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ARTICLE

Molecular Diagnostics

Multicentric validation of diagnostic tests based on BC-116 and BC-106 urine peptide biomarkers for bladder cancer in two prospective cohorts of patients

Lourdes Mengual[1,2], Maria Frantzi[3]✉, Marika Mokou[3], Mercedes Ingelmo-Torres[1], Michiel Vlaming[4], Axel S. Merseburger[5], Marie C. Roesch[5], Zoran Culig[6], Antonio Alcaraz[1], Antonia Vlahou[7], Harald Mischak[3,8] and Antoine G. Van der Heijden[4]

BACKGROUND: Non-invasive urine-based biomarkers can potentially improve current diagnostic and monitoring protocols for bladder cancer (BC). Here we assess the performance of earlier published biomarker panels for BC detection (BC-116) and monitoring of recurrence (BC-106) in combination with cytology, in two prospectively collected patient cohorts.
METHODS: Of the 602 patients screened for BC, 551 were found eligible. For the primary setting, 73 patients diagnosed with primary BC ($n = 27$) and benign urological disorders, including patients with macroscopic haematuria, cystitis and/or nephrolithiasis ($n = 46$) were included. In total, 478 patients under surveillance were additionally considered (83 BC recurrences; 395 negative for recurrence). Urine samples were analysed with capillary electrophoresis-mass spectrometry. The biomarker score was estimated via support vector machine-based software.
RESULTS: Validation of BC-116 biomarker panel resulted in 89% sensitivity and 67% specificity ($AUC_{BC\text{-}116} = 0.82$). A diagnostic score based on cytology and BC-116 resulted in good ($AUC_{Nom116} = 0.85$) but not significantly better performance ($P = 0.5672$). A diagnostic score including BC-106 and cytology was evaluated ($AUC_{Nom106} = 0.82$), significantly outperforming both cytology ($AUC_{cyt} = 0.72$; $P = 0.0022$) and BC-106 ($AUC_{BC\text{-}106} = 0.67$; $P = 0.0012$).
CONCLUSIONS: BC-116 biomarker panel is a useful test for detecting primary BC. BC-106 classifier integrated with cytology showing >95% negative predictive value, might be useful for decreasing the number of cystoscopies during surveillance.

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BACKGROUND

The high recurrence rate of bladder cancer (BC) [1] with the substantial risk of progression [2], poses a major healthcare challenge and makes BC one of the most expensive cancers to manage [3]. Most incident BC cases (~75%) are non-muscle-invasive (NMIBC) tumours, exhibiting a 5-year recurrence rate of 50–70% and 10–30% progression rate to muscle-invasive disease (MIBC), thus necessitating long-term patient monitoring [4]. Timely detection of primary BC and efficacious surveillance of BC recurrence are vital for optimal patient outcomes. Cystoscopy remains the gold standard for BC detection [5]. However, it is an invasive [6] and expensive approach, having also potential complications while subtle recurrences can be easily missed [7]. Urine cytology is still the most accurate non-invasive test for BC detection having high sensitivity (84%) in high-grade (G3) tumours, but low sensitivity (16%) in low-grade tumours (G1), while in experienced hands specificity can reach values >90% [5]. However, in recent large prospective multicenter studies, the performance of urine cytology in contemporary practice proves to be lower than previously reported [8]. Thus, a need for a non-invasive approach for BC detection and monitoring is evident.

Liquid biopsy has yielded enormous interest in the field of BC [9] with urine being particularly attractive due to its direct contact with the tumour and its simple collection [10]. Urinary profiling data acquired by capillary electrophoresis coupled to mass spectrometry (CE-MS) have been previously explored for detection of BC [11] and discrimination of NMIBC from MIBC [12], with multi-biomarker panels appearing advantageous compared to single biomarkers [13, 14]. Towards the need for reducing cystoscopies, we previously developed two urinary peptide biomarker panels for non-invasive detection of primary BC (BC-116) and for monitoring of recurrent BC (BC-106) in 1357 patients [15]. Both

[1]Laboratory and Department of Urology, Hospital Clinic de Barcelona, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain. [2]Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University of Barcelona, Barcelona, Spain. [3]Department of Biomarker Research, Mosaiques Diagnostics, Hannover, Germany. [4]Department of Urology, Radboud University Medical Center, Nijmegen, The Netherlands. [5]University Hospital Schleswig-Holstein, Campus Lübeck, Department of Urology, Lübeck, Germany. [6]Experimental Urology, Department of Urology, Medical University of Innsbruck, Innsbruck, Austria. [7]Systems Biology Center, Biomedical Research Foundation, Academy of Athens, Athens, Greece. [8]Institute of Cardiovascular and Medical Science, University of Glasgow, Glasgow, UK.
✉email: frantzi@mosaiques-diagnostics.com

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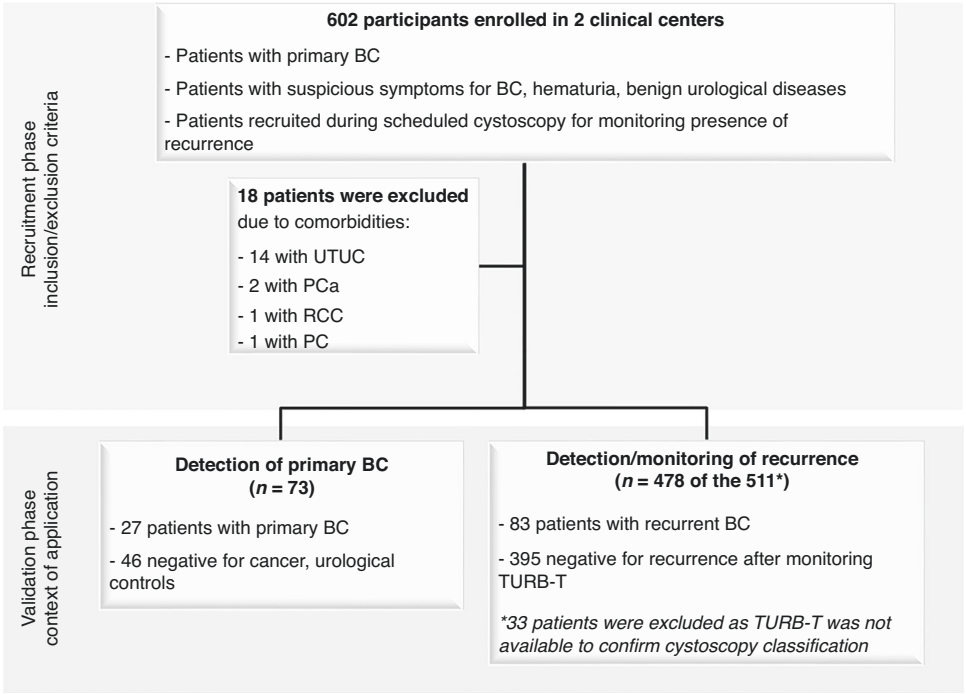


Fig. 1 Schematic representation of the study workflow. Patients with primary BC, recurrent BC and urological controls were enrolled at two different clinical centres (Hospital Clinic of Barcelona and Radboud University Medical Center). Patients encountering other comorbidities were excluded from this study ($n = 18$). A total number of 584 participants were considered for further analysis and stratified into two cohorts. The primary cohort was consisted of patients with primary BC ($n = 73$), whereas the recurrent cohort comprised patients with BC recurrences ($n = 83$) and those negative for recurrence controls ($n = 395$). BC bladder cancer, UTUC upper tract urothelial carcinoma, PC pancreatic cancer, PCa prostate cancer, RCC renal cell carcinoma, TURB-T transurethral resection of bladder tumour.

biomarker panels exhibited good performance, with BC-116 reaching 91% sensitivity and 68% specificity, and BC-106 87% sensitivity and 51% specificity, respectively. The area under the ROC curve values (AUCs) were 0.87 and 0.75, for detection of primary (BC-116) and recurrent (BC-106) urothelial BC [15], while BC-106 also demonstrated a prognostic potential for BC recurrence (HR:3.15; 1.73–5.70; 95% CI; $P = 0.0002$) [16]. Additionally, a pilot assessment showed added value of the biomarker panels when combined with cytology [15].

Considering this hypothesis, here we present a multicenter clinical study aiming to evaluate the diagnostic performance of the above biomarker panels. At first, the BC-116 biomarker panel was validated for the primary diagnosis of BC. In parallel, we aimed to validate the BC-106 panel for monitoring of recurrence in combination with cytology in a surveillance setting. To this end, 602 patients were recruited, those with suspicious symptoms that underwent cystoscopy to confirm the presence of BC, or those under surveillance that were scheduled for cystoscopic examination to investigate the presence of BC.

PATIENTS AND METHODS

Study population

A multicentric prospective analysis was conducted to investigate the study objectives according to the REMARK Reporting Recommendations [17] and the recommendations for biomarker identification and reporting in clinical proteomics [18]. The study protocol was drafted by the TransBioBC consortium, and power calculations were performed to estimate the sample size. Overall calculations were based on a binomial test for a single proportion, assuming a type I error of 5% (alpha = 0.05) and a power of 90% (beta = 0.10) in a two-sided test and improvement of sensitivity to 90% (reference sensitivity of cystoscopy was considered). Sample size estimation included 38 cases and 342 controls for the surveillance setting (assuming a prevalence of disease 0.10) and 20 cases and 46 controls for primary setting (assuming prevalence of disease 0.30).

In total, 602 patients with suspicious symptoms for BC (including the presence of macroscopic haematuria, and/ or suffering from other urological diseases (e.g., acute cystitis, nephrolithiasis) and those patients under surveillance were enrolled at the Radboud University Medical Center in Nijmegen (The Netherlands) and at the Hospital Clinic of Barcelona (Spain) between 2015 and 2016. Out of the 602 patients, 18 were excluded due to the presence of other cancers/comorbidities including: (i) upper urinary tract cancer ($n = 14$), (ii) prostate cancer ($n = 2$), (iii) renal cell carcinoma ($n = 1$) and (iv) pancreatic carcinoma ($n = 1$). Of the remaining 584 patients, 33 initially defined as positive for BC (based on cystoscopy) were further excluded as histopathological data based on white-light transurethral resection of the bladder tumour (TURB-T) or biopsy reported a benign lesion. Therefore, the positive cystoscopy result could not be confirmed. For the biomarker panel validation, the patients were stratified into two cohorts: (a) the primary group, including 73 patients positive for primary BC and those with suspicious symptoms scheduled for cystoscopy, and (b) the recurrent group, including 478 patients under surveillance, as presented in detail below.

Among the 551 eligible patients, the presence of bladder tumours was confirmed with cystoscopy and histological confirmation (biopsy or TURB-T) at the two clinical centres at the Hospital Clinic of Barcelona (366 patients) and at the Radboud University Medical Center (208 patients). Tumour grade and stage were determined according to WHO criteria [19] and Tumour Node Metastasis (TNM) classification [20], respectively. Tumours were classified according to their risk of recurrence and progression into high, intermediate, or low risk based on the EAU guidelines published in 2019 [5]. The study was performed in accordance with the Declaration of Helsinki. Informed consent processes adhered to Institutional Review Board-approved guidelines. Ethical approval for this study was obtained by the Ethics Committee at Medical School of Hannover (ID:3274–2016). A schematic representation of the study design is presented in Fig. 1.

Primary cohort

For assessing primary urothelial BC, 73 eligible participants were considered of those individuals presenting suspicious for BC symptoms and further underwent white-light cystoscopy to investigate the presence of malignancy. Forty- six patients were enrolled at the Hospital Clinic of Barcelona, whereas the remaining 27 were patients undergoing cystoscopy at Radboud

Table 1. Clinical and demographical characteristics of the patients screened for primary (Study Arm I) and recurrence BC (Study Arm II).

	Number of recruited patients
Study Arm I: evaluation of diagnostic score for detecting Primary BC	73
Primary BC patients (%)	27 (37.0)
Urological controls (%)	46 (63.0)
Median age (IQR)	66 (16.5)
Gender (M/F; %)	52 M (71.2)/21 F (28.8)
Stage classification (TNM) for primary BC*	
Ta (%)	14 (51.9)
T1 (%)	6 (22.2)
T3 (%)	1 (3.7)
Tis (%)	1 (3.7)
Tx (%)	5 (18.5)
Grade classification for primary BC*	
Low grade (%)	14 (51.9)
High grade (%)	10 (37.0)
Gx (%)	3 (11.1)
Risk assessment for primary BC*	
High risk (%)	11 (40.7)
Low risk (%)	15 (55.6)
Unknown (%)	1 (3.7)
Concomitant Tis (yes/no/unknown)	3 (11.1)/13 (48.2)/11 (40.7)
Cytology (positive/negative/NA)	14 (19.2)/44 (60.3)/15 (20.5)
Study Arm II: evaluation of diagnostic score for recurrent BC	478
Positive for recurrent BC (%)	83 (17.4)
Negative for recurrence (%)	395 (82.6)
Median age (IQR)	71 (15)
Gender (M/F; %)	381 M (79.7)/97 F (20.3)
Stage classification (TNM) for recurrent BC*	
Ta (%)	58 (69.9)
T1 (%)	11 (13.6)
T2 (%)	3 (3.6)
T4 (%)	1 (1.2)
Tis (%)	10 (12.0)
Grade classification for recurrent BC*	
Low grade (%)	55 (62.3)
High grade (%)	25 (30.1)
Gx (%)	3 (3.6)
Risk assessment for recurrent BC*	
High risk (%)	30 (36.1)
Intermediate risk (%)	27 (32.5)
Low risk (%)	25 (30.1)
Unknown (%)	1 (1.2)
Cytology (positive/negative/atypia/NA)	33 (6.9)/285 (59.6)/19 (4.0)/141 (29.5)
Excluded patients because of comorbidities	18
Excluded patients as TURB-T was not available to confirm cystoscopy	33
Total number of recruited patients	602

BC bladder cancer, *F* female, *IQR* interquartile range, *M* male, *NA* not available, *TNM* tumour, node, metastasis, *Tis* carcinoma in situ, *TURB-T* Transurethral resection of bladder tumour.
*Tumour grade and stage were determined according to WHO criteria [19] and TNM classification [20], while the BC tumours were classified according to their risk of recurrence and progression into high, intermediate or low risk based on the EAU guidelines published in 2019 [5].

University Medical Center. Among the 73 patients, 27 were diagnosed with primary urothelial BC, whereas the remaining 46 presented with suspicious symptoms for BC, including macroscopic haematuria, or suffered from other urological diseases (e.g., acute cystitis, nephrolithiasis) and served as urologic controls. At the time of recruitment, all patients with primary tumours did not have a prior history of urothelial cell carcinoma and underwent transurethral resection to radically remove the bladder tumour. BC was confirmed by cystoscopy and histological assessment. The cohort characteristics related to BC and subject demographics, among other age and gender variables, are summarised in Table 1, and the full list of patient clinical data is given in Supplementary Table S1.

Recurrent cohort

For the evaluation of BC recurrence, 511 patients scheduled for follow-up monitoring cystoscopies due to prior history of BC were analysed in compliance with the EAU guidelines and the European Organization for Research and Treatment of Cancer (EORTC) recurrence and progression scores [5]. Among these were 320 patients undergoing cystoscopy at the Hospital Clinic of Barcelona and 181 at the Radboud University Medical Center. In both clinical centres, BC recurrence was confirmed by cystoscopy and histological assessment. A negative score at cystoscopy was used to exclude recurrence and define controls. The recurrent cohort comprised 83 confirmed BC cases and 395 negative for recurrence controls. Of the 83 relapses, 79 were NMIBC (Ta, CIS, T1) and 4 MIBC (≥T2) cases. The cohort characteristics, including age and gender variables, are summarised in Table 1, and the full list of patient clinical data is given in Supplementary Table S2. Complete information about the clinicopathological tumour characteristics (including risk, stage and grade) for the previously diagnosed BC tumours is also provided (Supplementary Table S2).

Urine collection and cytologic evaluation

All urine samples were collected prior to cystoscopy. Voided urine samples were collected in sterile containers and immediately stored at −20 °C until further processing. From all patients and controls, only one single sample was included. Without being mandatory, patients were advised to have cytology at cystoscopy or during the period between cystoscopy and surgery. Urine cytology was performed according to Papanicolaou staining and evaluated by expert pathologists in each centre blinded to the patient's clinical history. The results were considered as positive or negative.

Sample preparation and CE-MS analysis

Urine sample preparation and CE-MS analysis were performed according to previous reports [15, 16, 21]. In detail, 700 µl of each urine sample were diluted with an equal volume of alkaline buffer containing 2 M urea, 10 mM NH_4OH and 0.02% SDS (pH 10.5). Thereafter, the samples were concentrated at 1.1 ml using Centrisart ultracentrifugation filters with a cut-off of 20 kDa (Sartorius, Göttingen, Germany) after centrifugation at 3000×*g*. The filtrate was then desalted using PD-10 columns (GE Healthcare, Munich, Germany) and equilibrated in 0.01% NH_4OH in HPLC-grade water. The peptide extracts were lyophilised and stored at 4 °C until further use. CE-MS analysis was performed using a P/ACE MDQ capillary electrophoresis system (Beckman Coulter, Fullerton, USA) online coupled to a MicroTOF MS (Bruker Daltonic, Bremen, Germany), as described previously [15, 16]. Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses using MosaiquesVisu software [22]. In addition, migration time and ion signal intensity (amplitude) were normalised using 29 internal peptide standards with excretion levels unaffected by any disease state [23]. The resulting peak list characterises each peptide by its molecular mass [kDa], normalised migration time (min), and normalised signal intensity. The detected peptides were annotated, matched and deposited in a Microsoft SQL database (Human Urinary Proteome Database [24, 25]) and used as input in this study. Accuracy, precision, selectivity, sensitivity, reproducibility and stability were reported previously [11, 21].

Statistical analysis

The biomarker scores were calculated via the support vector machine (SVM)-based software, MosaCluster (version 1.7.0) [15]. The list of scoring data is presented in Supplementary Table S3. The relationship of BC-116 and BC-106 panel with cytology was established using multiple linear regression analyses using MedCalc 12.7.5.0 (Mariakerke, Belgium). Additional clinical and demographic factors, like age, gender, previous grade and risk group, were also investigated but not found of added value to the

diagnostic score and thus were not considered (Supplementary Table S4). Sensitivity and specificity for the SVM-based peptide marker pattern were calculated based on the number of correctly classified samples, as defined by biopsy, considering the previously reported cut-off criteria. Receiver operating characteristic (ROC) plots and the respective confidence intervals (95% CI) were based on exact binomial calculations and were calculated in MedCalc 12.7.5.0 (Mariakerke, Belgium). The area under the ROC curve (AUC) was evaluated to estimate the overall accuracy independent of a particular threshold [26], and the values were then compared using DeLong tests. Statistical comparisons of the classification scores between stage an and grade groups were performed by the Kruskal–Wallis rank-sum test using MedCalc 12.7.5.0 (Mariakerke, Belgium) [27].

RESULTS

Validation of the BC-116 biomarker panel for detection of primary BC

For the validation of the BC-116 biomarker panel, 27 patients with primary urothelial BC and 46 patients presenting with non-malignant urological conditions were included. The AUC for BC-116 biomarker panel ($AUC_{BC\text{-}116}$) was estimated at 0.82 (0.71–0.90; 95% CI; $P < 0.0001$; Fig. 2a). At the validated cut-off level (−0.27), the sensitivity was estimated at 89% and the specificity at 67%. The classifier correctly classified 24 of 27 primary BC cases, whereas 15 of 46 urological controls were misclassified. Considering a prevalence rate of 37%, negative predictive value (NPV) was 91% (61–100%; 95% CI) while positive predictive value (PPV) was 61% (33–85%; 95% CI). The BC-116 biomarker panel significantly discriminated urothelial BC cases from controls ($P < 0.0001$; Kruskal–Wallis H test; Fig. 2b) but also separated controls from cases according to their TNM stage and grade ($P < 0.05$; Kruskal–Wallis H test; Fig. 2c, d). Considering high-grade BC, the $AUC_{BC\text{-}116}$ was estimated at 0.90 (0.78–0.96; 95% CI; $P < 0.0001$), NPV at 100% (49–100%; 95%CI) and PPV at 85% (18–100; 95% CI). For low-grade BC primary cases, the $AUC_{BC\text{-}116}$ was estimated at 0.76 (0.63–0.86; 95% CI; $P < 0.0005$), NPV at 93% (40–100%; 95% CI) and PPV at 54% (20–86; 95% CI). For 58 (of 73) patients with primary BC, cytology data were available (Supplementary Table 1). The sensitivity of cytology for detecting primary BC was estimated at 42.3%, while the sensitivity of the classifier was 88.5% in this subset of patients. Twelve of 26 primary BC cases were detected by the BC-116 biomarker panel but were missed by urine cytology. Out of twelve patients, eight were bearing low-grade BC tumours (TaG1) but were also three patients with high-grade NMBC (T1G3) and one with MIBC that were missed by cytology and detected by BC-116. Three cases that were not detected by the BC-116 panel were also missed by urine cytology (all patients bearing low-grade BC/TaG1). Along these lines, the NPV was higher for the BC-116 classifier (91.2%, 60.9–99.7%; 95% CI) than that of the urine cytology (73.2%; 49–90.2%; 95% CI). Nevertheless, PPV was higher for urine cytology (77.9%; 26.7–99.2; 95% CI) than for the BC-116 classifier (61.3%; 31.4–86.0%; 95% CI). Specificity of cytology (93.7%) was higher than that of the BC-116 panel (67.2%) respectively). Multivariate analysis showed that BC-116 biomarker panel performed significantly better than cytology in primary BC detection, presenting an AUC value 0.84 (0.72–0.92; 95% CI) for the classifier and 0.68 (0.55–0.80; 95% CI) for cytology ($P = 0.0195$; Fig. 3a).

Integrative diagnostic score including BC-116 and cytology to detect primary BC

An integrated diagnostic score based on the BC-116 urinary biomarkers and cytology was examined in the primary patient cohort. The AUC for the diagnostic score was estimated 0.85 (0.73–0.93; 95% CI; $P < 0.0001$; Fig. 3b) only slightly but not significantly higher than the BC-116 alone (0.84; $P = 0.5672$), yet significantly higher than cytology alone (0.68; $P = 0.0016$). At the optimal cut-off level (0.39), sensitivity was estimated at 84.6% and specificity at 75%. The diagnostic score correctly classified 23 of 26 primary BC cases, whereas nine of 32 controls were misclassified. Considering a prevalence rate of 37%, NPV was computed at 89.5% (60–99%; 95% CI) while PPV was estimated at 66.6% (35–90%; 95% CI).

Validation of the BC-106 biomarker panel for detection of recurrent BC

BC-106 biomarker panel for detection of BC recurrence was validated in 478 patients. 83 patients were bearing a histopathologically confirmed BC recurrence, and 395 were negative for recurrence (controls). The AUC was estimated at 0.67 (0.63–0.72; 95% CI; Fig. 4a). At the previously validated cut-off (−0.63), sensitivity was estimated at 90.4%, whereas specificity at 29.1%, meaning that 75 of the 83 recurrences were correctly classified by the BC-106 biomarker panel. However, the number of correctly classified negative controls was only 112. Accounting for a prevalence of 17.4% in the investigated population, NPV was estimated at 93.2% (73.3–99.6%; 95% CI) while the PPV was calculated at 21.1% (11.7–33.4%; 95% CI). Urine cytology was performed in 318 patients of which, based on cystoscopy, 36 were positive for recurrence cases, and 282 were negative for recurrence controls. The sensitivity of the BC-106 biomarker panel (91.6%) was higher than that of cytology (50.0%), while the specificity BC-106 (31.2%) was lower than that of cytology for the classifier (94.7%). The estimated AUCs were reported to be comparable between the two tests ($AUC_{BC\text{-}106}$: 0.67; AUC_{cyt}:0.72; $P = 0.3853$; Fig. 4b).

Integrative diagnostic score including BC-106 and cytology to detect recurrent BC

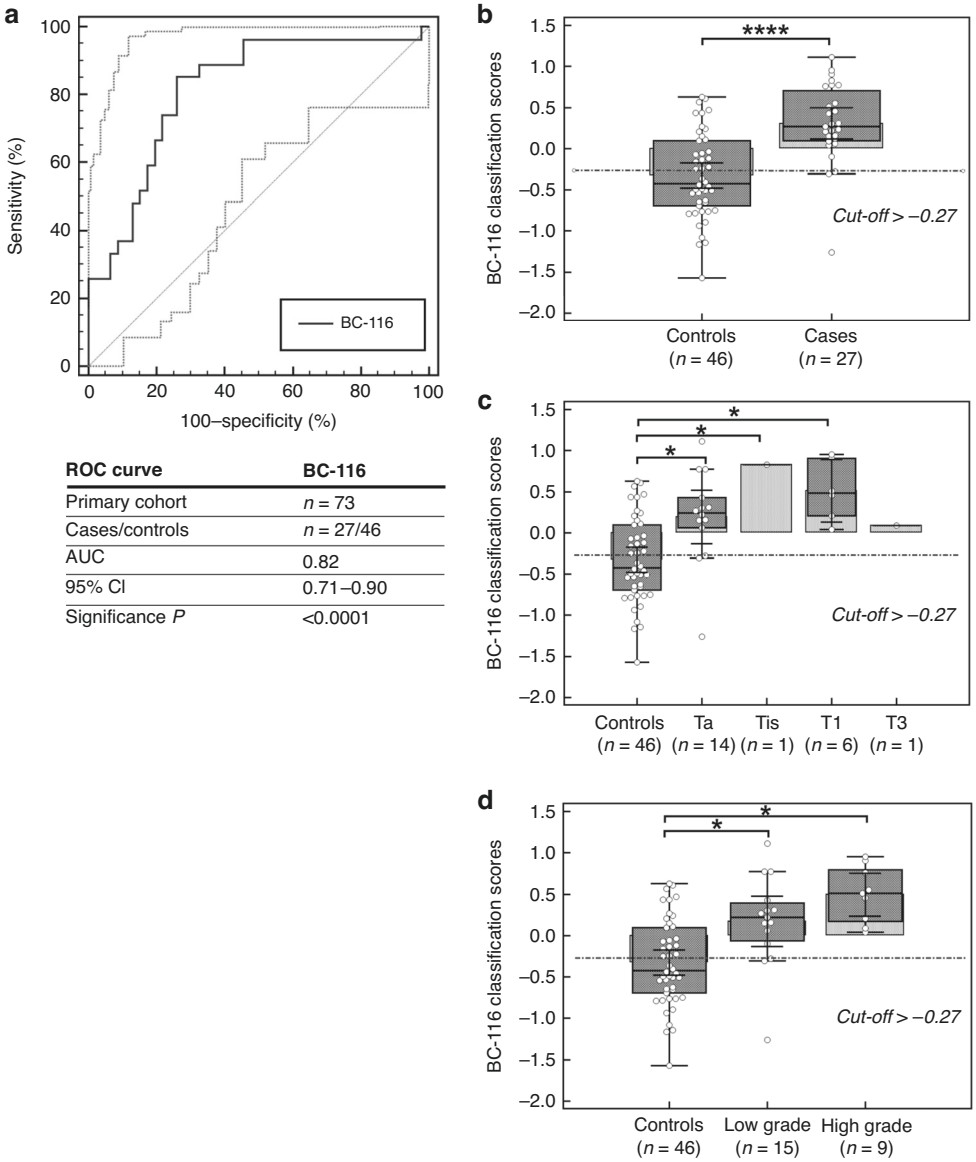
An integrative diagnostic score including the BC-106 CE-MS based biomarkers and cytology was validated in 318 patients for which cytology results were available. Improved diagnostic performance was observed (AUC = 0.82; 0.77–0.86; 95% CI, $P < 0.0001$; Fig. 5a), while at the optimal cut-off (0.06), sensitivity was estimated at 86%, whereas specificity at 61%. In all, 31 of the 36 recurrent BC cases and 172 of the 282 negative for recurrence patients were correctly classified. Accounting for a prevalence of 17.4%, NPV was estimated at 95.4% (74.9–99.9%; 95% CI) while the PPV at 31.7% (11.3–59.1%; 95% CI). The diagnostic score was found of significantly improved performance (AUC_{NOM106}:0.74; 0.69–0.78; 95% CI) compared to cytology alone (AUC_{cyt}:0.72; 0.67–0.77; $P = 0{,}0022$), but also to the BC-106 alone ($AUC_{BC\text{-}106}$:0.66; 0.61–0.72; 95% CI; $P = 0.0012$). The integrative diagnostic score significantly discriminated recurrent BC cases from negative for recurrence controls, ($P = 0.0005$; Kruskal–Wallis H test; Fig. 5b), but also separated patients with recurrent BC from those negative for recurrence according to their TNM stage and grade ($P < 0.05$; Kruskal–Wallis H test; Fig. 5c, d).

Reducing the number of follow-up cystoscopies by applying the integrative diagnostic score (BC-106/cytology)

In the participating clinical centres of the study, recurrence was detected in 15.6% of all follow-up cystoscopies, while 84% of the patients previously diagnosed with BC were without recurrence at the time of follow-up cystoscopy. At the cut-off point value of 0.06 of the integrative diagnostic score, sensitivity was 86%, whereas specificity at 61%. With a very high NPV value (estimated at 95.4%), 172 out of 282 patients truly did not bear any recurrence, while only 4 out of the 36 cases would have been missed. All four patients were carrying a low-grade BC (TaG1). Assuming that patients who present a negative classification based on the integrative diagnostic score ($n = 172$) will not undergo a cystoscopy, more than 60% of all cystoscopies could be prevented, at the cost of 14% of low-grade recurrences remaining undiagnosed.

DISCUSSION

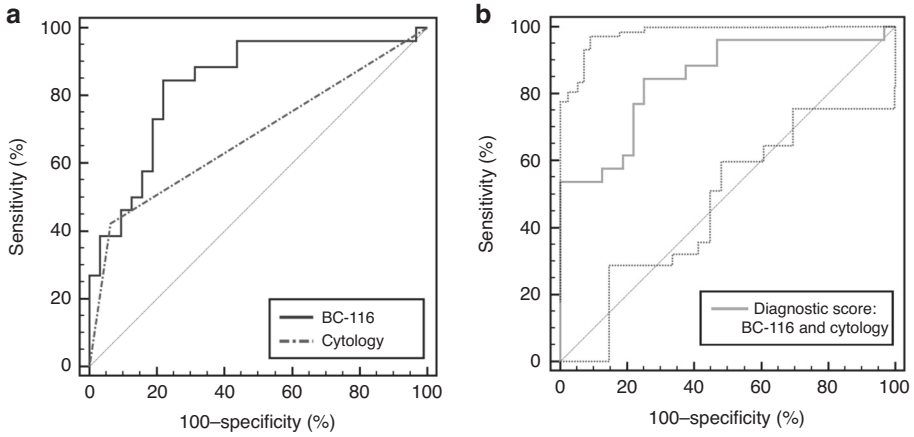
Several urinary biomarkers, including proteomics, have been developed for BC detection and monitoring (summarised in refs. [28, 29]), biomarkers [30]. To date, none of these has replaced



ROC curve	BC-116
Primary cohort	*n* = 73
Cases/controls	*n* = 27/46
AUC	0.82
95% CI	0.71–0.90
Significance *P*	<0.0001

Fig. 2 Performance characteristics of the urinary biomarkers in the primary cohort including 73 patients. a ROC curve analysis for the urinary biomarker panel BC-116 performed in the primary cohort. The AUC, 95% CI, and *P* value for the classification of the BC patients are also provided in the table shown in (**a**). **b**, **d** Classification scores presented in Box-and-Whisker displaying the level of discrimination between **b** the urothelial BC cases and the urologic controls, **c** BC cases and controls according to their TNM stage and **d** BC cases and controls considering their grade. The average rank differences were significantly different (****$P \leq 0.0001$ and *$P < 0.05$) as defined with Kruskal–Wallis H test.

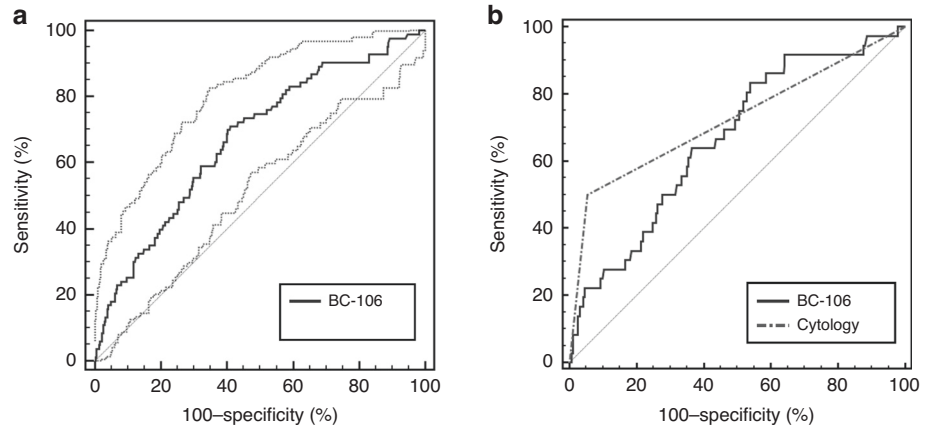
cystoscopy as the gold standard for diagnosis and surveillance or has been accepted for diagnosis or follow-up in routine practice [5]. The lack of validation studies presents a challenge in the expanding field of biomarker development, thus delaying their uptake in the clinical setting. In this multicentric prospective study, two previously published urinary peptide-based biomarker panels for detecting primary (BC-116 panel) and recurrent urothelial BC (BC-106) [15] were validated in prospectively collected patient cohorts. BC-116 biomarker panel for detection of primary BC [15] demonstrated excellent reproducibility in this multicentric validation study. BC-116 exhibited good performance (AUC_{BC-116}:0.82) that was comparable to the previously described estimates (AUC:0.87) [15]. Furthermore, BC-116 sensitivity (89%) and specificity (67%) were similar to those previously reported (91% and 68%, respectively) [15]. In this study, BC-116 again performed better than cytology and successfully classified most of BC cases (panel: 24/27 patients; cytology: 11/27 patients). Cytology missed 58% of the tumours, similarly to other studies [31] and did not identify any BC cases that were not previously identified by BC-116. Interestingly, 82% of the cases that were missed by cytology were correctly classified by the biomarker panel, while three cases that were missed by BC-116 were all low-grade BC. NPV of BC-116 was also higher than cytology's (91% versus 73%). An integrative diagnostic score including BC-116 and cytology was investigated, showing, however, only slightly increased performance over the BC-116 alone. BC-116 biomarker panel could be potentially applied in clinical practice prior to cystoscopy to guide the procedure. For detection of recurrence, the BC-106 panel exhibited similar performance than urine cytology, lower than previously reported [15]. The observed reduced performance is mostly attributed to the misclassification of almost half of the negative for recurrence patients. BC-106 reduced specificity could be attributed to differences in the study population and the demographics of the patients. In the initial study, patients with a minimum of 1-year of recurrence-free follow-up were



ROC curve	BC-116	Cytology
Primary cohort	*n* = 58	*n* = 58
Cases/controls	*n* = 26/32	*n* = 26/32
AUC	0.84	0.68
95% CI	0.72–0.92	0.55–0.80
Significance *P*	<0.0001	0.0008
Pairwise comparison	*P* = 0.0195	

ROC curve	BC-116 and cytology
Primary cohort	*n* = 58
Cases/controls	*n* = 26/32
AUC	0.85
95% CI	0.73–0.93
Significance *P*	<0.0001

Fig. 3 Comparative ROC curve analyses for the urinary biomarkers and the cytology. a ROC curves for the urinary BC-116 biomarker panel and the cytology as performed on those patients from the primary cohort with available cytological data. **b** ROC curve for the integrative diagnostic score, including the BC-116 biomarker panel and the cytology. The AUCs, 95% CI, and *P* values are provided in the respective tables.

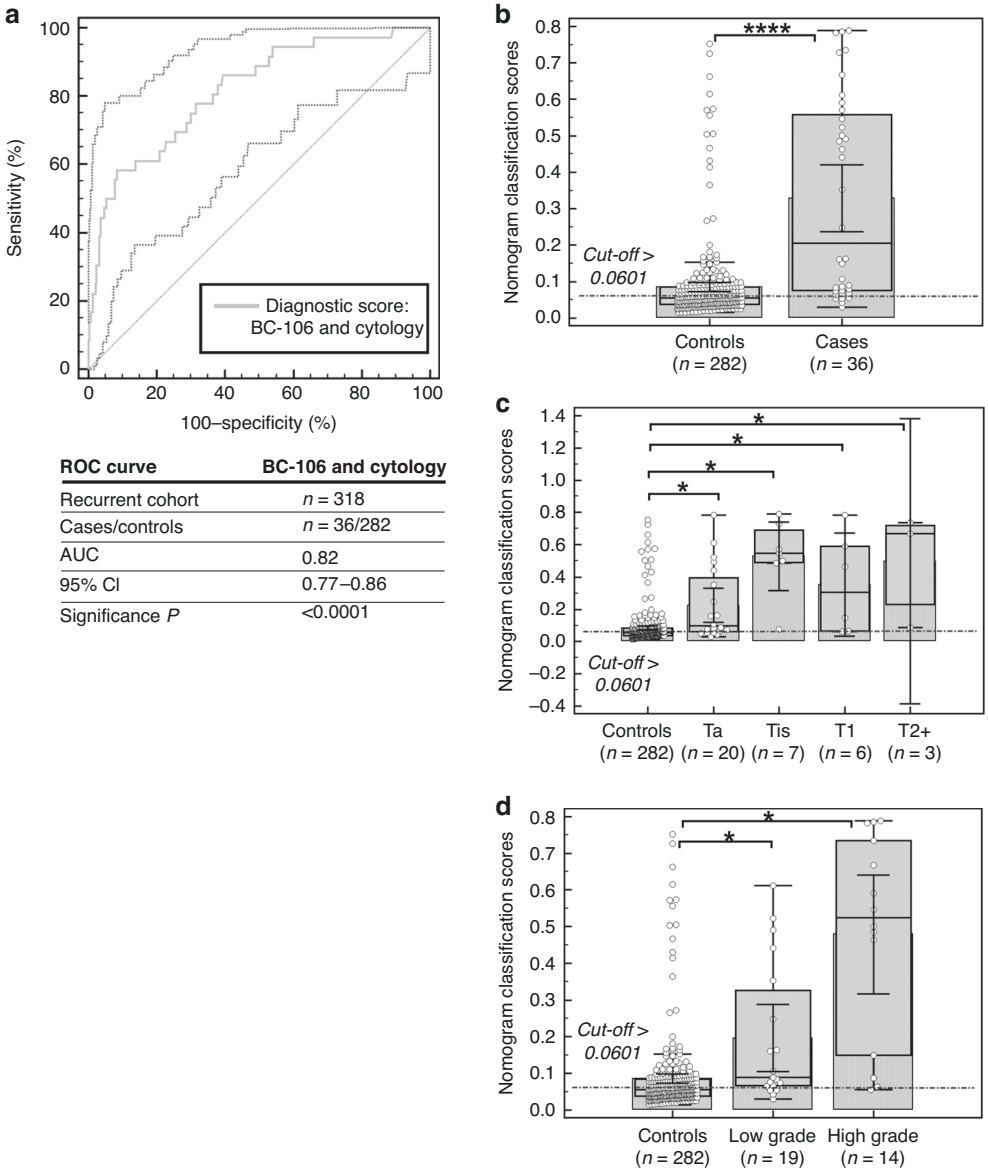


ROC curve	BC-106
Recurrent cohort	*n* = 478
Cases/controls	*n* = 83/395
AUC	0.67
95% CI	0.63–0.72
Significance *P*	<0.0001

ROC curve	BC-106	Cytology
Recurrent cohort	*n* = 318	*n* = 318
Cases/controls	*n* = 36/282	*n* = 36/282
AUC	0.67	0.72
95% CI	0.61–0.72	0.67–0.77
Significance *P*	0.0004	<0.0001
Pairwise comparison	*P* = 0.3853	

Fig. 4 Comparative ROC curve analyses for the urinary biomarkers and the cytology for the recurrence cohort. a ROC curve for the BC-106 urinary biomarker panel, consisting of 106 peptides as performed in the recurrent cohort. **b** ROC curve analysis for the urinary BC-106 biomarker panel as performed in those patients from the recurrence cohort with available cytological data. The AUCs, 95% CI and *P* values are provided for the classification of recurrent BC patients.

investigated, while in this prospective setting, any patient during the regular BC monitoring setting was included, mainly NMIBC (>98%). Importantly, BC-106 showed complementary value with cytology. The integrative diagnostic score including BC-106 and cytology demonstrated significantly increased performance compared to both cytology alone and the BC-106 alone. Accounting for NPV values of >90% for primary and >95% for surveillance, the availability of highly sensitive urine peptide markers, in combination with cytology allows for the reduction of the number of follow-up cystoscopies as in clinical practice only patients with a positive test undergoing cystoscopy, whereas for those with a negative test cystoscopy could be postponed/ skipped [32]. The integrative diagnostic score, including BC-106 classifier and cytology, ruled out 172 of the 282 patients, theoretically reducing the number of follow-up cystoscopies by factor 2. Based on the integrative BC-106 model, ~14% of the BC patients at follow-up were incorrectly misclassified as not having recurrence, but these were all low-grade recurrent BC. Yet, even cystoscopy which is the gold standard



ROC curve	BC-106 and cytology
Recurrent cohort	*n* = 318
Cases/controls	*n* = 36/282
AUC	0.82
95% CI	0.77–0.86
Significance *P*	<0.0001

Fig. 5 Performance characteristics of the integrative diagnostic score in the recurrence cohort. a ROC curve analysis for the integrative diagnostic score including the urinary BC-106 biomarker panel and cytology, as performed in 318 patients (consisted of 36 positive and 282 negative for recurrence controls) with available cytological data from the recurrent cohort. The AUC, 95% CI, and *P* value for the integrative diagnostic score are provided in the table in (**a**). **b**–**d** Classification scores presented in Box-and-Whisker displaying the level of discrimination between the recurrence BC cases and the negative for recurrence controls (**b**), the recurrence BC cases and the negative for recurrence controls among stage (**c**), and the recurrence BC cases and the negative for recurrence controls among grade groups (**d**). The average rank differences were significantly different (****$P \leq 0.0001$ and *$P = 0.05$) as defined by Kruskal–Wallis H test.

misses up to 15% of the papillary and up to 30% of the flat lesions [33]. Both BC-116 and BC-106 have similar performance as other tests approved by the U.S. Food and Drug Administration Food (FDA) and/or are currently under investigation in urinary tests, as recently reviewed in a metanalysis [34]. To date, there are six urinary tests approved by the FDA for clinical use in conjunction with cystoscopy [28, 29]. However, a direct comparison of our biomarkers with the FDA-approved based on the data currently available is complicated and difficult to be accomplished. A high NPV is important when the panel has a negative score and represents a point of reference to spare unnecessary cystoscopies. This value is critical especially in high-grade diseases where a negative biomarker test could result in a missed cancer, thus having detrimental effects with respect to disease progression [29]. In this study, the layout of the clinical settings closely represents a "real-clinical setting" situation. Considering the prevalence rate of each cohort in this study, the NPV was computed at 89.5% for the integrative diagnostic score of BC-116 classifier and cytology for detecting primary BC and at 95.4% for the integrative diagnostic score including BC-106 and cytology for detecting BC recurrence, comparable to the previous study [15]. Both panels demonstrated high NPV values that may offer benefit to patients with high-risk disease (either with haematuria at primary diagnosis or under surveillance) to spare unnecessary cystoscopies.

Limitations

One of the limitations of this study is that potential confounding factors such as clinical treatments before the last recurrence were not available for all patients and thus were not considered. Nevertheless, as shown in the initial study, prior treatment did not

affect the urinary peptidomic profiles [15]. Moreover, follow-up data were not accessible for all patients. Thus, correlation analysis of false-positives cases with later recurrences was not feasible. Therefore, no conclusion can be made on false positives of BC-116 and BC-106 biomarker panels since they could be attributed to early detection of subclinical recurrence that could not be detected by cystoscopy. This has been previously described for other FDA-approved urinary biomarkers [34]. Furthermore, no investigations were performed in patients that had a positive test but a negative cystoscopy to rule out the presence of upper tract tumours. Moreover, clinical application of such multi-parametric models is associated with increased analytical costs as it is based on an omics approach. In particular, the cost of mass spectrometry analysis is currently in the range of 500–1000€ per sample, also due to high instrument costs. In comparison to genomic testing that typically ranges between 300 to more than 10,000$ [35], the above costs are in fact lower. However, given the high accuracy of such tests, such a clinical application can be cost-effective when considering the benefits in BC patient management in terms of reducing the number of diagnostics and monitoring biopsies. From a practical point of view, a major advantage of analysing urine samples by mass spectrometry is that the sample can easily be shipped to a specialised laboratory. The feasibility and clinical applicability of capillary electrophoresis- mass spectrometry-based urinary peptidomics (CE-MS) has been demonstrated, among others, in multicentric randomised control trials [36, 37]. Among others, only a small volume (700ul) is required for the CE-MS analysis, and the samples can be shipped without the need of dry ice, by the use of boric acid tubes.

CONCLUSIONS

This multicentric study provides evidence on the clinical relevance of the BC-116 and BC-106 biomarker panels for BC monitoring. Such non-invasive biomarker panels can facilitate BC diagnosis (BC-116) and can be applied prior to cystoscopy in combination with cytology (BC-106) to reduce the number of follow-up cystoscopies as well as patient discomfort and financial burden.

DATA AVAILABILITY

All data (except raw files) generated or analysed during this study are included in this article (and its supplementary information files). Raw data are available upon request from the corresponding author.

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AUTHOR CONTRIBUTIONS

AGVdH had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: LM, MF, AV, HM and AGVdH; acquisition of the data: LM, MF, MI-T and MV; analysis and interpretation of the data: LM, MF, MM, MI-T, MV; drafting of the manuscript: LM, MF, MM; critical revision of the manuscript for important intellectual content: LM, MF, MM, MI-T, MV, ASM, MCR, ZC, AA, AV, HM and AGVdH; statistical analysis: LM, MF and MM; obtaining funding: MF; administrative, technical or material support: LM, AA, AV, HM and AGVdH; supervision: LM, ASM, MCR, ZC, AA, HM and AGVdH; other/software validation: MF and MM.

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COMPETING INTERESTS

Prof. HM holds ownership interest in Mosaiques Diagnostics GmbH. Dr. MF and Dr. MM are employed by Mosaiques Diagnostics GmbH. The remaining authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Informed consent forms were obtained and adhered to Institutional Review Board-approved guidelines. Ethical approval for this study was obtained by the Ethics Committee in Medical School of Hannover (ID:3274–2016). The study was performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

CONSENT TO PUBLISH

Not applicable.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Maria Frantzi.

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