof. Dr. med. K. Jung

2. Prof. Dr. med. D. Fahlenkamp

3. Priv.-Doz. Dr. med. A. Sokolowski

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Abbreviations:

ALAT	Alanine transaminase
AUC	Area under the ROC curve
bALP	Bone-specific alkaline phosphatase
BPH	Benign prostatic hyperplasia
CTX	C-terminal cross-linked telopeptide of type I collagen
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GGT	Gamma-glutamyl transferase
ICTP	Cross-linked carboxyterminal telopeptide of type I collagen
MMP	Matrix metalloproteinase
OPN	Osteopontin
PCa	Prostate cancer
PICP	C-terminal propeptide of type I procollagen
PINP	N-terminal propeptide of type I procollagen
PSA	Prostate specific antigen
RCC	Renal cell carcinoma
RGD	Arginine-Glycine-Aspartic acid sequence
ROC	Receiver operation characteristics
RR	Relative risk
RT	Room temperature
SVVYGLR	Serine-valine-valine-tyrosine-glycine-leucine-arginine sequence
tALP	Total alkaline phosphatase
uPA	Urokinase type plasminogen activator
VEGF	Vascular endothelial growth factor
95% CI	95% Confidence interval

1 Introduction and objectives

Cancer is a major public health problem in the world, causing millions of people to die every year. In fact, one in four deaths in the United States is due to cancer [1]. Cancer detected at an early stage, before it has metastasized, can often be treated successfully by surgery or local irradiation. In contrast, cancer diagnosed after it has developed metastases, treatments are much less successful and in most cases only palliative. Metastases, rather than primary tumors, are responsible for most cancer deaths. Therefore, improved ways of early detection of metastatic disease are urgently being sought. Development of biochemical markers, which are measurable in blood, easy repeatable, inexpensive, and safe for patients, is a promising strategy to improve the diagnosis of metastasis. Biochemical markers providing a clinician with both accurate diagnostic and prognostic information regarding cancer patients are most desirable. Prognostic value of biochemical markers will assist in identifying patients at risk in order to provide them with timely and appropriate treatment. Such stratification of patients into risk groups based on levels of biochemical markers will also enable clinicians to use diagnostic recourses such as radiography and scintigraphy more cost-effectively.

Recently there has been a focus of attention towards bone markers, which reflect subtle changes in bone metabolism like bone formation and resorption. In fact, once a tumor invades the bone it disturbs finely balanced processes of bone formation and resorption. These changes in bone metabolism can easily be assessed using bone markers in blood [2]. These markers are particularly useful to detect bone metastases from cancers, which preferentially metastasize to bone, such as prostate cancer (PCa) and breast cancer. Renal cell carcinoma (RCC) is also known to metastasize frequently to the bone. However, at present there is no ideal test for detecting bone metastases and there is still much room for the improvement of the diagnosis of bone metastases.

In the course of searching for a better and more reliable marker for cancer metastases, osteopontin (OPN) was examined in this study. OPN, a glycoprotein, was recently identified as a key protein in tumor genesis and progression [3]. OPN exists in a secreted form in all body fluids that makes it available for routine determinations in blood [4]. In addition, OPN is abundantly distributed in bone tissue and involved in the regulation of bone turnover [5-7]. This indicates that plasma OPN could provide

diagnostic information relating to skeletal metastases. Therefore, this study was undertaken to evaluate the clinical usefulness of plasma OPN in two urologic cancers: PCa and RCC with all patients classified into subgroups with distant bone and non-bone metastases, with metastases in regional lymph nodes, and organ-confined disease. Its diagnostic and prognostic performance was validated against the established markers for bone metastases such as bone formation markers: N-terminal propeptide of type I procollagen (PINP), bone-specific alkaline phosphatase (bALP), and bone resorption marker: cross-linked carboxyterminal telopeptide of type I collagen (ICTP).

This chapter functions as an introduction of the thesis and outlines statistical figures on PCa, RCC and their metastases. Furthermore, it describes aforementioned bone markers as well as structure and functions of OPN. The formulation of the objectives of the current study will conclude this chapter.

1.1 Prostate cancer and metastases

PCa is the most common malignancy to afflict elderly men. In 2006, PCa is estimated to cause 234,460 new cases and 27,350 deaths in the USA [1]. While most of the patients with organ-confined tumors can be curatively treated by radical prostatectomy, about 20% of patients experience tumor recurrence or metastatic tumor progression. The distinct predilection site of hematogenous spread of PCa is bone. Bone lesions from prostate cancer are characterized by increased osteoblastic reaction [8]. Bone metastases in PCa patients are associated with pain, impaired mobility, pathological fracture, spinal or nerve root compression, and bone marrow infiltration. Up to 70% of patients with advanced PCa have bone metastases, which significantly reduce quality of life and cause morbidity [9,10]. More than 85% of those patients who die of PCa have bone metastases [11]. The survival of patients is essentially determined by the extent of metastatic spread within the skeletal system [12]. These few figures underline the great challenge to detect bone metastases at an early stage or to classify patients as risk persons in order to provide timely, appropriate treatment and prognostic information.

1.2 Renal cell carcinoma and metastases

In 2006, RCC is estimated to cause 38,890 new cases and 12,840 deaths in the USA [1]. RCC is, most of the times, clinically asymptomatic and casually detected by routine ultrasonographic follow-up in persons otherwise in inconspicuous conditions [13]. However, at the time of initial presentation, about 50% patients have localized carcinoma, while 20% suffer from regional and another 20% from distant metastases [14]. Distant metastases most frequently occur in the lungs, bone, liver or brain. Bone metastases are found in 30% of patients with metastases either alone or in combination with metastases in other locations [15-17]. In contrast to PCa skeletal metastases from RCC are osteolytic [18]. Metastatic spread to bones accounts for high morbidity in these patients and is a poor survival factor [19,20]. These data indicate the importance of early detection of metastases in RCC patients.

In relation to histological types of RCC clear cell RCC is the most frequent one with an incidence of 70% followed by papillary and chromophobe types with an incidence of 10% and 5%, respectively. Histological feature of RCC provides prognostic information regarding tumor patients. Clear cell type has a worse prognosis for RCC patients compared to both papillary and chromophobe types [21]. In a recent study, a 5-year survival of patients with clear cell and chromophobe RCC types was 50% and 78%, respectively [22].

1.3 Bone markers as bone metabolic indicators

Although bone seems to be an inert tissue, in fact, it is a metabolically active one, which continuously undergoes turnover that consists of bone resorption and formation processes [23]. Bone markers are mainly represented by bone cell enzymes such as bALP or by-products liberated during synthesis and degradation of type I collagen such as PINP and ICTP. As mentioned earlier, bone markers bALP, PINP, and ICTP were used in this study to validate the diagnostic and prognostic significance of OPN. Therefore, in order to outline the origin of the above-mentioned bone markers, bone turnover and metabolism of type I collagen are described in this section. In addition, it also gives a short overview of the clinical utility of these bone markers in human malignancies.

1.3.1 Bone turnover

Bone tissue consists of three components: an organic matrix, or osteoid, bone mineral, and bone cells [24]. The cells responsible for resorption and formation are osteoclasts and osteoblasts, respectively. Under the physiological conditions, bone resorption takes approximately 10 days, which is then followed by formation that lasts for up to 3 months. These two processes are tightly coupled through well-coordinated mechanisms [23,25].

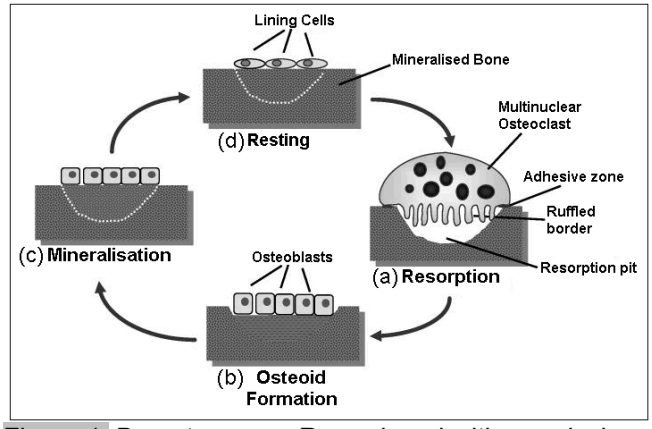


Figure 1. Bone turnover. Reproduced with permission from M. J. Seibel ref [25].

As shown in Figure 1, first, (a) osteoclasts should anchor to the bone matrix, which is mediated by an ariginine-glycine-asparic acid (RGD) cell-binding sequence of extracellular matrix (ECM) proteins such as OPN [6]. Osteoclasts dissolve bone mineral by massive acid secretion and also secrete specialized proteinases such as matrix metalloproteinases (MMPs) and cathepsin K that degrade the organic matrix, mainly type I collagen [26,27]. The resorption process takes place in an extracellular compartment covered by the ruffled border of the osteoclast and results in formation of the resorption pit [27]. (b) After the erosion of a cavity is completed by osteoclasts, osteoblasts fill the cavity with an equivalent amount of organic matrix. (c) Newly formed osteoid undergoes mineralization with hydroxyapatite and (d) the remodelled area then passes into a quiescent phase before a new cycle begins [28].

Therefore, this continuous process of bone turnover plays an important role in replacing old bone and maintaining homeostasis in bone tissue.

1.3.2 Bone-specific alkaline phosphatase

bALP is an enzyme synthesized by the osteoblasts in extremely high amounts during bone formation. Due to this fact bALP is considered as a reliable indicator of bone formation activity [28]. Total alkaline phosphatase (tALP) has been used widely as a marker of bone formation and is mainly composed of hepatic, renal, and bone isoenzymes. However, its diagnostic value is restricted since the bone isoform contributes to only about 40% of the total activity [23]. Therefore, measurement of bALP could be more accurate in the assessment of bone formation [29].

1.3.3 Propeptides and telopeptides of type I collagen

Type I collagen makes up 90% of bone matrix and the remaining 10% include proteins such as osteocalcin, osteonectin, and OPN [24]. Although type I collagen is found in connective tissue and some other tissues, bone has a distinctly higher proportion and turnover of this protein [28]. During the bone formation the osteoblast secretes into the extracellular space the type I procollagen molecules which form triple helixes each consisting of two chains of α1 and one chain of α2 procollagen [30]. N- and C-terminal portions of these triple helix molecules are cleaved by proteinases, which results in releasing two propeptides PINP and PICP (Figure 2). This cleavage allows molecules to aggregate into mature collagen fibrils by forming terminal cross-links [30,31]. Therefore, the cleaved by-products, PINP and PICP, directly reflect the rate of synthesis of type I collagen and thus of bone formation.

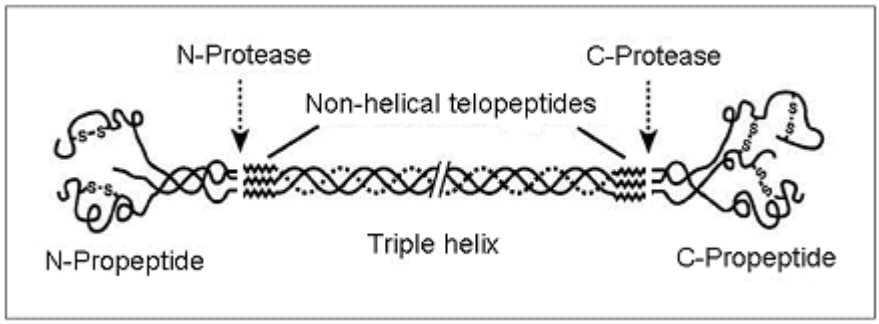


Figure 2. Schematic structure of collagen type I molecule. Reproduced with permission from Dr. S Robins, Aberdeen, Scotland.

In contrast, during bone resorption type I collagen undergoes degradation in which the collagen molecule is cleaved at both C- and N-terminal ends. This generates N- and C-telopeptides that reflect the rate of type I collagen degradation and thus of bone resorption. It is of interest that two different fragments are being generated on

each telopeptide end. This is due to the existence of different collagenolytic pathways. Indeed, ICTP collagen fragments are commonly produced by MMPs while cleavage by cathepsin K generates cross-linked carboxyterminal telopeptide of type I collagen fragments (CTX) [32,33]. As shown in Figure 3, CTX is a linear eight amino acid sequence of alfa1 chain, whereas ICTP, a cross-link-containing collagen peptide, is a larger fragment compared to CTX [32].

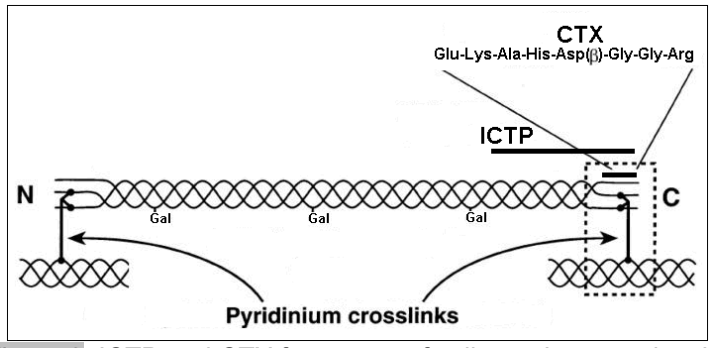


Figure 3. ICTP and CTX fragments of collagen I type molecule. Reproduced with permission from Dr S Robins, Aberdeen, Scotland. (with some modifications).

1.3.4 Clinical utility of bone markers in human malignancies

Metastatic spread of the tumor to bone alters these finely balanced processes of bone resorption and formation [2]. Skeletal metastases from PCa accelerate the bone formation rate and therefore are called osteoblastic [8]. In contrast, bone metastases from RCC are known to increase the rate of bone resorption and are termed osteolytic metastases [18]. These changes in bone metabolism caused by metastasis can be assessed by evaluating bone markers in blood. In this respect, Koizumi et al. [34] showed that PINP and bALP were effective markers in the detection of bone metastases in PCa patients and, moreover, PINP was reported as a more accurate diagnostic marker than bALP. This was also confirmed in another study, in which serum PINP in distinguishing PCa with bone metastases had a sensitivity and specificity of 100% and 87% compared to 90% and 82 % of bALP [35]. De la Piedra et al. [36] showed that serum PINP is an excellent marker for bone metastatic disease from PCa with a sensitivity and specificity of 100%. ICTP, a bone resorption marker, was also reported to be significantly elevated in PCa patients with bone metastases compared to those without bone metastases and BPH patients [34,37]. Moreover, all these markers: bALP, PINP, and ICTP correlate closely with Soloway's grading for bone scans reflecting the metastatic burden in PCa patients [37-39]. Besides PCa, in breast, lung,

and other malignancies ICTP and tALP were also useful in distinguishing patients with bone involvement and associated with the number of metastatic lesions in bone. In that study the overall specificity of these two markers was over 90% [40].

Bone markers were shown to be helpful in monitoring the response to hormonal therapy in PCa patients. In PCa patients with bone metastases, serum ICTP levels showed a downward trend along with a clinical response to hormonal treatment, and a significant decrease was observed after 12 weeks of treatment [37]. Yoshida et al. reported on an earlier response of ICTP levels to hormonal therapy in PCa patients after 8 weeks of initiation of treatment [39]. Bone markers also provide useful information in patients with bone metastases treated with bisphosphonates [41].

Bone markers may also be valuable in determining the prognosis in cancer patients. A recent study involving 153 metastatic PCa patients showed that the increased concentrations of PINP and bALP were strongly associated with shorter survival in those patients [38]. Prognostic significance of bALP and PINP related to survival in PCa patients was also confirmed in a large study involving 10 bone markers [42].

Therefore, bone markers are useful in the evaluation of cancer patients (i) to diagnose skeletal metastases, (ii) to assess their response to therapy, and (iii) to determine the prognosis.

1.4 Osteopontin

This section is meant to outline the results of the literature review carried out from PubMed concerning OPN and cancer. Furthermore it describes the structure and functions of OPN with particular stress on its implications in tumor progression and metastasis.

1.4.1 Literature review

OPN is a phosphorylated acidic glycoprotein with RGD sequence that interacts with cell surface integrin receptors and promotes cell adhesion, migration, and proliferation as well as cell survival. OPN exists as an immobilized ECM molecule in mineralized tissues and as a cytokine in body fluids. In bone tissue OPN is the most

abundant non-collagenous protein. Due to its multidomain structure OPN plays an important role in diverse physiological and pathological processes [4,7,43].

Senger et al. [44] first described the protein in 1979 as a marker of transformation of epithelial cells indicating its function in tumor biology. Later this protein was identified as a key non-collagenous protein in bone matrix and the name “osteopontin” was proposed to denote that it is a product of bone cells and that it can form bridge (“pons” is Latin for bridge) between cells and the mineral matrix [45]. However, the protein has also been shown to be important in various processes such as angiogenesis, wound healing and in inflammatory and immune response [4]. It was also named as an early T-lymphocyte activation 1 protein (Eta-1) in order to emphasize its importance in immune activity and bacterial resistance [46]. More than two and a half decades have passed since it was first described as a transformation-associated protein. However, there is still a considerable interest in the role of OPN in genesis and progression of human tumor.

In order to examine this tendency a PubMed search was performed using the keywords “osteopontin” and “cancer”, which retrieved 513 publications with the distribution in regard to the date of issue (Figure 4). The current literature review suggests that the number of publications involving OPN and cancer has been constantly increasing since 1987.

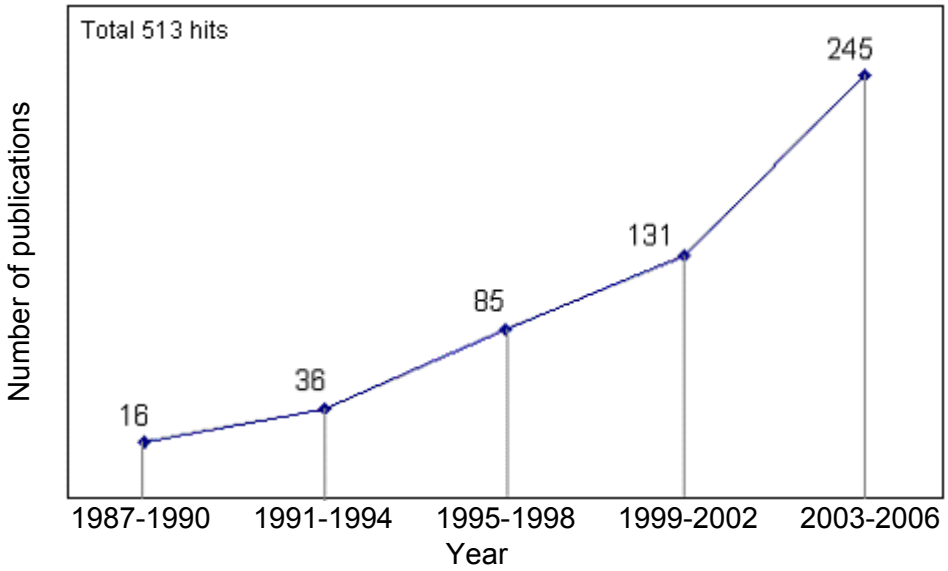


Figure 4. Medical literature review in PubMed on-line library specified by the following key words: “osteopontin” and “cancer” (August 2006).

Numerous studies in vitro and in animal models have clearly indicated that OPN can function to regulate tumor growth and metastatic spread. Studies on OPN tissue

expression have shown that OPN is elevated in a number of tumors compared to normal specimens. Moreover, intensity of OPN expression appears to correlate with patients' survival and clinico-pathological data [47-49]. Recent studies involving OPN-deficient mice [50,51] and techniques using OPN gene transfection [52] have considerably contributed to understanding the role of OPN in tumor invasion and metastasis.

Since the main objective of the current study was to investigate the full clinical potential of plasma OPN in patients with PCa and RCC, a more focused review of previously published findings involving plasma OPN in different malignancies was necessary. For this purpose a literature search was carried out from PubMed using the keywords "plasma osteopontin" and "cancer". Each subject-related publication was studied and used to construct Table 1, which gives an overview of the results of all presently available studies on plasma OPN in different human tumors.

Table 1. Summary of plasma OPN level in different human cancers: the association with clinico-pathological factors of patients and prognostic usefulness (August 2006)

Malignancy:	Without metastases[1]	With metastases:				Stage	Grade	Poor survival[3]	Prognostic predictor:	
		All kinds	Lymph nodes	Bone	Number of affected sites				Survival outcome[4]	Tumor recurrence
Prostate [53,54]				+				+++	+++	
Breast [53,55,56]	+++	+++			+			+++	+++	
Lung [53,57]	++					+				
Bladder [58]	+	+				+				
Liver [59,60]	+++		++			+	+	+++	+++	+
Multiple myeloma [61,62]	+++					+				
Ovarian [63-66]	+++					+				+++[2]
Pancreatic [67]	+++									
Uveal [68]	+++	+++								
Head and neck [69-71]	++		+			+		++	+	

Key: P value: +, <0.05; ++, <0.01; +++, <0.001.
[1]Compared to controls or/and respective benign disease.
[2]Only in combination with other established markers.
[3]Evaluated by Kaplan-Meier analysis.
[4]Evaluated by Cox regression model.
Note: numbers in parentheses after each tumor entity refer to original articles in reference list.

As shown in Table 1 plasma OPN has been found to be significantly elevated in a number of malignancies compared to healthy individuals or patients with benign disease. In several malignancies plasma OPN is suggested as a useful prognostic marker. Levels of OPN in plasma appear to correlate with pathological data such as stage or grade of tumor. In some malignancies OPN has a tendency to increase in plasma of patients with metastatic tumors. Moreover, in breast cancer plasma OPN is associated with the number of organ sites affected by metastases, reflecting the extension of the disease. All these findings suggest that plasma OPN is a promising diagnostic marker for primary tumor or metastases and, moreover, could be of prognostic value for cancer patients. Plasma OPN in PCa patients is mentioned only in two reports and, therefore, many aspects of the subject have not been extensively studied. In fact, important data concerning the behavior of plasma OPN in PCa patients with different clinico-pathological characteristics are still not available. In contrast, in patients with RCC plasma OPN has not been evaluated so far. This indicates that more extensive research on plasma OPN in PCa and RCC patients is needed to elucidate its full diagnostic and prognostic potential in these malignancies.

1.4.2 Structure of osteopontin

OPN is a negatively-charged acidic hydrophilic protein of approximately 300 amino acid residues detectable in all body fluids [4]. Its molecular weight ranges from 44 kDa to 75 kDa due to differences in post-translational modifications [72]. OPN is aspartic acid-rich and highly phosphorylated on serines and threonines, endowing the protein with a high acidic character [73]. Structurally, OPN contains several domains that suggest its various functions (Figure 5):

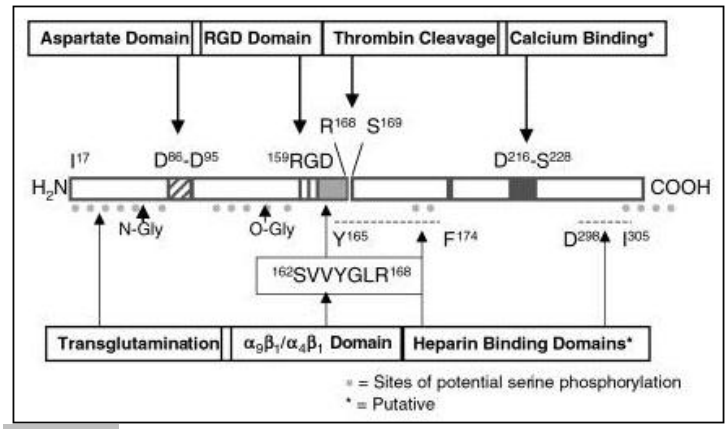


Figure 5. Structure of OPN. Reproduced with permission from D. T. Denhardt, ref. [43].

1.4.2.1 Arginine-glycine-aspartic acid domain - a ligand for cell integrin receptors

A central region of OPN contains a tri-peptide RGD sequence, which is responsible for adhesion to cell surface integrin receptors (Figure 5) [45]. Integrins comprise a large family of cell receptors composed of two subunits, α and β. To date, at least 15 α and 8 β integrin subunits have been identified and each combination mediates certain functions and elicits specific signaling pathways [74]. Integrin receptors are multifunctional molecules capable of transmitting biochemical signals from the ECM to the cells interior. In fact, the cytoplasmic tail of β subunit is connected to the specific components of the cytoskeleton such as talin and paxillin. Activated integrins and cytoskeletal proteins assemble into aggregates, which potentiate transmembrane signaling events. Integrins further activate protein tyrosine kinases, including focal adhesion kinase (FAK) and Src-family kinases. Such integrin-dependent interactions alter gene expression in cell and regulate cell motility, growth, and survival [75,76].

1.4.2.2 Thrombin cleavage site

OPN can be cleaved by thrombin in close proximity to the RGD cell-binding region (Figure 5). Cleavage of OPN occurs under physiological conditions and could serve as an important mechanism to regulate the bioactivity of OPN. Thrombin cleavage allows greater accessibility of the RGD domain to cell surface receptors. In the study by Senger et al. [77] thrombin-cleaved OPN promoted markedly greater cell attachment and spreading than intact molecule. This fact of cleavage by thrombin restricts the quantification of OPN to plasma samples. Indeed, as a preliminary preparation to this study OPN was assayed with ELISA in matched serum and plasma samples of healthy individuals and RCC patients. OPN was only measurable in plasma whereas in serum OPN was not detectable apparently due to the susceptibility of OPN to thrombin (unpublished results).

1.4.2.3 Serine-valine-valine-tyrosine-glycine-leucine-arginine sequence (SVVYGLR)

As shown in Figure 5, SVVYGLR sequence consists of seven residues of amino acid and is located between the RGD domain and the thrombin cleavage site. Two integrin receptors, $\alpha 9\beta 1$ and $\alpha 4\beta 1$, are known to bind to SVVYGLR sequence. This

domain is also termed cryptic because it is functional only after cleavage by thrombin [78,79].

1.4.2.4 Other domains

OPN contains two domains with heparin-binding properties that are likely to mediate its binding to ECM (Figure 5). Presence of putative Ca^{2+}binding motifs probably explains the ability of OPN to bind large amounts of Ca^{2+} and interact with hydroxyapatite with high affinity [73]. OPN is also a ligand for several splice variants of CD44 cell receptor such as CD44v3-v6. The domains of OPN responsible for binding the CD44v3-v6 variants have not been established [73,80].

1.4.3 Biological functions of osteopontin

OPN exists both as an immobilized ECM molecule in mineralised tissues and as a cytokine in body fluids [43]. Due to its multidomain structure OPN regulates various physiological and pathological processes (Figure 6).

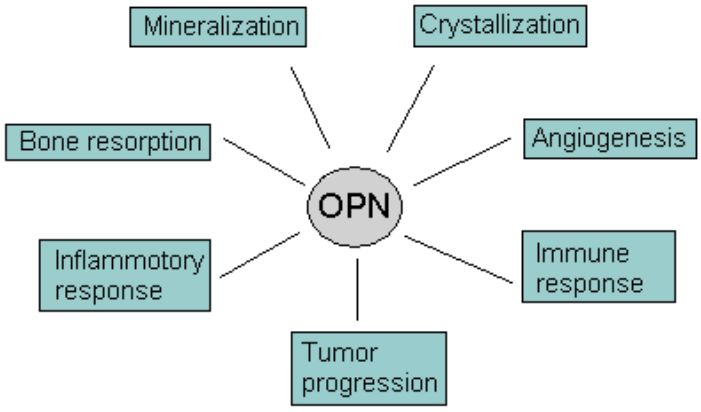


Figure 6. Biological functions of OPN.

1.4.3.1 Bone resorption

OPN is abundantly distributed in bone and is estimated to comprise approximately 2% of non-collagenous proteins in bone tissue [7,73]. OPN is involved in the regulation of bone turnover and secreted by both bone cells: osteoblasts and osteoclasts [5,73]. Osteoclasts are known to highly express $\alpha v\beta 3$ integrin. [81]. Through the interaction with $\alpha v\beta 3$ integrin OPN mediates migration and adhesion of osteoclasts

to bone matrix and, consequently, initiates a resorptive process [6,73]. In vitro and in vivo studies support the importance of the interaction between OPN and $\alpha v\beta 3$ integrin of osteoclasts. Interference with the function of OPN or $\alpha v\beta 3$ integrin using a variety of approaches leads to an inhibition of the adherence of osteoclasts and results in decreased bone resorption [82,83].

1.4.3.2 Mineralization and crystallization

OPN is assumed to play a role in regulating the deposition of mineral in bone and has been shown in vitro to inhibit hydroxyapatite crystal growth. The inhibitory activity of OPN is apparently due to both polyaspartic acid sequence and phosphate groups. In fact, interference with the phosphate groups or modification of carboxylate groups of aspartic acids reduced the inhibitory activity of OPN by a factor of 40 and 6, respectively [84].

OPN appears to be an important natural defense against renal crystallizations and nephrolithiasis. In vitro data indicate that urinary OPN may inhibit the formation of calcium oxalate crystals [85]. In a recent study with ethylene glycol-induced hyperoxaluria OPN knockout mice developed crystal formation and retention in kidney whereas wild types were completely unaffected [86]. Possibly due to its polyaspartic acid structure OPN also directs calcium oxalate (CaOx) crystallization to the CaOx dihydrate phase, which is markedly less adherent to renal tubular epithelial cells compared to the CaOx monohydrate [87,88]. OPN is present in human urine at levels that can efficiently inhibit CaOx crystallization [89]. Lower concentrations of OPN were found in the urine of patients with renal stone disease compared with normal individuals [90].

1.4.3.3 Inflammatory and immune response

OPN plays an important role during acute inflammation where it may be synthesized by infiltrating macrophages. OPN is involved in the recruitment and retention of immune cells to inflamed sites [4]. Using a rat model, Giachelli et al. [91] demonstrated macrophage-rich infiltration and high OPN expression at sites of subcutaneous injection of bacterial chemotactic peptide, N-phormyl-methionyl-leucyl-

phenylalanine and the inhibition of macrophage infiltration by application of OPN neutralizing antibodies.

In addition to acute inflammation, OPN is also involved in chronic inflammation initiated by T cell-mediated immunity. O'Regan et al. [92] demonstrated an extensive OPN expression in T-cells in granulomatous disease such as sarcoidosis. In the same study OPN fragments generated by thrombin cleavage enhance markedly the adhesion and migration of T-cells and macrophages in comparison with the native OPN. A recent experiment with OPN-null mice showed that OPN-deficient mice had a defective immune response and were more sensitive to viral and bacterial infection. Moreover, macrophage synthesis of the two major regulators of cell-mediated immunity interleukin-12 and interferon-γ was diminished in OPN-null mice compared to wild types. This indicates that OPN is a critical cytokine regulating the type 1 cell-mediated immune response.

1.4.3.4 Angiogenesis

Recently, there have been some reports indicating the importance of OPN in angiogenesis. Interaction between RGD region of OPN and αv integrin family of endothelial cells appears to play a crucial role in angiogenesis. Takagi et al. [93] showed a hypoxia-induced increase in expression of OPN as well as $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins in retina. This evidence suggests that co-upregulation of the αv integrin family and OPN may potentiate neovascularization in ischemic retina. A recent study has revealed another possible mechanism by which OPN regulates angiogenesis. According to that study OPN-derived synthetic peptide SVVYGLR not only promotes adhesion and migration of endothelial cells but also stimulates lumen formation in vitro as efficiently as vascular endothelial growth factor (VEGF) [94]. In addition, OPN delivers an antiapoptotic signal to the cell via the $\alpha v\beta 3$ integrin and thus promotes the survival of endothelial cells [95].

1.4.3.5 Osteopontin in tumor progression and metastasis

Metastasis is the process by which cancer cells detach from the primary tumor, travel to a distant site via the circulatory system and form a secondary tumor. Several

events are necessary for malignant cells to leave the primary tumor and proliferate at a distant site: cell proliferation, invasion (cell motility, ECM degradation), and angiogenesis [96]. OPN appears to be implicated in all these events and, therefore, is recognized as a key protein in tumor progression. In the following sections experimental evidence, which supports this view, will be reviewed.

Proliferation. OPN contains an RGD sequence that binds to integrins and is capable of promoting the proliferation of tumor cells. In this regard, Thalmann et al. [97] clearly demonstrated the stimulatory effect of OPN on the growth of PCa cells, in which OPN antibody inhibits the growth stimulatory effect by endogenous OPN and addition of exogenous OPN returns growth to a normal rate. Obviously tumor cells support their growth by secreting OPN and, as a consequence, OPN expression in tissue directly correlates with tumor size and stage [47,98]. On the other hand, OPN serves as a survival factor for tumor cells due to its ability to inhibit the synthesis of nitric oxide (NO) by activated immune cells. Generation and release of NO is known to be lethal for both host cell and tumor cell due to inactivation of critical metabolic pathways. Therefore, tumor cells that produce OPN may protect themselves from oxidative damage [99,100]. Moreover, recent data also showed that OPN delivers antiapoptotic signal to the cell [95]. Since tumor growth, either primary or metastatic, is merely defined by the number of cells in proliferation and cells that undergo apoptosis, the mechanisms described above could explain the association between OPN and tumor growth.

Invasion. Enhanced motility of tumor cells as well as the ability to intravasate into the vasculature are known to play a crucial role in tumor invasion and metastasis [101]. OPN was shown to induce cell migration in breast cancer cells [102] and facilitate intravasation of PCa cells [103]. Distinct increase in OPN expression found in metastatic lesions compared to that of primary tumor emphasizes the importance of OPN in the invasion and spreading of tumor cells [103]. On the other hand, degradation of ECM is also important for cellular migration and invasion. In this regard, ECM-degrading proteases such as urokinase type plasminogen activator (uPA) and MMPs appear to be of major importance [104,105]. Through its adhesive properties, OPN can induce changes in tumor cell gene expression including induction of proteolytic enzymes. In this respect, Tuck et al. [102] demonstrated that OPN induces uPA expression and cellular invasiveness of breast epithelial cells. In another study OPN and uPA expression was found to be higher in bone metastases and invasive carcinomas than in non-invasive or normal breast tissue [106]. In murine melanoma cells, OPN was also

shown to increase pro-MMP2 expression and activation, cell migration, and ECM invasion leading to enhanced tumorigenicity [107].

Angiogenesis. OPN appears to play an important role in tumor growth through the enhancement of angiogenesis [108]. OPN promotes adhesion, migration, and proliferation of endothelial cells, and, moreover, enhances their survival [93-95]. Generation of new blood vessels is important for the growth of both primary and metastatic tumors since cell proliferation requires continuous supply of oxygen and nutrients. A high degree of tumor vascularization also increases the chance for tumor cells to enter the circulatory system and metastasize [109]. Moreover, increased tumor vascularity is known to be associated with tumor progression and poor survival of tumor patients [110,111].

Tumor cell $\alpha v\beta 3$ integrin. Considerable evidence suggests the implication of the $\alpha v\beta 3$ integrin in increased malignancy of tumor cells. Tumorigenic and highly metastatic breast epithelial cells migrate toward OPN in an $\alpha v\beta 3$-dependent manner while non-malignant and less malignant epithelial cells do not express $\alpha v\beta 3$. Migration of the latter cells to OPN is mediated by $\alpha v\beta 5$ and $\alpha v\beta 1$ integrins [52,112]. Moreover, transfection of the less malignant cells lacking $\alpha v\beta 3$ with $\beta 3$ enhances cell adherence, migration and invasiveness in vitro and also results in increased tumorigenesis in vivo [52]. In other cell types such as PCa cells, the highly invasive tumor cells are shown to express $\alpha v\beta 3$ in contrast to non-invasive tumor cells [113]. Most interestingly, nearly all breast cancer and PCa cells that have metastasized to bone express $\alpha v\beta 3$ integrin [114,115]. Collectively, these observations indicate that OPN and interaction particularly with $\alpha v\beta 3$ is important for tumor progression and dissemination.

1.5 Objectives of study

As described earlier, OPN, a glycoprotein, with its multidomain structure participates in various physiological and pathological processes. In relation to tumor biology numerous experimental studies indicate the implication of OPN in tumor progression towards metastasis [3]. In brief, in vivo and in vitro data showed elevated expression of OPN in metastatic lesions [103] and in cancer cells with highly invasive properties [102]. OPN expression in tissue correlates with tumor stage [47] and survival of cancer patients [48]. In addition, OPN, a secreted protein, is present in all body fluids [4] and therefore is available for routine determinations in plasma. All this suggests that evaluation of OPN in plasma could be of diagnostic value in relation to metastasis and could provide prognostic information regarding cancer patients. As mentioned earlier, plasma OPN in RCC patients has not been evaluated so far, whereas in PCa patients it is only available in a limited number of reports.

Therefore, the current study was undertaken to investigate the diagnostic and prognostic usefulness of plasma OPN in PCa and RCC patients in comparison with the established bone markers such as ICTP, PINP, and bALP. The following aspects were examined:

1. Concentrations of plasma OPN and the bone markers in controls and different subgroups of PCa and RCC patients classified according to the TNM system.
2. Behavior of plasma OPN in PCa and RCC patients with different tumor stages and grades.
3. Correlation of plasma OPN with the bone markers.
4. Diagnostic accuracy of plasma OPN in comparison with the bone markers in the detection of distant metastases, especially bone metastases, in PCa and RCC patients.
5. Ability of plasma OPN in comparison with the bone markers to predict the probability of distant metastasis in PCa and RCC patients.
6. Possibility to increase diagnostic accuracy by combination of biomarkers using logistic regression approach.
7. Prognostic significance of plasma OPN in comparison with the bone markers to predict the survival outcome in PCa and RCC patients.

2 Materials and methods

2.1 Study population

2.1.1 Control groups

The control group for PCa patients consisted of 29 men whereas that for RCC patients included 27 females and 25 males (Tables 2 and 3). Participants in both control groups received no medication known to interfere with bone metabolism and had no signs of infection; gastrointestinal, hepatic, cardiac, or renal disease, tumors, or immunologic disease. In addition, liver and kidney diseases were excluded since all subjects had values of alanine aminotransferase and creatinine within the reference intervals.

2.1.2 Patients with benign prostatic hyperplasia

Thirty-five men who were classified as benign prostatic hyperplasia (BPH) patients received no treatment for prostatic disease at the time of blood sampling. The clinical diagnosis of BPH was histologically confirmed by examining prostatic specimens obtained by ultrasound-guided biopsies or after transurethral resection (Table 2).

2.1.3 Prostate cancer patients

There were 90 patients (median age 65 years, range, 38-77) with PCa (Table 2). PCa was diagnosed histopathologically by microscopic examination of prostatic specimens after biopsy or additionally at radical prostatectomy. Cancer stage was assigned according to the TNM system and histological grade was classified as grade 1, 2 or 3. Gleason score was not available in all PCa patients. Bone scintigraphy and, in special cases, X-ray, computerized tomography or magnetic resonance imaging were used to diagnose bone metastases. There were 28 patients with bone metastases (indicated as group M1). The 62 patients without distant metastases received surgical staging (pelvic lymphadenectomy) with histological examination and were therefore subdivided into groups without (pN0M0, n=32) and with (pN1M0, n=30) lymph node metastases. In the pN1M0 group, 19 patients were untreated and 11 received hormonal therapy (orchidectomy, luteinizing hormone-releasing analogs, and antiandrogens)

before sample collection (median 2.1 months, range 0.8-3.2). In the M1 group, 12 patients were untreated and 16 received hormonal therapy or had this treatment after radical prostatectomy or radiotherapy before sample collection (median 18.4 months; range 6.3 to 56).

Table 2. Characteristics of controls, BPH, and PCa groups

	Controls	BPH	PCa		
			Stage pN0M0	Stage pN1M0	Stage M1
Number of patients	29	35	32	30	28
Age (years)[1]	50 (41, 51)	60 (68, 71)	64 (58, 69)	68 (63, 72)	65 (59, 69)
Tumor stage					
T2			18	11	5
T3			14	19	20
T4			--	--	3
Tumor grade					
G1			2	1	--
G2			17	19	11
G3			13	10	17

[1]Values are medians, with lower and upper quartiles in parentheses.

2.1.4 Renal cell carcinoma patients

The RCC group included 80 patients (Table 3). Cancer stage and grade were assigned according to the TNM system. Data on histological types of RCC were available for 70 patients. According to the histological data, of those 70 patients 55 (79%) had clear cell RCC, 8 (11%) and 2 (3%) patients presented with papillary and chromophobe types of RCC. Another 5 (7%) patients had unclassified histological types of RCC. Bone scintigraphy, X-ray, computerized tomography, magnetic resonance imaging, and ultrasound diagnostics were used to diagnose metastases. Regional lymph node dissections with histological examinations were performed in certain cases for staging purposes. RCC patients were therefore subdivided into three groups: those without metastases (N0, n=32), patients with lymph node metastases (N1, n=11), and 37 patients with distant metastases (M1 group). The patients with distant metastases were in turn subdivided into groups with bone and without bone metastases. Table 3 also outlines the number and character of additional distant metastases in M1 group.

Table 3. Characteristics of controls and RCC groups

	Controls	RCC			
		Stage pN0M0	Stage pN1M0	Stage M1	
				With bone metastases	Without bone metastases
Number of patients	52	32	11	17	20
Female	27	17	5	8	7
Male	25	15	6	9	13
Age (years) [1]	52 (41, 60)	60 (58, 65)	62 (57, 67)	58 (57, 65)	62 (56, 68)
Tumor stage					
T1		19	4	4	3
T2		6	2	4	5
T3		7	4	8	11
T4		--	1	1	1
Tumor grade					
G1		2	1	--	--
G2		25	5	8	9
G3		5	5	9	11

Additional metastases in M1 group[2]	
With bone metastases	Without bone metastases
bone (2)	lung (7)
bone + lung (7)	liver (2)
bone + liver (1)	duodenum (1)
bone + mediastinum (1)	lung + liver (1)
bone + lung + mediastinum (1)	lung + CNS (1)
bone + lung + CNS (2)	thyroidal gland + mediastinum (1)
bone + lung + mediastinum + CNS (2)	lung + liver + pancreas (2)
bone + lung + liver + pancreas + skin (1)	lung + liver + duodenum (1)
	lung + liver + CNS + vagina (1)

[1]Values are medians, with lower and upper quartiles in parentheses.
[2]Number of patients with respective metastases in parentheses.
Abbreviation: CNS, central nerve system.

2.2 Collection of blood samples

Controls and patients in this study were investigated at the Department of Urology, Charité University Hospital. Blood samples were collected in plastic tubes containing K-EDTA for OPN determination or kaolin-coated granulate for the quantification of other analytes (Monovette systems, Sarstedt, Nümbrecht, Germany) between 7:00 and 9:00 a.m. and centrifuged at 2,000g for 10 min at 4°C within 2 hours after venipuncture. Supernatants were stored at –80°C for further analysis.

2.2.1 Prostate cancer

In PCa patients blood samples were collected before any treatment except in the groups pN1M0 and M1 as mentioned in 2.1.3. In all other cases, blood samples were

taken before any diagnostic procedure, transurethral resection of the prostate, prostatectomy or 4 weeks after digital rectal examination, prostatic biopsy or transrectal ultrasound.

2.2.2 Renal cell carcinoma

In RCC patients blood samples were collected before any treatment except in the group of patients with distant metastases. In the group of 17 patients with bone metastases, blood was taken from 11 patients one day before radical nephrectomy and from 26 patients between 2 and 72 months after radical nephrectomy at a control examination at our institution. In the group of 20 patients without bone metastases, blood samples were collected from 13 patients one day before surgery, from 3 patients 3-72 months after radical nephrectomy at a control examination, and from 4 patients at the time of the diagnosis of metastases.

2.3 Quantification of osteopontin

2.3.1 Sample preparation

The ELISA technique was used to quantify OPN in K-EDTA plasma from PCa and RCC patients and in respective controls. Samples were brought to a room temperature and rested till completely thawed. After short vortex and visual check, samples were centrifuged at 5000g and 4°C. Assay buffer provided in ELISA kits was used to dilute plasma samples to a desired proportion.

2.3.2 The ELISA procedure

Figure 7 schematically illustrates ELISA procedure used for OPN quantification: (a) each well of ELISA plate was coated with a capture antibody to human OPN; (b) standards and samples were diluted with assay buffer and added to the wells; OPN, if any present, bound to the immobilized antibody building antigen-antibody complex; (c) the plate was incubated and washed so that excess OPN and unbound non-specific antigens were washed away whereas captured OPN remained in the wells for further quantification; (d) enzyme-linked antibody was added to the wells and coupled to the previously formed antigen-antibody complex; (e) the plate underwent incubation and

wash for a second time so that labelled molecules that did not bind could be removed; (f) a colorless substrate was applied to the wells; (g) the reaction between the enzyme and the substrate converts the latter to generate color; (h) adding stop solution terminated the enzymatic reaction and (i) the color signal was finally estimated by spectrophotometry; the color intensity in each well was directly proportional to the concentration of OPN.

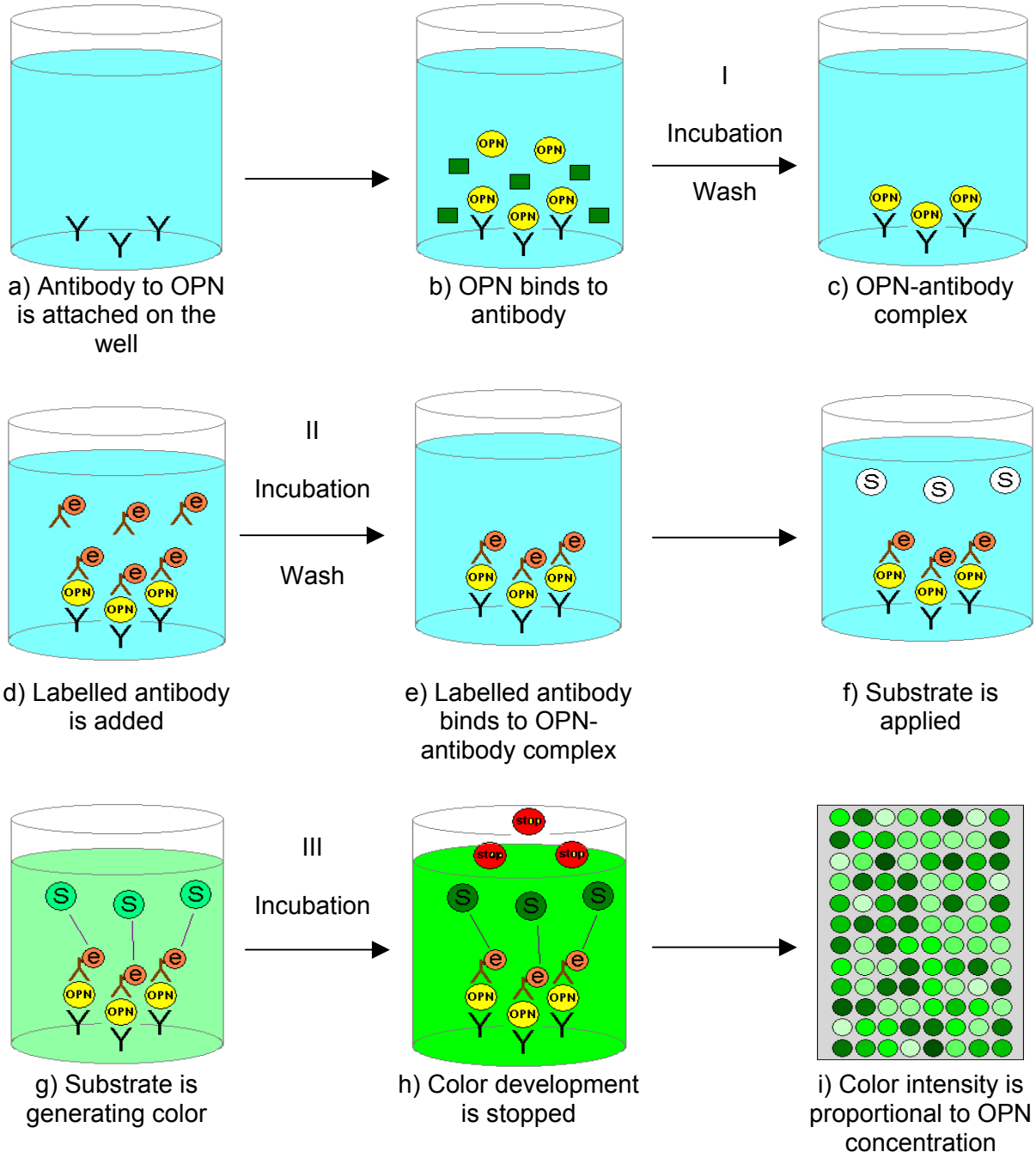


Figure 7. The ELISA procedure for OPN quantification.

Plasma OPN from PCa patients was quantified with ELISA kits manufactured by Calbiochem® which were later not available for purchase. Further quantification of plasma OPN from RCC patients proceeded with ELISA kits produced by TiterZyme®EIA. Therefore, detailed information on each ELISA assay performed in plasma from PCa or RCC patients is outlined in Table 4.

Table 4. ELISA assays used for OPN quantification in plasma of PCa and RCC patients

	PCa	RCC
Manufacturer	Calbiochem®. USA	TiterZyme®EIA. USA
Number of wells per plate	96	96
Antibody detects	whole OPN	epitop of OPN[1]
Standards, µg/L	5, 10, 20, 40, 80, 160, 320	2, 4, 8, 16, 32
Sample, dilution	Plasma, 1:10	Plasma, 1:10
Final standard or sample volume	100 µL	100 µL
Incubation I Wash I	1 hour, RT, on a shaker 7 x 400 µL	1 hour, RT, on a shaker 4 x 400 µL
Antibody Enzyme label	100 µL Horseradish peroxidase	100 µL Alkaline phosphatase
Incubation II Wash II	2 hours, RT, on a shaker 9 x 400 µL	1 hour, RT, on a shaker 4 x 400 µL
Substrate Incubation III	Tetramethyl benzidine, 100 µL 30 min, RT, on a shaker, dark	p-Nitrophenyl phosphate, 100 µL 30 min, RT, on a shaker
Stop solution	Sulphuric acid, 100 µL,	Trisodium phosphate, 25 µL

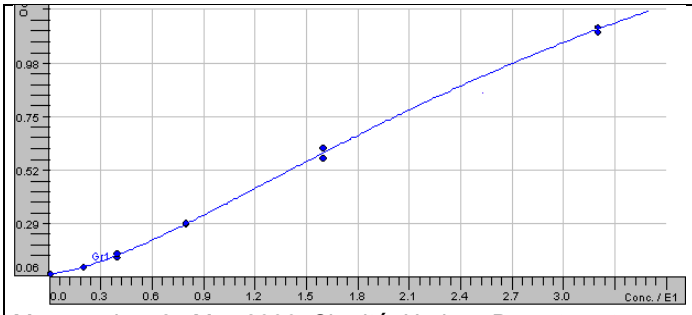
[1]Epitope is located after thrombin cleavage site and includes SVVYGLRSKSK sequence.

Note: Volume is given per well. Samples and standards were run in duplicate. TiterZyme®EIA kit required that two extra chemicals be added to the assay buffer in order to maintain OPN integrity in all samples and standards during the assay. Therefore Proteinase Inhibitor Cocktail (Sigma, St. Louis, MI; 0.5 µL/mL) and phenylmethylsulfonyl fluoride (1 mol/L) were added to the assay buffer.

2.3.3 Calculation of osteopontin concentration

The intensity of the color generated in the plate was measured optically with the spectrophotometer (Anthos HtII, Anthos Labtec Instruments, Salzburg, Austria) at 450

nm with the reference wavelength set at 620 nm. The spectrophotometer was interfaced to a personal computer to analyse data obtained with the software (MikroWin 3.0, Mikrotek Laborsysteme, Germany). Standard curves were constructed using 4-parameter logistic curve fitting approach with known OPN concentrations of standards and corresponding absorbance values (Figure 8).



Abscissa: Concentrations, µg/L (mean)		*Ordinate*: Optical Density (mean)
S0	0	0.072
S1	2.0	0.104
S2	4.0	0.152
S3	8.0	0.290
S4	16.0	0.594
S5	32.0	1.126
G362	1.70	0.096
G890	6.15	0.223

Figure 8. Example of standard curve for OPN.

Therefore, OPN concentrations of all samples within the plate could be determined with the standard curve. As shown in Figure 8 two samples of RCC patients with the coded numbers G362 and G890 had optical densities 0.096 and 0.223. Their concentrations calculated from the standard curve amounted to 1.70 and 6.15 µg/L. Taking into consideration that samples for the assay were diluted in proportion 1:10 (Table 4) their actual OPN plasma level should be 17.0 and 61.5 µg/L. All measurements described above were performed by the author.

2.4 Quantification of bone markers

tALP was measured with standard enzyme assay on the Modular analyzer (Roche, Mannheim, Germany). PINP (Total PINP-Assay, Roche) was measured on the Elecsys 2010 analyzer. The quantification of the above-mentioned markers was performed at the Institute of Laboratory Medicine and Pathobiochemistry at the Charité (Prof. Dr. E. Köttgen – at that time the director of the institute). bALP was determined by the Tandem-MP Ostase Immunoenzymetric Assay (Beckman Coulter, Fullerton, CA, USA), which specifically quantifies skeletal ALP with low immunoreactivity for liver/kidney isoforms. ICTP was quantified with ELISA (Orion Diagnostica, Espoo, Finland). These measurements were performed in the Research Laboratory of the Department of Urology, CCM with the kind assistance of Ms. Janet Reiche and Ms. Silke Klotzek to the

author of the dissertation. In RCC patients only ICTP and bALP were measured due to sample availability.

2.5 Routine clinical chemistry determinations

Alanine aminotransferase (ALAT) (upper reference limit 41 U/L), gamma-glutamyl transferase (GGT), C-reactive protein (5 mg/L), and creatinine (105 µmol/L) were measured by standard assays on the Modular analyser and were partly taken from the patient's records. Total prostate specific antigen (PSA) was quantified with the Immulite PSA kit (Diagnostic Products, Los Angeles, CA, USA).

2.6 Statistical analysis

Statistical calculations were performed with SPSS 13.0 for Windows (SPSS, Munich, Germany) and GraphPad Prism 4.3 (GraphPad, San Diego, CA). The following tests were used: the non-parametric Kruskal-Wallis ANOVA with Dunn's post-test, the Mann-Whitney U test, Spearman's rank correlation coefficients (r_s), and the distribution fitting procedure of Kolmogorov-Smirnov. Logistic regression approach was used to identify significant predictors of bone metastasis. The Kaplan-Meier product-limit method was used to determine survival probability for subgroups. Univariate and multivariate analyses of risk factors predicting PCa or RCC-specific death were performed using the Cox proportional hazards regression model. 1000 bootstrap re-samples with the software R, version 2.3.1 (www.r-project.org) were partly used to estimate the parameters of the models and to prevent an overfitting bias. Bootstrap calculations were performed by Dr. Keller, Addstats, Leipzig. Diagnostic accuracy was evaluated by receiver operation characteristics (ROC) curve analysis using the software MedCalc 9.0.1.0 (MedCalc, Mariakerke, Belgium). Reference intervals were calculated according to the recommended procedure of the International Federation of Clinical Chemistry using the program RefVal [116]. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Prostate cancer

3.1.1 Levels of osteopontin and bone markers

Figure 9 shows the scatter plots and medians of OPN and bone markers in controls, BPH patients, and PCa patients subdivided into the groups N0M0, N1M0, and M1. Statistical assessment of the data can be summarized as follows:

(i) Concentrations of all analytes did not differ among controls, BPH group, PCa with lymph node-negative and lymph node-positive groups except OPN where BPH patients showed a higher concentration than controls ($P < 0.01$).

(ii) OPN and all bone markers were significantly higher in patients with bone metastases compared to controls, BPH, and the N0M0 and N1M0 groups ($P < 0.05$ at least), showing their relationship with skeletal involvement.

(iii) Significant differences were observed for OPN and all bone markers between PCa patients with and without bone metastases.

Concentrations of OPN and bone markers in M1 group were evaluated in relation to the 95 percentile cutoffs of the controls. In this regard 79% of the M1 patients had increased OPN values compared to 71%, 68%, and 63% of patients with increased values of ICTP, bALP, and PINP, respectively.

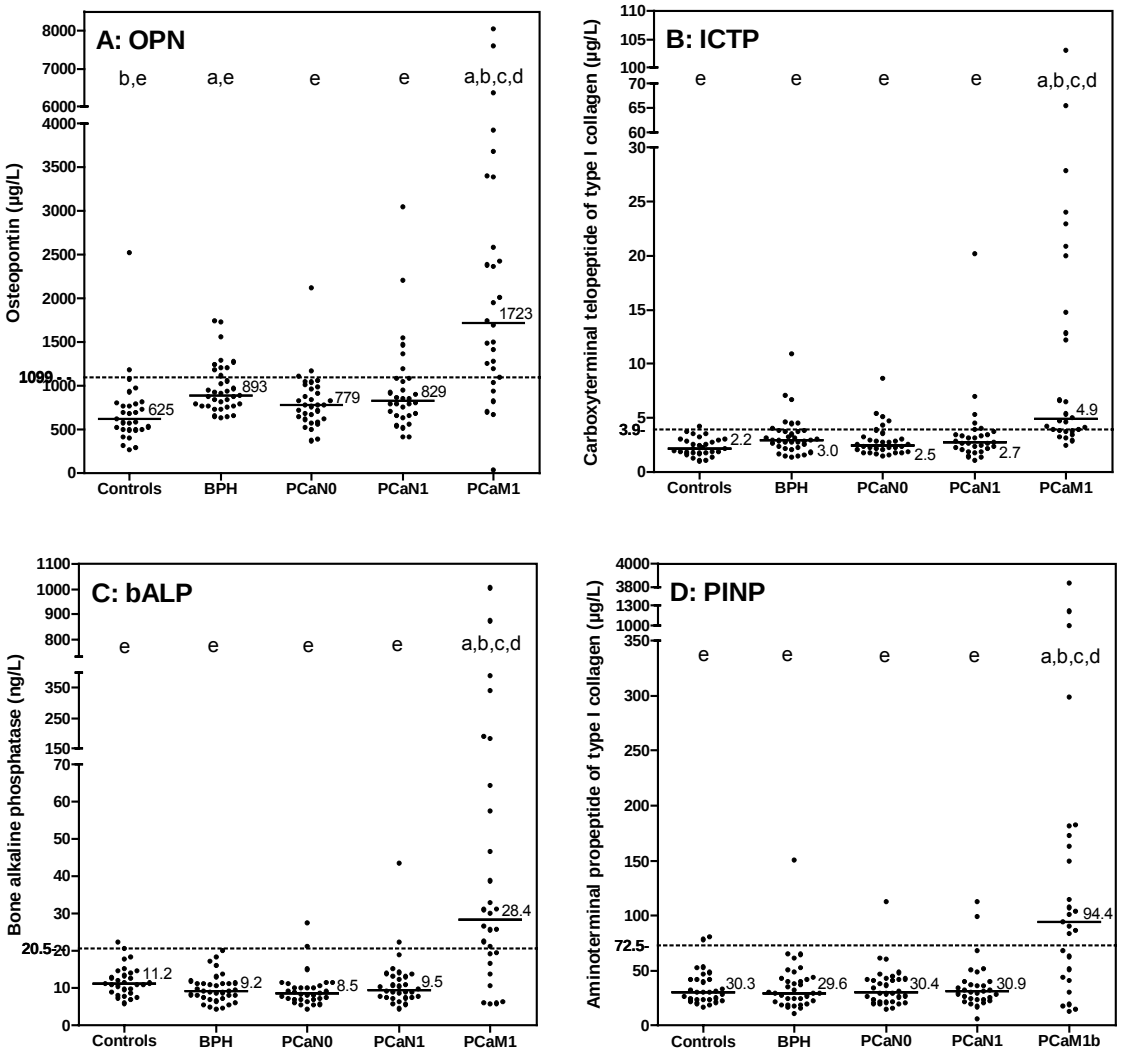


Figure 9. Scatter plots of OPN and bone markers in controls and patients with BPH or PCa. Median values of the groups are shown as horizontal lines with corresponding figures; dotted lines indicate the upper 95 percentiles of controls. Significant differences (Kruskal-Wallis non-parametric ANOVA with Dunn's post-test, P<0.05 at least) are shown by the following symbols; **a**, compared to controls; **b**, compared to BPH patients; **c**, compared to PCa patients without lymph node metastases (group pN0M0); **d**, compared to PCa patients with lymph node metastases (group pN1M0); **e**, compared to PCa patients with bone metastases (group M1).

3.1.2 Correlation between osteopontin, bone markers, and clinico-pathological data

Spearman's rank correlation analyses were performed with all PCa patients as shown in Table 5. Significant correlations were observed between OPN and all bone markers (r_s=0.43-0.79, all P <0.01). Moreover, OPN correlated with tumor grade whereas bALP correlated with tumor stage. Concentrations of OPN compared in different tumor grades of PCa patients ranged from 40 to 3388 µg/L with the median of

838 µg/L in G1-2 tumors and from 412 to 8057 µg/L with the median of 994 µg/L in G3 tumors (Mann-Whitney U test, P=0.044). Levels of bALP compared in different tumor stages of PCa patients ranged from 4.3 to 1006 ng/L with the median of 8.8 ng/L in stage II tumors and from 4.5 to 874 ng/L with the median of 10.9 ng/L in stage III+IV tumors (Mann-Whitney U test, P= 0.038). ICTP and PINP showed no association with tumor stage or grade. bALP correlated negatively with age. PSA significantly correlated with OPN and all bone markers (r_s=0.30-0.37, all P <0.01).

Table 5. Correlation between OPN, bone markers and clinico-pathological data

	ICTP	bALP	PINP	PSA	Age	T-Stage	Grading
OPN	0.54**	0.43**	0.49**	0.32**	0.17	0.21	0.23*
ICTP	1.00	0.48**	0.61**	0.37**	0.05	0.08	0.17
bALP		1.00	0.79**	0.37**	-0.24*	0.24*	0.16
PINP			1.00	0.30**	-0.13	0.14	0.07
PSA				1.00	-0.09	0.23*	0.29*
Age					1.00	-0.04	0.08

Significances: *, P <0.05; **, P <0.01.

The effect of the hormonal therapy on OPN and on the other markers was subsequently evaluated. For this purpose their concentrations were compared in patients with and without treatment in the groups pN1M0 and M1, whereas the pN0M0 group only included untreated patients. In the pN1M0 group, 19 patients were untreated and 11 had received hormonal therapy before sample collection (median 2.1 months, range 0.8 - 3.2) while 12 patients were untreated and 16 had received hormonal therapy in group M1 before sampling (median 18.4 months, range 6.3 – 56). In both groups, the concentrations of all markers did not differ between patients with and without hormonal treatment (Mann-Whitney U test; P values between 0.211 and 1.00). Consequently, all further calculations were performed with the data of all patients in the respective groups independently of the treatment.

3.1.3 Osteopontin and bone markers as diagnostic indicators of metastases

ROC analysis was used to assess the diagnostic usefulness of OPN and bone markers to differentiate PCa patients with and without bone metastases (Table 6). OPN and bone markers were effective for the detection of bone metastases with the largest area under the ROC curve (AUC) observed in ICTP, 0.88, followed by OPN, bALP, and

PINP, 0.85, 0.84, and 0.80 (all P <0.0001). There were no significant differences in AUC between the markers (P=0.164-0.937). However, at the cutoff level of 95% sensitivity, specificity of OPN outperformed that of bALP and PINP (P=0.0266 and 0.0009, McNemar test), but was less than that of ICTP (P=0.0002, McNemar test). At the same cutoff ICTP had the highest specificity. Similar to that, sensitivity of OPN and bone markers was examined at the cutoff level set at 95% specificity. However, at that point there were no differences in sensitivity of OPN and bone markers (P=0.30-1.0, McNemar test).

Table 6. Diagnostic sensitivity, specificity and area under the curve (AUC) of OPN and bone markers to distinguish PCa patients with and without bone metastases

Variable	Sensitivity (%)	Specificity (%)	AUC
OPN (µg/L)			
1192[a]	75 (55-89)	90 (80-96)	0.85 (0.76-0.91)
1099[b]	75 (55-89)	86 (74-93)	
659[c]	95 (82-99)	31 (20-44)	
ICTP (µg/L)			
3.7[a]	82 (63-94)	81 (69-90)	0.87 (0.79-0.93)
3.9[b]	71 (51-87)	82 (71-91)	
2.9[c]	95 (82-99)	63 (50-75)	
bALP (ng/L)			
15.2[a]	79 (59-92)	92 (82-97)	0.84 (0.75-0.91)
20.5[b]	68 (48-84)	95 (87-99)	
5.8[c]	95 (82-99)	11 (5-22)	
PINP (µg/L)			
61.1[a]	70 (50-86)	94 (84-98)	0.80 (0.71-0.88)
72.5[b]	63 (42-81)	95 (87-99)	
14.5[c]	95 (82-99)	3 (1-11)	
OPN+bALP			
0.2139[a]	89 (72-98)	87 (76-94)	0.93 (0.85-0.97)
0.1024[c]	95 (82-99)	63 (50-75)	

Sensitivity, specificity, and area under the curves (AUC) with 95% confidence intervals in parentheses of the various markers were calculated using either
[a]the cutoff level with the highest diagnostic accuracy obtained from ROC analysis performed with 62 patients without bone metastases and 28 patients with bone metastases or
[b]the cutoff level of 95 percentile of controls.
[c]Specificity calculated at the cutoff level of 95% sensitivity.

The possibility of increasing the diagnostic accuracy in the detection of bone metastases was examined by means of combination of markers. For this purpose the binary logistic regression approach was applied. To identify the significant predictors of bone metastasis in PCa patients, both univariate and multivariate logistic regression analyses were performed (Table 7).

Table 7. Logistic regression analysis of OPN and bone markers in relation to bone metastasis in PCa[1]

I. Univariate analysis

Variable	RR (95% CI)	P-value
OPN	1.002 (1.001-1.003)	<0.0001
ICTP	1.325 (1.092-1.607)	0.004
bALP	1.156 (1.075-1.243)	<0.0001
PINP	1.038 (1.019-1.057)	<0.0001

II. Multivariate analysis

	Inclusion selection		Stepwise selection	
Variable	RR (95% CI)	P-value	RR (95% CI)	P-value
OPN	1.001 (1.00-1.003)	0.039	1.001 (1.00-1.003)	0.011
ICTP	1.135 (0.92-1.393)	0.227	--	--
bALP	1.191 (1.03-1.378)	0.018	1.124 (1.05-1.207)	0.001
PINP	0.979 (0.94-1.020)	0.315	--	--

[1]Calculated with PCa patients with bone (n=28) and without (n=62) bone metastases.

Univariate regression model determined all four analytes OPN, bALP, PINP, and ICTP as significant factors. Significant variables were further analyzed in multivariate regression model to identify independent predictors of bone metastasis. OPN and bALP were the only independent predictors of bone metastasis in PCa patients. These results were also confirmed by multivariate analyses with stepwise selection where PINP and ICTP as insignificant variables were eliminated from the model while OPN and bALP remained in the model. The final regression equation was:

logit(p)= -4.581+0.001*(OPN)+0.117*(bALP) where ***p*** was defined as the probability of the occurrence of metastasis. The Wald statistics showed values of 6.507 and 10.653 for OPN and bALP, respectively, with corresponding significant P values of 0.011 and 0.001. The overall model fit was characterized by the Nagelkerke value of R^2 = 0.621 demonstrating a good predictive efficacy. An overall correct classification of 86% was obtained. The values obtained from the regression equation with these two analytes were used to construct a corresponding ROC curve. This two-marker

combination resulted in an increased AUC up to 0.93 compared to that of OPN (AUC, 0.85; P=0.026) or bALP (AUC, 0.84; P=0.008) alone as shown in Figure 10. Overoptimism of the model referring to overfitting was estimated by a validation procedure using bootstrapping with 1000 cycles [117,118]. Overoptimism for AUC was estimated to be only 0.007 leading to an AUC of the model of 0.924.

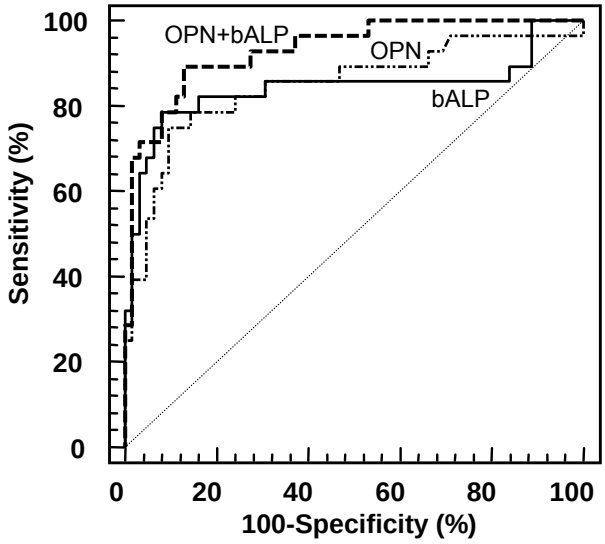


Figure 10. ROC curves for OPN, bALP and their combination to distinguish between PCa patients with and without bone metastases. AUC values ± standard errors were as follows: OPN, 0.85 ± 0.05; bALP, 0.84 ± 0.05; combination of OPN and bALP, 0.93 ± 0.03.

The AUCs of the OPN+bALP combination and ICTP were not significantly different (P=0.235). At the cutoff level of 95% sensitivity, the specificity of OPN and bALP as marker combination amounted to 63%, which was higher than that for OPN (31%) or for bALP (11%) alone, and achieved equal specificity as ICTP (Table 6).

3.1.4 Osteopontin and bone markers as predictors of survival outcome

Complete follow-up data were obtained in all 90 PCa patients, making each case eligible for survival analysis. Mean follow-up time was 39.2±18.2 months (range 2.7-88.4). The primary end point of this analysis was cancer-related survival, as measured from the date of surgery or visit to the last follow-up or cancer-related death. According to the death certificates and the information of general practitioners, 13 patients died from PCa. To determine whether the concentrations of OPN and markers correlated

with disease outcome, patients were stratified into 2 groups using the cutoff points of 95 percentiles of controls. To identify the significant prognostic factors associated with PCa-specific death, univariate and multivariate risk factor analyses were performed using the Cox proportional hazards regression model (Table 8).

Table 8. Univariate and multivariate Cox regression analyses of OPN, bone markers, and clinico-pathological factors in relation to PCa survival[1]

I. Univariate analysis

Variable	Dichotomous criteria[2]	RR (95% CI)	P-value
OPN	1099 µg/L	13.8 (3.04-62.9)	0.001
ICTP	3.9 µg/L	7.48 (2.06-27.2)	0.002
PINP	72.5 µg/L	11.9 (3.62-39.2)	<0.0001
bALP	20.5 ng/L	9.26 (2.83-30.3)	<0.0001
PSA	10 ng/mL	3.37 (0.74-15.4)	0.117
Age	60 years	1.36 (0.37-4.95)	0.639
Tumor stage	T1-2/T3-4	2.07 (0.55-7.82)	0.285
Tumor grade	G1-2/G3	2.39 (0.70-8.21)	0.167
Bone metastases	absence/presence	11.3 (3.03-42.3)	<0.0001

II. Multivariate analysis

		Inclusion selection		Stepwise selection	
Variable	Dichotomous criteria[2]	RR (95% CI)	P-value	RR (95% CI)	P-value
OPN	1099 µg/L	5.02 (0.76-33.4)	0.095	6.50 (1.17-36.2)	0.033
ICTP	3.9 µg/L	1.84 (0.44-7.79)	0.406	--	--
PINP	72.5 µg/L	1.82 (0.35-9.45)	0.477	4.48 (1.17-17.2)	0.029
bALP	20.5 ng/L	2.54 (0.42-15.3)	0.311	--	--
PSA	10 ng/mL	--	--		
Age	60 years	--	--		
Tumor stage	T1-2/T3-4	--	--		
Tumor grade	G1-2/G3	--	--		
Bone metastases	absence/presence	1.24 (0.18-8, 8)	0.878	--	--

[1]All 90 PCa patients were available for analysis of independent prognostic significance.
[2]Dichotomous criteria for each marker represents 95 percentile of the corresponding control group as also shown in Figure 9.

Univariate Cox regression analysis revealed that the markers OPN, ICTP, PINP, and bALP as well as the presence of metastases were potential prognostic factors for survival in PCa patients with P <0.05 (Table 8). In Kaplan-Meier analysis, patients with concentrations of the above-mentioned 4 markers higher than the cutoffs had significantly shorter overall survival time than patients with low concentrations (Figure 11). These significant variables were further evaluated in multivariate analysis with both inclusion and stepwise selection procedures. OPN and PINP were the only independent

negative predictors of survival in PCa after adjusting for the other factors significant in univariate analysis. In the stepwise elimination procedure (backward and forward), both OPN and PINP remained as significant prognostic factors in the model while other variables with less impact on survival were eliminated from the model (Table 8)

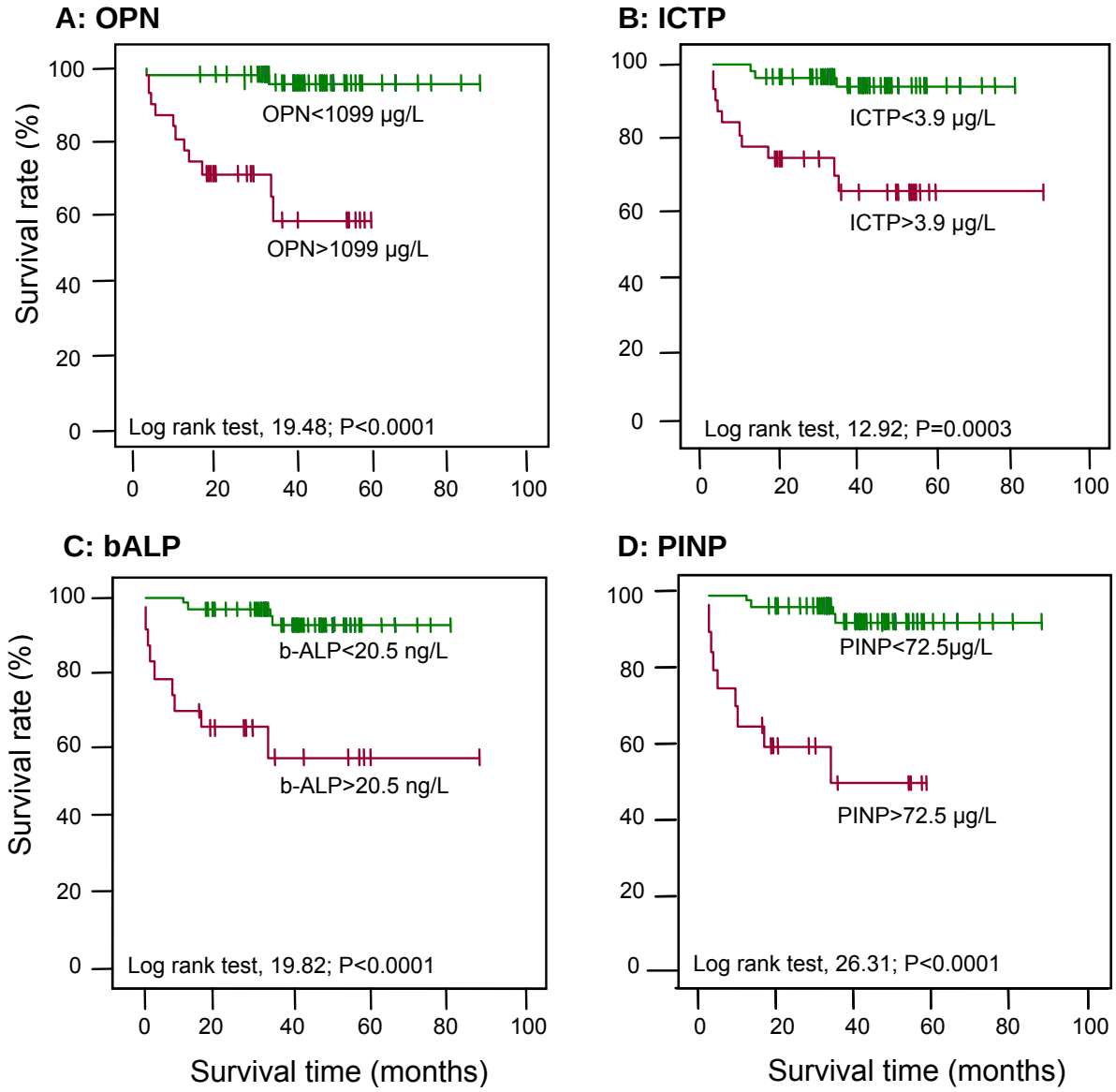


Figure 11. Cumulative cancer-related survival in PCa patients with OPN and bone marker concentrations below and above the cutoff points of 95 percentiles of controls. Cutoff points were taken from the data in Figure 9. Survival distributions were calculated using the Kaplan-Meier method and compared by the log rank test. All 90 PCa patients were included for survival analysis.

3.2 Renal cell carcinoma

3.2.1 Levels of osteopontin, bone markers, and enzymes

Levels of OPN, bone markers, and enzymes were compared in controls between groups with healthy men and women. Calculations showed that there were no differences in concentrations of biochemical markers between those two groups (P = 0.2255 - 0.8683, Mann-Whitney U test) and, therefore, the gender-dependent variation of the markers in this study is most probably excluded. Based on these results, further evaluations of biochemical markers were performed with the cutoff points set at 90 percentile of the control groups. Figure 12 shows the scatter plots and medians of OPN, bone markers, and enzymes in controls and RCC patients subdivided into the groups N0M0, N1M0, M1b and M1nb. Statistical assessment of the data can be summarized as follows:

(i) OPN and ICTP levels had no significant differences between controls and the N0 group.

(ii) Concentrations of OPN and ICTP in RCC groups with regional lymph node (N1M0) and distant bone and non-bone metastases (M1b, M1nb) were significantly higher than those in controls.

(iii) Compared to the RCC group without metastases (N0), OPN values were significantly elevated in both M1b and M1nb groups, whereas ICTP values were elevated only in M1nb.

(iv) Levels of the markers were not different between groups with distant bone (M1b) and non-bone (M1nb) metastases (P = 0.1384 - 0.9151, Mann-Whitney U test).

(v) bALP and ALAT did not vary among controls and all RCC groups.

(vi) GGT was elevated in the M1nb group in comparison to controls.

In relation to the 90 percentile cutoffs 73% of patients in the groups with distant metastases (M1nb and M1b) had increased OPN concentrations compared to 69%, 33%, 25%, and 8% of patients with increased values of ICTP, GGT, bALP, and ALAT, respectively.

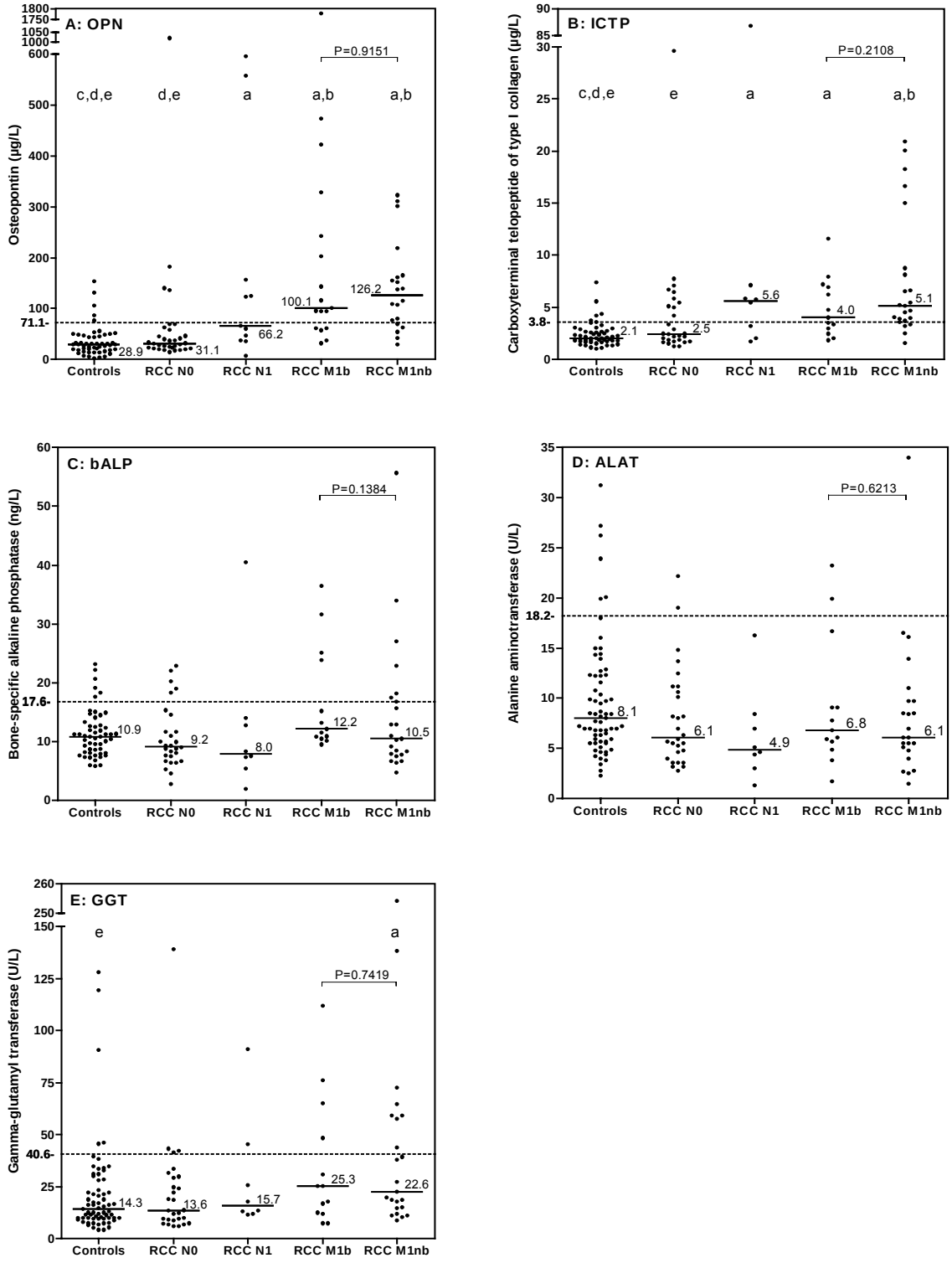


Figure 12. Scatter plots of OPN (A), bone markers (B-C) and enzymes (D-E) in controls and RCC patients: without metastases (N0), with regional lymph node metastases (N1), and with distant bone (M1b) and non-bone (M1nb) metastases. Median values of the groups are shown as horizontal lines with corresponding figures. The dotted line on each graph represents the 90 percentile of controls. Significant differences (P<0.05 at least) are shown by the following symbols; **a**, compared to controls; **b**, compared to N0 group; **c**, compared to N1 group; **d**, compared to M1b group; **e**, compared to M1nb group. Levels of the markers between RCC groups with bone (M1b) and non-bone (M1nb) metastases were compared by Mann-Whitney U test.

3.2.2 Correlation between osteopontin, bone markers, enzymes, and clinico-pathological data

Spearman's rank correlation analyses were performed with all RCC patients. As shown in Table 9, a significant correlation was observed between OPN and each bone marker. As for enzymes, GGT correlated strongly with OPN, whereas ALAT showed no association with OPN. In relation to tumor stage or grade of RCC patients, levels of OPN associated more strongly with tumor stage than with tumor grade. Concentrations of ICTP correlated with both stage and grade more closely than concentrations of bALP. Association with stage was also observed in levels of GGT.

Table 9. Correlation between biochemical markers and clinico-pathological data

	ICTP	bALP	ALAT	GGT	T-Stage	Grading
OPN	0.50**	0.37**	0.02	0.43**	0.50**	0.33*
ICTP	1.00	0.09	-0.28*	0.01	0.38**	0.37**
bALP		1.00	0.29*	0.48**	0.26*	0.30*
ALAT			1.00	0.45**	0.07	-0.08
GGT				1.00	0.29*	0.18

Significances: *, P <0.05; **, P <0.01.

Biochemical markers that showed correlation with tumor stage or grade were subsequently analyzed with regard to their concentration range and median in different tumor stages and grades (Table 10).

Table 10. Concentrations of OPN, bone markers, and GGT in different tumor stages and grades of RCC patients

	OPN	ICTP	bALP	GGT
I. Stage of RCC				
I	31 (14-557)[bc]	2.2 (1.2-86.8)[c]	8.8 (1.9-40.5)	13 (6-112)
II	82 (23-473)[a]	4.3 (1.8-16.6)	12.5 (5.3-31.7)	17 (6-48)
III+IV	116 (20-1778)[a]	5.1 (1.4-29.6)[a]	11.0 (2.8-55.7)	25 (7-254)
P value[1]	0.0002	0.003	0.0501	0.065
II. Grade of RCC				
G1-2	46 (14-473)	3.3 (1.2-29.6)	9.0 (2.0-27.1)	
G3	116 (32-1778)	5.8 (1.8-86.8)	15.4 (4.7-55.7)	
P value[2]	0.0377	0.0089	0.0010	

[1]Calculated with the Kruskal-Wallis overall test. Concentrations in different tumor stages were compared in pairs and significant difference (P<0.05 at least) shown as: **a**, compared to Stage I; **b**, compared to Stage II; or **c**, compared to Stages III+IV (Kruskal-Wallis with Dunn's post test).

[2]Calculated with the Mann-Whitney U test.

Note: values are medians with ranges in parentheses.

Plasma concentrations of OPN increased with progression of the malignancy. As shown in Table 10, OPN levels were significantly lower in RCC patients with stage I than in RCC patients with stage II or stages III+IV. Compared to OPN, a less significant difference was observed in ICTP levels in various tumor stages whereas concentrations of bALP and GGT did not differ in that manner. In addition, levels of OPN were higher in RCC patients with Grade 3 than in those with Grade 1 or 2. Compared to OPN, concentrations of ICTP and bALP differ more significantly in those two tumor grade groups.

OPN concentrations were examined with regard to the number of organ sites affected by metastases. The difference in median levels of plasma OPN in RCC patients with metastatic lesions in one, two, and three or more organs were not statistically significant (Table 11).

Table 11. Plasma OPN and number of organs affected by metastases in RCC patients

Number of organ sites affected by metastases	Number of cases	Median with range (µg/L)
one	12	116 (41.8-301)
two	13	152 (28.9-1778)
three and more	12	101 (31.5-473)
P=0.250 (Kruskal-Wallis overall test).		

In addition, plasma OPN levels were evaluated in relation to the histological types of RCC. Histological data were available in 70 cases. Plasma OPN compared in different histological types of RCC were not significantly different as shown in Table 12.

Table 12. Plasma OPN and histological types of RCC patients

Histological types	Number of cases	Median with range (µg/L)
Clear cell	55	61 (6.3-1778)
Papillary	8	51 (21.2-1019)
Chromophobe	2	35 (31.6-37.5)
Unclassified	5	69 (26.3-595.7)
P=0.4913 (Kruskal-Wallis overall test).		

3.2.3 Osteopontin and bone markers as diagnostic indicators of metastases

Since only OPN and ICTP levels showed statistically significant differences among RCC groups (Figure 12), their diagnostic accuracies to differentiate RCC patients without metastases from those with distant bone and non-bone metastases were evaluated subsequently. The area under the OPN curve was significantly larger by 0.181 in comparison with that of ICTP (Figure 13; P=0.018) and proved the superior diagnostic accuracy of OPN for the detection of distant metastases. At the cutoff for 95% sensitivity (Figure 13), the specificity of OPN amounted to 57.1% (95% CI, 37.2-75.5) and significantly outperformed (McNemar test, P=0.0309) that of ICTP with 25% (95% CI, 10.7-44.9). The points with the highest diagnostic accuracy were at the OPN concentration of 44.6 µg/L with 87.5% sensitivity and 78.6% specificity.

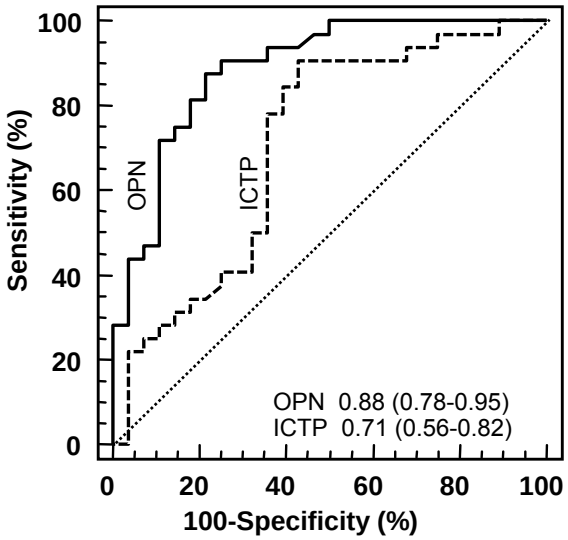


Figure 13. ROC curve to distinguish between RCC groups without metastases (N0) and with distant metastases (M1nb and M1b), 28 and 32 patients in each group, respectively. AUC with 95% CI in parentheses are shown in the lower right corner.

Further ROC analysis was performed in the same fashion as described above with the exception that each group with distant bone (M1b) or non-bone (M1nb) metastases was analyzed separately. This was aimed at evaluating whether OPN had different diagnostic performance in distinguishing RCC patients with distant bone (M1b) or non-bone (M1nb) metastases from those with organ-confined disease (N0). However, ROC analysis showed that AUCs of OPN in relation to distant bone or non-bone

metastases were almost equal and amounted to 0.86 (0.73-0.94) and 0.87 (0.75-0.95), respectively. ICTP was also evaluated using the same approach. In this respect, AUCs of ICTP in the detection of distant bone or non-bone metastases amounted to 0.69 (0.52-0.82) and 0.75 (0.61-0.86) and there was no difference between ROC curves (P=0.112).

OPN, bone markers, and enzymes were analyzed using univariate and multivariate logistic regression models in order to assess their ability to predict distant metastasis in RCC patients (Table 13). The univariate regression model determined OPN and bALP as significant factors related to the risk of distant metastasis whereas the other variables were less significant. All variables were further included in multivariate analyses with inclusion and stepwise selections in order to determine independent predictors of distant metastasis. OPN was proven in the multivariate model by both selections to be the only independent variable related to the risk of distant metastasis.

Table 13. Logistic regression analysis of OPN, bone markers, and enzymes in relation to distant metastasis in RCC[1]

I. Univariate analysis

Variable	RR (95% CI)	P-value
OPN	1.02 (1.01-1.04)	0.004
ICTP	1.12 (0.99-1.28)	0.098
bALP	1.09 (1.00-1.18)	0.047
ALAT	1.02 (0.94-1.12)	0.606
GGT	1.02 (1.00-1.04)	0.091

II. Multivariate analysis

	Inclusion selection		Stepwise selection	
Variable	RR (95% CI)	P-value	RR (95% CI)	P-value
OPN	1.03 (1.00-1.05)	0.019	1.02 (1.01-1.04)	0.004
ICTP	0.92 (0.79-1.09)	0.346	--	--
bALP	1.01 (0.90-1.15)	0.820	--	--
ALAT	1.05 (0.91-1.21)	0.524	--	--
GGT	1.01 (0.98-1.04)	0.588	--	--

[1]Calculated for RCC patients with distant metastases (M1b and M1nb groups) and without metastases (N0 group).

As described earlier in Chapter 3.1.3, in PCa patients, using the logistic regression approach, the combination of two significant variables was considered in order to increase diagnostic accuracy in the detection of distant metastases. In RCC patients all other variables except OPN were insignificant in the multivariate logistic

regression model (Table 13), therefore, the possibility of increasing diagnostic accuracy using the same approach was not feasible.

In order to investigate the diagnostic ability of OPN and the bone markers to group RCC patients according to different tumor stages and grades, biochemical markers were further examined with the ROC analysis. Based on the results from Table 10 the diagnostic accuracy of OPN, ICTP, and bALP to differentiate between tumor stages I and II-IV or between tumor grades G1-2 and G3 was further evaluated as shown on Figure 14 and Table 14.

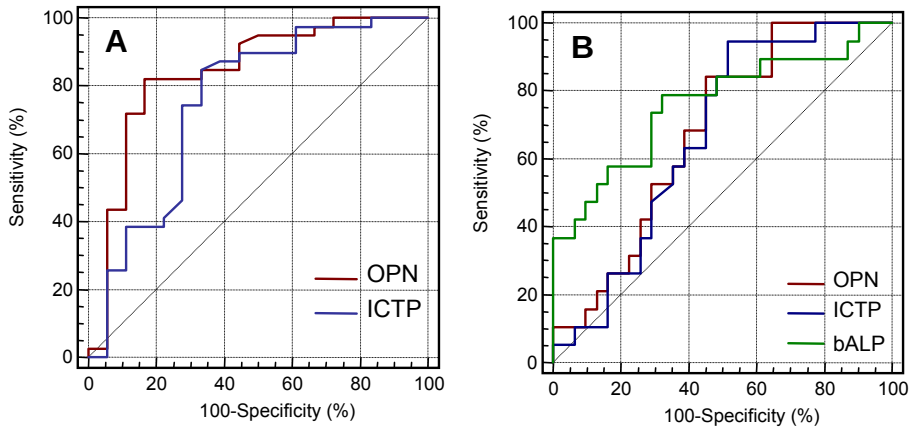


Figure 14 ROC curves of OPN, ICTP, and bALP to differentiate RCC patients with different tumor stages and grades.
A. Tumor stages: I versus II-IV.
B. Tumor grades: G1-2 versus G3.

In distinguishing RCC patients with tumor stage I from those with tumor stages II-IV, OPN with AUC of 0.84 was more accurate than ICTP with that of 0.75 (Figure 14 A, Table 14 A). Difference between AUCs of both markers was not significant (P=0.189). However, when both markers were compared at the 80% specificity, the sensitivity of OPN amounted to 82 % (67-92) and was 40% higher than that of ICTP (P=0.0044, McNemar test). However, above the sensitivity of 80% there was no difference in diagnostic specificity between OPN and ICTP (Figure 14 A). The diagnostic performance of biochemical markers in differentiating RCC patients with tumor grades G1-2 and G3 was less effective (Figure 14 B, Table 14 B). The AUCs of OPN, ICTP, and bALP ranged from 0.67 to 0.76 and there was no difference between the AUCs of the markers (P=0.335-0.901).

Table 14. Diagnostic sensitivity, specificity and AUC of OPN and bone markers to distinguish different tumor stages and grades in RCC patients

A. Tumor stages: I versus II-IV

Variable	Sensitivity (%)	Specificity (%)	AUC
OPN			
46.5 µg/L	82 (67-92)	83 (57-96)	0.84 (0.78-0.92)
ICTP			
2.5 µg/L	86 (74-94.3)	67 (43-85)	0.75 (0.62-0.86)

B. Tumor grades: G1-2 versus G3

Variable	Sensitivity (%)	Specificity (%)	AUC
OPN			
46.5 µg/L	84 (60-96)	55 (36-73)	0.68 (0.53-0.80)
ICTP			
2.5 µg/L	95 (76-99)	44 (29-60)	0.67 (0.52-0.79)
bALP			
10.0 ng/L	81 (58-94)	63 (47-77)	0.76 (0.62-0.87)

Sensitivity, specificity, and AUC with 95% confidence intervals in parentheses of the various markers were obtained from ROC analysis. Concentration of each marker corresponds to the point with the highest diagnostic accuracy on a ROC curve.

3.2.4 Osteopontin and bone markers as predictors of survival outcome

The mean and median follow-up time was 47.9 ± 28.9 months and 47.9 months, respectively (range = 1.0 – 91.3 months). The primary end point of the analyses was cancer-related survival as measured from the date of surgery or presentation in our institution to the time of the last follow-up or cancer-related death. According to the death certificates and to information provided by the general practitioners, 20 patients died from RCC. To determine whether variables correlated to the disease outcome, patients were stratified into two groups by means of the cutoff points using the 90 percentiles of the controls. To identify the significant prognostic factors associated with RCC-specific death, univariate and multivariate risk factor analyses were performed using the Cox regression model with the stratified groups (Table 15).

Table 15. Univariate and multivariate Cox regression analyses of biochemical markers and clinico-pathological factors in relation to RCC survival[1]

I. Univariate analysis

Variable	Dichotomous criteria[2]	RR (95% CI)	P value
Age	60 years	0.80 (0.39-1.67)	0.568
OPN	71.1 µg/L	3.55 (1.51-8.35)	0.004
ICTP	3.8 µg/L	2.74 (1.26-5.96)	0.011
bALP	17.6 ng/L	1.58 (0.71-3.54)	0.266
ALAT	18.2 U/L	0.04 (0.00-15.0)	0.293
GGT	40.6 U/L	1.61 (0.76-3.42)	0.217
Tumor stage	T1-2/T3-4	2.59 (1.19-5.64)	0.016
Tumor grade	G1-2/G3	2.34 (1.08-5.06)	0.031
Metastases[3]	absence /presence	5.77 (2.47-13.5)	0.0001

II. Multivariate analysis

		Inclusion selection		Stepwise selection	
Variable	Dichotomous criteria[2]	RR (95% CI)	P value	RR (95% CI)	P value
Age	60 years	-	-		
OPN	71.1 µg/L	2.08 (0.67-6.45)	0.206	2.92 (1.04-8.16)	0.041
ICTP	3.8 µg/L	1.90 (0.61-5.97)	0.271	-	-
bALP	17.6 ng/L	-	-		
ALAT	18.2 U/L	-	-		
GGT	40.6 U/L	-	-		
Tumor stage	T1-2/T3-4	1.64 (0.52-5.21)	0.401	-	-
Tumor grade	G1-2/G3	0.78 (0.29-2.13)	0.631	-	-
Metastases[3]	absence /presence	2.83 (0.92-8.71)	0.071	3.18 (1.13-8.93)	0.028

[1]The Cox proportional hazards regression model was calculated with all 80 RCC patients.
[2]Dichotomous criteria for each biochemical marker represents 90 percentile of the corresponding control group as also shown in Figure 12.
[3]Included all metastatic patients: with the regional (n=11) and distant cases (n=37).

The levels of OPN and ICTP, as well as the tumor stage, grade, and the presence of distant metastases were found to be significant univariate prognostic factors of death from RCC. Patients with levels of OPN and ICTP above the cutoff point had significantly shorter survival time than patients with the levels of those markers under the cutoff points (Figure 15). The multivariate Cox regression analysis of these univariate significant predictors showed that none of them was an independent predictor of cancer-related death in that model (Table 15). However, the result of the forward or backward stepwise calculation to set up a reduced model was that only OPN and the presence of distant metastases retained statistical significance in the model. Thus, OPN was shown to be an independent survival indicator.

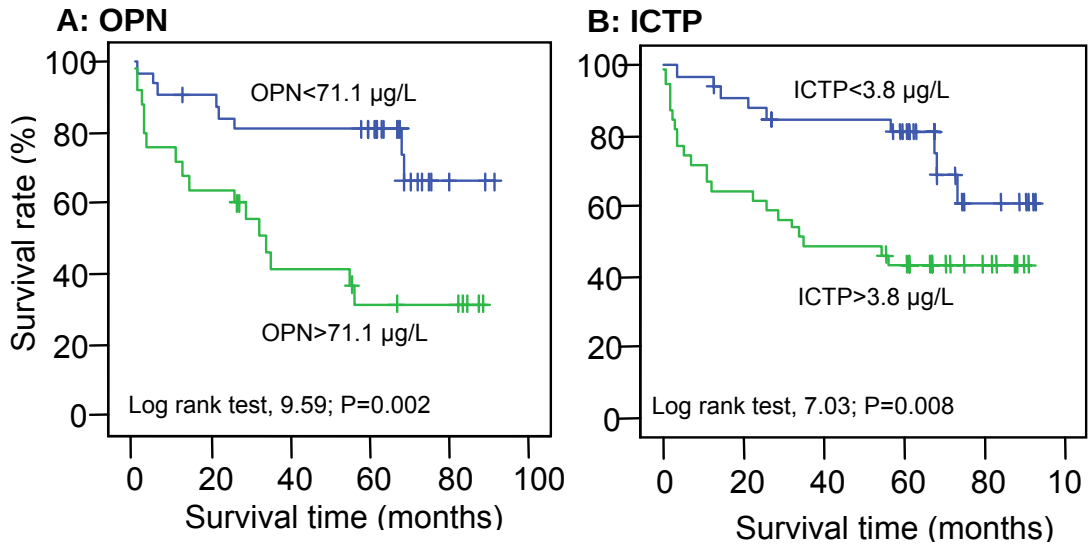


Figure 15. Cumulative cancer-related survival in RCC patients calculated with the Kaplan-Meier method and compared by the log rank test.

4 Discussion

This chapter discusses the diagnostic and prognostic significance of OPN and the bone markers for PCa patients followed by that for RCC patients. A brief conclusion on the clinical usefulness of OPN in both tumor entities will close the chapter.

4.1 Prostate cancer

PCa has a distinct predilection to metastasize to bone. The mechanisms underlying the preferential homing to the bone are unclear. Several factors have been proposed to determine the mechanisms responsible for the involvement of bone in metastatic PCa. For example, experimental data suggest that osteoblast-derived growth factors appear to stimulate the proliferation of PCa cells [119]. Thus, bone provides a favorable environment for the potential growth of PCa cells. Another study emphasizes the importance of bone marrow endothelium to which PCa cells preferentially adhere [120]. Additional data suggest that PCa cells acquire osteomimetic properties and behavior becoming more osteoblast-like in order to metastasize, survive, and thrive in the bone environment. Indeed, PCa cells were shown to synthesize and secrete bone-related proteins [121]. On the other hand, specific cell adhesion molecules such as OPN are also implicated in this multi-factorial process of preferential metastasis of PCa to bone. In fact, involvement of OPN in bone metastasis was demonstrated in vivo, where a 50% decrease in bone metastasis was observed in OPN-deficient mice compared to wild types [51]. Interaction of $\alpha v\beta 3$ integrin and RGD-binding sequence of OPN appears to play a crucial role in PCa metastasis to bone sites [122]. In vivo and in vitro OPN was demonstrated to stimulate the proliferation and induce the invasive ability of PCa cells possibly by interaction with $\alpha v\beta 3$ [97,103]. Most interestingly, PCa cells obtained from bone metastatic lesions were shown to express αv and $\beta 3$ integrin subunits [114]. Based on these observations it may be concluded that OPN, first, as the most abundant non-collagenous protein in bone and, second, as a ligand for $\alpha v\beta 3$ integrin, may mediate preferential adhesion, migration, and growth of PCa in bone.

4.1.1 Levels of osteopontin and bone markers

Significant elevation of OPN levels was found in PCa patients with bone metastases while OPN concentrations in patients without metastases were not different from controls (Figure 9). This distinct increase of plasma OPN in PCa patients with bone metastases clearly indicates its association with metastatic spread to bone. Moreover, compared to the bone markers OPN levels in patients with BPH were higher than in controls (Figure 9). In fact, BPH tissue specimens were also found to be positive for OPN [123]. These findings could be explained by the fact that BPH often has an inflammatory component, which is accompanied by additional OPN synthesis in immune cells [4,97].

The findings of unchanged OPN values in patients with localized cancer restrict the diagnostic capability of OPN to its application as a metastatic marker in PCa patients and classify OPN as an unsuitable marker for the early detection of PCa. That conclusion is in certain contrast to the suggestion of Fedarko et al. [53] who described OPN as a highly sensitive and specific marker with an AUC of 0.91 in discriminating PCa patients from controls. These authors determined total OPN using a protocol by which the complex of complement factor H and OPN was disrupted before OPN was measured. However, in that study the PCa patients were not characterized with regard to tumor stage and grade while most of the patients obviously had PSA >20 ng/ml. Moreover, these PSA concentrations are not typical for organ-confined PCa. Therefore the study population of Fedarko et al. [53] was presumably inappropriate to answer the question whether the assay of OPN, despite the use of the other method, is informative in the gray zone of PSA with concentrations between 2 und 10 ng/ml. In addition, the current data correspond to results of Hotte et al. [54] who showed OPN levels in hormone-refractory PCa patients with metastases. Since these authors did not present detailed data of patients with localized PCa, BPH, and healthy controls, the present data are the first results to allow a clear conclusion concerning the application of OPN as metastatic marker but not as detection marker in PCa patients.

4.1.2 Correlation between osteopontin, bone markers, and clinico-pathological data

Plasma OPN in PCa patients correlated with tumor grade (Table 5). PCa patients with G3 tumor grade tended to have higher concentrations of OPN in plasma compared to their counterparts with G1-2 tumor grades. Median concentrations in those two groups were 994 and 838 µg/L, respectively. Although the difference between these two tumor grade groups was marginal with P value of 0.044 these results suggest that plasma OPN may reflect tumor progression. Elevation of OPN levels in higher tumor grades was also reported in patients with hepatic malignancy [60]. Plasma OPN strongly correlates with bone markers such as PINP, bALP, and ICTP (Table 5). In several studies bone markers are reported as indicators of tumor extension. In fact, PINP, bALP, and ICTP correlate with the number of metastatic lesions on bone scans known as Soloway grades [37-39]. This fact suggests that plasma OPN is associated with skeletal extension of metastatic PCa.

4.1.3 Diagnostic performance of osteopontin and bone markers

The diagnostic accuracy of OPN in the detection of bone metastases was comparable with that of the bone markers (Table 6). AUC value of OPN was higher than that of PINP and bALP. OPN had the highest percentage of values (79%) above its cutoff level in the group with bone metastases compared to that of ICTP, PINP, and bALP. In other malignancies such as metastatic breast cancer, this figure is 69% [56]. As bone metastases of prostate cancer are predominantly osteoblastic [8], a bone formation marker is supposed to be a good indicator of bone involvement. However, in this study ICTP, a bone resorption marker, had the largest AUC value and highest specificity at 95% sensitivity compared to the typical bone formation markers PINP and bALP (Table 6). This might be explained by the fact that bone lesions in PCa are not purely osteoblastic. Biochemical [124] and histological [125] evidence suggests that despite the osteoblastic nature of most PCa bone metastases, increased bone resorption occurs as well. Since in logistic regression analyses OPN and bALP were the only significant independent markers in relation to bone metastasis (Table 7), a combination of these two analytes was examined to enhance diagnostic accuracy in detection of bone metastases. OPN and bALP, in combination, increased AUC value up

to 0.93 compared with OPN (AUC, 0.85) and bALP (AUC, 0.84) curves (Table 6, Figure 10). Therefore, combined measurement of OPN and bALP could provide a better diagnosis for bone metastases. Other authors have also considered the possibility of increasing the diagnostic accuracy via a combination of markers. For example, Withold et al. [126] obtained AUC of 0.84 for bALP and 0.76 for pyridinium cross-links (PYR) with respect to the detection of bone metastases. When these both markers were evaluated as a combination it resulted in an increase of AUC up to 0.89.

4.1.4 Prognostic significance of osteopontin and bone markers

Prognostic significance of OPN was evaluated in comparison to the bone markers. The association of survival was demonstrated in PCa patients with ALP, PINP, or ICTP corresponding to the extent of bone metastasis monitored by bone scans [12,124,127-129]. PSA was shown not to be directly associated with bone progression in several studies [127,129] and also in the current work (Table 8). In the present study, OPN and all bone markers were confirmed as significant predictors of death from PCa verified by the univariate Cox regression calculation (Table 8) and demonstrated by the survival curves of Kaplan-Meier analysis (Figure 11). Patients with marker levels below the cutoff of 95 percentile of the control group tended to have longer survival times compared to those with higher values of these markers. In the multivariate Cox regression analyses, OPN remained a significant independent prognostic factor of survival probability in PCa (Table 8). These data are in agreement with the findings obtained by Hotte et al. [54] that increased plasma OPN levels were associated with bone metastases and decreased survival in patients with PCa. Correlation of plasma OPN with decreased survival was also observed in malignancies including breast, esophagus, head, and neck cancers [56,69,70]. In addition, a recent study on OPN protein expression in PCa tissue showed that an increased level of OPN expression was significantly associated with reduced survival time of the patients [48]. These data, therefore, support the results of the current study, and those obtained by Hotte et al. [54].

4.1.5 Limitations of the study

Some limitations of this study should be recognized. First, the limited number of patients could be seen as the main limitation of the present study. In contrast to this limitation, it is remarkable that even with the low statistical power due to the sample size, significant results of OPN were obtained. Thus, the risk of type II error, a problem associated with small studies, does not exist in the current study. In addition, the possibility of an overfitting bias for the calculated models due to the small sample size could be excluded as far as possible by additional bootstrapping calculations as shown in the results [117,118]. There was only a low proportion of overoptimism for the AUC calculated with the final model. Second, the present study is limited by its retrospective nature with blood sampling at different times. However, all measurements were performed in a blinded manner. Third, the groups with positive lymph nodes and distant metastases included patients with and without hormonal treatment. It was shown that hormonal therapy influences bone turnover although the study data based on different treatment regimes and duration of treatments are not at all consistent concerning the various bone markers [37,39,130-133]. In the present study, the concentrations of OPN and bone markers did not significantly depend on the hormonal treatment status showing P values >0.2 so that an interfering effect of the hormonal treatment on the diagnostic and prognostic capability is most probably excluded. The duration of treatment, in addition to the limited number of patients, could be one reason that explains the finding of no differences between treated and untreated patients. The median treatment period of 2.1 months in the pN1M0 group before sample collection was probably too short to have any effect upon the bone marker concentrations. For example, changed concentrations of ICTP were only observed after an antiandrogen therapy of 12 weeks, while PINP did not change during such treatment [37,39]. The missing marker differences between the group of pN0M0 that consisted only of untreated patients and the group of pN1M0 additionally support this view.

4.2 Renal cell carcinoma

As described earlier in the Introduction, OPN contains an RGD sequence that binds to cell integrin receptors and is capable of promoting migration and proliferation of tumor cells [97,102,112]. Due to this property expression of OPN by tumor cells may play a role in tumor cell invasion and metastasis. Elevated expression of OPN found in metastatic lesions [103] and in cancer cells with high invasive properties [102] support the importance of OPN in tumor metastasis. Evidently, there are multiple mechanisms by which OPN could impact on the metastatic process. Through its adhesive properties OPN can induce changes in tumor cell gene expression including proteolytic enzymes, which in turn may lead to increased cell motility and invasion [102]. OPN also promotes angiogenesis, which is crucial for tumor growth and metastasis [93-95,108]. Consistent with these observations the current data, for the first time, show the association of plasma OPN with tumor progression and metastases in patients with RCC.

4.2.1 Levels of osteopontin, bone markers, and enzymes

OPN is one of the most abundant non-collagenous proteins in bone and plays an important role in bone remodeling [5,7]. In RCC, bone metastases are found in 30% of patients with metastases either alone or in combination with metastases in other locations [15-17]. In the current research design, the patients with distant metastases were classified according to those with bone and non-bone metastases, originally expecting that OPN could possibly be a marker for bone metastases in RCC as well. However, the results of the present study showed that neither plasma OPN nor the well-known bone resorption and formation markers such as ICTP and bALP were able to differentiate between non-bone and bone distant metastases in RCC. As shown in Figure 12, compared to controls and RCC patients without metastases (N0 group) plasma OPN levels were significantly elevated in RCC patients with distant metastases regardless of the presence or absence of bone metastases (M1nb and M1b groups). In addition, also GGT and ALAT were rather ineffective markers to discriminate between RCC patients with and without metastases (Figure 12). Similar results had been reported previously in a large study involving six bone markers. In that study bone

markers were not sensitive enough to differentiate between RCC patients with bone and non-bone metastases [134].

As shown in Figure 12, plasma OPN appears to reflect tumor extension towards metastasis in RCC, since the median value (31 µg/L) in a group with local tumor is two times lower than in a group with regional lymph node metastases (66 µg/L) with the highest values being in the two groups with distant metastases (100 and 126 µg/L). This fact of significant elevation of plasma OPN values in metastatic RCC patients suggests its possible clinical application as a non-invasive marker to diagnose metastasis in RCC patients. Recent data on OPN tissue expression support the present results. OPN expression in tissue samples of organ-confined RCC was significantly lower compared to those invading beyond kidney [98]. However, in regard to plasma OPN, it should be noted that various cell types including immune, endothelial, smooth muscle and nerve cells secrete OPN [72,135]. Therefore, besides malignancy other pathological conditions such as inflammation, neurological disorders or cardiovascular diseases may also contribute to the elevation of OPN level in plasma [3,136]. This fact should also be taken into consideration when evaluating the results. Interestingly, OPN derived from malignant cells has structural properties different from those of host immune cells apparently due to post-translational modification [72,135]. Obviously, OPN derived from host cells mediates migration, proliferation, and survival of immune cells whereas OPN molecules synthesized by tumor cells are involved in tumor progression and metastasis. In support of this view OPN intensity in malignant cells was associated with tumor aggressiveness and the survival time of cancer patients [137].

4.2.2 Correlation between osteopontin, bone markers, and clinico-pathological data

OPN concentrations correlated strongly with tumor stage (Table 9). Levels of OPN were significantly lower in RCC patients with tumor stage T1 compared to those with stage T2 or stages T3-4 with the medians 31, 82 and 116 µg/L, respectively (Table 10). Correlation of plasma OPN with tumor stage was earlier reported in lung, liver, and bladder cancers [57-59]. Moreover, plasma OPN in RCC patients was associated with tumor grade (Table 9). RCC patients with tumor grades I-II had lower concentrations of OPN in plasma compared to those with grade III with the medians 46 vs. 116 µg/L (Table 10). This close correlation with clinico-pathological data clearly indicates that

plasma OPN is associated with tumor progression in RCC patients. Recent data on OPN protein expression in RCC tissue samples are consistent with the current results. In those studies OPN expression correlated with tumor stage and was significantly higher in tumor stages III-IV compared to that of I-II stages [47,98]. Significant difference of plasma OPN levels in different tumor stages also allows to distinguish RCC patients with stage I tumor from those with the advanced stages. In this respect, plasma OPN was more accurate with AUC of 0.84 compared to 0.75 of ICTP (Figure 14 A, Table 14 A). On the other hand, when plasma OPN was examined in regard to the number of metastatic sites (one, two, and three or more), no differences in concentrations of OPN were observed between these groups (Table 11). In contrast to these results, plasma OPN in metastatic breast cancer patients appears to increase with the number of organs affected by metastasis reflecting the metastatic burden in these patients [56]. In the same study plasma OPN strongly (r_s=0.81) correlated with tALP. Similar to that, correlation analysis in the present study revealed a close correlation of plasma OPN with the bone markers ICTP and bALP (Table 9). Correlation of OPN with these bone markers, which are known to reflect the extent of disease, suggests that OPN could also be related to the tumor extension.

In relation to histological types clear cell RCC is the most frequent type with an incidence of 70% followed by the papillary and chromophobe types with 10% and 5% [21]. In accordance with these statistical data in the current study of 70 RCC patients with available histological data, 55 (79%) had clear cell type whereas 8 (11%) and 2 (3%) of RCC patients presented with papillary and chromophobe types. Characterization of histological types in RCC is known to provide prognostic information for these patients. Patients with clear cell RCC tend to have better prognosis compared to those with papillary or chromophobe RCC [21]. However, in the current study levels of plasma OPN showed no association with different histological types of RCC (Table 12).

4.2.3 Diagnostic performance of osteopontin and bone markers

As described previously in the current study and by other authors [37] ICTP is a reliable indicator of metastatic spread to bone in PCa patients. However, in RCC patients ICTP, a bone resorption marker, is ineffective in the diagnosis of bone

metastases (Figure 12) even though bone lesions in RCC are known to be osteolytic [18]. Since ICTP and OPN were elevated in both RCC groups with bone and non-bone distant metastases diagnostic performance of these markers was evaluated in relation to distant metastases. ROC analysis showed that OPN is more accurate compared to ICTP with the difference in AUC of 0.181 (P=0.018) (Figure 14).

Plasma OPN compared to other markers was confirmed in a multivariate logistic regression model as the only significant variable related to the probability of distant metastasis in RCC patients (Table 13). Therefore, a combination of two significant variables as described in Chapter 3.1.3. in order to increase diagnostic accuracy was not possible. The results of multivariate logistic regression analysis indicated that plasma OPN in comparison with the other variables had the best potential to identify RCC patients with distant metastases (Table 13).

4.2.4 Prognostic significance of osteopontin and bone markers

In addition to the use of OPN as diagnostic marker, plasma OPN was examined with regard to its prognostic significance. Several studies examining other cancer types proved increased OPN values to be a significant prognostic factor for the overall survival [54,55,60]. Since diverse ELISAs have been employing different techniques, antibodies, and calibrators, a comparison of the OPN cutoffs defined in these different studies as prognostic decision level is less reasonable. In the case of OPN, that technical aspect may be more important than the cutoff defined using either the arbitrary or data-derived methods [138]. In the present study univariate Cox regression analyses confirmed the well-known prognostic significance of tumor stage and grade as well as the presence of metastases and proved the current study groups as appropriate for further analyses (Table 15). Plasma OPN retained significance as a predictor of death from RCC verified by the univariate Cox regression calculation (P=0.004) and demonstrated by the survival curves of Kaplan-Meier analysis (Figure 15). Patients with plasma OPN values below the cutoff of 90 percentile of the control group tended to have longer survival times compared to those with higher OPN values. In a multivariate Cox regression model with stepwise forward or backward elimination procedure increased OPN was proven to be an independent prognostic factor of survival probability in RCC patients in addition to the risk factor of metastasis (Table 15). Thus,

increased OPN values were associated with an increase in risk of death of 2.92. In patients with metastatic breast cancer OPN was recently shown to be the variable with the highest prognostic value for poor survival with a relative risk of 3.26 [55]. In addition, prognostic significance of OPN was also confirmed on a tissue level. The intensity of OPN protein expression in RCC tissue samples inversely correlated with the survival of RCC patients [98].

4.3 Conclusion

Plasma OPN is an effective marker in the detection of bone metastases in PCa patients. Moreover, the combination of OPN with bALP significantly enhances diagnostic accuracy in relation to bone metastases. In RCC patients plasma OPN is useful in the diagnosis of distant bone and non-bone metastases and reflects tumor progression. In addition, evaluation of OPN in plasma has prognostic significance for both PCa and RCC patients.

5 Summary

Osteopontin (OPN) is a glycoprotein, which is present in all body fluids including plasma. Due to the presence of arginine-glycine-aspartic acid sequence (RGD) in its structure OPN is capable of binding to cell integrin receptors and promoting adhesion, proliferation, and survival in various cell types including tumor cells. Its involvement in tumor progression and metastasis has been indicated in a number of studies. For example, tumor cells with high invasive properties or obtained from metastatic lesions show elevated OPN expression and, moreover, OPN expression in tissue correlates with tumor stage and size as well as survival of cancer patients. All these findings suggest that elevation of OPN levels in blood could also reflect tumor progression towards metastasis and poor prognosis for cancer patients. In addition, OPN is abundantly distributed in bone tissue and involved in the regulation of bone turnover. This indicates that OPN in plasma could also be a sensitive indicator of skeletal metastasis, since the latter alters finely balanced processes of bone turnover. The PubMed literature review has shown that reports on plasma OPN in prostate cancer (PCa) are very limited whereas in renal cell carcinoma (RCC) no studies have been done so far. Therefore, the aim of this study was to evaluate the clinical usefulness of plasma OPN in patients suffering from PCa and RCC. Diagnostic and prognostic significance of plasma OPN was compared with the established bone formation markers: N-terminal propeptide of type I procollagen (PINP), bone-specific alkaline phosphatase (bALP) and the bone resorption marker: cross-linked carboxyterminal telopeptide of type I collagen (ICTP).

Prostate cancer. This study included 90 patients with PCa, 35 patients with benign prostatic hyperplasia (BPH) and 29 healthy men. Plasma OPN and bone markers were significantly elevated in PCa patients with bone metastases compared to those without bone metastases, BPH group, and controls ($P<0.05$ at least). OPN and bone markers were effective in the detection of bone metastases with AUC ranged from 0.80 to 0.88 (all $P<0.0001$). There were no significant differences between ROC curves of OPN and bone markers. However, at the cutoff level of 95% sensitivity, specificity of OPN outperformed that of bALP and PINP ($P=0.0266$ and 0.0009, McNemar test). Only OPN and bALP in the multivariate binary logistic model retained significant predictive value in relation to bone metastasis in PCa patients ($P=0.011$ and 0.001). Combination of these two markers using logistic regression approach in order to enhance the

diagnostic accuracy in the detection of bone metastases led to a distinct increase in AUC up to 0.93 compared to OPN (AUC, 0.85; P=0.026) and bALP (AUC, 0.88; P=0.008). At the cutoff with 95% sensitivity, the specificity of OPN and bALP in combination amounted to 63% and was greater than that for OPN (31%) and bALP (11%). OPN correlated closely with the bone markers (r_s=0.43-0.79, all P<0.05) and with tumor grade (r_s=0.23, P<0.05). OPN and all bone markers were associated with survival (Kaplan-Meier, P<0.0001). PCa patients with high concentration of biochemical markers had shorter survival time than those with lower concentrations of biochemical markers. OPN and PINP were identified in multivariate Cox regression model as independent predictors of survival outcome in PCa patients.

Renal cell carcinoma. This study included 80 patients with RCC and 52 controls. Compared to controls plasma OPN and ICTP were elevated in patients with distant bone and non-bone metastases (P<0.05 at least). Moreover, plasma OPN was also elevated in RCC patients with distant metastases compared to those with organ-confined disease (P<0.05 at least). OPN and ICTP were examined in ROC analysis in relation to distant metastases. ROC curve of OPN (AUC, 0.89) was larger than that of ICTP (AUC, 0.71, P=0.018). At the cutoff with 95 % sensitivity, the specificity of OPN (57%) outperformed (McNemar test, P=0.0309) that of ICTP (25%). OPN correlated closely with the bone markers (r_s=0.37-0.50, all P<0.05). Significant correlation was also observed between OPN and tumor stage (r_s=0.50, P<0.01) and grade (r_s=0.33, P<0.05). Levels of OPN and ICTP were associated with survival (Kaplan-Meier, P<0.0001). Patients with high concentrations of these two markers had shorter survival time than those with lower concentrations of OPN and ICTP. Logistic regression model determined OPN as a significant independent variable with predictive value related to distant metastasis in RCC patients (P=0.004). OPN was identified in Cox regression model as an independent factor related to the survival outcome in patients with RCC (P=0.041).

In conclusion, plasma OPN is an effective marker in the detection of bone metastases in PCa patients. Moreover, combination of OPN with bALP significantly enhances diagnostic accuracy in relation to bone metastases. In RCC patients plasma OPN is useful in the diagnosis of distant bone and non-bone metastases and reflects tumor progression. In addition, evaluation of OPN in plasma has prognostic significance for both PCa and RCC patients.

Zusammenfassung

Osteopontin (OPN) ist ein Glycoprotein, das in allen menschlichen Flüssigkeiten einschließlich Plasma vorkommt. Auf Grund der Arginin-Glycin-Asparaginsäure-Sequenz (RGD) in der Struktur des OPN-Proteins ist dieses fähig, sich an die Integrin-Rezeptoren der Zellen zu binden. Dadurch werden Adhäsion, Proliferation und das Überleben von verschiedenen Zellen, auch Tumorzellen positiv beeinflusst. Die Bedeutung des OPN-Proteins hinsichtlich Tumorprogression und Metastasierung wurde in zahlreichen Studien bewiesen. In invasiven Tumorzellen oder Tumorzellen aus Metastasen fanden sich erhöhte Mengen von OPN. Die OPN-Expression im Tumorgewebe korreliert mit Tumorstadium und Tumorgröße sowie mit der Überlebenszeit der Patienten. Alle diese Ergebnisse deuten darauf hin, dass ein Anstieg von OPN im Plasma die Tumorprogression zur Metastasierung und damit eine schlechte Prognose für den Patienten anzeigt. Durch das reichliche Vorkommen von OPN im Knochen und seiner Bedeutung für Regulierung beim Knochenumsatz, könnte ein erhöhter OPN-Wert im Plasma ein sensitiver Indikator der Knochenmetastasierung sein. Eine eigene PubMed-Literaturrecherche ergab nur wenige Publikationen über das Verhalten des Plasma-OPN bei Patienten mit einem Prostatakarzinom (PCa). Bei Patienten mit einem Nierenzellkarzinom (RCC) war dies bisher kein Gegenstand von Untersuchungen. Deshalb war das Ziel der Studie, die klinische Aussagekraft von Plasma-OPN bei PCa- und RCC-Patienten zu ermitteln. Die diagnostische und prognostische Bedeutung von Plasma-OPN wurde mit Markern des Knochenaufbaus, dem N-terminalen Propeptid vom Typ I Prokollagen (PINP) und der knochenspezifischen alkalischen Phosphatase (bALP) sowie mit dem Knochenabbaumarker, dem quervernetzten, karboxyterminalen Telopeptid vom Typ I Prokollagen (ICTP), verglichen.

Prostatakarzinom. Diese Studie umfasste 90 PCa-Patienten, 35 Patienten mit benigner Prostatahyperplasie (BPH) und 29 gesunde Männer. OPN und die Knochenmarker waren im Plasma von Patienten mit Knochen-Metastasen im Vergleich zu denen ohne Knochen-Metastasen, zu BPH-Patienten und Gesunden wesentlich erhöht ($P<0.05$ mindestens). Knochenmetastasen wurden bei den Patienten durch die Knochenszintigraphie sowie weitere Untersuchungen gesichert. OPN und Knochenmarker wiesen in der receiver operation characteristic-(ROC)-Analyse eine gute Diskrimination zwischen Patienten mit und ohne Knochenmetastasen auf. Die Flächen unter den ROC-Kurven (AUC) lagen zwischen 0.80 bis 0.88 (alle P-Werte <0.0001). Es gab keine entscheidenden Unterschiede zwischen den AUCs der ROC-Kurven von OPN und Knochenmarkern. Jedoch war beim Diskriminationspunkt von 95% Sensitivität die Spezifität von OPN höher als die Spezifität von bALP und PINP ($P=0.026$ und 0.0009, McNemar Test). OPN und bALP waren in der multivariaten

Auswertung mit der binären logistischen Regression signifikant unabhhängige Diskriminatoren in Bezug auf die Erfassung einer Knochenmetastasierung. Die Kombination dieser beiden Marker mit Hilfe der logistischer Regression ergab einen signifikant höheren AUC-Wert als für die Einzelmarker (AUC von 0.93 im Vergleich zu OPN mit AUC, 0.85; P=0.026 bzw. zu bALP mit AUC, 0.88; P=0.008). Beim Diskriminationspunkt von 95% Sensitivität erreichte die Kombination von OPN und bALP eine Spezifität von 63%. Diese war höher als die Spezifität von OPN (31%) und bALP (11%) für sich genommen. Es gab eine signifikant positive Korrelation von OPN zu den Knochenmarkern (r_s=0.43-0.79, alle P-Werte <0.05) und zum Tumorgrad (r_s=0.23, P<0.05). Die Konzentrationen von OPN und Knochenmarkern im Blut korrelierten negativ mit der Überlebenszeit der Patienten (Kaplan-Meier, P<0.0001). Je höher die Markerkonzentration, desto kürzer war die Überlebenszeit. OPN und PINP wurden mit Hilfe der multivariaten Cox-Regression als signifikante Indikatoren hinsichtlich Überlebenszeit von PCa-Patienten ermittelt.

Nierenzellkarzinom. Diese Studie umfasste 80 RCC-Patienten mit lokal begrenztem Tumor, mit Lymphknotenmetastasen bzw. Fernmetastasen sowie 52 gesunde Frauen und Männer als Kontrollgruppe. Im Vergleich zur Kontrollgruppe waren OPN und ICTP bei Patienten mit Fernmetastasen in Knochen und in anderen Organen (P<0.05 mindestens) erhöht. Erhöhte OPN-Werte wurden außerdem bei Patienten mit Fernmetastasen im Vergleich zu RCC-Patienten ohne Metastasen beobachtet (P<0.05 mindestens). Die Beziehung von OPN und ICTP bei Patienten mit Fernmetastasen wurde weiter mit der ROC-Analyse untersucht. Der AUC-Wert für OPN (0.89) war größer als der für ICTP (AUC, 0.71, P=0.018). Beim Diskriminationspunkt von 95% Sensitivität betrug die Spezifität für OPN 57%, die für ICTP lediglich 25% (McNemar Test, P=0.0309). OPN zeigte signifikante Korrelationen mit Knochenmarkern (r_s=0.37-0.50, alle P Werte <0.05). Die OPN-Konzentration korrelierte mit dem Tumorstadium (r_s=0.50, P<0.01) und Tumorgrad (r_s=0.33, P<0.05). Konzentrationen von OPN und ICTP wurden außerdem mit der Überlebenszeit von RCC-Patienten assoziiert (Kaplan-Meier, P<0.0001). In der multivariaten Cox-Regression erwies sich OPN als allein signifikanter Faktor hinsichtlich Überlebenszeit (P=0.041).

Die wesentliche Schlussfolgerung aus den hier vorgestellten Untersuchungen besteht darin, dass OPN im Plasma bei Patienten mit Prostatakarzinom und Nierenzellkarzinom als Metastasierungs- und Prognosemarker hinsichtlich des Überlebens eingesetzt werden kann. Die Daten belegen, dass die Durchführung einer prospektiven multizentrischen Studie, die auch andere z.Z. diskutierte neue Marker wie z.B. YKL-40 mit einschließen sollte, im Sinne der evidenzbasierten Medizin gerechtfertigt ist.

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Erklärung

„Ich, Azizbek Ramankulov, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Plasma osteopontin in comparison with bone markers as an indicator of distant metastases and a predictor of survival outcome in prostate cancer and renal cell carcinoma patients“ selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.“

Datum Dezember 2006

Unterschrift

Lebenslauf

Mein Lebenslauf wird aus Datenschutzgründen in der elektronischen Version meiner Arbeit nicht mit veröffentlicht.