EUROPEAN UROLOGY 75 (2019) 853–860

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology

Bladder Cancer

Prospective Validation of an mRNA-based Urine Test for Surveillance of Patients with Bladder Cancer

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Article info

Article history:
Accepted November 30, 2018

Associate Editor:
James Catto

Keywords:
Bladder cancer
Cystoscopy
Diagnostic test
mRNA
Non-muscle-invasive bladder cancer
Recurrence
Sensitivity
Specificity
Urine test

Abstract

Background: A fast, noninvasive test with high sensitivity (SN) and a negative predictive value (NPV), which is able to detect recurrences in bladder cancer (BC) patients, is needed. A newly developed urine assay, Xpert Bladder Cancer Monitor (Xpert), measures five mRNA targets (ABL1, CRH, IGF2, UPK1B, and ANXA10) that are frequently overexpressed in BC.
Objective: To validate Xpert characteristics in patients previously diagnosed with non-muscle-invasive BC.
Design, setting, and participants: Voided precystoscopy urine samples were prospectively collected at 22 sites. Xpert, cytology, and UroVysion were performed. If cystoscopy was suspicious for BC, a histologic examination was performed. Additionally, technical validation was performed and specificity was determined in patients without a history or clinical evidence of BC.
Outcome measurements and statistical analysis: Test characteristics were calculated based on cystoscopy and histology results, and compared between Xpert, cytology, and UroVysion.
Results and limitations: Of the eligible patients, 239 with a history of BC had results for all assays. The mean age was 71 yr; 190 patients were male, 53 never smoked, and 64% had previous intravesical immunotherapy (35%) or chemotherapy (29%). Forty-three cases of recurrences occurred. Xpert had overall SN of 74% (95% confidence interval [CI]: 60–85) and 83% (95% CI: 64–93) for high-grade (HG) tumors. The NPV was 93% (95% CI: 89–96) overall and 98% (95% CI: 94–99) for HG tumors. Specificity was 80% (95% CI: 73–85). Xpert

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https://doi.org/10.1016/j.eururo.2018.11.055

SN and NPV were superior to those of cytology and UroVysion. Specificity in non-BC individuals (n = 508) was 95% (95% CI: 93–97).
Conclusions: Xpert has an improved NPV compared with UroVysion and cytology in patients under follow-up for BC. It represents a promising tool for excluding BC in these patients, reducing the need for cystoscopy.
Patient summary: Xpert is an easy-to-perform urine test with good performance compared with standard urine tests. It should help optimize the follow-up of recurrent bladder cancer patients.

1. Introduction

Bladder cancer (BC) is associated with significant morbidity and mortality, with nearly 200 000 cases and >50 000 deaths occurring in 2016 in the USA and European Union [1,2]. Seventy-five percent of newly diagnosed BC is non-muscle-invasive BC (NMIBC) [3] and has a recurrence rate approaching 52% within 5 yr [4]. This high recurrence rate requires diligent and accurate monitoring of early detection of recurrence and treatment [5].

Following diagnosis, patients are monitored frequently over 5–10 yr, with the schedule depending on several factors that determine the risk of recurrence and progression [6,7]. White light cystoscopy (WLC) and urine cytology are still considered the best methods of surveillance following diagnosis. However, both methodologies have limitations. Although cytology has good specificity (SP) for BC, it has poor a sensitivity (SN) and negative predictive value (NPV). Cytology requires a review by a pathologist, is not performed on the same day as a clinic visit, and has associated inter- and intraobserver variability [8]. WLC demonstrates a lack of SN for flat lesions such as carcinoma in situ (CIS) [9]. In addition, cystoscopy is costly and uncomfortable, and carries a risk of infection. Improved monitoring is needed to reduce the morbidity and costs associated with cystoscopy, improve detection of CIS, as well as enhance compliance with follow-up schedules as advised by the European Association of Urology (EAU) NMIBC guideline [10].

A recently developed assay, the Xpert Bladder Cancer Monitor (Xpert; CE-IVD; Cepheid, Sunnyvale, CA, USA), measures the expression of five mRNA targets that are frequently overexpressed in NMIBC and can be detected in voided urine [11]. The assay is performed in a self-contained cartridge using the GeneXpert Systems (Cepheid) with hands-on time of < 2 min and turnaround time of 90 min. We aimed to validate the Xpert characteristics in patients undergoing surveillance for NMIBC.

2. Patients and methods

2.1. Patients

Individuals from 19 centers who had an initial diagnosis or recurrence of NMIBC within the proceeding 9 mo of enrollment and were scheduled for a standard of care cystoscopy were enrolled in a monitoring study. Previous enrollment in this study, first morning void urine or a volume of < 60 ml, and transurethral resection of bladder tumor (TURBT) or other bladder excision procedure within 90 d of cystoscopy were the exclusion criteria.

As the assessment of SP in a population already diagnosed previously with BC is problematic, a separate specificity study was conducted at another three sites. This specificity study included both patients referred for a urology consultation with no history or clinical evidence of BC and healthy volunteers. Both trials were approved by the appropriate institutional review board or ethics committee. All patients gave informed consent as required and, for California only, signed the Experimental Subjects Bill of Rights.

2.2. Xpert Bladder Cancer Monitor

Xpert detects five mRNA targets (*ABL1*, *ANXA10*, *UPK1B*, *CRH*, and *IGF2*) in a self-contained cartridge. The expression levels of the markers combined in a linear discriminate analysis (LDA) are used to classify samples as negative or positive using a linear model [11]. In addition, the cartridge contains a sample adequacy control, a probe check control, and an internal control to detect any sample-associated inhibition of the reverse transcriptase polymerase chain reaction (RT-PCR). The GeneXpert Systems automate and integrate cell lysis, nucleic acid amplification, and detection of the target sequences using real-time RT-PCR.

Voided urine samples were mixed with the Xpert urine transport reagent (UTR) within 1 h of urine collection, and Xpert, fluorescence in situ hybridization (FISH) analysis using UroVysion (Abbott Molecular, Des Plaines, IL, USA), and cytology were performed. For Xpert, 4 ml of treated urine was added to the cartridge and run in the GeneXpert Systems. External positive and negative controls (Maine Molecular Quality Controls, Inc., Saco, ME, USA) were run on each day that study specimens were tested.

2.3. Reproducibility study

A reproducibility study was conducted at three sites using a panel of five blinded samples prepared in a background matrix of 50% Xpert UTR and 50% urine, and spanning the reportable range of the assay (0–1.5). Two operators at each site, one with PCR experience and one without, tested one panel of five samples twice each day over nine testing days. Three lots of Xpert cartridges were tested.

2.4. Pathology, cytology, and UroVysion procedures

For all positive or suspicious cystoscopies, tissue was obtained through a cystoscopic biopsy or TURBT within 6 weeks of the original cystoscopy. All tissue specimens were assessed by the local histology laboratory. Two central pathologists independently evaluated all biopsy specimens using the same or an adjacent slide. If the central pathologists did not agree, a third central pathologist reviewed the slides.

All voided urine specimens were analyzed by cytology and UroVysion at the reference laboratory (J.B. at UNMC, Omaha, NE, USA). Cytology was

performed according to the Paris system within the reference laboratory's standard procedures. Positive and suspicious results were considered positive; atypical and negative results were considered negative. UroVysion testing was performed according to the manufacturer's instructions.

2.5. Statistical analysis

SN, SP, positive predictive value, and NPV including 95% confidence intervals (CIs) were calculated for any- and high-grade (HG) recurrences to assess clinical test performance. Urine tests were assessed for the outcome of BC as based on cystoscopy plus histology outcomes. The Xpert result, the LDA total, uses a linear regression model where the cycle threshold (Ct) results of the five markers are individually weighted and combined [11]. Assay performance in the reproducibility study was assessed using nested analysis of variance (ANOVA) to explore possible differences by site/instrument, lot, day, operator/run, and within assay for each of the five samples.

3. Results

3.1. Monitoring study

3.1.1. Assay performance

A total of 259 samples were tested by Xpert (Fig. 1A). Testing for 96% (249/259) of the samples was successful on the first attempt. Seven of the 10 indeterminate cases were retested. Six yielded valid results upon repeat assay. The overall rate of assay success was 99% (255/259). A total of 723 external, in vitro premanufactured control samples were run; 98% (709/723) gave a result on the first attempt. All other initially indeterminate controls were retested and gave valid results on the second attempt.

3.1.2. Patients

A total of 363 individuals were enrolled in the monitoring study, of whom 264 were eligible for inclusion (Fig. 1A). Data from nine patients were excluded due to invalid Xpert results ($n = 4$) or specimen shipping delay or damage ($n = 5$). Xpert results from these 255 patients are listed in Supplementary Table 1. For the performance analysis, a further 16 samples were excluded because of invalid cytology results ($n = 6$), invalid UroVysion results ($n = 8$), or both ($n = 2$). Characteristics of the 239 included patients are depicted in Table 1. Based on cystoscopy and histology, 43 cases of recurrences (prevalence 18%) were identified. Of these, 19 were of low grade (LG; all Ta). The remaining patients had HG Ta ($n = 13$), T1 ($n = 5$), CIS ($n = 4$), or T2 ($n = 2$).

3.1.3. Clinical performance

Xpert had overall SN and SP of 74% (95% CI: 60–85) and 80% (95% CI: 73–85), respectively (Table 2). SN was 83% (95% CI: 64–93) for HG (Table 3) and 63% (95% CI: 41–81) for LG tumors (data not shown). NPV was 93% (95% CI: 89–96) overall and 98% (95% CI: 94–99) for HG disease.

In patients treated with Bacillus Calmette-Guérin (BCG) within 90 d before enrollment ($n = 49$ with nine recurrences), SN (78%), SP (73%), and NPV (94%) were comparable with overall SN, SP, and NPV (74%, 80%, and 93%, respectively), suggesting that BCG treatment $\leq$ 90 d before the assay does not influence Xpert results.

Cytology SN was 30% (95% CI: 19–45) with SP of 90% (95% CI: 85–93; Table 2). SN for HG tumors was 50% (95% CI: 31–67; Table 3) and for LG tumors 5.3% (95% CI: 1–25). Overall NPV was 86% (95% CI: 81–90).

UroVysion SN was 51% (95% CI: 37–65), SP was 80% (95% CI: 73–85), and NPV was 88% (95% CI: 83–92; Table 2). SN was 75% for HG tumors (95% CI: 55–88; Table 3) and 21% for LG tumors (95% CI: 8.5–43).

Comparing the three urine tests side by side, Xpert could identify more recurrent cancers correctly and was significantly more sensitive in detecting LG tumors than cytology or UroVysion ($p < 0.001$ and $p = 0.021$, respectively, McNemar's test, Supplementary Tables 2 and 3). Xpert was more sensitive in detecting the most common recurrent tumors (Ta), independent of the grade.

3.2. Specificity study

A total of 537 individuals were initially enrolled, of whom 508 were included and had valid results for the final dataset (Fig. 1B). The included individuals were either patients without a history or clinical evidence of BC who were referred for urology consultation ($n = 434$) or healthy volunteers ($n = 74$; Table 1). Of these patients, 24/508 were positive by Xpert (of whom one healthy volunteer resulting in a SP of 95% [95% CI: 93–97]). The percentage of nonsmokers was higher than in the monitoring study (51% vs 24%), but SP in current smokers (98%) and nonsmokers (96%) was similar. The most common reasons for urologic consultation were urinary incontinence or overactive bladder ($n = 77$), benign prostate hyperplasia ($n = 73$), and prostate cancer ($n = 49$). SP values in these subgroups were 100%, 95%, and 96%, respectively. Slightly lower SP of 83% was observed in patients with kidney stones ($n = 35$).

3.3. Reproducibility study

Testing for 98% (529/540) of samples was successful on the first attempt. All the 11 indeterminate cases yielded valid results upon repeat assay testing.

The 95% CIs for observed LDA totals overlapped the expected LDA totals for all analyses (Supplementary Table 4 and Supplementary Fig. 1). In nested ANOVA analyses, Ct variability did not exceed 50% of the assay noise for the following factors: site/instrument, day, and operator/run. Lot-to-lot variability exceeded 50% of the assay noise at one level for the assay (expected LDA total 1.27), but the total standard deviation for this highlight concentrated sample was not as high as 0.08. The total standard deviation did not exceed 0.1 for any of the five samples that cover the dynamic range of the assay used in the study.

4. Discussion

The current diagnostic armamentarium for the detection of recurrent BC includes cystoscopy, cytology, and a variety of

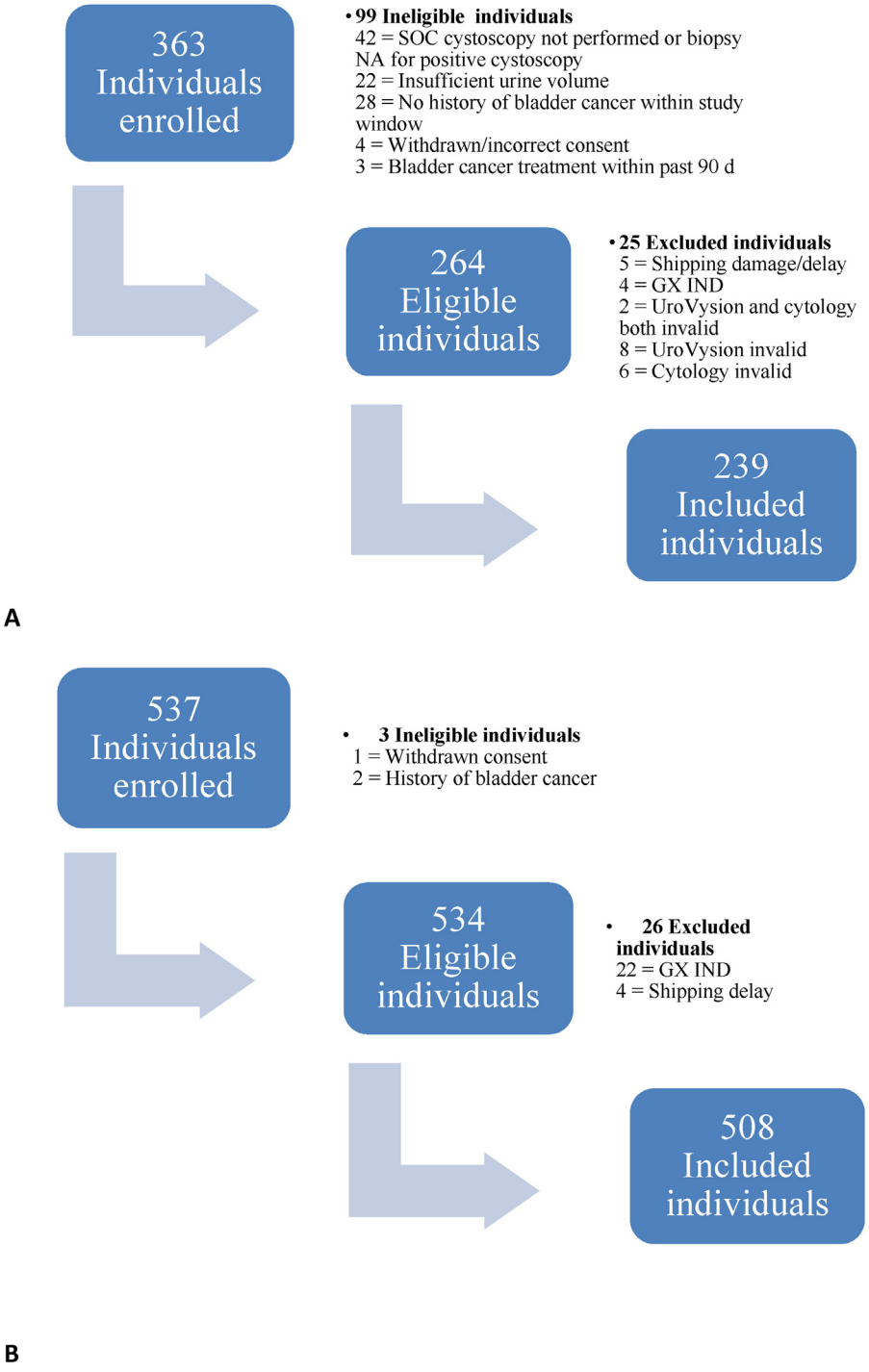

Fig. 1 – Accountability of patients in the (A) monitoring and (B) specificity studies. The specificity study was designed for NMIBC and specific testing of patients who had previous pelvic radiation for bladder cancer or cystectomy with no evaluation of urinary diversion. GX IND = indeterminate result of the GeneXpert System; NA = not available; NMIBC = non-muscle invasive bladder cancer; SOC = standard of care.

noninvasive assays that use protein (eg, ImmunoCyt/UCyt+, NMP-22) or DNA (UroVysion) markers [12–14]. Noninvasive assays come with limitations, some due to poor test performance for the detection or exclusion of BC (eg, high false-positive rates for NMP-22) and some because they are expensive as well as time and labor intensive (eg, UroVysion) [15].

Cytology is frequently used and is characterized by high SP but poor SN, especially for LG BC. In a systematic review of cytology performance in the recurrence monitoring setting, the median SN was 35% and median SP 94% [14], while in a meta-analysis of 56 studies with over 22 000 patients, pooled data showed SN of 44% and SP of 96% [12]. Other studies report that SP of urine cytology in

Table 1 – Patient characteristics of both the monitoring study with BC patients and specificity study with non-BC patients and healthy volunteers

Category	Detection study, *n* (%)	Specificity study, *n* (%)	*p* value
Sex			
Male	190 (80)	341 (67)	<0.001[a]
Female	49 (20)	167 (33)	
Race			
Caucasian	229 (96)		
Hispanic	2 (1)		
Black or African American	7 (3)		
Other	1 (0.4)		
Smoking history			
Current smoker	45 (19)	52 (10)	<0.001[a]
Former smoker	141 (59)	196 (39)	
Never smoked	53 (22)	260 (51)	
Intravesical therapy (BC patients only)			
Chemotherapy (eg, mytomycin)	69 (29)		
Immunotherapy (eg, BCG)	83 (35)		
No history of treatment	87 (36)		
Cancer history (non-BC patients only)			
History of non-BC		105 (21)	
No history of cancer		403 (79)	
Most common conditions (non-BC patients only)			
BPH		73 (14)	
Incontinence/overactive bladder		77 (15)	
Kidney stones		35 (6.9)	
Infection/inflammation		26 (5.1)	
Other		297 (59)	
Mean age, yr (range)			
Overall	71 (30–93)	62 (19–91)	<0.001[b]

BC = bladder cancer; BCG = Bacillus Calmette-Guérin; BPH = benign prostate hyperplasia.
[a] Pearson chi-square test is used for comparison.
[b] *T* test is used for comparison.

clinical routine is often lower than that reported historically [16].

The UroVysion assay uses FISH to detect intracellular chromosomal changes in BC cells [17]. At least 25 abnormal cells need to be detected, but interpretation and exact definition of positivity vary from study to study and are observer dependent. In systematic reviews, SN of 70–75% and SP of 70% are reported for UroVysion [12,15].

WLC is the current gold standard for NMIBC monitoring, but it has some limitations as well. WLC can have difficulty in detecting small papillary tumors and flat lesions such as CIS, consequently resulting in false-negative findings [3]. When compared with the more sensitive blue light cystoscopy, CIS is invisible by WLC in up to 50% of patients [18]. However, it is costly and time consuming [19]. In addition, all cystoscopic methods are unpleasant for patients to endure and increase the risk of urinary tract infection.

In this study, we investigated the performance characteristics of a newly developed five-marker mRNA expression signature in urine—Xpert Bladder Cancer Monitor—and its ability to correctly identify patients with a BC recurrence. We found higher SN and NPV compared with cytology and UroVysion. The SP was only minimally improved compared with UroVysion and was lower compared with cytology. Especially, the Xpert NPV of 93% and 98% for HG disease in particular might contribute substantially to NMIBC monitoring. A high NPV, implying that most negative test results are truly negative, allows one to reliably exclude disease. If used in the follow-up of NMIBC, a cystoscopy might be waived if the Xpert result is negative. The currently advised follow-up schedule for low-risk NMIBC consists of a cystoscopy at 3 and 12 mo after TURBT [6]. Nonetheless, cystoscopy is possibly more regularly performed within this time frame out of caution by urologists [20]. The Xpert test could partially replace WLC and thus help urologists adhere to the advised follow-up schedules. With an NPV of 98% for HG disease, Xpert might additionally help urologists reduce the amount of invasive cystoscopies for intermediate- to high-risk NMIBC, thus reducing costs and patient discomfort. The time to result of 90 min, the minimal training and hands-on time required to perform this test, and a high assay success rate contribute to its potential at the outpatient clinic.

The assay was shown to be robust in the reproducibility study and to have good SP in the specificity study in non-BC patients. The only condition where SP was lower (83%) was in patients with urolithiasis. However, the distinct clinical presentation and imaging of this condition should facilitate the discrimination of these patients. Finally, in a subset of patients recently ($\leq$ 3 mo) treated with BCG prior to enrollment, Xpert SN, SP, and NPV results were as good as those in the overall population.

Compared with cytology and UroVysion, Xpert was more sensitive for both HG and LG BC. It captured 100% of the T1 and T2 HG tumors (Supplementary Table 1), and the only

Table 2 – Xpert monitor, cytology, and UroVysion versus cystoscopy/histology cross tables and test characteristics for overall bladder cancer detection

	Cystoscopy/histology		
	Pos	Neg	Total
Xpert monitor			
Pos	32	40	72
Neg	11	156	167
Total	43	196	239
Sensitivity	74% (95% CI: 60–85)		
Specificity	80% (95% CI: 73–85)		
PPV	44% (95% CI: 34–56)		
NPV	93% (95% CI: 89–96)		
Accuracy	79% (95% CI: 73–83)		
Prevalence	18% (95% CI: 14–23)		

	Cystoscopy/histology		
	Pos	Neg	Total
Cytology			
Pos	13	19	32
Neg	30	177	207
Total	43	196	239
Sensitivity	30% (95% CI: 19–45)		
Specificity	90% (95% CI: 85–94)		
PPV	41% (95% CI: 26–58)		
NPV	86% (95% CI: 80–90)		
Accuracy	80% (95% CI: 74–84)		
Prevalence	18% (95% CI: 14–23)		

	Cystoscopy/histology		
	Pos	Neg	Total
UroVysion			
Pos	22	40	62
Neg	21	156	177
Total	43	196	239
Sensitivity	51% (95% CI: 37–65)		
Specificity	80% (95% CI: 73–85)		
PPV	36% (95% CI: 25–48)		
NPV	88% (95% CI: 82–92)		
Accuracy	75% (95% CI: 69–80)		
Prevalence	18% (95% CI:14–23)		

CI = confidence interval; Neg = negative; NPV = negative predictive value; Pos = positive; PPV = positive predictive value.

Table 3 – Xpert monitor, cytology, and UroVysion versus cystoscopy/histology cross tables and test characteristics for HG disease only (n = 239)

	Cystoscopy/histology		
	Pos	Neg[a]	Total
Xpert monitor			
Pos	20	52	72
Neg	4	163	167
Total	24	215	239
Sensitivity	83.3% (95% CI: 64.1–93.3)		
Specificity	75.8% (95% CI: 69.7–81.1)		
PPV	27.8% (95% CI: 18.8–39.0)		
NPV	97.6% (95% CI: 94.0–99.1)		
Accuracy	76.6% (95% CI: 70.8–81.5)		
Prevalence	10.0% (95% CI: 6.8–14.5)		

	Cystoscopy/histology		
	Pos[b]	Neg[a,c]	Total
Cytology			
Pos	12	20	32
Neg	12	195	207
Total	24	215	239
Sensitivity	50.0% (95% CI: 31.4–68.6)		
Specificity	90.7% (95% CI: 86.1–93.9)		
PPV	37.5% (95% CI: 22.9–54.7)		
NPV	94.2% (95% CI: 90.1–96.7)		
Accuracy	86.6% (95% CI: 81.7–90.4)		
Prevalence	10.0% (95% CI: 6.8–14.5)		

	Cystoscopy/histology		
	Pos	Neg[a]	Total
UroVysion			
Pos	18	44	62
Neg	6	171	177
Total	24	215	239
Sensitivity	75.0% (95% CI: 55.1–88.0)		
Specificity	79.5% (95% CI: 73.6–84.4)		
PPV	29.0% (95% CI: 19.2–41.3)		
NPV	96.6% (95% CI: 92.8–98.4)		
Accuracy	79.1% (95% CI: 73.5–83.8)		
Prevalence	10.0% (95% CI: 6.8–14.5)		

CI = confidence interval; Neg = negative; NPV = negative predictive value; Pos = positive; PPV = positive predictive value.
[a] Patients with low-grade disease were reclassified negative.
[b] Includes positive and suspicious results.
[c] Includes atypical and negative results.

CIS that was missed was also missed by both other methods, raising suspicion that the particular sample was an outlier. However, comparing the three urine tests, most 95% CIs partly overlap (Table 3), justifying caution in drawing conclusions.

The assessment of SP in the monitoring study was lower than that in the specificity study. It is possible that lesions too small to be visible by WLC could be detectable by molecular assays, as has previously been shown for UroVysion. In a study of 211 patients with negative cystoscopic examination results, 56 (27%) had positive FISH results, and in 35 (63%) of these patients, recurrent urothelial carcinoma developed within a median follow-up of 23 mo [21,22]. Two prospective clinical trials are ongoing to investigate this possibility for Xpert.

Recent developments in noninvasive detection of BC include DNA-based liquid biopsy. Although the detection of circulating tumor DNA markers holds great promise for monitoring of recurrence in many cancers, the methods used are less than ideal for clinical management. Techniques such as “Beaming” [23], CAPP-Seq [24], and the combination of next-generation sequencing and digital PCR [25] are promising tools, but they are complex and time consuming, and at present are more likely to be research tools rather than widely deployable clinical tools for outpatient care. Combined DNA-methylation- and mutation-based urine markers were described to be sensitive (93%) and specific (86%) with a good NPV (99%), and might be combined with the Xpert test to further optimize BC

recurrence prediction, although hands-on and turnaround times were much longer and hematuria patients instead of confirmed NMIBC follow-up patients were used [26].

The lack of long-term follow-up data is a limitation to our study. Consequently, it was not known whether unnecessary follow-up or treatment was performed, or whether early recurrences would develop. However, for our study aim, that is, to validate the Xpert characteristics, no follow-up was needed. Additionally, the study included a small number of cancer cases. Although the HG NPV was high, the NPV partly depends on the prevalence of disease, which was 10% for HG disease in our population. This prevalence implies that one would expect a HG NPV of at least 90%. Nonetheless, a HG NPV of 98% is a confident number to exclude HG disease and help reduce (not omit) the amount of follow-up cystoscopies. Lastly, different centers for the reproducibility and specificity studies were evaluated, potentially influencing test handling and execution. Operator and instrument variability did not differ significantly, although patient demographics for the SP cohort did.

5. Conclusions

Xpert Bladder Cancer Monitor is a fast, easy-to-use, robust assay that is used to analyze voided urine; it demonstrates fair SP for BC as well as enhanced SN relative to cytology and UroVysion. It has a high NPV in patients previously diagnosed with NMIBC and can be used to partially replace cystoscopy by excluding BC. This test will additionally help better adhere to the follow-up schedules advised by the EAU guideline on NMIBC. Further research on the long-term follow-up results is ongoing.

Author contributions: F. Johannes P. van Valenberg 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.

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Statistical analysis: Zhao, Ge.
Obtaining funding: Cepheid.
Administrative, technical, or material support: Wallace.
Supervision: Witjes.
Other: Biomarker evaluation and Xpert Bladder Cancer Monitor development: E. Wallace, R.G. Higuchi, M.P. Bates.

Financial disclosures: F. Johannes P. van Valenberg certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: Andrew M. Hiar, Ellen Wallace, Suling Zhao, Sijian Ge, Iris M. Simon, Scott A. Campbell, Brian Rhees, Michael P. Bates, and Russell G. Higuchi are employees of Cepheid. Franciscus Johannes P. van Valenberg, Julia A. Bridge, Wade J. Sexton, Alon Z. Weizer, John F. Danella, Barry Shepard, Kevin J. Cline, Michael B. Williams, Timothy J. Bradford, Fred N. Wolk, Andrew F. Trainer, Russell B. Egerdie, Bernard Goldfarb, Godfrey K. Jansz, Eric W. Klein, Donna J. Mayne, Karl R. Westenfelder, Joseph A. Zadra, J. Alfred Witjes: none. Yair Lotan: consultant for Pacific Edge, MDxHealth, PHotocure, and Cepheid; grant and research support from Abbott, Pacific Edge, MDxHealth, Genomedix, and Cepheid. Arnulf Stenzl: speaker for Janssen, Ipsen Pharma, Sanofi Aventis, CureVac, and Astellas; in advisory board of/consultant for Ipsen Pharma, Roche, Janssen, Alere; in the advisory board of Bristol-Myers Squibb; grant and research support from Johnson & Johnson, Roche, Cepheid, Amgen Inc, Bayer AG, CureVac, Immatics Biotechnologies GmbH, Novartis AG, and Karl Storz AG. Timothy A. Richardson: Bayer and Astellas.

Funding/Support and role of the sponsor: Cepheid (Sunnyvale, CA, USA) supported in the conduct of the trials and provided funding for the trials.

Acknowledgments: We would like to thank Ilona Koster, Yimin Sun, Hui Zhang, Janet Strome, Tanna Morris, Misty Pichardo, Bailey Polier, Nada L. Wood, Katherine Cole, Amy Nelson, Susan Wiley, Kris Wheeler, S. Rangaswamy, R. Schalm, G. Wagner, Karen Shirriff, Lea Renfro, Michelle Schindler, Jörg Hennenlotter, and Tilman Todenhöfer for their assistance in sample collection and local study coordination. We would like to thank Jill Birkmeier and Lizabeth Sauer for their assistance in manuscript review and coordination.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2018.11.055.

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