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RESEARCH

Validation of Circulating MMP-7 Level as an Independent Prognostic Marker of Poor Survival in Urinary Bladder Cancer

Tibor Szarvas · Tobias Jäger · Markus Becker · Stephan Tschirdewahn · Christian Niedworok · Ilona Kovalszky · Herbert Rübben · Süleyman Ergün · Frank vom Dorp

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Abstract Molecular marker analyses aiming a more accurate disease characterization and risk stratification of cancer patients provided several promising marker candidates in the last few years. However, recent reviews underlined the paramount importance of validation, since many of the initially promising results could not be confirmed in independent patient cohorts. If serum or plasma is a more appropriate sample to test for prognostic markers is a matter of debate. We recently found serum MMP-7 levels to correlate with poor patients' prognosis in urinary bladder cancer. In this study, we examined associations of the MMP-7 plasma levels with clinical follow-up data in an independent cohort of bladder cancer patients to validate our former results and to assess if plasma is also suitable for MMP-7 analysis. Plasma levels of 97 patients and 22 controls were analyzed, using enzyme-linked immunosorbent assay. Associations between MMP-7 plasma concentrations and clinical data were assessed applying both univariate and multivariate analysis. Plasma MMP-7 levels were significantly higher in patients than in controls. Similarly to our former findings in sera, high MMP-7 plasma levels proved to be significant and independent predictors of both overall and disease-specific survival. In addition, we observed a metastasis-specific difference in MMP-7 levels between serum and plasma. In summary, we confirmed the prognostic relevance of circulating MMP-7 levels in an independent cohort of patients and concluded that circulating MMP-7 levels may help to identify bladder cancer patients at high-risk of disease progression who could benefit from an adjuvant chemotherapy or from an extended lymph node dissection.

Keywords Bladder cancer · MMP-7 · Matrilysin · Metastasis · Plasma · Prognosis · Serum

T. Szarvas · T. Jäger · M. Becker · S. Tschirdewahn · C. Niedworok · H. Rübben · F. vom Dorp (✉)
Department of Urology, University of Duisburg-Essen,
Hufelandstr. 55,
45147 Essen, Germany
e-mail: frankvomdorp@web.de

I. Kovalszky
1st Department of Pathology and Experimental Cancer Research,
Semmelweis University,
Budapest, Hungary

S. Ergün
Institute of Anatomy, University Hospital of Essen,
Essen, Germany

Introduction

Prediction of patients' outcome is one of the most important challenges in clinical cancer research; enabling better choice of risk-related therapy. This is particularly true for muscle-invasive bladder cancer treated by radical surgery which is characterized by a remarkably heterogeneous clinical course. A considerable subset of bladder carcinoma patients (50%) demonstrates rapid metastatic progression and poor survival after radical cystectomy, while others enjoy a long disease-free survival [1–3]. Standard prognostic features such as pathologic stage and grade have limited ability to predict outcome in these patients. Therefore, a molecular marker that could identify a high-risk group of patients for disease progression would be of great clinical value.

The development of comprehensive, gene expression microarray technology has allowed the selection of relevant marker genes from a large pool of candidate genes in prognostic marker studies for various cancers including urinary bladder cancer [4]. Performing in silico data mining of published microarray data our group previously selected MMP-7 as a possible prognostic marker in bladder cancer. Analyzing frozen tissue and serum samples, we recently demonstrated that high tissue and serum levels of matrix metalloproteinase 7 (MMP-7) are significantly and independently associated with poor prognosis in bladder cancer patients [5].

Recent reviews suggested that single studies of a given biomarker within one cohort of patients are often inadequate to firmly establish its prognostic value [6]. Altmann et al. concluded that prognostic models are not generalizable until validated in an independent cohort of patients [7]. In accordance, Bleeker et al. found that prognostic models perform best when applied to datasets from which the prognostic model was originally constructed, in contrast they perform much less well when applied to external (independent) cohorts [8]. Therefore, validation of formerly tested models in an independent cohort of patients is a necessary step towards the implementation of molecular markers in everyday clinical practice.

There is no consensus if serum or plasma is a more appropriate medium for testing protein markers for their prognostic relevance. For example in case of vascular endothelial-derived growth factor (VEGF) it has been found that analysis of VEGF in serum samples provide a more accurate prognostic stratification as that of plasma samples [9]. In addition, some members of the matrix metalloproteinases such as MMP-1, MMP-8 and MMP-9 show considerable differences between their serum and plasma concentrations, suggesting that selection of the analyzed medium may influence the determinability of prognostic relevance.

Prompted by these, we initiated this study to validate the formerly determined prognostic performance of circulating MMP-7 levels in an independent cohort of bladder cancer patients. Furthermore, plasma samples were analyzed to test if blood sampling methods do have any impact on the prognostic performance of MMP-7 measurement.

Materials and Methods

Clinical Samples

Plasma samples of 97 bladder cancer patients were collected before surgery in the Department of Urology of the University Hospital of Essen between 1995 and 1998. The criteria for study enrolment were histopathological diagnosis of transitional cell carcinoma of the bladder, no history of other tumor, no chemotherapy before surgery, availability of sufficient plasma sample and the potential to follow-up. The study was performed in accordance with the ethical standards of the Helsinki Declaration and was approved by the ethical board of the hospital. Archive tissue sections from each patient were retrieved and reclassified by a pathologist according to the WHO classification of 2004 [10]. In addition, samples of 22 age-matched controls were also analyzed. Sixty one of 97 patients died until 07/2007; 52 bladder cancer-related (median survival: 10.5 months) and 9 not bladder cancer-related (median survival: 55 months). The primary endpoint of this study was cancer-specific survival. Cause of death was obtained from death certificates. Subjects were followed from baseline survey (date of surgery) until 07/2007.

Blood samples were collected into Monovette tubes coated with EDTA (Sarstedt, Nümbrecht, Germany) and were centrifuged at 1600 g for 15 min. at 4°C within 2 h after venipuncture. Separated plasma samples were stored at −80°C until analysis.

In addition, we prospectively collected both serum and EDTA-plasma samples at the same time before surgery from 20 bladder cancer patients (11 with and 9 without lymph node metastasis).

Measurement of Plasma MMP-7 Levels

Plasma levels of MMP-7 were quantified by sandwich enzyme-linked immunosorbent assay using Quantikine ELISA kit from R&D Systems (Wiesbaden, Germany), according to the manufacturer's instructions. All samples were examined in duplicate, and the mean values were used for statistical analysis. The intra-assay variability for duplicate measurement was 4.5% while the inter-assay variability was 7.7%.

Statistical Analysis

The lack of normal distribution of gene expression data (controlled by Shapio-Wilk test) indicated the use of non-parametric two-sided Wilcoxon rank sum test (Mann-Whitney test) for paired group comparisons. Univariate recurrence-free, metastasis-free and disease-specific survival analysis was done using both Kaplan-Meier log-rank test and univariate Cox analysis. For multiple analyses, the Cox proportional hazards regression model was used. According to the test of Schoenfeld's residuals, we observed no evidence for deviations from the proportional hazards assumption for each variable. In all tests the *P*-value of at least 0.05 was considered to be statistically significant. All statistical analyses were done with SPSS software package, version 17.0 (Chicago, IL, USA).

Table 1 Patients' characteristics and MMP-7 plasma concentrations

	n	MMP-7 plasma cc. median (range)	P
Age			
≤65	48	1.93 (0.00–26.49)	0.434
>65	49	2.26 (0.37–26.94)	
Gender			
Male	76	2.10 (0.00–26.94)	0.639
Female	21	1.78 (0.10–10.22)	
Stage			
Ta	17	1.66 (0.32–4.94)	0.065
T1	18	2.10 (0.34–14.44)	0.711
T2	27	1.93 (0.20–16.76)	0.791
T3	25	1.72 (0.00–26.94)	0.004
T4	10	8.32 (2.00–26.50)	
Non-inv. (Ta-T1)	35	1.86 (0.32–14.44)	0.144
Invasive (T2-T4)	62	2.35 (0.00–26.94)	
Grade			
G1	10	1.91 (0.37–8.02)	0.825
G2	32	1.69 (0.20–26.94)	0.073
G3	55	2.40 (0.00–26.49)	
Low-grade (G 1–2)	42	1.78 (0.20–26.94)	0.048
High-grade (G 3)	55	2.40 (0.00–26.49)	
Primer	56	1.97 (0.00–26.94)	0.844
Recurrent	41	2.13 (0.32–24.48)	
Lymph node status			
N+	19	3.14 (0.94–10.17)	0.283
N−	78	1.92 (0.00–26.94)	
Smoking			
yes	23	2.13 (0.32–24.48)	0.600
no	33	2.34 (0.00–13.95)	
unknown	41		
Control	22	0.96 (0.11–2.22)	<0.001
Tumor	97	2.06 (0.00–26.94)	

Results

Clinical Background

The main characteristics of patients are given in Table 1. The median follow-up time was 35 months with a mean of 47.5 months and a maximum of 148 months. All 62 patients with muscle-invasive bladder cancer were treated by radical cystectomy. Nineteen of 35 patients with Ta/T1 cancers were treated by transurethral resection, while in 16 patients with high-risk non-muscle-invasive cancer radical cystectomy was performed. In 19 patients metastatic disease was present at surgery, while in 18 patients metastatic progression was detected after surgery. Plasma samples of 22 age-matched healthy individuals with no history of cancer were used as controls. In the prospectively collected group, eleven patients were treated by radical cystectomy (metastatic cohort), and nine with transurethral resection (non-metastatic cohort).

Comparison of MMP-7 Plasma Levels Between Tumor and Control Samples

Ninety-seven preoperatively collected plasma samples of bladder cancer patients and 22 healthy controls were analyzed using MMP-7 enzyme-linked immunosorbent assay. The MMP-7 concentration was two-fold significantly elevated in plasma samples of tumor patients compared to healthy controls ($P<0.001$) (Table 1). There were no significant differences in MMP-7 concentration between males and females or between patients with primary or recurrent tumors. Plasma MMP-7 concentrations did not correlate with age and smoking consumption (Table 1).

MMP-7 Plasma Concentration and Clinicopathological Parameters

MMP-7 plasma levels were significantly higher in high-grade (G3) than in low grade carcinomas (G1-G2) ($P=0.048$). No such correlation was observed between plasma levels of patients with low-stage (Ta-T1) and high-stage tumors ($P=0.144$) (Table 1, Fig. 1). However, MMP-7 levels were higher in plasma samples of patients with lymph node positive bladder cancers than those without lymphatic metastasis, although this correlation failed to reach significance ($P=0.283$).

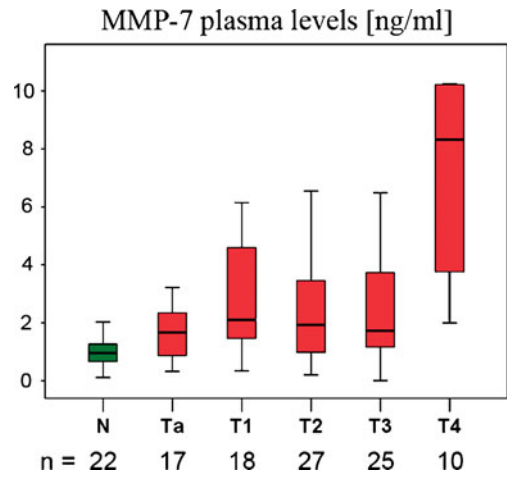

Fig. 1 MMP-7 plasma concentration in bladder cancer patients and controls. MMP-7 plasma levels are significantly higher in patients than in controls (P<0.001). The highest plasma MMP-7 levels were found in tumor stage 4

Univariate Analysis

Results of univariate analysis and prognostic endpoints (overall, disease-specific and metastasis-free survival) are listed in Table 2. Figure 2 shows the Kaplan-Meier survival curves. In analyses where dichotomization is necessary (Cox univariate and multivariate analyses), patients were subdivided into low or high groups for MMP-7 plasma concentration using the median value as cut-off (1.86 ng/ml) (Fig. 2b,d). Application of two cutpoints enables a more objective presentation of Kaplan-Meier survival analysis. Therefore, we displayed in Fig. 2 the results of Kaplan-Meier survival analysis performed also by using two cut-off values. The two cutpoints (1.26 ng/ml and 2.40 ng/ml) were established as the 33rd and 67th percentiles of patients (Fig. 2a,c).

Association of MMP-7 Plasma Levels with Patients' Prognosis

High patient age and female gender proved to be associated with reduced overall- and disease-specific survival (P=0.003, P=0.047, P=0.002 and P=0.009 respectively). In contrast smoking consumptions (yes vs. no) did not influence overall-, disease-specific or metastasis-free survival (Table 2). Furthermore, high tumor grade and the

Table 2 Cox univariate analysis of overall, disease-specific and metastasis-free survival

Variables	n	Overall survival			Disease-Specific survival			Metastasis-free survival		
		HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age										
≤65	48	ref.			ref.			ref.		
>65	49	2.223	1.309–3.778	0.003	2.475	1.385–4.425	0.002	1.858	0.690–5.005	0.218
Sex										
Female	76	ref.			ref.			ref.		
Male	21	0.550	0.306–0.991	0.047	0.448	0.245–0.821	0.009	0.482	0.155–1.499	0.207
Stage										
Non-inv. (Ta-T1)	35	ref.			ref.			ref.		
Invasive (T2-T4)	62	1.484	0.857–2.570	0.159	2.071	1.101–3.896	0.024	1.043	0.388–2.803	0.933
Grade										
Low-grade (G 1–2)	42	ref.			ref.			ref.		
High-grade (G 3)	55	2.065	1.201–3.552	0.009	2.816	1.518–5.225	0.001	2.263	0.822–6.233	0.114
Lymph node status										
N- / Nx	78	ref.			ref.			–		
N+	19	2.990	1.682–5.316	<0.001	3.535	1.950–6.408	<0.001	–	–	–
Prior Recurrence										
Primer	56	ref.			ref.			ref.		
Recurrent	41	1.510	0.906–2.518	0.114	1.278	0.735–2.220	0.385	1.796	0.668–4.830	0.246
Smoking										
no	33	ref.			ref.			ref.		
yes	23	0.850	0.461–1.565	0.601	0.855	0.432–1.684	0.650	1.039	0.341–3.162	0.947
unknown	41									
MMP-7 plasma cc.										
low	42	ref.			ref.			ref.		
high	55	2.305	1.322–4.021	0.003	2.175	1.201–3.937	0.010	1.697	0.616–4.674	0.307
MMP-7 plasma cc. Cystectomy[a]										
low	32	ref.			ref.			ref.		
high	46	2.264	1.235–4.148	0.008	1.906	1.006–3.614	0.048	2.037	0.625–6.636	0.238

HR hazard ratio, *CI* confidence interval, *Ref.* referent.

[a] MMP-7 plasma levles in patients treated by radical cysectomy

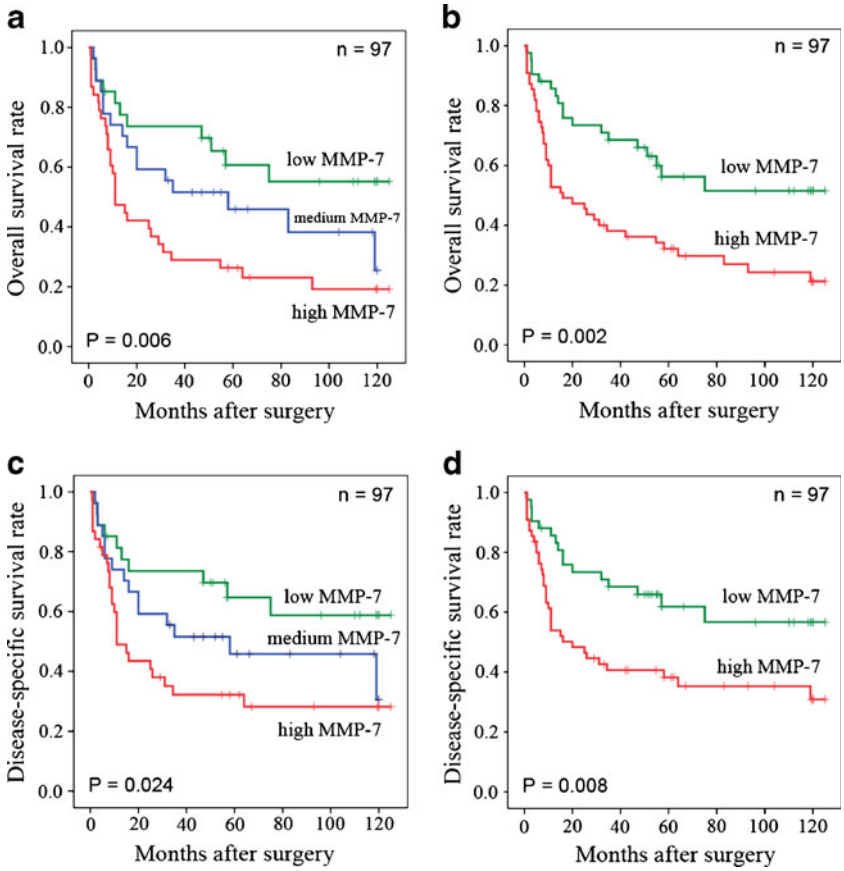

Fig. 2 Kaplan-Meier curves of overall and cancer-specific survival stratified by MMP-7 plasma concentration. Both overall (**a**–**b**) and disease-specific (**c**–**d**) survival is significantly shorter in patients with high MMP-7 plasma concentration. **a** and **c**) MMP-7 <1.26 ng/ml—low (green); 1.26 ng/ml<MMP-7 <2.40 ng/ml—medium (blue); MMP-7 >2.40 ng/ml—high (red). **b** and **d**) MMP-7 <1.86—low (green), MMP-7 >1.86—high (red)

presence of lymph node metastasis have a strong impact on overall- (P=0.009, P=0.001), and also disease-specific (P< 0.001 each) survival. Most important, high MMP-7 plasma concentration is a significant predictor of disease-specific and overall survival (P=0.010 and P=0.003 respectively). The risk stratification of patients treated by radical cystectomy is of particular interest. Therefore, we also analyzed the prognostic significance of MMP-7 levels focusing solely on this group. High MMP-7 plasma levels were significantly associated with poor overall- and disease-specific survival in patients treated by radical surgery (P=0.048, P=0.008) (Table 2). None of the analyzed variables correlated with metastasis formation after surgery.

Multivariate Analysis

Multivariate analysis revealed high MMP-7 plasma concentration as a stage- and grade-independent prognostic factor of disease-specific and overall survival (HR, 2.017; 95% CI, 1.110–3.665; P=0.021, HR, 2.235; 95% CI, 1.277–3.910; P=0.005 respectively) but not metastasis-free survival (HR, 1.612; 95% CI, 0.580–4.475; P=0.360) (Table 3).

Comparison of MMP-7 Concentration Between Serum and EDTA-plasma in Patients with and Without Metastasis

Analyzing MMP-7 serum and EDTA-plasma levels of independent patient cohorts we did not find such a

Table 3 Cox multivariate models of overall, disease-specific and metastasis-free survival

Variables	HR	95% CI	p
Overall survival			
Stage (T2-T4)	0.940	0.488–1.812	0.853
Grade (G2-G3)	2.047	1.072–3.908	0.030
MMP-7 plasma (>1.86 ng/ml)	2.235	1.277–3.910	0.005
Disease-specific survival			
Stage (T2-T4)	1.207	0.576–2.526	0.618
Grade (G2-G3)	2.437	1.185–5.015	0.016
MMP-7 plasma (>1.86 ng/ml)	2.017	1.110–3.665	0.021
Metastais-free survival			
Stage (T2-T4)	0.527	0.158–1.761	0.298
Grade (G3)	3.104	0.904–10.656	0.072
MMP-7 plasma (>1.86 ng/ml)	1.612	0.580–4.475	0.360

considerable difference as Jung et al. [11]. On the other hand, we formerly found MMP-7 significantly elevated in serum and urine samples of bladder cancer patients if lymph node metastasis was present. In the present study analyzing EDTA-plasma samples we did not observe such correlation. To address these two discrepancies, we analyzed matched serum and EDTA-plasma samples collected before surgery. In the 11 patients with known lymph node metastasis MMP-7 concentrations were significantly higher in serum than in plasma (P=0.003). In contrast, in patients with non-metastatic disease serum and plasma MMP-7 levels did not differed significantly (P=0.248). The difference between serum and plasma MMP-7 levels seems to be capable to clearly distinguish between patients with and without lymph node metastasis (Fig. 3).

Discussion

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes capable of cleaving extracellular matrix proteins which is essential for invasive tumor growth. Through its wide range of substrate specificity MMP-7 may be critically involved in several tumor promoting cellular processes [12–15]. Elevated MMP-7 tissue expressions were found to be correlated with poor patients' survival in various cancers such as colorectal, gastric, pancreatic and prostate cancer [16–19]. In accordance, high serum/plasma levels were found to be associated with poor prognosis in colorectal, renal cell and prostate cancer [20–23]. In bladder cancer, we recently observed significantly elevated MMP-7 tissue, serum and urinary levels in patients with metastatic disease [5, 24]. Furthermore, high tissue and serum levels proved to be independent predictors of poor metastasis-free and disease-specific survival in bladder cancer patients [5]. These results suggest that MMP-7 may be a valuable marker for predicting prognosis in bladder cancer. This information is of particular interest in patients with advanced bladder carcinoma treated by radical cystectomy. High preoperative MMP-7 levels may indicate the necessity of an extended lymph node dissection or early adjuvant chemotherapy in these high-risk patients. We formerly observed a significant decrease in MMP-7 serum concentration following radical cystectomy [5]. In patients with known metastasis MMP-7 also decreased but remained still high after radical surgery, suggesting that monitoring of circulating MMP-7 levels may allow patients follow-up after surgical treatment.

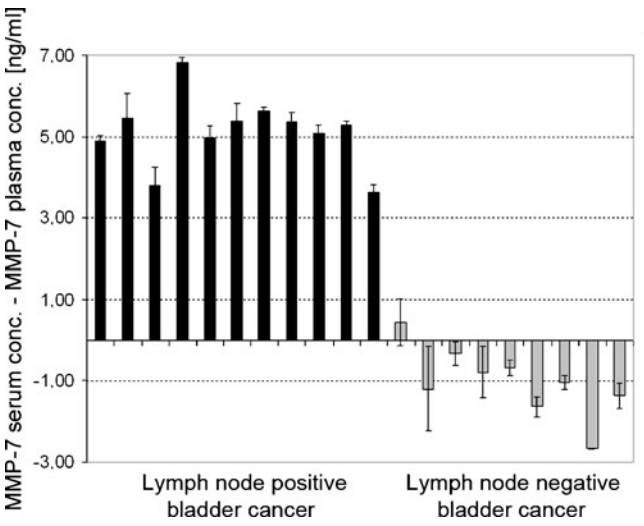

Fig. 3 Difference between matched serum and plasma MMP-7 levels in patients with metastatic and localized bladder cancer. MMP-7 levels are significantly higher in serum than in plasma samples of patients with metastatic disease (P=.0.003). In contrast no such difference is detectable in patients with localized disease (P=0.248)

Former reviews highlighted that promising results of prognostic marker studies could not always be confirmed in independent patient cohorts [6]. Therefore, validation of such results in a similar but independent cohort of patients is necessary before their implication in everyday practice. Whether serum or plasma is a more appropriate specimen for determination of MMPs is a matter of debate.

In the present study, we analyzed MMP-7 plasma levels in samples of bladder cancer patients. The results were compared to our former findings in serum samples of bladder cancer patients. The patient groups of the compared studies were similar in terms of gender, age, stage and grade distribution. Furthermore, we used the same ELISA kit with the same sample dilutions in both experiments. This allows a direct comparison of MMP-7 concentrations between the two studies. In contrast to Jung et al. we found MMP-7 concentrations to be comparable in serum and EDTA-plasma [11]. Similar to the findings in serum samples, plasma MMP-7 concentrations were significantly higher in tumor patients than in healthy controls. Most important, present data confirm the prognostic value of circulating MMP-7 levels in bladder cancer patients. Our former analysis demonstrated that the 10-year disease-specific survival rate is significantly higher in patients with low MMP-7 serum concentrations compared to those with high MMP-7 levels (59% vs. 29%). Similar difference in survival rates was found when analyzing plasma MMP-7 levels (62% vs. 36%). Since the risk stratification of patients treated by radical cystectomy is of particular interest, we also analyzed the prognostic significance of MMP-7 levels focussing solely on this group. Considering only patients treated by radical cystectomy the 10 year survival rates were 51% with low and 17% with high MMP-7 serum levels. Using plasma samples the difference was slightly smaller but still significant; 55% in patients with low and 33% in those with high MMP-7 levels.

Multivariate analysis demonstrated that the prognostic value of circulating MMP-7 levels in both serum and plasma are independent predictors of poor patients' prognosis. Present results confirm the prognostic significance of circulating MMP-7 levels in an independent patient cohort. This effect does not seem to be fundamentally influenced by the blood sampling procedures (serum/plasma). However, our results show that analysis of serum samples may provide a slightly more accurate prognostic stratification compared to that of plasma.

Interestingly, we could not observe significantly higher MMP-7 levels in *plasma* samples of patients with metastatic bladder cancer that we formerly found in *serum* and urine samples. Furthermore, in contrast to *serum* MMP-7 levels, *plasma* MMP-7 concentrations were not informative with regard to the prediction of metastatic spread. To address this obvious discrepancy, we prospectively collected and analyzed matched serum and EDTA-plasma samples of bladder cancer patients with and without metastasis. In samples of patients with metastatic cancer, serum MMP-7 levels were significantly higher than in plasma. In contrast, MMP-7 serum and plasma concentrations were similar in patients with localized cancer. This may explain why we found elevated MMP-7 only in serum but not in plasma samples of patients with metastatic disease. This observation may have a clear clinical relevance in the detection of metastasis. However, further analyses are needed to confirm these data in a larger number of patients. On the other hand, these results suggest the potential existence of metastasis-specific MMP-7 forms, or alternatively, metastasis-dependent degradation or uptake of MMP-7, which may be differently influenced by coagulation. A further possible explanation for this difference is that leukocytes and platelets also abundantly secrete some types of MMPs which secretion has shown to be stimulated upon addition of silicates, a component of some serum collection tubes. In addition, MMPs can also be released during the coagulation process, which can results in concentration difference between serum and plasma. Further analyses are needed to address this interesting question.

In summary, analyzing an independent cohort of bladder cancer patients we confirmed the prognostic significance of circulating blood MMP-7 levels in predicting disease-specific survival. Our results demonstrate that serum but not plasma MMP-7 levels could help to identify patients with present and of high risk of metastasis. The significant difference between plasma and serum MMP-7 levels we found may provide an even more accurate identification of patients with metastatic bladder cancer, and therefore may help to optimize therapy decisions. Further prospective studies are needed to evaluate if patients with elevated MMP-7 levels would benefit from an extended lymph node dissection or from early adjuvant chemotherapy.

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Conflict of Interests Statement None of the authors of this manuscript have a conflict of interest to declare.

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