Role and efficacy of current biomarkers in bladder cancer

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Abstract: In order to overcome the current limitations in the diagnosis, therapy and follow-up of bladder cancer (BCa), the detection of biomarkers in urine, tissue and blood has been proposed as essential components of the precision medicine. The introduction of these modern molecular tests may help to identify disease earlier, to risk-stratify patients, to improve the prediction of oncological outcomes and to optimize target therapies. On the other hand, the main limitation for their spread in clinical practice is the lack of evidence of a meaningful improvement in clinical decision-making due to their unsatisfactory performances. Recent Literature made a great effort with the aim to describe novel biomarkers and their potential role, but this jigsaw puzzle scenario makes difficult the orienteering into their correct uses and indications. We reviewed the current state of the art and the performance of available and most promising biomarkers. Despite some good results, especially regarding the improvement of sensitivity compared to urinary cytology in the diagnostic setting, the use of these molecules has not been yet introduced into daily clinical practice by international guidelines, because of both the difficulty in identifying the correct scenario of use and the lack of high-quality prospective trials, with a subsequent low level of evidence. Further prospective investigations and large international collaborations are needed to define the role of these promising biomarkers.

Keywords: Urinary biomarkers; tissue-based biomarkers; non-muscle invasive bladder cancer (NMIBC); response to treatment

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Introduction

Despite continuous research and improvements of available tools over the last years, above all endoscopic and imaging ones, the cornerstones for the diagnosis and surveillance of bladder cancer (BCa) have not changed and are still represented by cystoscopy and urine cytology (1,2). Cystoscopy is a routinely used exam performed almost in every urologic center and, due to its high sensitivity and specificity (2), is still considered the gold standard for BCa detection. However, this is not a side-effect free procedure: due to its invasiveness, it still represents an important cause of patients' discomfort that negatively affects patients' quality of life, and a costly procedure that severely impacts on the health-care system (3). Voided urinary cytology is a non-invasive test used in combination with cystoscopy in the diagnosis and follow up of high-grade tumors. Actually, urinary cytology has an overall high specificity and a good sensitivity for high-grade tumors, while its accuracy dramatically decreases in low-grade tumors (1). Recent innovations aimed to fill this lack by following the direction drawn by modern medical research towards the era of "precision medicine", which consists in taking into account patient's and tumor's variability, thereby allowing the personalization of therapy. In this scenario biomarkers play a key role. Precisely, biomarkers may potentially improve

Table 1 Proposed structured approach to systematic validation of biomarkers

Level	Sample details/bias	Target	Main features
Preclinical testing	*In vitro* or animal models (an appropriate model may be lacking)	A hypothesis-generating step	–
Phase 0	On a small group of patients (sample of population is limited)	Development of clinically reproducible preliminary assays	Do not take into account the potential benefit
Phase 1	On a small group of patients (defined from biomarker profile, sample of population is limited)	Determination of the benefit (define the marker, establish the prediction rules, and determine the assay cut-off points)	–
Phase 2	On a relevant, representative target population	Independent validation of the accuracy of the assay (reproducibility and robustness of the assay, determination of reference)	Retrospective design, internal validation
Phase 3	On large patient populations (multi-institutional collaboration)	To yield the sensitivity and specificity of the biomarker, to show an earning in clinical decision-making	Randomized trial, external validation
Phase 4	Define if burden of disease is reduced by the biomarker	Post-approval reporting and testing for other disease processes or disease stages	–

patients' risk stratification by increasing the accuracy of available diagnostic tools and by predicting oncological outcomes and response to therapies. Many biomarkers have been studied over the last years in different clinical scenarios, such as for screening, surveillance and follow-up. However, despite significant efforts dedicated to a better understanding of their principles and roles, none of them is, to date, endorsed by international guidelines, and therefore used in clinical routine (4). The main limitation for their spread in clinical practice is the lack of evidence of a meaningful improvement in clinical decision-making due to their unsatisfactory performances. This could be the consequence of multiple factors. As said before, biomarkers could be theoretically applied to different clinical scenarios such as BCa diagnosis, screening, risk stratification, prognostication of outcomes, and prediction of response to therapies. Obviously, different abilities are required for each setting: for example, to simplify, a biomarker used in a screening setting should have a high specificity in order to avoid unnecessary investigations in healthy patients, while during the follow-up of non-muscle invasive bladder cancer (NMIBC), a high sensitivity should be required, aiming to reduce the risk of missing a clinically significant tumor. Therefore, the first challenge in the detection of a reliable marker is to identify the correct scenario of application based on its accuracy. Moreover, as described by Goebell *et al.* (5), a complex process of validation should be followed by biomarkers to be introduced into clinical practice (*Table 1*): they have to be analyzed into pre-clinical exploratory studies, clinical assay development and validation studies, small clinical retrospective studies, external validation in larger cohorts, prospective clinical trials and post-approval studies. This rigorous validation process is rarely observed, and this deficiency may be identified as the second cause for the presence of numerous promising markers that have never been subsequently reproduced or validated: most of biomarkers either do not follow or fall behind this strict validation path. Despite all these limitations, and the difficulty to find orientation surfing the big wave of biomarkers in literature, their hypothetical role still remains interesting and potentially useful in the management of BCa. The aim of this review is to elucidate the current state of the art of the most promising biomarkers which may entry in clinical practice in the next future.

Different biomarkers for different clinical scenarios: screening, risk stratification and surveillance

For the detection of BCa and its first diagnosis, two main clinical scenarios can be identified: for screening purpose in high-risk population, such as heavy smokers and workers with occupational exposure to agents known to increase the risk of BCa, and for risk-stratification of patients with asymptomatic microhematuria (AMH). Because of the low prevalence of disease even in high-risk categories (6), in these scenarios the ideal urinary marker should have a

high positive predictive value (PPV) and high specificity. However, data from recent trials indicate that even in patients with high-risk of developing BCa the incidence is so low [from 0.1% to 3.3% in heavy smokers and from 0.01% to 8% in exposed workers (7,8)] that systematic screening cannot be clearly recommended. The second clinical scenario is represented by the risk-stratification of patients with AMH. In this population previous studies have suggested a significant risk of BCa [approximately 10% (9)], thus making cystoscopic workup mandatory. The critical point in this setting is the high prevalence of AMH in the adult population, from 10% to 18%, yet only 2% of which have BCa. Therefore, the introduction of urinary biomarkers for risk-stratification purposes maintains an increasing appeal, in order to identify those patients in whom cystoscopy, an exam not side-effect free as we already discussed, can be avoided. However, only limited prospective data on the use of molecular urinary biomarkers in patients presenting with AMH are available, and to date no adequate evidence is disposable. Another potential role of biomarkers in BCa consists in their application during surveillance and follow-up of patients with previous NMIBC. This may be perceived in two major usages: as substitute to cystoscopy for low/intermediate risk NMIBC patients or as an adjunct to the endoscopic workup for high-risk NMIBC patients.

Urinary biomarkers overview

Immunocytology (ImmunoCyt and uCyt)

Urine cytology is a well-studied test approved and strongly recommended by international guidelines which has in its limited sensitivity for low grade tumors and in its variability of interpretation the main points of weakness (1). In order to overcome the low sensitivity, ImmunoCyt and uCyt assays were developed. These immunocytological tests are based upon microscopic detection of high molecular weight form of carcinoembryonic antigen (CEA) and BCa associated-mucins contain fluorescein-labelled monoclonal antibodies M344 and LDQ10 (against mucin glycoproteins) and Texas red-linked antibody 19A211 (against CEA). The strength of these assays is represented by the higher sensitivity for low grade tumors compared to conventional urine cytology. On average, the detection rate for low-grade tumors was 75%, and sensitivity for G2 and high-grade tumors was approximately 85%. Overall specificity was 75% (10-12). Conversely, costs are higher, mostly due to lab equipment, long time of processing and reading the specimens and the high experience needed for interpretation of results (13). Reported confounding factors for the uCyt TM assay seem to be benign prostatic enlargement, hematuria, urolithiasis, and inflammatory conditions but recent reports suggest that the impact of these conditions on test performance seems to be limited (14).

UroVysion FISH

The UroVysion multicolor FISH test provides the detection of chromosomal aberrations (probes for chromosomes 3, 7 and 17 and the 9p21 locus) associated to BCa. This assay is approved by the Food and Drug Administration (FDA) for diagnosis and surveillance of BCa. Many cut-off levels for the positivity of the test have been introduced (15), but in general a minimum of 25 morphologically abnormal cells are analyzed and it is considered positive when a minimum of 4 cells have a gain of two or more of the aforesaid chromosomes or 12 of more cells have a loss of the 2 copies of locus 9p21. Schlomer *et al*. suggested to use the UroVysion FISH test when urine cytology provides dubious findings (16). The main limitations for the use of UroVysion are related to the high costs, the need of specialized laboratories for adequate interpretation, the significative rate of false-positive and non-informative findings in approximately 10% of the cases (17). Despite the broad range of accuracy found in literature for UroVysion FISH, probably affected by different experience of investigators and cut-off values, sensitivity seem to be set from 70% to 80% and specificity approximately around 80% (18-20). Part of the relatively high rate of false-positive could be explained in the follow-up scenario as anticipatory positive results, which means that a premalignant molecular change is detected by the assay even if no macroscopic alterations can be found endoscopically: indeed, two studies showed that 85% and 88% of patients with a false-positive test finally developed a bladder recurrence within 12 months. However, the interpretation of the anticipatory positive result still remains uncertain due to the high recurrence rate of BCa (21).

Nuclear matrix proteins (NMPs)

NMPs are important biomarkers and antigens of BCa. They contribute to the structure of the nucleus and are fundamental for supporting nuclear shape. One member of this jigsaw puzzle of NMPs is NMP22, which is much

more prevalent in malignant urothelial cells compared to normal ones, because of their higher rate of apoptosis. Both qualitative point-of-care tests and quantitative ELISA tests exist, and this assay is FDA approved. According to several meta-analyses, the benefit of these protein markers compared to urine cytology seems to be a higher sensitivity both for low (median of 50% *vs.* 27%) and high (median of 83% *vs.* 78%) grade tumors (22-24). Tumor volume, in terms of numbers of lesions or size, seems to reflect the positivity of the test, as described by Poulakis *et al.* who found sensitivities of 79%, 90% and 97% in patients with 1, 2–3 and >3 lesions, respectively (25). The main limitation for the NMP22 application in clinical practice is its lower specificity compared to urine cytology. Indeed, urological pathologic conditions, such as inflammation, infection, stones, hematuria and also diagnostic and therapeutic procedures such as endoscopic ones, could affect the assay, thus causing false-positive results. This is due by the multiple causes of apoptosis and subsequent release of NMPs in urine. Sharma *et al.* tried to exclude these clinical categories, with and improvement of specificity and PPV of NMP22 (95.6%, 87.5%). As for FISH, also for NMP22 was described an anticipatory positive result (26). A recent prospective trial on 1,303 patients identified NMP22 as the strongest predictor of BCa, higher than urine cytology, and its addition to a predictive model for BCa recurrence, significantly improved the accuracy of the model (27).

BTA test

Another test approved by FDA in adjunct to cystoscopy is BTA (Bladder Tumor Antigen), both in its two newer versions, BTA stat and TRAK (which differ from the original in detecting different proteins) (28). The qualitative point of care and the quantitative Elisa test detect the human complement factor H related protein (that is thought to help cells to evade the host immune system) and complement factor H in urine samples. BTA stat is a cheap, single-step, easy to reproduce, fast to perform test and requires minimal experience from the laboratory staff (29). BTA TRAK needs instead a more sophisticated technology and more time to be performed. Manufacturer recommended the cut-off of 14 U/mL for the positivity of the test (28). BTA stat and BTA TRAK are not approved by FDA for diagnostic and screening purpose, but in surveillance setting as adjunct to cystoscopy. Glas *et al.* in their meta-analysis, helped our understanding about BTA assays by reporting a sensitivity of 70% (95% CI: 66–74) and a specificity of 75% (95% CI: 64–84) for BTA stat, while for BTA TRAK test sensitivity and specificity were 66% (95% CI: 62–71) and 65% (95% CI: 45–81), respectively. Again, findings may be affected by concomitant urological pathologies and procedures. BTA tests in not recommended alone in the detection of BCa (30).

Urinary biomarkers for the screening of high-risk population

The low prevalence of BCa even in categories considered at high-risk of developing urothelial tumor together with the low cancer-specific mortality (CSM) and the incidence of low-grade tumors affect the effectiveness of screening programs (7,8). The low prevalence of disease inevitably affects biomarkers' PPV and negative predictive value (NPV) (*Table 2*). Lotan *et al.* tested NMP22 in high-risk population (1,175 men and 327 women with at least 10 years of smoking or 15 years of exposure to carcinogenic substances). Eighty-five (5.7%) subjects had a positive NMP22. Of these, 69 patients underwent further evaluation, and only 3 (3.5%) had a BCa diagnosis (all low-grade tumors). After 12 months, 2 of 1,309 (0.15%) participants developed low-grade BCa (24). Steiner *et al.* studied 183 heavy smokers and reported urine cytology, NMP22 and FISH levels. Forty-one percent of subjects had at least one positive biomarker, but only 5 cases of tumors and 12 cases of precancerous lesions were recorded (23). To date, no urinary biomarkers has met the criteria for screening purpose and, actually, guidelines do not advise their use, even if future large controlled trials may help to define their potential role in this setting.

Urinary biomarkers for the risk-stratification of asymptomatic microscopic hematuria

While painless gross hematuria is generally considered an indication for cystoscopy, the management of painless microhematuria is controversial and differs widely. However, the need of stratifying patients by the risk of presenting BCa is univocal. As said before, the lack of prospective studies and the not distinction of gross hematuria and AMH in many studies represent a big obstacle for studies' quality (*Table 3*). Cha *et al.* performed a retrospective analysis of 1,182 patients by testing a predictive model including cytology, imaging, cystoscopy, and immunocytology, and reported an accuracy for BCa detection of 90.8%. Within this nomogram, a positive immunocytology was the

Table 2 Summary of studies assessing the role of biomarkers for screening in high-risk populations

Study	Number of patients	Test	Study population	Results
Lotan *et al.* (24)	1,502	NMP22	Smokers (≥10 years), occupational exposure	Eighty-five positive tests (8.5%), of which 69 cystoscopies were performed, 3 with abnormal findings. After a median F-U of 78.4 months no case of MIBC in subpopulation of 925 patients
Steiner *et al.* (23)	183	Dipstick, NMP22, cytology, FISH	Smokers (≥40 pack years)	Seventy-five patients with at least one positive marker (40.9%), at least one marker positive in all tumor cases
Pesch *et al.* (8)	1,609	Dipstick, NMP22, FISH, cytology	Workers with occupational exposure	Four hundred and ninety-three positive tests, 8 cases detected by cytology. 20 abnormal findings
Bonberg *et al.* (31)	1,609	UroVysion	Workers with occupational exposure	Nine of 21 BCa detected in 20 cases had UroVysion test +, including 7 HG carcinomas and 7 overlapping results with cytology
Banek *et al.* (32)	1,609	FISH	Workers with occupational exposure	Sixteen incidental BCa detected and 5 recurrent tumors in 20 cases. FISH was positive in 9 BCa cases of which 7 were HG. FISH overlapped with cytology in 7 cases. Sensitivity was 45.0% and PPV 16.4% in all and 53.85% and 13.21% in HG tumors. Specificity and NPV were 96.97% and 99.26% in all BCa
Huber *et al.* (33)	1,772	NMP22	Workers with occupational exposure	Higher NMP22 concentrations in 224 patients, which correctly predicted BCa in 6 cases (sensitivity 97.2%, specificity 28.5%; NPV 99.04%, PPV 12.24%)

MIBC, muscle invasive bladder cancer; BCa, bladder cancer; PPV, positive predictive value; NPV, negative predictive value; NMP, nuclear matrix protein.

Table 3 Summary of studies assessing the role of biomarkers in patients presenting hematuria

Study	Number of patients	Test	Study population	Results
Cha *et al*. (34)	1182	Immunocytology	Hematuria (68% AMH)	Construction of a nomogram with 90.8% of accuracy of prediction of BCa (immunocytology was the strongest predictor)
Lotan *et al*. (35)	381	NMP22	Hematuria	Construction of a nomogram with 80.2 % of accuracy of prediction of BCa
Beukers *et al*. (36)	169	Methylation analysis of OSR1, SIM2, OTX1, MEIS1 and ONECUT2	Hematuria (86 AMH, 83 gross hematuria)	Construction of a nomogram with sensitivity of 85% and specificity of 87% for the prediction of BCa (performance of the model was better in patients with gross hematuria compared AMH)
Todenhöfer *et al*. (37)	2,365	Urovysion, NMP22, immunocytology	AMH	The extent of AMH significantly influences the performance of noninvasive urine markers, underlining the relevance of the grade of AMH for the interpretation of tests
Lucca *et al*. (38)	227	HDAC9, HDAC3, tRNA methyltransferase1, DNA methyltransferase 1	AMH	The addition of HDAC3 to a base risk factor model improved its accuracy by 1.4%
Davidson *et al*. (39)	571	CxbT	Hematuria	High NPV was found for the clinical pathway composed by imaging and CxbT, significantly reducing the need of cystoscopy

BCa, bladder cancer; NPV, negative predictive value; NMP, nuclear matrix protein; AMH, asymptomatic microhematuria.

strongest predictor of presence of BCa (34). Lotan *et al.* tested another nomogram including NMP22, and predictive accuracy decreased to 80.2% (35). Therefore, even if prospective trials are lacking, retrospective experience seems to suggest that some biomarkers could help risk-stratifying patients with AMH.

Urinary biomarkers for the surveillance of NMIBC

Some guidelines currently recommended the use of biomarkers in adjunct to cystoscopy, but not its replacement. Awareness of markers' positivity seems to improve the detection rate of BCa recurrence, as described in a prospective, single-blind, randomized multicenter trial, in which 448 patients were included. When urologists performing cystoscopy were informed about the results of urinary biomarkers, 42 recurrences were detected in 131 cystoscopies, whereas urologists that were not aware of the positivity of the marker, observed only 6 recurrences in 120 cases (40). Shariat *et al.* described a possible relationship between biomarkers' positivity and tumor aggressiveness at transurethral resection of the bladder (TURBT). Positive cytology and NMP22 were associated with a 33-fold risk of a ≥ pT1 tumor and a 21-fold risk of a G3 tumor (41). A multicenter study analyzed and compared the sensitivity and specificity of main urine biomarkers for the diagnosis of BCa recurrence (NMP22, Cxbladder Detect, UroVysion FISH). Cxbladder Monitor (designed to analyze the expression of five genes, *CDC2*, *HOXA13*, *MDK*, *IGFBP5* and *CXCR2*) resulted highly sensitive for the diagnosis of recurrent BCa and with a low rate of false negative results and, therefore, it outperformed the remaining markers (42). Based on these promising findings, Waitemata Disctrict Health Board in New Zealand (one of publicly funded health care providers) accepted Cxbladder Monitor as a substitute of cystoscopy in all low-risk NMIBC patients. The use of a panel of biomarkers, instead of only one of them, has been studied, and it has been shown that this approach, including voided urine cytology and other markers, does not improve the predictive value of a test that based on only one biomarker (high grade disease was diagnosed with both tests simultaneously) (43,44). Another possible approach is related to the use of biomarkers as reflex test (45). Instead of a simultaneous analysis of a panel of biomarkers, for those patients with negative result of one test, subsequent and highly sensitive markers were added, thus leading to an increased accuracy of follow up. The subsequent combination of two among the four widely adopted markers (cytology, immunocytology, FISH and NMP22) resulted in a sensitivity and NPV of 89.8% (ImmunoCyt + NMP22) and 92.1% (FISH + ImmunoCyt), respectively. If urine cytology is supplemented with any of the four tests, corresponding sensitivity and NPV are 86.7% (NMP22) and 91.3% (immunocytology), respectively. Adding FISH to conventional urine cytology is associated with a sensitivity of 80.5% (94.0% for high-risk tumors) and a NPV of 90.1% (98.8% for high-risk tumors). In conclusion, while European Association of Urology (EAU) and American Urological Associations (AUA) do not recommend urinary markers in the routine surveillance of patients with NMIBC (40,46), AUA Guidelines assess their use to define response to intravesical bacillus Calmette-Guérin (BCG) (UroVysion FISH) and adjudicate equivocal cytology (UroVysion FISH and ImmunoCyt) (47). Serial measurements of UroVysion FISH in patients treated with BCG revealed that abnormal results at baseline and during follow-up are significantly associated with both cancer recurrence and progression (cancer progressed after 2 years in half of those with positive FISH test and in only 3% of those who had normal result). The authors concluded that patients with a negative cystoscopy with abnormal FISH findings may be good candidates for clinical trials due their worse prognosis (48). FISH and ImmunoCyt were also investigated in patients with atypical cytology (49). UroVysion FISH has been shown to have 100% sensitivity and 100% NPV in those with negative cystoscopy, while ImmunoCyt was found to have 73% sensitivity in detecting recurrent bladder tumor in patients with atypical cytology with corresponding NPV of 80%. A summary of diagnostic performance of biomarkers in the detection of NMIBC can be observed in *Table 4*.

Urinary biomarkers for the prediction of response to intravesical BCG immunotherapy

As already anticipated, urinary biomarkers have been analyzed also as predictors of response to intravesical BCG (*Table 5*). In a systematic review, Kamat *et al.* concluded that, beside the standard clinicopathologic features (which still represent the most effective predictors of BCG response), single biomarkers, such as tumor p53 and urinary interleukin-2 (IL-2) expression, have limited success in predicting BCG response, while more comprehensive molecular panels (mostly urinary cytokines), have a robust correlation with response, assuming a potential future role for them in stratifying patients for outcomes after BCG

Table 4 Summary of diagnostic performance of biomarkers in the follow-up of NMIBC

Study	Number of patients	Sens (%)	Spec (%)	PPV (%)	NPV (%)
VUC					
Raitanen *et al.* (50)	445	19.2	98.3	82.8	73.6
Casetta *et al.* (51)	102	70.0	75.0	85.9	53.3
Raitanen *et al.* (52)	510	19.2	85.7	–	–
Nisman *et al.* (53)	154	61.9	96.2	28.6	94.3
Babjuk *et al.* (54)	88	19.8	99.0	89.5	74.9
Hosseini *et al.* (55)	144	44.2	83.7	60.5	72.6
Messing *et al.* (56)	327	23.0	93.0	–	–
NMP22					
Casetta *et al.* (51)	102	64.0	64.0	78.3	45.2
Serretta *et al.* (57)	137	71.5	61.0	44.7	82.8
Horstmann *et al.* (58)	221	68.0	49.0	57.0	60.0
Shariat *et al.* (59)	2,871	57.0	81.0	64.0	77.0
Grossman *et al.* (60)	668	49.5	87.3	–	–
Gupta *et al.* (61)	145	85.7	77.5	70.6	89.6
BTA stat					
Raitanen *et al.* (50)	445	56.0	85.7	63.1	81.7
Pode *et al.* (43)	162	73.7	67.6	69.8 (accuracy)	
Raitanen *et al.* (52)	510	56.0	85.7	42.5	81.4
Lokeshwar *et al.* (62)	70	60.7	74.1	87.9	37.7
BTA TRAK					
Casetta *et al.* (51)	102	60.0	60.0	75.9	39.6
Babjuk *et al.* (54)	88	38.5–53.8	83.9–88.6	58.6	81.1
Miyanaga *et al.* (63)	57	9.3	86.2	23.5	67.5
ImmunoCyt					
Vriesema *et al.* (64)	104	50.0	73.0	39.0	81.0
Sullivan *et al.* (65)	100	76.0	63.0	43.0	88.0
Horstmann *et al.* (58)	221	73.0	72.0	72.0	74.0
Messing *et al.* (56)	327	81.0	75.0	38.0	95.0
FISH					
Sarosdy *et al.* (66)	176	71.0	65.8	53.0	80.7
Horstmann *et al.* (58)	221	76.0	63.0	68.0	71.0
Youssef *et al.* (67)	123	23.5	94.3	40.0	88.5
Cxbladder monitor					
Lotan *et al.* (68)	803	91.0	–	–	96.0
Kavalieris *et al.* (69)	763	93.0	–	–	97.0

NMIBC, non-muscle invasive bladder cancer; VUC, voided urine cytology; PPV, positive predictive value; NPV, negative predictive value; NMP, nuclear matrix protein.

Table 5 Summary of studies assessing the role of biomarkers for the prediction of response to intravesical BCG immunotherapy

Study	Number of patients	Test	Results
Esuvaranathan *et al*. (70)	47	Rb	Underexpression of Rb may be predictive of non-response to BCG
Lima *et al*. (71)	94	Sialyl-Tn and sialyl-6-T	High expression leads to effective BCG treatment
Langle *et al*. (72)	11	FGFR3	Down regulation of FGFR3 predicts good response to BCG
Leibovici *et al*. (73)	519	IL-6	High levels related to tumor recurrence after BCG
Zhao *et al*. (74)	224	Glutathione peroxidase 1	Wild type had higher recurrence after BCG
Lima *et al*. (75)	204	Multiple genes	Multi-gene panel as predictor of outcomes after BCG

BCG, bacillus Calmette-Guérin.

treatment (48). In another analysis including 130 patients, a nomogram (CyPRIT) constructed using urinary levels of nine inducible cytokines (IL-2, IL-6, IL-8, IL-18, IL-1ra, TRAIL, IFN-γ, IL-12, and TNF-α) predicted the likelihood of recurrence with 85.5% accuracy (95% CI: 77.9–93.1%). This cytokine panel and nomogram was described as having a potential for identifying patients at risk of tumor recurrence during BCG treatment to guide modification of the dose and duration of BCG immunotherapy (76). To date current evidences are still inadequate to include biomarkers in clinical practice for the prediction of response to intravesical BCG, even if future randomized controlled trials could overcome this obstacle and so revealing their true potential.

Prognostic and predictive tissue-based biomarkers in NMIBC

Prediction of oncological outcomes, such as recurrence-free survival (RFS) and progression-free survival (PFS), as well as the prediction of response to intravesical BCG are the most important aims of tissue-based biomarkers in NMIBC. The predictive accuracy of currently existing risk stratification systems may be improved by their application and, therefore, could be used to develop personalized treatment and follow up schemes based on different risk of recurrence and progression. To date, biomarkers associated with pathways important for tumor growth and spread have been evaluated, including cycle-cell regulators, angiogenesis, apoptosis, signaling proteins and hormonal biomarkers.

Prognostic tissue-based biomarkers of recurrence

In general, tissue biomarkers reflect tumor aggressiveness and invasiveness and, therefore, they may be indicators of progression of disease rather than tumor recurrence. Numerous markers have been analyzed with heterogeneous results, and p53 was the most extensively studied tissue-based biomarker (*Table 6*). p53 is the most common oncosuppressor gene mutated in almost all human cancers and its role in BCa have led to conflicting results. Some researchers found a correlation between altered p53 and poorer outcomes of BCa, due to its association with features of tumor aggressiveness such as higher stage, higher grade and lympho-vascular invasion (LVI) (85,107). Alterations of p53 were also found in normal mucosa of patients with NMBIC who experienced recurrence, maybe due to the presence of premalignant lesions surrounding the cancer area (79). Shariat *et al*. found a positive correlation between mutant p53 and BCa recurrence (81). However, other authors did not confirm these findings (80,92). Due to these controversial findings, researchers shifted their attention from a single gene to a series of combined alterations of multiple genes. A retrospective study including 404 patients investigated a 26-gene signature that resulted negative for tumor recurrence (108), while another retrospective multicenter study investigated the prognostic role of fibroblast growth factor receptor 3 (FGFR3) status and three molecular markers (MIB-1, P53, and P27kip1) in 286 patients with NMIBC and a mean follow-up of 5.5 years. The combination of FGFR3 and MIB-1 was found to be an independent predictor of disease recurrence rate (109). Finally, also miRNAs have been investigated as biomarkers for tumor recurrence, but to date results are incomplete (110,111).

Prognostic tissue-based biomarkers of progression

Many tissue-based biomarkers have been studied as

Table 6 Summary of the roles of p53 as predictor of outcomes of BCa

Clinical setting	Study	Number of patients	Results
NMIBC			
Predictor of recurrence	Gontero *et al.* (77)	214	p53 has no role in prediction of recurrence of BCa
	Shariat *et al.* (78)	43	p53 has no role in prediction of recurrence of BCa
	Friedrich *et al.* (79)	53	p53 has only slight correlation with tumor rec, but its accumulation in healthy bladder mucosa correlated strongly with disease recurrence (maybe for premalignant alterations)
	Liukkonen *et al.* (80)	207	p53 may be a predictor of recurrence of BCa
	Shariat *et al.* (81)	83	p53 may help in stratifying patients with higher risk of rec
Predictor of progression	Wolf *et al.* (82)	30	p53 overexpression significantly decreases tumor-free survival
	Hermann *et al.* (83)	143	p53 overexpression should be considered as a criteria for immediate RC, because of higher progression rate
	Sarkis *et al.* (84)	43	BCas exhibiting nuclear overexpression of p53 protein have a higher probability of disease progression
	Serth *et al.* (85)	69	85.7% of patients with more than 20% of cells positive for p53 had disease progression with muscle-invasive growth compared with only 1.8% of patients negative for p53 ($P<0.01$); confirm of the role of p53 in the progression of BCa
Predictor of response to intravesical BCG	Lacombe *et al.* (86)	98	p53 overexpression before BCG therapy did not predict response to BCG therapy
	Caliskan *et al.* (87)	30	p53 appears to be a prognostic indicator of tumor unresponsive to BCG
	Pfister *et al.* (88)	60	p53 mutations have a useful predictive value for BCG therapy response in BCa
	Saint *et al.* (89)	102	p53 nuclear overexpression is associated with cancer death after BCG therapy
	Hegazy *et al.* (90)	88	p53 positivity is a predictor of recurrence and/or progression after BCG adjuvant therapy
MIBC			
Predictor of outcomes after RC alone	Shariat *et al.* (91)	692	p53 expression was independently associated with rec and CSM
	Malats *et al.* (92)	10,026	A prognostic value of p53 overexpression was observed in 9/34 studies (27%)
	Esrig *et al.* (93)	243	p53 status was an independent predictor of rec and OS
	Shariat *et al.* (94)	80	p53 is the strongest predictor of BCa outcomes in patients undergoing RC
	Shariat *et al.* (95)	91	Including p53 in standard predictors improves predictive accuracy of outcomes
Predictor of response to NAC	Chang *et al.* (96)	*In vitro*	Mutant p53 protein may enhance chemosensitivity
	Sarkis *et al.* (97)	90	p53 overexpression has independent prognostic value for survival in patients with MIBC treated with NAC
	Plimack *et al.* (98)	44	p53 mutation did not predict response or toxicity of NAC
Predictor of response to systemic chemotherapy	Stadler *et al.* (99)	499	The prognostic value of p53 was not confirmed
	Siu *et al.* (100)	118	Expression of p53 did not predict response to systemic therapy
	Qureshi *et al.* (101)	83	p53 did not predict response to systemic therapy, but it had prognostic significance within the chemoresistant subgroup
	Kong *et al.* (102)	89	Prognostic differences related to P53 expression in invasive BCas may be partly due to lower chemosensitivity

Table 6 (*continued*)

Table 6 (*continued*)

Clinical setting	Study	Number of patients	Results
Predictor of response to systemic immunotherapy	Not assessed for clinical, biological and pathophysiological reasons		
Predictor of response to radiotherapy	Rödel *et al.* (103)	70	p53 was found to be not predictive of radiosensitivity
	Lipponen *et al.* (104)	400	p53 may be predictive of radiosensitivity
	Koga *et al.* (105)	35	No significant association with chemoradiation response was observed for immunohistochemical expression p53
	Matsumoto *et al.* (106)	62	p53 was found to be not predictive of radiosensitivity

BCa, bladder cancer; NMIBC, non-muscle invasive bladder cancer; BCG, bacillus Calmette-Guérin; MIBC, muscle-invasive bladder cancer; RC, radical cystectomy; CSM, cancer-specific mortality; OS, overall survival; NAC, neoadjuvant chemotherapy.

predictors of tumor progression, but only few of them were founded to be clinically relevant. As already discussed, p53 is the most frequently studied biomarker and its overexpression or mutation seems to be correlated with tumor progression and aggressiveness. Wolf *et al*. showed how p53 may identify patients with most aggressive high-risk cancers, and therefore eligible for immediate radical cystectomy (RC) (82). Shariat *et al*. and Chan *et al*. focused their work on a combination of genes' alterations, all related to cell cycle regulation (p53, pRb, P21, P27, MMP-2, PAI-1), finding that a series of genes rather than a single gene may affect tumor progression (81,112). In its retrospective study, van Rhijn *et al*. described a correlation between FGFR3 mutation together with an increased MIB-1 expression and risk of tumor progression (109). Similarly, an important prospective study of van Kessel *et al*., including 1,239 patients, confirmed the important role of FGFR3 mutational status and methylation of GATA2 in improving the EAU NMIBC risk stratification for the prediction of tumor progression (113). Despite these interesting results, information obtained by molecular biomarkers is not sufficient alone to be introduced in clinical practice, and its integration with established clinico-pathologic variables remains mandatory.

Predictive tissue-based biomarkers of response to intravesical therapy

Numerous biomarkers have been tested as predictor of response to intravesical therapy such as BCG treatment. Malmström *et al*. in a multicenter trial evaluated the role of ezrin, CK20, and Ki-67 as predictors of response to BCG and intravesical chemotherapy (114). However, none of the analyzed biomarkers showed to be able to predict response and only tumor multifocality was associated with disease progression.

Prognostic and predictive markers in muscle-invasive bladder cancer (MIBC)

MIBC still remains an aggressive disease characterized by a generally unfavorable prognosis and high rates of progression to metastatic disease. The standard treatment of MIBC is RC, eventually preceded by neoadjuvant chemotherapy (NAC) (115,116). The current predictive models of prognosis, essential for patients' stratification and decision-making regarding perioperative systemic therapies are mainly based on clinico-pathologic variables such as clinical stage, presence of concomitant CIS, variant histology and LVI at TURBT, tumor grade and presence of pre-operative hydronephrosis. In this context, several biomarkers have been proposed in order to strengthen and improve the precision of these prognostic models.

Prognostic markers after RC alone

As for NMIBC, p53 has been the most studied biomarker for the prediction of outcomes also in patients with

MIBC. Shariat *et al*. demonstrated how its expression was independently associated with recurrence and CSM (91). These findings were confirmed by Malats *et al*. in its meta-analysis (a prognostic value of p53 was found in 27% of cases) (92). Human epidermal growth factor receptor 2 (HER2) has also been tested in patients with MIBC with contrasting results. Soria *et al*. found a correlation between overexpression of HER2 and worse anatomopathological features at RC such as lymph node involvement, but this did not translate into a worse prognosis (117); conversely, Bolenz *et al*. found an association between HER2 and both disease recurrence and CSM (118). The retinoblastoma protein (RB1) and PTEN, tumor suppressor genes, and their inactivation seem to be correlated with worse cancer-specific survival in MIBC, while their mutation seem to be absent or rare in low-grade and superficial tumors (of the 38 patients with muscle-invasive tumors, 13 had altered expression of Rb, and only one of 10 patients with superficial carcinomas had this alteration) (119). An important role of survivin was described by Shariat *et al*.: its expression was detected only in tumor sections and in none of the normal bladder specimens (both in cystectomy and nodes specimens). At multivariable analyses that adjusted for the effect of standard prognosticators, survivin expression was associated with disease recurrence (P=0.040), disease-specific mortality and all-cause mortality in patients treated with RC. The addition of survivin improved the accuracy of the model over standard clinico-pathologic features for prediction of disease recurrence and CSS and raised the potential for survivin-targeted therapy for BCa (120). Despite some interesting results coming from these biomarkers, their potential role as single predictors of outcomes remains poor since their accuracy over standard prognosticators has proven to be unsatisfactory. Some works tried to assess the role of a panel of established BCa biomarkers. A combined analysis of p53, pRB, p21, p27, and cyclin E1 in 191 patients with pTa3N0M0 BCa treated with RC and bilateral lymphadenectomy (median follow-up: 3.1 years) showed that the number of altered biomarkers had the highest predictive accuracy for both disease recurrence (76.8%, P<0.001) and CSM (78.3%, P<0.001), and its addiction increased the predictive accuracy of a nomogram based on the TNM staging system for disease recurrence and CSM by 10.9% (83.4% *vs.* 72.5%, P<0.001) and 8.6% (86.9% *vs.* 78.3, P<0.001), respectively (121,122). The Cancer Genome Atlas (TCGA) project reported an analysis of 131 urothelial carcinomas to provide a comprehensive landscape of molecular alterations, detecting recurrent mutations in 32 genes, including multiple genes involved in cell-cycle regulation, chromatin regulation, and kinase signaling pathways (123). RNA sequencing revealed four expression subtypes: the "cluster I" tumors presented a papillary-like morphology, with overexpression of FGFR3, decreased expression of miR-99a and miR-100 and high expression of GATA3 and FOXA1; cluster II tumors presented overexpression of uroplakins, E-cadherin, members of the miR-200 family, ERBB2 and estrogen receptor-β. Cluster III tumors, defined also as basal/squamous like, were characterized by overexpression of epithelial lineage genes. Starting from that, an international collaboration led by Kamoun *et al*. tried to facilitate the clinical use of molecular classes by the identification of six classes of MIBC on the basis of oncogenic mechanisms: luminal papillary (24%), luminal non-specified (8%), luminal unstable (15%), stroma-rich (15%), basal/squamous (35%), and neuroendocrine-like (3%). Luminal unstable, basal/squamous and neuroendocrine-like subtypes were associated with worse oncological outcomes after RC (124). These findings may have important future implications such as those related to targeted therapies, helping the development of MIBC precision medicine by providing a robust framework to connect clinical contexts to molecular findings.

Predictive markers of response to NAC

A plethora of biomarkers involved in cell cycle as DNA repair genes, regulators of apoptosis, receptor tyrosine kinases, genes involved in cellular efflux and molecular subtypes have been analyzed for understanding the response to cisplatin-based NAC. Regarding products of DNA repair genes, a significant pathological response (pT0-1) at RC after NAC was observed in 66% (24 of 39) of patients with low/intermediate BRCA1 levels compared to 22% (4 of 18) of patients with high BRCA1 levels (P=0.01). Median overall survival (OS) was 168 months in patients with low/intermediate levels and 34 months in patients with high BRCA1 levels (P=0.002). In the multivariable analysis for survival, only BRCA1 expression levels and the presence of LVI emerged as independent prognostic factors (125). Regarding ERCC1, results are conflicting. Some authors have denied a correlation between expression of ERCC1 and response to NAC (126), while others as Ozcan *et al*. reported a median OS of 9.3 *vs.* 26.7 months (P=0.058) in patients with low and high ERCC1 expression, respectively (127). Pathological complete response after

NAC (HR: 0.1, 95% CI: 0.012–0.842, P=0.034) and high ERCC1 expression (HR: 3.7, 95% CI: 1.2–11.2, P=0.019) were significantly associated with disease-free survival. Van Allen *et al*. performed a whole-exome sequencing on DNA from 50 patients with muscle-invasive urothelial carcinoma who received neoadjuvant cisplatin-based chemotherapy followed by RC (25 pT0/pTis "responders", 25 pT2+ "not-responders") and resulted that ERCC2 was the only significantly mutated gene enriched in the cisplatin responders compared with not-responders (128). In 2015 a prospective study showed how an alteration in one or more of the three DNA repair genes ATM, RB1, and FANCC predicted pathologic response (P<0.001; 87% sensitivity, 100% specificity) and better OS (P=0.007) after cisplatin-based chemotherapy for MIBC (98). The ERRB family of tyrosine kinase growth-factor receptors was also analyzed of response to NAC, and main results derived from a cohort composed of 38 patients with complete response (ypT0N0) and of 33 patients not-responders (higher than ypT2). Using a sequencing of 178 genes it was found that 9 of 38 complete responders had ERBB2 mutations, whereas none of 33 not-responders had ERBB2 mutations (P=0.003), thus concluding that ERBB2 may characterize a subgroup of MIBC patients with an excellent response to NAC (129). Regarding the regulators of apoptosis, patients with nuclear overexpression of p53 had a significantly higher proportion of cancer deaths and, at multivariable analysis, p53 overexpression was independently associated with survival (P=0.001; relative risk ratio, 3.1). Long-term survival was low (41%) in patients with p53 overexpression *vs.* 77% in those without p53 overexpression (P=0.007) (97). High affinity copper uptake protein 1 (CTR1) is a protein involved in cellular efflux and plays a role in the influx of platinum. An evaluation of CTR1 expression in 47 patients with MIBC treated with NAC seems to suggest that CTR1 expression may be associated with pathological response (130). Lastly, while past studies seemed to correlate response to NAC to basal, luminal and p53-like subtypes, based on different molecular expression, a recent international consensus about molecular classification of MIBC did not find a significant association between the molecular subtypes and response to NAC treatment (124).

Predictive markers of response to systemic chemotherapy

Regulators of the cell cycle have been studied also as predictors for response to systemic chemotherapy in advanced/metastatic setting. In this scenario, cyclins and cyclin-dependent kinases have a key-role (131). Chemotherapy was showed as particularly beneficial in patients with high nuclear CyclinD1 expression (100), and CCND1 amplification status and CyclinD1 expression are independent risk factors for developing metastatic BCa (132). Analyzing the role of mi-RNAs in 83 patients with advanced MIBC, higher levels of miR-21 (P=0.01, HR: 2.01) and miR-372 (P=0.05, HR: 1.70) were associated with a shorter PFS, even if this study did not confirm the role of mi-RNAs in modulating platinum sensitivity (133). P53 was studied also as predictor of response to systemic and adjuvant CT. Despite initial promising results (93), a phase III randomized controlled trial did not confirm the predictive role of p53 in invasive disease: in a cohort of 499 patients, overall 5-year probability of disease recurrence after CT was not related to the p53 status (99). Currently, the most promising biomarkers as predictors of response to CT are the proteins involved in DNA damage detection and repair (DDR), such as BRCA-1, BRCA-2, RAD51, PAR, PARP1, ERCC1, ERCC2, and RRM1. In a study including 100 patients treated with platinum-based chemotherapy, the ones with DDR alterations showed an improved PFS (9.3 *vs.* 6.0 months, P=0.007) and OS (23.7 *vs.* 13.0 months, P=0.006) (134). Finally, molecules involved in drug transport such as MDR1 gene, and in cell growth signaling such as EGFR, HER2 and FGF, were studied as predictors to therapy, with miscellaneous and not conclusive results (100,135).

Predictive markers of response to systemic immunotherapy

The recent approval of two classes of drugs (PD-L1 targeting agents and PD-1 blocking monoclonal antibodies) for the treatment of urothelial cancer by the FDA dramatically transformed the therapeutic landscape of advanced/metastatic BCa. Several biomarkers have been explored in this context and the evidence that a significant proportion of patients has a good response to treatment despite being tested negatively for a molecule (and vice-versa) underlines the need of larger prospective trials (136-140). PD-L1 expression has been widely studied also in BCa, with contrasting results. In a study of 315 patients the PD-L1 expression status on infiltrating immune cells (ICs) in the tumor microenvironment was defined by the percentage of PD-L1-positive ICs: IC0 (<1%), IC1 (≥1% but <5%), and IC2/3 (≥5%). Objective response rates were 26% (95% CI: 18–36) in the IC2/3 group, 18% (95% CI: 13–24) in the IC1/2/3 group, and 15% (95%

CI: 11–19) overall in all 310 patients (136). Conversely, the CheckMate 275 trial showed clinical responses to nivolumab independently of PD-L1 expression levels (137). Many causes can be identified as responsible of these discrepancies: lack of standardized testing and evaluation of PD-L1, variations in percentage cut-offs and, lastly, that PD-L1 level is frequently assessed on a single biopsy and is therefore unable to reflect the intratumoral variability of BCa. The IMvigor210 trial found that molecular subtypes of BCa were independently associated with response to atezolizumab treatment (136). PD-L1 immune cell prevalence was higher in the basal subtype rather than in the luminal one (60% *vs.* 23%, P<0.0001), with expression of 15% in papillary-like luminal cluster I, 34% in cluster II, 68% in squamous-like basal cluster III, and 50% in basal cluster IV. Response to atezolizumab was significantly higher in luminal cluster II (objective response rate of 34%, P=0.0017). Conversely, CheckMate 275 showed that basal 1 subtype had the highest proportion of responders (137). As other biomarkers, TGFβ was shown as attenuator of tumor response to PD-L1 blockade (136), while higher values of IFN-γ were associated with good response to nivolumab and higher PD-L1 expression (137). Finally, a correlation between high mutational burden and better responses to immunotherapeutic agents have been identified (136). Despite these interesting and promising results, more research is needed before tissue biomarkers could enter in the clinical practice and drive decision regarding systemic immunotherapy.

Predictive markers of response to radiotherapy

Currently, patients unfit for RC can benefit of radiotherapic treatment in the context of trimodal therapy (TMT), combined with transurethral tumor resection and chemotherapy, with quality of life and oncological outcomes apparently comparable to surgical treatment (141,142). It is mandatory to stratify patients on the basis of the level of response to radiotherapy, in order to identify optimal candidates for TMT. Rödel *et al*. correlated high Ki-67 index (≥8%) to complete response and local control with preserved bladder at 5 years after radiotherapy (103). Tanabe *et al*., in their retrospective analysis, demonstrated that, among various clinicopathologic variables, high Ki-67 expression (≥20%) was independently associated with better CSS (5-year CSS rate, 78% *vs.* 46% for high *vs.* low Ki-67 levels; P=0.019) (143). Matsumoto *et al*., describing the role of molecules involved in apoptotic pathways as p53, Bcl-2, Bax, found no correlation between their expression and response to radiotherapy (106). As already described with systemic chemotherapy, the prognostic impact of DDR gene alterations, most commonly identified in ERCC2, showed a trend for improved response to radiotherapy, with higher bladder (HR: 0.32, P=0.070) or metastatic (HR: 0.37, P=0.070) RFS after 2 years of follow-up (144). Differently from other experiences, no impact of MRE11 protein expression on outcomes was detected (145).

Conclusions

The great interest about biomarkers and the related amount of literature published in the last decade reflects the fact that we are moving from the era of "one fits all" to that of precision medicine. Despite tremendous and continuous research, to date, biomarkers are not yet recommended for their use in daily clinical routine, because of both low level of evidence due to the poor quality of available data and to the difficulty in identifying the correct scenario of use for each biomarker, as shown in this systematic review. Despite this, there is no doubt that the potential of these biomarkers is hugely significant and that the path followed is the correct one. It derives that it is essential to pour even more energies investing in randomized controlled trials and in large international collaborations to overcome the obstacles we have encountered so far. Until then, the role of biomarkers should be limited to experimental settings.

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