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Liquid biopsy in bladder cancer: State of the art and future perspectives

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ABSTRACT

Bladder cancer is the most common malignancy of the urinary tract. Cystoscopy represents the gold standard in the diagnosis of suspicious bladder lesions. However, the procedure is invasive and burdened by pain, discomfort and infective complications. Cytology, which represents an alternative diagnostic possibility is limited by poor sensitivity. Considering the limitations of both procedures, and the necessity to perform multiple evaluations in patients who are in follow-up for bladder cancer, an improved non-invasive methodology is required in the clinical management of this disease. Liquid biopsy, e.g. the detection of clinical biomarkers in urine, represent a promising novel and non-invasive approach that could overcome those limitations and be integrated into the current clinical practice. The aim of this review is to summarize the state of the art of this approach and the latest novelties regarding detection, prognosis and surveillance of bladder cancer.

1. Introduction

Bladder cancer (BC) is the most common malignancy of the urinary tract and the 9th most common cancer worldwide with 430000 incident cases and 165000 deaths per year (Cumberbatch and Noon, 2019). The age-standardized incidence rate of BC is estimated to be 26.9 and 5 per 100000 in men and women, respectively, with an overall higher incidence in North America and Western Europe (Mohammadian et al., 2020). Over 75 % of BC patients are diagnosed with non-muscle invasive bladder cancer (NMIBC), with 10–25 % that eventually develop muscle-invasive bladder cancer (MIBC) (Sanli et al., 2017). The most common diagnosed histological subtype is the urothelial carcinoma (up to 90 % of cases), followed by the squamous cell carcinoma (SCC) (5%), the adenocarcinoma (0.5–2 %) and the small cell carcinoma (<1%) (Martin et al., 2016; Alderson et al., 2020). The diagnosis of BC is usually performed, after episodic macroscopic haematuria, through ultrasound scan (US), cytology evaluation, and cystoscopy, which represent the current gold standard. A certain diagnosis is however obtained only via histopathological reports performed on tissue sampled with a transurethral bladder resection (TURB), which also permits, in non-muscle invasive tumours, the eradication of the neoplasm (DeGeorge et al., 2017).

Although cystoscopy represents the best diagnostic tool to inquire about a suspicious US or an episode of haematuria, the procedure is still invasive and burdened by pain and infective complications (Roth et al., 2021). Conversely, the use of US and urine cytology for the diagnosis of BC, despite practical advantages such as non-invasiveness, limited costs and easily execution, suffer from limited sensibility and sensitivity and a low degree of reproducibility (Yafi et al., 2015). Other imaging techniques, like computed tomography (CT) scan, or magnetic resonance imaging (MRI), although presenting optimal diagnostic capabilities in detecting BC, are limited, in clinical practice, to the staging of disease, due to ionizing radiations and/or increased costs (Bouchelouche et al., 2012; Galgano et al., 2020). Moreover, in addition to the first BC diagnosis, patients treated for NMIBC require a strict follow-up in order to evaluate recurrence of the disease, which is, despite transurethral resection of bladder (TURB) and adjuvant chemo-immunotherapy, particularly frequent (31–78 % at 5 years) (Kassouf et al., 2016). As

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result, multiple cystoscopies are performed after TURB (every 3–6 months for 5 years), according to stage, grade and recurrence, as reported by urological guidelines (Babjuk et al., 2020). The necessity of multiple invasive procedures as cystoscopies, with all the related risks, pain and discomfort associated, in a third of patients, has underlined the necessity of new surveillance methods for NMIBC (Van Der Aa et al., 2008).

Liquid biopsy refers to the non-invasive analysis of biomarkers in biological fluids (such as blood, plasma, urine, liquor and saliva) in order to allow the detection, and the longitudinal follow up of cancer evolution, avoiding the limitations of invasive procedures and, contextually, obtaining enough molecular information as could be derived from tissue biopsies (Serrano et al., 2020; Ferro et al., 2021). One of the advantages of liquid biopsy is the possibility to obtain multiple analytes as circulating tumour cells (CTCs), circulating cell-free tumour DNA (ctDNA), circulating cell-free tumour RNA (ctRNA), proteins, peptides and metabolites, even from a single specimen, which could similarly be utilized in multiple assays (Soda et al., 2019; Crocetto et al., 2021). Another advantage of liquid biopsy is the possibility to reduce or eliminate the intra-tumoral heterogeneity, overcoming the variability of molecular information obtained by tissue analysis which could be dependent on location and accessibility of tumour. Finally, the possibility to obtain serial monitoring of tumoral biomarkers permit the evaluation of tumour progression and, therefore, the choice of a tailored therapy (Geeurickx and Hendrix, 2020).

Although blood is the most commonly described fluid adopted in liquid biopsy for several cancers due to the high abundancy of tumor marker proteins and the close correlation between markers and diseases, urine represents, for BC, the best choice, as it is constantly in contact with bladder mucosa and bladder tumour, is easily acquired, do not require particular compliance of the patient and has fewer contaminants compared to blood (Jain et al., 2019). Differently from blood, indeed, urine is a less complex and relatively clean and cell-free biofluid, with a negligible amount of proteins and comparable levels of ctDNA and ctRNA (Michela, 2021; Oshi et al., 2021). Considering that the vast majority of diagnosed BC are transitional/urothelial (90 %) and squamous (5%) and that both histological types are characterized by a propensity to exfoliate, urine represent a resourceful specimen for liquid biopsy (Ringsrud, 2001; Satyal et al., 2019).

The aim of this review is to summarize the current landscape of urinary biomarkers and their clinical applications in BC.

2. Materials and methods

A systematic search was conducted using MEDLINE, Scopus and Web of Science databases in August 2021, according to the general guidelines recommended by the Primary Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) statement (Fig. 1) (Page et al., 2021). The following terms with synonyms were combined in a title-abstract search to retrieve all relevant publications: bladder cancer, non-muscle-invasive bladder cancer, urinary biomarker, liquid biopsy. Articles were screened by two independent reviewers in order to select studies, extract data and remove duplicates. In addition, other articles were manually retrieved from references and urological guidelines (European Association of Urology and American Urological Association) reporting currently utilized urinary biomarkers. We excluded from the search any biomarkers not urine-based, and studies published before 2000, preferring, where possible, studies of the last 10 years. Articles retrieved were analysed and narratively reported. A comprehensive table summarizes the urinary biomarkers reported in this review (Table 1).

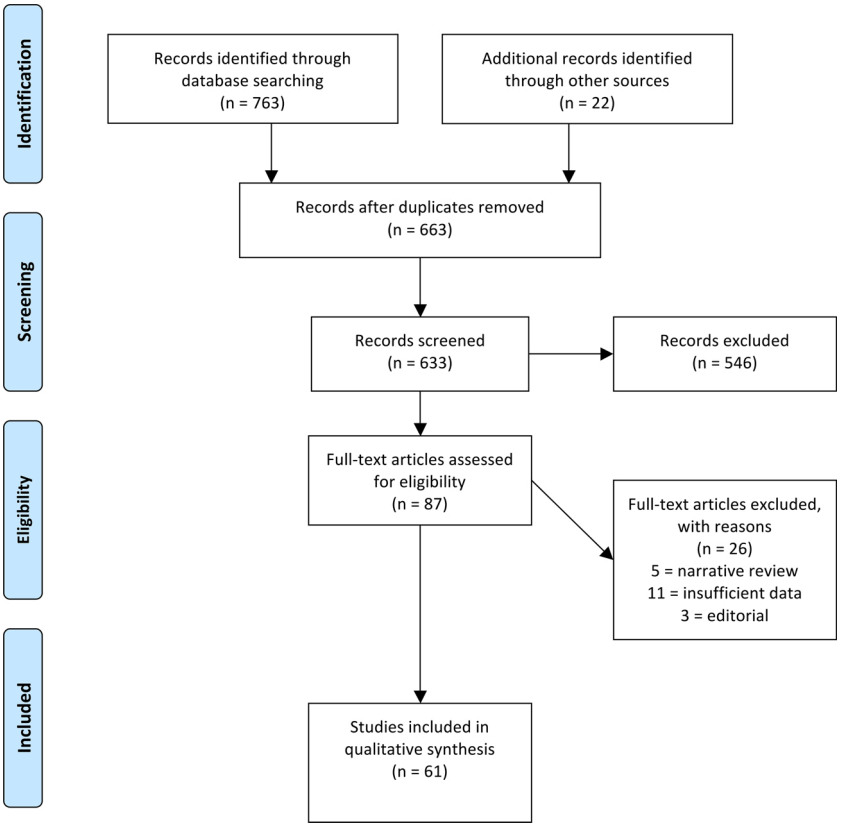

Fig. 1. PRISMA flowchart.

Table 1
Summary of Urinary biomarkers. Abbreviations - CEA: Carcinoembryonic Antigen; MAUB: Mucin Antigen of the Urinary Bladder; FDA: Food and Drug Administration; FISH: Fluorescence In Situ Hybridization; CE: Conformitè Europëenne; NGS: Next Generation Sequencing; BS-Seq: Bisulfite Sequencing; PCR: Polymerase Chain Reaction; SafeSeqS: Safe-Sequencing System; HTS: High-Throughput Sequencing; RT: Real Time; MASO: Multiplex Allele-Specific, Oligonucleotide; qPCR: quantitative Polymerase Chain Reaction; ELISA: Enzyme-Linked Immunosorbent Assay; POC: Point Of Care.

Test name	Producer	Variables	Assay type	Molecular Target	Sensibility/ Specificity	Approval	Cost	Reference
ImmunoCyt	DiagnoCure and Scimedx	CEA, MAUB	Immunofluorescence cytology	Sediment cells	72 %/65 %	FDA	80$	(Crocetto et al., 2021; Geeurickx and Hendrix, 2020; Jain et al., 2019)
Urovysion	Abbot	Chromosome 3-7-9-17	FISH	Sediment cells/DNA	69 %/76 %	FDA/CE	800 $	(Michela, 2021; Oshi et al., 2021; Ringsrud, 2001; Satyal et al., 2019; Page et al., 2021; Fradet and Lockhard, 1997; Odisho et al., 2013)
Uromark	Kelly-Feber and Abbot	Epigenetic alterations	NGS + BS-Seq PCR	Sediment cells/DNA	95 %/96 %	FDA	N/A	(He et al., 2016; Nagai et al., 2021)
Uromonitor	U-monitor Lda	*FGFR3, TERT, KRAS*	PCR	DNA	73.5 %/93.2 %	FDA/CE	N/A	(Ikeda et al., 2020; Chou et al., 2015)
Uroseek	John Hopkins University	*TERT, FGFR3, TP53, CDKN2A, ERBB2, HRAS, PIK3CA, METH, BHL, MLL*	SafeSeqS	DNA	95 %/93 %	No	750 $	(Sassa et al., 2019; Iwata et al., 2021; Ward et al., 2016)
Uromutert	International Agency for Research on Cancer	*TERT*	NGS PCR	DNA	87.1 %/94.7 %	No	N/A	(Feber et al., 2017; Tan et al., 2017; Cappellen et al., 1999)
uCAPP-Seq	Standford University	*TERT, PLEKHS1, TP53, FGFR3, ERBB2, RB1*	HTS	DNA	84 %/96 %	No	N/A	(Ouerhani et al., 2013; Jebar et al., 2005)
Bladder Epicheck	Nucleix	DNA methylations	RT-PCR	DNA	81 %/83 %	FDA/CE	150 $	(Sieverink et al., 2020; Kinde et al., 2011; Springer et al., 2018; Rodriguez Pena et al., 2020)
Urodiag	Oncodiag	*FGFR3, HS3ST2, SEPT9, SLIT2*	DNA methylation + MASO-PCR	DNA	95.5 %/75.9 %	CE	100 $	(Eich et al., 2019; Avogbe et al., 2019; Hosen et al., 2020b)
AssureMDx	MDxHealth	*FGFR3, TERT, HRAS, OTX1, ONECUT2, TWIST1*	DNA methylation + PCR	DNA	93 %/86 %	No	500 $	(Dudley et al., 2019; Chen et al., 2021)
CxBladder	Pacific Edge	*CDK1, MDK, HOXA13, IGFBP5, CXCR2*	qPCR	mRNA	82 %/85 %	CE	300 $	(Babbra et al., 2020; Witjes et al., 2018; Mancini et al., 2020; Trenti et al., 2019)
Xpert BC	Cepheid	*ABL1, UPK1B, CRH, ANXA10, IGF2*	RT-PCR	mRNA	76 %/85 %	FDA/CE	165 $	(Pierconti et al., 2021; Roperch and Hennion, 2020; Kompier et al., 2010; Porten, 2018; Roperch et al., 2016; Kessel et al., 2016)
NMP22 Bladder Chek	Abbott	NMP22	ELISA + POC immunoassay	Protein	59 %/93 %	FDA/CE	25$	(Beukers et al., 2017; Konety et al., 2019; O'Sullivan et al., 2012)
BTA	Polymedco and Sysmex	BTA	Immunochromatography + ELISA	Protein	56 %/85.7 %	FDA/CE	40$	(Pichler et al., 2018; Valenberg et al., 2021; Cancel-Tassin et al., 2021; D'Elia et al., 2021; Hurle et al., 2020)
ADXBLADDER	Arquer	MCM	ELISA	Protein	73 %/68.4 %	CE	50$	(Liu et al., 2021; Nguyen and Jones, 2008; Hatzichristodoulou et al., 2012; Miyake et al., 2012a; Wang et al., 2017; Pichler et al., 2017)
CYFRA 21.1	CIS Bio International and Fujirebio Diagnostics	Cytokeratin 19	ELISA	Protein	82 %/80 %	No	450 $	(Raitanen and The FinnBladder, 2008; Miyake et al., 2012b; Babjuk et al., 2008)
UBC Rapid	IDL Biotech	Cytokeratin 8 and 18	POC immunoassay	Protein	70.8 %/61.4 %	No	500 $	(Dudderidge et al., 2020; Roupret et al., 2020; Gontero et al., 2021; Białek et al., 2021; Anastasi et al., 2020)
Oncuria	Nonagen	ANG, APOE, A1AT, CA9, IL8,	Immunoassay	Protein	85 %/81 %	No	N/A	(Andreadis et al., 2005; Huang et al., 2015;

(*continued on next page*)

Table 1 (*continued*)

Test name	Producer	Variables	Assay type	Molecular Target	Sensibility/ Specificity	Approval	Cost	Reference
		MMP9, PAI1, SDC1, VEGF						Fernandez-Gomez et al., 2007)

3. Urinary biomarkers for detection and surveillance of bladder cancer

3.1. Improved cytology

3.1.1. ImmunoCyt

ImmunoCyt (uCyt+) is an immunofluorescence test developed by Fradet and Lockhard in 1997 based on the use of three monoclonal antibodies aimed at urothelial cells in voided urine, in particular at two tumoral antigens (a glycoform of the carcinoembryionic antigen, i.e CEA, and a mucin antigen associated with bladder, i.e MAUB) (Fradet and Lockhard, 1997). Despite the early origin of the test, its use in clinical settings has been quite limited and overlooked. Odisho et al. suggested the use of uCyt + as a second-level test to clarify results of atypical cytology, reporting a good and homogeneous sensitivity for low and high-grade disease (75 %) while specificity was lacking (only 49 %) (Odisho et al., 2013). In 2016, a large meta-analysis by He et al. performed on seven separate studies, for a total of 1602 BC patients, reported an overall sensitivity for uCyt + of 72 % with a specificity of 65 %, suggesting the use of uCyt + in combination with cytology due to the evident limitations related to specificity (He et al., 2016).

3.1.2. Urovysion

Urovysion is a multitarget fluorescence in situ hybridization (FISH) assay performed on exfoliated cells in voided urine which deliver a dichotomous response based on criteria including chromosomal (3, 7, 9 and 17) and morphologic changes of cells (Nagai et al., 2021). Recently a sensitivity of 67–69 % and a specificity of 72–76 % has been reported, although a sustained variability has been shown in other studies, especially in the setting of atypical urothelial cells scenario, with a sensitivity and specificity of, respectively, 44–48 % and 78–81 % (Lavery et al., 2017; Virk et al., 2017). Due to the peculiar characteristics of Urovysion, the test has been used in several clinical applications. In a follow-up setting for assessing the risk of recurrence of BC, Urovysion well performed, reporting, in a two-consecutive testing, respectively 16.5 % (one positive test) and 33.3 % (two positive tests) of BC recurrence after transurethral resection of bladder tumor (TURB) (Ikeda et al., 2020). In addition, Urovysion has been successfully used in the clarification of atypical urothelial cells reported in urinary cytology, identifying in 17.9 % of cases high grade (HG) BC (Miki et al., 2017). Despite promising results, Urovysion has a lower sensitivity for low-grade tumors compared to Immunocyt (Sullivan et al., 2009; Chou et al., 2015). Finally, promising results are reported in the utilization of Urovysion in detecting urothelial cancer of the upper urinary tract (Sassa et al., 2019; Iwata et al., 2021).

3.1.3. Uromark

Uromark assay is a non-invasive test performed on urine that analyses a panel of 150 epigenetic alterations through next-generation DNA sequencing (NGS) techniques and RainDrop BS-Seq (bisulfite sequencing), a microdroplet-based polymerase chain reaction (PCR) amplification of bisulfite converted DNA. Compared to other PCR techniques, NGS permit to obtain and extract DNA from almost all urine samples, due to low input requirements (Ward et al., 2016). The test was developed and validated on different cohorts for a total of 274 patients reporting sensitivity and specificity of, respectively, 95 % and 96 % (Feber et al., 2017). Currently, two multicentric studies are evaluating the performance of the Uromark assay (DETECT I and DETECT II) both in first diagnosis and recurrent BC (Tan et al., 2017).

3.2. DNA mutations

3.2.1. Uromonitor

Uromonitor is a real-time PCR assay that detects oncogene hotspot mutations in BC tumour exfoliated cells in urine, particularly fibroblast growth factor receptor 3 (*FGFR3*), which accounts for 35 % of BC, and telomerase reverse transcriptase (*TERT*) promoter (Cappellen et al., 1999; Liu et al., 2013). A first technical validation for NMIBC recurrence detection comprehended 331 urine samples, while a subsequent clinical validation involved 185 patients, reporting a sensitivity of 73.5 % and a specificity of 93.2 % (Batista et al., 2019). An updated version of Uromonitor (Uromonitor-V2), added the detection of *KRAS* (Kirsten rat sarcoma) hotspot mutations which, although have a key role in bladder cancer pathogenesis, are mutually exclusive with *FGFR3* (Ouerhani et al., 2013; Jebar et al., 2005). The new Uromonitor-V2 reported on 97 patients involved, a sensitivity of 93.1 % while specificity reached 85.4 %. The test presented, among the advantages of a high detection rate, a short time to yield results (6−8 h), limited costs and a dichotomic response (Sieverink et al., 2020). However, due to the limited size of patients involved and the limited results in terms of high-grade NMIBC detection, further studies are required.

3.2.2. Uroseek

Uroseek is a urine-based assay that applies massive parallel safe sequencing system (SafeSeqS), a novel PCR approach able to identify mutations present in small fraction of DNA templates, to detect BC mutations affecting *TERT* promoter and 10 additional genes which comprehend *FGFR3*, *TP53* (Tumour Protein P53), *CDKN2A* (Cyclin Dependent Kinase Inhibitor 2A), *ERBB2* (v-Erb-B2 Avian Erythroblastic Leukemia viral oncogene homolog 2), *HRAS* (Harvey Rat sarcoma), *KRAS*, *PIK3CA* (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha), *METH* (Methionine synthase), *VHL* (Von Hippel-Lindau Tumor suppressor), *MLL/KMT2A* (Lysine Methyltransferase 2A) (Kinde et al., 2011). A first study by Springer et al., reported, for Uroseek plus cytology, on two different cohorts (early detection and surveillance) a sensitivity and specificity of 95 % and 93 %, respectively, in 570 patients of the early detection cohort; whereas a worse performance was reported for 322 patients belonging to the surveillance cohort, yielding a sensitivity and specificity of 71 % and 80 %, respectively (Springer et al., 2018). A similar study by Rodriguez Pena et al. assessed the performance of Uroseek only on the two previously reported cohorts, reporting sensitivity and specificity of 96 % and 88 %, respectively, for 496 patients belonging to the early cohort; analogously, sensitivity and specificity for 348 patients of the surveillance cohort were lower, reaching 74 % and 72 % respectively (Rodriguez Pena et al., 2020). Interestingly, Eich et al. reported, in 527 patients, an overall 92 % positivity for at least one genetic alteration detected by Uroseek panel, confirming the comprehensive coverage and the potentiality of this assay in the diagnosis and surveillance of BC (Eich et al., 2019).

3.2.3. Uromutert

Uromutert is based on an ultra-deep next-generation sequencing of partial *TERT* promoter which detects, via an algorithm, low-allelic fractions mutations in various body fluids, including urine. A first validation test performed by Avogbe et al. reported, on 93 primary and recurrent BC and 94 controls, an overall sensitivity of 87.1 % while specificity was 94.7 % (Avogbe et al., 2019). Interestingly, Hosen et al. reported in a prospective case-control study on 30 asymptomatic individuals and 101 matched controls the detection, in the first cohort of

TERT promoter mutations in 14 patients up to 10 years the clinical diagnosis of BC (sensitivity 46.7 %, specificity 100 %) (Hosen et al., 2020a). A successive study from the same authors, which increased the size of patients involved for a total of 287 (143 cases and 144 controls), compared a digital droplet polymerase chain reaction (ddPCR) assay detecting *TERT* promoter mutations with Uromutert, reporting comparable results (Hosen et al., 2020b). Due to the fast processing time, the affordable cost and the independence from extensive bioinformatics post-processing, the ddPCR assay could however more easily permit the large scale implementation of *TERT* mutation analysis (Perkins et al., 2017).

3.2.4. uCAPP-Seq

uCAPP-Seq (urine Cancer Personalized Profiling by Deep Sequencing) is a novel high-throughput sequencing (HTS) method for the detection of tumour DNA in urine. This type of sequencing, which has been already successfully used, with plasma, for lung cancer, reported, in a recent study by Dudley et al. on 67 healthy subjects and 118 NMIBC patients, a sensibility of 84 % with 96 % of specificity. *TERT* and *PLEKHS1* (Pleckstrin Homology Domain Containing S1) promoters mutations were found, respectively, in 74 % and 46 % of cases, followed by *TP53*, *FGFR3*, *ERBB2* and Retinoblastoma Transcriptional Corepressor 1 (*RB1*) mutations. Interestingly, detection of urinary tumour DNA preceded clinical disease recurrence in 92 % of patients by a median of 2.7 months (Dudley et al., 2019). Although the recent application of uCAPP-Seq in BC, its possible use also in MIBC before radical cystectomy, neoadjuvant therapy and as a prognostic biomarker is promising (Chen et al., 2021; Babbra et al., 2020).

3.3. DNA methylations

3.3.1. Bladder Epicheck

Bladder Epicheck test is a non-invasive assay based on the detection of DNA methylation status of 15 different genomic loci through Real time (RT)-PCR and further analysed via specific software, delivering a numerical value (Episcore) which ranges from 0 to 100; values >60 are considered positive for bladder cancer (Witjes et al., 2018). The test reports a higher sensitivity in higher stages and grades (delivering a sensitivity of 81 % for Ta, 91 % for in situ carcinoma, i.e CIS) and up to 100 % for T1-T2) while specificity was reported to reach up to 83 %. In addition, it has been evaluated that a one-point increase in Episcore yielded a 4% increase of any grade BC and 8% increase in high grade NMIBC (Mancini et al., 2020). Considering the promising results in terms of sensitivity also for low stage/grade BC, Bladder Epicheck expresses the maximum diagnostic power in those cases while, in high-grade NMIBC, the test could be efficiently utilized to increase the interval between follow-up cystoscopies (Trenti et al., 2019; Pierconti et al., 2021).

3.3.2. Urodiag

Urodiag is a novel test proposed by Roperch and Hennion, which associates the detection of *FGFR3* mutations with DNA methylation assay via an ultra-sensitive multiplex PCR assay denominated Mutated Allele-Specific Oligonucleotide-PCR (MASO-PCR) (Roperch and Hennion, 2020). The rationale of this test lies in the evidence of *FGFR3* mutations as highly reported in BC, with, in particular, four mutations (p.G372C, p.R248C, p.S249C and p.Y375C) which accounted for over 95 % of cases (Kompier et al., 2010). Similarly, epigenetic modifications as DNA methylation has been already reported to have a pivotal role in this disease (Porten, 2018). Urodiag is, indeed, a multiplex PCR kit that detects *FGFR3* somatic mutations and quantifies three DNA methylation markers (*HS3ST2*, *SEPT9* and *SLIT2,* i.e, respectively: Heparan Sulfate-Glucosamine 3-Sulfotransferase 2, Septin9 and Slit Guidance Ligand 2) by stable multiplex PCR in urine. In a previous study on 263 patients, the panel including *FGFR3* mutations and hypermethylation of previously cited DNA markers yielded a sensitivity of 95.5 % and a specificity of 75.9 % (Roperch et al., 2016) in NMIBC. This however the only study currently reporting data on mutations and hypermethylations detected by Urodiag.

3.3.3. AssureMDx

AssureMDx is, similarly to Urodiag, a novel non-invasive, urine-based test that combines epigenetic and mutation biomarkers. In particular, the assay analyses mutations in *FGFR3*, *TERT* and *HRAS* genes and methylations in *OTX1* (Orthodenticle Homeobox 1), *ONECUT2* (One Cut Homeobox 2) and *TWIST1* (Twist Family BHLH Transcription Factor 1) genes (Kessel et al., 2016). In a multicentric study involving 200 patients undergoing cystoscopy for haematuria, the test yielded a sensitivity and specificity of 93 % and 86 %, respectively, with higher AUC (Area Under the Curve) in high-grade tumours compared to low-grade tumours, leading to a 77 % reduction of diagnostic cystoscopies (van Kessel et al., 2017). Similarly, a recent multicenter study by Beukers et al. reported on 977 patients a sensitivity of 57 % in primary low-grade NMIBC and 83 % in high grade and MIBC (Beukers et al., 2017).

3.4. MRNA signatures

3.4.1. CxBladder

CxBladder is a clinically validated mRNA test which measures the concentration of five genes (*CDK1*, *MDK*, *HOXA13*, *IGFBP5* and *CXCR2,* i.e, respectively: Cyclin Dependent Kinase 1, Midkine, Homeobox A13, Insulin Like Growth Factor Binding Protein 5 and C-X-C Motif Chemokine Receptor 2) in unfractionated urine, utilizing a quantitative PCR assay. The test presents two versions (Detect and Monitor) which permit the identification of high-risk patients that require a full urological work-up or monitoring low-risk patients in case of haematuria (Darling et al., 2017). CxBladder outperforms cytology in terms of negative predictive value (NPV) (97 % compared to 93 % of cytology) and sensitivity, missing only 8.5 % of tumours at cystoscopy versus 63 % of cytology, additionally sparing 35 % of patients from an unnecessary cystoscopy (Konety et al., 2019). Although a reported variability in terms of detection rate, the overall sensitivity and specificity of the test is, respectively, 82 % and 85 % (O'Sullivan et al., 2012). Moreover, the use of CxBladder could further improve the follow up of low-risk patients, identifying a high proportion of subjects that could be safely managed with only one annually cystoscopy, lowering the economic burden and contextually increasing patient's compliance (Koya et al., 2020).

3.4.2. Xpert BC

Xpert BC is a novel qualitative mRNA test for detection and follow up of bladder cancer that measures five target mRNA (*ABL1*, *UPK1B*, *CRH*, *ANXA10* and *IGF2,* i.e, respectively: ABL Proto-Oncogene 1 Non-Receptor Tyrosine Kinase, Uroplakin 1B, Corticotropin Releasing Hormone, Annexin A10 and Insulin Like Growth Factor 2) through RT-PCR assay which are overexpressed in the urine of patients with BC. Using an automatic nucleic acid amplification, the test detects target sequences in approximately 90 min, with an estimated sensitivity and specificity of, respectively, 76 % and 85 % (Pichler et al., 2018; Valenberg et al., 2021). Results are classified as "positive" or "negative" upon the proprietary linear regression algorithm built into the assay software, with a cut-off value, defined as linear discriminant analysis, of 0.5 or above (Cancel-Tassin et al., 2021). The test, however, presents a variable sensitivity based on the presence of low-grade or high-grade BC. In particular, Xpert BC yielded an overall sensitivity of 45.2 % for low-grade BC while this percentage rose to 80.9 % for high-grade BC, with an overall specificity of 78.4 % (D'Elia et al., 2021). In addition, Xpert BC arises as a reliable assay that permits to avoid, for a cut-off <0.4, 33.7 % of cystoscopies, with only a 9% of failures for low-grade BC (Hurle et al., 2020). Finally, a recent meta-analysis by Liu et al., reported, on 8 studies, an overall sensitivity and specificity of,

respectively, 71 % and 81 %, with an AUC of 0.84 and a higher detection rate for high-grade BC (sensitivity of 86 % compared to 59 % for low-grade BC), confirming the reliability of Xpert in the non-invasive diagnosis and follow up of BC (Liu et al., 2021).

3.5. Protein-based assays

3.5.1. NMP22 Bladder Chek

NMP22 (nuclear maxtrix protein 22) is a nuclear matrix protein overexpressed in urothelial cancer cells and excreted in urine as result of necrotic and apoptotic processes related to tumorigenesis. Due to the quantitative nature of NMP22 levels, which seems to correlate with the degree of differentiation of bladder cancer cells, many efforts have been directed towards the creation of a reliable test (Nguyen and Jones, 2008). Two NMP22 tests have been approved by FDA (Food and Drug Administration) for BC which are the NMP22 BC ELISA (enzyme-linked immunoassay) test kit (Alere NMP22) and the point-of-care NMP22 BladderChek. In a comparison between the two tests, NMP22 ELISA kit reported overall a lower sensitivity compared to NMP22 Bladder Chek (40–42 % versus 59 %), while specificity was similar for both tests (93–100 %) (Hatzichristodoulou et al., 2012). In addition to a lower sensitivity, the disadvantages and limitations of the ELISA version, which included different cut-off values, results dependent on operator experience and not immediately available, shifted the choice to the qualitative counterpart (Miyake et al., 2012a). An updated meta-analysis performed on 23 studies, evaluating the accuracy of NMP22 BladderChek, confirmed an overall pooled sensitivity of 56 % while specificity reached 88 % (Wang et al., 2017). However, although promising results, the use of NMP22 BladderChek alone or in combination with urinary cytology, delivered a sensitivity in the detection of low-grade urothelial cancer of 50 %, with a maximum specificity of 77.3 %, thus impeding the replacing of cystoscopies in the diagnostic algorithm (Pichler et al., 2017).

3.5.2. BTA

BTA stat/BTA TRAK are two assays FDA approved for diagnosis and follow-up of BC, measuring the Bladder tumor antigen (BTA), an human complement factor H-related protein: the first is an immunochromatographic assay while the second is a quantitative ELISA assay with comparable estimated ranges of sensitivity and specificity (Miremami and Kyprianou, 2014). BTA stat reported indeed an overall sensitivity of 56 % while specificity reached 85.7 %, although urine infection, haematuria and BCG instillations could influence the rate of false positive (Raitanen and The FinnBladder, 2008; Miyake et al., 2012b). A similar result was reported by Babjuk et al. for BTA TRAK, in a comparison with urinary cytology, whereas the first test reached a sensitivity of 53.8 % and a specificity of 83.9 % (Babjuk et al., 2008). A large meta-analysis consisting of 3462 patients finalized a sensitivity for BTA stat assay of 67 % while specificity was estimated to be 75 %, sensibly lower compared to the specificity of urinary cytology (Guo et al., 2014). Although the non-invasiveness of BTA tests, most studies criticized the correlation with haematuria which could limit the diagnostic capabilities of both assays, as well as the lowered sensitivity for low-grade BC, limiting, therefore, the use of those urinary biomarkers in the clinical practice. Regarding head-to-head comparisons, no differences were reported in sensitivity or specificity between NMP22 BladderChek and BTA (Chou et al., 2015; Babjuk et al., 2008). Conversely, a lower sensitivity of BTA was reported, compared to ImmunoCyt (Toma et al., 2004).

3.5.3. ADXBLADDER

AdXBLADDER is an ELISA test utilizing the detection of mini-chromosome maintenance protein (MCM) 5 via antibodies in urine. MCM5 are highly expressed in cancer cells therefore the presence of an urothelial cancer provokes the shedding of MCM5 proteins in urine, making the detection through antibodies a suitable alternative to urine cytology (Wolfs et al., 2021). The first evaluation of the performance of ADXBLADDER was effected by Dudderigde et al. on 856 patients, reporting an overall sensitivity of 73 % and an overall specificity of 68.4 %, with best results for T1 and above BC (Dudderidge et al., 2020). In another similar larger study on 1431 patients, ADXBLADDER delivered an overall sensitivity of 44.9 %, which increased to 75.6 % when low-grade BCs were was excluded, and an overall specificity of 71.1 % (Roupret et al., 2020). Although limitations related to false positives (as renal stones or urinary infections), ADXBLADDER outperformed the sensitivity of urinary cytology for all tumour types, yielding a sensitivity of 51.9 % compared to 16.7 % of urinary cytology (Gontero et al., 2021). However, if promising results are reported for the detection of recurrence of BC, with peaks of sensitivity reported to be up to 73.5 %, the use of ADXBLADDER in a primary diagnosis of BC is still limited (Białek et al., 2021). Nevertheless, Anastasi et al. reported, in the initial diagnosis of BC an overall sensitivity and specificity of this assay of, respectively, 60 % and 88.2 % with improved results for detection of high-grade tumours or in combination with urinary cytology (Anastasi et al., 2020).

3.5.4. CYFRA 21.1

CYFRA 21.1 (Cytokeratin Fragment 21.1) assay represent an ELISA test that detects soluble cytokeratin 19 fragments both in urine, expressed in epithelial cells and regarded as a tumour marker for cancer diagnosis, through two monoclonal antibodies (BM 19.21 and KS19.1) (Andreadis et al., 2005). A meta-analysis by Huang et al. involving 16 studies for a total of 2495 patients, reported a pooled sensitivity of 82 % while pooled specificity was 80 % (Huang et al., 2015). However, due to the intrinsic properties of CYFRA 21.1, which could be unproperly detected in patients with a history of previous BCG therapy and radiotherapy, and multiple biases reported in the studies analysed by Huang et al., the role of this assay in BC as a surveillance test is controversial (Fernandez-Gomez et al., 2007; Nisman et al., 2009; Guo and Long, 2016).

3.5.5. UBC Rapid

UBC Rapid assay is a point-of-care (POC) immunoassay detecting cytokeratin fragments 8 and 18 in urine, two soluble fragments related to early bladder tumorigenesis (Barak et al., 2020; Hakenberg et al., 2004). A prospective multicentre phase II study reported a sensitivity of 70.8 % and a specificity of 61.4 %, with significantly higher values in patients with high-risk group while a recent meta-analysis by Lu et al. reported on 8 studies involving 1237 patients, a sensitivity of 59 %, with a specificity reaching 76 % (Styrke et al., 2017; Lu et al., 2018). Better results were furthermore obtained for carcinoma in situ and increased tumour size (Agreda Castañeda et al., 2020; Ecke et al., 2017). However, due to the wide difference in performance reported and the lower specificity compared to standard cytology, further studies are required in order to fully assess the potential of this biomarker. Interestingly, a coupling of UBC with the new survivin ELISA has been proposed in order to improve the diagnostic performance of both assays (Gleichenhagen et al., 2018).

3.5.6. Oncuria

Oncuria is a recent developed multiplex bead-based immunoassay that monitors the concentrations of ten proteins (ANG, APOE, A1AT, CA9, IL8, MMP9, MMP10, PAI1, SDC1, VEGF, i.e, respectively: Angiogenin, Apolipoprotein E, Alpha-1 Antitrypsin, Carbonic Anhydrase 9, Interleukin 8, Matrix Metallopeptidase 9, Matrix Metallopeptidase 10, Plasminogen Activator Inhibitor type 1, Syndecan 1, Vascular Endothelial Growth Factor) overexpressed in voided urine of BC patients. Firstly reported sensitivity and specificity were, respectively, 85 % and 81 % (Furuya et al., 2020). In a large multi-institutional cohort of 362 patients, however, Oncuria reached a higher sensitivity and specificity (93 % for both entries), showing encouraging diagnostic performance in the setting of non-invasive follow-up of BC patients (Hirasawa et al.,

2021). In addition, in an ex-vivo experimental model issued on healthy subjects, Oncuria well performed even in the discrimination of false positive (e.g. macroscopic haematuria), reporting thus promising results also for first diagnosis patients (Murakami et al., 2021).

4. Limitations of urinary biomarkers

The use of urinary biomarkers in clinical practice is a fascinating perspective, as a tool to reduce or even avoid the complications and the discomfort related to multiple cystoscopies and, at the same time, provide a safe, easily available and innovative diagnostic method in the detection and surveillance of BC. Despite the several advantages, many flaws are currently impeding the wide diffusion of liquid biopsy in BC as a preferred diagnostic methodology. First, the urine is a dynamic body fluid and, therefore, the concentrations of potential biomarkers could vary with hydration status, renal pathologies and effect of medications; as result, a high degree of variability is still possible intra and inter patients (Vlachostergios and Faltas, 2019). Second, the specificity of urinary biomarkers is still limited compared to urinary cytology, although recent improvements in the detection rate of false positives. In addition, the limitations related to sensitivity and specificity are highlighted in the case of lower clinical stages (0a, 0is and 1), thus partially limiting the use of urinary biomarkers in recurrence detection. Third, excluding few biomarkers designed for the analysis with conventional PCR techniques, many assays require highly qualified personnel and state-of-art laboratories; this means the necessity of large investment in terms of resources and time to upgrade facilities and the quality of training; for this reason, it is probable that only large centers could provide those requirements (Schwarze et al., 2020). Fourth, the costs related to the use and the processing of particular urinary biomarkers could overcome the cost of a single cystoscopy; however, considering the initial investment and development of further cost-efficient techniques, the overall price could be lower or comparable to a single cystoscopy. Fifth, large prospective multi-centers studies are required to properly evaluate the performance of liquid biopsy compared to urinary cytology and cystoscopy. Finally, many potential useful and better urinary biomarkers are not currently approved by FDA, thus slowing the research in this field.

5. Conclusion

Liquid biopsy is increasingly utilized for the diagnosis and the follow-up of BC patients and it is foreseeable a larger role of non-invasive urinary tests in the clinical work-up of this disease. To date, urinary tests are quite efficient in the detection of advanced BC while performances are lacking in the initial screening of suspicious patients, especially in a low-grade BC setting. In order to improve the early detection of tumours, the role of liquid biopsy could be crucial, permitting to provide the best care for patients and avoiding the human and economical costs of a delayed treatment. Further studies are however required in order to validate FDA approved and not urinary biomarkers in order to implement the use of liquid biopsy in bladder cancer in the clinical practice and in the diagnostic alghorithm.

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Declaration of Competing Interest

The authors declare no competing financial interests.

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