Circulating tumor cells and serum levels of MMP-2, MMP-9 and VEGF as markers of the metastatic process in patients with high risk of metastatic progression

Marketa Skerenova[a], Veronika Mikulova[a], Otakar Capoun[c], Tomas Zima[a], Petra Tesarova[b]

Background and Aims. Metastases are a severe complication in cancer patients and biomarkers predicting their progression are still lacking for specific groups of patients. HER2 positive breast cancer (HER2 BC) patients on trastuzumab therapy are at risk of the development of unpredictable and often fatal central nervous system (CNS) metastases and castration resistant prostate cancer (CRPC) patients urgently need a marker of disease progression during therapy. Proposed metastatic markers: circulating tumor cells (CTC), serum levels of matrix metalloproteinase 2 (MMP-2), 9 (MMP-9) and vascular endothelial growth factor (VEGF) were prospectively studied to confirm their utility in these two narrowly defined groups of cancer patients.

Patients and Methods. The groups comprised 44 advanced HER2 BC, 24 CRPC patients and 42 healthy controls. An immunomagnetic separation method followed by PCR and electrophoretic detection (AdnaGen, Germany) were used for CTC determination. Serum marker levels were determined by the ELISAs (R&D System, USA).

Results. MMP-2 serum level was significantly higher in HER2 BC patients who developed CNS metastases, especially if there were also bone metastases. CTCs were a negative predictive marker for overall survival in HER2 BC patients. MMP-9 serum level was significantly higher in CRPC patients in whom disease progression occurred. CTC vanished from the blood of most of the CRPC patients (from 88% to 37%) during chemotherapy.

Conclusion. MMP-2 serum level and CTCs show the potential to predict CNS metastases and overall survival in BC patients. CTCs and MMP-9 serum level could be a promising therapy response marker in CRPC patients.

Key words: cancer biomarkers, circulating tumor cells, matrix metalloproteinases, vascular endothelial growth factor, breast cancers, prostate cancer

[a]*Institute of Medical Biochemistry and Laboratory Diagnostics, General University Hospital in Prague and First Faculty of Medicine, Charles University, Prague, Czech Republic*
[b]*Department of Oncology, General University Hospital in Prague and First Faculty of Medicine, Charles University, Prague, Czech Republic*
[c]*Department of Urology, General University Hospital in Prague and First Faculty of Medicine, Charles University, Prague, Czech Republic*
Coresponding author: Marketa Skerenova, e-mail: jancikova.market@gmail.com

INTRODUCTION

Activation of tumor cell invasiveness and subsequent angiogenesis required for metastatic growth are crucial hallmarks of cancer cells[1]. Metastases formation is mediated by circulating tumor cells (CTCs) i.e. cells that have escaped from the primary tumor. Several studies have proven that CTCs can serve as a powerful marker in advanced breast and prostate cancer[2-6]. However, the escape of tumor cells from the primary tumor, their entry into the circulation and subsequent metastasis is a complex process. Gelatinases, also known as matrix metalloproteinase 2 (MMP-2) and 9 (MMP-9), are important factors in this process. Gelatinases have been widely studied as potential cancer markers for their ability to degrade extracellular matrix and basement membrane[7,8]. They help tumor cells enter the circulation and they may also support their extravasation and metastasis[9-11]. Moreover, MMP-9 is closely linked with angiogenesis which is essential for tumor and metastasis growth[12]. One way by which MMP-9 can modulate angiogenesis is through vascular endothelial growth factor (VEGF) (ref.[11]). VEGF participates in embryogenesis, organ development and wound healing[13]. The involvement of VEGF in tumor cell invasion and survival has also been suggested[14]. However, VEGF is mainly studied in connection with tumor-vessel formation. It is considered to be the main angiogenic factor enabling tumor and metastasis growth.

Higher VEGF expression in tumor cells correlates with poorer disease prognosis[15,16]. The ability of VEGF to increase the permeability of vessels may facilitate tumor cell entry into the circulation as well as extravasation at the place of a new metastasis[17-20].

We wanted to explore the predictive potential of the chosen markers in two well-defined groups of advanced cancer patients in which a metastatic process play a crucial role and which urgently need a predictive biomarker. Patients with human epidermal growth factor receptor 2 positive breast cancer (HER2 BC) receiving trastuzumab therapy are at increased risk of central nervous system (CNS) metastasis[21,22]. The aim of this study was to determine the relationship between the development of metastases and metastatic serum marker levels/ CTCs. Most castration resistant prostate cancer (CRPC) patients

already have multiple metastases. A prostate-specific antigen (PSA) decline of >30% may be associated with therapy efficacy, although the results of studies are conflicting[23]. The introduction of additional markers should help the clinician to recognize those patients who would benefit from chemotherapy from those who would not. We would like to study the applicability of the proposed metastatic markers to these unanswered clinical questions.

Many different studies have investigated the serum levels of MMP-2, MMP-9 and VEGF in cancer patients with different and sometimes contradictory results[8,24-32]. We wanted to incorporate these arguable markers into specific clinical events of two narrowly defined groups of cancer patients to prove or disprove their clinical utility.

MATERIALS AND METHODS

Patient characteristics

Two well-defined groups of advanced cancer patients endangered by metastatic progression were prospectively monitored for metastatic markers and compared with control subjects. The groups comprised 44 patients with advanced HER2 BC, 24 patients with CRPC and 42 healthy volunteers. The study was approved by the Ethics Committee of the General University Hospital in Prague. All participants in the study signed their informed consent.

The BC patients were selected according to the following criteria: advanced breast cancer; HER2 positive status; and trastuzumab or lapatinib palliative treatment. All of these patients were at increased risk of CNS metastases although the duration of treatment differed. The median duration of therapy was 30 months (from 3 to 83). For this reason it was impossible to take samples in the same therapy cycle for every patient. Blood samples were taken once in the course of treatment preceding trastuzumab or oral lapatinib in the therapy cycle following the patient's enrolment in the study.

The prostate cancer patients' enrolment was based on castration resistant stage criteria according to the European Association of Urology guidelines[33]. All the CRPC patients were enrolled at the time of CRPC diagnosis. For this reason, blood was sampled twice, at the time of indication to docetaxel therapy (the first measurement) and in the course of the therapy after the third cycle of chemotherapy (the second measurement), to evaluate the therapy response.

The control group comprised 42 healthy volunteer (21 men and 21 women). The median age of the control group was 41 (from 23 to 63) years. The median age of men and women was 42 (from 23 to 53) and 40 (from 24 to 63) years respectively. CTC presence was not investigated in the blood of the healthy controls. It was presumed, based on the literature, that CTCs would not be present in healthy people[5,34,35]

Methods

Whole blood for CTC analysis and serum for the determination of serum markers were collected from each patient. The serum was divided into aliquots and stored at -20 °C. The serum levels of MMP-2, MMP-9 and VEGF were determined by Quantikine® ELISA Human MMP-2, MMP-9 and VEGF respectively (R&D Systems, USA). CTC presence was determined by the AdnaTestBreast/Prostate Cancer Select/Detect (AdnaGen, Germany). CTCs were enriched by an immunomagnetic separation from the 5 mL of whole blood. The enriched cells were washed three times and lysed. mRNA from the lysate was isolated by the immunomagnetic separation and transcribed to the cDNA by a reverse transcription (Qiagen, Germany). Afterwards, a Multiplex-PCR and on-chip capillary electrophoresis (2100 Bioanalyzer, Agilent, USA) were used to check the presence of PCR products originated from three tumor-associated and one control gene transcripts (AdnaGen, Germany; Qiagen, Germany). The monitored PCR products were HER2, Mucin and Epithelial cell adhesion molecule (EpCAM) in breast cancer and epidermal growth factor receptor (EGFR), PSA and prostate-specific membrane antigen (PSMA) in prostate cancer patients. Actin was used as a control fragment in both cases. According to manufacturer, the detection of at least one of the tumor-associated PCR products at a concentration higher than 0.15 ng/mL proved the patient CTC positive.

Statistical analysis

Collected data were analyzed using SAS software (SAS Institute Inc., Cary, NC, USA). Basic statistical data were computed for the whole study group. A Wilcoxon test or Kruskal-Wallis test were used to compare the differences in variables among defined groups. The relations between the variables were examined using Spearman's correlation coefficients. The optimal cut-offs were determined both by a receiver-operating characteristic (ROC) analysis and an odds ratio (risk evaluation). The differences between variables were tested using a Chi-squared test or Fisher's exact test. Overall survival (OS) was determined using Kaplan-Meier survival curves. The impact of the monitored factors on OS was tested using the log-rank test and Cox regression analysis (Hazard Ratio). The statistical significance (P) was set to 0.05.

RESULTS

Characterization of study groups

The study group characteristics are summarized in Table 1. In the HER2 BC group, CTC presence was determined in 29 (66%) of the patients. Thirteen patients were enrolled in a small preliminary study where only serum markers were determined and two samples were unevaluable. The performance status of patients was 0-1. Twelve patients in the study developed CNS metastases, three patients before their enrolment and nine during the follow-up period. The time between blood sampling and CNS metastases detection varied from 1 to 20 months. Fourteen patients (32%) died during the project.

In the CRPC group, CTCs were determined in 24 (100%) and 21 (88%) of the patients in the first and

Table 1. Study group characteristics.

a) HER2 positive breast cancer

Variable	n	%
All patients	44	100%
Median age (range)	53 (33-72)	
Median follow-up	3 years 6 months	
Stadium of BC at the time of diagnosis		
Localized	6	14%
Locally advanced	21	48%
Metastatic	17	39%
Unknown	1	2%
Best response		
Complete response	9	20%
Partial response	7	16%
Stable disease	13	30%
Progressive disease	15	34%
Metastases*		
CNS	12	27%
Bone	12	27%
Other	20	45%
None	7	16%
Lapatinib therapy		
Yes	8	18%
No	36	82%
Unknown	1	2%
Hormone receptor status*		
ER	16	36%
PR	16	36%
Unknown	1	2%
CTC status		
Positive	5	11%
Negative	24	55%
Unknown	15	34%

* presence of more than one positive
characteristic in one patient is possible

b) Castration resistant prostate cancer

Variable	n	%
All patients	24	100%
Median age (range)	70 (54-82)	
Median follow-up	1 years 4 months	
Stadium of PC at the time of diagnosis		
Localized	15	63%
Locally advanced	3	13%
Metastatic	6	25%
Best response		
Complete response	0	33%
Partial response	8	33%
Stable disease	9	38%
Progressive disease	3	13%
Unknown	4	17%
Metastases		
Bone	19	79%
None	3	13%
Unknown	2	8%
Previous therapy		
Radical prostatectomy	6	24%
Radiotherapy	6	24%
None	12	52%
Gleason score		
6-7	14	58%
8-10	8	33%
Unknown	2	8%
CTC status before docetaxel therapy		
Positive	21	88%
Negative	3	13%
CTC status after the 3rd therapy cycle		
Positive	9	38%
Negative	12	50%
Unknown	3	13%
PSA serum level [ng/mL] median (range)		
Before docetaxel therapy	124 (16-749)	
After the 3rd therapy cycle	49 (4-1243)	

second measurements respectively. Two patients did not participate in the second measurement and the CTC determination failed in one case. The performance status of the patients at the time of blood taking ranged from 0 to 2. Fifteen patients (63%) died during the project.

Serum marker levels in HER2 BC and CRPC patients in comparison with the control group

Since no statistical significant differences were found in the serum marker levels between men and women the control group was not divided according to sex. Similarly, no correlation of serum markers with age was found in any studied group. The only exception was MMP-9 serum level in the control group which showed a weak positive correlation with age (correlation coefficient=0.375; P=0.016). The serum marker levels are presented in Table 2.

In the HER2 BC group, serum marker levels were measured in 42 (96%) patients; two serum samples were not measurable because of haemolysis. In the CRPC group, serum marker levels were determined in 17 (71%) and 20

Table 2. Serum marker levels in the HER2 BC patiens, CRPC patients and control group.

Serum marker levels	MMP2 Median (min-max) [ng/mL]	MMP9 Median (min-max) [ng/mL]	VEGF Median (min-max) [pg/mL]
Control group-women (N=21)	193 (163-262)	650 (3-1113)	237 (102-636)
Control group-men (N=21)	202 (145-319)	442 (3-912)	239 (19-589)
Control group (N=42)	201 (145-319)	597 (3-1113)	238 (19-636)
HER2 BC (N=42†)	249** (143-407)	294 (65-1070)	245 (0-2800)
CRPC First measurement (N=17†)	243* (147-342)	385 (47-785)	332 (125-1050)
CRPC Second measurement (N=20†)	235** (171-344)	544 (8-2003)	346* (128-1099)

Women and men both separately and all together.
Statistically significant differences from the control group are marked with stars (*$P<0.050$; ** $P<0.001$).
† The numbers of cases differed slightly between the groups because a few patients lacked serum marker level measurement.
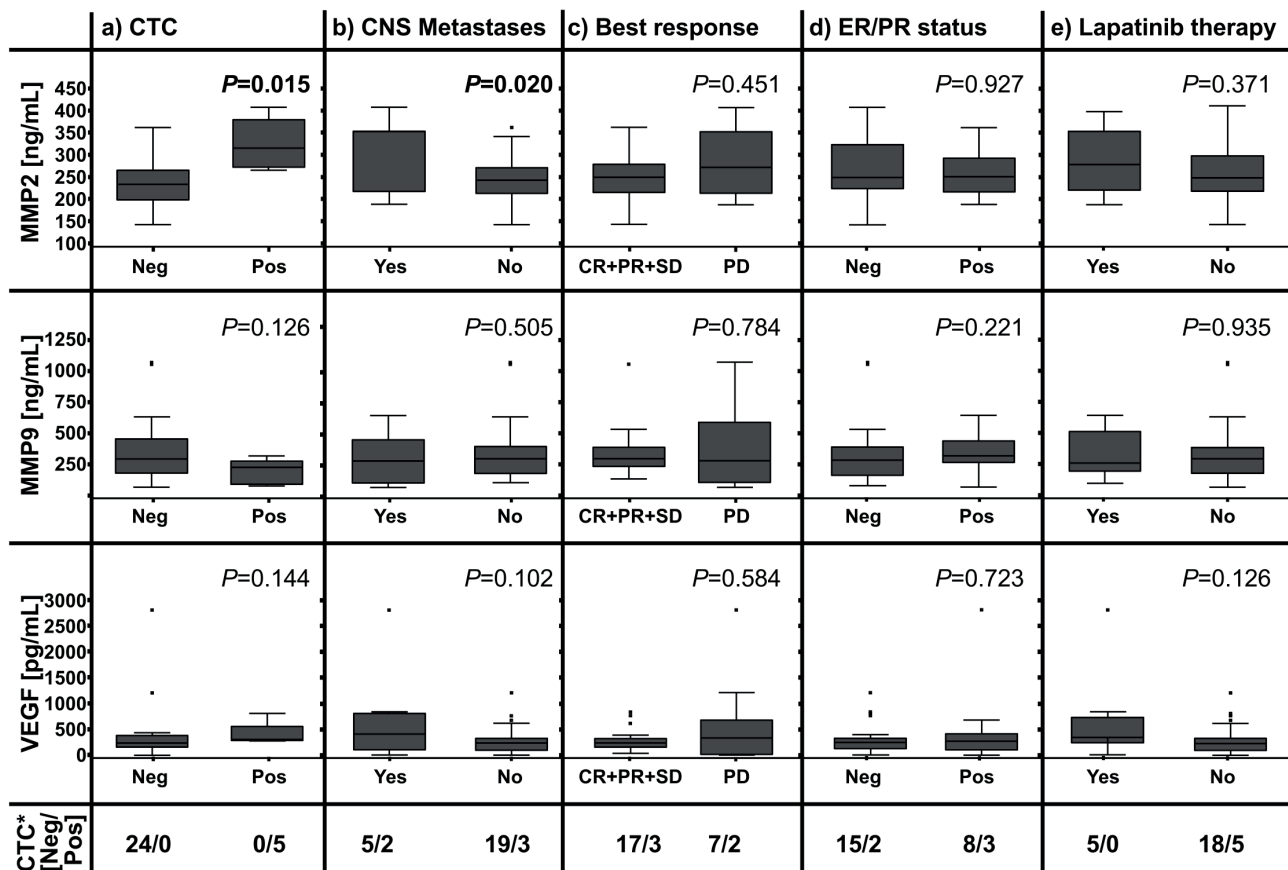
Fig. 1. The comparison of MMP-2, MMP-9 and VEGF serum levels and CTC presence in HER2 BC patients.
a) CTC negative (N=23) vs. CTC positive (n=4)
b) with (n=11) vs. without (n=31) CNS metastases
c) without (n=27) vs. with (n=15) disease progression
d) without (n=24) vs. with (n=17) primary tumor ER and/or PR positivity
e) with (n=8) vs. without (n=33) lapatnib therapy. Statistically significant differences are bold ($P<0.05$)
Neg-negative; Pos-positive; CNS-central nervous system; ER-oestrogen receptor positive primary tumor; PR-progesterone receptor positive primary tumor; PD-progressive disease, SD-Stable disease, PR-partial response, CR-complete response; N-number of performed serum marker measurements. *Number of CTC measurement can differ from number of serum marker measurement.
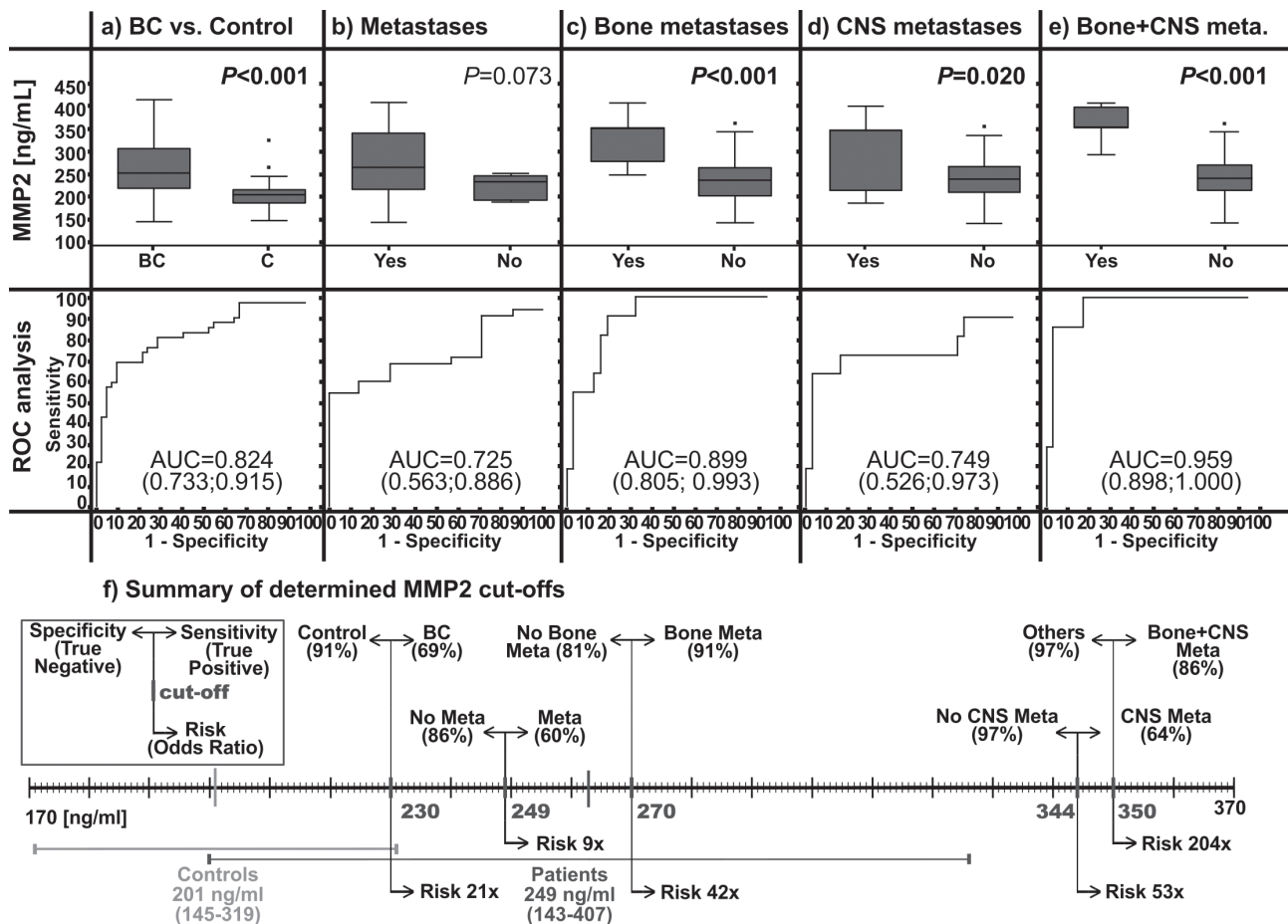
Fig. 2. Serum levels and ROC analysis of MMP-2 in the HER2 BC patients (n=42).

a) vs. control group (n=42)

b) with (n=35) vs. without (n=7) metastases

c) with (n= 11) vs. without (n=31) bone metastases

d) with (n= 11) vs. without (n=31)CNS metastases

e) with bone and CNS metastases (n=7) vs. other patients (n=35)

f) concluding diagram with the cut-offs, specificity, sensitivity and risk values for different pathologic states. Statistically significant differences are bold (P<0.05)

BC-breast cancer; C-control group; CNS-central nervous system; Meta-metastases; AUC-area under curve; N-number of performed serum marker measurements.

(83%) of CRPC patients in the first and second measurements respectively. The lack of serum marker measurement was caused either by the sample haemolysis or the loss of the patient during the study.

Serum marker levels and CTC in HER2 positive breast cancer patients

The results for the HER2 BC patients, divided according to monitored characteristics are presented in Fig. 1.

Although the difference in MMP-2 serum level between patients with and without metastases was not statistically significant at the 0.05 level (Fig. 2b), the MMP-2 serum level in the patients who developed CNS metastases significantly differed from those who did not (Fig. 2d). Moreover, the patients with bone metastases also showed significantly elevated MMP-2 serum levels in comparison with patients without bone metastases (Fig. 2c). In agreement with these results, the highest MMP2 serum level

was measured in seven patients who developed CNS metastases and who had bone metastases (Fig. 2e).The MMP-2 serum level cut-offs, specificity, sensitivity and risks determined by ROC analyses are summarized in Fig. 2f.

The CTC positive patients had worse overall survival than the CTC negative ones. However, the difference was not significant probably due to the limited number of patients and high survival rate in the HER2 BC group (Fig. 3a).

Prostate cancer

Patients who had unfavourable CTC status, those who turned or stayed CTC positive, in the second measurement had shorter overall survival. However, because of the limited number of patients the difference was not statistically significant (Fig. 3b).

No correlation between CTCs, serum markers and PSA serum level was found either pre-therapy or in the

course of therapy or in the change in their levels between the first and the second measurements (data not shown). Moderately strong significant positive correlation was found in the MMP-2 serum levels between the first and second measurements (correlation coefficient=0.616; P=0.025). The results for serum marker levels and CTCs in the CRPC patients, divided according to monitored clinical characteristics, are presented in Fig. 4.

DISCUSSION

To the best of our knowledge, this study is the first to focus on the joint determination of CTCs and MMP-2, MMP-9 and VEGF serum levels in advanced cancer patients. We presumed that these markers could predict metastasis formation and therapy efficiency in the two well-defined groups of HER2 BC and CRPC patients at risk of metastatic progression. However the narrow specification of the groups diminished the number of patients involved in the study. Since the connection of all the monitored serum markers with the metastatic process have been claimed in many previous works and our patient groups consisted of advanced cancer patients it was surprising that only MMP-2 serum level showed significant differences between the patients and control group. The VEGF serum level of CRPC patients was also significantly higher but only in the samples taken in the course of therapy-the second measurement (Fig. 4). The pre-therapy VEGF serum level -the first measurement-was not statistically significant (P=0.072). Furthermore, only MMP-2 and in one case MMP-9 showed some discriminating power to distinguish between the groups of patients with monitored clinical characteristics (P<0.05; Fig. 1, Fig. 4).

An elevated MMP-2 serum level in BC patients has already been connected with a poor prognosis[26] and the role of MMP-2 in the development of CNS metastases has been also proposed in animal experiments[36-38] According to our results, the MMP-2 serum level was significantly elevated in HER2 BC patients with bone and/or CNS metastases (Fig. 2). We found out that the MMP-2 serum level has the best predictive power for the development of CNS metastases in HER2 BC patients suffering from bone metastases. The cut-off value of 350 ng/mL determined for these patients was significantly higher than the normal MMP-2 serum levels and proved to be a strong discriminator (Fig. 2e). All these data are summarized in Fig. 2f. Since CNS metastases represent a very serious and still unpredictable complication in HER2 BC our findings warrant verification in a larger group of patients.

Despite the frequency of metastases, only five HER2 BC patients (17%) exhibited CTC positivity in our study. This can be explained by the efficacy of HER2 targeted therapy. A similar rate of CTC positivity in HER2 positive metastatic BC patients has been reported by Cristofanlli. In common with Cristofanilli, the overall survival of the CTC positive BC patients in our study was shorter in comparison with the CTC negative group. CTCs in the patients more than doubled their risk of death. However,
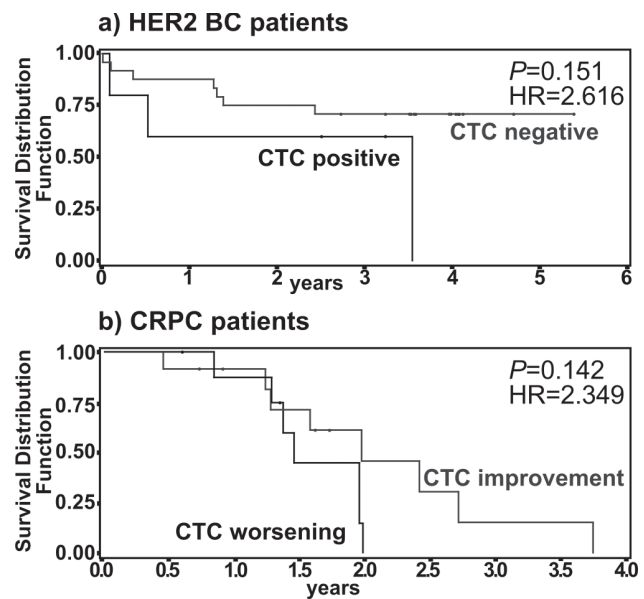

Fig. 3. Kaplan-Meier analysis of the overall survival and Hazard ration analysis (HR).
a) HER2 BC patients with vs. without CTCs
b) CRPC patients with CTC status worsening (Neg→Pos or Pos→Pos) vs. CTC status improvement (Pos→Neg or Neg→Neg) in the course of therapy

the results were not statistically significant because two thirds of patients were still alive at the end of the follow-up period (Fig. 3) (ref.[5]).

A significantly elevated MMP-2 serum level in the CTC positive patients (P=0.015) was found. Moreover, 4/5 CTC positive patients had bone metastases. Despite the small number of cases, these results support other studies' findings that CTCs are more closely related to bone metastases than to the other metastatic sites[4,39]. Surprisingly, only 2/7 patients with bone and CNS metastases were CTC positive but four patients missed the CTC test (Fig. 1). There was no correlation between CTC positivity and CNS metastases but this may be due to the small number of CTC positive BC patients in our study. No significant differences in serum marker levels were found between patients on trastuzumab and those who were switched from trastuzumab to lapatinib therapy due to resistance to trastuzumab suggesting that the development of trastuzumab resistance causes no serum marker or CTC elevation.

In the CRPC patients, the MMP-2 serum level seems to follow the initial risk stratification of the patients according to the biopsy Gleason score. Additionally, MMP-9 serum levels measured in the course of therapy showed the predictive value for the patients' therapy response evaluation (Fig. 4). The CRPC patients with an elevated MMP-9 serum level in the second measurement had a worse overall response to docetaxel therapy (P=0.044). Yet, MMP-9 serum levels in the CRPC patients did not significantly differ from the healthy controls; whereas the VEGF serum level differed significantly for CRPC patients and the control group, consistent with previous
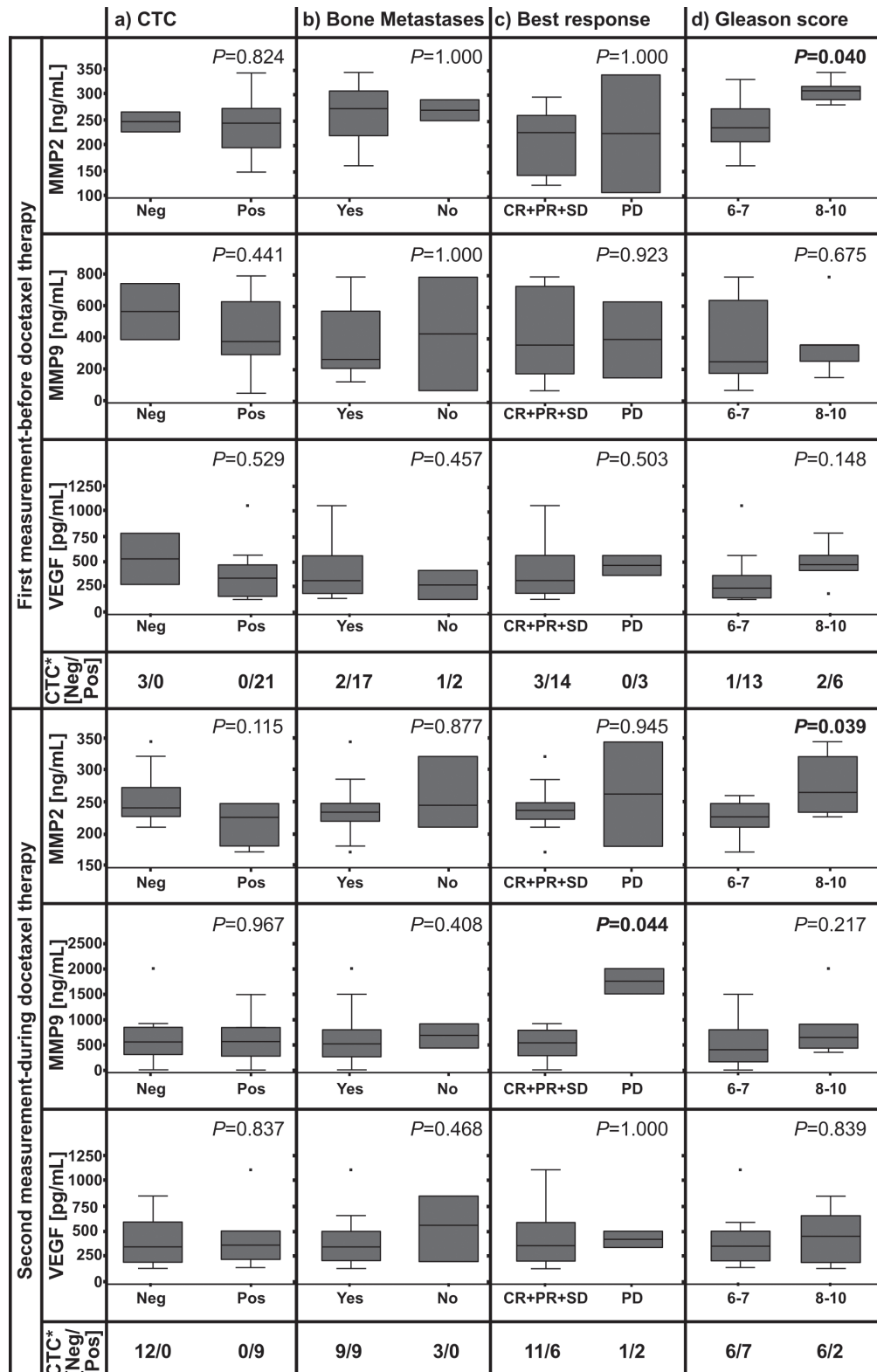
Fig. 4. The comparison of MMP-2, MMP-9 and VEGF serum levels and CTC presence in CRPC patients before and during docetaxel therapy.

a) CTC negative ($n_{1st}=13$, $n_{2nd}=17$) vs. CTC positive ($n_{1st}=2$, $n_{2nd}=3$)

b) with ($n_{1st}=13$, $n_{2nd}=17$) vs. without ($n_{1st}=2$, $n_{2nd}=3$) bone metastases

c) without ($n_{1st}=11$, $n_{2nd}=16$) vs. with ($n_{1st}=2$, $n_{2nd}=2$) disease progression

d) with lower ($n_{1st}=10$, $n_{2nd}=14$) vs. higher ($n_{1st}=5$, $n_{2nd}=6$) biopsy Gleason score

Statistically significant differences are bold ($P<0.05$).

Neg-negative; Pos-positive; PD-progressive disease, SD-Stable disease, PR-partial response, CR-complete response; N_{1st}- N-number of performed serum marker measurements before the therapy; N_{2nd}- N-number of performed serum marker measurements during the therapy *Number of CTC measurement can differ from number of serum marker measurement.

findings[40], but failed to distinguish between the CRPC patient groups divided according to the various clinical characteristics (Table 1, Fig. 4). In contrast with the HER2 BC patients, bone metastases seem to have had no impact on the serum marker levels in the CRPC patients. Since the MMP-2 serum level has been shown by Gohji to be elevated in the PC patients with bone metastases, our results may be influenced by the small number of CRPC patients without bone metastases in our study group[27]. The percentage of CTC positive CRPC patients decreased during the therapy from 88% to 37% (Fig. 4). Although the patients who stayed or became CTC positive during treatment had shorter overall survival and twice the risk of death, the difference was not statistically significant (Fig. 3).

The weak points of our study were limited numbers of patients and a younger control group but, gelatinases and VEGF serum levels do not correlate with age[32,40-42]. Hence age is probably not important. In this study, only the MMP-9 serum level in the control group showed a weak positive correlation with age (correlation coefficient=0.375; P=0.016). Consistent with other studies, there were no significant differences in serum marker levels between men and women in the control group[29,41].

CONCLUSION

We can conclude that i) MMP-2 serum levels are significantly higher in HER2 BC patients with CNS metastases; ii) the power of MMP-2 serum level to predict CNS metastases is strongest in patients with bone metastases; iii) elevated MMP-2 levels and CTCs seem to be connected with bone metastases in HER2 BC patients; iv) CTCs are found only in small percentage (17%) of HER2 BC patients treated with trastuzumab; v) CTCs are linked to shorter overall survival in HER2 BC patients; vi) MMP-9 serum levels are significantly higher in CRPC patients during disease progression; vii) the disappearance of CTCs from the blood of CRPC patients during therapy is connected with longer overall survival; viii) the wide range of the MMP-9 and VEGF serum levels are an unfavourable parameter for their use as clinical markers. These conclusions warrant verification in a larger study.

Author contributions: MJ: study conception and design, acquisition of data, data analysis and interpretation, manuscript drafting; VM: study conception and design, data analysis and interpretation, critical revision; OC: acquisition of data, critical revision; TZ: critical revision; PT: acquisition of data, critical revision

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