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Epimarkers for Bladder Cancer diagnosis and monitoring (EpiBlaC)

Mariana Filipa Silva Ferreira

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Mariana Filipa Silva Ferreira

Epimarkers for Bladder Cancer diagnosis and monitoring (EpiBlaC)

Dissertação de Candidatura ao grau de **Mestre em Oncologia** – Especialização em Oncologia Laboratorial submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

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“Around here, however, we don’t look backwards for very long. We keep moving forward, opening up new doors and doing new things, because we’re curious… and curiosity keeps leading us down new paths.”

Walt Disney

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Para a minha mãe

RESUMO

Introdução: O cancro da bexiga (BlCa) é o décimo cancro mais incidente no mundo e o quinto na Europa. O diagnóstico inicial do BlCa é confirmado por cistoscopia combinada com citologia urinária. No entanto, mesmo após a conclusão de um tratamento bem sucedido, estima-se que aproximadamente 50% dos casos recidivam ou progridem para uma doença mais agressiva nos 10 anos subsequentes. Por conseguinte, os doentes são monitorizados utilizando as mesmas técnicas de diagnóstico que para a deteção primária durante estes anos, o que representa um considerável encargo financeiro para os sistemas de saúde e inflige um forte desconforto aos doentes. Para fazer face a esta situação, foram investigadas várias alternativas não invasivas, baseadas na deteção de biomarcadores específicos de doença em biópsias líquidas. A metilação de DNA, por exemplo, tem sido amplamente estudada para a deteção de BlCa, dado o seu papel no desenvolvimento e progressão do tumor. No entanto, nenhum marcador de metilação de DNA é ainda recomendado por qualquer diretriz clínica para a deteção não invasiva de BlCa.

Objetivos: Na Parte I, pretendemos encontrar os biomarcadores de metilação de DNA mais promissores para a deteção de BlCa utilizando amostras de urina de doentes, através de uma revisão sistemática e meta-análise. Posteriormente, na Parte II, pretendemos validar analiticamente a precisão dos marcadores selecionados numa coorte independente de doentes com BlCa e diferentes tipos de controlos.

Materiais e Métodos: Na Parte I, foi realizada uma pesquisa bibliográfica nas plataformas PubMed, Scopus e Biblioteca Cochrane, para obtenção de estudos relevantes publicados até 31 de dezembro de 2022. Após a seleção dos estudos, foi realizada a meta-análise, utilizando uma abordagem univariada para o *Diagnostic Odds Ratio* (DOR) e uma abordagem bivariada para a análise da sensibilidade e especificidade. Na Parte II, a acuidade dos marcadores identificados na meta-análise foi validada por PCR específico de metilação (qMSP) em sedimentos de urina existentes no Biobanco do Serviço de Anatomia patológica do IPO Porto. Designadamente, amostras de 80 doentes com cancro da bexiga não-músculo invasivo (NMIBC), 25 doentes com cancro da próstata (PCa), 25 doentes com cancro renal (RCa), 25 dadores saudáveis (HD) e 25 amostras de doentes com condições inflamatórias (IC) do trato urinário da Universidade de Córdoba, Espanha.

Resultados: A revisão sistemática resultou em 2297 estudos, dos quais 68 foram incluídos na análise final. Tendo o valor DOR como medida comparativa, os níveis de metilação dos cinco genes mais promissores utilizados como biomarcadores para a deteção de BlCa revelaram-se ser o *SALL3*, *PENK*, *ZNF154*, *VIM* e *POU4F2*. No estudo de validação, os genes *SALL3*, *ZNF154* e *VIM* apresentaram níveis de metilação dos seus

promotores significativamente mais elevados entre doentes com BlCa e doentes com IC. Os painéis que combinam estes três marcadores alcançaram sensibilidades e especificidades que variaram entre 48-55% e 84-92%, respetivamente, para distinguir NMIBC de IC.

Discussão e Conclusões: Embora vários marcadores baseados na metilação de DNA tenham obtido uma elevada acuidade para a deteção de BlCa na meta-análise, quando os mais promissores foram validados analiticamente na Parte II desta Dissertação, os resultados foram ligeiramente discrepantes. No entanto, algumas das diferenças observadas entre o presente estudo de validação e os estudos incluídos na meta-análise, podem refletir o tipo de casos e controlos utilizados, bem como fórmulas de cálculo aplicadas. Assim, o presente trabalho apoia a importância de estudos de validação de biomarcadores em diferentes contextos clínicos, com o objetivo final de melhorar o diagnóstico e a gestão dos doentes BlCa.

ABSTRACT

Background: Bladder cancer (BlCa) is the 10th most incident cancer in the world, and 5th in Europe. The initial diagnosis of BlCa is confirmed by cystoscopy combined with urinary cytology. However, even after completing successful treatment, it is estimated that approximately 50% of the cases relapse or progress into a more aggressive disease in up to 10 years. Therefore, the patients are monitored using the same diagnostic techniques as for primary detection for long periods of time, which heavies a financial burden on healthcare systems and inflicts strong discomfort to patients. To tackle this, several non-invasive alternatives have been researched, relying on the detection of disease-specific biomarkers in liquid biopsies. DNA Methylation, for instance, has been extensively studied for the detection of BlCa, given its role in tumor development and progression. Nevertheless, no DNA methylation marker is yet recommended by any clinical guidelines for the non-invasive detection of BlCa.

Aims: In Part I, we aim to find the most promising DNA Methylation-based biomarkers to detect BlCa using patients' urine samples, through a systematic review and meta-analysis. Subsequently, in Part II, we aim to analytically validate the accuracy of the selected markers in an independent cohort of BlCa patients and different types of controls.

Material and Methods: In Part I, bibliographic research was conducted on PubMed, Scopus and Cochrane Library platforms, to obtain relevant studies published up to the 31st of December of 2022. After the selection of the studies, the meta-analysis was performed, using a univariate approach for the Diagnostic Odds Ratio (DOR) and a bivariate approach for the sensitivity and specificity analysis. In Part II, the two markers identified in the meta-analysis were validated by methylation-specific PCR (qMSP) in existing urine sediments in the Biobank of IPO Porto's Department of Pathology. Specifically, samples of 80 patients with non-muscle invasive prostate cancer (NMIBC), 25 patients with prostate cancer (PCa), 25 patients with renal cancer (RCa), 25 healthy donors (HD) and 25 samples of patients with conditions inflammatory diseases (IC) of the urinary tract of the University of Córdoba, Spain.

Results: The systematic review resulted in 2297 studies, of which 68 were included in the final analysis. Using the DOR value as a comparative measure, the methylation levels of the five most promising genes used as biomarkers for BlCa detection turned out to be *SALL3*, *PENK*, *ZNF154*, *VIM* and *POU4F2*. In the validation study, the *SALL3*, *ZNF154* and *VIM* genes showed significantly higher methylation levels of their promoters among BlCa patients compared to IC patients. Panels combining these three markers achieved

sensitivities and specificities ranging from 48-55% and 84-92%, respectively, for distinguishing NMIBC from IC.

Discussion and Conclusions: Although several DNA methylation-based markers achieved high accuracy for the detection of BlCa in the meta-analysis, when the most promising ones were analytically validated in Part II of this Dissertation, the results were slightly discrepant. However, some of the differences observed between this validation study and the studies included in the meta-analysis may reflect the type of cases and controls used, as well as the calculation formulas applied. This study therefore supports the importance of biomarker validation studies in different clinical contexts, with the ultimate aim of improving the diagnosis and management of BlCa patients.

TABLE OF CONTENTS

INDEX OF FIGURES

General Introduction

Part I

Part II

INDEX OF TABLES

General Introduction

Part I

Part II

LIST OF ABBREVIATIONS

5

5caC – 5-carboxylcytosine
5fC – 5-formylcystein
5hmC – 5-hydroxycytosine
5mC – 5-methylcytosine

A

ACTB – β-Actin
AUC – Area Under the Curve

B

BCG – Bacillus Calmette-Guérin
BER – Base Excision Repair
BlCa – Bladder Cancer
BSP – Bisulfite Specific PCR

C

CE – Conformité Européene
CI – Confidence Interval
CIS – Carcinoma *in situ*
CpG – Cytosine phosphate Guanine
CYSTO – Urethrocystoscopy

D

ddPCR – Droplet Digital PCR
DNA – Deoxyribonucleic Acid
DNMT – DNA Methyltransferase
DOR – Diagnostic Odds Ratio
DSL – DerSimonian and Laird
DTA – Diagnostic Test Accuracy

E

EAU – European Association of Urology
ELISA – Enzyme-linked Immunosorbent Assay
EMT – Epithelial-Mesenchymal Transition
EORTC – European Organization for Research and Treatment of Cancer
EU – European Union

F

FDA – Food and Drug Administration
FISH – Fluorescence *in situ* Hybridization
FN – False Negative
FP – False Positive

H

hCFHrp – human Complement Factor-H Related Protein
HD – Healthy Donors
HG – High-Grade

L

LG – Low-grade

I

IC – Inflammatory Conditions
ICI – Immune Checkpoint Inhibitor
IVD – *In Vitro* Diagnostic
IVDR – *In Vitro* Diagnostic Regulation

M

MBD – Methyl-Binding Domain
MeCP – Methyl-CpGs Binding Protein
MIBC – Muscle-invasive Bladder Cancer
MMC – Mitomycin C
MSP – Methylation-Specific PCR

N

NAC – Neoadjuvant Chemotherapy
ncRNA – non-coding Ribonucleic Acid
NGS – Next-Generation Sequencing
NLR – Negative Likelihood Ratio
NMIBC – Non-muscle-invasive Bladder Cancer
NMP22 – Nuclear Matrix Protein 22

NPV – Negative Predictive Value

O

OS – Overall Survival

P

PBS – Phosphate-Buffered Saline
PCa – Prostate Cancer
PCR – Polymerase Chain Reaction
PDD – Photodynamic Diagnosis
PICO – Patients, Index Test, Comparator and Outcomes
PLR – Positive Likelihood Ratio
POC – Point of Care
PPV – Positive Predictive Value
PRISMA – Preferred Reporting Items for Systematic Reviews and Meta-analyses
PSQ – Pyrosequencing
PUNLMP – Papillary Urothelial Neoplasm of Low Malignant Potential

Q

qMSP – Quantitative Methylation-Specific PCR
QUADAS-2 – Quality Assessment of Diagnostic Accuracy Studies 2

R

RC – Radical Cystectomy
RCa – Renal Cancer
RFS – Recurrence-Free Survival
RNA – Ribonucleic Acid
ROC – Receiver Operating Characteristics

S

SAH – S-adenosyl-homocysteine
SAM – S-adenosyl-methionine
SEER – Surveillance, Epidemiology, and End Results
Se – Sensitivity
SI – Single Instillation
Sp – Specificity
STARD – Standards for Reporting Diagnostic Accuracy Studies

T

TDG – Thymine DNA Glycosylase
TET – Ten-Eleven Translocation
TN – True Negative
TNM – Tumor-Node-Metastasis
TP – True Positive
TURBT – Transurethral Resection of the Bladder Tumor

U

USA – United States of America
UTI – Urinary Tract Infection

GENERAL INTRODUCTION

BLADDER CANCER

Epidemiology and Etiology of Bladder Cancer

In 2020, bladder cancer (BlCa) accounted for 573,278 new cases and 212,536 fatalities, becoming the 10th most common cancer and 13th leading cause of death by cancer in the world (1). Considering only the male population, BlCa rises to sixth most diagnosed cancer, since BlCa is about three times more frequent in men than in women. Regarding geographical distribution, the incidence and mortality rate of BlCa also differs across countries, being higher in developed countries. This may be caused by different risk factors or by dissimilarities in health care, such as detection methodologies or therapeutic approaches (2). Particularly in Europe BlCa was the fifth most incident cancer type, and in Portugal was is the sixth, where 2,608 BlCa patients were diagnosed in 2020 (1).

Concerning risk factors, tobacco smoking may be perceived as the most preeminent one, responsible for about 50% of cases (3). Age is also a very important element to consider since 80% of BlCa patients are older than 65 years (4). Some professional activities also can bear an associated risk, specifically in industrial environments that operate with dye, textile, rubber, leather, metal or petroleum, since workers are exposed to carcinogens such as aromatic amines (3). Fortunately, safety measures have been taken by developed industries to reduce the occupational exposure of their employees to these agents, successfully diminishing the risk of developing bladder neoplasms (2). Another factor with great impact, and that may be also responsible for the differences in the prevalence of BlCa across the globe, is pathogens infection. For instance, schistosomiasis is a parasitic infection disease found mostly in Africa and other southern regions and significantly increases the risk of developing squamous cell BlCa (5). Nonetheless, patients with benign chronic conditions such as urinary tract infections (UTI), kidney or bladder stones, among others, hold a higher risk of developing cancer as well (3, 6). Previous localized radiation therapy also contributes as a risk factor, and other aspects, such as family history and drinking habits, seem to have little influence on the development of the tumor (2, 3).

Pathology of Bladder Cancer

The bladder wall may be histologically organized into four main layers: specialized stratified epithelium or urothelium, lamina propria, detrusor muscle and adventitia (7). Most bladder tumors occur from transitional cells of the urothelium, accounting for approximately 90% of all bladder cancer cases (4, 8). Less frequent subtypes of BlCa include squamous cell carcinoma, adenocarcinoma, micropapillary, small cell and plasmacytoid (6). Bladder tumors are staged by the Tumor-Node-Metastasis (TNM) system, classifying tumors from

Ta to T4 according to the extent of invasion of the bladder wall. When the malignancy presents as a flat non-invasive lesion is designated a *carcinoma in situ* (CIS) and classified as Tis; Ta is a non-invasive papillary tumor; T1, when the tumor invades the *lamina propria*; T2, if the muscle is invaded; T3, when it occurs perivesical invasion; and finally a T4, when adjacent organs as prostate stroma, seminal vesicles, uterus, vagina pelvic wall or abdominal wall are invaded (9). Having this in consideration, BICa can be clinically categorized into two major subgroups: non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), which arise from different molecular pathways and possess very distinct prognoses and treatments (Figure 1) (10). For instance, overall survival is strongly related to stage of the disease at time of diagnosis. According to the Surveillance, Epidemiology, and End Results (SEER) database, while tumors confined to the mucosa present a 5-year survival rate close to 96%, it drops to 70% if the disease is diagnosed as localized, lowers to 36% if the cancer invades surrounding regions, and to 7% if the tumors have already spread to distant organs, hence the importance of early diagnosis (11). Nevertheless, the majority of the tumors (70-80%) are non-muscle invasive at diagnosis (12).

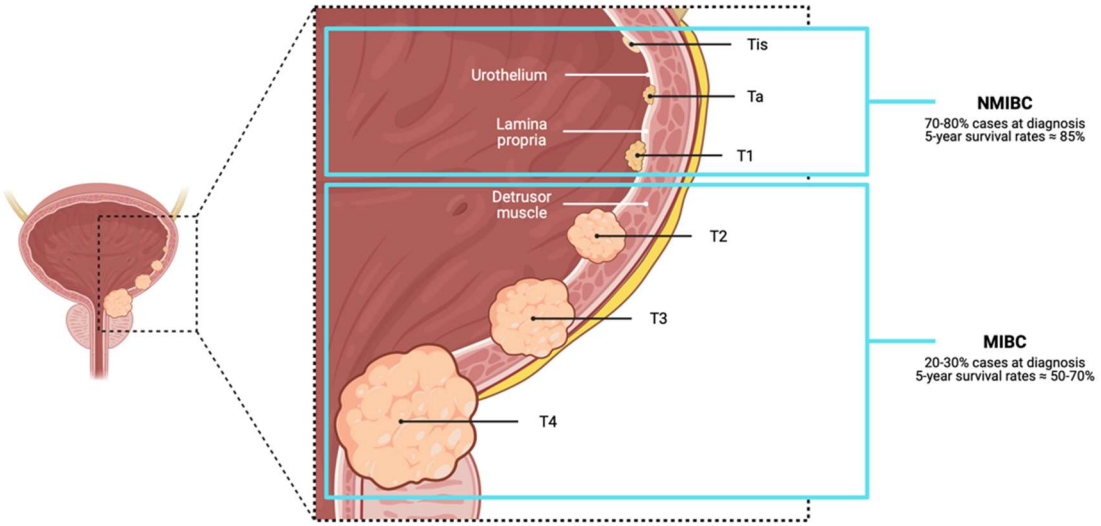

Figure 1. Illustration of the layers composing the bladder wall and representation of cancer staging in accordance with the Tumor, Node, Metastasis (TNM) system. The NMIBC can be presented as Tis (or carcinoma *in situ*, CIS), Ta, or T1, and represent 70 to 80% of the diagnosed cases. On the other hand, MIBC can be classified as T2, T3, or T4, and around 30% of the cases are diagnosed in these stages. Created with BioRender.com.

Besides staging, histological grading of urothelial tumors is also pivotal for the decision-making of the best therapeutic approach in non-invasive neoplasms. According to the WHO 2004/2016 classification, bladder tumors can be divided into Papillary Urothelial Neoplasm

of Low Malignant Potential (PUNLMP), low-grade (LG) and high-grade (HG) papillary urothelial carcinoma, based on tumor architecture and cellular atypia (2, 13, 14). Besides being flat and non-invasive, CIS is always HG due to its ability to easily spread to other bladder regions. Other NMIBC stages (Ta and T1) can be either LG or HG, whereas muscle-invasive tumors (T2, T3 and T4) are always classified as HG tumors (2).

Signs, Symptoms and Diagnosis

The most common sign found to be associated with bladder neoplasms is hematuria, but other presentations of the disease also include dysuria and/or polyuria (15). Hematuria can be either gross or microscopic, with visible hematuria being usually associated with more advanced stages and grades of the disease (16). Patients that present these symptoms are usually suggested for imaging exams and urinary cytology examination, but the final diagnosis is settled based on bladder cystoscopy and biopsy evaluation of a sample obtained from transurethral resection of the bladder tumor (TURBT) (2). Particularly, CIS can be presented as a reddish area like inflammation, or be completely invisible in cystoscopy, but can be detected with a sensitivity ranging 66-83% by cytology (17, 18). In these cases, when a patient presents suspicious lesions or possesses positive cytology with no signs of disease, several mapped biopsies from different areas of the bladder are recommended (2). Nevertheless, new methods of visualization have become more frequently used, as Photodynamic Diagnosis (PDD) using blue-light flexible cystoscopy, which constitutes a more sensitive alternative to standard white-light cystoscopy (18). A locally advanced disease can be easily noticed resorting to vaginal or rectal bimanual palpation under anesthesia or by imaging techniques, but the ultimate diagnosis is still cystoscopy and histological evaluation of the mass (15).

Management and Treatment Options

In NMIBC, the goal of the transurethral resection is to perform a complete resection of the tumor(s). To assure a good prognosis, it is crucial to assess the quality of the resection when the histological evaluation of the specimens is performed (19). The presence of detrusor muscle is required for a good-quality resection, otherwise it is not possible to ensure the complete resection of the tumor, increasing the risk of progression and/or recurrence due to possible residual disease (20). After TURBT, an immediate single instillation (SI), usually with Mitomycin C (MMC), is performed within 24 hours, which significantly reduces the risk of recurrence in the first three months (21).

Additional therapeutic approaches for NMIBC are then chosen upon scoring models that predict recurrence and/or progression risk. Several models are available, and the most

commonly considered factors include age, number of tumors, tumor size, stage, grade, concomitant CIS and previous recurrences (22). The 2006 European Organization for Research and Treatment of Cancer (EORTC) scoring model allows for an easy way to calculate the individual probability of recurrence and progression of a bladder tumor at one and five years, having in consideration tumor size, stage, multifocality, prior recurrences, concurrent CIS and the WHO 1973 classification system (23). In turn, the EAU (European Association of Urology) NMIBC 2021 scoring model take into consideration both WHO 2004/2016 and WHO 1973 classification systems, besides all other parameters mentioned, and stratifies patients into four main risk groups of progression: low, intermediate, high and very high (2).

Low-risk patients do not benefit from adjuvant therapy since the progression risk is less than 1% even in a 5-year period, so active surveillance is the best option after TURBT and SI (21). Intermediate-risk patients usually undergo adjuvant intravesical chemotherapy instillations with MMC, increasing recurrence-free survival (RFS). Bacillus Calmette-Guérin (BCG) instillations for one year of maintenance seem to better prevent recurrences than chemotherapy in intermediate-risk patients but are also responsible for greater side effects (24, 25). High- and very high-risk patients are treated with BCG instillations given in a maintenance schedule for three years to delay disease progression. However, a significant group of patients never get to the end of the therapy due to treatment inefficacy (26). For instance, it is expected that more than 50% of very-high risk patients will relapse in a 10-year period, so radical cystectomy (RC) may be considered and suggested to the patients who might benefit from the procedure (Figure 2) (2).

In this setting, it becomes crucial to implement an efficient surveillance system for NMIBC patients. The gold standard procedures rely on cystoscopy combined with urinary cytology every 3-6 months in the first year, continuing the same schedule or once every year for five years, depending on the risk stratification. In intermediate- and high-risk patients there is a significant risk of recurrence up to 10 years, so life-long monitorization is recommended.

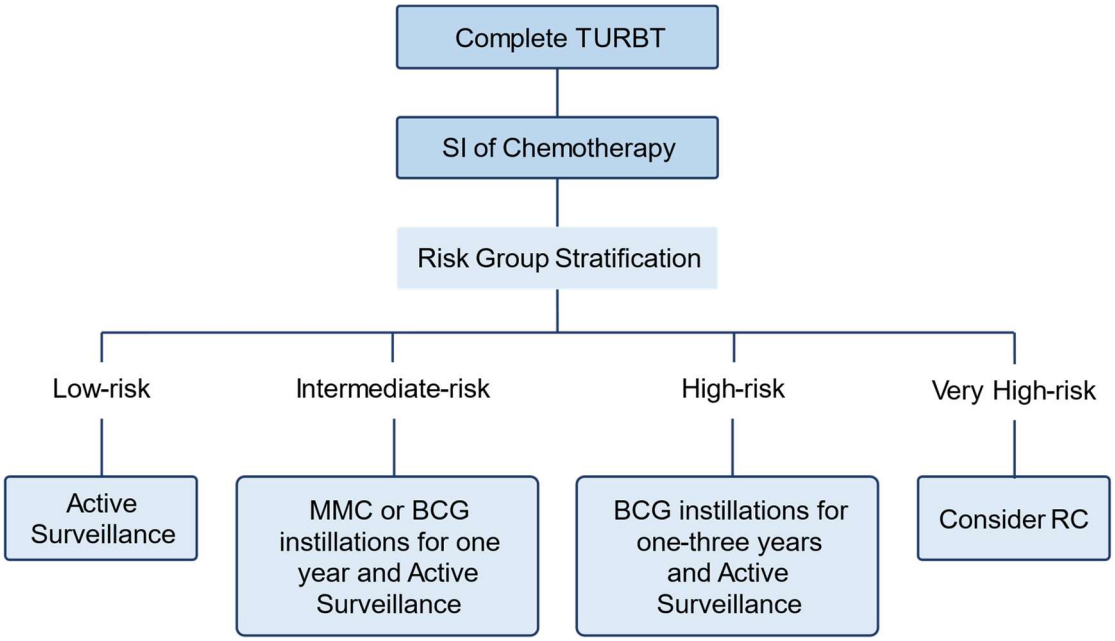

Figure 2. Graphical representation of therapeutic scheme for NMIBC patients. After being submitted to TURBT and a subsequent single instillation of chemotherapy, all patients will undergo long active surveillance, but follow-up frequency and further therapy approach will depend on the risk group stratification.

On the other hand, RC is the gold standard treatment for MIBC. In organ-confined or locally advanced non-metastatic BlCa, *i.e.*, T2-4N0M0 patients, cisplatin-based neoadjuvant chemotherapy (NAC) is recommended for improving overall survival (OS) since it helps to reduce tumor size and micrometastases. Adjuvant chemotherapy may be considered in patients in which neoadjuvant therapy was not previously administrated. However, MIBC patients who recur after treatment or patients that present metastatic disease at diagnosis, lack an effective therapy, starting a palliative-intended treatment with cisplatin-based chemotherapy as first-line, and approved immune checkpoint inhibitors (ICIs) as second-line treatment for resistant patients.

Problems and limitations in BlCa management

Unfortunately, several problems can be identified relating to BlCa management. For instance, cystoscopy and cytology are, as mentioned, the gold standard to detect BlCa either at diagnosis or recurrence, but both hold several disadvantages. Cystoscopy is a highly invasive and expensive procedure, and since it requires instrumentation, it is also associated with increased comorbidities such as hematuria, and frequent and painful urine voiding (27, 28). Despite its high sensitivity, considering it is an operator-dependent imaging technique, it can also miss-detect smaller tumors, as Ta or Tis (2). Urine cytology is a highly specific method with a specificity ranging between 73-100% for all BlCa stages, and it is a

good complement to cystoscopy, especially regarding Tis because it presents a particularly high sensitivity for high-grade tumors (28, 29). However, its sensitivity significantly decreases (3-34%) in the detection of low-grade tumors (29-31). Despite relying on the expertise of the pathologist and, consequently, being subjected to a high inter- and intra-observer variability for that reason alone, the results of urinary cytology might be influenced by the presence of other benign urological conditions as well (32, 33). This results in a large group of miss-diagnosed and thus untreated Ta and Tis tumors, leading to possible disease progression (34). Besides, due to the high risk of progression, these procedures are performed routinely for years or for the lifetime of the patient, as mentioned before, which entails a heavy burden on financial and human resources for health institutions, making BlCa one of the most expensive cancers to treat (35, 36). Therefore, there is an urgent need to identify biomarkers that can detect BlCa or can reliably exclude the presence of malignancy, to decrease the number of cystoscopies performed.

EPIGENETICS

Epigenetics as a Hallmark of Cancer

In 2000, Hanahan and Weinberg proposed six key biological pathways as the principal capabilities cells obtain upon neoplastic transformation, designated as the Hallmarks of Cancer (37). The acquirement of successive hallmarks seems to increase cells' malignant potential and weightily contribute to tumorigenic mechanisms. This model was updated later in 2011, with four more proposed hallmarks, and again, recently, in 2022 (38, 39). In the latest revision, new four emerging hallmarks were introduced in the model, culminating in a total of fourteen Hallmarks of Cancer (Figure 3) (39).

One of the most recently added hallmarks consist in "Nonmutational Epigenetic Reprogramming". It is well known that DNA instability is foundational for malignant progression and tumor development (39). For many years, genomic mutations have been the main focus of study regarding this topic, and only in the last decades other mechanisms of genomic reprogramming started to be intensely explored, such as epigenetic alterations (40).

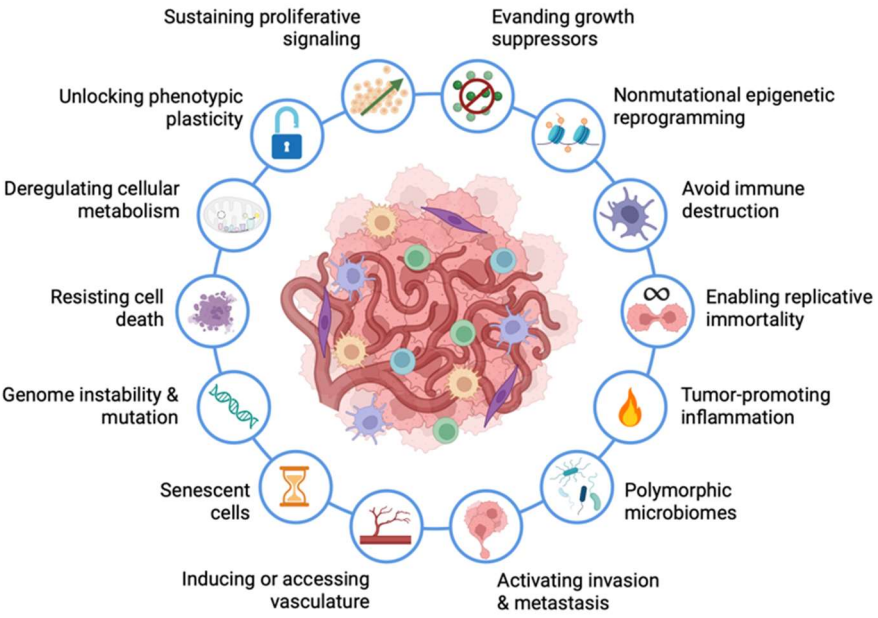

Figure 3. Hallmarks of Cancer. Adapted from (Hanahan, 2022) (39). Created with BioRender.com.

A broad definition for epigenetics is a reversible phenomenon that alters the expression or outcome of a genomic region, without modifying its DNA sequence, and it might occur through several different mechanisms, such as DNA Methylation, Histone Post-translational Modifications or the action of non-coding RNAs (ncRNA) (41, 42). Epigenetics' role in embryonic development has been well established for several decades, since it regulates cell mechanisms crucial for key processes such as differentiation and organogenesis (39). Moreover, epigenetics also operates different roles in normal adult cells such as correct organization of chromatin, silencing of repetitive elements and triggering of gene expression switches, as inactivation of X chromosome-linked genes and imprinted genes (41).

Nevertheless, these epigenetic alterations might be manipulated and used in a beneficial way by cells during malignant transformation and predispose to a more aggressive phenotype (40). For example, epigenetically regulated mechanisms such as the ones responsible for cell turnover from an epithelial phenotype to a more mesenchymal one, known as Epithelial-Mesenchymal Transition (EMT) which favors cell plasticity and mobility in embryonic development, might also be a central enabler of cell migration in cancer and metastasis formation (43). Epigenetics also seems to provide numerous other advantages to tumors cells, related to cells' stemness, metabolism and even tumor microenvironment.

DNA Methylation

DNA Methylation is an epigenetic mechanism that consists of the addition of a methyl group to the carbon on position 5 of a cytosine ring, a reaction catalyzed by DNA

Methyltransferases (DNMTs), resulting in 5-methylcytosine (5mC). Generally, the methyl group is provided by the universal methyl donor SAM (S-adenosyl-methionine), which is consequently converted to SAH (S-adenosyl-homocysteine) (44). This modification usually occurs in CpG (Cytosine phosphate Guanine) dinucleotides and it is estimated that 70-80% of CpGs in the human genome are methylated (45). Nevertheless, CpG islands, which consist of clusters of CG dinucleotides with a C-G content above 50% most frequently present in genes' promoters, are generally unmethylated in normal cells (40).

As mentioned before, DNA Methylation plays a key role in several cell processes as the regulation of imprinted genes, silencing of X chromosome and repetitive sequences (40). Also, during the embryonic development, cells go through several phases and pathways where there is a need to migrate and to differentiate and for that it is crucial to have a flexible and efficient reprogramming of the epigenome, accomplished by the correct balance between *de novo* methylation and demethylation (46).

The establishment of *de novo* methylation is carried out by the complex DNMT3A and DNMT3B while the maintenance of methylation marks during DNA replication is done by DNMT1, which recognizes hemimethylated cytosines. Afterwards, to affect gene expression, the methylation marks need to be recognized. Methyl-CpGs Binding Proteins (MeCPs) are protein complexes responsible for the readout of methylation marks. Usually, these proteins contain a methyl-binding domain (MBD) which specifically binds to methylated DNA, which in turn inhibits the binding of transcription factors, therefore, repressing gene expression (46, 47). Concerning demethylation of DNA, the removal of the methyl group alone would require the breakage of covalent C-C bonds which demand high levels of energy. Thus, demethylation of DNA involves the removal of not only the methyl group but the whole methylated cytosine (46). Bearing this in mind, DNA repair mechanisms play a crucial role in this process. Active DNA demethylation is carried out by TETs (Ten-Eleven Translocation) methylcytosine dioxygenases which convert 5mC to oxidized forms as 5-hydroxycytosine (5hmC), 5-formylcystein (5fC) and 5-carboxylcytosine (5caC). The DNA repair enzyme TDG (thymine DNA glycosylase) then recognizes the oxidized and deaminated forms of cytosine and 5mC allows the repair by BER (Base Excision Repair) DNA repair mechanism (Figure 4) (46, 47).

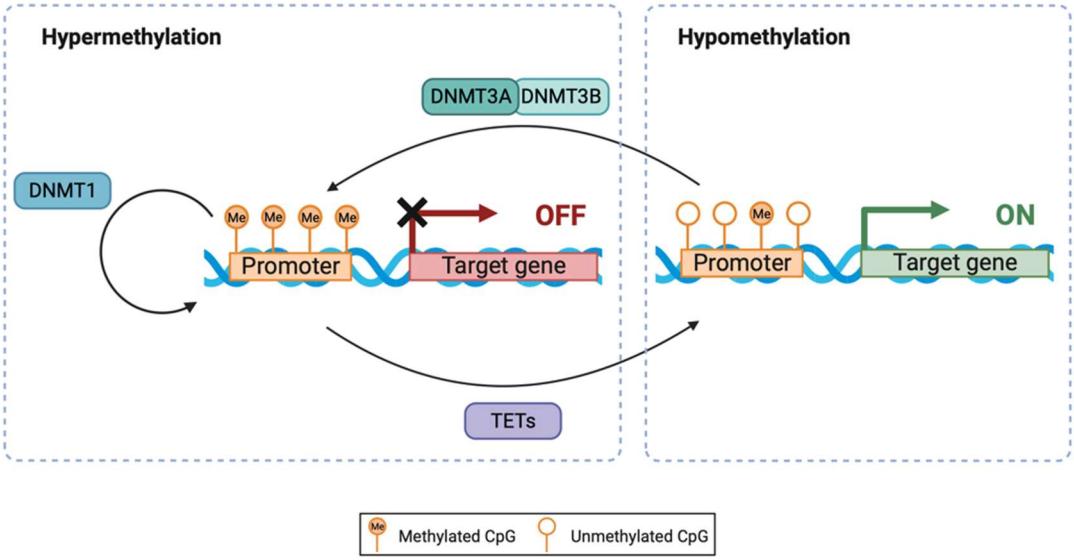

Figure 4. DNA Methylation machinery. When a gene promoter is unmethylated the gene expression is activated. However, DNMT3A and B may promote *de novo* methylation on the promoter, which will repress the gene expression. This methylation is maintained by the DNMT1. To go back to the unmethylated state, TETs are responsible for removing the methyl group from the cytosines, leading to the reactivation of the gene expression. Created with BioRender.com.

In the context of tumorigenesis, as previously stated, epigenetic alterations might grant cells several neoplastic abilities and contribute with advantages for tumor progression and aggressiveness. For instance, aberrant DNA Methylation is the most frequent epigenetic alteration observed in cancer cells. Abnormal hypomethylation usually occurs in methylated sites in normal tissue, which may lead to loss of imprinting or genomic instability when imprinted genes and repetitive sequences are unmethylated, respectively. Less frequently, hypomethylation also may happen in some promotors that usually leads to oncogene re-activation and transcription. In turn, hypermethylation most commonly occurs in unmethylated sites in normal tissue, such as CpG islands, which frequently correspond to the promotor of tumor suppressor genes (46). According to Knudson two-hit hypothesis, the inactivation of a tumor suppressor gene demands the loss of function of both alleles of the gene, and it might happen by both genetic mutation and epimutations. Specifically in inherited cancer, where the first hit is a genetic mutation, the second hit very frequently consists of DNA methylation. Therefore, DNA hypermethylation can cause the inactivation of tumor suppressor genes, leading to cancer (48).

DNA Methylation in Bladder Cancer

DNA Methylation is the most studied epigenetic modification in BlCa (49). Over the years, numerous genes have been identified as abnormally methylated, and thus differently expressed, in BlCa, affecting pathways related to cell metabolism, cell cycle, cell adhesion,

DNA repair, DNA transcription, apoptosis, among others (50). Nearly 50-90% of bladder cancers present DNA hypermethylation in the promoter site of a gene contributor to one of these mechanisms (49). For the record, DNA hypermethylation is settled to be one of the possible former events to induce urothelial carcinogenesis, being already established in the most initial stages of the disease, and appears to bear an important role in the promotion of BlCa development and progression (49, 51). Moreover, DNA methylation showed to be associated with important clinicopathological features of the tumor such as subtype, stage and prognosis (52).

NON-INVASIVE DIAGNOSTIC ALTERNATIVES FOR BLADDER CANCER

Liquid Biopsies for the study of Biomarkers

A disease-specific biomarker is a measurable indicator of a certain phenotype or biological state, and may consist of any molecule, genetic or epigenetic alteration. In the oncological context, it can be used to diagnose malignancies and monitor disease relapse, as well as to monitor therapeutic responses (53). The ideal tumoral marker used for clinical practice should have good sensitivity and specificity, should be cost-efficient and easy to assess, resorting to the use of minimally invasive approaches (54).

Liquid biopsies consist of body fluids that carry relevant molecular information that can be used as biomarkers and are obtained easily through minimal or non-invasive procedures (55). Therefore, its use is associated with a reduced morbidity of the patients, allowing the increase of schedule frequency, which facilitates a continuous monitorization of the disease (55). Other advantage presented by liquid biopsies is the fact that it better represents the heterogeneity of the tumor, since it contemplates material derived from more than one clone, which a small fraction of the tissue obtained by biopsy cannot account (56).

For instance, urine consists of a very appealing liquid biopsy for the management of BlCa since it is in direct contact with the tumor, consequently being enriched with material derived from the tumor microenvironment, and is accessed through a completely non-invasive way (57).

All urine fractions, such as full voided urine, urine supernatant and urine sediment, already proved to be useful liquid biopsies for BlCa, however, one of them might be better than the other depending on the target biomarker in study. If the aim is to study methylation status of DNA, the urinary pellet seems to be the best option, since it is expected that the exfoliated tumor cells hold higher quantity and quality DNA (58).

IVD tests Approved for the Detection of Bladder Cancer

For any *In Vitro* Diagnostic (IVD) test to be eligible, it is obligatory to comply with *In Vitro* Diagnostic Regulation (IVDR), according to EU2017/746 in EU and FDA (Food and Drug Administration) in USA (United States of America). Hence, to get approval for clinical use, all tests should go through the same process with clear-cut phases. First, the target population must be defined, and research studies shall select the most clinically promising biomarker(s) for the defined purpose. Then, it is necessary to develop the technology needed to detect the biomarker(s). And lastly, it is mandatory to analytical and clinically validate the developed test, to generate sustained evidence on the diagnostic power of the biomarker(s) in clinical practice (59).

Indeed, several urine-based tests using biomarkers of different molecular sources, such as DNA, RNA, proteins and metabolites, have been developed. After undergoing clinical validation in large studies, some of them achieved FDA Approval or European Conformity Approval (CE) marking, which allowed their commercialization (Table 1).

Table 1. List of commercially available tests with FDA Approval or European Conformity Approval (CE) marking for the detection of Bladder Cancer.

Test Name	Approval	Biomarker	Purpose	Pooled Se (%)	Pooled Sp (%)	Reference
Cytology	FDA & CE Mark	Cell phenotype	Diagnosis & Surveillance	34	99	(60)
NMP22® BladderChek®	FDA & CE Mark	Peptides	Diagnosis & Surveillance	56	88	(61)
BTA Stat®	FDA	Proteins	Diagnosis & Surveillance	67	75	(62)
UroVysion™	FDA & CE Mark	DNA	Diagnosis & Surveillance	72	83	(63)
ImmunoCyt/uCyt+™	FDA	Antigens	Surveillance	73	66	(64)
Xpert® Bladder Cancer Monitor	CE Mark	mRNA	Surveillance	73	77	(65)
Uromonitor	CE Mark	Tumoral DNA	Surveillance	93	79	(66)
Bladder EpiCheck™	FDA & CE Mark	DNA Methylation	Surveillance	74	84	(66)
ADXBLADDER™	CE Mark	Proteins	Diagnosis & Surveillance	57	62	(66)

Se: Sensitivity; Sp: Specificity; FDA: Food and Drug Administration; CE Mark: European Conformity Mark

There are two available tests that target the nuclear matrix protein 22 (NMP22), the NMP22 Bladder Cancer ELISA (enzyme-linked immunosorbent assay) and the NMP22 BladderChek test, similar to what happens to the tests that detect the human complement factor-H related protein (hCFHrp), BTA TRAK and BTA Stat, which are quantitative and qualitative tests, respectively. These tests are approved for both the initial diagnosis of BlCa as well as its detection in relapse. The two proteins are usually elevated in cancer cells and both quantitative tests rely on ELISA to detect the respective protein in urine, while the other two qualitative tests are point-of-care (POC) tests. NMP22® BladderChek® test (Alere Scarborough, Inc., Waltham, MA, USA) and BTA Stat® (Polymedco, Cortlandt, NY, USA) are the most commonly used and studied, since by being POC tests they are easier to perform and more practical to use in clinical routine (67). The pooled sensitivities and specificities achieved in meta-analyses for these tests are 56% and 88% for NMP22® BladderChek® and 67% and 75% for BTA Stat®, respectively (61, 62).

Another test approved for the diagnosis and surveillance of BlCa is UroVysion™ (Abbott Molecular, Chicago, IL, USA), which identifies frequent alterations in the chromosomes 3, 7, and 17, as well as the deletion of 9p21, which is commonly seen in BlCa, by fluorescent in situ hybridization (FISH). Its estimated pooled sensitivity and specificity is 72% and 83%, respectively (63).

ImmunoCyt/uCyt+™ (Scimedx Inc., Denville, NJ, USA) is the only FDA-approved test for BlCa follow-up. It relies on the immunofluorescence of three monoclonal antibodies that target three antigens highly expressed in tumoral exfoliated cells present in urine, M344, LDQ10 and 19A11. The pooled sensitivity and specificity obtained by meta-analysis for this test is 73% and 66%, respectively (64).

More recently developed but promising tests, such as Xpert® Bladder Cancer Monitor, Uromonitor, and ADXBLADDER™ have limited number of research articles and are not yet approved by FDA but are already commercially available for clinical practice in EU, possessing the CE Mark (66). Except for ADXBLADDER™, which is approved for both diagnosis and surveillance of BlCa, all other three kits aim the detection of bladder cancer during recurrence monitoring. Uromonitor (U-Monitor, Porto, Portugal), despite being the one with the highest sensitivity (93%), only has two published studies which could lead to a biased interpretation of its performance, so further validation studies should be carried out to validate its diagnostic accuracy (66). In turn, Bladder EpiCheck™ (Nucleix, Rehovot, Israel) has been extensively studied in the last year, got the CE Mark in 2017 and was just recently approved by FDA. Despite the lower sensitivity for Ta-LG tumors, it has very robust results and can accurately “rule out” high-grade BlCa recurrence. In fact, it achieved a 99% Negative Predictive Value (NPV), which means it can ensure with a 99% certainty that, in

face of a negative test, the patient does not carry a high-grade tumor (68). Moreover, the performance of Bladder EpiCheck™ doesn't seem to be influenced by benign conditions or previous therapy, which is crucial for a biomarker used in BlCa monitoring (68, 69).

DNA Methylation as a Cancer Biomarker

Until the date, DNA methylation seems to be the only epigenetic alteration sufficiently stable to be used in IVD setting (59).

DNA methylation has been studied as a biomarker for BlCa for several years and for various purposes, in the context of detection and prognosis. Since DNA methylation may be an early event in bladder tumorigenesis, it could be promising for the early detection of the malignancy. Over the years, the accuracy of the methylation status of the promoter of certain genes such as *TWIST1, NID2, RASSF1A, DAPK, RARβ, BCL2, VIM*, among many others, to indicate presence of bladder neoplasm, separately or in panels, have been frequently and extensively studied (70-72). Some studies achieved thriving results, yet specificity is often relatively low. Hence, in our research group, several methylation biomarkers have been addressed, such as the three-gene panel *GDF15*, *TMEFF2* and *VIM*, which achieved a sensitivity and specificity of 94% and 100% respectively, and a two-gene panel *miR-663a* and *VIM* that detected BlCa with 80% sensitivity and 75% specificity (73, 74).

Nevertheless, research studies that aim for biomarker discovery most likely cannot demonstrate solid evidence regarding its application in clinical context. Therefore, well-designed prospective studies, with well-defined cohorts representative of the target population must be carried out to assess the real performance of the discovered biomarker (75).

Problems and Limitations of Non-Invasive Biomarkers

Despite being commercially available, only in the most recent EAU guidelines 4 tests were mentioned as promising. Cxbladder™, ADXBLADDER™, Xpert® Bladder and Bladder EpiCheck™. Even so, no test seems to be reliable enough to replace cystoscopy for diagnosis or follow-up of BlCa (2).

The majority of the biomarkers have higher sensitivities compared to cytology, however specificities are usually lower (67). This reflects a high rate of false positives most typically resulting from the presence of benign conditions such as UTIs, hematuria, cystitis and inflammation, which is concerning since these conditions are commonly seen in clinics but might also be symptomatic manifestations of BlCa, so it would be fundamental to reliably

distinguish benign from malignant disease (76, 77). Most of the biomarkers are influenced by these factors, as NMP22®, BTA® and uCyt+™ (77). Conversely, UroVysion™ is not affected by benign conditions, but tetraploidy may exist in normal mitosis or inflammatory proliferation, as well as in other primary tumors, which also decreases specificity (78). Other causes of BlCa tests' low specificity may be the the influence of previous therapies (77). For instance, the specificity of BTA stat® and uCyt+™ diminishes in patients previously treated with BCG, while the performance of Bladder EpiCheck™ remains similar (69, 79, 80).

Moreover, although the sensitivities are higher than cytology, they usually vary according to tumor stage and/or grade, with usually reduced sensitivities in detecting low-grade tumors, which also implies a problem regarding early detection (77). Nonetheless, in surveillance context, there is a greater urge to detect high-grade tumors due to the high risk of progression, enhancing the need for earlier diagnosis and treatment, whilst a low-grade recurrence does not require such prompt detection in order to be successfully treated (2).

An alternative solution to reduce invasive procedures could be the combination of the proposed biomarker with urinary cytology, which has been largely studied in parallel with the validation of the isolated biomarkers (81-83). Usually the sensitivity of the two combined surpasses their sensitivity individually, with the cost of a lower specificity than the provided by cytology alone (29). Nevertheless, the cost-effectiveness of this combination needs to be addressed and further assessed.

Therefore, the discovery of new reliable biomarkers and/or the proper validation of biomarkers already present in various research studies constitutes an emergent need for BlCa management.

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AIMS

Aims

Abnormal patterns of DNA methylation have been extensively studied in bladder cancer through the years, as well as their potential as non-invasive biomarkers. Despite that, currently, methylation biomarkers are still not recommended in clinical guidelines for the management of bladder cancer.

Having this in mind, the aims of this dissertation are:

- To understand where we stand regarding the studies and applications of DNA methylation-based biomarkers in clinical practice for bladder cancer detection;
- To unveil the most promising DNA methylation biomarkers for detecting primary and/or recurrent bladder cancer through a systematic review and meta-analysis of Diagnostic Test Accuracy (DTA) studies;
- To validate the diagnostic accuracy of a panel of the most promising biomarkers in a training retrospective cohort of Portuguese Institute of Oncology of Porto (IPO Porto) bladder cancer patients.

PART I

Diagnostic test accuracy of urinary DNA methylation-based biomarkers for detection of primary and recurrent bladder cancer: a systematic review and meta-analysis

Manuscript Under Submission

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ABSTRACT

Introduction: Approximately 50% of bladder non-invasive papillary carcinomas recur up to 10 years after first curative-intended treatment, entailing periodic clinical surveillance. Diagnosis of primary and relapsed tumors is accomplished by urethrocystoscopy, an invasive procedure, combined with urinary cytology, with low sensitivity, resulting in substantial financial burden. Thus, non-invasive biomarkers, assessed in urine, have been investigated, among which DNA methylation has shown promise.

Aim: In this systematic review and meta-analysis, we sought to assess the diagnostic accuracy of DNA methylation biomarkers reported in literature for bladder cancer detection, pinpointing the most informative.

Materials and Methods: Search was conducted on PubMed, Scopus, and Cochrane Library for relevant studies published until December 31, 2022, focused on sensitivity and specificity of DNA methylation markers in urine.

Results: Among 2297 studies retrieved, 68 were included in final analysis. Using Diagnostic Odds Ratio (DOR) as comparative measure, the five most promising methylated genes (sensitivity, specificity, and DOR, estimated at 95% confidence interval, respectively) were *SALL3* (61%, 97%, 55.67), *PENK* (77%, 93%, 47.90), *ZNF154* (87%, 90%, 45.07), *VIM* (82%, 90%, 44.81) and *POU4F2* (81%, 89%, 34.89). Urinary cytology, in contrast, identified bladder cancer with 55% sensitivity, 92% specificity and 14.37 DOR. Notwithstanding, only *SALL3* and *PENK* depicted significantly higher accuracy than cytology.

Discussion and Conclusion: DNA methylation-based biomarkers disclose high accuracy for bladder cancer detection in urine. Nonetheless, validation studies in different clinical settings are scarce and limited information on accuracy hampers clinical use. Those biomarkers should be prioritized in future validation studies, improving bladder cancer detection and management.

INTRODUCTION

Bladder Cancer (BlCa) is the tenth most diagnosed cancer worldwide, predominantly affecting men, with approximately 573,000 new cases and 213,000 deaths reported in 2020. Incidence rates in both sexes are highest in Southern Europe, Western Europe and Northern America (1). In Portugal, BlCa is the seventh most incident cancer and the fourth among the male population. The variability of BlCa incidence and mortality rates across

countries is most likely due to differences in risk factors, detection practices, and treatment availability. The majority of newly diagnosed BlCa (75-80%) are non-muscle invasive (NMIBC, pTa and pT1) whereas 20-25% initially present muscle invasion (MIBC, pT2-pT4) (2). There are also intra-epithelial, high-grade (HG) tumors confined to the mucosa classified as CIS (pTis) (3).

Because up to 50% of high grade NMIBC may recur or progress to MIBC, regular and long-term urethrocystoscopy (CYSTO) complemented with voided urine cytology is mandatory for monitorization. Even though these two methods are currently the recommended strategy for patient surveillance, CYSTO is an expensive invasive procedure, with non-negligible complications (2, 4). Although voided urine cytology is non-invasive and discloses high sensitivity for high-grade cancers detection (84%), it has limited sensitivity for low-grade tumors (16%), and widely variable performance for CIS detection (5, 6). Importantly, clinical guidelines are mostly on retrospective studies, and, thus far, little data is available on the efficacy of reducing the frequency of follow-up CYSTO. Considering all these caveats and that BlCa is the costliest neoplasm to treat and monitor (7), there is an urgent need to implement new, more affordable and minimally invasive follow-up strategies, ideally based on molecular biomarkers.

In this setting, urine constitutes an optimal sample for BlCa diagnosis and monitoring, considering its enrichment in biomarkers, including DNA, RNA, secreted proteins as well as with dissociated tumor cells. Indeed, several commercially available urine-based tests validated in prospective multicentered studies, such as CxBladder (8, 9), ADX-Bladder™ (10, 11), Xpert Bladder® (12, 13) and Bladder EpiCheck™ (14, 15), have demonstrated high sensitivity and negative predictive values for HG disease detection. Until now, however, none of these biomarkers replaced patients' monitoring with CYSTO or have been validated to increase follow-up interval (16, 17), and may only be considered as a surveillance method in case CYSTO cannot be performed (2).

Epigenetic mechanisms, such as aberrant DNA methylation, histone modifications and non-coding RNAs, are a promising source of biomarkers for BlCa detection and follow-up. DNA methylation alters genes switch, affecting its reading and transcription, without changing the underlying DNA sequence, potentially leading to cancer development, progression and possibly impacting on therapy. These epimutations may occur at initial stages of disease (18), preceding the emergence of an overtly malignant phenotype. They are stable in body fluids and in tissue preparation, and their levels are also mostly constant in healthy people (*i.e.*, are not noticeably affected by gender, body mass index, or other similar traits) (19). Thus, altered methylation patterns may also help to predict recurrence. Over the years, numerous research teams, including ours, have extensively investigated

DNA methylation-based biomarkers for BlCa detection, achieving favorable preliminary results. Costa et al. (20) identified a three-gene promoter methylation panel (*GDF15*, *TMEFF2*, and *VIM*) to detect BlCa in both tissue and urine samples with outstanding sensitivity (94%) and specificity (100%). Notwithstanding the methylation-based tests approved for *In Vitro* Diagnostic (IVD) of BlCa, the lack of strong evidence and validation of performance limits their use in daily clinical care.

Considering the plethora of candidate biomarkers reported in the literature, only a systematic analysis complemented with robust statistics of all relevant findings concerning assessment of urinary DNA methylation-based markers for BlCa detection might allow for solid conclusions about its impact and potential for clinical application. Hence, the aims of this meta-analysis are to (1) review the most pertinent literature concerning DNA methylation biomarkers in urine for BlCa diagnosis and/or surveillance, and (2) identify and compare the sensitivity, specificity and accuracy of the different DNA methylation biomarkers, to advise on prioritization in subsequent validation studies.

METHODS

Protocol and registration

This systematic review and meta-analysis were devised following the guidelines of Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) of Diagnostic Test Accuracy (DTA) studies (Supplementary Table 1). The study protocol was registered on the International Prospective Register of Systematic Reviews (PROSPERO), which granted the registration ID CRD42023397703.

Search strategy

To assemble all relevant studies regarding BlCa detection using methylation-based biomarkers, scientific databases including PubMed, Scopus and Cochrane Library were searched for publications up to December 31, 2022. Search strategy included combination of terms such as ‘bladder cancer’ and ‘DNA methylation’ and ‘diagnosis’ or ‘recurrence’. Full queries used in each database are depicted in Supplementary Data.

The reference lists of previously published reviews and/or meta-analyses on the topic were also thoughtfully scanned to identify any additional records of interest (21, 22).

Eligibility criteria

After the removal of duplicates, the retrieved records were screened by title and abstract, based on eligibility criteria, by two independent investigators (Silva-Ferreira M. and Carvalho J.A.). In case of disagreement, a meeting was scheduled to find a consensus. The

principal endpoint of the study was to evaluate the diagnostic test accuracy of methylation-based biomarkers in urine samples, used independently or in panel, for detecting primary or recurrent urothelial carcinoma of bladder. Therefore, PICO framework (patients, index test, comparator, and outcomes) was structured accordingly. Patients enrolled in the studies that (1) carried suspicious symptoms (hematuria) or had a confirmed primary diagnosis of bladder urothelial carcinoma, or (2) were undergoing surveillance due to a previously treated bladder cancer. The index test comprised DNA methylation markers for BlCa detection, independently of the technology used. Moreover, the malignancy had to be diagnosed by a reliable standard diagnostic method such as cystoscopy and/or histological evaluation. Respecting exclusion criteria, case reports, case series, editorials, commentaries, notes, letters, brief correspondence, meeting reports, and other reviews or meta-analyses were excluded. Studies published in languages other than English or Portuguese were not considered. Furthermore, methylation analysis in urinary samples was mandatory, but studies that did not report sensitivity and specificity in this body fluid were excluded. If the target population of a study included patients with histological subtypes other than urothelial carcinoma and did not provide an independent analysis for urothelial carcinoma were also excluded.

Data extraction

The full texts of the remaining records were further assessed for eligibility. All causes leading to the exclusion of a study were noted. The two researchers independently extracted all relevant data from the selected studies, including first author name and affiliations, year of publication, study design, demographic information on the study population, sample size, number of primary vs. recurrent tumors, tumors' stage and grade, previous treatment, DNA methylation marker(s) assessed, detection methodology, cut-off value of each biomarker, as well as true positive (TP), true negative (TN), false positive (FP), false negative (FN), sensitivity (Se) and specificity (Sp) for the primary outcomes. When TP, TN, FP, and FN were reported, two-by-two tables were constructed and Se and Sp were calculated using the following formulas: Se=TP/(TP+FN) and Sp=TN/(TN+FP). When TP, TN, FP, and FN were not disclosed, these values were estimated from the provided Se and Sp. If some of these data could not be extracted nor calculated, the authors of the study were contacted by e-mail, and if no conclusive response was achieved, the study was excluded from the analysis for incomplete retrieval.

Risk of bias assessment

The quality of the studies was appraised by resorting to adapted criteria from the available tool QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies 2), which

assesses the risk of bias and applicability concerns in four major domains (patient selection, index test, reference standard, and flow and timing). An additional item was added to the index test domain, which evaluated the potential bias that the detection method could induce. Specifically, a qualitative method was considered to foster a higher risk of bias whilst a quantitative method was considered to entail a lower risk of bias.

Data synthesis and statistical analysis

The meta-analyses in this study were conducted based on different techniques and sample types used in each individual study. Sensitivity, specificity, diagnostic odds ratio (DOR), positive likelihood ratio (PLR), and negative likelihood ratio (NLR) were determined along with a 95% confidence interval using true positive (TP), true negative (TN), false positive (FP), and false negative (FN) rates extracted from the studies included.

For statistical analysis, when a study was carried out in several cohorts (test, training and validation sets), the most clinically representative cohort was selected and further analyzed. Nevertheless, if a marker was studied in a preliminary search state, e.g., training set, and not in the selected cohort, e.g., validation set, the data related to that marker was not disregarded and the information of the studied cohort was used for the analysis of the marker alone.

Considering the heterogeneity of methodologies among studies, a random effects model was employed. PLR, NLR, and DOR were computed using a univariate approach based on the methodology proposed by DerSimonian and Laird (DSL) (23). Since sensitivity and specificity are interrelated, meaning that as the cutoff value is adjusted to increase sensitivity, the specificity often decreases, a bivariate approach to the meta-analysis of diagnostic accuracy was used. All calculations were performed using the R package mada (24) for statistical analysis.

To compare the diagnostic accuracy of different markers, DOR was considered as a single global measurement for the interpretation of markers' performance (25). For easier graphical visualization, logarithm of DOR was displayed in forest plots.

RESULTS

Study characteristics

The search resulted in a total of 2297 records, of which 1785 were retrieved from PubMed, 499 from Scopus, 5 from Cochrane Library and 8 were manually included.

After removing duplicates, 2164 titles and abstracts were screened. Of these, 2071 studies were excluded based on the criteria mentioned above. Ninety-three full texts were

then reviewed, leading to exclusion of additional 25 studies, since 12 did not provide results in urine samples, 5 did not report sufficient data for methylation markers alone, 1 had a different outcome, 3 included other subtypes of BlCa and 4 did not identify the methylation markers assessed. Hence, 68 studies were included. Seven of them reported both primary and recurrent detection as outcomes whereas 49 reported detection at diagnosis alone and 12 only investigated detection of relapsed tumors. The Flow Diagram in Figure 1 provides detailed information on the study selection process.

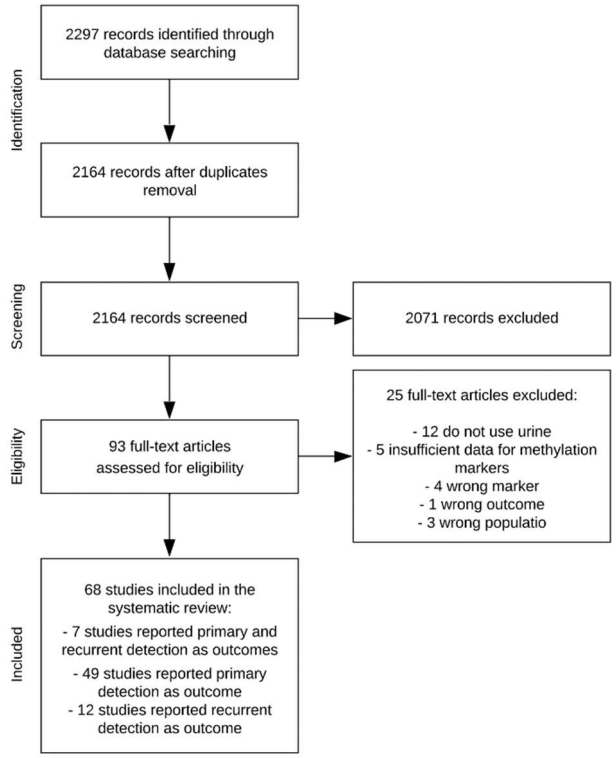

Figure 1. Flowchart of the literature selection process for the included studies, according to Preferred Reporting Items of Systematic Reviews and Meta-Analyses (PRISMA) guidelines.

Thus, we obtained a broad overview of a 20-year period of research on BlCa detection based on DNA methylation biomarkers, with studies published from 2002 to 2022, encompassing a total of 12,696 participants from diverse regions around the globe, with a higher representation of the western population, 5,557 of which harbored urothelial carcinoma of bladder.

Whereas 39 of the included studies were case-control, 25 were cohort studies and 4 were clinical trials. Moreover, 38 disclosed results for the markers individually and in panels, 12 only assessed the performance of the markers alone and 18 only evaluated panels of markers. Additionally, most of the studies used quantitative technologies to detect DNA methylation-based markers, but 14 used qualitative methods and 6 used semi-quantitative approaches. Descriptive information of the included studies is summarized in Table 1.

Table 1. Summary table of the characteristics of the included studies, and respective analyzed cohorts, appraising the diagnostic accuracy of urinary biomarkers for the primary or recurrent diagnosis of bladder cancer.

ID	Author and year of publication	Objective	Number of cases	Number of controls	DNA methylation markers	Single or Panel or Both	Platform	Ref.
A1	Chan M.W. 2002	D	22	17	*RARβ, DAPK, CDH1, p16*	S	MSP	(26)
B1	Chan M.W. 2003	D	14	10	*RASSF1*	S	MSP	(27)
C1	Friedrich M.G. 2004	D	37	20	*BCL2, TERT, DAPK*	B	qMSP	(28)
D1	Urakami S. 2006	D	24	20	*SFRP1, SFRP2, SFRP4, SFRP5, DKK3, WIF1*	S	MSP	(29)
D2	Yates D.R. 2006	D	35	69	*RASSF1, APC, CDH1, RARβ, p14, p16, DAPK, GSTP1*	B	qMSP	(30)
E1	Yu J. 2007	D	132	23	*SALL3, CFTR, ABCC6, HPP1, BCL2, ALX4, RUNX3, ITGA4 RASSF1, MYOD1, MT1A, DRM, GDF10, CCNA1, CDH13, RPRM, APBA1, BRCA1, DISP3, TMS1, GSTP1*	B	MSP	(31)
F1	Cebrian V. 2008	D	40	124	*SYNPO2*	S	MSP	(32)
F2	Rouprêt M. 2008	R	15	25	*RASSF1, CDH1, APC, DAPK, MGMT, BCL2, TERT, EDNRB, WIF1, TNFRSF25, IGFBP3*	B	qMSP	(33)
G1	Sun J. 2009	D	82	15	*SFRP1, FANCF, LOXL1, p16, XAF1, CDH1, LOXL4, TIMP3, RARRES1, SOX9, SALL3, CFTR*	B	MSP	(34)
H1	Costa V.L. 2010	D	51	59	*GDF15, HSPA2, TMEFF2, VIM*	B	qMSP	(20)
H2	Lin H.H. 2010	D	57	20	*CDH1, p16, p14, RASSF1*	B	MSP	(35)
H3	Renard I. 2010	D	35	57	*TWIST1, NID2*	P	qMSP	(36)
I1	Cabello M.J. 2011	D	49	25	*TP73, MSH6, VHL, RARβ, ESR1, CDKN2A, PAX5A, PTEN, MGMT, PAX6, WT1, CD44, GSTP1, ATM, IGDF4, CHFR, BRCA2, RB1, THBS1, PYCARD, CDH13, TP53, BRCA1, STK11, GATA5*	S	MS-MLPA	(37)
I2	Chen P-C. 2011	D	30	19	*DAPK, IRF8, p14, RASSF1, SFRP1*	B	qMSP	(38)
I3	Costa V.L. 2011	D	50	48	*PCDH17, TCF21*	P	qMSP	(39)

I4	Reinert T. 2011	D	115	59	*ZNF154, POU4F2, HOXA9, EOMES*	B	MS-HRM	(40)
I5	Serizawa R.R. 2011	D	111	33	*APC, DBCCR1, RARβ, RASSF1, SFRP1, SFRP2, SFRP4, SFRP5*	B	qMSP	(41)
I6	Vinci S. 2011	D	108	105	*BCL2, TERT, DAPK*	B	qMSP	(42)
J1	Berrada N. 2012	D	31	12	*BIRC5, RARβ, APC*	B	MSP	(43)
J2	Eissa S. 2012	D	77	116	*RARβ*	S	MSP	(44)
J3	Fernandez C.A. 2012	R	48	275	*TWIST1, NID2*	P	MSP	(45)
J4	Reinert T. 2012	D	184	35	*EOMES, HOXA9, POU4F2, TWIST1, VIM, ZNF154*	*S*	*qMSP*	(46)
		R	139	19				
J5	Scher M.B. 2012	D	42	22	*BCL2, CDKN2A, NID2*	B	qMSP	(47)
J6	Zhao Y. 2012	D	212	41	*VAX1, KCNV1, ECEL1, TMEM26, PROX1, TAL1, SLC6A20, LMX1A, CFTR*	B	MSP + BSP	(48)
J7	Zuiverloon T.C. 2012	R	65	29	*APC, TERT, EDNRB*	P	MS-MLPA	(49)
K1	Chihara Y. 2013	D	73	18	*SOX1, TJP2, MYOD1, HOXA9, VAMP8, CASP8, SPP1, IFNG, CAPG, HLA-DPA1, RIPK3*	B	PSQ	(50)
K2	García-Baquero R. 2013	D	100	28	*PRDM2, RUNX3, RARβ, HLTF, SCGB3A1, ID4, TWIST1, SFRP4, DLC1, SFRP5, BNIP3, H2AFX, CCND2, CACNA1G, TGIF1, BCL2, CACNA1A, TIMP3*	S	MS-MLPA	(51)
K3	Kandimalla R. 2013	R	101	70	*OTX1, MEIS1, ONECUT2, SIM2, FOXA1, ZNF503, HOXA9, OSR1*	S	SNaPshot	(52)
			95	130	*OTX1, ONECUT2, OSR1*	P		
K4	Shimizu T. 2013	D	34	11	*miR-137, miR-124-2, miR-124-3, miR-9-3*	B	MSP + PSQ	(53)
K5	Yegin Z. 2013	D	24	15	*TWIST1, NID2*	B	MSP	(54)
L1	Abern M.R. 2014	B	24	87	*TWIST1, NID2*	P	qMSP	(55)
L2	Hayashi M. 2014	D	20	20	*VGF*	S	qMSP	(56)

L3	Maldonado L. 2014	D	73	60	*CCNA1*	S	qMSP	(57)
			148	56	*CCND2, CALCA*			
L4	Su S-F. 2014	R	34	56	*SOX1, IRAK3, L1-MET*	P	MSP + PSQ	(58)
M1	Yeh C-M. 2015	D	33	28	*ZNF671*	S	qMSP	(59)
			26	19	*ZNF671, IRF8, SFRP1*	B		
N1	Dahmcke C.M. 2016	B	99	376	*ONECUT2, VIM, SALL3, CCNA1, BCL2, EOMES*	B	qMSP	(60)
N2	Roperch J-P. 2016	D	167	105	*HS3ST2, SEPTIN9, SLIT2*	B	qMSP	(61)
N3	Van Kessel K.E. 2016	D	74	80	*TWIST1, OTX1, ONECUT2*	S	MSP + SNaPshot	(62)
N4	Wang K. 2016	D	112	27	*p14, RUNX3, RARβ, DAPK, CDH1, HPP1, ITGA4*	B	MSP	(63)
N5	Wang Y. 2016	D	58	90	*EOMES, GDF15, NID2, PCDH17, POU4F2, TCF21, ZNF154*	B	qMSP	(64)
O1	Fantony J. J. 2017	B	51	121	*TWIST1, NID2*	P	qMSP	(65)
O2	Feber A. 2017	B	55	133	Uromark	P	RainDrop BS-seq	(66)
O3	Pietrusiński M. 2017	D	113	100	*p16, p14, DAPK, RASSF1, APC*	S	MSP	(67)
O4	Van Kessel K.E. 2017	D	97	103	*TWIST1, OTX1, ONECUT2*	B	MSP + SNaPshot	(68)
P1	Shindo T. 2018	R	26	181	*miR-137, miR-124-2, miR-124-3, miR-9-3*	P	PSQ	(69)
P2	Van Der Heijden A. 2018	D	111	57	*SALL3*, *CFTR*, *TWIST1*	P	MSP + PSQ	(70)
		R	173	285				
P3	Witjes J.A. 2018	R	44	309	Bladder EpiCheck™	P	qMSP	(15)
Q1	Bosschieter J. 2019	D	70	733	*MAL, GHSR, PRDM14, SST, ZIC1, miR-129, miR-935, miR-148, PHACTR3, FAM19A4, miR-137, miR-124-2, miR-181, CADM1*	B	qMSP	(71)

Q2	D'Andrea D. 2019	R	49	308	Bladder EpiCheck™	P	qMSP	(14)
Q3	Trenti E. 2019	R	69	146	Bladder EpiCheck™	P	qMSP	(72)
R1	Chen X. 2020	D	109	66	*OTX1*, *SOX1-OT*	B	utMeMa	(73)
		R	38	43		P		
R2	Hentschel A.E. 2020	D	14	12	*FAM19A4, GHSR, MAL, miR-129, miR-935, PHACTR3, PRDM14, SST, ZIC1*	B	qMSP	(74)
R3	Hermanns T. 2020	D	211	102	*TWIST1, NID2*	B	qMSP	(75)
R4	Monteiro-Reis S. 2020	D	27	24	*VIM*, *miR663a*	S	qMSP	(76)
			100	174		P		
R5	Rose M. 2020	D	130	81	*ECRG4, ITIH5, NID2*	B	qMSP	(77)
R6	Steinbach D. 2020	D	30	30	GynTect ®	B	qMSP	(78)
R7	Wu Y. 2020	D	53	58	*HOXA9, PCDH17, POU4F2, ONECUT2*	B	MS-HRM PCR	(79)
S1	Georgopoulos P. 2021	D	44	22	*DAPK, RARβ, TERT, RASSF1, APC*	B	qMSP	(80)
S2	Piatti P. 2021	D	77	136	Bladder CARE™	P	qMSP	(81)
S3	Ruan W. 2021	D	34	140	*ONECUT2, VIM*	P	qMSP	(82)
T1	Cochetti G. 2022	R	18	22	Bladder EpiCheck™	P	qMSP	(83)
T2	Deng L. 2022	D	100	137	*PENK*	S	qMSP	(84)
			79	304	*DMRTA2*			
T3	Fang Q. 2022	D	107	100	*PCDH17, POU4F2, PENK*	B	qMSP	(85)
T4	Hentschel A.E. 2022	D	108	100	*GHSR, MAL*	P	qMSP	(4)

T5	Oh T.J. 2022	D	16	35	*DMC1, SIM2*	S	PSQ	(86)
			55	106	*PENK*	S	qMSP	
T6	Peña K.B. 2022	R	59	11	Bladder EpiCheck™	P	qMSP	(87)
T7	Pharo H.D. 2022	D	26	86	BladMetrix™	S	ddPCR	(88)
			93	180		P		
T8	Ragonese M. 2022	R	71	160	Bladder EpiCheck™	P	qMSP	(89)

D: Diagnosis; R: Recurrence; S: Single; P: Panel; B: Both; MSP: Methylation-Specific PCR; qMSP: quantitative Methylation-Specific PCR; MS-MLPA: Methylation-Specific Multiplex Ligation-dependent Probe Amplification; MS-HRM: Methylation-Sensitive High Resolution Melting; BSP: Bisulfite Specific PCR; PSQ: Pyrosequencing; ddPCR: Droplet Digital PCR; Ref: References

Risk of bias and applicability

QUADAS-2 was the tool used to assess the quality of the primary studies. After careful tailoring, the signaling questions of each item were answered for each study (Supplementary Table 2). The risk of bias was low in most studies in all parameters, excepting for flow and timing, as a large proportion of the studies were retrospective, or the workflow was not clear (Figure 2). Another domain which attained high risk of bias (22% of studies) was patient selection domain, either as a result of the failure to use random or consecutive patient samples, or because the design of the study was case-control.

Regarding applicability concerns, the index test consisted of the domain with the highest concern rate, mostly due to the detection technology used in each study.

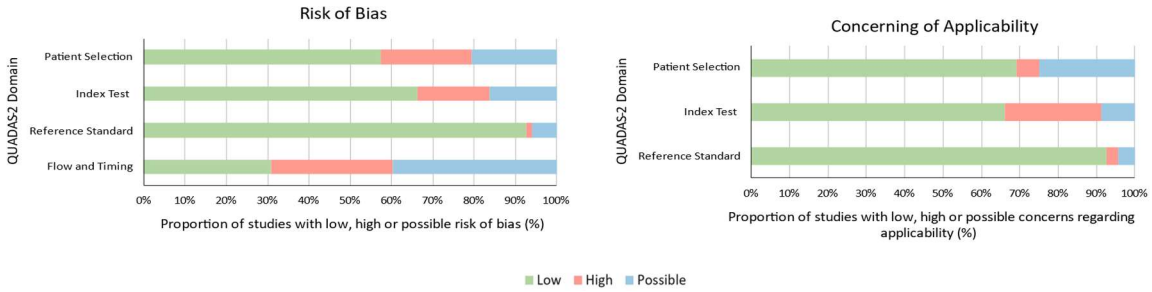

Figure 2. Summary of risk of bias and concerns of applicability for the included studies, obtained through QUADAS-2 evaluation.

Meta-analysis

Diagnostic accuracy of single genes methylation

When individually analyzed, 103 out of 156 DNA methylation markers were only reported in one study. Therefore, only a descriptive analysis is appropriate to assess their diagnostic accuracy. Additionally, biomarkers reported in two studies (25 genes out of 156), did not have enough evidence to perform the meta-analysis. Indeed, only genes that were investigated in more than three eligible studies were included in the meta-analysis (Table 2).

Table 2. Pooled diagnostic accuracy measures for the 28 markers included in the statistical analysis.

Marker	Studies	Sensitivity (95% CI)	Specificity (95% CI)	PLR (95% CI)	NLR (95% CI)	DOR (95% CI)
RASSF1	10	0.41 [0.31-0.52]	0.89 [0.73-0.96]	3.02 [1.53-5.97]	0.68 [0.57-0.82]	5.02 [2.04-12.39]
DAPK	9	0.20 [0.13-0.28]	0.93 [0.89-0.96]	2.79 [1.72-4.52]	0.88 [0.81-0.95]	3.51 [1.88-6.55]
RARβ	8	0.32 [0.14-0.58]	0.91 [0.84-0.95]	3.74 [2.18-6.42]	0.68 [0.56-0.82]	7.09 [3.00-16.75]
TWIST1	8	0.77 [0.66-0.88]	0.77 [0.63-0.87]	2.88 [1.96-4.24]	0.29 [0.17-0.49]	10.70 [5.32-21.55]
BCL2	7	0.42 [0.23-0.63]	0.96 [0.93-0.98]	9.48 [2.69-33.40]	0.55 [0.40-0.77]	17.99 [3.71-87.33]
APC	6	0.33 [0.25-0.43]	0.95 [0.82-0.99]	5.59 [1.84-17.02]	0.71 [0.61-0.83]	8.97 [2.44-33.04]
CDH1	6	0.21 [0.07-0.48]	0.87 [0.66-0.96]	1.25 [0.49-3.14]	0.89 [0.72-1.10]	1.59 [0.44-5.80]
HOXA9	6	0.77 [0.62-0.88]	0.86 [0.73-0.93]	6.28 [2.88-13.68]	0.27 [0.18-0.40]	22.45 [11.81-42.66]
NID2	6	0.71 [0.59-0.81]	0.77 [0.62-0.87]	3.18 [2.06-4.93]	0.37 [0.27-0.51]	8.93 [5.14-15.53]
POU4F2	6	0.81 [0.74-0.87]	0.89 [0.75-0.95]	6.43 [3.31-12.48]	0.22 [0.16-0.30]	34.89 [15.49-78.56]
VIM	6	0.82 [0.74-0.88]	0.90 [0.78-0.96]	10.86 [3.73-31.66]	0.21 [0.15-0.29]	44.81 [19.37-103.68]
EOMES	5	0.79 [0.58-0.91]	0.88 [0.71-0.96]	9.09 [3.03-27.25]	0.24 [0.13-0.44]	33.22 [17.47-63.18]
ONECUT2	5	0.70 [0.56-0.81]	0.92 [0.90-0.94]	8.87 [6.17-12.75]	0.32 [0.21-0.48]	27.83 [13.64-56.78]
p14	5	0.25 [0.17-0.36]	0.99 [0.96-1.00]	13.69 [3.70-50.69]	0.79 [0.66-0.94]	18.11 [4.73-69.29]
p16	5	0.18 [0.10-0.30]	0.97 [0.88-0.99]	4.02 [1.19-13.63]	0.87 [0.78-0.98]	5.02 [1.37-18.42]
SFRP1	5	0.36 [0.19-0.57]	0.95 [0.87-0.98]	6.57 [2.88-14.98]	0.66 [0.50-0.85]	11.16 [4.42-28.16]
OTX1	4	0.77 [0.67-0.84]	0.73 [0.53-0.87]	2.78 [1.79-4.32]	0.34 [0.28-0.41]	9.02 [5.49-14.81]
TERT	4	0.23 [0.07-0.56]	0.98 [0.95-0.99]	15.26 [4.75-49.06]	0.72 [0.52-0.99]	19.67 [4.48-86.44]
ZNF154	4	0.87 [0.70-0.95]	0.90 [0.56-0.98]	4.20 [2.12-8.32]	0.16 [0.08-0.35]	45.07 [16.77-121.15]
CCNA1	3	0.49 [0.17-0.82]	0.95 [0.79-0.99]	8.53 [2.27-32.04]	0.49 [0.24-0.97]	20.82 [5.05-85.81]
GSTP1	3	0.03 [0.01-0.06]	0.99 [0.93-1.00]	2.18 [0.37-12.96]	0.98 [0.94-1.02]	2.23 [0.36-13.72]
ITGA4	3	0.44 [0.30-0.59]	0.89 [0.56-0.98]	4.08 [0.87-19.23]	0.65 [0.50-0.86]	6.64 [1.11-39.57]
PCDH17	3	0.58 [0.40-0.74]	0.88 [0.70-0.96]	4.60 [2.06-10.28]	0.48 [0.32-0.72]	10.47 [3.31-33.06]
PENK	3	0.77 [0.71-0.81]	0.93 [0.90-0.96]	11.77 [7.81-17.74]	0.25 [0.20-0.31]	47.90 [28.33-81.00]
RUNX3	3	0.43 [0.27-0.60]	0.91 [0.75-0.97]	4.94 [1.33-18.42]	0.61 [0.44-0.86]	8.60 [1.67-44.16]
SALL3	3	0.61 [0.55-0.67]	0.97 [0.94-0.98]	19.11 [11.35-32.19]	0.40 [0.35-0.46]	55.67 [29.10-106.52]
SFRP4	3	0.14 [0.02-0.59]	0.93 [0.80-0.98]	2.53 [1.08-5.92]	0.91 [0.77-1.08]	3.08 [0.87-10.90]
SFRP5	3	0.32 [0.08-0.72]	0.93 [0.84-0.97]	4.78 [1.60-14.23]	0.78 [0.61-1.00]	9.01 [1.47-55.22]

PLR: Positive Likelihood Ratio; NLR: Negative Likelihood Ratio; DOR: Diagnostic Odds Ratio; CI: Confidence Interval

By providing a balance between sensitivity, specificity and DOR the plausibly best-performing genes were *SALL3*, *PENK*, *ZNF154*, *VIM*, *POU4F2* and *EOMES*, with pooled sensitivities and specificities ranging from 61-87% and 88-97%, respectively, and pooled DORs ranging from 33.22 to 55.67.

The methylation status of *SALL3* disclosed a pooled specificity of 97% with narrow confidence interval (95%CI 94-96%). Despite having 61% sensitivity (95%CI 55-67%), *SALL3* achieved the highest DOR among all other single markers (Figure 3).

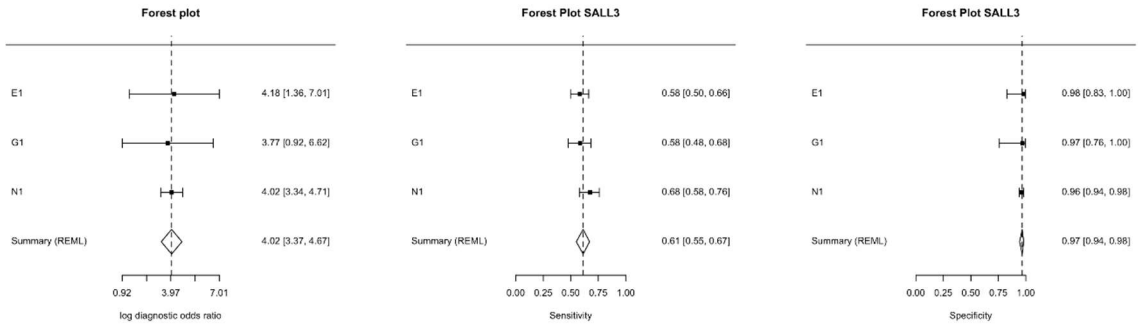

Figure 3. *SALL3* marker's forest plots for sensitivity, specificity, and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

PENK also proved to be highly specific in each of the three studies, reaching pooled 93% specificity (95%CI 90-96%) and 77% sensitivity (95%CI 71-81%). This marker obtained the second highest DOR among all single markers (Figure 4).

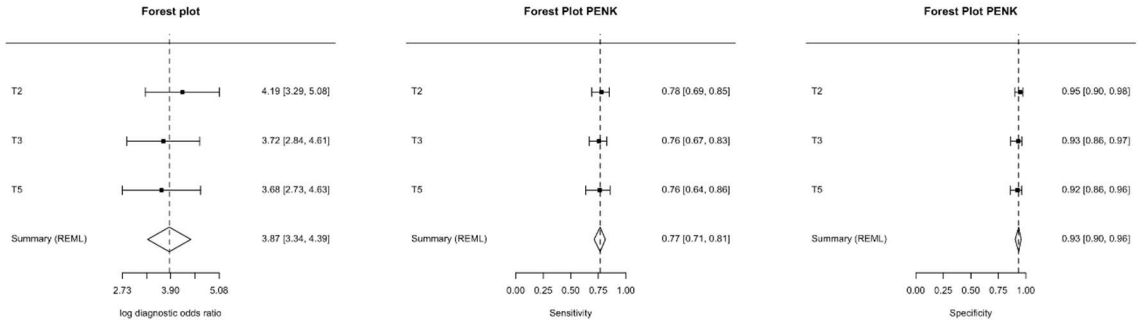

Figure 4. *PENK* marker's forest plots for sensitivity, specificity, and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

ZNF154 depicted rather variable performance among the reports, resulting in larger confidence intervals. Herein, study J4 was considered twice as it provided performance data for diagnostic and recurrence outcomes separately. Nevertheless, *ZNF154* attained pooled 87% sensitivity (95%CI 70-95%) and 90% specificity (95%CI 56-96%), and the third highest DOR (Figure 5).

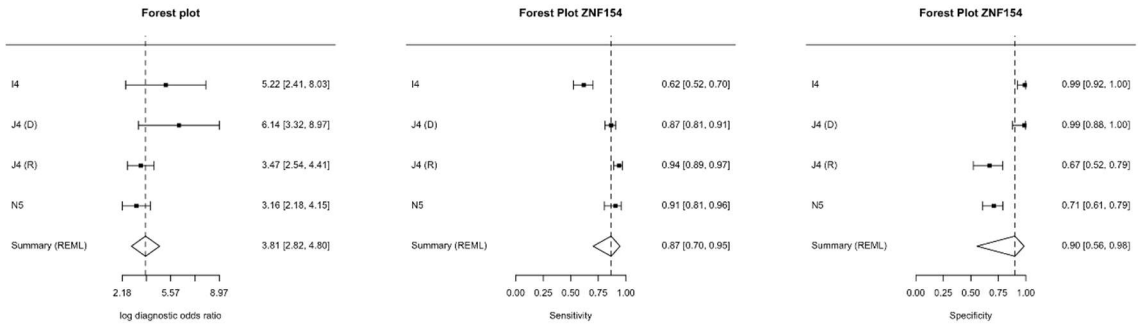

Figure 5. *ZNF154* marker's forest plots for sensitivity, specificity, and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

The gene *VIM* was appraised in six studies, reaching consistent results for primary bladder cancer detection in all, but not for recurrence in J4. Nonetheless, *VIM* displayed 82% sensitivity (95%CI 74-88%), 90% specificity (95%CI 78-96%) and 44.81 DOR (95%CI 19.37-103.68) (Figure 6).

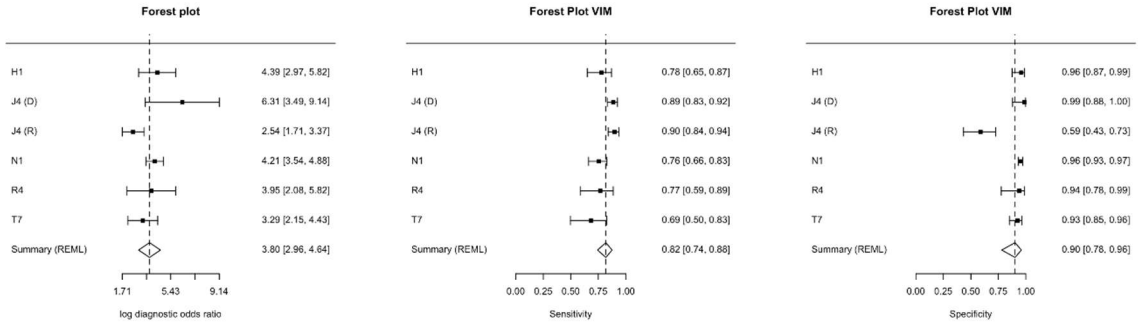

Figure 6. *VIM* marker's forest plots for sensitivity, specificity and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

Similarly, *POU4F2* methylation status was analyzed in six studies, also revealing a considerably lower specificity in the recurrence study described in J4, although pooled sensitivity and specificity reached 81% (95%CI 74-87%) and 89% (95%CI 75-95%), respectively (Figure 7).

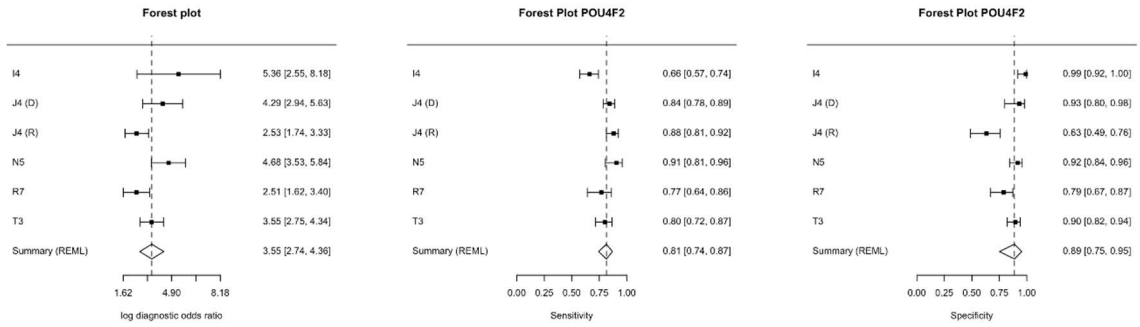

Figure 7. *POU4F2* marker's forest plots for sensitivity, specificity and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

Contrarily to *SALL3*, *PENK*, *VIM* and *POU4F2*, *EOMES* showed highly variable performance among the five evaluated studies. Nonetheless, *EOMES* displayed pooled 79% sensitivity (95%CI 58-91%) and 88% specificity (95%CI 71-96%) and the sixth highest DOR (Figure 8).

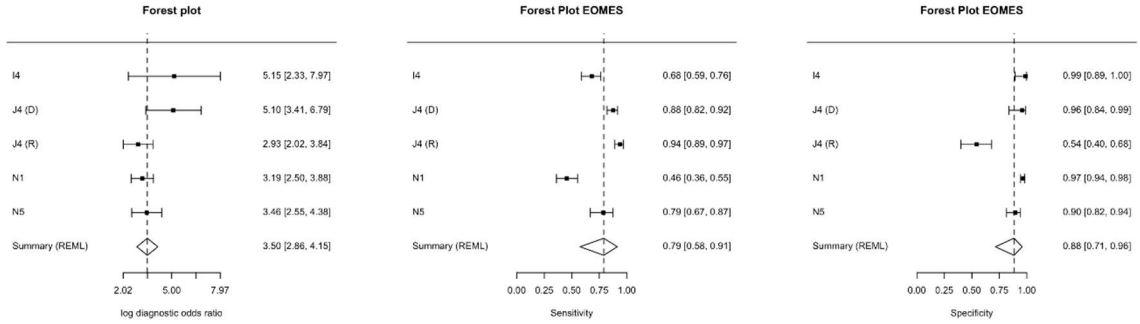

Figure 8. *EOMES* marker's forest plots for sensitivity, specificity and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

Other genes reached equally promising results, although they have been assessed in just one study, namely *DMRTA2*, *SOX1-OT*, *TMEFF2*, as well as those included in the BladMetrix™ panel.

Diagnostic accuracy of gene methylation panels

Regarding gene methylation panels, statistical analysis was conducted with two reports. The panel comprising *TWIST1* and *NID2* was the most widely studied, assessed in five different studies, attaining pooled sensitivity, specificity and DOR of 79% (95%CI 56-92%), 84% (95%CI 71-91%) and 15.94 (95%CI 4.57-55.58), respectively (Figure 9). The other analyzed panel was Bladder EpiCheck™, a commercially available IVD test, that accomplished pooled 74% sensitivity (95%CI 63-83%), 87% specificity (95%CI 85-89%) and 18.57 DOR (95%CI 10.73-32.13) (Figure 10).

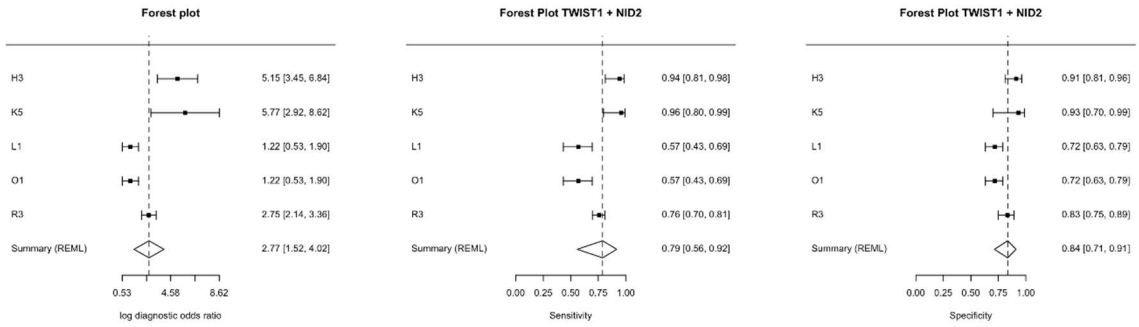

Figure 9. *TWIST1* / *NID2* panel's forest plots for sensitivity, specificity and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

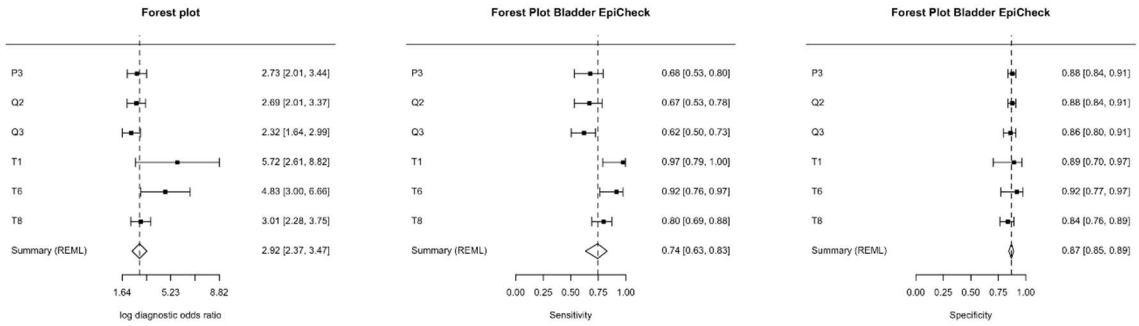

Figure 10. Bladder EpiCheck™ Test's forest plots for sensitivity, specificity and logarithm of diagnostic odds ratio (DOR) in detecting bladder cancer.

Other panels, including different combinations of genes, also exhibited promising results, but little evidence is available, and no statistical analysis was performed (Table 3).

Table 3. Sensitivity, specificity and diagnostic odds ratio (DOR) for the highest-performing panels not included in the statistical analysis.

Panel	Studies	Sensitivity	Specificity	DOR	Ref.
SOX1 / TJP2 / MYOD1 / HOXA9_1 / HOXA9_2 / VAMP8 / CASP8 / SPP1 / IFNG / CAPG / HLA-DPA1 / RIPK3	1	100.00%	100.00%	INF	(50)
CDH1 / RASSF1 / p14 / p16	1	82.46%	100.00%	INF	(35)
Uromark™	1	96.36%	96.99%	854.63	(66)
RUNX3 / RARβ / DAPK / HPP1 / p14	1	98.21%	88.89%	440	(63)
RUNX3 / RARβ	1	96.43%	88.89%	216	(63)
Bladder CARE™	1	93.51%	92.65%	181.44	(81)
BladMetrix™	1	92.13%	93.33%	164	(88)
BCL2 / TERT	1	75.93%	98.10%	162.42	(42)
VIM / TMEFF2 / GDF15	1	94.12%	89.83%	141.33	(20)
IRF8 / SFRP1 / ZNF671	1	96.15%	84.21%	133.33	(59)
POU4F2 / EOMES / HOXA9 / ZNF154	1	83.93%	96.15%	130.56	(40)
IRF8 / SFRP1 / p14	1	86.67%	94.74%	117	(38)
PCDH17 / POU4F2 / PENK	1	77.57%	97.00%	111.82	(85)
PCDH17 / POU4F2	2	91.38% 77.57%	93.33% 95.00%	148.4 65.71	(64, 85)

DOR: Diagnostic Odds Ratio; Ref: References

Interestingly, *SOX1* / *TJP2* / *MYOD1* / *HOXA9*_1 / *HOXA9*_2 / *VAMP8* / *CASP8* / *SPP1* / *IFNG* / *CAPG* / *HLA-DPA1* / *RIPK3* was the only panel achieving the ideal 100% sensitivity and specificity, whereas *CDH1*, *RASSF1*, *p14* and *p16* panel disclosed 100% specificity and 82% sensitivity. Nonetheless, both panels lack further validation.

Furthermore, three tests – BladMetrix™, Uromark™ and Bladder CARE™ (the last two already approved for IVD applications) – disclosed performances above 90% sensitivity and specificity.

Detection setting

As previously mentioned, seven studies investigated the performance of respective biomarkers for BlCa detection and follow-up, but only three provided separate analysis. Moreover, the only detection tests that complied with the established criteria were *TWIST1*, *HOXA9* and urinary cytology. Nonetheless, no significant differences were reported in performance for detecting primary or recurrent bladder cancer (Figure 11).

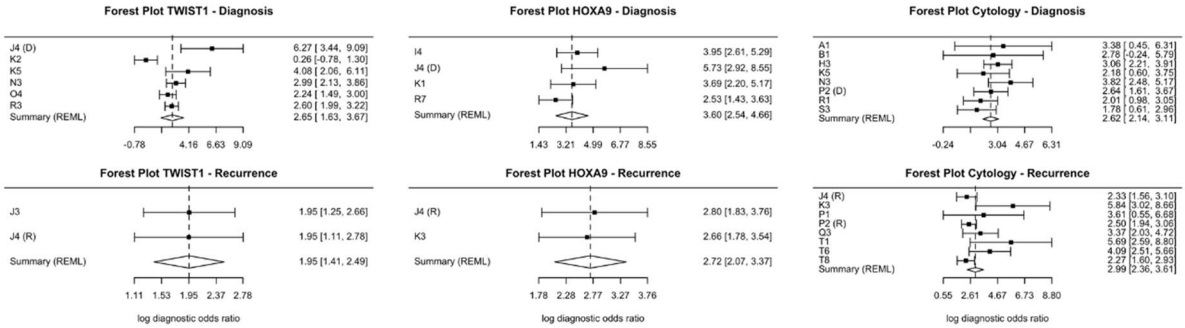

Figure 11. *TWIST1*, *HOXA9* and urinary cytologys' forest plots for logarithm of diagnostic odds ratio (DOR) in detecting primary and recurrent bladder cancer.

Tumor stage

Overall, regarding the subgroup analysis for tumor stage, superior sensitivity for detecting high-grade (HG) tumors was apparent, although only Bladder EpiCheck™ and urine cytology disclosed significant differences. Indeed, the sensitivity of Bladder EpiCheck™ sharply increased to 87% (95%CI 80-92%) when low-grade (LG) tumors were excluded from analysis, outperforming cytology in the same scenario (66% sensitivity; 95%CI 54-75%) (Figure 12 and Supplementary Table 3).

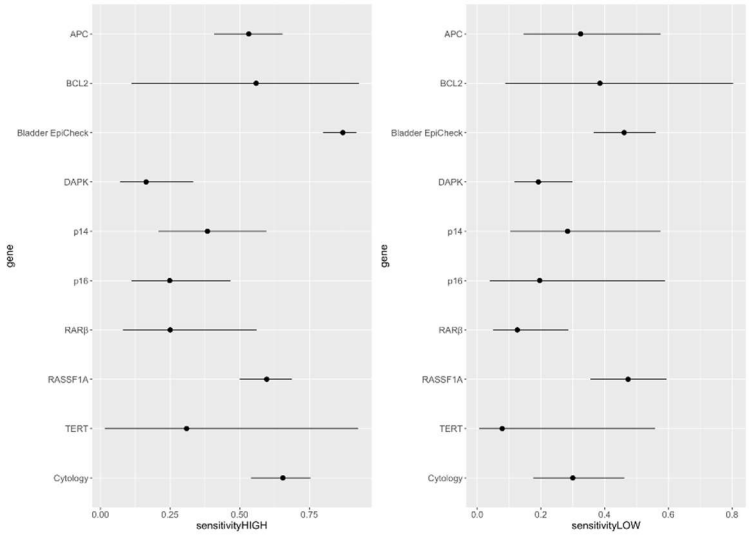

Figure 12. Forest plots for the sensitivity of ten markers in detecting low-grade or high-grade bladder cancer.

Urinary Cytology

In addition to assessment of DNA methylation markers performance, 17 studies also assessed urine cytology (the current standard test) performance for detecting BlCa cancer. In this meta-analysis, cytology achieved 55% sensitivity (95%CI 45-64%), 92% specificity (95%CI 87-95%) and a DOR of 14.37 (95%CI 9.76-21.17) (Supplementary Figure 1). In a subgroup analysis, urine cytology depicted significant differences in sensitivity for detecting low- or high-grade tumors [30% (95%CI 18-46%) and 66% (95%CI 54-75%), respectively] (Figure 12). Although some genes disclosed better sensitivity than cytology, only three also displayed higher specificity. Regarding DOR, 14 markers outperformed cytology, although only *SALL3* and *PENK* achieved statistical significance (Figure 13).

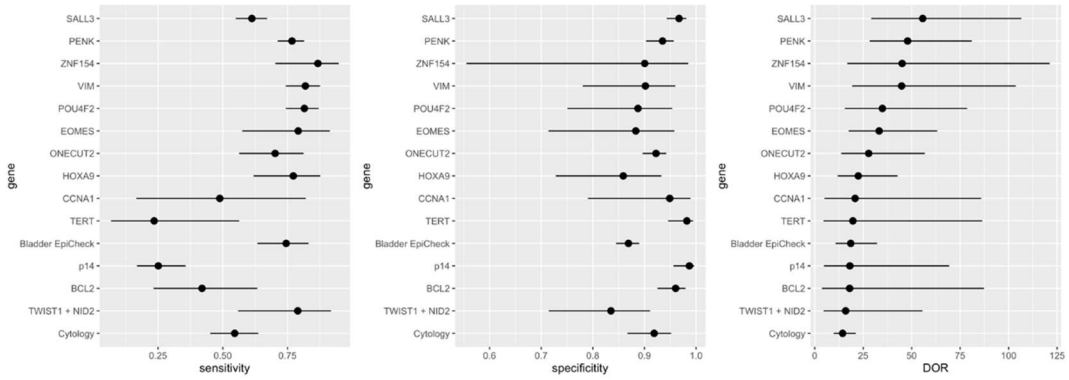

Figure 13. Forest plots for pooled sensitivity, specificity and diagnostic odds ratio (DOR) of the 14 markers with higher DOR values than urinary cytology for the detection of bladder cancer. Urinary cytology is also included in the graphs for better visualization and comparison.

DISCUSSION

Bladder cancer is a prevalent disease worldwide, with high propensity for relapse and progression (90). Currently, urethrocystoscopy complemented with voided urine cytology remains standard of care for patient diagnosis and monitoring. This strategy, however, has many drawbacks including invasiveness, economic burden, and low accuracy for diagnosing CIS and low-grade disease (91). Thus, minimally invasive molecular biomarkers constitute an attractive research target but despite the many reported, none has replaced urethrocystocopy or reduced its frequency (2), probably due to lack of robust validation studies.

This meta-analysis aimed to disclose the most promising methylated gene promoters which might be used as biomarkers for BlCa detection, either in diagnosis or surveillance settings. Remarkably, a lack of recurrently studied genes was noticed. Indeed, some individual genes and gene panels seemed very promising but were only studied once (e.g., *DMRTA1*, *SOX1-OT*, *TMEFF2*, as well as BladMetrix™, Bladder CARE™ and Uromark™) (20, 66, 73, 81, 84, 88). Among these, the most intriguing case was that of the panel *SOX1* / *TJP2* / *MYOD1* / *HOXA9_1* / *HOXA9_2* / *VAMP8* / *CASP8* / *SPP1* / *IFNG* / *CAPG* / *HLA-DPA1* / *RIPK3* since it managed to diagnose all cancer cases without any false positives, using pyrosequencing (50), but no further investment on performance validation seems to be undertaken. The panel consists of 12 markers, including two different loci for *HOXA9*, but despite the high accuracy they present together, the same is not observed when they are assessed separately. Furthermore, controls consisted only of urine samples from 18 healthy volunteers, which might contribute to study bias: not only this is a small number of controls, but it also does not encompass the full range of clinical conditions which constitute differential diagnosis with BlCa.

Control samples are, indeed, a relevant potential source of bias. Only 35 studies (half of the studies included) used patients with benign diseases or other urological cancers as controls in addition to healthy individuals, and only 26 had a sample size superior to 30. This might constitute a problem for interpretation of specificity, since non-invasive tests would be especially helpful in discriminating between cancer patients and individuals with similar clinical manifestations, but which do not harbor a bladder cancer. Thus, it is of paramount importance to determine biomarker performance when the control group includes hematuria or urinary tract infection.

Uromark™ was one of the best-performing tests, with sensitivity and specificity reaching 96% and 97%, respectively, although the available results were derived from a single study

(66). This test assesses the methylation status of 150 loci using droplet digital PCR (ddPCR) followed by next-generation sequencing (NGS), a technique called RainDrop BS-Seq. One protocol is available for two multicenter prospective clinical trials aiming to validate this test for detecting BlCa in patients presenting hematuria: DETECT I and DETECT II (92). However, results from these studies have not been disclosed, yet. Despite being highly promising, even if its accuracy is validated, cost analyses should be carried out to evaluate cost-effectiveness of the test, considering that the methodology used is expensive. Likewise, the diagnostic accuracy of Bladder CARE™ and BladMetrix™ should also be validated in larger prospective cohorts, since favorable results were found in the studies included in this meta-analysis (81, 88).

Regarding accuracy analysis of single biomarkers, the best-performing genes, having DOR as a comparator measurement, were *SALL3*, *PENK*, *ZNF154*, *VIM*, *POU4F2* and *EOMES*. Besides being included in three studies, *SALL3* and *PENK* were the only markers which showed a DOR significantly superior to urine cytology. Moreover, although less investigated, they disclosed quite consistent performance across the three studies, as reflected by the narrow confidence interval for sensitivity and specificity. Both markers are highly specific, although only moderately sensitive. Nevertheless, it is important to bear in mind that despite *SALL3* high specificity, the control groups were not very representative of the clinical scenario. *ZNF154*, *EOMES*, *VIM* and *POU4F2*, with an increasing number of additional studies, are more balanced concerning sensitivity and specificity and disclose high DOR values, as well. Interestingly, when included in gene panels, high performance is retained, but no panel combining these top-performing markers was ever tested. Considering the other single gene biomarkers and despite being extensively studied throughout the years, *RASSF1*, *DAPK*, *RARβ* and *TWIST1* did not obtain such thriving results. Moreover, some genes that were explored solely in two of the included studies disclosed inconsistent results, such as *FAM19A4*, *GHSR*, *MAL*, *miR-124-2*, *MYOD1*, and *WIF1*. This highlights, once again, the importance of validating the same markers in different studies and different contexts.

Some other inconsistencies between studies were also identified. For instance, Vinci S. et al. investigated *BCL2*, *TERT* and *DAPK* as well as their combinations for BlCa diagnosis using quantitative methylation-specific PCR (qMSP) (42). The *BCL2* / *TERT* panel disclosed 76% sensitivity and 98% specificity, whereas the addition of *DAPK* only increased sensitivity by 2% and specificity decreased by 8%. Nonetheless, this three-gene panel had already been studied by MSP in a small cohort, in 2003, by Chan M. W. Y. et al., which obtained a similar sensitivity of 78% but a specificity of 100% (27). Several reasons may lead to the differences found in both studies, including sample size, types of controls used or detection

method. Whereas the 2003 study used only 17 healthy volunteers as controls, the study of Vinci S. et al. used 105 controls, which consisted of urine from healthy volunteers and patients with urinary infection or other benign diseases. Moreover, using qualitative or quantitative approaches may also significantly impact the results. Vinci S. et al assessed the panel performance using a quantitative methylation-specific PCR multiplex approach, which is a more sensitive technique, and becomes more specific when probes are used, allowing for threshold adjustment, whereas qualitative PCR is not as sensitive and has lower resolution. Although some authors opted for different detection approaches, the vast majority chose qMSP.

The *PCDH17* and *POU4F2* panel also depicted inconsistent results in the two included studies. First, it was explored by Wang Y. et al. in 2016, disclosing 91% sensitivity and 93% specificity in the training set (64). Later, in 2022, Fang Q. et al. obtained a similar specificity (95%) but a considerably lower sensitivity (78%) (85). This demonstrates that even the use of the same technology (qMSP in this case) does not guarantee comparable results, since many other factors influence biomarker performance. Examples include the quantity of DNA used in PCR, the annealing temperature and time, primer sequence and concentration, targeted CGs, and even the fluorescence method to detect the PCR product (SYBR Green vs. TaqMan probes). This enlightens another difficulty which is reproducibility of methodologies among users and laboratories, even when test performance is validated, which reinforces the need for transparency and detailed reporting of methods in scientific publications. A paradigmatic example is that 23 of the included articles did not report TP, TN, FP, and FN values, further impairing the desired homogeneity among studies. Hence, adopting data report guidelines, such as the “Standards for Reporting Diagnostic Accuracy Studies” (STARD) guidelines, constitutes a relevant step forward, although only Pharo et al. have done it (88, 93).

Another important pitfall found was the surprisingly low number of studies addressing recurrence, which would be the most important scenario to reduce the number of urethrocystocopy performed. Thus, the subgroup analysis performed to evaluate whether the markers performed differently in the detection of primary tumor or recurrence, was only possible for the few markers that were studied in both scenarios: *TWIST1*, *HOXA9* and cytology. No significant differences between settings were found, however. Moreover, very few studies reported the impact of previous therapies, such as intravesical BCG or mitomycin C, in urinary molecular biomarkers performance, which precluded an additional subgroup analysis.

Furthermore, very few articles performed a translation between WHO 1973 (G1, G2, G3) and WHO 2004/2022 classification system [Papillary Urothelial Neoplasm of Low Malignant Potential (PUNLMP), Low-Grade (LG), High-Grade (HG)], making unclear which proportion of G2 cases were assigned to LG or HG categories in most of the studies (94, 95). Subgroup analysis regarding tumor stage only considered studies that displayed results for LG and HG tumors. Moreover, only Bladder EpiCheck™ test and urinary cytology depicted significant differences in sensitivity for detecting LG or HG tumors, with both tests being more sensitive for HG tumors. Although no statistically significantly difference was observed for LG tumors detection, Bladder EpiCheck™ significantly outperformed urine cytology in HG tumors detection. These findings might have significant impact depending on the clinical context. For initial diagnosis there is a more urgent need to detect the tumor at early stages, being of utmost interest to detect LG tumors. Looking for recurrence in follow-up, however, if the goal is to reduce urethrocystoscopies, alternating with non-invasive tests, missing a LG tumor might not be as clinically relevant as missing a HG lesion, since a LG is less likely to progress in the interval until the next urethrocystoscopy (2). In this scenario, Bladder EpiCheck™, an IVD test that assesses the methylation status of 15 biomarkers and is approved for BlCa surveillance, seems to be the most appropriate. However, because very few studies could be included in this subgroup analysis, the likelihood of bias is significant. In future studies, it would be very interesting to also know the results of urinary biomarkers only atypical urothelial cells are found in urine cytology, as well as the subsequent outcome.

Interestingly, urine cytology achieved 55% sensitivity, 92% specificity and DOR of 14.37. Although several biomarkers were more sensitive than cytology, none disclosed a significantly higher specificity. Having DOR as a comparative measure, *SALL3* and *PENK* were the only markers to achieve a higher accuracy than urinary cytology. For the record, urine cytology performs well at finding high-grade BlCa urothelial carcinoma, especially CIS, yet its major drawback emerges when it comes to detecting low-grade BlCa. Although it is practical, unexpensive and has no adverse effects for patients, cytology highly depends on the individual pathologist's proficiency (96). Thus, combining urine cytology with molecular biomarkers might provide an interesting tool for follow-up, eventually emulating CYSTO performance with the added benefit of not carrying adverse effects and being solely non-invasive.

Urinary biomarkers are likely beneficial both for initial detection and relapse of BlCa. Although a few tests have been approved, including methylation-based ones, no marker has been recommended for BlCa diagnosis or follow-up in routine clinical practice by any clinical guidelines, since no test seems able, thus far, to replace urethrocystoscopy (2). Nevertheless, non-invasive biomarkers could still be very useful in an initial diagnostic

phase to “rule-in” or “rule-out” patients for urethrocystoscopy. Also, a well-structured BlCa screening program adjusted to age and, eventually other risk factors, does not exist at present but it would be an ideal scenario to test urine-based molecular biomarkers that would likely reduce time to BlCa diagnosis and decrease disease-related mortality, especially among those with identifiable risk factors such exposed to tobacco smoke or occupational hazards (aromatic amines, polycyclic aromatic hydrocarbons, and chlorinated hydrocarbons).

CONCLUSION

In a global view, the results of this meta-analysis emphasize that several DNA methylation-based biomarkers disclose high accuracy for BlCa detection but are highly understudied in different clinical scenarios, especially those in which they might prove more useful. This is translated into low quality evidence regarding performance, hampering its approval for clinical use. Notwithstanding, our study clearly identified the best performing biomarkers, as well as proposed some scenarios in which biomolecular testing is more likely to outperform currently defined strategies. Thus, a strategy is set and might be implemented to finally validate the most promising candidates and definitively deliver the promise of molecular testing to improve diagnosis and management of BlCa suspects and patients.

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PART II

Aberrant DNA methylation of *SALL3*, *ZNF154* and *VIM* in urine samples accurately distinguish Bladder Cancer from Benign Conditions of the Urinary Tract

In preparation

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ABSTRACT

Introduction: Notwithstanding most bladder tumors (BlCa) are diagnosed in non-muscle invasive (NMIBC) stages, one of the biggest dilemmas is the lack of reliable biomarkers to provide non-invasive early detection tools of this malignancy. The gold-standard diagnostic procedure for BlCa is the combination of cystoscopic examination of the bladder and urinary cytology, which can be invasive and expensive. In a meta-analysis conducted by our research group, five most promising DNA methylation urinary biomarkers were unveiled – *SALL3*, *PENK*, *ZNF154*, *VIM* and *POU4F2* for BlCa detection.

Aim: Herein, we aim to analytically validate the diagnostic accuracy of the five markers retrieved by the above-mentioned meta-analysis.

Material and Methods: The performance of the selected markers was validated in a retrospective series of urine sediments from 80 non-muscle invasive BlCa (NMIBC) patients, 25 healthy donors (HD), 25 prostate cancer (PCa) patients, 25 renal cancer (RCa) patients, and 25 patients with inflammatory conditions (IC) of the urinary tract, by quantitative methylation-specific PCR (qMSP).

Results: Even though significant methylation differences were found between BlCa patients and HD, PCa and RCa patients' urines for all genes' promotors, only *SALL3*, *ZNF154* and *VIM* presented significantly higher methylation levels in BlCa urine samples than in IC patients. Panels combining two of these three markers achieved sensitivities and specificities ranging 48-55% and 84-92%, respectively.

Discussion and Conclusions: The current study appraised the diagnostic accuracy of *SALL3*, *PENK*, *ZNF154*, *VIM* and *POU4F2* methylation levels as BlCa early detection biomarkers, since NMIBC patients' samples were tested. Furthermore, the study closely mirrors real-world clinical scenarios, where individuals displaying suspicious urinary tract symptoms might be initially ruled out as BlCa cases. Therefore, this work constitutes a preliminary biomarkers validation that if further validated may perfect non-invasive early diagnostic of BlCa.

INTRODUCTION

The neoplasia of the bladder is the second most incident urological cancer in the world, and the seventh most prevalent cancer among all types (1). Particularly in Europe, Bladder Cancer (BlCa) is the fifth most diagnosed malignancy and the eighth deadliest. Many bladder tumors arise from urothelium cells and around 75-80% are usually still non-muscle invasive bladder carcinomas (NMIBC) upon diagnosis (2). Nevertheless, even after successful curative-intended treatments, it is expected that up to 50% of the tumors will relapse or eventually progress into a muscle-invasive carcinoma (MIBC), which is a much more aggressive presentation of the disease with the highest probability of metastasization (3, 4). In order to detect recurrent tumors, the more promptly possible, routine follow-ups are performed up until 10 years after initial treatment, or for the rest of the patient's lifetime (5, 6). Surveillance relies on the same techniques as the standard first diagnosis approach, which consists of cystoscopic examination combined with the evaluation of exfoliated cells through urinary cytology. When a suspicious lesion is identified, a biopsy is taken, or a transurethral resection of the tumor (TURBT) is performed for later histological appreciation of the resected tissue (5). However, urethrocystoscopy is a highly invasive procedure, associated with patients' comorbidities and adverse events, whilst urine cytology presents limited sensitivity in detecting low-grade tumors (7, 8). So, the frequency and extensiveness of these procedures over time contribute to a heavy burden on healthcare systems, which unduly affects financial sustainability and patients' life quality (9). Moreover, the most frequently associated symptom of BlCa, the macroscopic hematuria, is more evident in advanced stages of the disease, ruling-in patients for further clinical examination (6). However, hematuria can also be present in patients with benign urinary tract infections (UTIs) or non-infectious inflammatory conditions (10). Therefore, the urge to find reliable non-invasive alternative diagnostic tools, that accurately discriminate malignant bladder tumors from benign conditions and detect BlCa in its early stages has been one of the main research drivers.

Over the years, epigenetics has unraveled to be extremely relevant for malignant tumors development and progression, being acknowledged as a hallmark of cancer (11). Namely, DNA methylation has been largely studied in BlCa, particularly its application as non-invasive biomarkers (12, 13). Moreover, DNA methylation is the only epigenetic modification that seems to be sufficiently stable to be used as a biomarker in *In Vitro* Diagnostic (IVD) setting (14). Over the last years, our research group has been assessing many disease-specific urinary DNA methylation biomarkers for the diagnosis of BlCa. In 2010, the three-gene panel *GDF15*, *TMEFF2* and *VIM* showed a high sensitivity and specificity of 94% and

100%, respectively (15). Then, in 2017, the promoter methylation of two microRNAs (*miR129-2* and *miR663a*) detected upper and lower tract urothelial carcinoma with a sensitivity of 87.8% and specificity of 82.7% (16). Later, in 2020, the accuracy of the multiplex assessment of promoter methylation levels of *miR663a* and *VIM* was further explored, which discriminated BlCa from patients with urinary tract inflammatory conditions with a sensitivity of 80% and specificity of 75.3% (17). Many other research groups and authors extensively studied the potential of DNA methylation biomarkers, but the ideal accuracy is hardly achieved, and the rate of false positive results is usually high (13, 18).

In an effort to bring together all the relevant work in this area and find the best markers for validation and implementation in a clinical context, our group conducted a meta-analysis including 68 studies of diagnostic test accuracy of DNA methylation markers in urine (Part I). This analysis uncovered the five most promising biomarkers reported in the literature to detect BlCa – *SALL3*, *PENK*, *ZNF154*, *VIM* and *POU4F2*. As mentioned, the lack of validation in independent cohorts has been hampering its clinical implementation. Therefore, the present study aims to validate and test the specificity of those biomarkers in a different set of samples.

MATERIAL AND METHODS

Patients and Urine Samples' Preparation

The study was conducted in voided urines from 84 patients with primary NMIBC consecutively diagnosed with gold standard procedures (histological evaluation after TURBT complemented with cytology) between 2006 and 2021 at the Portuguese Institute of Oncology of Porto (IPO Porto), along with the voided urine of 100 age and gender-matched controls. The control groups were composed of 25 healthy donors (HD), collected at Farmacy Faculty of the University of Porto (FFUP), 25 prostate cancer (PCa) patients, 25 renal cancer (RCa) patients from IPO Porto, and 25 patients with benign urinary inflammatory conditions (IC), diagnosed between 2008 and 2014 at University Hospital of Cordoba (UHC). Detailed patients' and controls clinicopathological and demographic data is displayed in Table 1. In patients, all urines were obtained at time of diagnosis, before any treatment was administrated. This study was approved by the ethics committee of IPO Porto, FFUP and UHC (CES-IPO 18/023 and CES-IPO 019/08, FFUP). Urine samples were processed within four hours upon collection by a 20-minute centrifugation at 4000 rpm and

a double *pellet* wash with phosphate-buffered saline (PBS), and were finally stored at -80 °C.

Table 1. Demographic and clinicopathological features of the patients in the retrospective cohort.

Demographic and Clinicopathological characteristics	NMIBC	Controls			
		HD	PCa	RCa	IC
Patients, *n*	84	25	25	25	25
Mean age, yrs (range)	74.3 (42-99)	74.4 (58-90)	74.3 (56-92)	74.4 (56-92)	72.9 (48-92)
Gender, *n*					
Males	65	19	25	19	19
Females	19	6	n.a.	6	6
Grade, *n*					
PUNLMP	1	n.a.	n.a.	n.a.	n.a.
LG	31	n.a.	n.a.	n.a.	n.a.
HG	41	n.a.	n.a.	n.a.	n.a.
Pathological stage, *n*					
pTis	2	n.a.	0	0	n.a.
pTa	52	n.a.	0	0	n.a.
pT1	30	n.a.	1	9	n.a.
pT2	0	n.a.	11	1	n.a.
pT3	0	n.a.	7	5	n.a.

NMIBC: Non-muscle Invasive Bladder Cancer; HD: Healthy Donors; PCa: Prostate Cancer; RCa: Renal Cancer; IC: Inflammatory Conditions; PUNLMP: Papillary Urothelial Neoplasm of Low Malignant Potential; LG: Low-grade Tumor; HG: High-grade Tumor; yrs: years; n.a.: non applicable

DNA Isolation and Bisulfite Conversion

DNA was extracted from urine sample by the phenol-chloroform isolation method and its concentration was measured by Qubit 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Then, bisulfite modification was carried out using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's protocol. The DNA was eluted in different volumes of bi-distilled water so that the final concentration of modified DNA was always 2 ng/μL.

Quantitative Methylation-Specific PCR (qMSP)

Two multiplex reactions (1) *SALL3*, *POU4F2* and *β-Actin*, and (2) *PENK*, *VIM* and *ZNF154* were performed in triplicates and analyzed by quantitative Methylation-Specific PCR (qMSP) in 96-well plates using the Applied Biosystems 7500 Sequence Detector (Perkin Elmer, Waltham, CA, USA). *β-Actin* (ACTB) was used as a housekeeping gene for prior normalization. Primers and probes sequences were designed with the Methyl Primer

Express v1.0 (Supplementary Table 4). Briefly, each well contained 1.5 μL of modified DNA template, 5 μL of Xpert Fast Probe Master Mix (GRiSP, Porto, Portugal) with ROX, an optimized volume of primer (F+R) and probe at 10 μM for each gene in the panel, and a variable volume of sterile bi-distilled water to make up to a final volume of 10 μL. Moreover, five serial dilutions (dilution factor of 5x) of fully methylated bisulfite modified universal DNA (Human HCT116 DKO Methylated DNA, D5014-2; Zymo) were included in each plate, in duplicates, to generate a standard curve and assure similar run efficiencies among plates. The relative DNA methylation levels for each sample and gene were calculated by the ratio between the targeted gene/ACTB mean quantities multiplied by 1000. The “Standards for Reporting Diagnostic Accuracy Studies” (STARD) guidelines were followed for clearer data reporting (Supplementary Table 5).

Statistical Analysis

The non-parametric tests, namely Mann-Whitney test and Kruskal-Wallis test adjusted for multiple comparisons using a Dunn’s test, were used to assess differences in methylation levels of the targeted genes among two or more groups, respectively, using GraphPad Prism (version 9.0) (GraphPad Software, San Diego, CA, USA). The non-parametric Spearman’s correlation was applied to evaluate the association between the methylation levels of target genes’ promoters and patients’ age at diagnosis.

The receiver operator characteristic (ROC) curves were designed by plotting the true positive rates (sensitivity) in function of the false positive rates (1 - specificity), and the respective areas under the curve (AUC) were estimated. To evaluate the performance of the methylation status of the genes’ promoters as biomarkers, cases were sorted as methylated or non-methylated based on cutoff values attained using Youden's J index (maximum (sensitivity + specificity - 1)), which is a value that combines the highest sensitivity and specificity derived from the ROC curve analysis (19).

Standard diagnostic accuracy estimates as sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy were calculated to appraise the individual performance of the biomarkers in detecting BlCa. Panels with multiple combinations of the different markers were further explored to improve the diagnostic power of the markers. In this regard, a sample was considered positive if at least one of the included markers was positive in compliance with its own pre-established cutoff value.

RESULTS

Samples' description

Although the initial selection included 184 samples, 10 samples were excluded from the analysis due to insufficient DNA, and 1 sample did not show adequate ACTB amplification. Therefore, the final retrospective cohort resulted in a total of 173 individuals, from which urine samples with enough DNA was available; 80, 22, 23 and 25 from BlCa, PCa, RCa and IC patients, respectively, while 23 were obtained from HD individuals. PCa, RCa and IC patients served as specificity controls.

Methylation Analysis and Performance of the Individual Targeted Genes

The promoter regions of all five analyzed genes were significantly more methylated in BlCa urine samples than in healthy donors and patients with other urological malignancies. Nonetheless, methylation levels of healthy donors and prostate and renal cancer patients were similar. Moreover, only *SALL3*, *VIM* and *ZNF154* presented significantly higher methylation levels between BlCa patients and IC patients (p=0.0060, p=0.0259 and p=0.0054, respectively).

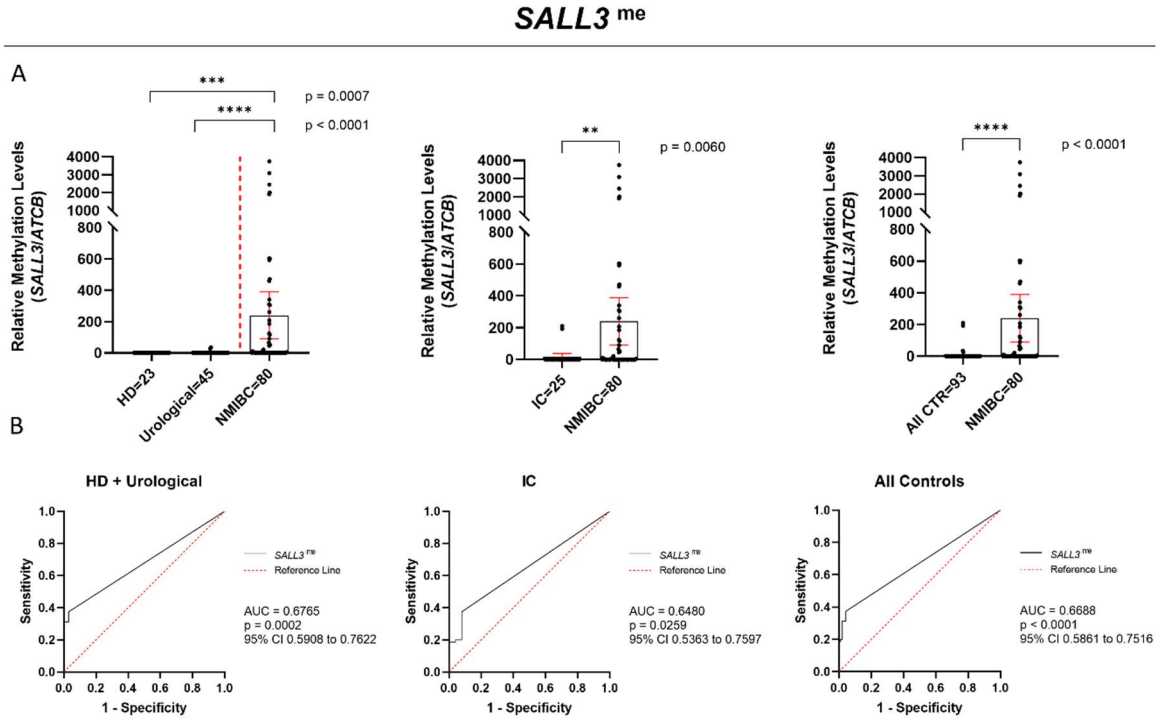

Figure 1. *SALL3's* promoter methylation status in BlCa and control groups. (A) Distribution of *SALL3*^{me} levels in the urine samples of bladder cancer (NMIBC; n=80) patients, healthy donors (HD; n=23), other urological malignancies (Urological; n=45), benign inflammatory conditions (IC; n=25) and all controls combined (All CTR; n=93). (B) Respective receiver operator characteristic (ROC)

curves evaluating the performance of *SALL3*^{me} marker in distinguishing BICa from the control groups. (ACTB: *β-Actin*; AUC: Area Under the Curve; CI: Confidence Interval).

SALL3 promoter methylation levels were significantly higher in BICa patients' urines than in the controls (Figure 1). Urinary *SALL3* methylation levels discriminated BICa from HD and other urological tumors with 37.50% sensitivity and 97.06% specificity. While the sensitivity was maintained for distinguishing between BICa and IC, the specificity declined to 92%. *SALL3* was the most specific BICa marker.

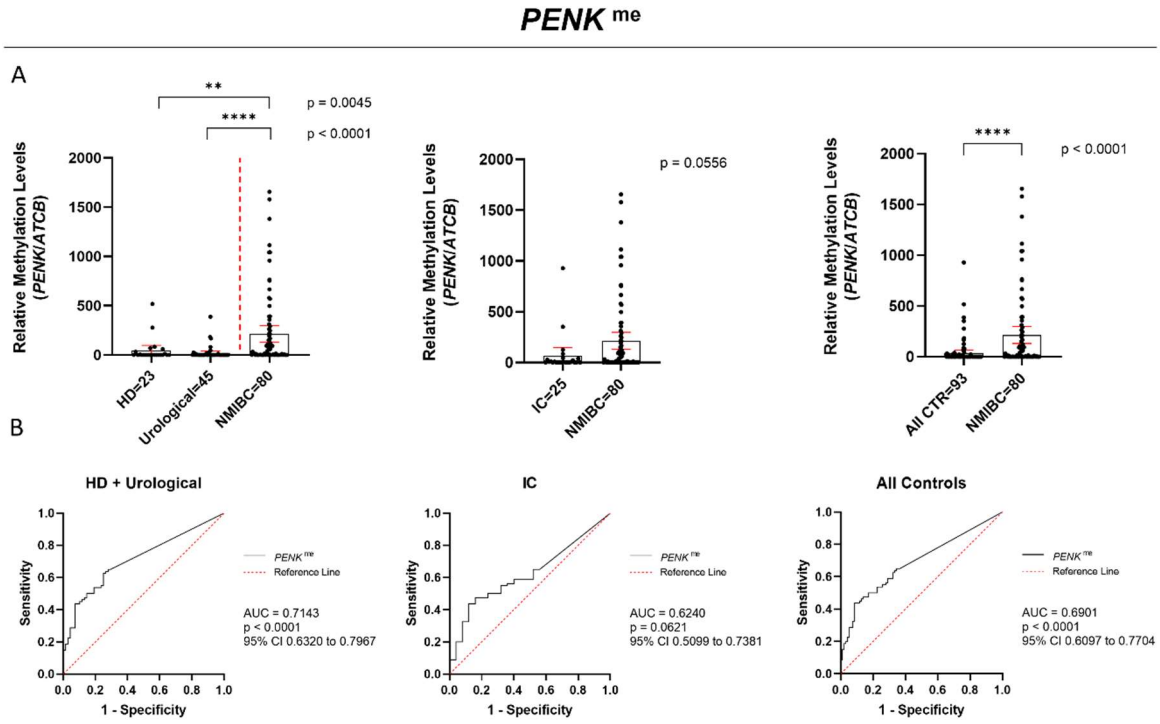

Figure 2. *PENK*'s promoter methylation status in BICa and control groups. (A) Distribution of *PENK*^{me} levels in the urine samples of bladder cancer (NMIBC; n=80) patients, healthy donors (HD; n=23), other urological malignancies (Urological; n=45), benign inflammatory conditions (IC; n=25) and all controls combined (All CTR; n=93). (B) Respective receiver operator characteristic (ROC) curves evaluating the performance of *PENK*^{me} marker in distinguishing BICa from the control groups. (ACTB: *β-Actin*; AUC: Area Under the Curve; CI: Confidence Interval).

PENK promoter methylation levels were significantly different in BICa patients from HD, PCa and RCa patients, but not from IC patients (p=0.556) (Figure 2). Considering HD and other urological cancer types as controls, *PENK* was able to identify BICa with 62.50% sensitivity and 75% specificity, achieving an AUC of 0.7143.

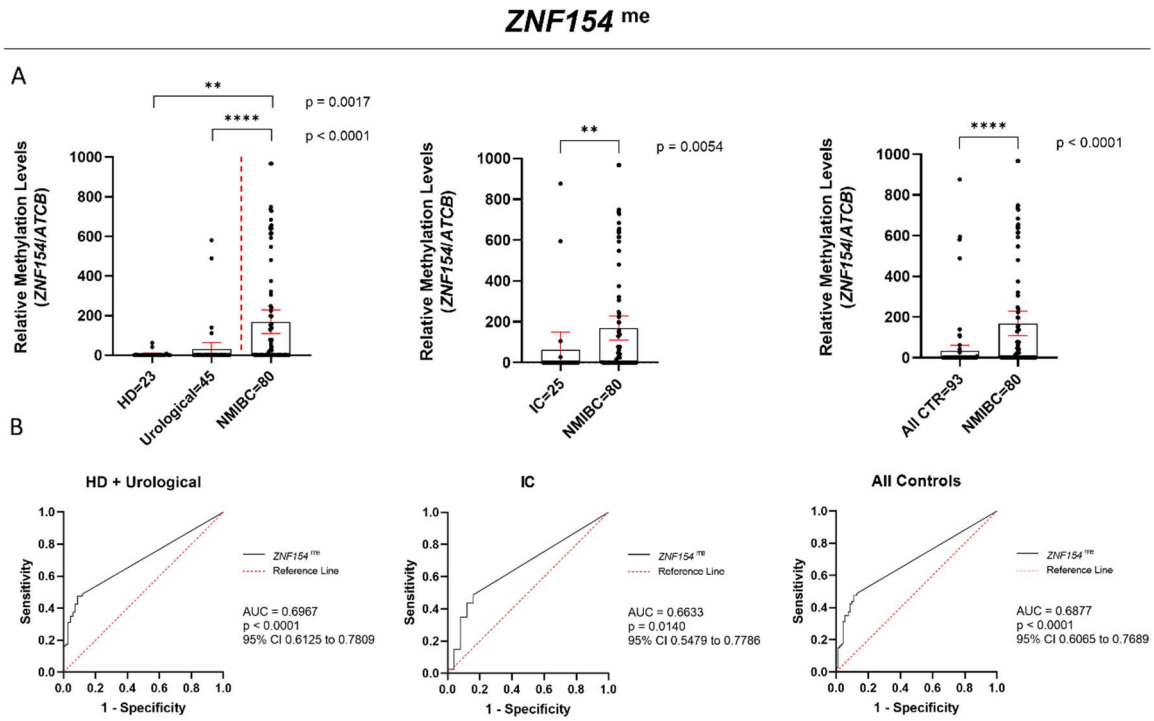

Figure 3. *ZNF154*'s promoter methylation status in BlCa and control groups. (A) Distribution of *ZNF154*^{me} levels in the urine samples of bladder cancer (NMIBC; n=80) patients, healthy donors (HD; n=23), other urological malignancies (Urological; n=45), benign inflammatory conditions (IC; n=25) and all controls combined (All CTR; n=93). (B) Respective receiver operator characteristic (ROC) curves evaluating the performance of *ZNF154*^{me} marker in distinguishing BlCa from the control groups. (ACTB: *β-Actin*; AUC: Area Under the Curve; CI: Confidence Interval).

Concerning *ZNF154,* methylation levels were significantly higher in BlCa urine sediments than all urine sediments of the controls (Figure 3). *ZNF154* displayed the highest AUC (0.6633) in distinguishing BlCa patients from benign inflammatory conditions patients', corresponding to a sensitivity and specificity of 48.75% and 84%, respectively.

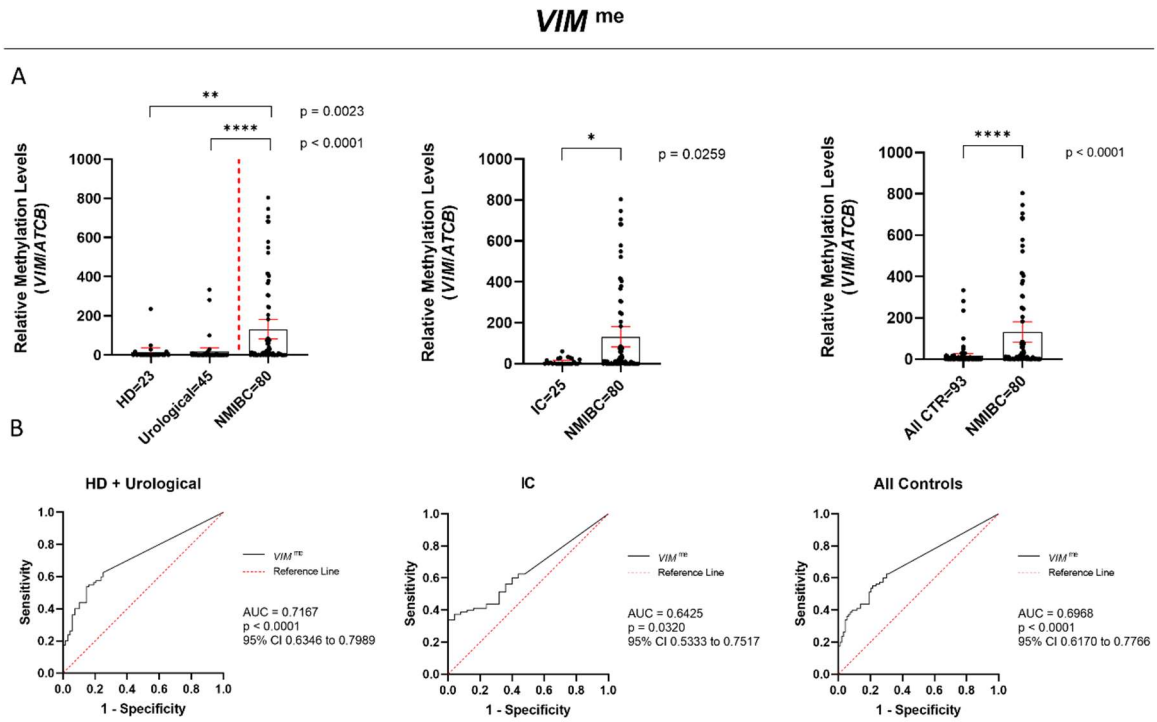

Figure 4. *VIM*'s promoter methylation status in BlCa and control groups. (A) Distribution of *VIM*^{me} levels in the urine samples of bladder cancer (NMIBC; n=80) patients, healthy donors (HD; n=23), other urological malignancies (Urological; n=45), benign inflammatory conditions (IC; n=25) and all controls combined (All CTR; n=93). (B) Respective receiver operator characteristic (ROC) curves evaluating the performance of *VIM*^{me} marker in distinguishing BlCa from the control groups. (ACTB: *β-Actin*; AUC: Area Under the Curve; CI: Confidence Interval).

Likewise, *VIM* promoter methylation levels were also significantly higher in BlCa patients than all other control groups (Figure 4). When using only HD, PCa and RCa patients' urines as controls, *VIM*^{me} detected BlCa with 53.75% sensitivity and 85.29% specificity. *VIM*^{me} differentiated BlCa from inflammatory disease urines with 100% specificity, although with a limited sensitivity of 33.75%.

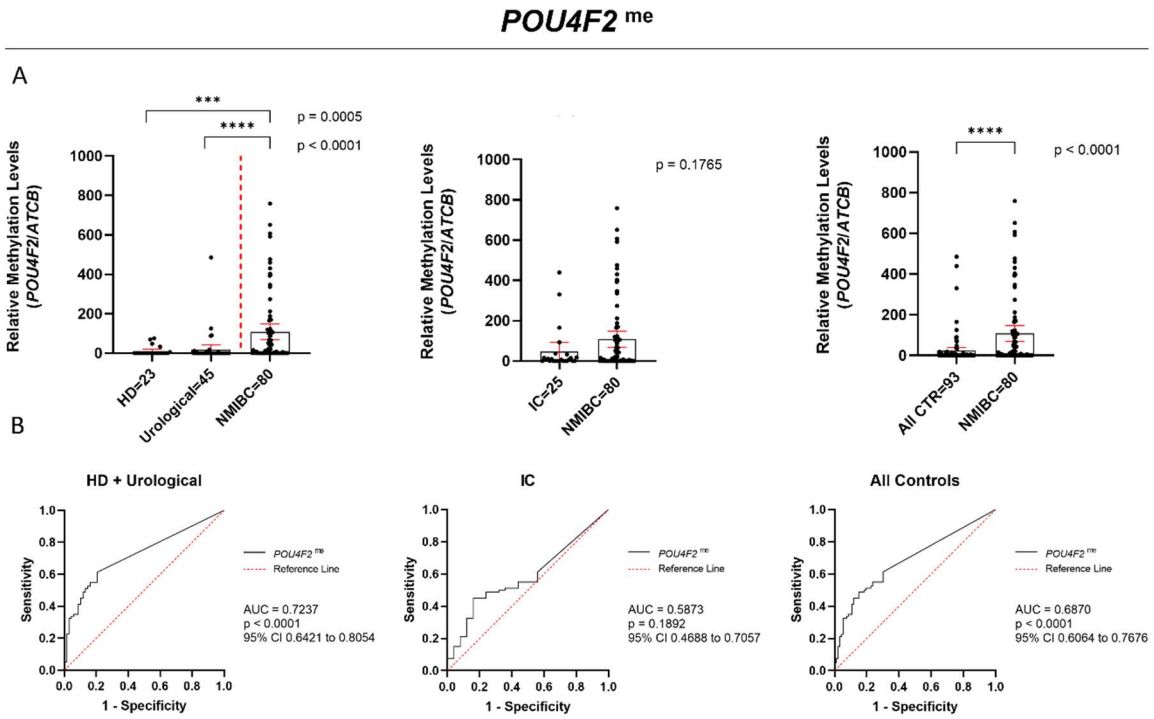

Figure 5. *POU4F2*'s promoter methylation status in BlCa and control groups. (A) Distribution of *POU4F2*^{me} levels in the urine samples of bladder cancer (NMIBC; n=80) patients, healthy donors (HD; n=23), other urological malignancies (Urological; n=45), benign inflammatory conditions (IC; n=25) and all controls combined (All CTR; n=93). (B) Respective receiver operator characteristic (ROC) curves evaluating the performance of *POU4F2*^{me} marker in distinguishing BlCa from the control groups. (ACTB: *β-Actin*; AUC: Area Under the Curve; CI: Confidence Interval).

Lastly, *POU4F2*'s promoter methylation levels in BlCa patients significantly differed from HD and other urological cancer patients, but not from patients with bladder inflammatory conditions (p=0.1765) (Figure 5). Nevertheless, *POU4F2* methylation levels discriminated BlCa from HD, PCa and RCa patients' urines (AUC 0.7237) and a sensitivity and specificity of 61.25% and 79.41%, respectively.

The accuracy values of the five markers are reported in detail in Table 2. As methylation levels of HD, PCa and RCa patients were similar, they were lumped in one control group (HD + Other Urological Cancers). In general, all markers showed similar accuracies and AUCs comparing with the former control group, although lower accuracies were obtained when testing urines from bladder inflammatory conditions.

Table 2. Performance of *SALL3*^{me}, *PENK*^{me}, *ZNF154*^{me}, *VIM*^{me}, and *POU4F2*^{me} in distinguishing bladder cancer from all the tested control groups (healthy donors and patients with other urological malignancies, and from individuals with inflammatory conditions).

Healthy Donors & Other Urological Malignancies											
Gene	**TP**	**TN**	**FP**	**FN**	**Cutoff**	**Se**	**Sp**	**PPV**	**NPV**	**Accuracy**	**AUC**
SALL3	30	66	2	50	2.397	37.50%	97.06%	93.75%	56.90%	64.86%	0.6765
PENK	50	51	17	30	3.860	62.50%	75.00%	74.63%	62.96%	68.24%	0.7143
ZNF154	38	62	6	42	11.62	47.50%	91.18%	86.36%	59.62%	67.57%	0.6967
VIM	43	58	10	37	9.305	53.75%	85.29%	81.13%	61.05%	68.24%	0.7167
POU4F2	49	54	14	31	0.5598	61.25%	79.41%	77.78%	63.53%	69.59%	0.7237
Only Inflammatory Conditions											
Gene	**TP**	**TN**	**FP**	**FN**	**Cutoff**	**Se**	**Sp**	**PPV**	**NPV**	**Accuracy**	**AUC**
SALL3	30	23	2	50	2.397	37.50%	92.00%	93.75%	31.51%	50.48%	0.648
PENK	35	22	3	45	86.56	43.75%	88.00%	92.11%	32.84%	54.29%	0.624
ZNF154	39	21	4	41	1.103	48.75%	84.00%	90.70%	33.87%	57.14%	0.6633
VIM	27	25	0	53	60.22	33.75%	100.00%	100.00%	32.05%	49.52%	0.6425
POU4F2	36	21	4	44	39.87	45.00%	84.00%	90.00%	32.31%	54.29%	0.5873
All Controls											
Gene	**TP**	**TN**	**FP**	**FN**	**Cutoff**	**Se**	**Sp**	**PPV**	**NPV**	**Accuracy**	**AUC**
SALL3	30	89	4	50	2.397	37.50%	95.70%	88.24%	64.03%	68.79%	0.6688
PENK	35	85	8	45	86.56	43.75%	91.40%	81.40%	65.38%	69.36%	0.6901
ZNF154	38	83	10	42	11.62	47.50%	89.25%	79.17%	66.40%	69.94%	0.6877
VIM	44	73	20	36	8.239	55.00%	78.49%	68.75%	66.97%	67.63%	0.6968
POU4F2	39	79	14	41	18.67	48.75%	84.95%	73.58%	65.83%	68.21%	0.687

TP: True positive; TN: True Negative; FP: False Positive; FN: False Negative; Se: Sensitivity; Sp: Specificity; PPV: Positive Predictive Value; NPV: Negative Predictive Value; AUC: Area Under the Curve

Methylation Analysis and Performance of Panels of the Targeted Genes' Promoters in Urines of BlCa Patients and Patients with Inflammatory Conditions of the Bladder

After the individual analysis of each target gene, all combinations of markers that were differently methylated in IC control group were assessed. The cutoff established for each gene was set accounting BlCa and IC patients' ROC Curve methylation analysis. In the panel combining the three assessed markers, several cutoffs were investigated (if at least 1, 2 or more, or the 3 markers were considered as positive).

The panels' performance in discriminating malignant from benign/inflammatory bladder disease were slightly higher than the performance of each marker alone (Table 3). The most sensitive panel was *SALL3* / *ZNF154* (55%), while the most specific was *SALL3* / *VIM*

(92%). The specificity of the three-gene panel increased with higher cutoff levels, reaching 100% specificity but lower sensitivity than *VIM*^{me} alone, also demonstrating maximal specificity.

Table 3. Performance of various panels combining *SALL3*^{me}, *ZNF154*^{me} and/or *VIM*^{me} in distinguishing bladder cancer from controls with inflammatory conditions.

Gene Panels								
Panel	**Cutoff**	**TP**	**TN**	**FP**	**FN**	**Se**	**Sp**	**AUC**
SALL3 / ZNF154	>1	44	21	4	36	55.0%	84.0%	0.7214
SALL3 / VIM	>1	38	23	2	42	47.5%	92.0%	0.7219
ZNF154 / VIM	>1	41	21	4	39	51.3%	84.0%	0.7221
SALL3 / ZNF154 / VIM	>1	45	21	4	35	56.3%	84.0%	0.7332
SALL3 / ZNF154 / VIM	>2	33	23	2	47	41.3%	92.0%	0.7332
SALL3 / ZNF154 / VIM	>3	17	25	0	63	21.3%	100.0%	0.7332

TP: True positive; TN: True Negative; FP: False Positive; FN: False Negative; Se: Sensitivity; Sp: Specificity; AUC: Area Under the Curve

Associations between genes' Methylation levels and Bladder Cancer Clinicopathological Features

Globally, significantly positive weak or moderate association was found for all the tested genes between patients' age and the promoter methylation levels (Supplementary Table 6). Contrarily, only *SALL3* methylation levels were significantly different between male and female gender (p=0.0230), being higher in male patients.

Regarding the differences in methylation levels across distinct pathological stages, except for *SALL3*, significantly higher methylation levels was displayed by pTa and pT1 tumors (p=0.1129) (Figure 6). Conversely, no differences were observed among pTis and other stages, although only two cases were staged as pTis.

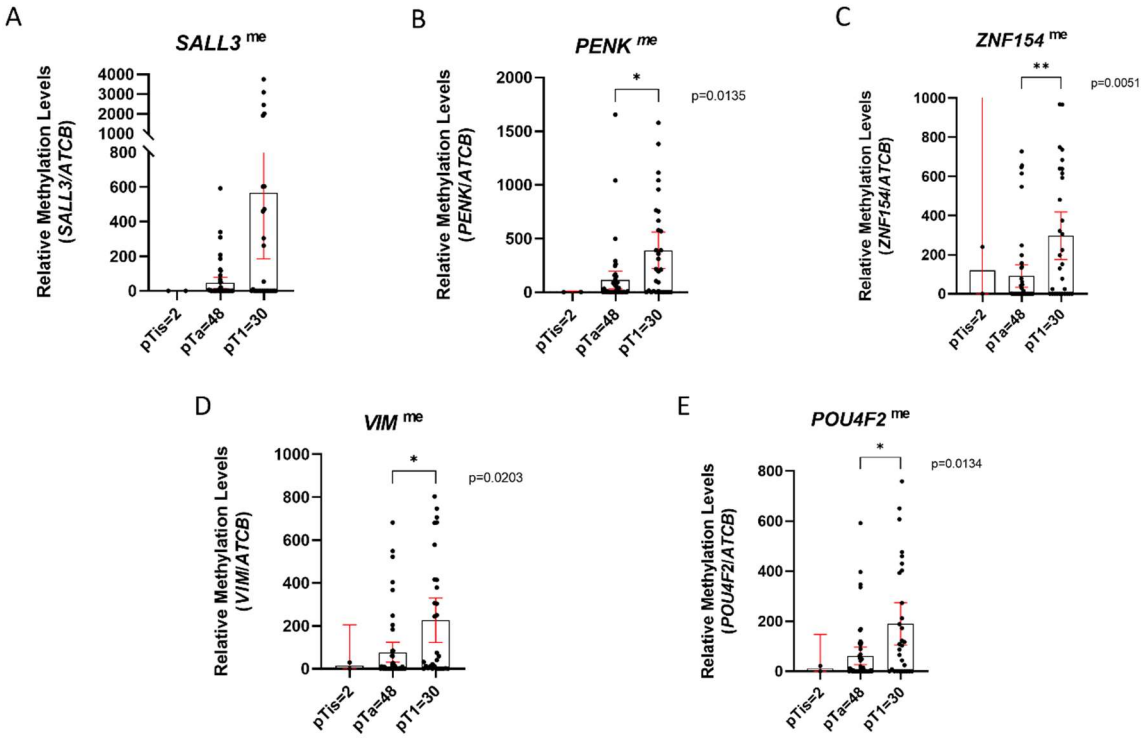

Figure 6. Distribution of *SALL3*^{me} (A), *PENK*^{me} (B), *ZNF154*^{me} (C), *VIM*^{me} (D), and *POU4F2*^{me} (E) levels across the different pathological stages of non-muscle invasive bladder cancer – pTis (n=2), pTa (n=48) and pT1 (n=30).

Nevertheless, all markers presented higher sensitivity in identifying pT1 tumors in comparison to pTa. On the other hand, sensitivities for detecting pTa-staged BlCa were between 27-38% and considerably improved to 42-46% when matched in panels (Table 4).

Table 4. Sensitivity of *SALL3*^{me}, *PENK*^{me}, *ZNF154*^{me}, *VIM*^{me}, and *POU4F2*^{me} and assessed panels for detecting non-muscle invasive bladder cancer in different pathological stages.

Stage	Gene	Cutoff	Sensitivity % (n positive / n total)
pTis	*SALL3*	2.397	0% (0/2)
	PENK	86.56	0% (0/2)
	ZNF154	1.103	50.00% (1/2)
	VIM	60.22	0% (0/2)
	POU4F2	39.87	0% (0/2)
	SALL3 / ZNF154	>1	50.00% (1/2)
	SALL3 / VIM	>1	0% (0/2)
	ZNF154 / VIM	>1	50.00% (1/2)
pTa	*SALL3*	2.397	33.33% (16/48)
	PENK	86.56	33.33% (16/48)
	ZNF154	1.103	37.50% (18/48)
	VIM	60.22	27.08% (13/48)
	POU4F2	39.87	35.42% (17/48)
	SALL3 / ZNF154	>1	45.83% (22/48)
	SALL3 / VIM	>1	43.75% (21/48)
	ZNF154 / VIM	>1	41.67% (20/48)
pT1	*SALL3*	2.397	46.67% (14/30)
	PENK	86.56	63.33% (19/30)
	ZNF154	1.103	66.67% (20/30)
	VIM	60.22	46.67% (14/30)
	POU4F2	39.87	63.33% (19/30)
	SALL3 / ZNF154	>1	70.00% (21/30)
	SALL3 / VIM	>1	56.67% (17/30)
	ZNF154 / VIM	>1	66.67% (20/30)

Regarding BlCa grading, no apparent differences were noticed on genes' promoters' methylation levels for all the tested genes (Figure 7).

Moreover, no single gene or panel was able to identify PUNLMP lesions using the pre-established cutoffs obtained when using the IC control group.

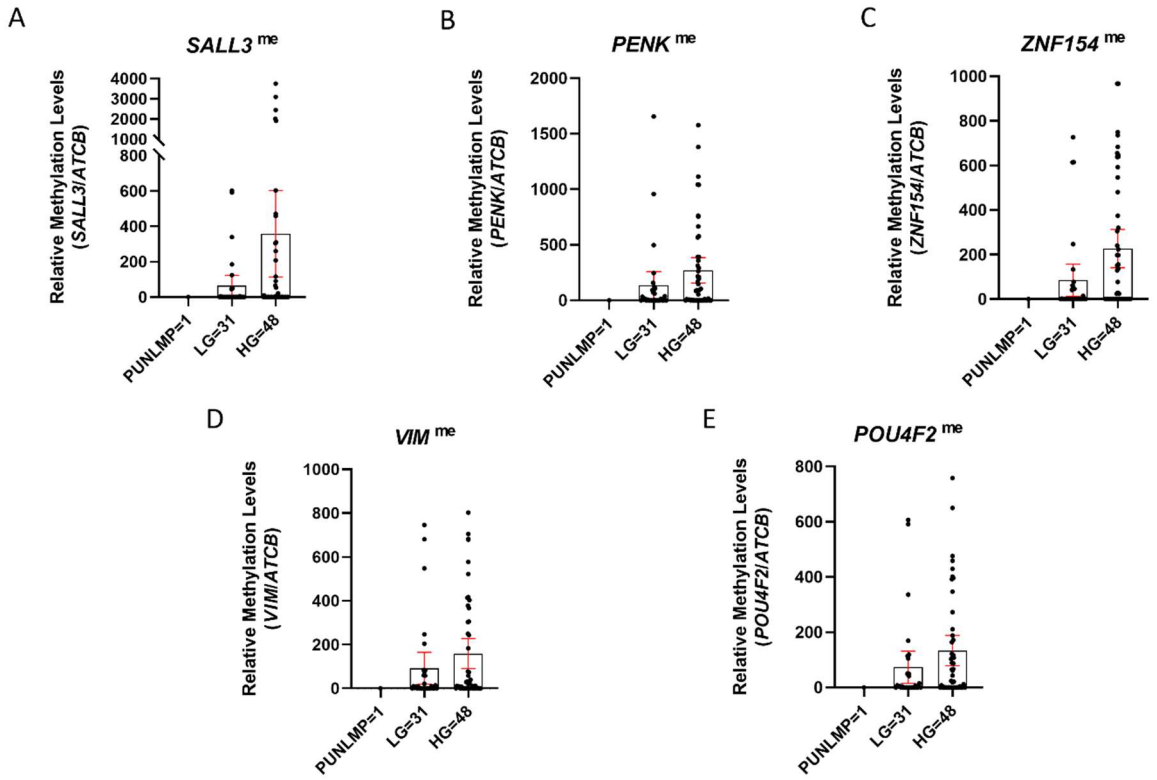

Figure 7. Distribution of *SALL3*^{me} (A), *PENK*^{me} (B), *ZNF154*^{me} (C), *VIM*^{me} (D), and *POU4F2*^{me} (E) levels across the different grading groups of bladder cancer – PUNLMP (n=1), LG (n=31) and HG (n=48). (PUNLMP: Papillary Urothelial Neoplasm of Low Malignant Potential; LG: Low-Grade; HG: High-Grade).

Nonetheless, excepting *SALL3* and *VIM* alone*,* most markers identified at least 50% of HG tumors. Particularly, when combining *SALL3* and *ZNF154* in the same panel, sensitivity reached 64.58%. For LG tumors detection, sensitivities of individual markers ranged 29-38%, while improved to 42% when genes were aggregated in panels (Table 5).

Table 5. Sensitivity of *SALL3*^{me}, *PENK*^{me}, *ZNF154*^{me}, *VIM*^{me}, and *POU4F2*^{me} and assessed panels for detecting different grading groups of non-muscle invasive bladder cancer.

Stage	Gene	Cutoff	Sensitivity % (n positive / n total)
PUNLMP	*SALL3*	2.397	0% (0/1)
	PENK	86.56	0% (0/1)
	ZNF154	1.103	0% (0/1)
	VIM	60.22	0% (0/1)
	POU4F2	39.87	0% (0/1)
	SALL3 / ZNF154	>1	0% (0/1)
	SALL3 / VIM	>1	0% (0/1)
	ZNF154 / VIM	>1	0% (0/1)
LG	*SALL3*	2.397	32.26% (10/31)
	PENK	86.56	29.03% (9/31)
	ZNF154	1.103	38.71% (12/31)
	VIM	60.22	29.03% (9/31)
	POU4F2	39.87	35.48% (11/31)
	SALL3 / ZNF154	>1	41.94% (13/31)
	SALL3 / VIM	>1	41.94% (13/31)
	ZNF154 / VIM	>1	41.94% (13/31)
HG	*SALL3*	2.397	41.67% (20/48)
	PENK	86.56	54.17% (22/48)
	ZNF154	1.103	56.25% (27/48)
	VIM	60.22	37.50% (18/48)
	POU4F2	39.87	52.08% (25/48)
	SALL3 / ZNF154	>1	64.58% (31/48)
	SALL3 / VIM	>1	52.08% (25/48)
	ZNF154 / VIM	>1	58.33% (28/48)

PUNLMP: Papillary Urothelial Neoplasm of Low Malignant Potential; LG: Low-Grade; HG: High-Grade

DISCUSSION

In oncological care, a primary challenge lies in promoting early detection to facilitate more effective disease management (20). Specifically regarding BlCa, even following diagnosis and successful treatment, patients undergo continuous surveillance due to the elevated risk of relapse (6). Furthermore, a significant research focus revolves around identifying dependable non-invasive alternatives for BlCa diagnosis. This emphasis stems from the invasive and expensive nature of current diagnostic techniques, posing a substantial health concern (3). Although great efforts have been made over the years to discover new biomarkers for the detection of BlCa, yet none have proven to be capable of replacing the current invasive detection methods, largely due to the lack of validation in different clinical scenarios (5). Thus, this study aimed to take the first step of a robust validation of the five most promising BlCa detection methylation markers – *SALL3*, *PENK*,

ZNF154, *VIM* and *POU4F2* attained in a preliminary meta-analysis carried out by our research team.

Overall, all genes obtained relatively similar performances, with AUCs between 0.67-0.70 and accuracies between 67-69%. As noted in the meta-analysis conducted by us, the lack of published studies testing the specificity of these markers for BlCa is a huge pitfall. Healthy individuals and patients suffering from inflammatory diseases are expected to have significantly less molecular alterations than cancer patients, while other malignancies that release cells for urine may also contain alterations. Hence, to accurately demonstrate that these DNA methylation-based markers were bladder cancer specific, herein voided urines encompassing all those conditions were tested.

All markers showed different methylation levels in the exfoliated cells of BlCa patients' urine samples in comparison to HD and PCa or RCa patients' urine samples. Sensitivity values ranged from 37.50% to 61.25%, specificity ranged between 75-97.06%, and AUCs were around 0.70. As these two groups presented similar methylation levels, they were lumped together for further analysis. In clinical practice, a diagnostic biomarker would prove most beneficial when a patient with suspicious signs or symptoms of BlCa can be accurately forward to cystoscopy avoiding unnecessary procedures in case of other pathologies (21). Hence, studies that exclusively involve healthy individuals as the control group fail to accurately represent the "at-risk" population for whom the biomarker would be intended. Indeed, *PENK* and *POU4F2* methylation levels did not significantly differed between BlCa patients' urine samples and IC patients. Conversely, *SALL3*, *ZNF154* and *VIM's* methylation levels in BlCa patients' urinary sediments were significantly higher than in patients with inflammatory diseases. However, as anticipated, when considering only individuals affected by inflammatory conditions of the urinary tract as controls, the accuracies and AUCs of all markers for detecting BlCa noticeably decrease. This underscores the paramount importance of including such patients in biomarker studies, making it a mandatory requirement for validation studies. Nonetheless, it is worth noting that *VIM* methylation levels achieved an impressive specificity of 100%.

Following the selection of optimal cutoff values for each marker (with consideration given to only the IC control group), the combined performance of *SALL3*, *ZNF154*, and *VIM* in panels was evaluated. Indeed, panels showed considerable increased performance than each marker alone. For instance, the panel combining *SALL3* and *VIM* was the most specific panel (92%), while being the less sensitive (47.5%) in detecting BlCa. Nevertheless, the sensitivity of the panel surpasses that of both individual markers by more than 10%. In comparison, *ZNF154* and *SALL3* attained a specificity of 84%, mirroring the performance

of the panel comprising *ZNF154* / *VIM*, while exhibiting the highest sensitivity at approximately 55%.

Importantly, the combination of any three genes in a panel showed lower performance. In fact, the inclusion of a new marker in the panel would escalate associated costs and hinder reproducibility. It should be strongly advocated only if accompanied by a significant enhancement in performance, which, regrettably, is not observed in this case. When adopting a cutoff of at least one gene being methylated, the specificity is at its lowest (84%) with only a marginal increase of 1.3% in sensitivity compared to the *SALL3* and *ZNF154* panel. Conversely, when the criterion of at least two genes needed to be methylated to consider a sample positive, the specificity increased to 92%, but the sensitivity was lower than of the *SALL3* and *VIM* panel, which is equally specific. Lastly, the specificity in detecting BICa achieves 100% if all genes were simultaneously methylated, but at the cost of sensitivity that drops to 21.3%, much inferior to the sensitivity displayed by *VIM* methylation levels alone (33.75%), that was also is 100% specific.

It is important to highlight that the choice of a specific marker or panel may vary depending on the defined objective. In various clinical contexts, there might be a priority for high sensitivity over high specificity, or vice versa. For example, in a diagnostic setting, high sensitivity holds greater significance because the primary goal is to detect and avoid missing any potential malignant cases. Conversely, in the context of surveillance for potential recurrences, where an "at-risk" population is under observation, a very high specificity will be prioritized over sensitivity (22). Hence, all the individual markers or panels assessed in this study may address distinct clinical questions and prove valuable in various scenarios.

Furthermore, one of the biggest pitfalls of early detection is that most of the markers show low sensitivity in earlier and milder phenotypes of the disease, such as low grades (23). Indeed, urinary cytology is also burdened by this drawback, as it exhibits reduced sensitivity for low-grade tumors and early stages (8). Herein, regarding staging, with the exception of *SALL3*, the methylation levels of the examined genes were higher in pT1 compared to pTa. It's noteworthy that for pTa tumors, no marker achieved a sensitivity of 40%, whereas more than 60% of the tumors invading the lamina propria (pT1) were detected by most of the markers. Contrarily, no methylation differences were found among LG and HG tumors. Notwithstanding, there were subtle variations in the markers sensitivity when detecting LG or HG tumors. For example, *PENK*, *ZNF154*, and *POU4F2* exhibit 20% higher sensitivity in detecting HG tumors compared to LG tumors. However, the lack of significant differences suggests that the markers could detect LG tumors as reliably as HG

tumors. This is noteworthy, as it often represents a limitation for the majority of markers. Additionally, while the overall markers' sensitivity were not very impressive, it is crucial to bear in mind that this study exclusively involved Non-Muscle Invasive Bladder Cancer (NMIBC) patients.

Nevertheless, the small number of individuals with different non-BlCa conditions may represent a limitation. Moreover, the inclusion of solely NMIBC may have affected the sensitivity of the markers. However, it is crucial to remember that the most essential non-invasive markers are precisely those for detecting the early stages of BlCa, while there are numerous studies with MIBC lesions incorporated into their cohorts, significantly enhancing sensitivity due to their typically elevated methylation levels compared to NMIBC lesions. Therefore, if a study's objective is to identify reliable biomarkers for the early detection of BlCa, it is of utmost importance to exclusively test those markers in NMIBC patients' urines.

The study employed a retrospective case-control design, including 80 urine samples from NMIBC patients at the time of diagnosis. The next step entails enlarging the series of urine samples and conducting the analysis in a longitudinal series with multiple follow-up time points. This will involve comparing the molecular results with conventional cytology, which remains the standard of care.

CONCLUSION

In this study, we conducted the validation of five methylation biomarkers identified through a meta-analysis, demonstrating accuracies approaching 70% in specifically detecting BlCa. Among these, three genes proved high accuracy in distinguishing bladder malignancy from benign diseases in patients exhibiting suspicious symptoms – an analysis notably absent in previous studies, yet crucial in the realm of non-invasive early BlCa detection. Combinations of these markers could prove beneficial in various clinical contexts and generally exhibited superior performance compared to individual use. Further, larger-scale studies are now imperative to reliably validate the accuracy of these markers and pave the way for their application in the clinical management of BlCa.

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RESULTS SUMMARY

RESULTS SUMMARY

Part I – Diagnostic test accuracy of urinary DNA methylation-based biomarkers for detection of primary and recurrent bladder cancer: a systematic review and meta-analysis

- The most promising urinary DNA methylation-based biomarkers for the detection of primary and recurrent BlCa retrieved by a systematic review and meta-analysis of the published literature, having DOR as a comparative measure, are *SALL3*, *PENK*, *ZNF154*, *VIM* and *POU4F2*;
- For detecting BlCa, at initial diagnosis or relapsed disease, the diagnostic accuracy measures obtained for the markers were the following:
  - *SALL3* achieved a pooled sensitivity and specificity of 61% and 97%, respectively;
  - *PENK* presented a pooled sensitivity of 77% and specificity of 93%;
  - *ZNF154* demonstrated a pooled sensitivity of 87% and specificity 99%;
  - *VIM* revealed a pooled sensitivity and specificity of 82% and 90%, respectively;
  - *POU4F2* obtained a pooled sensitivity of 81% and specificity of 89%.
- Urinary cytology disclosed a pooled sensitivity of 55% and a specificity of 92%.

Part II – Aberrant DNA methylation of *SALL3*, *ZNF154* and *VIM* in urine samples accurately distinguish Bladder Cancer from Benign Conditions of the Urinary Tract

- In the validation study, all markers proved to be specific of bladder disease since all their promoters showed significantly different methylation levels between urines derived from patients with BlCa and urine samples from HD and patients with PCa and RCa;
- Nevertheless, *SALL3*, *ZNF514* and *VIM* were the only markers to show significantly different methylation levels between patients with bladder cancer and patients with benign inflammatory conditions of the bladder;
- In a cohort with 80 NMIBC urine samples and 25 urine samples from patients with IC:
  - *SALL3* obtained 37.5% sensitivity and 92% specificity;
  - *ZNF154* revealed a sensitivity of 48.75% and specificity of 84%;
  - *VIM* achieved a sensitivity and specificity of 33.75% and 100%, respectively.
- Panels combining *SALL3*, *ZNF514* or *VIM* achieved sensitivities and specificities ranging 47.5-55% and 84-92%, respectively, for detecting NMIBC in a cohort containing only patients with inflammatory conditions of the urinary tract as controls.

GENERAL DISCUSSION

GENERAL DISCUSSION

Bladder neoplasms are the 4th most common malignancy diagnosed in European men and the 7th cause of cancer-related death. Worldwide, BlCa is still one of the leading most incident cancer types and causes of cancer mortality (1). Current gold-standard diagnosis is based on cystoscopy, associated with the cytologic appraisal of exfoliated cells in urine (2). However, numerous problems are linked to the diagnosis methods of BlCa, as these procedures are usually only offered to patients who already present suspicious symptoms, such as hematuria. Nevertheless, only a small fraction of individuals who experience hematuria are diagnosed with BlCa, since benign conditions as inflammation or UTIs may also cause blood in the urine, so these patients undergo medical procedures unnecessarily (3, 4). Moreover, as approximately 50% of HG BlCa are expected to recur up to 10 years, cystoscopy and cytology are routinely performed following a surveillance scheme based on patient-specific clinicopathological criteria (2, 5). Yet, as patients need to be monitored through several years or for the rest of their lives, this entails a hefty financial load on healthcare systems (6). Additionally, two other big drawbacks of the gold-standard diagnostic methods currently used are their adverse events and limited accuracy. For instance, cystoscopy not only can miss-detect initial stages of BlCa, such as CIS, which is a flat small lesion yet highly probable to evolve into an invasive disease, but is also rather invasive causing patients discomfort and comorbidities (5, 7). Likewise, even though urinary cytology does not cause adverse effects since it is non-invasive, its sensitivity for low-grade tumors is very limited, not surpassing 30% (8). Therefore, the urge to discover reliable non-invasive urine-based biomarkers for the early detection and effective management of BlCa has been growing among researchers and clinicians.

In this regard, DNA methylation is the most common epigenetic alteration found deregulated in BlCa and its extent has already been associated with different clinicopathological features of the disease (9, 10). Indeed, DNA hypo- and hypermethylation are observed in early stages of BlCa, suggesting the great potential as biomarkers of early diagnosis (10).

Thus, in **Part I** of this Dissertation, through a meta-analysis, we deeply investigated the accuracy of all urinary DNA methylation-based biomarkers used to detect primary or relapsed BlCa published in the literature. After analysis and selection of the most promising markers, in **Part II** we focused on the preliminary validation of the markers' performance previously obtained by theoretical methodologies.

When collecting data for the meta-analysis, numerous difficulties emerged due to the lack of detail and homogeneity in data reporting among studies. This consists of a major

limitation for subgroup analysis and to truly draw accurate conclusions. For instance, subgroup analysis based on BlCa gradings was limited by the fact that few studies included this key information. Moreover, while some studies used the WHO 1973 (G1, G2, G3) classification system, some others used the WHO 2004/2022 system (PUNLMP, LG, HG). As the translation between these two systems is not straightforward, we could not lump them, so the classification reported in most studies was prioritized. Additionally, because of this limitation, we were not able to carry out any further subgroup analysis as initially intended. Also, we were unable to explore potential differences in the markers' performance in distinguishing stages of BlCa or whether if intravesical therapy influenced the accuracy of the target markers. Furthermore, our findings led us to the conclusion that many studies primarily focus on the initial diagnosis. Biomarkers are rarely reevaluated in follow-up studies, limiting our ability to draw meaningful conclusions for the surveillance setting. This may be attributed to the increased difficulty in obtaining follow-up samples at specific timepoints. Even when available, these samples are typically utilized for the clinical validation of already well-established and reliable biomarkers, rather during the discovery phase. Nevertheless, this is a big hurdle for the development and implementation of biomarkers for disease monitoring. Furthermore, around one-third of the included studies did not report True Positive, True Negative, False Positive, and False Negative values, critical for conducting a meta-analysis. To tackle this, we suggested the use of guidelines to report data, such as the STARD guidelines, which could help to minimize the confusion variables among studies and missing data (11). Therefore, we applied this suggestion later in our validation study in Part II.

One of the biggest limitations we encountered in discovery and analytical validation studies of diagnostic biomarkers was the types of controls used by most of them. Indeed, in an initial discovery phase and posterior analytical validation, most studies are retrospective case-control studies that use healthy donors (HD) as controls, essential to assess the specificity of the biomarker to the target population and also its sensitivity in the disease detection. However, these studies typically feature small sample sizes and may introduce potential confounding factors if the further study population differs (12). For instance, diagnostic biomarkers would only be offered to asymptomatic healthy individuals in a screening setting. Particularly for BlCa, as no screening strategy is implemented, a biomarker would be the most informative and useful for a population “at risk”, which present symptoms of the urinary tract, as hematuria. However, as mentioned before, hematuria may be caused by numerous pathologies, so including patients with benign conditions or other neoplasms of the urinary tract as controls is essential to truly determine the diagnostic accuracy of a marker in a more defined clinical context. In fact, that would be the main

objective of the next phase of biomarkers development, the clinical validation. In the clinical validation phase, well-designed blinded prospective studies and clinical trials should be carried out to robustly evaluate the usefulness of the biomarker (or developed test) in clinics (13). Nevertheless, very few studies include this particular setting. Indeed, if a biomarker is only tested in HD controls, there is a high probability that its accuracy will significantly decrease when applied in a population “at risk”. Therefore, it is of utmost importance to take into consideration the future practical application of the biomarker and include in the control group a sample set of symptomatic individuals without BlCa in pre-clinical studies. In this regard, in Part II of the Dissertation we were able to include a small sample set of such controls.

In the systematic review and meta-analysis conducted in Part I, we undertook the discovery phase of the most promising urinary DNA methylation biomarkers for the detection of BlCa, either at diagnosis or follow-up. While we were unable to investigate the biomarkers used for monitoring due to the scarcity of studies in this context, the top five markers used for both settings were selected for further analytical validation. The diagnostic odds ratio (DOR), a single diagnostic measure, not dependent of prevalence, which simultaneously considers sensitivity and specificity data was used to sort these markers (14, 15). DOR values can vary from 0 to infinity, and usually the higher the DOR, the higher is the sensitivity and specificity of the marker. However, we cannot solely assess the DOR independently, as two biomarkers may share the same DOR value but exhibit markedly different sensitivities and specificities, and all these parameters should be considered. Nevertheless, DOR is commonly employed as a comprehensive measure in meta-analyses of diagnostic test accuracy studies. This approach enables a comparison of the diagnostic efficacy of multiple markers (14).

The marker with higher DOR in the meta-analysis was *SALL3* (55.87), which was probably achieved with the great influence of its high pooled specificity of 97%, as the sensitivity was not as high (61%). *SALL3*, however, was only evaluated in three studies, so little evidence was available. In the analytical validation of this marker, we proved that it is in fact highly specific for BlCa, as it could distinguish BlCa patients from patients with IC with 92% specificity and 37.5% sensitivity. Then, *PENK* obtained the second highest DOR by meta-analysis (47,90), also most likely due to its elevated pooled specificity (93%). However, distinct from *SALL3*, the specificities achieved for *PENK* in Part II were not as remarkable as expected. In fact, *PENK* did not show significantly different methylation levels between urine samples from patients with BlCa and patients with IC, hence *PENK* would most likely not be very informative in clinical practice. The other three selected genes in the meta-analysis were *ZNF154*, *VIM* and *POU4F2*, with respective DORs of 45.07, 44.81 and

34.89. Contrarily to the two previously mentioned genes, these markers displayed more balanced sensitivities and specificities. In the same line, *POU4F2* methylation levels did not differ between BlCa urine samples and inflammatory conditions. Given the limited size of the used sample set for patients with inflammation of the urinary tract (n=25), it may pose a constraint in obtaining statistically significant results on this topic. Regarding *ZNF154* and *VIM*, the results achieved in our validation study followed the expected specificities obtained by meta-analysis, yet with lower sensitivities. Outstandingly, *VIM* detected BlCa with 33.75% sensitivity and 100% specificity, accounting only inflammatory controls.

Even though all markers attained lower sensitivities than expected compared to the meta-analysis results, it should be noted that only NMIBC cases were used in our validation study, contrarily to most of the studies included in the meta-analysis. Currently, approximately 70% of bladder tumors are diagnosed as NMIBC, while nearly 30% are identified as MIBC (2). Hence, diagnosis studies typically align with these statistics in their cohorts and encompass a limited sample set of these patients. However, if we aim to find and validate a marker for early-detection of BlCa, it is fundamental to assess its accuracy in detecting particularly NMIBC. Unfortunately, this aspect could not be statistically examined in the meta-analysis due to the absence of data, as previously mentioned. Consequently, we lacked information on how these markers would perform in detecting this specific group of patients. Nonetheless, we chose to assess the diagnostic efficacy of the selected markers exclusively in NMIBC patients' urine samples. Moreover, this may well be another explanation for the lack of differences of *PENK* and *POU4F2* methylation levels attained in the urine samples from patients with BlCa and patients with IC. Although most of the studies that analyzed *PENK* or *POU4F2* also included as controls patients with other bladder disorders, all of them also included MIBC cases. Indeed, the inclusion of this patient group may introduce bias into the interpretation of the discriminatory power of these markers.

Moreover, the performances of panels combining *SALL3*, *ZNF154* and *VIM* were further assessed. In general, the combination of two genes improved performance, while the combination of the three genes in a panel was not beneficial. Indeed, diagnostic panel's construction must be strongly reasoned as it may difficult further implementation, due to reproducibility and cost-effectiveness issues. Therefore, the panels combining *SALL3* / *ZNF154* and *SALL3* / *VIM* showed the best performance, with the first being most sensitive and the second most specific. As sensitivity or specificity may be prioritized over the other in different clinical contexts, both panels could be advantageous. For instance, in a surveillance setting, a highly specific marker or panel would be the most informative and, thus, useful rather than a marker with high sensitivity but lower specificity (16). Therefore,

it is very important to define the clinical purpose before selecting a marker. In our preliminary validation study, as the obtained specificity was rather impressive, they may be the most helpful for treated BlCa patients monitorization, which would be interesting to further investigate.

Furthermore, considering the results obtained in Part II, *SALL3*, *ZNF154* and *VIM* methylation levels exhibited very high specificities, quite comparable to urinary cytology performance. Although the results of cytology for BlCa patients were not appraised in Part II, in our meta-analysis urine cytology demonstrated a pooled sensitivity of 55% and specificity of 92%. Particularly in a subgroup analysis, cytology attained a sensitivity of 30% for detecting LG BlCa and 66% for HG BlCa. Nevertheless, we should keep in mind that the sensitivity of the markers in Part II was assessed exclusively for NMIBC, whereas the studies included in the meta-analysis to calculate cytology's performance, and specifically the sensitivity for HG tumors, also included MIBC. As previously reported and confirmed in our study, there are differences in methylation levels across pathological stages of BlCa, so the inclusion of MIBC cases may lead to a biased comparison of these results (9). Hence, considering only LG tumors, *SALL3* and *VIM* achieved similar sensitivity to cytology, while *ZNF154* and all two-gene panels demonstrated 9% and 12% increased sensitivity, respectively.

Curiously, the biological function of these genes is very little or absent. Although alterations in these genes' functions were already reported in BlCa and other cancer models, excepting for *VIM,* data is missing regarding the implication of each promotor's hypermethylation in BlCa development (17-19). The impact of *VIM* epigenetic deregulation was already investigated by our research group. Indeed, we were able to demonstrate that VIM overexpression associated with a more aggressive phenotype including tumor cells invasive capability, being a key player in epithelial-mesenchymal transition (EMT) (20). Moreover, we were able to link *VIM's* expression switch from normal to malignant cells, by promoter methylation modulation, further supporting the effect of *VIM* epigenetic deregulation in BlCa neoplastic transformation. Nevertheless, it would be interesting to further explore the biological role of the other studied genes in bladder malignancy.

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CONCLUSIONS AND FUTURE PERSPECTIVES

CONCLUSIONS AND FUTURE PERSPECTIVES

This Dissertation provides an updated overview of the use of DNA methylation biomarkers for BlCa detection through urine samples and evaluates their diagnostic accuracy. Numerous gaps and inconsistencies were identified in the assessed studies, particularly in data reporting. This served as a significant obstacle to conducting a thorough analysis and drawing conclusions from all published data, as well as for subsequent replication. Additionally, the absence of marker validation in a surveillance setting poses a substantial limitation to the progress of biomarker research in this clinical context. Nonetheless, we successfully assessed the diagnostic accuracy of 28 DNA methylation-based markers, two panels, and urinary cytology through a meta-analysis approach.

A significant bottleneck identified in the validation of biomarkers for BlCa detection pertains to the type of control individuals included in the studies. Only a handful of studies incorporate samples from patients reflecting the population "at risk" – those who would genuinely benefit from a non-invasive alternative, such as patients with suspicious symptoms of the urinary tract. To meticulously validate the markers selected by the meta-analysis, we utilized healthy donors, patients with other genitourinary cancers, and patients with bladder inflammatory conditions as controls. This approach allowed us to firmly establish the specificity of the markers in detecting BlCa. However, we observed that the achieved sensitivity values were not as high as estimated by others, likely due to the exclusive use of urine samples from patients with NMIBC, contrary to the studies addressed in the meta-analysis.

Nevertheless, *SALL3*, *ZNF154* and *VIM* methylation levels particularly excelled in distinguishing bladder cancer from benign conditions. The performance of these DNA methylation-based biomarkers was further enhanced when they were combined into two-gene panels. For instance, two panels combining two of these markers (*SALL3* / *ZNF154* and *SALL3* / *VIM*) exhibited performances similar to those obtained by urinary cytology, with MIBC patients' samples, in the meta-analysis (48-55% sensitivity and 84-92% specificity). Therefore, it would be intriguing to assess the diagnostic accuracy of urinary cytology in the patients included in our validation study.

While promising results were obtained, a more organized and extensive prospective multicenter study should be conducted in the future. This would involve increasing the number of patients that truly represent the target population, thereby allowing for a reliable assessment of the utility of these markers in clinical practice.

APPENDIX

Part I Supplementary Data

Full literature search strategy

The search was conducted in PubMed, Scopus and Cochrane Library for articles published until the 31st of December 2022.

- **PubMed search strategy**

The query used for PubMed search was the following: ((("bladder cancer"[title/abstract]) OR ("bladder urothelial carcinoma"[title/abstract]) OR ("lower tract urothelial carcinoma"[title/abstract])) AND (("nucleic acids"[MeSH Terms]) OR ("CpG islands"[MeSH Terms]) OR ("DNA Methylation"[MeSH Terms]) OR ("Epigenomics"[MeSH Terms]) OR ("methylation"[MeSH Terms]) OR ("methylation"[title/abstract])) AND (("diagnosis"[MeSH Terms]) OR ("cancer diagnosis"[title/abstract]) OR (“recurrence"[MeSH Terms]) OR ("cancer recurrence"[title/abstract]) OR ("cancer monitoring"[title/abstract]) OR ("cancer surveillance"[title/abstract])))

- **Scopus search strategy**

The query used for Scopus search was the following: TITLE-ABS-KEY ((("bladder cancer") OR ("bladder urothelial carcinoma") OR ("lower tract urothelial carcinoma")) AND (("nucleic acids") OR ("CpG islands") OR ("DNA Methylation") OR ("epigenetics") OR ("methylation")) AND (("cancer diagnosis") OR ("cancer detection") OR ("cancer recurrence") OR ("cancer monitoring") OR ("cancer surveillance"))

- **Cochrane Library search strategy**

The query used for Cochrane Library search was the following: ((("bladder cancer") OR ("bladder urothelial carcinoma") OR ("lower tract urothelial carcinoma")) AND (("nucleic acids") OR ("CpG islands") OR ("DNA Methylation") OR ("epigenetics") OR ("methylation")) AND (("cancer diagnosis") OR ("cancer detection") OR ("cancer recurrence") OR ("cancer monitoring") OR ("cancer surveillance"))):ti,ab,kw

Supplementary Table 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses for Diagnostic Test Accuracy Studies (PRISMA-DTA) Checklist.

Section/topic	#	PRISMA-DTA Checklist Item	Reported on page #
TITLE / ABSTRACT			
Title	1	Identify the report as a systematic review (+/- meta-analysis) of diagnostic test accuracy (DTA) studies.	48
Abstract	2	Abstract: See PRISMA-DTA for abstracts.	50
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	51
Clinical role of index test	D1	State the scientific and clinical background, including the intended use and clinical role of the index test, and if applicable, the rationale for minimally acceptable test accuracy (or minimum difference in accuracy for comparative design).	51 & 52
Objectives	4	Provide an explicit statement of question(s) being addressed in terms of participants, index test(s), and target condition(s).	52
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	52
Eligibility criteria	6	Specify study characteristics (participants, setting, index test(s), reference standard(s), target condition(s), and study design) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	53
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	53
Search	8	Present full search strategies for all electronic databases and other sources searched, including any limits used, such that they could be repeated.	53
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	53
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	53 & 54
Definitions for data extraction	11	Provide definitions used in data extraction and classifications of target condition(s), index test(s), reference standard(s) and other characteristics (e.g. study design, clinical setting).	54
Risk of bias and applicability	12	Describe methods used for assessing risk of bias in individual studies and concerns regarding the applicability to the review question.	54
Diagnostic accuracy measures	13	State the principal diagnostic accuracy measure(s) reported (e.g. sensitivity, specificity) and state the unit of assessment (e.g. per-patient, per-lesion).	54

Synthesis of results	14	Describe methods of handling data, combining results of studies and describing variability between studies. This could include, but is not limited to: a) handling of multiple definitions of target condition. b) handling of multiple thresholds of test positivity, c) handling multiple index test readers, d) handling of indeterminate test results, e) grouping and comparing tests, f) handling of different reference standards	54
Meta-analysis	D2	Report the statistical methods used for meta-analyses, if performed.	54 & 55
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	54 & 55
RESULTS			
Study selection	17	Provide numbers of studies screened, assessed for eligibility, included in the review (and included in meta-analysis, if applicable) with reasons for exclusions at each stage, ideally with a flow diagram.	55 & 56
Study characteristics	18	For each included study provide citations and present key characteristics including: a) participant characteristics (presentation, prior testing), b) clinical setting, c) study design, d) target condition definition, e) index test, f) reference standard, g) sample size, h) funding sources	56-61
Risk of bias and applicability	19	Present evaluation of risk of bias and concerns regarding applicability for each study.	62
Results of individual studies	20	For each analysis in each study (e.g. unique combination of index test, reference standard, and positivity threshold) report 2x2 data (TP, FP, FN, TN) with estimates of diagnostic accuracy and confidence intervals, ideally with a forest or receiver operator characteristic (ROC) plot.	63-66
Synthesis of results	21	Describe test accuracy, including variability; if meta-analysis was done, include results and confidence intervals.	63-66
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression; analysis of index test: failure rates, proportion of inconclusive results, adverse events).	67-69
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence.	70-72
Limitations	25	Discuss limitations from included studies (e.g. risk of bias and concerns regarding applicability) and from the review process (e.g. incomplete retrieval of identified research).	73-74
Conclusions	26	Provide a general interpretation of the results in the context of other evidence. Discuss implications for future research and clinical practice (e.g. the intended use and clinical role of the index test).	74
FUNDING			
Funding	27	For the systematic review, describe the sources of funding and other support and the role of the funders.	-

Supplementary Table 2. Quality assessment of each included study through Quality Assessment of Diagnostic Accuracy Studies 2 (QUADAS-2).

Author, year, journal	Study ID	Risk of bias																Concerns of applicability		
		Patient selection					Index test					Reference standard				Flow and timing				P	I	R
		P1	P2	P3	P4	P	T1	T2	T3	T4	T	R1	R2	R3	R	F1	F2	F3	F			
Abern M.R. et al. 2014 Urologic Oncology	L1	U	Y	Y	U	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Low	Low	Low
Berrada N. et al. 2012 Cellular and Molecular Biology	J1	Y	U	N	U	Possible	Y	N	U	N	High	Y	Y	Y	Low	U	U	Y	Possible	Possible	High	Low
Bosschieter J. et al. 2019 Epigenomics	Q1	Y	U	N	Y	Possible	Y	U	U	Y	Low	Y	Y	Y	Low	Y	U	Y	Low	Possible	Low	Low
Cabello M.J. et al. 2011 The Journal of Molecular Diagnostics	I1	Y	Y	Y	Y	Low	Y	U	Y	U	Low	Y	Y	Y	Low	U	U	Y	Possible	Low	Low	Low
Cebrian V. et al. 2008 Journal of Pathology	F1	U	Y	Y	U	Low	Y	N	Y	N	High	Y	Y	Y	Low	U	Y	Y	Low	Low	High	Low
Chan M.W. et al. 2002 Clinical Cancer Research	A1	Y	N	N	U	High	Y	N	Y	N	High	Y	Y	Y	Low	U	N	Y	High	Possible	High	Low
Chan M.W. at el. 2003 International Journal of Cancer	B1	Y	N	N	U	High	Y	N	Y	N	High	Y	Y	Y	Low	U	N	Y	High	High	Low	Low
Chen P-C. et al. 2011 BMC Medical Genomics	I2	Y	N	N	U	High	Y	U	Y	Y	Low	Y	Y	Y	Low	U	N	Y	High	Possible	Low	Low
Chen X. et al. 2020 The Journal of Clinical Investigation	R1	Y	U	Y	U	Low	Y	U	U	U	Possible	N	Y	Y	Possible	U	U	U	High	Low	Possible	Possible
Chihara Y. et al. 2013 BMC Cancer	K1	Y	N	Y	U	Possible	Y	N	Y	Y	Low	Y	Y	Y	Low	N	U	Y	High	Possible	Low	Low
Cochetti G. et al. 2022 Urologic Oncology	T1	Y	Y	Y	N	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Low	Low	Low
Costa V.L. et al. 2010 Clinical Cancer Research	H1	Y	Y	N	Y	Low	Y	N	Y	Y	Low	Y	Y	Y	Low	U	N	Y	High	Possible	Possible	Low
Costa V.L. et al. 2011 Epigenetics	I3	Y	U	N	U	High	Y	N	Y	Y	Low	Y	Y	Y	Low	U	N	Y	High	Possible	High	Low
D'Andrea D. et al. 2019 BJU International	Q2	Y	U	Y	Y	Low	Y	Y	Y	Y	Low	Y	U	Y	Low	Y	Y	N	Possible	Low	Low	Possible
Dahmcke C.M. et al. 2016 European Association of Urology	N1	Y	Y	Y	N	Low	Y	Y	U	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Low	Low	Low
Deng L. et al. 2022 BMC Cancer	T2	Y	U	N	Y	Possible	Y	N	Y	Y	Low	Y	Y	Y	Low	U	U	Y	Possible	Possible	Low	Low
Eissa S. et al. 2012 Clinical Biochemistry	J2	U	Y	Y	Y	Low	Y	U	Y	N	Possible	Y	Y	Y	Low	U	U	Y	Possible	Low	High	Low
Fang Q. et al. 2022 BMC Cancer	T3	Y	U	Y	U	Low	Y	U	U	Y	Low	Y	Y	Y	Low	U	N	Y	High	Low	Low	Low
Fantony J.J. et al. 2017 Cancer Biomarkers	O1	Y	U	Y	U	Low	Y	Y	U	U	Low	Y	Y	Y	Low	Y	Y	Y	Low	Low	Low	Low

Feber A. et al. 2017 Clinical Epigenetics	O2	Y	Y	Y	U	Low	Y	U	Y	Y	Low	Y	Y	Y	Low	U	N	Y	High	Low	Low	Low
Fernandez C.A. et al. 2012 Research and Reports in Urology	J3	U	U	Y	Y	Low	Y	U	Y	N	Possible	Y	Y	Y	Low	U	U	Y	Possible	Low	High	Low
Friedrich M.G. et al. 2004 Clinical Cancer Research	C1	N	N	N	U	High	Y	N	U	Y	Possible	N	Y	Y	Possible	N	N	Y	High	High	Low	High
García-Baquero R. et al. 2013 The Journal of Urology	K2	Y	Y	Y	Y	Low	Y	Y	Y	U	Low	Y	Y	Y	Low	U	Y	Y	Low	Low	Low	Low
Georgopoulos P. et al. 2021 Urology Journal	S1	Y	U	N	U	High	Y	U	Y	Y	Low	U	Y	Y	Low	Y	Y	Y	Low	Low	Low	Low
Hayashi M. et al. 2014 Oncotarget	L2	U	N	N	U	High	Y	N	U	Y	Possible	N	U	Y	High	N	N	U	High	High	Low	High
Hentschel A.E. et al. 2022 BMC Clinical Epigenetics	T4	Y	Y	Y	N	Low	Y	U	U	Y	Low	Y	Y	Y	Low	Y	U	Y	Low	Low	Low	Low
Hentschel A.E. et al. 2020 Cancers	R2	Y	U	N	Y	Possible	Y	U	Y	Y	Low	Y	Y	Y	Low	U	U	Y	Possible	Low	Low	Low
Hermanns T. et al. 2020 Urologic Oncology	R3	Y	Y	N	U	Low	Y	Y	U	Y	Low	Y	Y	Y	Low	U	Y	Y	Low	Low	Low	Low
Kandimalla R. et al. 2013 Clinical Cancer Research	K3	Y	Y	Y	U	Low	Y	N	U	U	High	Y	Y	Y	Low	Y	U	Y	Low	Low	Possible	Low
Lin H.H. et al. 2010 Urologic Oncology	H2	Y	Y	N	U	Possible	Y	U	Y	N	High	Y	Y	Y	Low	U	N	Y	High	Possible	High	Low
Maldonado L. et al. 2014 Oncotarget	L3	Y	N	N	U	High	Y	N	U	Y	Possible	Y	Y	Y	Low	U	U	U	Possible	Possible	Low	Low
Monteiro-Reis S. et al. 2020 Journal of Clinical Medicine	R4	Y	U	N	Y	Possible	Y	U	Y	Y	Low	U	Y	Y	Low	N	N	U	High	Low	Low	Low
Oh T.J. et al. 2022 BMC Cancer	T5	Y	U	N	Y	Possible	Y	U	Y	Y	Low	U	Y	Y	Low	N	N	U	High	Low	Low	Low
Peña K. et al. 2022 Journal of Clinical Medicine	T6	Y	U	Y	U	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	N	Y	Y	Possible	Low	Low	Low
Pharo H.D. et al. 2022 Clinical Epigenetics	T7	Y	Y	Y	Y	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Low	Low	Low
Piatti P. et al. 2021 Clinical Epigenetics	S2	Y	U	Y	Y	Low	Y	U	Y	Y	Low	Y	Y	Y	Low	U	U	Y	Possible	Low	Low	Low
Pietrusiński M. et al. 2017 Cancer Biomarkers	O3	Y	Y	Y	Y	Low	Y	N	Y	N	High	Y	Y	Y	Low	U	N	Y	High	Low	High	Low
Ragonese M. et al. 2022 Clinical Genitourinary Cancer	T8	Y	Y	Y	U	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	N	Y	Y	Possible	Low	Low	Low
Reinert T. et al. 2012 Plos One	J4	Y	Y	Y	N	Low	Y	U	Y	Y	Low	Y	Y	Y	Low	Y	U	Y	Low	Low	Low	Low
Reinert T. et al. 2011 Clinical Cancer Research	I4	Y	U	N	N	High	Y	U	Y	Y	Low	U	Y	Y	Low	U	U	Y	Possible	Possible	Low	Low
Renard I. et al. 2010 European Association of Urology	H3	U	Y	Y	Y	Low	Y	U	Y	Y	Low	Y	Y	Y	Low	U	U	U	Possible	Low	Low	Low
Roperch J-P. et al. 2016 BMC Cancer	N2	Y	Y	Y	Y	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	Y	Low	Low	Low	Low

Rose M. et al. 2020 International Journal of Molecular Sciences	R5	Y	U	Y	U	Low	Y	U	Y	Y	Low	U	Y	Y	Low	U	U	U	Possible	Low	Low	Possible
Rouprêt M. et al. 2008 BJU International	F2	Y	U	Y	Y	Low	Y	U	U	Y	Low	Y	Y	Y	Low	U	U	Y	Possible	Low	High	Low
Ruan W. et al. 2021 Clinical Epigenetics	S3	Y	Y	Y	U	Low	Y	Y	U	Y	Low	Y	Y	Y	Low	U	U	Y	Possible	Low	Low	Low
Scher M.B. et al. 2012 The Journal of Urology	J5	Y	U	Ý	U	Low	Y	U	Y	Y	Low	Y	Y	Y	Low	U	N	Y	High	Low	Low	Low
Serizawa R.R. et al. 2011 International Journal of Cancer	I5	Y	U	N	Y	Possible	Y	N	U	Y	Possible	Y	Y	Y	Low	U	U	Y	Possible	Low	Low	Low
Shimizu T. et al. 2013 European Association of Urology	K4	Y	U	N	U	High	Y	N	Y	U	Possible	Y	Y	Y	Low	U	Y	Y	Low	Possible	High	Low
Shindo T. et al. 2018 Urology	P1	Y	Y	Y	Y	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	U	Y	Y	Low	Low	Low	Low
Steinbach D. et al. 2020 Clinical Genitourinary Cancer	R6	U	U	N	U	High	Y	U	Y	Y	Low	U	Y	Y	Low	U	U	Y	Possible	Possible	Low	Low
Su S-F.et al. 2014 Clinical Cancer Research	L4	Y	U	Y	U	Low	Y	U	Y	U	Low	Y	Y	Y	Low	U	U	Y	Possible	Low	High	Low
Sun J. et al. 2009 Journal of Cancer Research and Clinical Oncology	G1	Y	Y	N	Y	Low	Y	N	Y	N	High	Y	Y	Y	Low	U	N	Y	High	Possible	High	Low
Trenti E. et al. 2019 Cancer Cytopathology	Q3	Y	Y	Y	N	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	U	Y	Y	Low	Low	Low	Low
Urakami S. et al. 2006 Clinical Cancer Research	D1	N	U	N	U	High	Y	N	Y	N	High	Y	Y	Y	Low	U	N	Y	High	Possible	High	Low
Van Der Heijden A. et al. 2018 Clinical Epigenetics	P2	Y	Y	Y	N	Low	Y	U	Y	U	Low	Y	Y	Y	Low	U	Y	Y	Low	Low	Possible	Low
Van Kessel K.E. et al. 2017 The Journal of Urology	O4	Y	N	Y	U	Possible	Y	U	Y	Y	Low	Y	Y	Y	Low	Y	U	Y	Low	Low	High	Low
Van Kessel K.E. et al. 2016 The Journal of Urology	N3	Y	U	Y	Y	Low	Y	U	Y	U	Low	U	Y	Y	Low	U	U	Y	Possible	Low	Possible	Low
Vinci S. et al. 2011 Urologic Oncology	I6	Y	Y	N	U	Possible	Y	U	U	Y	Low	Y	Y	Y	Low	U	Y	Y	Low	Low	Low	Low
Wang K. et al. 2016 Clinical Laboratory	N4	U	U	Y	U	Possible	Y	N	Y	N	High	Y	Y	Y	Low	U	Y	Y	Low	Possible	High	Low
Wang Y. et al. 2016 Oncotarget	N5	N	U	Y	U	High	Y	U	Y	Y	Low	Y	Y	Y	Low	U	U	Y	Possible	Possible	Low	Low
Witjes J. et al. 2018 European Association of Urology	P3	Y	Y	Y	N	Low	Y	Y	Y	Y	Low	Y	Y	Y	Low	Y	Y	N	Possible	Low	Low	Low
Wu Y. et al. 2020 European Urology Focus	R7	Y	U	Y	U	Low	Y	U	U	Y	Low	N	Y	Y	Possible	U	N	U	High	Low	Low	Low
Yates D.R. et al. 2006 Oncogene	D2	U	Y	N	Y	Possible	Y	N	U	Y	Possible	Y	Y	Y	Low	U	U	Y	Possible	Low	Low	Low
Yegin Z. et al. 2013 DNA and Cellular Biology	K5	N	U	N	U	High	Y	N	Y	N	High	Y	Y	Y	Low	U	U	Y	Possible	High	High	Low

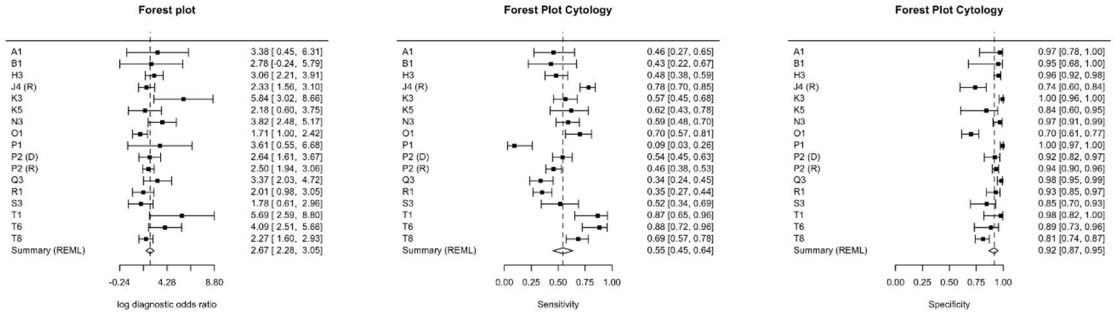
Yeh C-M. et al. 2015 Oncotarget	M1	U	U	N	Y	High	Y	U	Y	Y	Low	U	U	Y	Possible	U	U	U	Possible	Low	Low	Low
Yu J. et al. 2007 Clinical Cancer Research	E1	Y	Y	Y	Y	Low	Y	N	Y	N	High	Y	Y	Y	Low	U	U	Y	Possible	Low	High	Low
Zhao Y. et al. 2012 PLoS One	J6	Y	Y	Y	Y	Low	Y	N	Y	U	Possible	U	Y	Y	Low	U	U	U	Possible	Low	Possible	Low
Zuiverloon T.C. et al. 2012 BJU International	K7	Y	U	N	Y	Possible	Y	N	Y	U	Possible	Y	Y	Y	Low	U	N	Y	High	Low	Low	Low

QUADAS items: P1= Participant selection is fully described; P2= A consecutive or random sample of patients was selected; P3= A case-control design was avoided; P4= Study avoided inappropriate exclusions; P= Risk of Bias on Patient Selection; T1= The index test was well described; T2= The index test results were interpreted without knowledge of the results of reference standard; T3= The threshold used was pre-specified; T4= The technology used to assess the test does not induce bias; T= Risk of Bias on Index Test; R1= The reference test was well described; R2= The reference standard was likely to correctly identify the target; R3= The reference standard results were interpreted without knowledge of the results of index test; R= Risk of Bias on Reference Standard; F1= Description of patients lost-to-follow up; F2= Appropriate interval between index and reference test; F3= All the patients received the same reference standard; F= Risk of Bias on Flow and Time; Y= Fulfilled; N= Not fulfilled; U= Unclear

Supplementary Table 3. Sensitivity for *APC*, *BCL2*, Bladder EpiCheck™, *DAPK*, *p14*, *p16*, *RARβ*, *RASSF1*, *TERT* and urinary cytology for detecting low-grade and high-grade bladder cancer.

Marker	Studies	LG Sensitivity (95% CI)	HG Sensitivity (95% CI)
APC	2	0.32 [0.15-0.58]	0.53 [0.41-0.65]
BCL2	3	0.39 [0.09-0.80]	0.56 [0.11-0.93]
Bladder EpiCheck™	5	0.46 [0.37-0.56]	0.87 [0.80-0.92]
DAPK	3	0.19 [0.12-0.30]	0.16 [0.07-0.33]
p14	2	0.28 [0.10-0.58]	0.38 [0.21-0.60]
p16	2	0.20 [0.04-0.59]	0.25 [0.11-0.47]
RARβ	2	0.13 [0.05-0.29]	0.25 [0.08-0.56]
RASSF1	4	0.47 [0.36-0.59]	0.60 [0.50-0.69]
TERT	2	0.08 [0.01-0.56]	0.31 [0.02-0.93]
Urinary Cytology	10	0.30 [0.18-0.46]	0.66 [0.54-0.75]

LG: Low-Grade; HG: High-Grade; CI: Confidence Interval



Supplementary Figure 1. Forest plots for sensitivity, specificity and logarithm of diagnostic odds ratio (DOR) for urinary cytology in detecting bladder cancer.

Part II Supplementary Data

Supplementary Table 4. Sequence of primers and probes used in qMSP.

Primer Set		Sequence (5'-3')	$T_{Annealing}$, °C	Threshold
ACTB	**F Primer**	TGGTGATGGAGGAGGTTTAGTAAGT	60	0.007
	R Primer	AACCAATAAAACCTACTCCTCCCTTAA		
	Probe	Cy5 – ACCACCACCCAACACACAATAACAAACACA – BHQ		
PENK	**F Primer**	GTTGTTGTTGTTCGGTTTCGG	60	0.025
	R Primer	CGACCGAACGCACTAAACG		
	Probe	6-FAM – AACTACACGTCGCGCAAT – BHQ		
POU4F2	**F Primer**	TTGTGCGAAGTTGAGTTTATTCG	60	0.016
	R Primer	CCGTTCAAACTAACAACAAAAACG		
	Probe	HEX – TTCGGATTTTGTACGTTTGATTTCGGTTA – BHQ		
SALL3	**F Primer**	GTTCGCGTAGTCGTCGTC	60	0.02
	R Primer	TACTCGAAAACCCCGTCA		
	Probe	6-FAM – ACGACGCGAAACGACCTAACG – BHQ		
VIM	**F Primer**	TTCGGGAGTTAGTTCGCGTT	60	0.006
	R Primer	ACCGCCGAACATCCTACGA		
	Probe	Cy5 – TCGTCGTTTAGGTTATCGT – BHQ		
ZNF154	**F Primer**	TCGGATTAGAGATAGTAGAGCGTCG	60	0.01
	R Primer	ACGTAAATCCCCCAAAACGAC		
	Probe	HEX – AACGACGACTCCCCTCACGC – BHQ		

$T_{Annealing}$: Annealing Temperature; F Primer: Forward Primers; R Primer: Reverse Primer

Supplementary Table 5. Standards for Reporting Diagnostic Accuracy (STARD) checklist.

Section & Topic	No	Item	Reported on page #
TITLE OR ABSTRACT			
	1	Identification as a study of diagnostic accuracy using at least one measure of accuracy (such as sensitivity, specificity, predictive values, or AUC)	86
ABSTRACT			
	2	Structured summary of study design, methods, results, and conclusions (for specific guidance, see STARD for Abstracts)	86
INTRODUCTION			
	3	Scientific and clinical background, including the intended use and clinical role of the index test	87 & 88
	4	Study objectives and hypotheses	88
METHODS			
Study design	**5**	Whether data collection was planned before the index test and reference standard were performed (prospective study) or after (retrospective study)	88
Participants	**6**	Eligibility criteria	88 & 89
	7	On what basis potentially eligible participants were identified (such as symptoms, results from previous tests, inclusion in registry)	88 & 89
	8	Where and when potentially eligible participants were identified (setting, location and dates)	88
	9	Whether participants formed a consecutive, random or convenience series	88
Test methods	**10a**	Index test, in sufficient detail to allow replication	89 & 90

	10b	Reference standard, in sufficient detail to allow replication	89
	11	Rationale for choosing the reference standard (if alternatives exist)	-
	12a	Definition of and rationale for test positivity cut-offs or result categories of the index test, distinguishing pre-specified from exploratory	90
	12b	Definition of and rationale for test positivity cut-offs or result categories of the reference standard, distinguishing pre-specified from exploratory	-
	13a	Whether clinical information and reference standard results were available to the performers/readers of the index test	-
	13b	Whether clinical information and index test results were available to the assessors of the reference standard	89
Analysis	**14**	Methods for estimating or comparing measures of diagnostic accuracy	90
	15	How indeterminate index test or reference standard results were handled	91
	16	How missing data on the index test and reference standard were handled	91
	17	Any analyses of variability in diagnostic accuracy, distinguishing pre-specified from exploratory	90
	18	Intended sample size and how it was determined	-
RESULTS			
Participants	**19**	Flow of participants, using a diagram	-
	20	Baseline demographic and clinical characteristics of participants	89
	21a	Distribution of severity of disease in those with the target condition	89
	21b	Distribution of alternative diagnoses in those without the target condition	89
	22	Time interval and any clinical interventions between index test and reference standard	88
Test results	**23**	Cross tabulation of the index test results (or their distribution) by the results of the reference standard	96 & 97
	24	Estimates of diagnostic accuracy and their precision (such as 95% confidence intervals)	96 & 97
	25	Any adverse events from performing the index test or the reference standard	-
DISCUSSION			
	26	Study limitations, including sources of potential bias, statistical uncertainty, and generalisability	104
	27	Implications for practice, including the intended use and clinical role of the index test	103 & 104
OTHER INFORMATION			
	28	Registration number and name of registry	-
	29	Where the full study protocol can be accessed	-
	30	Sources of funding and other support; role of funders	-

Supplementary Table 6. Sperman's Correlation between methylation levels of the target genes' promoters and patients' age.

		POU4F2^{me}	***SALL3^{me}***	***VIM^{me}***	***PENK^{me}***	***ZNF154^{me}***
Age	**Correlation Coefficient**	0.307	0.325	0.300	0.319	0.423
	p-value	0.006*	0.003*	0.007*	0.004*	<0.001*