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High VEGF-D and Low MMP-2 Serum Levels Predict Nodal-Positive Disease in Invasive Bladder Cancer

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Background: To investigate stromal variables including angiogenesis, lymphangiogenesis, and matrix metalloproteinase (MMP) in the serum of patients with urothelial carcinoma of the bladder (UCB) and to evaluate their association with histopathological characteristics and clinical outcome.

Material/Methods: Protein levels of vascular endothelial growth factors-A, -C, -D (VEGF-A/-C/-D), their receptors- VEGF-R2 and -R3 (VEGF-R2/-R3), and matrix metalloproteinases 2, -3, and -7 (MMP-2, MMP-3, MMP-7) were quantified in the blood serum samples of 71 patients with UCB before radical cystectomy (RC). Samples of patients with non-invasive UCB or no history of UCB were investigated as controls (n=20). Protein levels in the serum were measured using a flow cytometric cytokine assay.

Results: A positive association for VEGF-D ($p<0.001$) and an inverse association for MMP-2 ($p=0.017$) were observed in patients with positive lymph node (LN) status at the time of RC. VEGF-A ($p<0.001$), VEGF-C ($p<0.001$), MMP-2 ($p<0.001$), and MMP-7 ($p=0.005$) serum levels were different in serum of patients with invasive UCB compared with non-invasive UCB or healthy individuals. None of the serum markers were associated with disease progression.

Conclusions: High VEGF-D and low MMP-2 serum levels predict LN metastasis in patients with UCB at the time of RC. VEGF-A, VEGF-C, MMP-2, and MMP-7 serum levels varied significantly between invasive and non-invasive disease as well as in comparison with healthy individuals. Clinical implementation of these marker serum measurements may be valuable to select high-risk patients with more invasive or nodal-positive disease.

MeSH Keywords: **Biological Markers • Matrix Metalloproteinase 2 • Urinary Bladder Neoplasms • Vascular Endothelial Growth Factor D**

Abbreviations: **CSS** – cancer-specific survival; **ECM** – extracellular matrix; **LN** – lymph node; **LVI** – lymphovascular invasion; **ml** – milliliters; **MMP** – matrix metalloproteinases; **OS** – overall survival; **RC** – radical cystectomy; **rpm** – revolutions per minute; **ROC** – receiver-operating characteristic; **TIMP-2** – tissue inhibitor metalloproteinase-2; **TURB** – transurethral resection of the bladder; **UCB** – urothelial carcinoma of the bladder; **VEGF** – vascular endothelial growth factor; **VEGF-R** – vascular endothelial growth factor receptor

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Background

Urothelial carcinoma of the urinary bladder (UCB) is one of the most frequent types of cancer worldwide, with an estimated 74 690 new cases and 15 580 deaths in the United States in 2014 [1]. Of all newly diagnosed cases of UCB, about 70% present as superficial tumors (stages Ta, T1, or tumors *in situ* [Tis]), but as many as 50–70% of those superficial tumors will recur and roughly 10–20% will progress to muscle-invasive disease (T2–4) [2]. Radical cystectomy (RC) with extended pelvic lymph node (LN) dissection is the standard treatment for localized muscle-invasive bladder cancer [3] and provides local control and long-term cancer-specific survival of the patients [4].

Angiogenesis and lymphangiogenesis play a critical role in tumor growth and systemic dissemination of cancer cells and seem to be of prognostic relevance in UCB [5–7]. Nevertheless, the mechanisms of angiogenesis and lymphangiogenesis are complex. Vascular endothelial growth factors (VEGFs) and their receptors (VEGF-R) are some of the key angiogenic factors that stimulate the formation of new blood vessels and tumor growth [7,8]. In recent years, several studies have shown that VEGFs and VEGF-Rs have an influence on metastatic spread and disease recurrence in various carcinomas including UCB [9–13].

Matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases collectively capable of degrading essentially all components of the extracellular matrix (ECM) [14]. MMPs have important functions in pathologic conditions characterized by excessive degradation of ECM, such as tumor invasion and metastasis [15,16]. In the present study, we focused on those MMPs having the most impact on bladder carcinogenesis, such as: the gelatinases MMP-2 [17,18], the stromelysin MMP-3 [16] and the matrilysin MMP-7 [14,16].

MMPs and VEGFs are important players in tumor angiogenesis and tumor growth [19] and there is some evidence that MMPs may interact with proteins of the VEGF-family [20,21]. Both, MMPs and the VEGF-family may therefore serve as potential prognostic and/or therapeutic targets in like bladder cancer.

The relevance of all the mentioned stromal variables as prognostic parameters for UCB is unclear and none of them are implemented in daily routine practice. Furthermore, not all have been investigated and contemporaneously tested in serum of patients with UCB.

This study was designed to evaluate the expression of various stromal variables involved in angio- and lymphangiogenesis in serum samples of patients with UCB undergoing RC and to compare them with clinical and histopathological characteristics as well as with clinical outcome.

Material and Methods

Patients

A total number of 91 individuals divided in three different groups were included in this study: The main cohort consisted of 71 serum samples from patients with aggressive UCB who underwent RC with bilateral pelvic LN dissection at the University Hospital Zurich between 2009 and 2013. Additional blood serum samples of patients were investigated as controls: One group consisted of 10 patients with histologically confirmed non-invasive UCB (pTa) during transurethral resection of the bladder (TURB). The other group comprised 10 patients with normal white light cystoscopy and no history of UCB. Serum samples were all taken preoperatively or before white light cystoscopy.

All pathological specimens were processed according to standardized institutional protocols. TURB and RC specimens were staged according to the TNM classification [3]. For this study, only post-RC patients were followed on a regular basis. Every 6 to 12 months a CT scan was performed to exclude metastatic disease. The median follow-up period for the RC cohort (n=71) was 17.2 months (range 0.4 to 48.5 months). The study was approved by the local scientific ethics committee Kantonale Ethikkommission Zürich (*http://www.kek.zh.ch/*, approval no.: StV-Nr. 02/2009 & 0352/2012). Each patient signed an informed consent.

Serum level measurements

Serum samples were obtained preoperatively or before white light cystoscopy. Venous blood (10 ml) was collected and centrifuged at 900 rpm for 10 minutes. The serum was then separated, aliquoted in 2 ml fractions and stored at -80°C until further use. Protein serum levels were assessed by the multiplexed particle-based flow cytometric cytokine assay (Vignali, 2000). Kits for VEGF-A, VEGF-C and VEGF-D were purchased from Millipore (Zug, Switzerland), for MMP-2, MMP-3 and MMP-7 from R&D Systems (Oxon, UK) and for VEGF-R2 and VEGF-R3 from eBioscience (Vienna, Austria). The procedures closely followed the manufacturer's instructions. The analysis was conducted using a conventional flow cytometer (Guava EasyCyte Plus, Millipore, Zug, Switzerland) [22].

Statistical analysis

All statistical analyses were performed using SPSS Statistics version 22 (SPSS, IBM Corp., Armonk, NY). Associations between measured parameters were assessed using the chi-square test for categorical variables and the Mann-Whitney U test for continuous variables. In order to find the optimal cut-off point for continuous variables with significant differences

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between clinicopathological features, receiver-operating characteristic (ROC) curves were created for different outcome measures at interest. The optimal value for each outcome parameter was identified by minimising the distance from the ROC curve to the top left corner of the ROC plot [23]. To analyze different groups of patients regarding the serum expression of investigated markers, the ANOVA test was used. Time to cancer-specific survival (CSS) was calculated from the date of RC to the date of death (death only from primary cancer). Time to overall survival (OS) was calculated from the date of RC to the date of death (death from all causes). Differences between survival estimates were evaluated by the log-rank test or by Cox regression analyses. Differences were considered significant at $p<0.05$.

Results

Clinicopathologic charateristics and their association with prognosis

All clinicopathologic characteristics of the RC cohort are summarized in Table 1. The median age of patients at the time of diagnosis was 72.4 years (range 45–87) and 55 (78%) were male. Among the 71 patients analyzed, organ-confined tumor stages (pTis to ≤pT2) were present in 34 (48%) and extravesical disease (pT3 and pT4) in 37 (52%).

Univariate analysis of clinicopathologic features for CSS and OS is provided in Table 2. Altogether, 28 deaths were documented (39.4%). CSS occured in 20 cases (28.2%). The presence of LVI, LN metastasis, venous invasion, and higher tumor stage were all associated with shorter CSS and OS in univariate analysis. LVI and positive LN staging ($p<0.001$ and $p=0.001$, respectively) were the best predictors for CCS and OS.

Expression of stromal variables in blood serum and its association with clinicopathologic parameters and prognosis

Protein levels of VEGFs, VEGF-Rs, and MMPs detected in in serum were compared with clinicopathologic characteristics (age, sex, tumor stage, adjacent carcinoma *in situ*, LVI, venous invasion, LN staging) by using the Mann-Whitney U Test. VEGF-D serum levels were positively associated with positive LN stage ($p=0.002$) and LVI ($p=0.019$), whereas MMP-2 serum levels showed an inversive association with positive LN stage ($p=0.062$). None of the other markers were associated with any of the tested clinicopathologic characteristics (data not shown). After defining optimal cut-off points for VEGF-D and MMP-2 regarding the outcome LN metastasis, the parameters were again compared with LN metastasis and with survival. The results are depicted in Table 3. In keeping with previous

Table 1. RC cohort patient and tumor characteristics and results of investigated stromal variables.

Variable	Categorization	n analyzable *	%
Total (n=71)*			
Clinicopathologic data:			
Month of follow-up (mean, range)		18.83 (0.4–48.5)	
Age at diagnosis (median, range)		72.4 (45–87) years	
	<70 years	33	47
	≥70 years	38	53
Sex			
	Female	16	22
	Male	55	78
Tumor stage (WHO 1973**)			
	pTis	3	4
	pTa	2	3
	pT1	13	18
	pT2	16	23
	pT3	20	28
	pT4	17	24
Histologic grade (WHO 2004***)			
	Low grade	0	0
	High grade	71	100
Adjacent carcinoma *in situ*			
	No	61	86
	Yes	10	14
Lymphovascular invasion			
	No	48	68
	Yes	23	32
Venous Invasion			
	No	57	80
	Yes	14	20
Lymph node positive			
	No	49	69
	Yes	22	31
Investigated serum markers			
VEGF-A (pg/mL) (median, range)		260 (40–892)	
VEGF-C (pg/mL) (median, range)		126 (61–313)	
VEGF-D (pg/mL) (median, range)		368 (25–2284)	
VEGF-R2 (ng/mL) (median, range)		17.2 (5.7–33.2)	
VEGF-R3 (ng/mL) (median, range)		98.6 (35.2–199.1)	
MMP-2 (ng/mL) (median, range)		117 (54–535)	
MMP-3 (ng/mL) (median, range)		12.3 (4.9–38.6)	
MMP-7 (pg/mL) (median, range)		1993 (828–19057)	

* All patients who underwent RC; ** Staging and grading according to the 1973 WHO classification system; *** Staging and grading according to the 2004 WHO classification system.

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Table 2. Univariate analysis of clinicopathological features for cancer specific survival and overall survival (n=71).

Variable	Categorization	Cancer specific survival (CSS)			Overall survival (OS)		
		n	Events	p*	n	Events	p*
Age at diagnosis							
	<70 years	33	10	0.397	33	12	0.818
	≥70 years	38	10		38	16	
Sex							
	Female	16	6	0.228	16	8	0.218
	Male	55	14		55	20	
Tumor stage (WHO 1973**)							
	<pT3	34	5	**0.001**	34	9	**0.003**
	≥pT3	37	15		37	19	
Adjacent carcinoma *in situ*							
	No	61	18	0.289	61	26	0.09
	Yes	10	2		10	2	
Lymphovascular invasion							
	No	48	7	**<0.001**	48	14	**<0.001**
	Yes	23	13		23	14	
Venous Invasion							
	No	57	14	**0.026**	57	20	**0.019**
	Yes	14	6		14	8	
Lymph node positive							
	No	49	6	<0.001	49	14	**0.001**
	Yes	22	14		22	14	

* Log Rank test (2-sided); bold face representing p-values <0.05; ** Staging and grading according to the 1973 WHO classification system.

Table 3. Comparison of VEGF-D and MMP-2 (dichotomized) with lymph node stage and survival (n=71).

Variable	Categorization	VEGF-D			MMP-2		
		<300 pg/mL	≥300 pg/mL	p	<100 ng/mL	≥100 ng/mL	p
Lymph node positive*							
	No	23	26	**<0.001**	12	37	**0.017**
	Yes	1	21		12	10	
Cancer-specific death (20 events)**							
	No	6	14	0.463	7	13	0.488
	Yes	18	33		17	34	
Overall death (28 events)**							
	No	11	17	0.772	10	18	0.493
	Yes	13	30		14	29	

* Fisher's exact Test (2-sided); bold face representing p-values <0.05; ** Log Rank test (2-sided); bold face representing p-values <0.05.

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Supplemental Table 1. Univariate cox regression analyses of clinicopathological parameters and tested serum markers for cancer-specific survival.

Variable	Univariate analysis			
	HR	95% CI		p value
Clinico-pathological parameters				
Age (<70 *vs.* ≥70 years)	0.674	0.269	1.688	0.400
Tumor Stage (WHO1973) (<T3 *vs.* ≥T3)	5.188	1.706	15.778	**0.004**
Adjacent carcinoma *in situ*	0.462	0.107	1.997	0.301
Lymphovascular invasion	8.979	3.116	25.874	**<0.001**
Venous Invasion	2.875	1.084	7.626	**0.034**
Lymph node positive	10.188	3.332	31.15	**<0.001**
Serum markers				
VEGF-A (pg/mL)	1.001	0.998	1.003	0.708
VEGF-C (pg/mL)	0.998	0.990	1.006	0.619
VEGF-D (pg/mL)	1.000	0.999	1.001	0.383
VEGF-R2 (ng/mL)	0.967	0.894	1.046	0.404
VEGF-R3 (ng/mL)	1.000	0.988	1.012	0.989
MMP-2 (ng/mL)	1.000	1.000	1.000	0.737
MMP-3 (ng/mL)	1.000	1.000	1.000	0.478
MMP-7 (pg/mL)	1.000	1.000	1.000	0.711

Univariate cox regression analyses of clinicopatholgical parameters and tested serum markers for CSS. Significant associations were found between CSS and higher tumor stage (p=0.004), LVI (p<0.001), venous invasion (p=0.034) and positive LN stage (p<0.001). None of the tested serum markers showed any association with CSS in univariate cox regression analysis.

findings, VEGF-D serum levels (cutoff ≥300 pg/mL) correlated positively with LN stage (p<0.001) and with LVI (p=0.015). Furthermore, low MMP-2 serum levels (cutoff ≥100 ng/mL) were associated with LN metastasis (p=0.017). Using these cut-off points, VEGF-D showed a high sensitivity (0.95) and low specificity (0.47), while MMP-2 showed good specificity and low sensitivity (sensitivity=0.55, specificity=0.76) for the detection of LN metastasis. To determine if a correlation between high VEGF-D and low MMP-2 serum levels exists, linear regression was performed. Linear regression showed no association between serum levels of VEGF-D and MMP-2 by giving a low coefficient of determination (R^2=0.027).

No associations were found between these 2 tested serum markers and CSS and overall deaths, respectively (Table 3). Additionally, further cut-off points for VEGF-D (≥500, ≥1000 and ≥1500 pg/mL) and MMP-2 (≥200, ≥300, and ≥400 ng/mL) have been evaluated for its association with disease course. None of the cut-off points for these 2 markers showed any association with CSS (data not shown). VEGF-D and MMP-2, as well as all other tested serum markers, showed no association with CSS when tested as continuous variables in Cox regression analysis (Supplemental Table 1).

Serum levels of stromal variables considering grade of invasiveness

For the analysis of serum levels in patients with invasive UCB in comparison with non-invasive UCB or healthy individuals, additional blood serum samples of patients with non-invasive UCB or no history of UCB were investigated. Figure 1 shows boxplots of all investigated stromal markers.

VEGF-A (p<0.001), VEGF-C (p<0.001) and MMP-7 (p=0.005) were elevated in serum of patients with invasive UCB compared to patients with non-invasive UCB or healthy individuals. In contrast, lower MMP-2 (p<0.001) serum levels were found in the invasive UCB group compared to the non-invasive UCB group or negative controls. No significant differences between the expression in UCB patients, non-invasive UCB patients, and healthy individuals were found for VEGF-D (p=0.588), VEGF-R2 (p=0.168), VEGF-R3 (p=0.074), or MMP-3 (p=0.509).

Discussion

Lymphatic metastasis is a common early event in aggressive UCB, and the presence of LN metastasis is associated with

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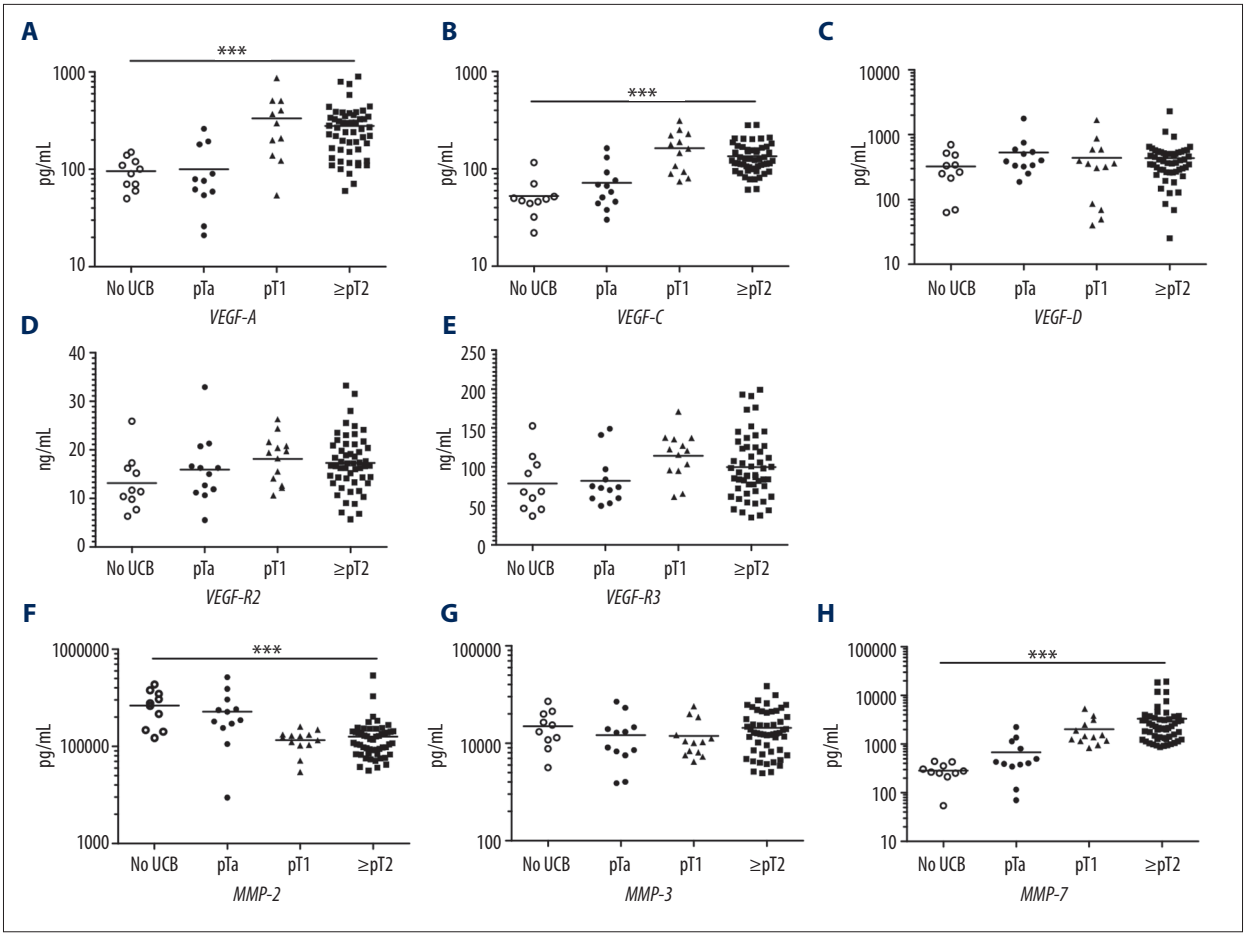

Figure 1. Markers were measured in serum of patients with invasive UCB (n=66) and in patients with pTa UCB or no history of UCB (n=22). For VEGF-A, only 85 samples could be evaluated. Dots represent patient samples. Horizontal lines represent mean values. *** p<0.001; ** p<001; * p<0.05. VEGF-A (**A**) and VEGF-C (**B**) were elevated in serum of patients with invasive UCB compared to patients with non-invasive (pTa) UCB or no history of UCB. VEGF-D (**C**), VEGF-R2 (**D**), VEGF-R3 (**E**) were equal in serum of patients with invasive UCB compared to patients with non-invasive (pTa) UCB or no history of UCB. MMP-2 (**F**) was lower in serum of patients with invasive UCB compared to patients with non-invasive (pTa) UCB or no history of UCB. MMP-3 (**G**) was equal in serum of patients with invasive UCB compared to patients with non-invasive (pTa) UCB or no history of UCB, whereas MMP-7 (**H**) was elevated in serum of patients with invasive UCB compared to patients with non-invasive (pTa) UCB or no history of UCB.

poor outcome following radical surgery. The presence of LVI and lymphangiogenesis can be considered as morphologic precursors for the ocurrence of micrometastasis in clinically localized forms of UCB [12,24]. A range of experimental studies have suggested that various stromal variables involved in lymphangio- and angiogenesis may serve as biomarkers for the diagnosis and prognosis of patients with UCB [25]. However, few studies have been conducted to investigate the clinical and prognostic value of these markers in patients' sera. Our study is the first comprehensive analysis of the VEGF family and MMPs in the serum of patients with bladder cancer. We found that high VEGF-D levels and low MMP-2 serum levels were more common in LN-positive disease. Furthermore, 4 investigated markers (VEGF-A, VEGF-C, MMP-2, and MMP-7) were changed in invasive UCB compared to non-invasive UCB or negative controls and might therefore be potential candidates for the prediction of invasive bladder cancer.

The expression of VEGF-D plays a major role during the metastatic process in various cancers, including UCB [8,9]. A recent study by von Hardenberg et al. [12] on the expression and predictive value of lymph-specific markers in UCB specimens of 119 patients showed that VEGF-D overexpression was significantly associated with LVI, LN involvement, and reduced CSS. Another study, by Herrmann et al. [26], investigated the role of VEGF-D in RC specimens of patients with UCB. VEGF-D overexpression correlated with higher stage, LN metastasis, and reduced CSS. Unlike these publications, Miyata et al. [6] reported that VEGF-D was not associated with disease outcome. Similar to these tissue-based results, we observed a correlation

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of VEGF-D serum levels with LVI and LN metastasis. However, because VEGF-D did not show any association with more invasive disease or disease outcome, it might be not an ideal serum biomarker for UCB. In our study, VEGF-D serum levels were associated with low specificity but had high sensitivity for the prediction of LN metastasis. Preoperative low VEGF-D serum levels might therefore put patients at very low risk of having LN-positive disease. However, more studies with more patients are needed to confirm this hypothesis.

Matrix metalloproteinases are the most important proteolytic enzymes catalyzing the degradation of ECM, which is essential for tumor invasion and metastasis [16]. An important representative of MMPs, MMP-2, physiologically degrades typ IV collagen, the major component of the basement membrane. Conflicting results have been reported regarding the prognostic value of MMP-2 in bladder cancer. Earlier studies by Vasala et al. [17] and by Kanamaya et al. [27] claimed high mRNA and protein levels to be significant risk factors for poor disease-specific survival, while Hara et al. [28] and Grignon et al. [29] found no correlation between MMP-2 levels and prognosis. MMP-2 levels in serum were observed to be similar in bladder cancer patients and healthy controls, but higher in patients at increased risk for disease recurrence and cancer-associated death [30]. Others found MMP-2 plasma levels to be elevated in patients with UCB compared to healthy controls, but without prognostic relevance [18]. Vasala et al. [31] showed that MMP-2 serum expression in tumor tissue was decreased in comparison with controls, but no correlation was found with pathological parameters. Considering our cohort, we observed that decreased serum levels of MMP-2 correlated inversely with more invasive and LN-positive disease. These controversial results may be explained by the fact that MMP-2 is also present in the serum as a proactive zymogen and in complexed form with tissue inhibitor metalloproteinase-2 (TIMP-2). Serum levels of these inactive MMP-2 variants were shown to be independent favorable prognostic factors in bladder cancer, indicating that endogenous inhibition of MMP-2 activity has not only biological but also prognostic relevance [17,31]. Furthermore, another study by Vasala et al. showed that a decrease in circulation MMP-2: TIMP-2 complex levels was associated with unfavorable survival in bladder cancer patients [32]. Our results seem to be in line with these findings, as the assay we used in our study can also measure the proactive form of MMP-2. Another reason for our findings may be based on the fact that we assessed MMP-2 serum levels by a multiplexed particle-based flow cytometric cytokine assay and not an ELISA assay. To the best of our knowledge, the only work that investigated MMP-2 serum levels in UCB by multiplexed particle-based flow cytometric cytokine assay found that MMP-2 serum levels were decreased in less invasive UCB, which is in line with our investigation [33]. Further studies, testing different methods and antibody kits for the measurement of MMP-2 in the serum of bladder cancer patients are needed to address this issue.

For MMP-7, we found elevated serum levels in more invasive UCB but no assocation with disease course. However, others have assessed MMP-7 serum levels in UCB and found that elevated MMP-7 serum levels were associated with lymph node metastasis [14] and shorter CSS [14,33,34].

Here, we propose that LN-positive UCB is characterized by high VEG-D serum levels and low MMP-2 serum levels. Both VEGF-D and MMP-2 are considered to be involved in angiogenesis. There is some evidence that MMPs interact with the VEGF-family through different signaling pathways [21,35,36]. Recently, it was postulated that VEGF may regulate MMP-2 in glioblastoma [21]. However, there are no reports of a direct connection or interaction between VEGF-D and MMP-2.

VEGF-A and VEGF-C are other key players in the processes of angiogenesis and lymphangiogenesis [8,9]. Miyata et al. [6] reported that expression of VEGF-A and VEGF-C in tissue samples of 126 patients with nonmetastatic bladder cancer correlated positively with tumor stage and grading. In line with these findings, we found that both VEGF-A and VEGF-C serum expression levels had a significant association with tumor invasiveness. However, no correlation was found between serum marker levels and nodal-positive disease.

For VEGF-R2 and VEGF-R3 serum levels we found no associations with clinico-pathological features or prognosis in bladder cancer. There is no literature available regarding VEGF-Rs in serum of UCB. However, one study has reported that higher VEGF-R3 expression in tumours is associated with reduced CSS in UCB [12].

An enhanced identification of patients who are at high risk of disease progression or cancer-specific death despite radical surgery remains an important challenge in the treatment of UCB. Taken together, the results of our study suggest that some of the investigated stromal variables indicate LN-positive disease and/or a higher stage of tumor invasiveness. As a consequence, clinical implementation of serum assays might be valuable to better select patients at high risk for more invasive disease than assessed preoperatively by TURB. Moreover, stromal variable serum measurements might have potential for identifying patients with presumable LN metastasis who may benefit from more aggressive treatment strategies. However, further studies need to be conducted to confirm this hypothesis and to assess the prognostic value of these tested markers in larger cohorts.

Our study has some limitations, including its rather small number of patients and the relatively short follow-up period, which

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limits the ability to draw firm conclusions. However, our cohort is representative, as all well known pathological indicators for poor prognosis worked well to predict CSS or OS. The lack of tissue analysis in our study for these proteins has to be considered as another limitation.

Further studies with more patients are needed to clarify the role of VEGF-D and MMP-2 in bladder cancer. It would be of interest to perform different methods for protein serum measurements and to look at the expression levels in tumor tissue.

Conclusions

We showed that higher VEGF-D serum levels are more common in patients with LVI or LN metastasis at the time of RC. Furthermore, low serum levels of MMP-2 defined invasive UCB and were more frequent in patients with node-positive disease. In addition, VEGF-A, VEGF-C and MMP-7 serum levels varied significantly between invasive and non-invasive disease, as well as in comparison with healthy individuals. However, none of the investigated markers were of prognostic value. Our findings suggest that implementation of these serum measurements might be useful to select high-risk patients with more invasive or nodal-positive disease.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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