Personalized Bladder Cancer Management

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COLOFON

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PERSONALIZED BLADDER CANCER MANAGEMENT

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The body of this thesis is based on articles published in different scientific journals. Dissimilarities in exact wording and abbreviations between the text of this thesis and the text of the published articles may exist due to editorial changes and linguistic differences. Permission to reproduce the individual chapters in this thesis was obtained from the publishers of the various scientific journals.

General introduction and scope of the thesis

THE URINARY TRACT

The urinary tract encompasses an upper tract (the renal pelvis and ureters) and a lower tract (the bladder and urethra) (figure 1). The function of the urinary tract is to collect and to dispose urine. Urine produced by the kidneys is collected in the renal pelvis, and then flows via the ureters into the urinary bladder. Here, the urine is stored until urination. On average an adult person will urinate 1.4 liters of urine per 24 hours [1]. In a clinical setting, a urine output of at least 0.5mL/kg body weight/hour is considered adequate. The largest component of urine is water (91-96%), therefore the variation in total urine output is primarily due to fluid intake. Several chemical components of urine are: Nitrogen (mostly in the form of urea), Phosphorus, Potassium, Calcium and Creatinine [1]. Lastly, urine also contains organic components, e.g. inflammatory cells, urothelial cells and sometimes bacteria.

The urinary tract is histologically made up of different cell layers; its inner lining consists of a specialized epithelium of urothelial cells, referred to as urothelium and is approximately 3-6 cells thick [3]. The urothelial cell layer that is in contact with urine consists of large and flat umbrella cells that prevent components from urine to enter the bladder wall (figure 2) [3]. The urothelium maintains the tissue barrier as the bladder expands and contracts. The urothelium is a regenerative cell layer and is separated from the lamina propria by a basement membrane (figure 2). The lamina propria consists of connective tissue containing stromal cells, small blood vessels and occasional smooth muscle fibers. The small bundles of smooth muscle fibers in the lamina propria are referred to as the muscularis mucosae [3]. Beneath the lamina propria lie large bundles of muscle fibers. The thickness of this muscularis propria or musculus detrusor varies at different locations in the bladder wall. Normally, the muscle is relaxed allowing expansion of the bladder enabling it to fill with urine [4]. During urination, the musculus detrusor contracts (figure 2) and the bladder is emptied. The outer most layer of the urinary bladder, the perivesical tissue, is a layer of fat.

The urinary bladder is a unique organ to study, since it is hollow and able to store a body fluid for a number of hours. Because of these features, the urinary bladder can be inspected via the urethra using a cystoscope and operations of the bladder can often be done transurethrally. Moreover, the urinary bladder can be flushed with therapeutic agents thereby avoiding systemic treatment. Finally, the urine provides doctors and researchers with a nearly endless supply of study material.

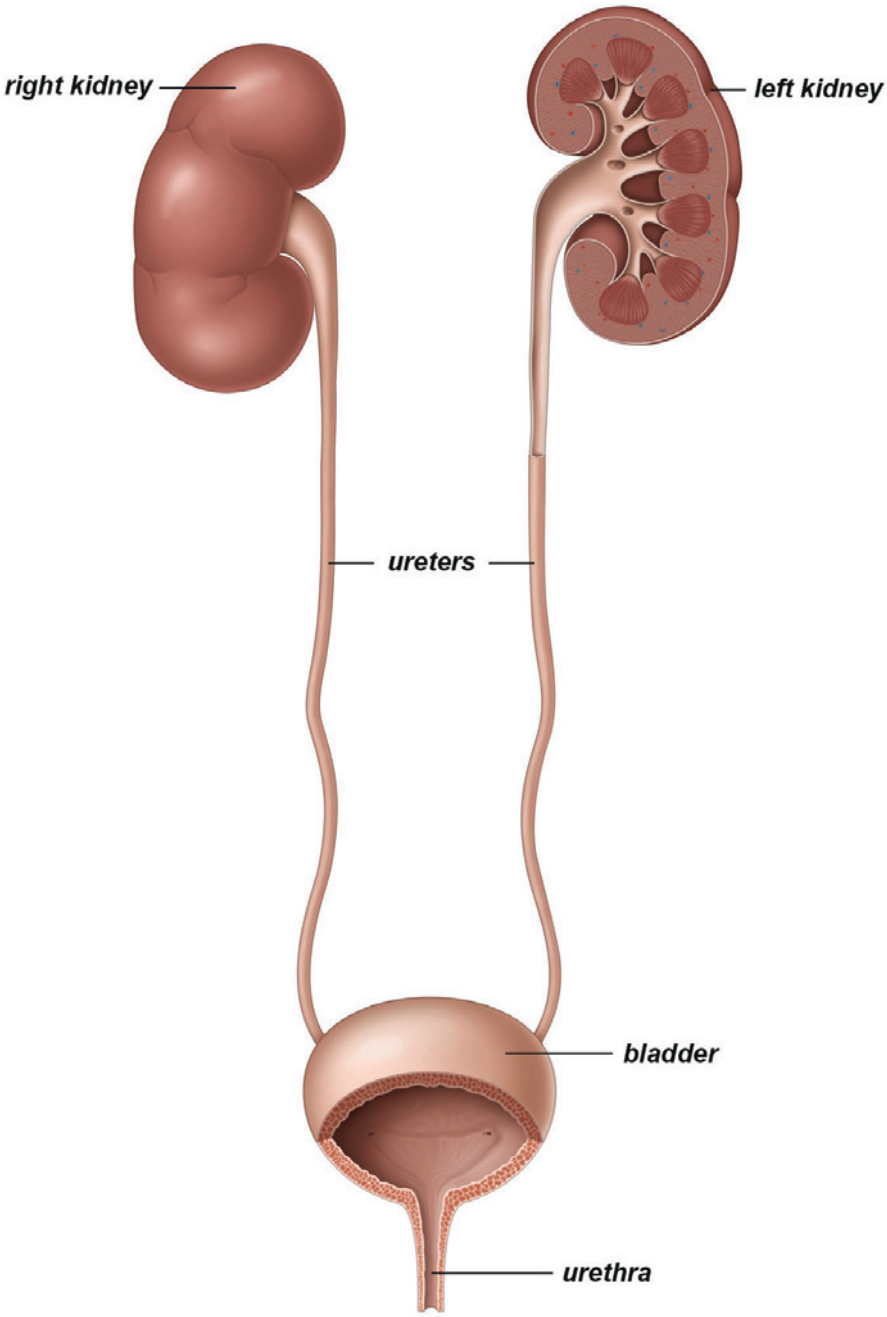

Figure 1. A schematic representation of the upper urinary tract (the renal pelvis and ureters) and the lower urinary tract (the bladder and urethra) [2]. *Reprinted with permission from the European Association of Urology.*

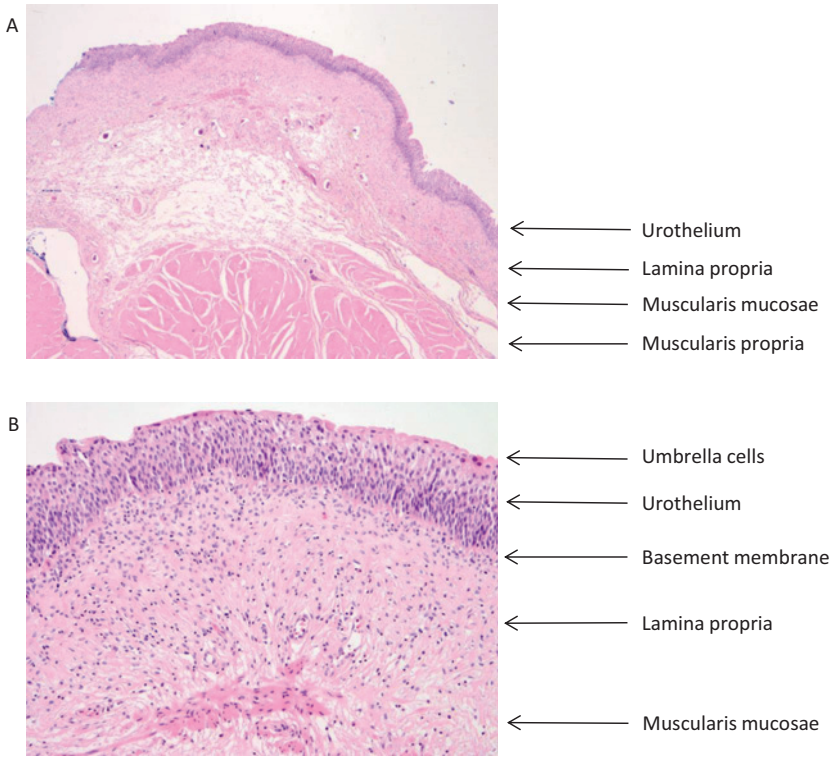

Figure 2. Cross-sectional histology slides with hematoxylin and eosin (H&E) staining showing the different cell layers of the urinary bladder wall at 20x magnification (A) and at 100x magnification (B).

BLADDER CANCER

The incidence of bladder cancer has increased more than 1.5-fold since the 1990s, with currently over 7,000 new cases each year in The Netherlands [5,6]. The incidence increases with age and peaks between 50-70 years of age [7]. The male-female ratio is 3:1, making it the fourth most common solid cancer in males, with only prostate, lung and colorectal cancer occurring more frequently [8,9]. Besides gender and age, tobacco smoking is the most important risk factor; current cigarette smokers have a three times higher risk than never-smokers [9,10]. Most bladder cancers (>95%) arise from the inner lining of the urinary tract; the urothelium [7]. Bladder cancer is therefore also referred to as urothelial carcinoma. A small percentage of cancers in the bladder originate from other cell types. Since the urothelium lines the entire urinary tract, urothelial cancer can occur in the lower urinary tract but also in the upper urinary tract.

Diagnosis

Symptoms of bladder cancer may include dysuria (unpleasant sensation during urination), frequency and urgency (sudden urge to urinate). However, by far most patients present with hematuria: blood cells in their urine. Hematuria can be either microscopic (not visible to the eye) or macroscopic (visible to the eye). Patients with microscopic hematuria are usually identified by coincidence, because of routine urine analysis. In women, the differentiation between hematuria and vaginal bleeding as the cause of blood cells in the urine is sometimes difficult to make.

In general, causes of hematuria can be benign or malignant. Examples of benign causes include; trauma, exercise induced hematuria, stones, urinary tract infections, vascular malformations or benign prostate hyperplasia. Malignant causes of hematuria include kidney cancer, prostate cancer or bladder cancer [11]. Only 3–28% of the patients presenting with hematuria will be diagnosed with bladder cancer [12, 13]. Hence, more than 70% of the patients have different causes of hematuria and most of these are non-malignant causes.

Cystoscopy accompanied by histological confirmation of the presence of tumor cells in a biopsy is considered the gold standard in bladder cancer diagnostics [8]. During a cystoscopy, the urologist visually inspects the bladder by inserting a flexible or rigid scope through the urethra into the bladder. Urine cytology is used in high-risk patients to confirm suspicion of the presence of a bladder tumor. In cytology, the pathologist inspects cells obtained from urine under a microscope. In addition, imaging of the upper urinary tract by computed tomography (CT) scan or ultrasound can aid in the diagnosis of upper urinary tract abnormalities.

Treatment

Patients found to have a suspicious lesion in their bladder will undergo a transurethral resection (TUR). During this procedure instruments are inserted through the urethra and the urologist will scrape the bladder tumor off the bladder wall. A pathologist will analyze the removed tissue in order to confirm the diagnosis of bladder cancer and to determine the histological subtype of the bladder tumor.

The treatment and follow-up of a bladder tumor is dependent on the pathological diagnosis, which is described according to the Tumor, Node, Metastasis (TNM) classification system (table 1, figure 3) [14]. If a bladder tumor is confined to the urothelium (stage T*is* or Ta) or the tumor shows micro-invasive growth into the lamina propria of the bladder (T1), the tumor is called a non-muscle-invasive bladder cancer (NMIBC). When the tumor grows deeper into the bladder wall, invading the musculus detrusor (T2), the

perivesical tissue (T3) or invades surrounding organs (T4), the bladder tumor is called a muscle-invasive bladder cancer (MIBC). Positive nodal disease can be in a single lymph node (N1) or multiple locoregional lymph nodes (N2), or more distant from the bladder (N3). Distant metastases (e.g. lung, bone, liver or brain metastasis) are classified as being absent (M0) or present (M1) (table 1) [14].

Table 1. TNM classification of malignant tumors of the urinary bladder [14].

	T - Primary Tumor
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Non-invasive papillary carcinoma
T*is*	Carcinoma *in situ*: "flat tumor"
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscle
	T2a Tumor invades superficial muscle (inner half)
	T2b Tumor invades deep muscle (outer half)
T3	Tumor invades perivesical tissue:
	T3a microscopically
	T3b macroscopically (extravesical mass)
T4	Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
	T4a Tumor invades prostate stroma, seminal vesicles, uterus, or vagina
	T4b Tumor invades pelvic wall or abdominal wall
	N - Regional Lymph Nodes
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph-node metastasis
N1	Metastasis in a single lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N2	Metastasis in multiple lymph nodes in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N3	Metastasis in common iliac lymph node(s)
	M - Distant Metastasis
M0	No distant metastasis
M1	Distant metastasis

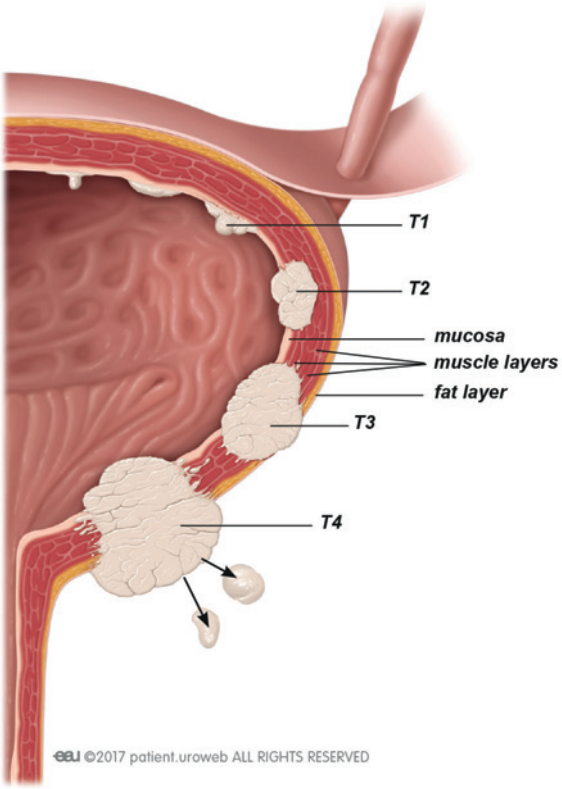

Figure 3. A schematic representation of the invasion depth of different T-stages of bladder cancer [15]. *Reprinted with permission from the European Association of Urology.*

Over two thirds of new patients are diagnosed with NMIBC, the remaining one third of patients are diagnosed with MIBC [8]. Patients diagnosed with NMIBC are treated by TUR and sometimes additional intravesical instillation of chemotherapeutic or immunotherapeutic drugs [8]. If the bladder tumor has grown deeper into the detrusor muscle, additional more invasive treatment modalities are indicated.

The preferred curative treatment for MIBC is radical cystectomy with pelvic lymph node dissection, which is sometimes preceded by four courses of systemic cisplatin-based neoadjuvant chemotherapy (NAC) [16]. During a radical cystectomy, the whole urinary bladder is removed and a continent or incontinent urinary diversion is created from the small bowel. NAC results in only 6% increase in 10-year survival, because the response to treatment differs highly between patients. Only 20-38% of patients are without residual disease after NAC (ypT0N0) [17, 18]. Patients who do not respond to NAC experience

a delay in time to potentially curative surgery and in addition may suffer from toxic side effects of chemotherapy. Compared to non-NAC treated patients, NAC treated patients with no response to NAC (f.i. pT2-T4 versus ypT2-T4) have worse survival after radical cystectomy [19]. Other potentially curative treatments include chemo-radiation, partial cystectomy with brachytherapy, or external beam radiotherapy, however, these treatment modalities are reserved for a selected group of patients.

Prognosis and surveillance

The overall and cancer-specific survival of NMIBC is >90%, however the recurrence rate is high (>50%) and these recurrences may progress to MIBC (in approximately 5-10% of cases) [8, 20]. The survival of MIBC is highly dependent on radical surgery and accompanying lymph node status. The 5-year survival rate for stage pT2N0 is around 73.5% but it drops to a mere 46.1% for stage pT4N0. Patients with node-positive disease at radical cystectomy have a poor outcome with a 5-year disease specific survival of only 22.4% [21]. A large study on recurrence after cystectomy showed that around 80% of the cases with pathological node-positive disease (pN1-3) recurred versus only 20% of pathological node-negative organ-confined disease [22].

Surveillance of NMIBC patients is based on their European Association of Urology (EAU) risk stratification for recurrence and progression (low, intermediate or high risk) (table 2) [8, 20]. Risk stratification is based on clinico-pathological parameters and was first proposed by the European Organisation for Research and Treatment of Cancer (EORTC) (table 3 and 4). These patients follow different surveillance schemes, ranging from cystoscopic evaluation every year to 3-monthly cystoscopies. Besides cystoscopy, cytology is sometimes used in the follow-up of bladder cancer patients. Also, ultrasound or CT imaging to visualize the upper urinary tract may be necessary. Finally, some patients will undergo random biopsies of their bladder wall to exclude the presence of T*is*, which is sometimes difficult to detect by cystoscopic inspection only [8].

MIBC patients have a very different surveillance schedule. These patients will undergo frequent CT scans and blood tests to analyze their physical well-being and disease status [16]. More and more attention is given to the mental and emotional challenges of having to undergo a radical cystectomy and the difficulties accompanied by living with a urinary diversion. Specialized uro-oncology nurses provide practical and emotional guidance. Also, the impact on the sexuality of patients treated for bladder cancer (e.g. due to urine leakage or erectile dysfunction) is addressed.

Overall, surveillance of both NMIBC and MIBC will take at least 5 years but mostly far longer.

Table 2. European Association of Urology (EAU) guidelines on non-muscle invasive urothelial carcinoma, risk group stratification [13].

Low-risk tumors	Primary, solitary, Ta, LG/G1, < 3 cm, no *CIS*
Intermediate-risk tumors	All tumors not defined in the two adjacent categories (between the category of low and high risk)
High-risk tumors	Any of the following: • T1 tumor • HG/G3 tumor • *CIS* • Multiple and recurrent and large (>3 cm) Ta G1G2 tumors (all conditions must be present in this point)

CIS = carcinoma in situ; HG = high grade; LG = low grade.

Table 3. European Organisation for Research and Treatment of Cancer (EORTC) nomogram, weights used to calculate the recurrence and progression scores [19].

Factor	**Recurrence**	**Progression**
Number of tumors		
Single	0	0
2 to 7	3	3
≥ 8	6	3
Tumor size		
< 3 cm	0	0
≥ 3 cm	3	3
Prior recurrence rate		
Primary	0	0
≤ rec/yr	2	2
> 1 rec/yr	4	2
T category		
Ta	0	0
T1	1	4
CIS		
No	0	0
Yes	1	6
Grade		
G1	0	0
G2	1	0
G3	2	5
Total score	**0–17**	**0–23**

Table 4. Probability of recurrence and progression according to the total EORTC risk scores [19].

	Probability of recurrence	
Recurrence score	**1 year % (95% CI)**	**5 years % (95% CI)**
0	15 (10 - 19)	31 (24 - 37)
1–4	24 (21 - 26)	46 (42 - 49)
5–9	38 (35 - 41)	62 (58 - 65)
10–17	61 (55 - 67)	78 (73 - 84)
	Probability of progression	
Progression score	**1 year % (95% CI)**	**5 years % (95% CI)**
0	0.2 (0 - 0.7)	0.8 (0 - 1.7)
2–6	1.0 (0.4 - 1.6)	6 (5 - 8)
7–13	5 (4 - 7)	17 (14 - 20)
14–23	17 (10 - 24)	45 (35 - 55)

Costs and awareness

The long duration of surveillance, combined with (sometimes) invasive surgical procedures, frequent imaging and invasive cystoscopies contribute to bladder cancer being one of the costliest cancers in western countries. Bladder cancer accounts for 3% of the entire cancer care costs in the European Union (€ 143 billion in 2012) [23]. The high incidence and recurrence rate of NMIBC, together with the poor survival rate of MIBC and the immense costs make bladder cancer a serious public health problem. Still, bladder cancer is desperately understudied and awareness in the general population is shortcoming. A lack of funding opportunities is a major cause of this problem [24]. For the future, this absence of awareness and the shortage of research funding implicate an important responsibility and challenge for all people involved in the field of urology.

BASIC GENETICS

The entire genetic information of an organism is stored in the DNA (deoxyribonucleic acid). DNA consists of two complementary strands of millions of bases on a backbone of deoxyribose sugar and phosphate groups [25]. The four bases are: Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). A base combined with its ribose and phosphate group is called a nucleotide. The double stranded structure of DNA is achieved by the formation of G-C and A-T pairs. The human genome consists of 3,000,000,000 of these base pairs and codes for ~25.000 genes. Each gene encompasses a unique sequence of bases.

The DNA is situated in the cell nucleus, consisting of 23 chromosome pairs [25]. When active, genes are transcribed and mRNA is produced, a process called transcription. The mRNA also consists of bases that are copied based on the DNA sequence but it is single stranded. The mRNA is subsequently translated into a protein (translation). A protein is a string of amino acids. A sequence of 3 bases in the DNA (and mRNA) codes for one amino acid in a protein. This is called the genetic code. Proteins function as the cell's work force and also as signaling molecules within and between cells.

The influence of genes can be altered by turning the gene on or off via activating or inhibitory proteins. Other ways to influence gene expression may be activating or silencing mutations. Different types of point mutations are: silent mutations, missense mutations, or nonsense mutations. In silent mutations the nucleotide alteration does not change the amino acid and thus does not change the protein structure. In missense mutations the nucleotide alteration results in a different amino acid, leading to a different protein. In a nonsense mutation, the nucleotide alteration leads to a premature stop codon, leading to an incomplete and usually nonfunctional protein. Hence, DNA mutations can lead to changes that increase activity of the produced proteins; gain-of function mutations. On the contrary, mutations may also lead to a reduction of activity or complete inactivity of the produced proteins; loss-of-function mutations.

Furthermore, the DNA sequence can be altered by deletion of nucleotides or (parts of) genes. All genes occur in diploid in a cell (positioned on paired chromosomes), one maternal copy and one paternal copy, referred to as alleles. Sometimes a whole section of a chromosome or gene is lost, a process referred to as Loss of Heterozygosity (LOH). In contrast, in healthy cells, large numbers of sections of the genome are repeated. These sections of repeats can be short repeats or long repeats. Copy Number Variation (CNV) is a phenomenon that describes the differences in the length of repeats between different individuals [25, 26]. The differences in repeats lead to variety in the population and may also be altered in cancer cells.

Methylation is another way to influence gene expression. It is an epigenetic process in which a methyl-group (CH^3-group) is added to a Cytosine in the DNA by the enzyme DNA-methyltransferase [27]. A Cytosine followed by a Guanine often cluster in very CG-rich regions in the DNA, called CpG islands. Hypermethylation of certain CpG islands is thought to alter gene expression by, for instance, preventing the transcription complex from binding to the promotor site [27]. Hypermethylation may lead to Allelic Imbalance (AI), a process that leads to different expression levels of a certain allele or gene in a cell [25, 28]. Besides hypermethylation also other alterations in f.i. regulatory regions of genes may cause AI.

CANCER EVOLUTION

Human life starts with one fertilized egg cell. For development during pregnancy and adulthood, cells need to divide and generate daughter cells. The daughter cells start to differ from each other, divide further and form the different organs and tissues. Eventually over 200 differentiated cell types form the organs, tissues and blood cells. An adult human body contains about 40,000,000,000 cells. In the adult certain cells, such as brain cells do not divide anymore and in other tissues there is a certain level of cell renewal, which is required for the function of the organ. However, it is mandatory that the number of cells in an adult does not increase further. Tightly regulated processes of cell division and cell death accomplish this. A cancer develops when cells start to divide uncontrollably or do not die when necessary, leading to tumor formation or overgrowth of normal cells and eventually destruction of normal architecture of tissues and dissemination throughout the body. Cells have to overcome certain safety mechanisms and barriers in order to become a cancer-cell. The capabilities a cell has to acquire to develop into a cancer are referred to as the hallmarks of cancer. Six hallmarks of cancer have been formulated by D. Hanahan and R.A. Weinberg in 2000 [29]. Ever since, new discoveries have unveiled an increasing complexity of cancer development, leading to the recently expanded hallmarks of cancer [30]. Some important hallmarks are:

Sustained proliferative signaling: Growth factors bind to cell-surface receptors (growth factor receptors), which stimulate intracellular signaling pathways that stimulate progression through the cell cycle [30]. In normal cells, the presence of growth signals is strictly controlled by adjusting the production and release of growth factors. Cancer cells are able to disturb these signals by for instance: stimulation of surrounding cells to release more growth factors, upregulation of the number of receptors on the cell surface, or structurally alter receptors to render them independent of the growth factor ligand [29]. In cancer cells, activating mutations are often found in genes coding for growth factor signaling molecules, so called gain-of-function mutations.

Evading growth suppressors: A way to maintain a good balance between cell renewal and the total number of cells in healthy tissues, tumor suppressor genes produce growth suppressors [29]. Cancer cells can circumvent these genes in order to sustain proliferation. Two well-known tumor suppressor genes are *TP53* and *RB*. Nicknamed the guardian of the genome, *TP53* receives input from stress and cell damage. If damage occurs, *TP53* will either initiate a repair mechanism or it will trigger apoptosis (cell death)[29]. In cancer cells, inactivating mutations can be found in tumor suppressor genes, so called loss-of-function mutations.

Enabling replicative immortality: Normally, cells only have a limited number of cell cycles until division stops: this is called the Hayflick limit [30]. Telomeres are protective repetitive nucleotide sequences at the end of a chromosome and shorten at every cell cycle. Telomerase is crucial for cancer cell survival; it increases telomere length at the chromosome ends. Without telomerase replicative senescence and genomic instability would occur. In normal cells, telomerase is active in stem cells but becomes inactive in differentiated cells [31].

Invasion and metastases: During cancer progression, tumors alter in shape and slowly lose their connection to surrounding cells and extracellular matrix [32], leading to local invasion and cell spread throughout the circulatory system (blood or lymphatic vessels). At a distant organ site, the tumor cells may exit from the vessels (extravasation) and metastases will start to form [32].

GENETIC ALTERATIONS IN BLADDER CANCER

On a DNA level, bladder cancer is known to have a high mutational burden. This means, that most bladder cancer tumor cells harbor many different mutations. Of all cancer types, the mutational burden in bladder cancer is amongst the highest, only slightly lower than in lung cancer and melanoma [33, 34]. Besides DNA mutations, other DNA alterations can occur in bladder cancer, such as; copy number alterations (CNAs, deletions or gain of (parts) of a gene) and genomic rearrangements (coupling of two unrelated genes).

Oncogenic mutations

Some of the most affected signaling pathways in cancer are the RTK-RAS-MAPK pathway and the RTK-PI3K-Akt pathway (figure 4) [35]. RTK stands for Receptor Tyrosine Kinase. Specific growth factors (for example, cytokines or hormones) bind to the extracellular domain of an RTK, thereby activating the intracellular kinase domain of the receptor. The activation results in the stimulation of downstream cascades that affect cell proliferation and growth resulting in cell division. Many different types of RTKs exist, of which Fibroblast Growth Factor Receptor 3 (*FGFR3*) is especially important in bladder cancer. Approximately two-thirds of all NMIBCs have activating *FGFR3* mutations [36]. In MIBC, <15% of tumors have *FGFR3* mutations, although >40% of MIBCs overexpress FGFR-3 [37]. Tumors with an *FGFR3* mutation have a favorable disease course; they grow slowly and are less likely to progress to MIBC than *FGFR3* wild type tumors [4, 37-39].

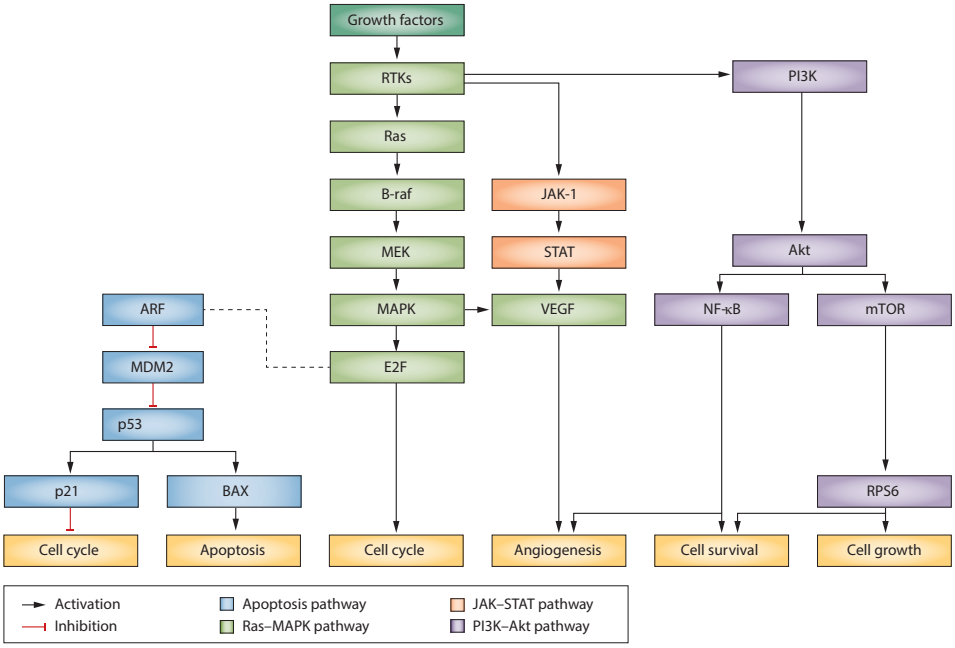

Figure 4. Flow diagram of the most affected signaling pathways in cancer, including the RTK-RAS-MAPK pathway and the RTK-PI3K-Akt pathway [35].

There are three known *RAS* genes, which code for *KRAS*, *NRAS* and *HRAS*. All RAS proteins are small GTPases that are involved in signal transduction. RAS proteins can be either bound to guanosine diphosphate (GDP); resulting in an inactivated state, or can be bound to guanosine triphosphate (GTP); resulting in an activated state [40]. Oncogenic mutations result in a permanently activated state, leading to persistent downstream signaling activation (figure 4). In general, *KRAS* mutations are most frequent in cancer. For instance, *KRAS* is mutated in 90% of pancreatic cancers and 45% of colorectal cancers [41]. Although in general *HRAS* mutations occur less frequently in cancer, in bladder cancer *HRAS* is the most commonly mutated *RAS* gene. Still, mutations in the RAS family of genes are rare in bladder cancer; *HRAS* mutations are present in only 5% of bladder tumors [34].

Approximately 20% of (N)MIBC tumors have a mutation in the *PIK3CA* gene [34]. PI3K stands for phosphatidylinositol-3-kinase, and consists of a regulatory subunit and a catalytic subunit. This catalytic subunit (phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit alpha) is encoded by the *PIK3CA* gene [42]. PI3K is activated by

RTKs, but can also be activated via crosstalk with the activated RTK-RAS-MAPK pathway. Downstream signaling of PIK3CA, via Akt and mTOR results in cell growth and cell survival (figure 4).

In 2014, the importance of the Telomerase reverse transcriptase (*TERT)* gene in bladder cancer became clear, >70% of bladder tumors were found to harbor a *TERT* promotor mutation [31]. The presence of a *TERT* mutation was not associated with stage or grade of the tumors, but was found to be more frequent in tumors that also harbored *FGFR3* mutations [31]. The presence of mutations in these genes is thus not always mutually exclusive; in fact significant overlap between different mutations occurs.

Gene hypermethylation

Genome-wide methylation analyses have identified several genes that are significantly hypermethylated in bladder cancer cells versus normal urothelial cells [43]. Bladder cancer specific methylation of *OTX1, ONECUT2*, *TWIST1* and several other genes was found to be useful for the diagnosis of bladder cancers [44-46]. A combination of methylation markers *OTX1, ONECUT2,* and *OSR1* together with mutation marker *FGFR3* could detect the presence of bladder cancer cells in urine samples with 74% sensitivity [44]. Another combination of methylation markers *OTX1*, *ONECUT2*, *OSR1* with *SIM2* and *MEIS1* showed that these methylation markers combined with age, gender and type of hematuria could detect bladder cancer cells in urine samples with a sensitivity of 82% [45]. Finally, *TWIST1* and *NID2* gene were identified to be hypermethylated in urine sediments. The two-gene panel was 90% sensitive for detecting primary bladder cancer [46].

In addition, hypermethylation of the *GATA2, TBX2, TBX3,* and *ZIC4* genes was shown to be associated with progression of NMIBC to MIBC [43]. Furthermore, methylation of *TBX2* and *TBX3* can, for instance, be used to stratify Ta tumors into three distinct molecular grades, with different risks of progression [47], with a 5-year progression rate of 8% for the low molecular grade group versus 29% and 63% for the intermediate and high molecular grade groups. Finally, hypermethylation of *ZIC4* was shown to be an independent predictor of progression to MIBC in patients with Ta bladder tumors [47].

Gene expression

In recent years, our knowledge of the molecular alterations in bladder cancers has increased substantially. Several research groups in the world have identified genetically distinct tumors that appeared associated with different clinical behaviors and therapy responsiveness [33, 48-50]. In NMIBCs, one group described three different classes based on gene expression analyses [51]. Another group found two different classes of tumors based

on copy-number variations and loss of chromosome 9q [52]. Both groups reported an overall lower mutational burden in NMIBC tumors compared to MIBC tumors (approximately 3-fold). Also, both groups described an enrichment of APOBEC mutational signatures in high-risk NMIBC tumors [51, 52].

The Cancer Genome Atlas (TCGA) initiative strived to analyze a large set of multiple tumor types, in order to provide a better overview of frequently occurring DNA, RNA and protein alterations. In 412 chemo-naïve non-metastatic MIBC from 36 different tissue source sites a total of 58 frequently mutated genes was identified [34]. Different mutational signatures were found to be related to differences in tumor behavior. Interestingly in MIBC, an APOBEC gene signature was correlated to a favorable prognosis. Furthermore, clustering of MIBC tumors based on their gene expression profile revealed five different gene expression subclasses. The five identified subclasses were 'luminal-infiltrated', 'luminal-papillary', 'luminal', 'basal/squamous', and 'neuronal' [34]. These findings will certainly play a major role in upcoming clinical trials and future research to optimize personalized management of both NMIBC and MIBC patients.

BIOMARKERS AND BLADDER CANCER

A biomarker is a feature (like a molecule or gene) that can be detected and measured in a patient to identify certain pathological or physiological processes in the body. In bladder cancer patients, biomarkers can aid in the diagnosis, prognosis, treatment and surveillance. We identified biomarkers in either tissue derived DNA, tissue derived RNA or urine derived DNA and evaluated their use in bladder cancer management.

LABORATORY TECHNIQUES

In the research described in this thesis, different laboratory techniques were used. In short the most important features of these techniques are described below.

Snapshot assay, single-nucleotide extension reaction

In a Snapshot assay one can interrogate a single nucleotide on a specific position on the DNA strand. First, the DNA sequence of interest (containing the specific nucleotide position you wish to interrogate) is multiplied by polymerase chain reaction (PCR), using specific primers. Then, specific probes will bind to the DNA adjacent to the nucleotide under interrogation. Next, fluorescently labelled nucleotides (called dideoxynucleotides or ddNTPs) are added. Only one of such nucleotides can bind to the probe. Then, all excess DNA and ddNTPs are removed and the remaining DNA is

analyzed on a sequencer. The fluorescent label will tell which nucleotide was able to bind to the probe, and thus which nucleotide is present at the opposite specific position on the amplified DNA (figure 5). This technique is used to identify hotspot mutations or methylation of specific CGs.

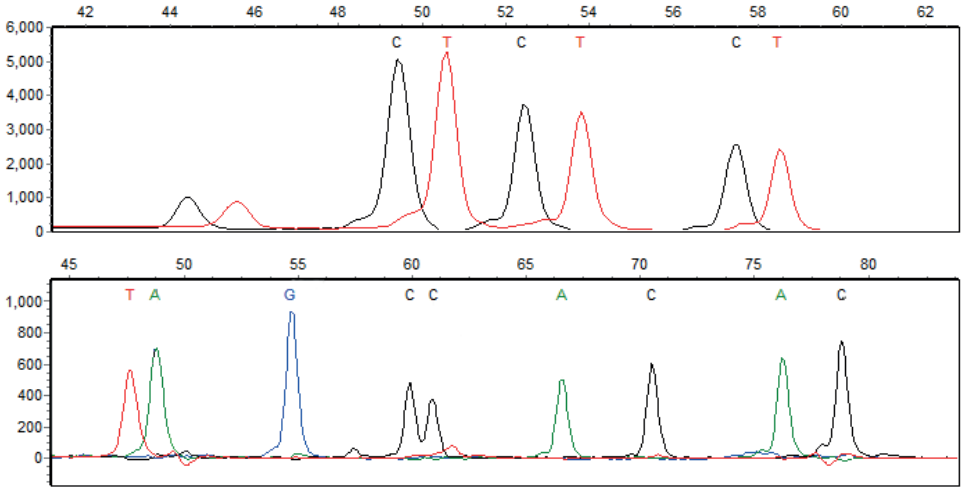

Figure 5. Output from a Snapshot analysis of methylation of the *OTX1* gene (upper panel, the C-peak depicts the methylated Cytosine, the T-peak depicts the unmethylated Cytosine) and mutation analysis of the *FGFR3* gene (lower panel, showing a wild type gene = no mutation present).

Methylation Specific Polymerase Chain Reaction (MSP)

MSP is a technique that uses methylation specific primers to amplify the DNA. The DNA is first treated with bisulfite. During the bisulfite conversion, methylated cytosines are protected from bisulfite modification because of their methyl-group, whereas unmethylated cytosines will be affected and transformed to uracils. After a PCR to amplify the DNA region of interest, the uracils will be transformed into thymines. Primers for MSP are specifically designed to cover regions of a chosen CpG island (region with many CG nucleotides). If a majority of the DNA of interest is unmethylated, the methylation-specific primer cannot anneal to the DNA and no PCR product (amplified DNA) will be formed. This technique is used to identify a region of methylated CGs in the DNA.

Quantitative real-time polymerase chain reaction (qRT-PCR)

A qRT-PCR is used to quantitatively measure the amount of a certain mRNA (product of a certain gene). In a qRT-PCR the enzyme reverse transcriptase is first employed to generate complementary DNA (cDNA) from the mRNA. Next, a PCR is used to multiply the cDNA. By comparing expression of genes of interest to household genes, differences in gene expression can be measured, expressed as delta C_t (ΔC_t). This indicates the difference between the number of PCR cycles between the product of the gene of interest and the household gene standard. In figure 6 an example of an output of a dilution series of a qRT-PCR reaction is given. This figure nicely shows that a smaller number of templates require more cycles to reach the threshold value. Note that duplicate samples completely overlap.

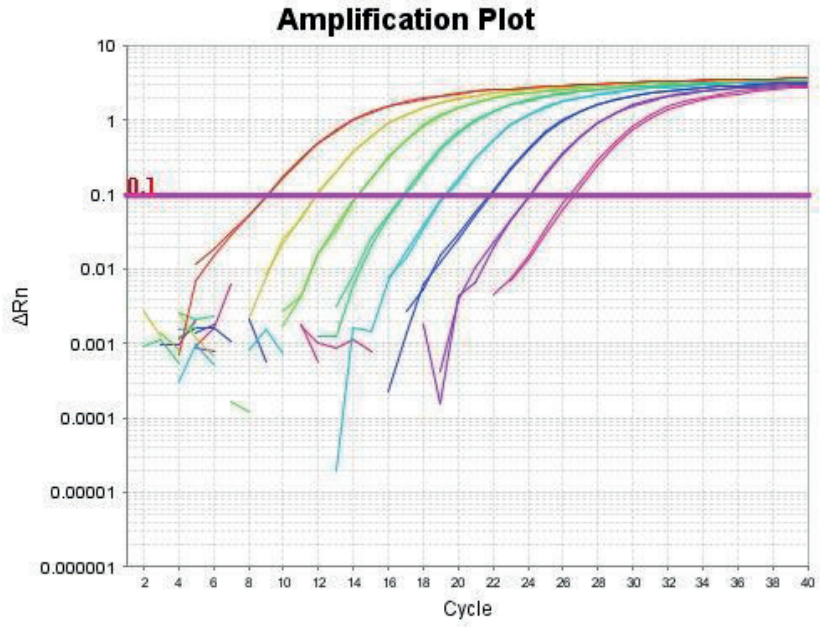

Figure 6. Dilution series of pooled T24 cell line RNA qRT-PCR.

AIMS OF THIS THESIS

The general aim of this thesis is to evaluate and validate the use of (epi)genetic biomarkers in personalized bladder cancer management; including diagnosis of primary disease, prognosis of disease, tools for treatment allocation and their use in individualized surveillance regimens. More specifically the aims of this thesis are:

- To develop and validate a non-invasive diagnostic urine assay for the primary diagnosis of bladder cancer
- To optimize NMIBC risk stratification for progression using molecular biomarkers
- To evaluate biomarker tools for the prediction of response to chemotherapy and the risk of disease progression in MIBC
- To provide insight into both the challenges and promises of the preclinical development of new targeted therapies for bladder cancer patients
- To evaluate the feasibility of implementing urine analysis for NMIBC surveillance

SCOPE OF THE THESIS

The research performed is outlined in the following parts.

Part I. Diagnosis of bladder cancer.

Almost all patients that present with hematuria to the urologist will undergo a cystoscopy to evaluate the cause of hematuria. A cystoscopy is invasive, uncomfortable and costly. Since, bladder cancer is only found in a minority of patients presenting with hematuria, biomarkers could aid in optimizing the current diagnostic protocol. In **chapter 2,** the development of a methylation and mutation based urine assay is explored to improve the selection of patients who should undergo a cystoscopy. We validated this urine assay in a retrospective international cohort, as described in **chapter 3**. In **chapter 4,** we elaborate on the validation of the assay in a large prospective regional cohort.

Part II. Prognosis of patients diagnosed with bladder cancer.

NMIBC patients can be stratified into different risk categories according to clinico-pathological parameters described in the EAU guidelines. This risk stratification does not include any molecular biomarkers of recurrence or progression (table 2 and 3). In **chapter 5,** we investigate the added effect of four different methylation markers (*GATA2*, *TBX2*, *TBX3* and *ZIC4*) and four different mutation markers (*FGFR3, TERT, PIK3CA* and *RAS*) to the EAU NMIBC risk stratification.

NAC results in a modest 6% increase in 10-year survival, because the response to treatment differs highly between patients. Patients who do not respond to NAC experience a delay in time to curative surgery and might experience toxic side effects of chemotherapy. Tools for the prediction of disease progression could potentially aid in the decision whether or not to offer NAC. In **chapter 6**, we investigate the neutrophil-to-lymphocyte ratio as a predictor of poor outcome in MIBC patients treated with pre-operative chemotherapy. Furthermore, in **chapter 7** we attempted to evaluate the clinical applicability a previously published gene expression model designed to predict the presence of lymph node positive disease at radical surgery in MIBC patients that were preoperatively staged as being clinically node negative.

Part III. Treatment and surveillance of bladder cancer patients.

Targeted therapies target specific molecules in different cellular processes of cancer cells to stop the cells from dividing or even stimulate cell death (figure 7). Numerous targeted therapies are available for the treatment of many different types of cancer. However, for bladder cancer, up to January 2017, no targeted therapies were approved.

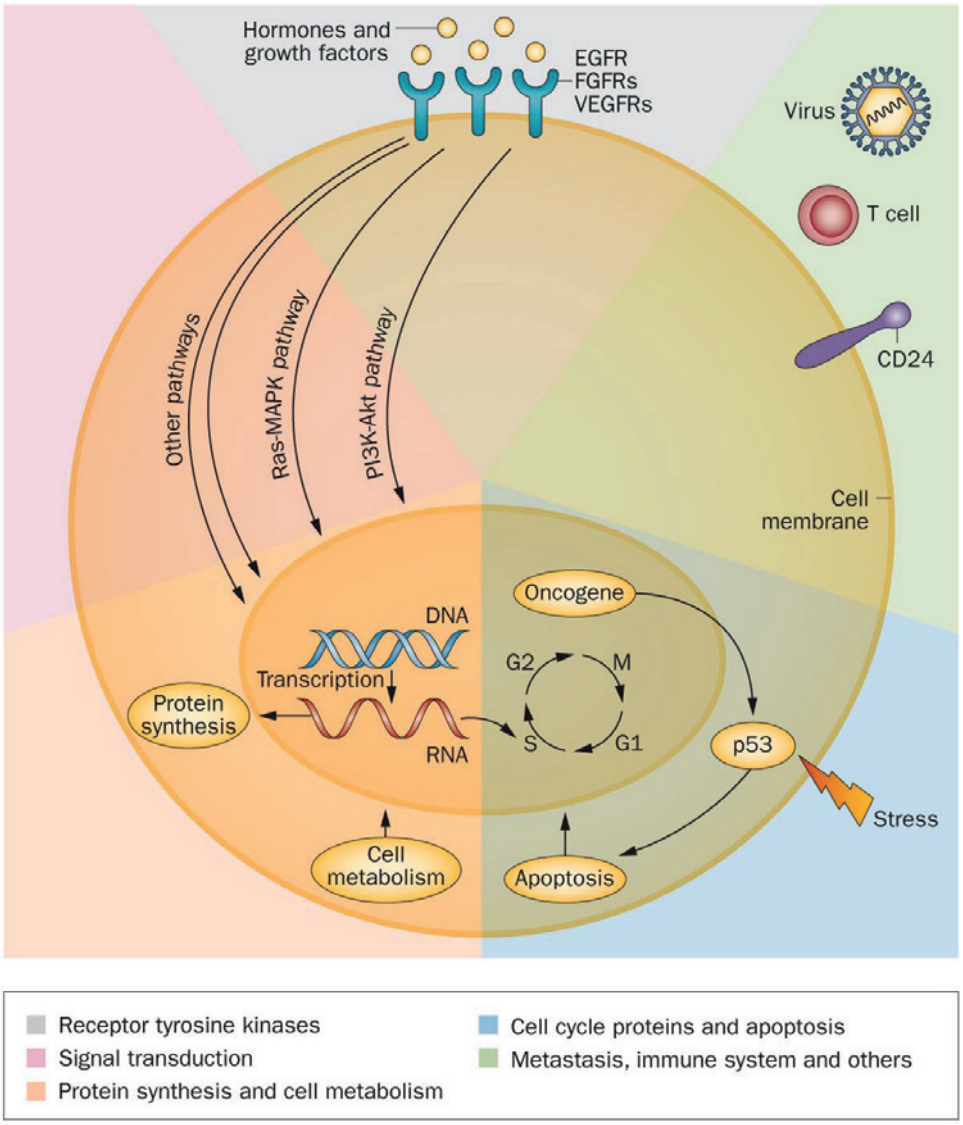

Figure 7. Different cellular processes involved in cancer development. The different processes show the diversity of potential targets for therapies [35].

In **chapter 8**, we reviewed studies published since 2009 reporting on the evaluation and the development of targeted therapies in animal bladder cancer models (*in vivo* studies). In this systematic review, we tried to analyze the reasons for the scarcity of new targeted therapies for bladder cancer patients.

Due to the high recurrence rate of NMIBC and the risk of progression to MIBC, patients treated for NMIBC are kept under strict surveillance. Reducing the amount of invasive cystoscopies in the surveillance of NMIBC could be a fruitful strategy to reduce the care costs for bladder cancer. In **chapter 9**, we performed a cost-effectiveness study to evaluate the safety and potential cost-reduction of implementing urine based surveillance strategies that would partially replace cystoscopies.

Part IV. General discussion and summary

In **chapter 10,** a bird's eye view of the thesis is given and where possible, overall conclusions are drawn. Finally, challenges for the research field, implications for clinical practice, future perspectives and aspirations are discussed.

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Part I: Diagnosis

Evaluation of an epigenetic profile for the detection of bladder cancer in patients with hematuria

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ABSTRACT

Purpose: Many patients enter the care cycle with gross or microscopic hematuria and undergo cystoscopy to rule out bladder cancer. Sensitivity of this invasive examination is limited, leaving many patients at risk for undetected cancer. To improve current clinical practice more sensitive and noninvasive screening methods should be applied.

Materials and methods: A total of 154 urine samples were collected from patients with hematuria, including 80 without and 74 with bladder cancer. DNA from cells in the urine was epigenetically profiled using 2 independent assays. Methylation specific polymerase chain reaction was performed on *TWIST1*. SNaPshot methylation analysis was done for different loci of *OTX1* and *ONECUT2*. Additionally, all samples were analyzed for mutation status of *TERT* (telomerase reverse transcriptase), *PIK3CA, FGFR3* (fibroblast growth factor receptor 3), *HRAS, KRAS* and *NRAS*.

Results: The combination of *TWIST1, ONECUT2* (2 loci) and *OTX1* resulted in the best overall performing panel. Logistic regression analysis on these methylation markers, mutation status of *FGFR3, TERT* and *HRAS*, and patient age resulted in an accurate model with 97% sensitivity, 83% specificity and an AUC of 0.93 (95% CI 0.88e0.98). Internal validation led to an optimism corrected AUC of 0.92. With an estimated bladder cancer prevalence of 5% to 10% in a hematuria cohort the assay resulted in a 99.6% to 99.9% negative predictive value.

Conclusions: Epigenetic profiling using *TWIST1, ONECUT2* and *OTX1* results in a high sensitivity and specificity. Accurate risk prediction might result in less extensive and invasive examination of patients at low risk, thereby reducing unnecessary patient burden and health care costs.

INTRODUCTION

Hematuria, which accounts for up to 20% of all urological visits, is the presence of red blood cells in urine.[1] Urological cancer is found to be the cause of hematuria in 5% of patients with microscopic hematuria and in up to 20% with macroscopic hematuria.[2,3] Therefore, it is an important clinical question to discriminate between a malignant and nonmalignant cause of hematuria since early detection of bladder cancer correlates with an increased likelihood of bladder preservation and improved overall survival.[4]

The typical diagnostic analysis of hematuria includes cystoscopy, which has 68% to 83% sensitivity to detect BC recurrence. For the primary diagnosis of BC sensitivity was described to be 87% in patients with gross hematuria.[5] Cystoscopy is invasive and causes pain and discomfort.[6] Cytology is considered specific but with low sensitivity and it is typically used in combination with cystoscopy.[7] Patients diagnosed with early stage BC undergo frequent monitoring currently based on cystoscopy and cytology, resulting in Ba becoming one of the most costly of all cancers to manage.[8]

To improve current clinical practice noninvasive and more sensitive screening methods should be applied to improve patient treatment and reduce the cost, morbidity and mortality of the sixth most common cancer.[9] Much effort has focused on trying to identify urine based markers for the diagnosis of BC. While urine cytology is specific but lacks sensitivity, especially for low grade BC,[10,11] and with cystoscopy being only 68% to 83% sensitive and invasive as well as costly and often associated with discomfort[12] an accurate, noninvasive assay could complement current clinical practice.

Cancer results from interactions between the environment, and genetic and epigenetic factors. Although genetic mutations are often the subject of investigation,[13] such genetic alterations account for only a small percent of most cancers.[14] The epigenetic component of cancer represents the most frequent DNA alteration that can lead to the development and progression of cancer.[15] More specifically DNA hypermethylation occurs when DNA becomes methylated at CpG-rich regions located in the gene promoter regions, leading to gene inactivation. DNA methylation of critical genes such as tumor suppressors is a frequent and early event in neoplastic development.[16]

MSP is widely used to detect hypermethylated genes originating from cancer cells in tissues and body fluids such as serum, urine, stool and saliva.[17] A large number of studies have identified methylated genes linked to BC.[18-24]

The aim of the current study was to combine 2 previously published methylation marker panels to assess their complementarity and validate the minimal panel with the best clinical performance. Detection of the methylated *TWIST1* gene in urine sediments using MSP provides a greater than 90% sensitive and specific noninvasive approach to detect primary BC.[20,21,25,26] Methylation of *OSR1, SIM2, OTX1, MEIS1* and *ONECUT2* was shown to be a significant predictor of the presence of urothelial cell carcinoma.[27]

MATERIALS AND METHODS

Patients and urine samples

Voided urine samples were collected from 154 patients undergoing cystoscopy for the diagnostic evaluation of hematuria. Urine samples were collected at Erasmus Medical Center, The Netherlands, between 2006 and 2013. These samples were used according to The Code for Proper Secondary Use of Human Tissues in the Netherlands (http://www.federa.org/). All patients were primary referrals with no history of any urinary tract malignancy. Of these 154 patients 74 were diagnosed with BC and 80 had a benign cause of hematuria (table 1).

Table 1. Characteristics of patients and tumors included in analysis.

		Urothelial Carcinoma	**Benign**
No. pts		74	80
Age (range)	Mean	68 (38-91)	58 (21-86)
		N (%)	**N (%)**
Gender (%)	Male	59 (80)	50 (63)
	Female	15 (20)	30 (37)
Stage (%)	Ta	36 (49)	
	T1	14 (19)	
	T2-4	24 (32)	
Grade	G1	11 (13)	
	G2	29 (39)	
	G3	34 (46)	

Urine samples were collected prior to cystoscopy and stored at 4°C. The samples were centrifuged at 3,000 rpm. The cell pellets were washed with 10 ml phosphate buffered saline twice and spun down for 10 minutes at 3,000 rpm. The cells were then

resuspended in 1 ml phosphate buffered saline, transferred to an Eppendorf vial and centrifuged for another 5 minutes. Supernatant was discarded and the cell pellet was stored at -80°C until DNA isolation.

ANALYSES

Methylation

DNA was isolated from urine sediments using the QIAamp® DNA Micro Kit and quantified with the Quant-iT™ PicoGreen® dsDNA Quantification Kit according to manufacturer instructions. DNA (100ng) was used for bisulfite modification with the EZ DNA Methylation-Lightning™ Kit. Modified DNA was eluted into 20µl tris-HCl (1mM, pH 8.0) and stored at -80°C for further processing.

Blinded MSP analyses were performed using a multiplex reaction for *TWIST1* on the antisense chain (Integrated DNA Technologies, Coralville, Iowa). Supplementary table 1 lists the sequences. Quantitative PCR mix (Quantitect® Multiplex Mastermix) was used and reactions were run on a Rotor-Gene® Q. Copy numbers were calculated based on linear regression of a standard curve obtained from plasmid DNA containing the bisulfite modified sequences of interest (GeneArt™ Gene Synthesis).

SNaPshot assays consisted of multiple probes covering 4 CpG sites in *OTX1* and 5 sites in *ONECUT2*. After bisulfite specific PCR for the sites of interest for *ONECUT2* and *OTX1* SNaPshot analysis was performed using primers that annealed to the PCR product adjacent to the cytosine (in a CpG dinucleotide) of interest and the SNaPshot Multiplex Kit. Probes were extended with labeled dideoxynucleotides and the products were analyzed on an automatic sequencer (ABI PRISM® 3100 Genetic Analyzer). The label indicated the presence or absence of a methylated cytosine as previously described. Supplementary table 1 lists details of the primers and probes used. The Supplementary Appendix A shows a more detailed description of the methods.

SNaPshot methylation data were also normalized for each gene by calculating a methylation percent. We divided the height of the methylated peak by the sum of the heights of the methylated and unmethylated peaks multiplied by hundred.

Mutation

DNA from cell pellets was analyzed for mutations in *FGFR3*, *TERT*, *PIK3CA*, *HRAS*, *NRAS* and *KRAS* as described previously.[28] Supplementary table 2 lists details of the primers and probes used.

Statistical

Statistical analyses were performed with SPSS® and R (https://www.r-project.org). Univariate and multivariate logistic regression analyses were done to calculate the association between BC and predictor variables. A limited number of variables were entered on multivariate analysis, selected based on performance on univariate analysis. The optimal probe combination for the genes *OTX1* and *ONECUT2* was selected based on overall performance on univariate logistic regression analysis. An average methylation ratio was calculated for this optimal probe combination per gene and then entered in the multivariate logistic regression model to account for multicollinearity of the different probes in each gene. Cutoffs to dichotomize the methylation marker results were chosen based on the desired sensitivity-specificity tradeoff as visualized by the ROC curve. The predictive accuracy of the multivariate model was determined by the AUC. The bootstrap procedure was used to internally validate the predictive model with $p < 0.05$ considered statistically significant. Different cutoffs (ie a patient was or was not selected for cystoscopy) of the optimal multivariate model were used to calculate the effect on sensitivity, specificity and NPV.

RESULTS

Assay robustness

Six urine samples (4%) with fewer than 750 copies of the neutral *TWIST1* assay were removed from the data set since the DNA yield was too low to be considered for accurate methylation or mutation analysis. All other methylation assays produce a valid result in more than 97% of samples. Similar robustness was observed for all 6 mutation assays with a dropout rate less than 3%.

Univariate logistic regression

On univariate analysis all variables were significantly associated with the presence of BC (table 2). Adding multiple probes in the *OTX1* assay did not add to the predictive capacity of the marker. Probe 2 performed best (OR 12.49, 95% CI 5.691-27.429). For the *ONECUT2* assay combining probes 1 and 4 enhanced the marker slightly, increasing maximal sensitivity from 84% to 85%. Because of high specificity, the best mutation assays were combined into a single mutation index. This index included *FGFR3, TERT* and *HRAS* mutation status, which resulted in 72% sensitivity and 93% specificity.

Table 2. Univariate logistic regression analyses

	OR (95% CI)		*P* value	% Sensitivity	% Specificity	AUC (95% CI)
Age	1.060	(1.031-1.090)	0.000	64.3	61.5	0.690 (0.606-0.774)
Gender	2.240	(1.062-4.725)	0.034	80	35.9	0.579 (0.488-0.671)
FGFR3	33.250	(4.330-255.297)	0.001	30.4	98.7	0.646 (0.555-0.737)
TERT	22.292	(7.950-62.511)	0.000	61.8	93.2	0.775 (0.695-0.856)
FGFR3/TERT/HRAS	35.074	(12.257-100.365)	0.000	72.1	93.2	0.826 (0.753-0.899)
Cytology	55.500	(6.976-441.537)	0.000	60	97.4	0.787 (0.687-0.887)
Twist	20.417	(8.364-49.837)	0.000	70	89.7	0.799 (0.723-0.874)
OTX1 probe 1	4.148	(1.780-9.668)	0.001	71.8	62	0.669 (0.563-0.774)
OTX1 probe 2	12.494	(5.691-27.429)	0.000	78.3	77.6	0.779 (0.701-0.858)
OTX1 probe 3	2.819	(1.198-6.638)	0.018	0	100	0.618 (0.510-0.726)
OTX1 probe 4	3.241	(1.347-7.800)	0.009	0	100	0.631 (0.524-0.738)
ONECUT2 probe 1	31.636	(12.771-78.370)	0.000	84.1	85.7	0.849 (0.781-0.916)
ONECUT2 probe 2	6.159	(2.949-12.862)	0.000	61.8	79.2	0.705 (0.618-0.792)
ONECUT2 probe 3	8.000	(3.804-16.827)	0.000	75	72.7	0.739 (0.656-0.822)
ONECUT2 probe 4	35.259	(13.858-89.708)	0.000	82.4	88.3	0.853 (0.786-0.921)
ONECUT2 probe 5	12.166	(5.417-27.326)	0.000	83.8	70.1	0.770 (0.691-0.849)
OTX1 all combined	3.667	(1.596-8.424)	0.002	69.2	62.0	0.656 (0.549-0.763)
ONECUT2 all combined	15.900	(7.039-35.915)	0.000	77.9	81.8	0.799 (0.723-0.875)
ONECUT2 pr 1 + 4	43.822	(16.674-115.171)	0.000	85.3	88.3	0.868 (0.804-0.932)

Cytology and mutations

Urine cytology was available for 56% of all urine samples. While cytology proved to be specific in this cohort, ie 97%, sensitivity was lacking at 60%. Despite missing data best case scenarios (all samples with unknown cytology predicted correctly) and worst case scenarios (all samples with unknown cytology predicted falsely) could be made. It was determined that when combining the methylation assay with cytology, sensitivity would range from 91% to 97%.

With regard to mutation analyses 1 *FGFR3* and 2 *TERT* mutations were detected in 3 Ta samples. Mutations in these 2 genes were observed 5 times for *TERT* and once for *FGFR3* in urine samples from control patients (supplementary table 3). Not a single *NRAS* mutation assay was positive in cases or controls (supplementary table 3).

Stage specific analyses

Table 3 shows stage specific analyses of the methylation assay and mutations of *FGFR3, TERT* and *HRAS*. Interestingly methylation and mutation seem to perform best in T1 or higher stage tumors. In general methylation performs better in higher stage disease than in Ta tumors, although the difference is minor. However, mutation analyses contributed most in stage Ta tumors.

Multivariate logistic regression

Multivariate regression analysis resulted in a predictive model including age, mutation status of *FGFR3, TERT* and *HRAS*, and the methylation markers *OTX1, ONECUT2* (2 loci) and *TWIST1* (table 4). NPV ranged from 99.9% at a 5% cancer prevalence in a general hematuria population to 99.6% at a 10% prevalence in a more macroscopic hematuria population. The predictive capacity of the overall model was 0.93 as represented by the AUC (fig. 1). Bootstrapping showed the robustness of the model with an AUC of 0.92 (table 4). Although methylation and mutation assays each performed well, combining them increased the predictive capacity of the model.

By choosing different cutoffs for the optimal model the optimal sensitivity could be chosen (table 5 and fig. 1), preferably maximizing sensitivity without a major reduction in specificity. The lowest cutoff was selected based on the optimal sensitivity-specificity tradeoff as visualized by the ROC curve. The higher cutoffs were chosen based on visual inspection of the ROC curve while maximizing sensitivity. The preferred cutoff, defined as missing the least amount of bladder cancers based on optimal sensitivity-specificity, would result in overall sensitivity of 97% and specificity of 83%, ie sensitivity of 91% for Ta and 91% for T1 or higher stage tumors (table 5 and fig. 2).

Table 3. Stage specific multivariate logistic regression analysis including methylation and mutation variables

Model (variables)	OR (95% CI)		*P* value	AUC (95% CI)
Ta tumors, methylation only				
ONECUT2 probes 1+4	14.005	(3.687-53.199)	0.000	0.871 (0.785-0.958)
OTX1 probe 2	1.597	(0.434-5.883)	0.481	
TWIST	3.507	(0.923-13.329	0.065	
Ta tumors, methylation + mutation				
ONECUT2 probes 1+4	9.660	(2.286-40.822)	0.002	0.900 (0.827-0.973)
OTX1 probe 2	1.424	(0.345-5.886)	0.625	
TWIST	1.226	(0.227-6.616)	0.813	
FGFR3/TERT/HRAS	7.886	(1.600-38.862)	0.011	
T1 + T2-4 tumors, methylation only				
ONECUT2 probes 1+4	12.992	(2.992-57.557)	0.001	0.927 (0.866-0.988)
OTX1 probe 2	4.616	(1.036-20.564)	0.045	
TWIST	3.863	(0.949-15.729)	0.059	
T1 + T2-4 tumors, methylation + mutation				
ONECUT2 probes 1+4	6.698	(1.257-35.689)	0.026	0.923 (0.860-0.987)
OTX1 probe 2	3.184	(0.635-15.964)	0.159	
TWIST	3.014	(0.692-13.127)	0.142	
FGFR3/TERT/HRAS	4.245	(0.849-21.227)	0.078	

Table 4. Multivariate logistic regression analyses

Model (variables)	OR (95% CI)		*P* value	AUC (95% CI)	Optimism corrected AUC
Optimal model					
Age	1.044	(0.997-1.093)	0.067	0.930 (0.883-0.978)	0.91665
FGFR3/TERT/HRAS	8.574	(2.176-33.780)	0.002		
ONECUT2 probes 1 + 4	6.802	(1.881-24.589)	0.003		
OTX1 probe 2	1.569	(0.434-5.670)	0.492		
Twist	1.796	(0.464-6.948)	0.396		
Optimal model without mutation					
Age	1.033	(0.992-1.075)	0.115	0.918 (0.870-0.965)	0.9031
ONECUT2 probes 1 + 4	11.886	(3.589-39.360)	0.000		
OTX1 probe 2	2.070	(0.641-6.683)	0.224		
Twist	3.587	(1.094-11.759)	0.035		
Optimal model without methylation					
Age	1.065	(1.023-1.108)	0.002	0.898 (0.844-0.953)	0.8974
FGFR3/TERT/HRAS	37.197	(11.945-15.837)	0.000		
Methylation only					
ONECUT2 probes 1 + 4	14.569	(4.531-46.851)	0.000	0.900 (0.843-0.956)	0.8926
OTX1 probe 2	2.034	(0.641-6.456)	0.228		
Twist	3.680	(1.131-11.971)	0.030		

Table 5. Effect on sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) at optimal multivariable model cutoffs

Optimal multivariable cutoff		0.1213372	0.1917196	0.3547327	0.4975214
Sensitivity (%)		98.5	97.0	90.9	86.4
Specificity (%)		57.7	83.1	85.9	90.1
PPV	5% prevalence	11.1	23.4	25.7	46.8
PPV	10% prevalence	20.7	39	41.9	49.4
NPV	5% prevalence	1	99.9	99.5	99.3
NPV	10% prevalence	99.8	99.6	98.9	98.4

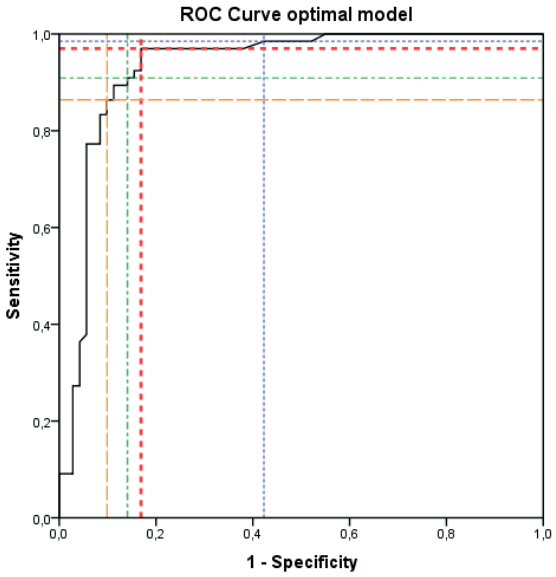

Figure 1. ROC curve with different cutoff values of optimal multivariable prediction model result, including cutoffs 0.1213372 (blue dotted line), 0.1917196 (red dashed line), 0.3547327 (green dotted and dashed line) and 0.4975214 (orange dashed line)

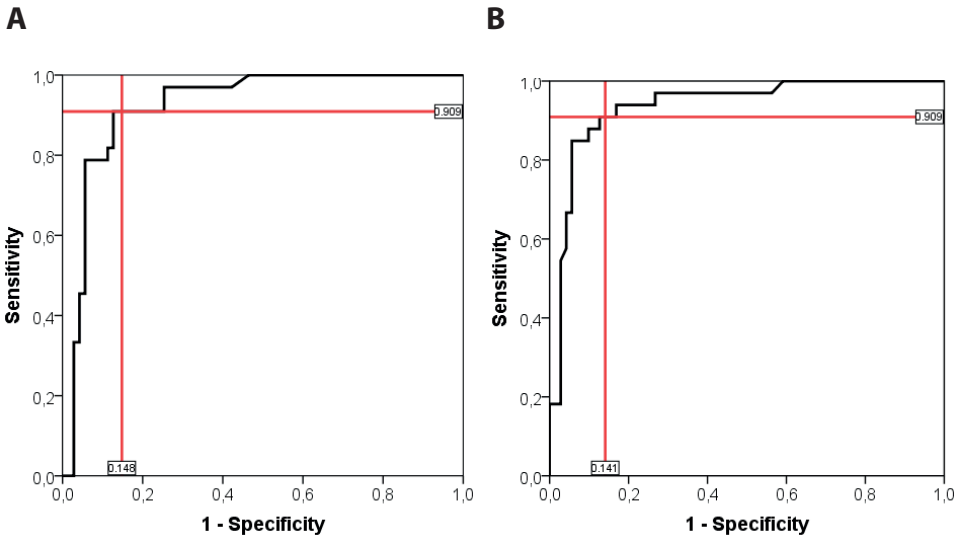

Figure 2. ROC curves with optimal cutoff values, and resulting sensitivity and specificity for stages Ta (A) and T1-T4 (B) disease

DISCUSSION

Epigenetic biomarker panels were generated and evaluated based on overall accuracy. A combination of the 3 genes *TWIST1*, *ONECUT2* (2 loci) and *OTX1* combined with mutation analyses of *FGFR3, TERT* and *HRAS* as well as the age parameter resulted in the best overall performance with 97% sensitivity and 83% specificity. The methylation assay performed with high sensitivity across all stages (table 2). Including mutation analyses of *FGFR3, TERT* and *HRAS*, and age the sensitivity was 91% for Ta and higher stage tumors (T1-T4). The prevalence of BC was estimated to range from 3% in microscopic hematuria cases to 20% in gross hematuria cases. As a general prevalence in a population of patients with hematuria at risk for BC, a 5% prevalence rate was assumed. In this population a NPV of 99.2% was reached with the methylation assay alone. When mutations were added in and the effect of cytology was estimated, the NPV could further increase to 99.9%. With low prevalence rates it is of crucial importance to obtain a high NPV to better diagnose patients so as to not subject them to unnecessary and potentially harmful invasive procedures.

Several urine biomarker assays have been suggested over the years (eg NMP22® , Immunocyt™ , Aura Tek FDP Test™ , BTA stat® , etc). Many have been proposed for surveillance of patients treated for BC. Most assays have not proved to be sufficiently sensitive and/or specific to be able to replace cystoscopy.[29] In the primary diagnostic setting patients must be ruled in for cystoscopy. Therefore, high sensitivity of the biomarker assay is desired. Our proposed biomarker assay showed convincing results with 97% sensitivity and 83% specificity, supporting the clinical usefulness of the assay in reducing superfluous cystoscopy in patients presenting with hematuria.

While the combination of *FGFR3, TERT* and *HRAS* mutations combined could detect 72% of all tumors with high specificity, sensitivity was insufficient for the combination to be used alone as a diagnostic tool. Multivariate analysis confirmed these results. However, while mutations of *TERT* or *FGFR3* showed a higher OR than the methylation assays, this could be assigned to the high specificity of mutations. Methylation remains the most significant risk factor and the complementarity of the methylation and mutation markers was validated in the multivariate logistic regression model. In addition, age had a small but significant contribution, serving as a risk factor in the multivariate model.

Limitations of the study were the relatively small sample size, limited cytology data and lack of data on the type of hematuria. Furthermore, the patient cohort was enriched for cases to enable sufficient events to reach statistical significance. We will now perform a

prospective validation study in a natural cohort of patients presenting with hematuria. We strongly believe that the data described in the current work is sufficiently promising to embark on this prospective study.

CONCLUSIONS

Epigenetic profiling in noninvasive urine samples based on the biomarkers *TWIST1*, *ONECUT2* and *OTX1* resulted in high sensitivity and specificity. The methylation assays yielded high predictive potential in a cohort of patients with hematuria. While the methylation assay was the best diagnostic tool, it could be further improved when complemented with the detection of mutation of *FGFR3, TERT* and *HRAS*, and the clinical parameter age, resulting in 97% sensitivity and 83% specificity. Further, cytology could potentially add to the model. However, neither mutations nor cytology have the diagnostic potential of epigenetics when considered alone. Accurate risk prediction might result in less extensive and invasive examination of patients at low risk, thereby reducing the unnecessary patient burden and health care costs.

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SUPPLEMENTARY APPENDIX A

Methods

Methylation analysis

DNA was isolated from urine sediments using the QIAamp DNA micro kit (Qiagen, Venlo, The Netherlands) and quantified using the Quant-iT PicoGreen dsDNA Quantification Kit (Molecular Probes, Eugene, OR, USA) according to the manufacturer's instructions. One hundred ng of DNA was used for the bisulfite modification using a commercially available kit (EZ DNA Methylation-Lightning Kit, Zymo D5030, ZymoResearch, Orange, CA, USA). This reaction selectively deaminates unmethylated cytosine residues, resulting in a conversion to uracil, while 5-methyl cytosine residues are not modified. The modified DNA was eluted into 20 µl Tris-HCl (1mM, pH 8.0), then stored at -80°C for further processing.

Blinded, MSP analyses were performed using a multiplex reaction for *TWIST1*, on the antisense chain. The multiplex also contained a methylation-neutral region without CpG-sites in the *TWIST1* promoter region, allowing for normalization relative to an input control. The reactions were performed in a total volume of 20 µl using methylation-specific primers and probes for each analyte (Integrated DNA Technologies, see table 2 for the sequences). A commercially available qPCR mix was used (Quantitect multiplex mastermix, Qiagen, 204745), with an input of 2 µl of bisulfite converted DNA. Reactions were run on a Rotor-Gene Q (Qiagen), under the following conditions 95°C for 15 minutes, 45 cycles of 95°C for 30 seconds, 57°C for 30 seconds, 72°C for 30 seconds. Copy numbers were calculated based on linear regression of a standard curve obtained from plasmid DNA containing the bisulfite-modified sequences of interest (GeneArt Gene Synthesis, Life Technologies). In vitro methylated DNA (Chemicon,S7821, Temecula, CA, USA) and the HCT116 DKO cell line (lacking methylation) were included in each run as positive and negative controls, respectively. HL60 and LNCAP cell lines with known methylation status for *TWIST1* served as a positive and negative control.

SNaPshot assays consisted of multiple probes covering four CpG-sites in *OTX1* and five sites in *ONECUT2*. DNA was treated with sodium bisulfite, followed by bisulfite-specific PCR for the sites of interest for *ONECUT2* and *OTX1*. For each reaction 20 ng of input DNA and a PCR primer concentration of 20 pM was used. After PCR, a SNaPshot analysis was performed, using primers that annealed to the PCR product adjacent to the cytosine (in a CpG dinucleotide) of interest (ABI PRISM SNaPshot Multiplex kit, Applied Biosystems). Probes were extended with labeled dideoxynucleotides and the products were analyzed on an automatic sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems), with the label indicating the presence or absence of a methylated cytosine, as previously described (See supplementary table S1 for details on the primers and probes used). SNaPshot methylation data was also normalized for each gene, by calculating a methylation percentage by dividing the height of the methylated peak by the sum of the heights of the methylated and unmethylated peaks multiplied by hundred.

Supplementary table 1. Sequences of the primers and the probes used in the methylation assays and size of the generated amplicons

TWIST	Forward primer	5′-GTTAGGGTTCGGGGGCGTTGTT-3′
	Reverse primer	5′-CCGTCGCCTTCCTCCGACGAA-3′
	Amplicon size	77 bp
	Probe	5′-/FAM/CGGCGGGGAAGGAAATCGTTTC/BHQ1/-3′
TWIST Neutral	Forward primer	5′-GTGGAAGGAAATTTAGTTTATGGGAAAGGT-3′
	Reverse primer	5′-CCACAAAATCTCTCCCTTAAATTAACATTACC-3′
	Amplicon size	113 bp
	Probe	5′-/ROXN/TAGAGTTAGAGGGGGGTAG/BHQ2/-3′
OTX1	Forward primer	5'-TTTTGAGAGGTATAGAGAGGGGTAGT-3'
	Reverse primer	5'-CCCCTAACAAACCCAAATCTC-3'
	Amplicon size	172 bp
	Probe 1	5′-TTTTTTTTTTTTTTTTTTATAGAGAGGGGTAGTTTT-3′
	Probe 2	5′-TTTTTTTTTTTTTTTTTTTTTATTTGTGGTTTTTTAGGTT-3′
	Probe 3	5′-TTTTTTTTTTTTTTTTTTTTTTTTTGGAGATATTGGTTTTTAGT-3′
	Probe 4	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGAGTTTTTTTTATTTTTAGTT-3′
ONECUT2	Forward primer	5'-GGGGTTTTTTGTTTTTTGTATTTTT-3'
	Reverse primer	5'-TCATTTTCAAACTTAAACTTAATCACC-3'
	Amplicon size	206 bp
	Probe 1	5′-TTTTTTTTTTTTTGTTTGGGYGGTTGGGTT-3′
	Probe 2	5′-TTTTTTTTTTTTTTTTTTGTATTATTTGTTTTTGTG-3′
	Probe 3	5′-TTTTTTTTTTTTTTTTTTTTTTCAAAATAATTAAAAAAACTC-3′
	Probe 4	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTGTGYGTATATATTGTTATTTG-3′
	Probe 5	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTATATATTGTTATTTGYGTTTT-3′

Supplementary table 2. Sequences of the primers and the probes used in the mutation assays and size of the generated amplicons

Pan-*RAS* PCR Primer mix			
Primer	**Sequence (5'->3')**	**Product size (bp)**	**Concentration in PCR mix (µM)**
HRAS exon1 Fw	CAGGAGACCCTGTAGGAGG	139	0.6
HRAS exon1 Rev	TCGTCCACAAAATGGTTCTG		0.6
HRAS exon2 Fw	GGAGACGTGCCTGTTGGA	140	0.3
HRAS exon2 Rev	GGTGGATGTCCTCAAAAGAC		0.3
KRAS exon1 Fw	GGCCTGCTGAAAATGACTG	163	0.3
KRAS exon1 Rev	GGTCCTGCACCAGTAATATG		0.3
KRAS exon2 Fw	CCAGACTGTGTTTCTCCCTT	155	0.3
KRAS exon2 Rev	CACAAAGAAAGCCCTCCCCA		0.3
NRAS exon1 Fw	GGTGTGAAATGACTGAGTAC	128	0.3
NRAS exon1 Rev	GGGCCTCACCTCTATGGTG		0.3
NRAS exon2 Fw	GGTGAAACCTGTTTGTTGGA	103	0.3
NRAS exon2 Rev	ATACACAGAGGAAGCCTTCG		0.3
***PIK3CA* PCR Primer mix**			
Primer	**Sequence (5'->3')**	**Product size (bp)**	**Concentration in PCR mix (µM)**
PIK3CA ex9-Fw	AGTAACAGACTAGCTAGAGA	139	1
PIK3CA ex9-Rev	ATTTTAGCACTTACCTGTGAC		1
PIK3CA ex20-Fw	GACCCTAGCCTTAGATAAAAC	109	0.7
PIK3CA ex20-Rev	GTGGAAGATCCAATCCATTT		0.7
***FGFR3* PCR Primer mix**			
Primer	**Sequence (5'->3')**	**Product size (bp)**	**Concentration in PCR mix (µM)**
FGFR3 RI Fw	AGTGGCGGTGGTGGTGAGGGAG	115	1.2
FGFR3 RI Rev	GCACCGCCGTCTGGTTGG		1.2
FGFR3 RII Fw	CAACGCCCATGTCTTTGCAG	138	0.7
FGFR3 RII Rev	AGGCGGCAGAGCGTCACAG		0.7
FGFR3 RIII Fw	GACCGAGGACAACGTGATG	160	0.7
FGFR3 RIII Rev	GTGTGGGAAGGCGGTGTTG		0.7
***TERT* PCR Primer mix**			
Primer	**Sequence (5'->3')**	**Product size (bp)**	**Concentration in PCR mix (µM)**
hTERT Fw	AGCGCTGCCTGAAACTCG	155	0.5
hTERT Rev	CCCTTCACCTTCCAGCTC		0.5

Supplementary table 3. Outcomes of the mutation analyses

Variable (N)		Urothelial carcinoma	Benign	% Sensitivity	% Specificity
FGFR3 (146)	Mutant	21	1	30.4	98.7
	Wild Type	48	76		
TERT (142)	Mutant	42	5	61.8	93.2
	Wild Type	26	69		
PIK3CA (144)	Mutant	9	0	13.0	100
	Wild Type	60	75		
HRAS (147)	Mutant	4	0	5.7	100
	Wild Type	66	77		
NRAS (147)	Mutant	0	0	-	-
	Wild Type	70	77		
KRAS (147)	Mutant	1	0	1.4	100
	Wild Type	69	77		

Validation of a DNA methylation-mutation urine assay to select patients with hematuria for cystoscopy

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ABSTRACT

Purpose: Only 3% to 28% of patients referred to the urology clinic for hematuria are diagnosed with bladder cancer. Cystoscopy leads to high diagnostic costs and a high patient burden. Therefore, to improve the selection of patients for cystoscopy and reduce costs and over testing we aimed to validate a recently developed diagnostic urine assay.

Materials and methods: Included in study were 200 patients from a total of 3 European countries who underwent cystoscopy for hematuria, including 97 with bladder cancer and 103 with nonmalignant findings. Voided urine samples were collected prior to cystoscopy. DNA was extracted and analyzed for mutations in *FGFR3*, *TERT* and *HRAS*, and methylation of *OTX1*, *ONECUT2* and *TWIST1*. Logistic regression was used to analyze the association between predictor variables and bladder cancer.

Results: Combining the methylation and mutation markers with age led to an AUC of 0.96 (95% CI 0.92-0.99) with 93% sensitivity and 86% specificity, and an optimism corrected AUC of 0.95. The AUC was higher for T1 or greater tumors compared to Ta tumors (0.99 vs 0.93). The AUC was also higher for high grade tumors compared to low grade tumors (1.00 vs 0.93). Overall negative predictive value was 99% based on the 5% to 10% prevalence of bladder cancer in patients with hematuria. This would lead to a 77% reduction in diagnostic cystoscopy.

Conclusions: Analyzing hematuria patients for the risk of bladder cancer using novel molecular markers may lead to a reduction in diagnostic cystoscopy. Combining methylation analysis (*OTX1*, *ONECUT2* and *TWIST1*) with mutation analysis (*FGFR3*, *TERT* and *HRAS*) and patient age resulted in a validated accurate prediction model.

INTRODUCTION

Approximately 20% of all patients presenting to a urology clinic are referred for macroscopic or persistent microscopic hematuria.[1] The origin of hematuria can be renal (e.g. benign and malignant renal masses, pyelonephritis and nephropathies) or from the upper and/or lower urinary tract (e.g. cystitis, bladder or prostate malignancies and benign prostatic hyperplasia). More general causes may be trauma, urolithiasis or infection.

Only 3% to 28% of the patients presenting with hematuria will be diagnosed with BC.[2] Risk factors for urinary tract malignancies in patients with hematuria include male gender, age greater than 35 years and smoking.[3-5] Most urological guidelines recommend performing cystoscopy in nearly all patients with hematuria.[1,2,4,6-8] An exception is the Swedish guideline, which advocates its use only in macroscopic hematuria cases.[9] The AUA (American Urological Association) and ACR (American College of Radiology) guidelines advise cystoscopy and abdominal computerized tomography in all patients with risk factors[4,6].

The Canadian guidelines recommend cystoscopy and upper urinary tract imaging in all patients with risk factors as well.[7] This leads to many negative cystoscopies and subsequent upper urinary tract imaging procedures, resulting in high diagnostic costs and substantial patient burden. In the United Kingdom Rodgers *et al* estimated that by reducing negative cystoscopies approximately US$627 per patient could be saved.[10] To better select patients for diagnostic cystoscopy and thereby decrease cost and over testing, there is a clear clinical need for new noninvasive diagnostic tests.

Oncogene mutations play an important role in the malignant transformation of urothelial cells. However, not all bladder tumors harbor mutations in the most commonly altered oncogenes.[11-15] Thus, to reach satisfactory sensitivity a new diagnostic test should include multiple biomarkers. Cancer associated hypermethylation of cytosines in CpG islands of genes shows great potential to identify the presence of malignant cells, and numerous studies in BC have been performed and led to the proposal of candidate markers.[16-18] Previously we described a diagnostic urine test that showed high sensitivity (97%) and specificity (83%).[19] The mutation assay included the *FGFR3*, *TERT* and *HRAS* genes, and the methylation assay included the *OTX1*, *ONECUT2* and *TWIST1* genes.

In the current study we aimed to validate the previously published diagnostic urine assay in an independent, international, multicenter cohort.

PATIENTS AND METHODS

Patients and urine samples

Any patient referred to the urology clinic and who underwent cystoscopy for microscopic or macroscopic hematuria with no prior history of bladder cancer was eligible for study inclusion. A total of 200 patients were prospectively, nonconsecutively included in the study, of whom 97 were diagnosed with BC and 103 were considered to have nonmalignant hematuria. Patients included in the previous development study of the assay were excluded from the current study.[19]

The DNA yield and quality of 163 patients (81.5%) were sufficient to complete all assays. Otherwise part of the assays were run with a result for *FGFR3* in 97% of patients, for *TERT* in 97.5%, for *HRAS* in 94%, for *TWIST1* in 92.5%, for *OTX1* in 93.5% and for *ONECUT2* in 96%. Samples were collected at Uppsala University Hospital, Sweden, Hospital del Mar, Barcelona, Spain, the Spanish National Cancer Research Centre, Madrid, Spain, and the Erasmus University Medical Center, The Netherlands. Informed consent was obtained from all Swedish and Spanish patients. Urine sampling at Erasmus Medical Center was done according to the code of conduct for responsible use of leftover materials.[20]

Voided urine samples were collected prior to cystoscopy and stored at 4°C. Within 12 hours after collection, the urine samples were processed in the laboratories at the participating centers. Cells were pelleted by centrifugation for 10 minutes at 2,500 rpm. Cells were then resuspended in 1 ml phosphate buffered saline, transferred to an Eppendorf vial and spun down for 5 minutes at 6,000 rpm. Supernatant was discarded and the cell pellet was stored at -80°C until DNA isolation. Cell pellets were shipped to Erasmus Medical Center for molecular analyses. *TWIST1* methylation analysis was performed at MDxHealth.

DNA isolation and molecular analyses

DNA was isolated using the QIAmp® DNA Mini-Kit according to the manufacturer protocol. Samples were analyzed for hotspot mutations in *FGFR3*, *TERT* and *HRAS* as described previously.[14,15,21,22] Supplementary table 1 shows primer and probe sequences.

For methylation analysis DNA was first treated with sodium bisulfite using the EZ DNA Methylation-Gold™ Kit according to the manufacturer protocol. After PCR for *OTX1* and *ONECUT2*, a multiplex SNaPshot® assay using the SNaPshot Multiplex Kit was performed per gene with probes for *OTX1* and *ONECUT2* that annealed adjacent to the different CpGs to be interrogated. Supplementary table 1 lists the sequences of the primers and probes used in the assay.

The methylation ratios of each CpG were calculated by dividing the height of the methylated peak by the sum of the height of the methylated and unmethylated peaks multiplied by 100. We have previously reported that reproducibility of the interpretation of the methylation assays is good (R^2 = 0.99 for *OTX1* and R^2 = 0.98 for *ONECUT2*).[23] For *TWIST1* methylation specific PCR was done via a multiplex reaction (IDT®) using QuantiTect® Multiplex PCR Master Mix for quantitative PCR (supplementary table 1). Reactions were run on a Rotor-Gene® Q. Copy numbers were calculated based on linear regression of a standard curve obtained from plasmid DNA containing the bisulfite modified sequences of interest using GeneArt® Gene Synthesis.

Statistical analyses

Statistical analyses were performed using IBM® SPSS® Statistics for Windows®, version 22.0 and R statistical software (https://www.r-project.org/foundation/). Characteristics of the urine samples were compared between patients with malignant and nonmalignant hematuria using the Mann-Whitney U test and the Fisher exact test. Previously determined cutoff values were used to dichotomize the methylation markers (supplementary table 2).[19] Univariable and multivariable logistic regression analysis was done to calculate the association between outcome and variables. The AUC was used to determine the predictive accuracy of the model and compare predictive accuracy to that of the model previously developed.[19] The optimism corrected AUC was used to illustrate model robustness.

RESULTS

Patient characteristics, and mutation and methylation analyses

Supplementary table 3 lists patient characteristics. Patients with a nonmalignant diagnosis were younger than patients diagnosed with BC (mean age 62 vs 71 years, p <0.01, supplementary table 3). Men were also more commonly represented among subjects without BC (p <0.01). Of the BCs diagnosed 83% were noninvasive, 8% were invasive and T stage was unknown in 9%. The average DNA concentration was 26.1 ng/µl (range 1.17 to 110) for tumor-positive urine samples and 16.7 ng/µl (range 1.29 to 104) for nonmalignant samples (p = 0.02). The average DNA concentration did not significantly differ between males and females (20.3 ng/µl, range 1.17 to 110 and 30.9, range 3.49 to 110, respectively, p = 0.30). *TERT* mutations were most prevalent, with 70 of 96 positive assays (73%) in BC urine samples and 3 of 99 positive assays (3%) in

the urine samples of patients without BC (supplementary table 3). The second most prevalent mutations were *FGFR3* mutations with 33 of 95 positive results (35%) in urine samples from patients with BC. Only 2 samples had a *HRAS* mutation.

The combined mutation data from *FGFR3*, *TERT* and *HRAS* were highly informative (OR 107.7, AUC 0.87, p <0.01). When dichotomized, a positive result of the methylation assays was significantly more frequent in urine samples from patients with BC (68% to 89% for the different genes) than in the urine samples from patients without BC (10% to 44%) (supplementary table 3 and fig. 1). Supplementary table 2 shows cutoffs for dichotomization. On univariate analysis methylation and mutation analysis had comparable predictive capacity (AUC 0.88, 95% CI 0.83-0.94 and AUC 0.87, 95% CI 0.81-0.93, respectively, table 1).

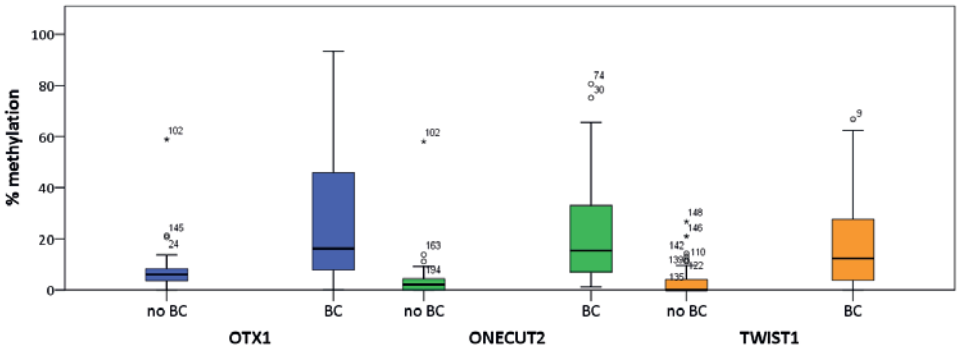

Figure 1. Percent methylation in nonmalignant hematuria samples versus malignant hematuria samples for *OTX1*, *ONECUT2* and *TWIST1*.

Table 1. Univariate logistic regression analysis of the association between predictor variables and bladder cancer in voided urine samples from patients presenting with hematuria (p<0.01).

Variable	OR (95% CI)	AUC (95% CI)
Age	1.10 (1.07-1.14)	0.75 (0.68-0.82)
Mutation combined	107.67 (30.76-376.84)	0.87 (0.81-0.93)
Methylation combined	6.15 (3.78-9.99)	0.88 (0.83-0.94)

Supplementary table 2 lists dichotomization cutoffs.

Multivariable analyses

The previously published optimal multivariable model[19] was applied to this independent cohort. The variables included in this model were age, combined mutation and combined methylation status. Supplementary table 4 shows the algorithm and overall results per sample and per marker. The ORs of the original model and the current model proved to be similar (table 2). Mutation status alone and methylation status alone predicted the presence of BC well but combining mutation status with methylation status increased predictive capacity, as depicted by the AUC (table 2). The AUC of the optimal model was 0.96 compared to 0.93 in the previous study.[19] Internal validation using the bootstrap procedure showed the robustness of each model as shown by the optimism corrected AUCs (table 2).

Table 2. Multivariable logistic regression analysis validating the optimal combination of best performing predictor variables from previous studies and bladder cancer in voided urine samples from patients presenting with hematuria with bladder cancer in 97 and no malignancy in 103 ($p<0.01$).

	OR (95% CI)	AUC (95% CI)	Optimism corrected AUC
Optimal model			
Age	1.12 (1.05-1.19)	0.96 (0.92-0.99)	0.95
Mutation combined	69.46 (11.06-436.22)		
Methylation combined	2.47 (1.30-4.70)		
Optimal model, no mutation			
Age	1.11 (1.06-1.17)	0.92 (0.88-0.97)	0.92
Methylation combined	5.95 (3.49-10.14)		
Optimal model, no methylation			
Age	1.11 (1.05-1.17)	0.93 (0.89-0.98)	0.93
Mutation combined	183.08 (35.75-937.66)		
Methylation only			
Methylation combined	6.15 (3.78-9.99)	0.88 (0.83-0.94)	0.88

To be able to select appropriate patients for initial cystoscopy, the outcome of the multivariable model has to be dichotomized (cancer vs no cancer). Using the cutoffs of the previously published optimal model, the multivariable model could predict the presence of bladder cancer with 93.2% sensitivity and 85.6% specificity in this cohort (table 3 and fig. 2). Depending on the estimated prevalence of 5% to 10% BCs in a

cohort of patients referred for hematuria, the NPV was 99.2% to 99.6%. These results were similar to those of the previously published model (AUC 0.93, 97% sensitivity, 83% specificity and 99.6% to 99.9% NPV).[19]

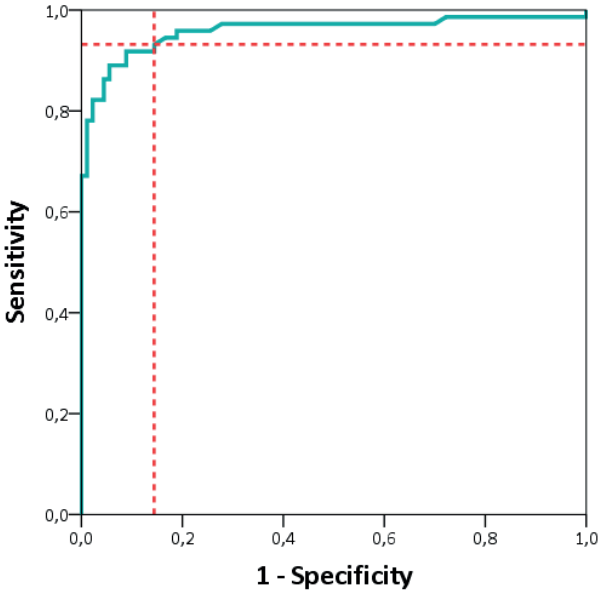

Figure 2. ROC curve of the optimal multivariable prediction model. Red dashed lines indicate cutoff of 93.2% sensitivity of 93.2% and 85.6% specificity (see table 3).

Stage and grade specific analyses

On the stage specific univariate and multivariable analyses the mutation and methylation markers seemed to show an increasing trend in AUC toward higher stage disease (AUC 0.87 for Ta tumors vs 0.98 for T1 and T2-4 tumors, supplementary table 5). The clinical parameters age and gender performed equally over all stages on univariate analysis. Based on overall accuracy, the proposed optimal model also performed better toward higher stage disease (AUC 0.93 for Ta tumors vs 0.99 for T1 and T2-4 tumors, supplementary table 5). Further, on the grade specific univariate and multivariate analyses all markers performed better in high than in low grade tumors. The model including age, mutation and methylation markers also had the highest predictive capacity for high grade tumors (AUC 1.00 vs 0.93).

Table 3. Effect on sensitivity, specificity, false-positive and false-negative findings, positive and negative predictive values, and cystoscopy reduction (fig. 2)

Cutoff	0.1917196
% Sensitivity	93.2
% Specificity	85.6
No. false-pos/1,000 tests†	
5%	136
10%	129
No. false-neg/1,000 tests†	
5%	3
10%	6
% Pos predictive value†	
5%	25.7
10%	42.2
% Neg predictive value†	
5%	99.6
10%	99.2
% Cystoscopy decrease†	
5%	81.7
10%	77.7

* Based on van Kessel *et al.*[19]
† Calculated for estimated 5% or 10% prevalence of bladder cancer in patients referred for hematuria.

DISCUSSION

Patients with hematuria represent an important burden in urology clinics (20%) and no definite consensus exists despite the existence of many different guidelines to analyze the cause of hematuria.[1,4,6-9] Mostly, cystoscopy is recommended. However, only 3% to 28% of patients with hematuria will be diagnosed with BC depending on other risk factors.[2]

In this study we were able to establish that the combination of mutation and methylation markers is a robust tool to select patients with hematuria for cystoscopy workup. The optimal prediction model, which comprised 3 methylation assays, 3 mutation markers and patient age, resulted in 93.2% sensitivity and 85.6% specificity. Previously, we have found good reproducibility of the interpretation of the methylation assays ($R^2 = 0.99$ for *OTX1* and $R^2 = 0.98$ for *ONECUT2*).[23] Also, the reproducibility of the mutation analyses was

robust. In an earlier study we noted that the *FGFR3* mutation analysis could be repeated at 2 independent laboratories in 2 countries with a 100% agreement in a total of 81 samples.[12] Implementing this assay for all patients with hematuria could potentially lead to a 77.7% to 81.7% reduction in cystoscopies in the primary diagnostic workup, although we acknowledge that some cystoscopies may still be necessary for alternative diagnosis. Further, since the assay takes several days to complete, the application of this assay in acute hematuria cases will not be feasible.

The assay can be performed in any molecular pathology laboratory. The minimum DNA input for the proposed assay combination is approximately 50ng (i.e. the amount of DNA present in 8,000 cells). Total material costs do not exceed US$23 per patient. Labor costs would depend on the volume of patients that could be analyzed simultaneously. In contrast, negative cystoscopies would lead to an extra cost of US$627 per patient.[10] Further, we have previously reported that the sensitivity of cystoscopy increases when the urologist is aware of a positive urine test.[24] In this regard, it is also relevant to mention that the sensitivity (68% to 84%) and the specificity (31% to 93%) of standard white light cystoscopy are not perfect, although that method is the gold standard.[25]

Some urinary biomarker tests are FDA (Food and Drug Administration) approved, of which one is the NMP22® BladderChek® Test.[26] In 2014 the prospective evaluation of the accuracy of the test in a cohort of patients referred for hematuria evaluation was published.[27] This study included 381 patients, of whom 6% were diagnosed with bladder cancer. This proportion is comparable to the 5% expected prevalence that we used to calculate the NPV in the current study and it more closely resembles a natural cohort of patients than our case enriched cohort. The predictive accuracy of the test was 80.2%. In our study we achieved 96% predictive accuracy. In this study the exclusion criteria were urinary tract infections, urinary retention or stone disease, kidney failure, ureteral stents, nephrostomy tubes, bowel interposition or recent genito-urinary instrumentation. In contrast, in our study the only exclusion criterion was a prior history of BC.[27]

Interestingly, we detected *FGFR3* and *TERT* mutations in 4 patients who were diagnosed with nonmalignant hematuria. In 2 patients with a *TERT* mutation and in 1 with a *FGFR3* mutation no bladder tumor was diagnosed after updated follow-up (follow-ups was 1, 3.5 and 4 years, respectively). In the remaining patient with a *TERT* mutation carcinoma in situ of the bladder was diagnosed 18 months after the primary diagnostic workup.

A limitation of our study is that information on microscopic vs macroscopic hematuria and cytology was not available. Since the Swedish guidelines recommend cystoscopy only in patients with macroscopic hematuria, these samples can be considered

macroscopic.[9] Analysis of the Swedish cohort showed no major difference in overall performance of the optimal model (AUC 0.96, 95% CI 0.92-1.00) compared to the total cohort. Further, in this case enriched series 94 patients (47%) were diagnosed with BC, a proportion that is much higher than would be expected in a urology clinic population of patients presenting with hematuria. Therefore, a multicenter, prospective validation of the study results is currently being performed. In that study we will also collect data on the type of hematuria and cytology. In conclusion, we validated a urine assay based on the methylation markers *OTX1*, *ONECUT2* and *TWIST1* combined with mutation markers and patient age to predict the presence of BC in patients presenting with hematuria.

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Supplementary table 1. Sequences of the primers and probes and size of the generated amplicons in the methylation analysis for different CpGs in the *OTX1*, *ONECUT2* and *TWIST1* genes

	OTX1
Forward primer	5'-TTTTGAGAGGTATAGAGAGGGGTAGT-3'
Reverse primer	5'-CCCCTAACAAACCCAAATCTC-3'
Amplicon size	172 bp
Probe 2	5'-TTTTTTTTTTTTTTTTTTTTTTATTTGTGGTTTTTTAGGTT-3'
	ONECUT2
Forward primer	5'-GGGGTTTTTTGTTTTTTGTATTTTT-3'
Reverse primer	5'-TCATTTTCAAACTTAAACTTAATCACC-3'
Amplicon size	206 bp
Probe 1	5'-TTTTTTTTTTTTTGTTTGGGYGGTTGGGTT-3'
Probe 4	5'-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGYGTATATATTGTTATTTG-3'
	TWIST1
Forward primer	5'-GTTAGGGTTCGGGGGCGTTGTT-3'
Reverse primer	5'-CCGTCGCCTTCCTCCGACGAA-3'
Amplicon size	77 bp
Probe	5'-/FAM/CGGCGGGGAAGGAAATCGTTTC/BHQ1/-3'
	***TWIST1* Neutral**
Forward primer	5'-GTGGAAGGAAATTTAGTTTATGGGAAAGGT-3'
Reverse primer	5'-CCACAAAATCTCTCCCTTAAATTAACATTACC-3'
Amplicon size	113 bp
Probe	5'-/ROXN/TAGAGTTAGAGGGGGGTAG/BHQ2/-3'

Supplementary table 2. Cutoffs used for dichotomization of the methylation markers based on van Kessel *et al*.[19]

Methylation markers	**Cutoff**
OTX1 probe 2	6.5444
ONECUT2 probe 1	6.9711
ONECUT2 probe 4	7.3936
TWIST1	0.1318

Supplementary table 3. Sociodemographic, tumor and urine characteristics of patients presenting with hematuria (n=200) included in the study

		BC (n=97)	Nonmalignant (n=103)	*P*-value
Patient characteristics				
Age	Mean (range)	71 (38-110)	62 (50-82)	<0.01
		N (%)	N (%)	
Gender	Male	80 (83)	101 (98)	<0.01
	Female	17 (18)	2 (2)	
Tumor characteristics				
Clinical Stage	Ta	52 (54)		
	T1	28 (29)		
	T2-4	8 (8)		
	Tx†	9 (9)		
Grade	G1	25 (26)		
	G2	24 (25)		
	G3	38 (39)		
	Gx†	10 (10)		
Urine characteristics				
DNA yield (ng/ml)	DNA concentration	26.1 (1.17-110)	16.7 (1.29-104)	0.02
	Positive test			
Mutation analyses	*FGFR3*	33 / 95	1 / 99	<0.01
	TERT	70 / 96	3 / 99	<0.01
	HRAS	2 / 90	0 / 98	0.23
	Mutation combined	68 / 88	3 / 98	<0.01
Methylation	*TWIST1*	77 / 87	43 / 98	<0.01
	OTX1	74 / 91	44 / 96	<0.01
	ONECUT2	70 / 94	6 / 98	<0.01
	Methylation combined	77 / 81	65 / 92	<0.01

† Tx and Gx; stage and/or grade not specified

Supplementary table 4. Results per sample for each marker and composite model given the following algorithm: -10.156+0.110*Age+4.241*Mutation+0.905*Methylation, which is Intercept+βAge+βMutation+βMethylation. Blanks represent missing values.

Study ID	Case	Age	Mutation†	Methylation*	Combined model result	Classified as case by model
1	1	60	1	3	0,96769	1
2	1	89	1			
3	1	79	1	2	0,98988	1
4	1	70	0	1	0,1747	2
5	1	81	1	3	0,99669	1
6	1	82	1	3	0,99704	1
7	1	83	1	2	0,99346	1
8	1	78	1	3	0,99541	1
9	1	80	1	3	0,99631	1
10	1	75	1	2	0,98438	1
11	1	50	1	2	0,80128	1
12	1	77	1	3	0,99488	1
13	1	69	0	3	0,53703	1
14	1	69	1	3	0,98774	1
15	1	81	1	3	0,99669	1
16	1	80	1	2	0,99093	1
17	1	75	0			
18	1	67	1	3	0,98477	1
19	1	56	1	3	0,95072	1
20	1	71	1	3	0,99014	1
21	1		1	1		
22	1	80	0	0	0,20448	1
23	1	73	1	3	0,99207	1
24	0	82	0			
25	1		1	3		
26	1	82	1	2	0,9927	1
27	1		0	3		
28	1	86	0	3	0,88265	1
29	1	74	1	3	0,99289	1
30	1	76	1	3	0,99428	1
31	1	82	1	2	0,9927	1
32	1	84	0	3	0,85788	1

Study ID	Case	Age	Mutation†	Methylation*	Combined model result	Classified as case by model
33	1	81	1	3	0,99669	1
34	1			3		
35	1	62	1	3	0,9739	1
36	1	78	1	3	0,99541	1
37	1	77				
38	0	75	1	1	0,96224	1
39	1	67	1	3	0,98477	1
40	1	60	1	3	0,96769	1
41	1	81	1	3	0,99669	1
42	1	50	0	0	0,0094	0
43	1	81	1			
44	1	71	1	3	0,99014	1
45	1	83	0	2	0,68619	1
46	1	69		3		
47	1	62	1	3	0,9739	1
48	1	84	1	3	0,99762	1
49	1	70	1	3	0,989	1
50	1	66	1	3	0,98303	1
51	1	83	1	3	0,99734	1
52	1	65	1	3	0,9811	1
53	1	52	0	3	0,15174	0
54	1	56	0	1	0,04343	0
55	1	53	1	3	0,93275	1
56	1	70	1	2	0,97324	1
57	1	81	1	3	0,99669	1
58	1	81	1	3	0,99669	1
59	1	78		2		
60	1	110	0	1	0,94511	1
61	1	81	0	1	0,41507	1
62	1		1	3		
63	1	89	0	0	0,40882	1
64	1	68	1	3	0,98634	1
65	1	81	1	2	0,99186	1
66	1	61	0	3	0,3249	1
67	1	73	1	3	0,99207	1

Study ID	Case	Age	Mutation†	Methylation*	Combined model result	Classified as case by model
68	0		1	1		
69	1	77	0	1	0,31369	1
70	0		0			
71	1	61	1	3	0,97096	1
72	1	78		2		
73	1	57	1	1	0,77878	1
74	1	79	1			
75	1	82	1	3	0,99704	1
76	1	62	1	2	0,93784	1
77	1	61	1	3	0,97096	1
78	1	38	1	2	0,51869	1
79	1	57	1	2	0,89698	1
80	1	56	1	3	0,95072	1
81	1	65	1	3	0,9811	1
82	1	77	1	3	0,99488	1
83	1	86	1	1	0,98843	1
84	1	76	1	3	0,99428	1
85	1	72	1	3	0,99116	1
86	1	66	1			
87	1	69	1			
88	1	54	1			
89	1	58	1			
90	1		1			
91	1	59	1	3	0,96407	1
92	1	82	1	3	0,99704	1
93	1	74	0	0	0,1173	0
94	1	51	0			
95	1	76	0	2	0,50316	1
96	1	77	1	3	0,99488	1
97	1	53				
98	1	48				
99	1	57				
100	1	74				
101	1	72	0			
102	0	58				

Study ID	Case	Age	Mutation†	Methylation*	Combined model result	Classified as case by model
103	0	50	0	2	0,05487	0
104	0	57	0	1	0,04824	0
105	0	62	0	0	0,0343	0
106	0	67	0	1	0,1321	0
107	0	58	0	1	0,05354	0
108	0	53	0	1	0,03161	0
109	0	69	0	1	0,15941	0
110	0	55	0			
111	0	52	0			
112	0	58	0	1	0,05354	0
113	0	54	0	1	0,03516	0
114	0	55	0	1	0,03909	0
115	0	70	0	2	0,34363	0
116	0	55	0	1	0,03909	0
117	0	59				
118	0	58				
119	0	57	0	1	0,04824	0
120	0	59	0	1	0,0594	0
121	0	57	0	2	0,11138	0
122	0	57	0	1	0,04824	0
123	0	54	0	2	0,08267	0
124	0	57	0	1	0,04824	0
125	0	68	0	2	0,29586	1
126	0	58	0	2	0,12274	0
127	0	57	0	2	0,11138	0
128	0	56	0	1	0,04343	0
129	0	56	0	2	0,10096	0
130	0	56	0	0	0,01803	0
131	0	57	0	0	0,02008	0
132	0	54	0	0	0,01452	0
133	0	57	0	0	0,02008	0
134	0	54	0	0	0,01452	0
135	0		0	2		
136	0	53	0	1	0,03161	0
137	0	57	0	2	0,11138	0

Study ID	Case	Age	Mutation†	Methylation*	Combined model result	Classified as case by model
138	0	55	0	2	0,0914	0
139	0	58	0	2	0,12274	0
140	0	56	0			
141	0	60	0	0	0,02771	0
142	0	56	0	1	0,04343	0
143	0	59	0	2	0,13508	0
144	0	58	0	1	0,05354	0
145	0	55	0	1	0,03909	0
146	0	54	0	2	0,08267	0
147	0	58	0	2	0,12274	0
148	0	55	0	1	0,03909	0
149	0	57	0	1	0,04824	0
150	0	60	0	1	0,06585	0
151	0	59	0	1	0,0594	0
152	0	62	0	1	0,08074	0
153	0	64	0	1	0,09864	0
154	0	60	0	1	0,06585	0
155	0	61	0	0	0,03083	0
156	0	61	0	0	0,03083	0
157	0	60	0	0	0,02771	0
158	0	54	0	1	0,03516	0
159	0	64	0	0	0,04237	0
160	0	61	0	0	0,03083	0
161	0	66	0	0	0,05225	0
162	0	60	1	1	0,8304	1
163	0	65	0	1	0,10886	0
164	0	62	0	1	0,08074	0
165	0	63	0	1	0,08929	0
166	0	64	0	0	0,04237	0
167	0	66	0	0	0,05225	0
168	0	65	0	1	0,10886	0
169	0	60				
170	0	67				
171	0	67	0	0	0,05797	0
172	0	66	0	1	0,11999	0

Study ID	Case	Age	Mutation†	Methylation*	Combined model result	Classified as case by model
173	0	68	0	0	0,06428	0
174	0	69	0	0	0,07122	0
175	0	71	0	2	0,36884	1
176	0	70	0	1	0,1747	0
177	0	71	0	0	0,08721	0
178	0	75	0	1	0,26838	1
179	0	54	0	1	0,03516	0
180	0	74	0	0	0,1173	0
181	0	71	0	3	0,59105	1
182	0	72	0	1	0,20871	1
183	0	69	0	0	0,07122	0
184	0	73	0	1	0,22745	1
185	0	71	0	0	0,08721	0
186	0	72	0	2	0,39479	1
187	0	69	0	0	0,07122	0
188	0	74	0	1	0,24735	1
189	0	69	0			
190	0	55	0	1	0,03909	0
191	0	74	0	0	0,1173	0
192	0	70	0	1	0,1747	0
193	0	72	0	0	0,09637	0
194	0	73	0	3	0,64296	1
195	0	55	0	1	0,03909	0
196	0	67	0	0	0,05797	0
197	0	65	0	1	0,10886	0
198	0	75	0	2	0,47569	1
199	0	69	0	1	0,15941	0
200	0	53	0	0	0,01303	0

†Mutation: any mutation present in either *FGFR3*, *TERT* and/or *HRAS* = 1, no mutations present = 0
*Methylation: one gene methylated = 1, two genes methylated = 2, three genes methylated = 3

Supplementary table 5. Univariate and multivariable stage-specific analysis for Ta tumors (a) or T1 and T2-4 tumors (b) and grade specific analysis for low grade (G1+G2) tumors (c) or high grade (G3) tumors (d).

a) Ta tumors (n=52)					
Variable	**OR**	**95% CI**	***P*-value**	**AUC**	**95% CI**
Age	2.20	1.06-1.15	<0.01	0.72	0.61-0.82
Gender	12.02	2.53-57.23	<0.01	0.59	0.49-0.69
Mutation combined	75.51	20.19-282.46	<0.01	0.84	0.75-0.92
Methylation combined	5.48	3.12-9.64	<0.01	0.85	0.77-0.93
Ta tumors, methylation and mutation					
Mutation combined	26.23	6.07-113.32	<0.01	0.87	0.79-0.96
Methylation combined	2.52	1.27-4.99	<0.01		
Ta tumors, optimal model					
Age	1.11	1.04-1.19	<0.01	0.93	0.87-1.00
Mutation combined	48.86	7.39-323.05	<0.01		
Methylation combined	2.14	1.04-4.42	0.04		

b) T1 and T2-4 tumors (n=36)					
Variable	**OR**	**95% CI**	***P*-value**	**AUC**	**95% CI**
Age	1.15	1.08-1.21	<0.01	0.78	0.69-0.87
Gender	8.15	1.51-44.08	0.02	0.56	0.45-0.67
Mutation combined	245.42	52.04-1157.28	<0.01	0.93	0.86-0.99
Methylation combined	28.35	8.03-100.09	<0.01	0.96	0.92-1.00
T1 and T2-4 tumors, methylation and mutation					
Mutation combined	140.93	10.15-1957.03	<0.01	0.98	0.95-1.00
Methylation combined	20.53	3.65-115.50	<0.01		
T1 and T2-4 tumors, optimal model					
Age	1.12	0.94-1.32	0.20	0.99	0.98-1.00
Mutation combined	101.02	7.03-1450.91	<0.01		
Methylation combined	13.85	2.56-74.91	<0.01		

c) Low-grade tumors (n=49)					
Variable	**OR**	**95% CI**	***P*-value**	**AUC**	**95% CI**
Age	1.09	1.04-1.14	<0.01	0.68	0.57-0.79
Gender	14.62	3.10-69.02	<0.01	0.60	0.50-0.70
Mutation combined	83.49	21.80-319.67	<0.01	0.85	0.76-0.93
Methylation combined	5.72	3.20-10.24	<0.01	0.86	0.77-0.94
Low-grade tumors, methylation and mutation					
Mutation combined	30.61	6.83-137.06	<0.01	0.88	0.79-0.97
Methylation combined	2.51	1.23-5.16	0.01		
Low-grade tumors, optimal model					
Age	1.10	1.01-1.18	0.02	0.93	0.86-1.00
Mutation combined	40.75	6.62-250.81	<0.01		
Methylation combined	2.30	1.10-4.83	0.03		

d) High-grade tumors (n=38)					
Variable	**OR**	**95% CI**	***P*-value**	**AUC**	**95% CI**
Age	1.17	1.10-1.23	<0.01	0.81	0.72-0.90
Gender	7.65	1.42-41.31	0.02	0.56	0.44-0.67
Mutation combined	168.89	39.91-714.71	<0.01	0.91	0.83-0.98
Methylation combined	28.15	8.20-96.71	<0.01	0.96	0.92-0.99
High-grade tumors, methylation and mutation					
Mutation combined	144.45	9.44-2210.06	<0.01	0.98	0.96-1.00
Methylation combined	29.57	5.09-171.75	<0.01		
High-grade tumors, optimal model					
Age	1.36	0.95-1.96	0.09	1.00	0.995-1.00
Mutation combined	23867.81	0.134-…..	0.10		
Methylation combined	235.20	0.28-201420.91	0.11		

A non-invasive diagnostic urine assay to safely reduce the need for diagnostic cystoscopy in patients presenting with hematuria

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ABSTRACT

Background: Microscopic hematuria is reason for referral to the urology clinic. Only 2-5% of these patients are diagnosed with a urothelial carcinoma. Still the vast majority of all hematuria patients undergo cystoscopic evaluation to rule out bladder tumor presence. Hence, a non-invasive diagnostic test to rule in (micro)hematuria patients for cystoscopy is an unmet clinical need. In addition, a urothelial carcinoma diagnosis in women may be delayed as they are often treated for cystitis first, before they are referred to the urology clinic. An accurate rule in assay could reduce this diagnostic delay.

Objective: To assess the accuracy to detect bladder cancer of a previously developed molecular assay in a large prospective cohort of patients referred for hematuria.

Design, Setting and Participants: We prospectively included 1003 patients referred to the urology clinic for hematuria whom received a cystoscopy. Mutation status of the *FGFR3*, *TERT* and *HRAS* genes and methylation of the *OTX1*, *ONECUT2* and *TWIST1* genes was determined.

Outcome Measurements and Statistical Analysis: The predictive capacity of the urine assay, potential confounders and the association between potential predictor variables and the detection of bladder cancer were determined via logistic regression analyses.

Results: Of all patients, 59% were male and 41% female, 55% presented with macroscopic hematuria compared to 45% with microscopic hematuria. A total of 115 patients was diagnosed with urothelial cancer. The assay resulted in an AUC of 0.95 and a sensitivity of 93%, a specificity of 81% and a NPV of 99%. All 4 upper tract tumors were identified.

Conclusion: In patients referred for hematuria the urine assay was able to predict the absence of bladder cancer, especially in the microhematuria patient population with very high accuracy. This assay seems ready to be implemented clinically to select patients for diagnostic cystoscopy.

Patient Summary: Microscopic hematuria is of frequent occurrence, however in only a small percentage a malignancy will be the cause. This urine assay detected all 12 high risk tumors in microscopic hematuria patients. Therefore, we conclude that the urine assay proved accurate in this group and can be used to select microhematuria patients for diagnostic cystoscopy. The urine assay could potentially reduce the diagnostic delay of bladder cancer in female patients presenting with hematuria.

Take home message: Clinical implementation of this assay will result in a reduction of cystoscopies and a subsequent reduction of patient burden and costs. Primary care use of the assay could potentially reduce the diagnostic delay in female patients with hematuria.

INTRODUCTION

Hematuria is a frequent finding in daily clinical practice; in a health screening study among 80.000 individuals the presence of microscopic hematuria ranged from 2.4% - 31.1%.[1, 2] Persistent microscopic or macroscopic hematuria is a reason for referral to the urology clinic. Hence, 20% of all visits to the urologist are because of hematuria.[3] The cause of hematuria can either be renal (malignant or benign masses, nephropathies or pyelonephritis) or urological (stones, cystitis, strictures, benign prostatic hyperplasia, urological malignancies or trauma). Global consensus on how to evaluate hematuria patients is lacking. Most of the clinical guidelines advise a diagnostic cystoscopy in all hematuria patients, irrespective of the type of hematuria.[4-7] For example, the AUA and the Canadian guidelines recommend cystoscopy in combination with imaging in all patients with risk factors (age, smoking history). The prevalence of a bladder tumor is 10-28% in macroscopic hematuria patients and only 2-5% in microscopic hematuria patients.[7] This implies that 70-90% of patients has a different, mostly benign, cause of hematuria. Cystoscopy could be abandoned in these benign or idiopathic patients. Moreover, cystoscopy is not 100% sensitive and cannot detect upper urinary tract tumors.[8] In addition, cystoscopy is a costly and invasive diagnostic procedure, often causing discomfort and pain.[9] Hence, there is a clear clinical need for a noninvasive diagnostic test to improve selection of hematuria patients for diagnostic cystoscopy and thereby reducing costs, overtesting and patient burden.[10, 11] A screening method to rule out patients for cystoscopy would be particularly valuable in the microscopic hematuria patient population.[1, 2] Moreover, a cystitis is often thought to be the cause of microhematuria in women, leading to treatment with antibiotics, prior to referral to the urology clinic. Hence, a bladder cancer diagnosis in female patients may be erroneously delayed.[12] A urine assay that can accurately select patients for imminent urological evaluation because of the high suspicion of bladder cancer, could improve current referral policy at the level of the general practitioner.

Previously, we developed a non-invasive urine assay that was found to accurately detect bladder cancer in two case enriched series of patients presenting with hematuria.[13] This assay included methylation of the *OTX1*, *ONECUT2* and *TWIST1* genes and mutation of the *FGFR3*, *TERT* and *HRAS* genes, and showed a 93% sensitivity and 86% specificity for the detection of bladder cancer.[14] The aim of the present study was to prospectively validate this assay and evaluate its efficiency to select patients presenting with hematuria for cystoscopy, especially for the microhematuria population, and to present a simple assay to general practicioners to facilitate earlier detection of bladder cancer in women. Therefore, we collected voided urine samples before diagnostic cystoscopy

of more than 1000 patients presenting with hematuria in six Dutch hospitals. We show that in this prospective, non-case enriched cohort the assay is even more efficient when adding the micro- or gross hematuria status to our prediction model.

MATERIALS AND METHODS

Patient selection and data collection

We aimed to prospectively include at least 1000 patients referred to the urologist for evaluation of macroscopic or microscopic hematuria in six hospitals in The Netherlands. After written informed consent, patients provided a voided urine sample prior to cystoscopy. Patients younger than 18 years of age and/or with a prior history of bladder cancer were excluded from the study. Urine was collected in an 80ml container, containing a preservation tablet (Sedi-tect™, Global Scientific inc.) and was stored at 4°C. This study was approved by the institutional review board of Erasmus MC (MEC-2015-274) and additionally approved in all participating centers.

Sample processing

Samples were collected once a week from all participating centers and processed at the Erasmus MC Pathology laboratory. First, urine dipstick analysis was done on all urine samples. Samples were then spun down at 2,500 rpm for 10 minutes. Urine supernatant was discarded; the cell pellet was re-suspended in 900µl PBS and transferred to a 1.5ml Eppendorf vial. The Eppendorf vials were centrifuged once more at 6,000 rpm for 5 minutes. Excess fluids were removed and the cell pellets were stored at -20°C until DNA isolation.

Molecular analyses

DNA was isolated using the Puregene DNA Isolation kit (Fischer Scientific) and aliquoted for two separate analyses (mutation and methylation analyses). Hotspot mutation analyses for *FGFR3*, *TERT* and *HRAS* were done at the Erasmus MC Pathology laboratory in Rotterdam, The Netherlands, via single-nucleotide extension reaction (Snapshot). See supplementary table 1 for primer and probe sequences. Probes were designed to cover the 5 most frequent mutations in *FGFR3*, the 2 most frequent mutations in *TERT* and the two most frequent mutations in *HRAS*. For *FGFR3* these were; R248C and S249C (exon7); G327C, Y375C and A393E (exon 10). For *TERT* these comprised of; C228T/A, C242T and C250T, and for *HRAS* these included; G12C/S, S12D/V, G13C/R, Q61K and Q61L/R. Methylation analyses for *OTX1, ONECUT2* and *TWIST1* were done at the MDxHealth laboratory in Irvine, California, using methylation-specific PCR (MSP) analyses.

Mutation analyses (Erasmus MC Pathology laboratory) and methylation analyses (MDxHealth laboratory) were performed blinded from each other. The clinical data was also collected without knowledge of the results of the urine assay.

Statistical analysis

Patient and urine characteristics were compared between benign and malignant, by the Mann Whitney-*U*-test and the Fisher's exact test. Methylation and mutation markers were dichotomized using the same cutoff values based on previous work.[15, 16] The association between outcome and potential predictor variables was determined by univariate and multivariate logistic regression analysis. The previously published optimal multivariable logistic regression model and its cutoff were used for prospective validation.[14] The predictive capacity of the model was illustrated by the Area Under the Receiver Operating Characteristic (ROC) Curve (AUC). Further, recursive partitioning was applied to obtain a decision rule model, applicable for clinical practice. Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0, R statistical software (R Foundation for Statistical Computing, Vienna, Austria) and WEKA – University of Waikato software.

RESULTS

Inclusion and patient characteristics

Voided urine samples of 1092 patients were prospectively collected. Inclusion criteria were not met by 89 patients. For 118 patients, mutation/methylation status could not be determined for one or more of the biomarkers, resulting in 885 patients included in the final analyses (supplementary figure 1). A total of 115 patients was diagnosed with histopathologically confirmed urothelial cancer. Of the 885 patients included, 525 (59%) were male and 360 (41%) were female. Urothelial carcinoma was significantly more present in male patients (91 vs 24, $p<0.01$). Macroscopic hematuria was present in 485 patients (55%), microscopic hematuria in 400 patients (45%) and urothelial carcinoma was significantly more present in macroscopic hematuria patients (101 vs 14, $p<0.01$). See table 1 for the patient, urine and tumor characteristics of the study population.

Table 1. Patient, urine and tumor characteristics

		Urothelial carcinoma (*n*=115)	Non-malignant (*n*=770)
Patient characteristics			
Age	Median (range)	70 (39-96)	64 (19-76)
		***N* (%)**	***N* (%)**
Gender	Male	91 (79.1))	434 (56.4)
	Female	24 (20.9)	336 (43.6)
Smoking	Current	37 (32.2)	190 (24.7)
	Former	45 (39.1)	156 (20.3)
	Never	19 (16.5)	256 (33.2)
	Unknown	*14 (12.2)*	*168 (21.8)*
Urine characteristics			
Volume (ml)	Median (range)	40 (5-80)	40 (2-100)
DNA concentration (ng/ml)	Mean (range)	19.3 (0.33-120)	14.2 (0.33-400)
		***N* (%)**	***N* (%)**
Type of hematuria	Microscopic	14 (12.2)	386 (50.1)
	Macroscopic	101 (87.8)	384 (49.9)
Cytology	Not done	79 (68.8)	512 (66.5)
	No abnormalities	13 (11.3)	212 (27.5)
	Suspect	3 (2.6)	5 (0.6)
	Low Grade	2 (1.7)	1 (0.1)
	High grade	13 (11.3)	2 (0.3)
	Atypic	2 (1.7)	13 (1.7)
	No diagnosis possible	2 (1.7)	16 (2.1)
	Unknown	*1 (0.9)*	*9 (1.2)*
Tumor characteristics			
Localization	Bladder	111 (96.5)	
	Upper Urothelial Tract	4 (3.5)	
Clinical stage	Ta	61 (53.0)	
	T1	32 (27.8)	
	At least T2	12 (10.5)	
	T*is*	10 (8.7)	
Grade	G1	33 (28.7)	
	G2	24 (20.9)	
	G3	48 (41.7)	
	Gx	10 (8.7)	

		Urothelial carcinoma (*n*=115)	Non-malignant (*n*=770)
Concomitant *CIS*	Yes	15 (13.0)	
	No	86 (74.8)	
	Unknown	*14 (12.2)*	

Validation and refit

Previously, we developed a prediction model in a case enriched discovery cohort, which was subsequently validated in an external case enriched cohort (93% sensitivity and 86% specificity). This optimal multivariate prediction model contained the variables age, mutation combined and methylation combined.[13, 14] Applying this prediction model and its predefined cutoff to the present prospectively collected cohort for external validation, resulted in a sensitivity of 88%, a specificity of 80%, a negative predictive value (NPV) of 98% and a predictive capacity of 0.92 (95% CI: 0.89-0.96), as presented by the AUC. In summary, this model proved robust in this independent cohort. Next, the model was refitted on the current cohort by running a multivariate logistic regression analysis, including the same three variables as in the previous model. This refit led to a prediction model with a similar predictive capacity (optimism corrected AUC: 0.93).

Optimization of the prediction model

Univariate logistic regression analysis also identified type of hematuria and gender as possible predictor variables in this dataset. Consequently, the prediction model was optimized by adding these to the multivariate logistic regression model. This so called full model (age, mutation combined, methylation combined, gender and type of hematuria) was compared with the refit of the previous model (age, mutation combined and methylation combined) in this cohort and showed a significantly improved goodness-of-fit (Likelihood Ratio Test (LRT): P=0.0005) with a higher predictive capacity (optimism corrected AUC: 0.95). Results depicted in table 2. Hereafter, we compared this improved full model with a reduced model, from which age and gender were left out. This reduced model (mutation combined, methylation combined and type of hematuria) showed the same predictive capacity (optimism corrected AUC: 0.95) and no significant difference in goodness-of-fit (LRT: p=0.5686). The reduced model was then considered to be the optimal regression model as it showed the same performance as the full model, whilst containing only 3 predictor variables instead of 5. Figure 1 shows the ROC curve of this optimal prediction model with possible cutoffs and their corresponding sensitivity/specificity. Using the 0.049 cutoff results in a 93% sensitivity, a 81% specificity and a 99% NPV for the detection of bladder cancer in this cohort.

Table 2. Logistic regression analyses validating and optimizing the multivariable prediction model. The optimal prediction model contains the type of hematuria, mutation combined and methylation combined variables.

External Validation					
Variables	**Coefficients (β)**		**AUC**	**95% CI**	
Validation of the previously developed logistic regression model in this prospective cohort					
Intercept	-10.156		0.9246	0.8925 - 0.9567	
Age	0.110				
Mutation combined (binary)	4.241		Cutoff: 0.1917196		Sensitivity 88%
Methylation combined	0.905				Specificity 80%
Univariate analysis					
Variables	**Coefficients (β)**		**AUC**	**95% CI**	
Association between predictor variables and urothelial carcinoma					
Type of Hematuria	7.252	<0.0001	0.6898	0.6550 - 0.7246	
Gender	2.935	<0.0001	0.6140	0.5726 - 0.6550	
Multivariate analysis					
Variables	**Coefficients (β)**		**AUC**	**95% CI**	
Refit variables of previous model					
Age	1.022	0.0861	0.9320	0.8999 - 0.9642	0.9311
Mutation combined (binary)	65.326	<0.0001			
Methylation combined	2.091	<0.0001			
Full model containing all predictor variables					
Age	1.014	0.3022	0.9492	0.9249 - 0.9735	0.9461
Mutation combined (binary)	58.174	<0.0001			
Methylation combined	2.058	<0.0001			
Type of Hematuria	4.921	0.0003			
Gender	0.898	0.7749			
Optimal model (without age and gender)					
Mutation combined (binary)	56.746	<0.0001	0.9479	0.9229 - 0.9729	0.9473
Methylation combined	2.145	<0.0001	Cutoff: 0.04902711		Sensitivity 93%
Type of Hematuria	5.072	0.0002			Specificity 81%

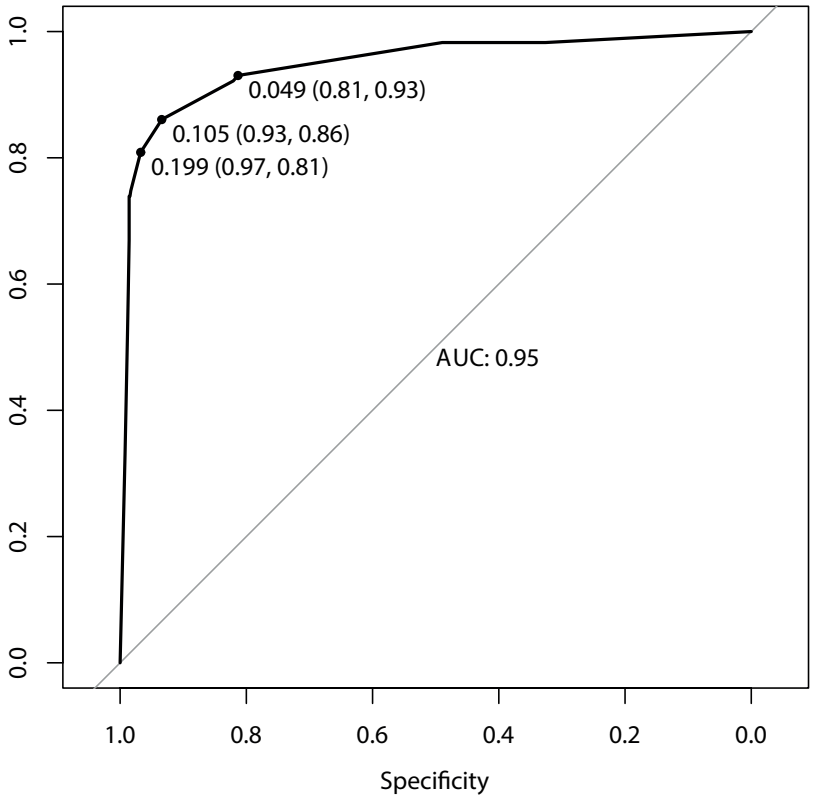

Figure 1. Receiver Operating Characteristic (ROC) curve of the optimal multivariable prediction model with 3 different cutoff values and their corresponding sensitivity/specificity. The 0.049 cutoff results in a 93% sensitivity, 81% specificity. AUC is the Area under the ROC curve expressing the predictive capacity of the optimal prediction model.

Generation of a decision model

To facilitate use of the model in the clinic, we used recursive partitioning analysis ('PART' classifier in WEKA software) for a clinically applicable decision rule model (figure 2). Decision rules were constructed after 10-fold cross-validation and a cost matrix was set to 25 on the false negatives (i.e. false negatives were considered 25 times worse than false positives) to maximize sensitivity. The variables included in the analysis were the same as included in the optimal logistic regression model (mutation combined, methylation combined and type of hematuria), all contributing to the performance of the clinical decision model. Applying the constructed decision rule model to this cohort resulted in exactly the same sensitivity, specificity and NPV (93%, 81% and 99%) as when the optimal logistic regression model was applied.

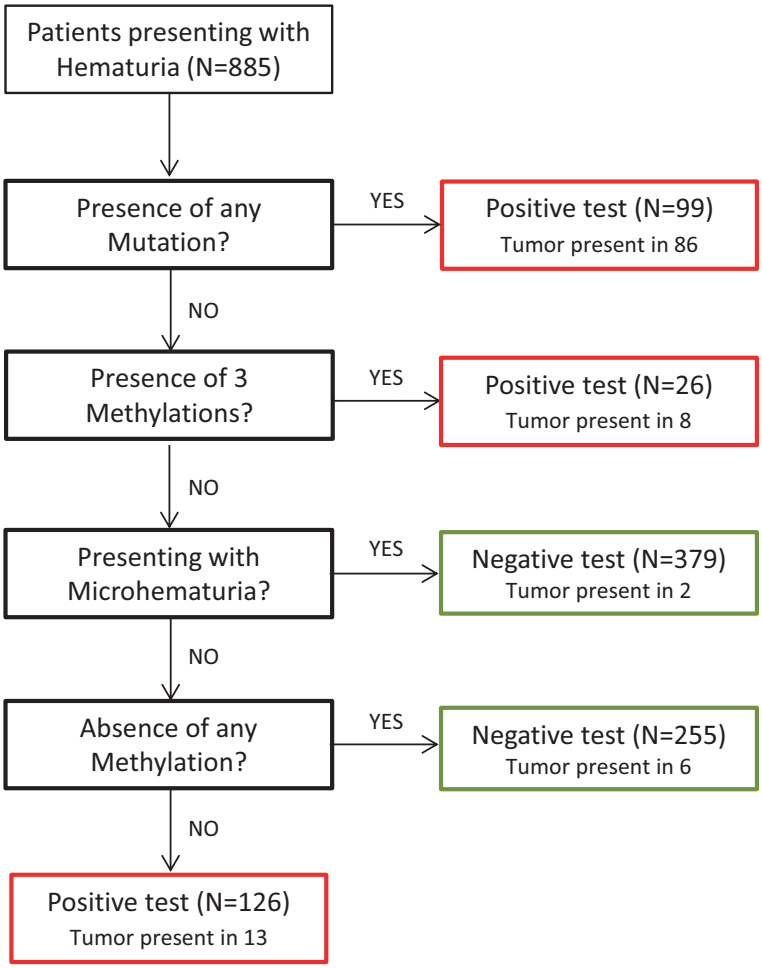

Figure 2. The constructed decision rule model. When applied to the study population leading to a 93% sensitivity, 81% specificity and 99% NPV. 107 of the 115 urothelial cancer cases had a positive test result when following the decision rule model.

DISCUSSION

Bladder cancer is a serious public health problem necessitating long term follow-up or cystectomy. One prominent symptom of bladder cancer is hematuria and most patients presenting with hematuria will undergo a cystoscopy and/or CT-scan to rule out the presence of a bladder tumor. However, by far the majority of patients presenting with hematuria does not have bladder cancer.[7] Consequently, a sensitive urine test to select

patients for cystoscopy could greatly reduce the percentage of cystoscopies. The purpose of this multicenter prospective cohort study was to provide evidence that a diagnostic urine assay based on methylation and mutation can identify patients with bladder cancer in an unselected cohort of 885 hematuria subjects with sufficient accuracy. Here we show that the assay, combined with type of hematuria (either macroscopic or microscopic) identifies the 111 bladder cancer cases as well as the 4 upper urinary tract urothelial tumors, that are not visible by cystoscopy, with a sensitivity of 93%, a specificity of 81% and a NPV of 99%. These results suggest that a reduction of 71% of cystoscopies is possible by replacing cystoscopy with the urine test.

It could be argued that patients presenting with macroscopic hematuria are not the ideal target for a urine assay since cystoscopy is mostly done as soon as possible, which may leave insufficient time for a urine assay that takes 2-4 days.[17] Cystoscopies are thereby often necessary for alternative diagnosis in macrohematuria patients. In this study, we show that macroscopic and microscopic hematuria are equally distributed in our patient population. Only a small proportion of patients with microscopic hematuria will ultimately be diagnosed with urothelial cancer (14/400, 3.5% in this cohort), while in current clinical practice a cystoscopy for urologic evaluation is performed on all. The urine assay detected all 12 high risk tumors, but missed 2 low risk (TaG1) urothelial cancer cases in the microscopic hematuria group. For these reasons, we conclude that the urine assay proved to be clinically useful in the microscopic hematuria group and can be introduced in the clinic to rule in microscopic hematuria patients for diagnostic cystoscopy. Implementation of this assay will result in a reduction of cystoscopies and thus a reduction of patient burden and costs. In addition, when the urine test becomes available for primary care, this could reduce the diagnostic delay in female patients presenting with hematuria. Moreover, we have previously reported an increase in the sensitivity of a cystoscopy when the urologist is aware of a positive urine test.[18] This could even further increase sensitivity when the urine test is used to rule in hematuria patients for cystoscopy.

A limitation of our study was that for 118 patients, mutation/methylation status could not be determined for 1 or more of the biomarker genes. In 27 patients, both the mutation and the methylation status could not be determined at all. A probable cause for these unknown assay results is a too small yield of DNA isolated from the urine samples. Indeed, the DNA concentration was significantly lower for the 118 patients that did not show an assay result for 1 or more biomarker genes, compared to the 885 patients that did show a result in all 6 genes ($p<0.01$). The DNA concentration was also significantly lower for 27/118 patients, from which both the mutation/methylation status could not be determined, when compared to the 91/118 patients that either did

show a methylation or a mutation result ($p<0.01$). In clinical setting, this limitation can be solved by requesting an additional urine sample from these patients to increase the DNA yield, as shown in previous research.[19] In addition, the urine DNA concentration was significantly higher in urothelial cancer patients ($p<0.01$), which suggests that the probability of tumor presence is smaller in patients with a low DNA concentration.

In conclusion, we proved that a diagnostic urine assay based on methylation and mutation markers is able to identify patients with urothelial carcinoma in this prospective cohort of 885 hematuria subjects with sufficient accuracy. The assay is clinically useful, especially in the microhematuria patient population, and can be implemented in the clinic to rule in patients for diagnostic cystoscopy.

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Supplementary table 1. Sequences of the primers and probes in the analysis for *FGFR3*, *TERT* and *HRAS* genes

	Primer sequence
HRAS	
HRAS exon1 Fw	5′-CAGGAGACCCTGTAGGAGG-3′
HRAS exon1 Rev	5′-TCGTCCACAAAATGGTTCTG-3′
HRAS exon2 Fw	5′-GGAGACGTGCCTGTTGGA-3′
HRAS exon2 Rev	5′-GGTGGATGTCCTCAAAAGAC-3′
TERT	
hTERT Fw 3	5′-AGCGCTGCCTGAAACTCG-3′
hTERT Rev 1	5′-CCCTTCACCTTCCAGCTC-3′
FGFR3	
FGFR3 RI Fw	5′-AGT GGC GGT GGT GGT GAG GGA G-3′
FGFR3 RI Rev2	5′-GCA CCG CCG TCT GGT TGG -3′
FGFR3 RII Fw	5′-CAA CGC CCA TGT CTT TGC AG-3′
FGFR3 RII Rev2	5′-AGGCGGCAGAGCGTCACAG-3′
	Probe sequence
HRAS	
HRAS pos.34	5′-TTTTTTTTTTTTTTTTTCTGGTGGTGGTGGGCGCC-3′
HRAS pos.182	5′-TTTTTTTTTTTTTTTTTTGCATGGCGCTGTACTCCTCC-3′
HRAS pos.35	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGCACTCTTGCCCACACCG-3′
HRAS pos.181	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCATCCTGGATACC GCCGGC-3′
HRAS pos.37	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCAGC GCACTCTTGCCCACAC-3′
TERT	
hTERT 1295228 Fw (37)	5′-TTTTTTTTTTTTTTTTTTTTGGCTGGGAGGGCCCGGA-3′
hTERT 1295242 Fw (44)	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGAGGGGGCTGGGCCGG-3′
hTERT 1295250 Fw	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCTGGGCCGGGGACCCGG-3′
FGFR3	
FGFR3-SNP R248C	5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCGTCATCTGCCCC CACAGAG-3′
FGFR3_S249C 55BP	5′-T36 TCT GCC CCC ACA GAG CGC T-3′
FGFR3-SNP G372C	5′-T29 GGT GGA GGC TGA CGA GGC G-3′
Y375C-63BP Fw	5′-T43 ACG AGG CGG GCA GTG TGT-3′
FGFR3-SNP A393E	5′-T34 CCT GTT CAT CCT GGT GGT GG-3′

Part II: Prognosis

Molecular markers increase precision of the European Association of Urology non-muscle invasive bladder cancer progression risk groups

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ABSTRACT

Purpose: The European Association of Urology (EAU) guidelines for non-muscle-invasive bladder cancer (NMIBC) recommend risk stratification based on clinicopathological parameters. Our aim was to investigate the added value of biomarkers to improve risk stratification of NMIBC.

Experimental design: We prospectively included 1,239 patients in follow-up for NMIBC in six European countries. Fresh-frozen tumor samples were analyzed for *GATA2*, *TBX2*, *TBX3* and *ZIC4* methylation and *FGFR3, TERT, PIK3CA* and *RAS* mutation status. Cox regression analyses identified markers that were significantly associated with progression to muscle-invasive disease. The progression incidence rate (PIR=rate of progression per 100 patient-years) was calculated for subgroups.

Results: In our cohort, 276 patients had a low, 273 an intermediate and 555 a high risk of tumor progression based on the EAU NMIBC guideline. Fifty-seven patients (4.6%) progressed to muscle-invasive disease. The limited number of progressors in this large cohort compared to older studies is likely due to improved treatment in the last two decades. Overall, wild type *FGFR3* and methylation of *GATA2* and *TBX3* were significantly associated with progression (HR 0.34, 2.53 and 2.64, respectively). The PIR for EAU high-risk patients was 4.25. On the basis of *FGFR3* mutation status and methylation of *GATA2* this cohort could be reclassified into a good class (PIR=0.86, 26.2% of patients), a moderate class (PIR=4.32, 49.7%) and a poor class (PIR=7.66, 24.0%).

Conclusions: We conclude that the addition of selected biomarkers to the EAU risk stratification, increases its accuracy and identifies a subset of NMIBC patients with a very high risk of progression.

Translational relevance: The EAU guideline for non-muscle-invasive bladder cancer (NMIBC) recommends risk stratification based on clinicopathological parameters only. This large prospective international study shows that the addition of *FGFR3* mutation status and *GATA2* methylation status to this risk stratification reclassifies EAU high-risk patients in good, moderate or poor progression risk subsets. Based on this biomarker subclassification of EAU high-risk patients, these patients could be allocated to different treatment strategies. Patients at very high risk of progression could receive more intensive surveillance and additional treatments or may even be considered for early cystectomy. We conclude that addition of selected biomarkers to the EAU risk stratification increases its accuracy and that the use of these progression markers has potential for implementation in the EAU NMIBC guidelines. Further validation is however recommended.

INTRODUCTION

Patients with non-muscle-invasive bladder cancer (NMIBC) have a recurrence rate of approximately 70% and in up to 15% of cases, the tumor progresses to muscle-invasive bladder cancer (MIBC) [1-3]. Hence, patients with NMIBC need to be monitored frequently for many years. This contributes to the fact that the management of patients accounts for 3% of all cancer costs in the EU (€ 143 billion in 2012) [4].

The European Association of Urology (EAU) has developed widely adopted guidelines for the treatment and follow-up of NMIBC patients. In these guidelines, patients are stratified into low-, intermediate- and high-risk groups based on clinicopathological characteristics [5]. These guidelines are based on European Organisation for Research and Treatment of Cancer (EORTC) nomograms [1]. Patients included in this large study were recruited between 1979 and 1989. Treatment and follow-up differed from current practice. For instance, BCG was not FDA approved until 1990 and the mandatory retransurethral resection (TUR) for high-risk tumors was not standard practice in the 1980s. Over the past decades, these items have changed NMIBC management, and insight on the molecular architecture of bladder tumors has dramatically increased. The EAU risk stratification does not include molecular markers, and it is important and challenging to determine whether the molecular knowledge can improve the management of NMIBC patients, particularly those at high risk of progression.

Previous work showed that activating point mutations in the *FGFR3* gene were associated with a lower chance of progression of pTa and pT1 tumors [6-8]. Methylation markers for progression of NMIBC (*GATA2*, *TBX2*, *TBX3* and *ZIC4)* were identified and validated in two small patient series [9]. Further validation of the four genes was done by Beukers and colleagues on 192 formalin-fixed paraffin-embedded (FFPE) bladder cancer samples enriched for progressing cases [10]. In this large international prospective study of NMIBC (FP7: UROMOL), we aimed to evaluate the added value of these markers to the well-established risk stratification of the EAU NMIBC guidelines [5].

MATERIALS AND METHODS

Patient selection and data collection

A total of 1,239 patients in follow-up for NMIBC (urothelial carcinoma) were prospectively included in hospitals in Denmark (n=581), Germany (n=386), Serbia (n=77), Spain (n=75), Sweden (n=84), and the Netherlands (n=36) (table 1). Inclusion criteria were patients diagnosed with NMIBC and not previously diagnosed with MIBC. Of these primary and

recurrent patients, 884 were in follow-up for stage pTa disease, 310 for pT1 disease and 45 for pTis. Only one tumor per patient was included in this study. Because treatment and follow-up regimens were based on the original pathology reports, we used these for staging and grading. Fresh-frozen tumor tissue was collected. Sections with at least 50% tumor cells were selected for DNA isolation. Clinicopathological parameters were entered in an online database. There was no attempt to modify current clinical practice at the participating centers; follow-up took place according to the national guidelines. The study was approved by the Central Denmark Region Committees on Biomedical Research Ethics (#1994/2920); the ethics committee of the University Hospital Erlangen (Erlangen, Germany; #3755); the ethics committee of the technical University of Munich (Munich, Germany; #2792/10); Medical Ethics Committee of Erasmus MC (Rotterdam, The Netherlands; MEC#168.922/1998/55); the Uppsala Region Committee on Biomedical Research Ethics (#2008/252); the Ethical Committee of Faculty of Medicine, University of Belgrade (Belgrade, Serbia; #440/VI-7); the Ethics Committee (CEIC) of Institut Municipal d'Assistència Sanitària/Hospital del Mar (Barcelona, Spain; 2008/3296/I); the ethics committee of the University Hospital Jena (Jena, Germany; #4774-4/16). Patients either gave their written informed consent, or samples were used according to "The Code for Proper Secondary Use of Human Tissues in the Netherlands" (http://www.federa.org/). The study was conducted according to the principles of the Declaration of Helsinki. In all centers standardized procedures were applied for sampling, freezing and shipment of the samples [11].

Molecular analyses

DNA was isolated using Puregene DNA Isolation kit (Thermo Fischer Scientific). Methylation of the *GATA2, TBX2, TBX3* and *ZIC4* genes was determined as described by Kandimalla and colleagues [9]. In short, DNA was converted with bisulfite (EZ-DNA Methylation Gold 30TM, Zymo Research Corp, Orange, CA, USA). The converted DNA samples were amplified in a bisulfite specific PCR. Primer and probe details can be found in supplementary table 1. After completion, the samples were treated with an Exonuclease I (EXOI)/Shrimp Alkaline Phosphatase (SAP) mixture in order to remove excess primers and dNTPs. Next, a single-nucleotide probe extension SNaPshot analysis was performed. Then, the sample was placed in an automatic sequencer (ABI PRISM 3130 XL Genetic Analyzer, Applied Biosystems). SNaPshot data was analyzed by use of GeneMarker version 2.4 (SoftGenetics). Point mutations in the *FGFR3, PIK3CA, TERT* and *RAS* oncogenes were likewise determined using a probe extension SNaPshot analysis following PCR of selected regions [12, 13].

Table 1. Patient and tumor characteristics of all included patients (N=1,239).

Patient characteristics		
Age	Mean (range)	70 (21-96)
		n (%)
Gender	Male	961 (77.6)
	Female	278 (22.4)
Smoking	Never	154 (12.4)
	Former	390 (30.8)
	Current	312 (25.2)
	Unknown	*383 (30.9)*
Country of inclusion	Denmark	581 (46.9)
	Germany	386 (31.2)
	Netherlands	36 (2.9)
	Serbia	77 (6.2)
	Spain	75 (6.1)
	Sweden	84 (6.8)
Ever diagnosed with *CIS*	Yes	191 (15.4)
	No	1048 (84.6)
Intravesical instillation	Yes	464 (37.4)
(BCG/MMC/Chemo ever)	No	774 (62.5)
	Unknown	*1 (0.1)*
Tumor characteristics		
Tumor type	Primary	583 (47.1)
	Recurrent	656 (52.9)
Stage	pTa	884 (71.3)
	pT1	310 (25.0)
	pT*is*	45 (3.6)
Grade[†]	Low grade	849 (68.5)
	High grade	353 (28.5)
	PUNLMP	12 (1.0)
	Unknown	*25 (2.0)*
Multiplicity	Solitary	879 (70.9)
	Multiple	344 (27.8)
	Unknown	*16 (1.3)*
Tumor Size	<3cm	663 (53.5)
	≥3cm	162 (13.1)
	Unknown	*414 (33.4)*

Tumor characteristics		
EAU risk category	Low	276 (22.3)
	Intermediate	273 (22.0)
	High	555 (44.8)
	Unknown	*135 (10.9)*
Prior recurrence rate	<1/year	157 (12.4)
	>1/year	86 (6.8)
	Unknown	*1028 (82.9)*
Outcome		
Progression to T2	Yes	57 (4.6)
	No	1182 (95.4)
Months of follow up	Median (range)	27.0 (0-81)
		Positive test
Mutation analyses	*FGFR3*	424 / 762 (55.6)
	TERT	571 / 770 (74.2)
	PIK3CA	171 / 778 (22.0)
	RAS	58 / 774 (7.5)
Methylation dichotomized	*GATA2*	241 / 792 (30.4)
	TBX2	447 / 792 (56.4)
	TBX3	273 / 792 (34.5)
	ZIC4	242 / 792 (30.6)

[†] Low grade included 'low grade', 'grade 1' and 'grade 2'; high grade included 'high grade', 'grade 3' and 'grade 4'.

Statistical analysis

Analyses were performed retrospectively on the data that was collected in real time. Each methylation marker was dichotomized as hypomethylated versus hypermethylated. The ROC curve was used to set a cutoff for each methylation marker by determining the optimum between sensitivity and specificity of the methylation ratio for predicting progression to MIBC. Progression-free survival (PFS) curves were estimated using the Kaplan-Meier method, followed by log-rank analysis to determine the difference between both groups. Progressive disease is defined as progression to stage T2 or higher stage disease, development of nodal or distant metastases or death of disease. Patients that died of other cause prior to progression were censored at time of death. Univariate and multivariable Cox regression analyses were performed to test the prognostic relevance of the different variables. Harrell c-statistic was defined to measure the predictive capacity. The progression incidence rate (PIR) was used to determine the

impact of a new risk stratification. The PIR is the number of progressors divided by the amount of person-years in that risk group, times 100 and can be interpreted as the rate of progression per 100 person-years of follow-up and is cumulative (14). Statistical analyses where performed by use of IBM SPSS Statistics 21 (IBM Corp.). Two-sided *P* values lesser than 0.05 were considered significant.

RESULTS

Patient and tumor characteristics

Over 47% of patients were included with a primary tumor and 37.4% received any type of intravesical instillation (table 1). Age and gender distribution was in accordance to the literature [2]. Numbers of tumors with methylation or mutations in the analyzed genes are depicted in table 1. The determined cutoffs, sensitivities and specificities for all methylation markers are listed in supplementary table 2. Distribution of clinical characteristics per country are depicted in supplementary table 3.

Relation of potential predictor variables to progression of NMIBC

According to the EAU risk stratification 276 NMIBC patients had a low-risk, 273 an intermediate-risk and 555 patients had a high-risk tumor (table 1)[5]. Progression to muscle-invasive disease was seen in one (0.4%) of low-risk tumors, eight (2.9%) of intermediate-risk tumors and 45 (8.1%) of high-risk patients, the remaining nine progressions occurred in patients of an unknown EAU risk category. In all included patients, univariate Cox regression analysis identified a significantly higher hazard ratio of progression for increasing age (HR 1.04, p=0.004) and EAU risk category (HR 5.92, p<0.001) (table 2a).

Many of the clinical parameters included in the EAU risk category were significantly correlated to progression (Carcinoma in situ (*CIS*), stage, grade and tumor size). Of the biomarkers, *FGFR3* mutations were associated with a lower hazard ratio for progression (HR 0.34, p=0.002) and methylation of *GATA2* and *TBX3* with a significantly higher hazard ratio (HR 2.53, p=0.003 and HR 2.64, p=0.002, respectively). All other potential biomarkers; mutation status of *TERT, PIK3CA, RAS,* and methylation status of *TBX2* and *ZIC4*, were not significantly associated with progression. Overall, c-statistics were highest for EAU risk category (0.70), grade (0.70), stage (0.69), *FGFR3* mutation status (0.66) and methylation of *GATA2* (0.62) (table 2a). PFS was significantly poorer in patients with higher EAU risk categorization, *FGFR3* wild-type and *GATA2* and *TBX3* methylated tumors (supplementary figure 5).

Table 2a. Univariate Cox regression analysis of potential predictor variables and time to progression in all patients (N=1,239).

Variable	n*	HR (95% CI)	*P*	c-statistic
Age	1,034	1.04 (1.01-1.07)	**0.004**	0.58
Gender	1,038	0.79 (0.40-1.56)	0.489	0.51
Smoking (ever)	794	1.13 (0.51-2.54)	0.760	0.52
Intravesical instill.	1,037	0.62 (0.36-1.07)	0.084	0.59
Primary / recurrent	1,038	1.12 (0.67-1.89)	0.664	0.51
Ever *CIS*	1,038	2.23 (1.25-3.97)	**0.007**	0.57
Stage	1,038	1.73 (1.31-2.29)	**<0.001**	0.69
Grade	1,020	4.80 (2.81-8.18)	**<0.001**	0.70
Tumor Size	742	2.12 (1.14-3.94)	**0.018**	0.56
Multiplicity	1,025	1.59 (0.94-2.70)	0.086	0.53
EAU risk category (low + interm. = reference)	946	5.92 (2.89-12.11)	**<0.001**	0.70
Mutation markers				
FGFR3	659	0.34 (0.17-0.68)	**0.002**	0.66
TERT	667	2.23 (0.87-5.73)	0.095	0.55
PIK3CA	676	1.21 (0.59-2.49)	0.605	0.49
RAS	671	0.44 (0.06-3.20)	0.416	0.51
Methylation markers				
GATA2	688	2.53 (1.36-4.71)	**0.003**	0.62
TBX2	688	1.90 (0.96-3.73)	0.064	0.56
TBX3	688	2.64 (1.41-4.92)	**0.002**	0.59
ZIC4	688	1.50 (0.79-2.81)	0.213	0.54

*n, number of patients included in that specific univariate analysis

The combination of EAU risk category, *FGFR3* mutation status, *GATA2* and *TBX3* methylation status resulted in an overall predictive capacity of 76% as calculated by the Harrell c-statistic (table 2b) and this was 0.72 for the biomarker combination without EAU risk category.

Table 2b. Multivariable Cox regression analysis of potential biomarker predictor variables and time to progression in all patients (patients with missing values were excluded).

Variables in model	HR (95% CI)	*P*	Complete model *P*	c-statistic
Biomarkers + EAU category (*n*=614)				
EAU category (low + interm. = reference)	3.08 (1.31-7.23)	0.010	<0.001	0.76
FGFR3 mutation	0.57 (0.28-1.20)	0.139		
GATA2 methylation	1.90 (0.98-3.66)	0.057		
TBX3 methylation	1.68 (0.84-3.34)	0.141		
Biomarkers (*n*=659)				
FGFR3 mutation	0.43 (0.21-0.87)	0.019	<0.001	0.72
GATA2 methylation	2.23 (1.16-4.31)	0.016		
TBX3 methylation	1.85 (0.94-3.65)	0.076		

Potential predictor variables for progression of high risk NMIBC

Because progression to MIBC was most prominent in the EAU high-risk group and a more personalized risk stratification for this group is of most benefit for patients, we next focused on this group. In this subgroup, age was again significantly associated with progression, even though the HR was low (HR 1.04, p=0.021) (table 3a). Furthermore, grade and hypermethylation of *GATA2* resulted in a significantly higher hazard ratio for progression (HR 2.28, p=0.018 and HR 2.04, p=0.046, respectively). In contrast, intravesical instillations and *FGFR3* mutations were associated with a lower hazard ratio of progression to MIBC (HR 0.35, p=0.002 and HR 0.31, p=0.010). The c-statistic was moderate for both biomarkers (*FGFR3* 0.63 and *GATA2* 0.58) (table 3a). PFS was significantly poorer in patients that did not receive intravesical instillation. In addition, higher grade, *GATA2* hypermethylation or *FGFR3* wild type were associated with a higher risk of progression (see supplementary figure 6).

Multivariable analysis showed that combining both *FGFR3* mutation status and *GATA2* methylation status resulted in an overall significant model (p=0.005), with a predictive capacity of 67% (table 3b). The PFS curves diverged significantly, p<0.01 (figure 1: good class "hypomethylated *GATA2* and mutated *FGFR3",* moderate class "either hypermethylated *GATA2* and mutated *FGFR3"* or "hypomethylated *GATA2* and wild-type *FGFR3"* and poor class "hypermethylated *GATA2* and wild-type *FGFR3"*). Supplementary figures 7 and 8 show further comparisons of EAU low-risk versus EAU high-risk good class patients and a comparison to overall high-risk EAU patients. Table 4 illustrates the distribution of characteristics of the subclassified patients.

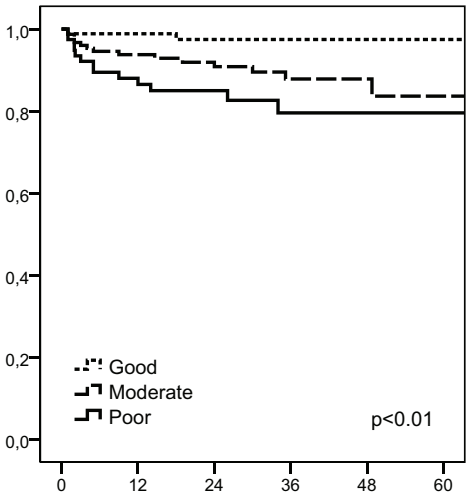
Table 3a. Univariate Cox regression analysis of potential predictor variables and time to progression in patients with high-risk tumors according to the EAU guidelines (*n*=555).

Variable	n*	HR (95% CI)	*P*	c-statistic
Age	465	1.04 (1.01-1.07)	**0.021**	0.57
Gender	467	1.00 (0.50-2.02)	0.999	0.51
Smoking (ever)	366	0.72 (0.30-1.73)	0.460	0.50
Intravesical instill.	467	0.35 (0.18-0.68)	**0.002**	0.64
Primary / Recurrent	467	1.22 (0.67-2.19)	0.518	0.53
Ever *CIS*	467	1.00 (0.54-1.84)	0.997	0.50
Stage	467	1.10 (0.76-1.59)	0.629	0.57
Grade	453	2.28 (1.15-4.52)	**0.018**	0.61
Tumor Size	283	1.28 (0.64-2.56)	0.483	0.52
Multiplicity	455	1.28 (0.70-2.36)	0.423	0.52
Mutation markers				
FGFR3	321	0.31 (0.13-0.75)	**0.010**	0.63
TERT	322	2.16 (0.75-6.20)	0.151	0.55
PIK3CA	328	1.04 (0.43-2.53)	0.933	0.50
RAS	323	0.49 (0.07-3.63)	0.494	0.52
Methylation markers				
GATA2	333	2.04 (1.01-4.10)	**0.046**	0.58
TBX2	333	1.36 (0.65-2.82)	0.413	0.52
TBX3	333	1.71 (0.86-3.43)	0.129	0.55
ZIC4	333	1.43 (0.70-2.89)	0.325	0.54

*n, number of patients included in that specific univariate analysis

Table 3b. Two multivariable Cox regression analysis of potential predictor variables and time to progression in patients with high-risk tumors according to the EAU guidelines (patients with missing values were excluded).

Variables in model	HR (95% CI)	*P*	Complete model *P*	c-statistic
Markers only (*n*=321)				
FGFR3 mutation	0.33 (0.13-0.80)	0.014	0.005	0.67
GATA2 methylation	1.88 (0.91-3.89)	0.087		
Biomarker predictor variables + grade (*n*=312)				
Grade	1.60 (0.64-4.01)	0.318	0.010	0.69
FGFR3 mutation	0.40 (0.15-1.06)	0.064		
GATA2 methylation	1.83 (0.86-3.86)	0.116		



Good	95	77	64	38	10	2	2
Moderate	180	115	86	52	21	12	15
Poor	87	58	42	26	10	1	13

Figure 1. PFS curve in patients with EAU high-risk tumors of a combination of *FGFR3* and *GATA2* status. Dotted line, good status (hypomethylated *GATA2* and mutated *FGFR3*); dashed line, moderate status (either hypermethylated *GATA2* and mutated *FGFR3* or hypomethylated *GATA2* and wild-type *FGFR3*); or solid line, poor status (hypermethylated *GATA2* and wild-type *FGFR3*). Progressive disease is defined as progression to stage T2 or higher stage disease. *P* value is based on log-rank test.

Addition of biomarkers improves EAU high-risk stratification precision

In order to compare the EAU high-risk group to the subclassified risk groups, the PIRs per risk group were calculated. In figure 2 and supplementary table 4, the PIRs for all risk groups are shown. The original EAU high-risk group had an PIR of 4.25. Using a combination of *GATA2* methylation status and *FGFR3* mutation status, 26.2% of the EAU high-risk patients could be subclassified in a good class (PIR 0.86). This is a 4.8 times lower progression risk than the original EAU high-risk stratification. In contrast, 24.0% of the original EAU high-risk patients would be subclassified as having a very high risk for progression, with an PIR of 7.66 (figure 2, supplementary table 4). Overall, the proportion of high-grade tumors and the proportion of patients ever diagnosed with *CIS* increased over the increasing risk groups (table 4). Progression to muscle-invasive disease was seen in 2.1% of the good class patients, increasing to 14.9% in the poor class patients (table 4). Based on this biomarker subclassification of EAU high-risk patients, these patients could be allocated to different management strategies (figure 2).

Table 4. Patient and tumor characteristics of all reclassified patients based on a combination of *FGFR3* mutation status and *GATA2* methylation status (*n*=362). Only high-risk patients with known marker results and known time of follow-up were included.

EAU risk group		**High risk (*n*=362)**		
Newly suggested risk groups		**Good (*n*=95)**	**Moderate (*n*=180)**	**Poor (*n*=87)**
Patient characteristics				
Age	Mean (range)	69 (47-90)	68 (37-96)	72(51-93)
		***n* (%)**	***n* (%)**	***n* (%)**
Gender	Male	69 (72.6)	139 (77.2)	72 (82.8)
	Female	26 (27.4)	41 (22.8)	15 (17.2)
Intravesical instill.	Yes	36 (37.9)	82 (45.6)	45 (51.7)
	No	59 (62.1)	98 (54.4)	42 (48.3)
Tumor characteristics				
Tumor type	Primary	59 (62.1)	87 (48.3)	51 (58.6)
	Recurrent	36 (37.9)	93 (51.7)	36 (41.4)
Stage	pTa	29 (30.5)	65 (36.1)	34 (39.1)
	pT1	62 (65.3)	104 (57.8)	49 (56.3)
	pT*is*	4 (4.2)	11 (6.1)	4 (4.6)
Grade	Low grade	59 (62.1)	60 (33.3)	14 (17.5)
	High grade	34 (35.8)	111 (61.7)	73 (83.9)
	Unknown	*2 (2.1)*	*9 (5.0)*	-
Ever *CIS*	Yes	13 (13.7)	64 (35.6)	32 (36.8)
	No	82 (86.3)	116 (64.4)	55 (63.2)
Follow-up				
Progression to T2	Yes	2 (2.1)	15 (8.3)	13 (14.9)
	No	93 (97.9)	165 (91.7)	74 (85.1)

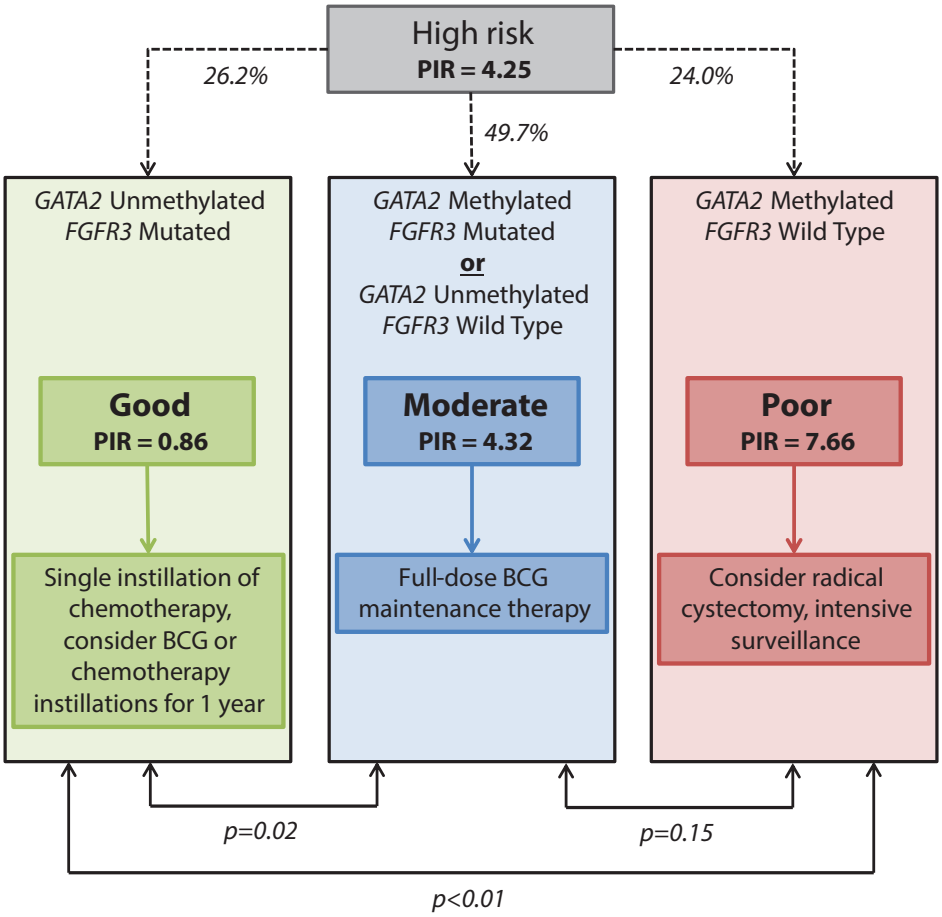


Figure 2. Reclassification of EAU high-risk group patients identifying a group with very low and very high risk, suggesting that treatment for these patients should be changed. The PIRs are depicted in the box of each risk group. The PIR is the number of progressors divided by the amount of person-years in that risk group, times 100, and can be interpreted as the rate of progression per 100 person-years. The percentages at each arrow indicate the proportion of reclassified patients that were originally classified in the EAU high-risk group. *P* values are based on log-rank test.

DISCUSSION

Currently, the EAU risk groups are used to stratify NMIBC patients for treatment and follow-up. Scoring according to this system is based on the clinicopathological parameters: tumor size, multiplicity, primary tumor, stage, grade, and *CIS* [5]. In particular, grading has been shown to be subject to interobserver variation and not all variables are always taken into account [15]. Furthermore the recommendation of the recent WHO classification (2016) to grade bladder cancer in two categories only (low and high grade) eliminates the importance of distinguishing Grade 2 from Grade 3 tumors for estimating progression in NMIBC [16]. Robust biomarkers that can improve risk assessment are therefore needed.

The purpose of this international prospective study was to investigate the previously discovered and validated methylation of *GATA2*, *TBX2, TBX3* and *ZIC4* as well as the hotspot mutations in the *FGFR3, PIK3CA, RAS* and *TERT* genes for the risk of progression of NMIBC [9, 10]. We investigated whether these biomarkers are of added value to the EAU risk groups for the stratification of NMIBC patients for the treatment and follow-up protocol. Here, we confirm that methylation of *GATA2* and mutation of *FGFR3* was associated with progression. Furthermore, a combination of *GATA2* and *FGFR3* status stratified EAU high-risk patients into three classes with different progression risks. The rate of progression per 100 patient-years (PIR) was calculated and compared among EAU high-risk plus biomarker subgroups. These data demonstrated that adding the *FGFR3* and *GATA2* biomarkers resulted in an improved prediction model. For instance, in the 24.0% of EAU high-risk patients was found to have an PIR of 7.66, which is 1.8 times higher than the entire high-risk group and almost 9 times higher than patients with the lowest risk of progression (26.2% of high-risk patients, who were subclassified in a good class). This finding suggests that these patients should receive more intensive surveillance and additional treatments or should even be considered for early cystectomy. Thus, we show that by adding the biomarkers, stratification of high-risk patients can be improved.

The value of this cohort is, its large sample size; its prospective nature; its multi-institutional and multinational character. From a statistical point of view, the low number of progressing patients (4.6% overall), is a limitation. However, the patients in this cohort were recruited between 2008 and 2013 and their progression risk is that of NMIBC patients in a 21st century urology clinic. In the large retrospective study by Sylvester and colleagues which formed the basis for the EAU risk group stratification, 11% of patients progressed to MIBC [1]. The latter patients were recruited between the years 1979 and 1989. Obviously, treatment and follow-up in those years differed considerably from current practice. In fact, the low numbers of patients with progression in our study

is most probably the result of improved treatment and surveillance protocols, e.g. MMC and BCG instillations, improved quality of endoscopic instruments and re-TUR in pT1 cases.

There is robust literature on the favorable disease course of NMIBC patients having *FGFR3* mutant tumors [6, 7, 17, 18]. In addition, van Rhijn and colleagues described the importance of *FGFR3* mutation status in pT1 tumors; *FGFR3* mutations occurred in 28% of pT1 tumors and multivariable analysis showed a hazard ratio of 2.2 for nonprogression in the multivariable analysis (p = 0.05)[19]. Although we found a higher mutation frequency in pT1 tumors (45%), we confirmed the favorable outcome of *FGFR3* mutant tumors.

Recently, a progression score based on expression of 12 genes was shown to add prognostic information beyond the EORTC risk score described by Sylvester and colleagues [1, 20]. This 12-gene signature was developed on a subset of the patient cohort in the current study. Univariate Cox regression analysis of the 12 gene score resulted in an HR of 2.39 (p<0.001) for the continuous and HR 5.08 (p<0.001) for the dichotomized risk score. Moreover, the 12-gene progression score was also able to predict PFS within the EORTC high risk patients (p=0.035 or p=0.041, depending on the cutoff used) [20]. In this study, using *FGFR3* and *GATA2* we were able to identify three groups with different progression risks within the EAU high risk patients (figure 1, p<0.01). Based on these data, we conclude that both gene expression profiling and *FGFR3*+*GATA2* analysis can be very useful to further differentiate risk groups in the EAU highest risk category. Both type of assays can be performed in a molecular pathology laboratory.

In this study, DNA was isolated from fresh-frozen tissue; therefore, we had to redetermine cutoffs for the methylation markers. Still, for clinical implementation an FFPE-based analyses is more suitable. That the assays perform well also on FFPE tissue was already shown in the study by Beukers and colleagues [10]. The biomarker assays that we used are not very expensive (€20 material cost/sample), however, a cost-effectiveness analysis of the proposed stratification should be conducted. Finally, given the number of progressing patients in the EAU high-risk group, it would be wise to further validate the combination of *FGFR3* mutations and *GATA2* methylation in a case-enriched series.

CONCLUSION

Addition of *FGFR3* mutation status and *GATA2* methylation status to the EAU risk stratification, increases its accuracy and, moreover, identifies a subset of NMIBC patients with a very high yearly risk of progression. In these very high risk patients intensive

surveillance is warranted and early cystectomy should be considered. We conclude that the use of these progression markers has potential for implementation in the EAU NMIBC guidelines. Further validation in high-risk NMIBC patients is recommended.

Acknowledgements

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Supplementary table 1. Sequences of the primers and probes used in the methylation analyses for the different CpGs in the *GATA2, TBX2, TBX3* and *ZIC4* genes. All sequences are noted from 5' to 3'.

	GATA2
Forward primer	GYGGTTTGATAATTGGTAAAT
Reverse primer	AACAAACAAAATTCRAAAACC
Amplicon size	131 bp
Probe	(T20)† ACAAACAAATTATACCTAAC
	TBX2
Forward primer	GYGAGTTAGYGGTTTGGATA
Reverse primer	CCTTACCRCCACCACCAAC
Amplicon size	113 bp
Probe	(T45)† GTTGATGGATATTGTAGT
	TBX3
Forward primer	TTTGTTAATGGTTTGTAAATTT
Reverse primer	AAGAAAAATTCRCACCTTCCC
Amplicon size	128 bp
Probe	(T13)† AATTTTGGGATTAAAAG
	ZIC4
Forward primer	TTTTTATTTTTTGAGGGTAAATATTTAGTA
Reverse primer	ATCTCCAAAAAACCTCTAAAACAAC
Amplicon size	127 bp
Probe	(T11)† AAATTTTGTTTAAATTTTT

† T20, T45, T13 and T11 represent poly-T tails of 20, 45, 13 and 11 T's, respectively. These tails are added to distinguish the different probes in order to allow a multiplex sequence run.

Supplementary table 2. Cutoffs for dichotomization of the methylation marker results were chosen based on the desired sensitivity-specificity trade-off for progression to MIBC as visualized by the receiver-operating characteristic curve (ROC curve); the point on the curve closest to the upper left corner.

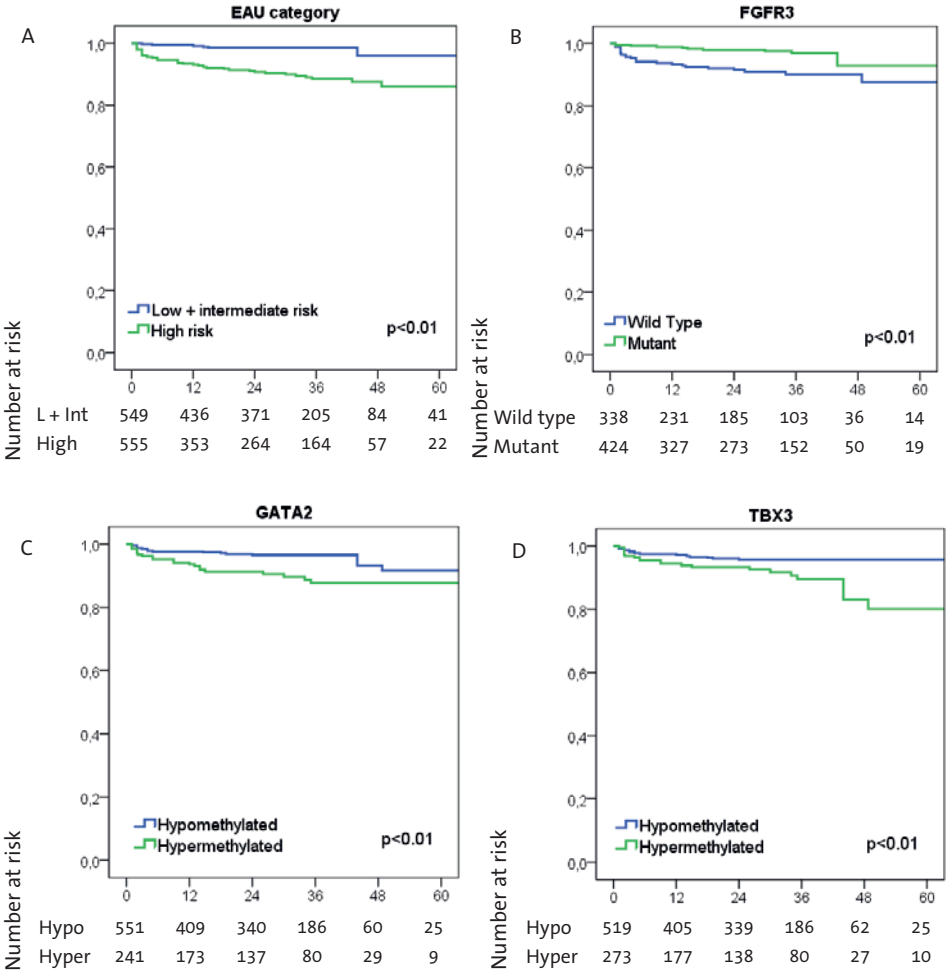
Methylation markers			
Gene	**Cutoff**	**Sensitivity**	**Specificity**
GATA2	47.4982	56.3	63.0
TBX2	7.7190	65.6	39.7
TBX3	14.0013	53.1	58.3
ZIC4	46.8911	40.6	67.9

Supplementary table 3. Distribution of the EAU category and administration of intravesical instillations per country. Between brackets the percentage (%) within each country is given.

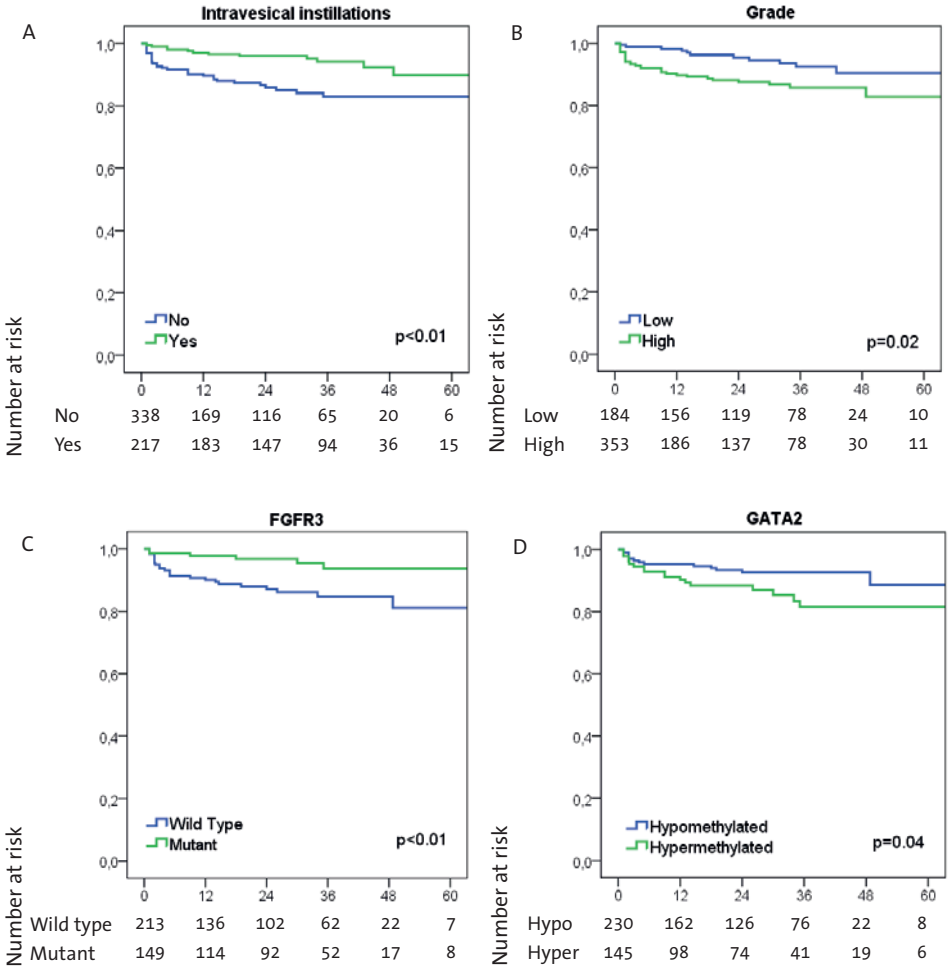
Variable	HR	Denmark *n*=581	Germany *n*=386	Netherlands *n*=36	Serbia *n*=77	Spain *n*=75	Sweden *n*=84
EAU risk category	Low	159 (27.4)	96 (24.9)	0 (0.0)	0 (0.0)	9 (12.0)	12 (14.3)
	Intermediate	163 (28.1)	70 (18.1)	5 (13.9)	0 (0.0)	6 (8.0)	29 (34.5)
	High	245 (42.2)	147 (38.1)	13 (36.1)	77 (100.0)	45 (60.0)	28 (33.3)
	Unknown	*14 (2.4)*	*73 (18.9)*	*18 (50.0)*	*0 (0.0)*	*15 (20.0)*	*15 (17.9)*
Intravesical instill.	Yes	221 (38.0)	106 (27.5)	0 (0.0)	22 (28.6)	61 (81.3)	54 (64.3)
	No	360 (62.0)	279 (72.3)	36 (100.0)	55 (71.4)	14 (18.7)	30 (35.7)
	Unknown	*0 (0.0)*	*1 (0.3)*	*0 (0.0)*	*0 (0.0)*	*0 (0.0)*	*0 (0.0)*
Progression to T2	Yes	35 (6.0)	16 (4.1)	1 (2.8)	0 (0.0)	2 (2.7)	3 (3.6)
	No	546 (94.0)	370 (95.9)	35 (97.2)	77 (100.0)	73 (97.3)	81 (96.4)

Supplementary table 4. Number of patients progression to MIBC and person-time per risk group. Suggested risk groups are based on a combination of *GATA2* methylation and *FGFR3* mutation status for EAU high-risk tumors.

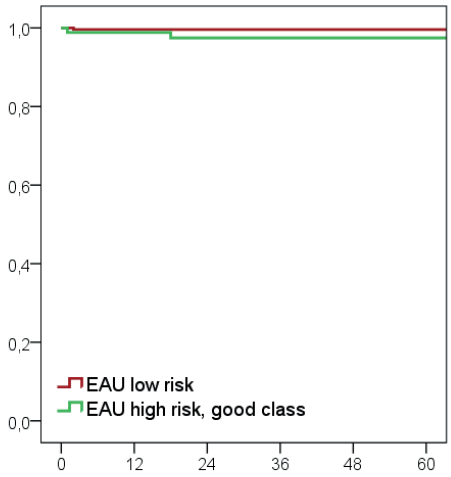
	EAU risk group	Suggested risk groups		
	High	Good	Moderate	Poor
N	555	95	180	87
Cases (progressors)	45	2	15	13
Person-time (months)	12695	2780	4162	2036
Person-time (years)	1057.92	231.67	346.83	169.67
PIR per year	0.04254	0.00863	0.04324	0.07662
% PIR per year	4.25	0.86	4.32	7.66



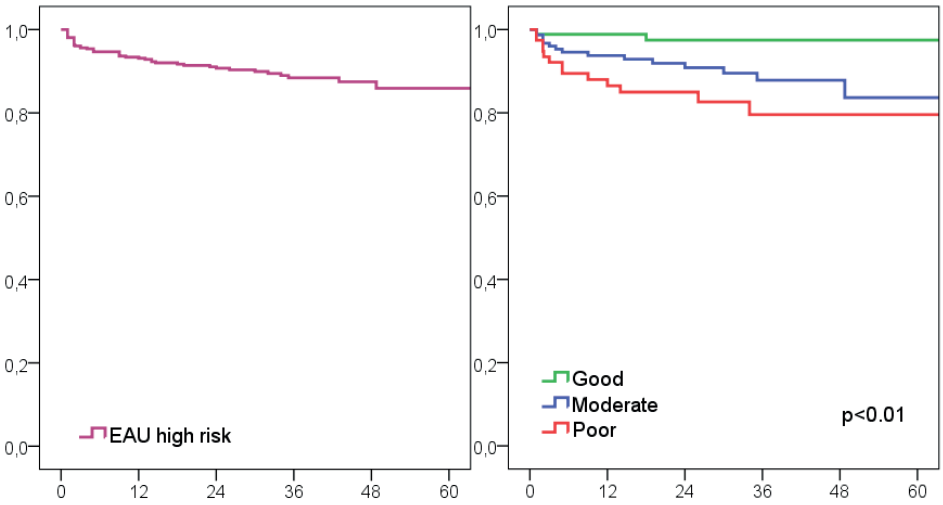
Supplementary figure 5. Progression-free survival curves in all patients for (A) EAU risk category, (B) *FGFR3*, (C) *GATA2* and (D) *TBX3*. Progressive disease is defined as progression to stage T2 or higher stage disease. *P* values (log-rank test) are indicated in each figure.



Supplementary figure 6. Progression-free survival curves in EAU high risk patients for (A) Intravesical instillations, (B) Grade, (C) *FGFR3* and (D) *GATA2*. Progressive disease is defined as progression to stage T2 or higher stage disease. *P* values (log-rank test) are indicated in each figure.



Supplementary figure 7. Progression-free survival curve in patients with all EAU low-risk tumors and EAU high-risk patients with a good class tumor (good class: hypomethylated *GATA2* and mutated *FGFR3*). Progressive disease is defined as progression to stage T2 or higher stage disease.



Supplementary figure 8. Progression-free survival curve in patients with EAU high-risk tumors of a combination of *FGFR3* and *GATA2* status. Blue: good status (hypomethylated *GATA2* and mutated *FGFR3*), green; moderate status (either hypermethylated *GATA2* and mutated *FGFR3* or hypomethylated *GATA2* and wild-type *FGFR3*) or red: poor status (hypermethylated *GATA2* and wild-type *FGFR3*) compared to a progression free survival curve in all EAU high-risk patients. Progressive disease is defined as progression to stage T2 or higher stage disease.

Elevated derived neutrophil-to-lymphocyte ratio corresponds with poor outcome in patients undergoing pre-operative chemotherapy in muscle-invasive bladder cancer

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ABSTRACT

Background: Platinum-based pre-operative chemotherapy (POC) for muscle-invasive bladder cancer (MIBC) increases the complete pathological response rate at cystectomy and improves overall survival. However, 60% of MIBC patients still has muscle-invasive disease at cystectomy despite POC. Therefore, accurate prediction of response to POC is an important clinical need. We hypothesized that an elevated neutrophil-to-lymphocyte ratio (NLR) corresponds with adverse outcome in patients undergoing POC and radical cystectomy.

Objective: To explore the correlation between the NLR and outcome in MIBC patients treated by POC and radical cystectomy.

Methods: In 123 MIBC patients (urothelial carcinoma) who were treated by platinum-based POC and radical cystectomy, the derived NLR (dNLR) was retrospectively calculated by dividing the neutrophil count by the difference between leukocytes and neutrophil counts, prior to the start of chemotherapy. The correlation of the dNLR with pathological response at cystectomy and survival was analyzed by logistic regression analysis or the Kaplan-Meier method.

Results: The complete pathological response (ypT0N0Mx) rate was 28.5%, 8.9% obtained a partial response (ypTa/T1/TisN0Mx), and 62.6% were non-responders (stage ≥ ypT2 and/or N+). An elevated dNLR (>2.21) correlated with non-response to POC (OR 2.70, 95% confidence interval: 1.15–6.38, $p = 0.02$) but this effect was nullified when corrected for clinically node-positive disease and clinical T stage. Patients with an elevated dNLR had shorter progression-free and overall survival albeit non-significant ($p = 0.42$, and $p = 0.45$, respectively).

Conclusions: An elevated dNLR corresponded with poor outcome in terms of survival and non-response to POC in MIBC patients undergoing radical surgery. However, after correction for well-known prognostic factors, such as positive lymph node status at diagnostic imaging and clinical T stage, the correlation for the dNLR was nullified. Therefore, we conclude that the dNLR is insufficient to predict response to POC in this heterogeneous patient population.

INTRODUCTION

The standard curative treatment for patients with non-metastatic MIBC is radical cystectomy, which can be preceded by pre-operative chemotherapy (POC): neoadjuvant chemotherapy (NAC) for clinical stage T2-T4aN0 or induction chemotherapy in case of N+disease[1]. The prognosis of MIBC is poor; the 5-year survival rate for stage pT2 UC is around 62% but it drops to a mere 5.5% for stage pT4[2].

Cisplatin-based chemotherapy is the current standard neoadjuvant regimen for MIBC[3]. A large multicenter randomized trial showed that a neoadjuvant regimen of cisplatin, methotrexate and vinblastine (CMV) gave a reduction in the risk of death (HR: 0.84) and an absolute increase in 10-year survival from 30% to 36% in MIBC patients[4]. In addition, another randomized trial showed that patients who received NAC of methotrexate, vinblastine, doxorubicin and cisplatin (MVAC) prior to radical cystectomy had a longer median overall survival than patients who underwent radical cystectomy alone (77 and 46 months respectively, $p = 0.06$)[5]. Despite level I evidence, controversies on the use of NAC in MIBC remain because the survival benefit is small, patients who do not respond to NAC are exposed to treatment-related toxicity[5-7]. On the contrary, the 25% of patients who do have a complete pathological response (no residual tumor) at radical cystectomy after NAC, have excellent outcomes (5-year survival rate of 80%)[8,9]. Therefore, an accurate marker that can predict response to POC in MIBC is clearly needed. Recently, genetic aberrations, such as oncogenic mutations and gene expression profiling have been reported to correspond with response to NAC[10-13]. Additionally, evidence is emerging on the causal relationship between inflammation and cancer. As part of the tumor microenvironment, neutrophils and lymphocytes both play prominent regulatory roles in tumor progression. The neutrophil-to-lymphocyte ratio (NLR) is a marker of systemic inflammatory response that reflects the balance of the inflammatory system and immune system. Several studies reported that the presence of neutrophils in tumor stroma is associated with a poor prognosis, while lymphocyte infiltration surrounding a tumor is associated with an improved prognosis[14]. Therefore, the NLR could be of prognostic value and an elevated NLR in the peripheral blood has proven to be an independent predictor of adverse outcome in several malignancies including colon, prostate and genito-urinary tract cancers[14-17]. Because of the important clinical need to accurately predict who will benefit from POC and who will not, the aim of this study was to assess the correlation between the NLR and the outcome of MIBC patients undergoing POC and radical cystectomy.

MATERIALS AND METHODS

Patient selection

The medical records from patients diagnosed with histologically proven non-metastatic MIBC (pure or predominant urothelial carcinoma) undergoing POC and radical cystectomy between 1990 and 2014 were retrospectively reviewed. A total of 283 patients was eligible for the study. Patients (n = 148) without documented neutrophil or leukocyte count at start of POC were excluded, as were patients who did not receive cisplatin- or carboplatin-based chemotherapy (n = 4), and patients who had completed insufficient cycles of POC (<3 cycles) (n = 8). Finally, 123 patients were eligible for analysis. These patients originated from three different hospitals in the Netherlands (Erasmus Medical Center, Antoni van Leeuwenhoek Hospital, Sint Franciscus Gasthuis).

Data collection and definitions

Clinicopathological parameters were collected retrospectively (Tables 1 and 2). The pathological response at cystectomy was classified as: i) complete response (CR, ypT0N0Mx); ii) partial response (PR, ypTa/T1/TisN0Mx); iii) non-response (NR, stage ≥ypT2 and/or N+). In the Netherlands, white blood cell and neutrophil counts are routinely measured in clinical practice, but this is less frequent for the lymphocyte count. Therefore, instead of the NLR, the derived NLR (dNLR) was calculated. Since peripheral white blood cells count consists almost exclusively of neutrophils and lymphocytes, the dNLR can be calculated by dividing the absolute neutrophil count by the difference between leukocytes and neutrophil counts[16]. The dNLR has been demonstrated to provide similar prognostic value as the NLR[18]. Baseline hemoglobin (HB), creatinine, Glomerular Filtration Rate (GFR) and dNLR before start of POC treatment were collected. Progression-free survival (PFS) was defined as time from POC initiation to date of cancer progression. Overall survival (OS) was defined as time from POC initiation to death from any cause. Patients were examined every three months and underwent a CT-scan of the thorax and abdomen every six months for the first two years after radical cystectomy. Lymphadenopathy at diagnostic imaging was defined as a lymph node with a short axis diameter of >10 mm in dimension on CT scan. Patients who were alive with or without disease and patients who were lost to follow up at the last follow-up date were censored.

Statistical analysis

Statistical analyses were performed using SPSS version 21 for Windows (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) and R statistical software (R Foundation for Statistical Computing, Vienna, Austria). Clinicopathological characteristics were compared between patients having a CR and

the NR group, using the Mann Whitney-U-test and the Fisher's exact test. The dNLR was dichotomized by choosing a cutoff based on the optimal sensitivity-specificity trade-off as visualized by the receiver operating characteristic (ROC), being the point on the curve which is situated closest to the upper left corner. The association of the dNLR and other predictor variables with POC response was evaluated by univariable and multivariable logistic regression analysis. The multivariable models were formed using backward elimination at the 5% level. To determine if there was a difference between the pathological response outcomes at cystectomy, uni- and multivariable analyses were performed with CR versus NR as an endpoint and CR plus PR versus NR as an endpoint. Input variables were selected based on their performance in the univariable analysis or their potential confounding effect. The predictive accuracy of the model was determined by the area under the curve (AUC) and c-statistic. Internal validation was done using the bootstrapping method with 1000 bootstrap samples. Based on the multivariable models, a POC treatment decision-making model was formed. A cutoff was determined based on the optimal sensitivity-specificity trade off of whether a positive advice concerning POC treatment should be given to patients or a negative advice. Survival curves were estimated using the Kaplan–Meier method and the difference between these curves was assessed by the log-rank test. *P* values<0.05 were considered statistically significant.

RESULTS

Baseline characteristics

The baseline characteristics of the individual response groups are depicted in table 1. The mean age of the 123 patients was 61 years of whom 91 (74%) patients were male. Of all patients, 87 (70.7%) had received 3 or 4 cycles of a cisplatin-based POC regimen (Gemcitabine/Cisplatin, Methotrexate/Vinblastine/Adriamycin/Cisplatin (MVAC), dose dense MVAC, Cisplatin/Methotrexate/Vinblastine (CMV) or Cisplatin/Paclitaxel), whereas 36 (29.3%) had received a carboplatin-based POC regimen (Gemcitabine/Carboplatin). Median time from diagnostic TUR (dTUR) to surgery was five months and median time from start of POC to surgery was three months, which both did not significantly differ between complete or non-responders ($p = 0.30$ and $p = 0.67$), respectively (Table 1).

Table 1. Baseline characteristics of patients originating from three different Dutch hospitals (Erasmus Medical Center, Antoni van Leeuwenhoek Hospital, Sint Franciscus Gasthuis) who underwent POC and radical cystectomy for MIBC (N = 123). Patients are categorized by response to POC.

Variables	CR (n=35)	PR (n=11)	NR (n=77)	*P*-value CR vs NR
Age, years (median, range)	58 (45-77)	57 (34-75)	63 (35-76)	0.76
Gender				0.82
Male	27 (77.1%)	7 (63.6%)	57 (74%)	
Female	8 (22.9%)	4 (36.4%)	20 (26%)	
Smoking status				0.74
Never	5 (14.3%)	-	10 (13.0%)	
Former	9 (25.7%)	1 (9.1%)	18 (23.4%)	
Current	9 (25.7%)	3 (27.3%)	12 (15.6%)	
Unknown	*12 (34.4%)*	*7 (63.6%)*	*37 (48.1%)*	
Charlson Comorbidity Index				0.72
3	1 (2.9%)	1 (9.1%)	-	
4	4 (11.4%)	2 (18.2%)	7 (9.1%)	
5	4 (11.4%)	-	6 (7.8%)	
6	2 (5.7%)	2 (18.2%)	4 (5.2%)	
7	-	-	2 (2.6%)	
Unknown	*24 (68.6%)*	*6 (54.5%)*	*58 (75.3%)*	
Karnofsky Performance Score				0.20
70	-	1 (9.1%)	1 (1.3%)	
80	-	-	5 (6.5%)	
90	8 (22.9%)	2 (18.2%)	8 (10.4%)	
100	3 (8.6%)	2 (18.2%)	5 (6.5%)	
Unknown	*24 (68.6%)*	*6 (54.5%)*	*58 (75.3%)*	
Tumor presentation				0.30
Primary	23 (65.7%)	10 (90.9%)	58 (75.3%)	
Recurrence	9 (25.7%)	1 (9.1%)	13 (16.9%)	
Unknown	*3 (8.6%)*	-	*6 (7.8%)*	
Clinical tumor stage				0.11
T1	3 (8.6%)	-	1 (1.3%)	
T2	8 (22.9%)	3 (27.3%)	16 (20.8%)	
T3	17 (48.6%)	6 (54.5%)	31 (40.3%)	
T4	7 (20%)	2 (18.2%)	28 (36.4%)	
Unknown	-	-	*1 (1.3%)*	
Extravesical extension at diagnostic imaging				0.18
Positive	10 (28.6%)	2 (18.2%)	33 (42.9%)	
Negative	17 (48.6%)	7 (63.6%)	29 (37.7%)	
Unknown	*8 (22.9%)*	*2 (18.2%)*	*15 (19.5%)*	
Lymphadenopathy at diagnostic imaging				<0.01
Positive	8 (22.9%)	4 (36.4%)	40 (51.9%)	
Negative	19 (54.3%)	5 (45.5%)	25 (32.5%)	
Unknown	*8 (22.9%)*	*2 (18.2%)*	*12 (15.6%)*	

Variables	CR (n=35)	PR (n=11)	NR (n=77)	*P*-value CR vs NR
Hydronephrosis at diagnostic imaging				0.17
Positive	9 (25.7%)	5 (45.5%)	32 (41.6%)	
Negative	18 (51.4%)	4 (36.4%)	32 (41.6%)	
Unknown	*8 (22.9%)*	*2 (18.2%)*	*12 (16.9%)*	
Hemoglobin level, mmol/L (median, range)	8.3 (5.5-10.3)	8.6 (7.3-9.5)	8.0 (5.5-10.0)	0.12
Creatinine level , µmol/L (median, range)	75 (56-209)	76 (65-122)	92 (46-186)	0.28
Glomerular Filtration Rate (GFR), ml/min (median, range)	87 (35-115)	80 (53-90)	72 (44-124)	0.56
POC regimen				0.51
Cisplatin based	26 (74.3%)	10 (90.9%)	51 (66.2%)	
Carboplatin based	9 (25.7%)	1 (9.1%)	26 (33.8%)	
Recurrence after cystectomy				<0.01
Yes	7 (20%)	2 (18.2%)	48 (62.3%)	
No	28 (80%)	9 (81.8%)	29 (37.7%)	
Last follow up status				<0.01
Alive, no evidence of disease	25 (71.4%)	8 (72.7%)	22 (28.6%)	
Alive, with recurrent disease	3 (8.6%)	-	6 (7.8%)	
Dead of non-disease	3 (8.6%)	1 (9.1%)	5 (6.5%)	
Dead of disease	3 (8.6%)	2 (18.2%)	39 (50.6%)	
Lost to follow up	1 (2.9%)	-	5 (6.5%)	
Time from dTUR to surgery, months (median, range)	5.0 (3-8)	5.0 (4-6)	5.0 (2-19)	0.30
Time from POC to surgery, months (median, range)	3.0 (2-4)	3.0 (2-5)	3.0 (1-9)	0.67

CR, complete response; NR, non-response; PR, partial response; POC, pre-operative chemotherapy

Complete pathological response was seen in 35 (28.5%) patients, 11 (8.9%) showed a partial response and 77 (62.6%) were classified as non-responders. In the NR group, positive lymphadenopathy prior to cystectomy was more frequent ($p = 0.01$), recurrence after cystectomy occurred more frequently ($p < 0.01$) and the median PFS and the OS were significantly shorter than in the CR group (33 vs 12 months, $p < 0.01$ and 33 vs 17 months, $p < 0.01$). The other clinicopathological parameters did not significantly differ between the two groups.

The median dNLR of all patients was 2.10 at baseline. Based on the ROC curve, the optimal cutoff value for the dNLR was determined at 2.21 (51.1% sensitivity and 71.4% specificity for predicting NR).

Pre-operative chemotherapeutic response at cystectomy

In the univariable analysis, an elevated dNLR corresponded with NR to POC in the CR vs NR analysis (OR 2.70, 95%-CI: 1.15–6.38, $p = 0.02$), but not in the CR+PR vs NR analysis (OR 1.98, 95%-CI: 0.92–4.26, $p = 0.08$) (Table 2). Extravesical extension and lymphadenopathy on diagnostic imaging before cystectomy significantly correlated with a higher non-response rate to POC in CR+PR vs NR analysis (OR 2.37, 95%-CI: 0.99–5.66, $p = 0.05$ and OR 3.30, 95%-CI: 1.38–7.87, $p = 0.01$, respectively) (table 2). To analyze the correlation between the dNLR and response to NAC and not POC, clinically node-positive patients were excluded from the analysis. In this NAC-only cohort ($n = 49$), none of the proposed predictor variables significantly correlated with NR. Also, the correspondence between dNLR and non-response was no longer statistically significant (OR 1.70 $p = 0.31$ in CR vs NR and OR 1.62 $p = 0.33$ in CR+PR vs NR).

In the multivariable analysis for both CR vs NR as for CR+PR vs NR, dNLR was removed from the model by backward elimination (table 3). The optimal multivariable model A for CR vs NR included lymphadenopathy and clinical T stage. The optimal multivariable model B for CR+PR vs NR included lymphadenopathy and extravesical extension at diagnostic imaging (table 3). The predictive capacity, represented by the AUC of the optimal model A for CR vs NR was 0.75 (96% CI 0.64–0.86, c-statistic 0.75) with an optimism-corrected AUC of 0.72 and 0.71 (95% CI 0.60–0.82, c-statistic 0.70) with an optimism-corrected AUC of 0.68 for CR+PR vs NR; model B. The type of POC was also removed by back-ward selection. However, because carboplatin-based POC is not the regimen of choice in MIBC the analyses were repeated excluding all patients who received carboplatin-based chemotherapy, leading to similar results (AUC model A: 0.79 (95% CI 0.67–0.90) and AUC model B: 0.73 (95% CI 0.60–0.86)).

Table 2a. Univariable logistic regression analyses assessing the association between potential predictive variables and response to POC at cystectomy.

	CR (n= 35) vs. NR (n=77)			CR (n=35) + PR (n=11) vs. NR (n=77)		
	OR *(95%CI)*	***P* value**	**AUC *(95% CI)***	**OR *(95% CI)***	***P* value**	**AUC *(95%CI)***
Age	1.00 *(0.95-1.05)*	0.93	0.52 *(0.39-0.65)*	1.02 *(0.98-1.07)*	0.28	0.58 *(0.46-0.69)*
Gender	1.18 *(0.36-2.69)*	0.72	0.52 *(0.40-0.63)*	1.09 *(0.47-2.53)*	0.85	0.51 *(0.40-0.62)*
Diagnostic imaging						
Extravesical extension	1.93 *(0.77-4.89)*	0.16	0.58 *(0.45-0.71)*	2.37 *(0.99-5.66)*	0.05	0.60 *(0.49-0.72)*
Lymphadenopathy	3.80*(1.45-9.98)*	0.01	0.66 *(0.54-0.78)*	3.30 *(1.38-7.87)*	0.01	0.64 *(0.53-0.76)*
Hydronephrosis	2.00 *(0.78-5.11)*	0.15	0.58 *(0.46-0.71)*	1.35 *(0.58-3.10)*	0.49	0.54 *(0.42-0.66)*
Concomitant (<50%) non-urothelial cell type at dTUR	0.66 *(0.24-1.84)*	0.43	0.47 *(0.35-0.59)*	0.74 *(0.2-1.84)*	0.54	0.48 *(0.37-0.59)*
Concomitant *CIS* at dTUR	1.04 *(0.35-3.13)*	0.94	0.50 *(0.38-0.63)*	1.48 *(0.51-4.29)*	0.48	0.53 *(0.41-0.65)*
Hemoglobin at baseline	0.70 *(0.45-1.08)*	0.11	0.40 *(0.28-0.53)*	0.66 *(0.44-1.00)*	0.05	0.39 *(0.27-0.50)*
Creatinine at baseline	1.00 *(0.99-1.02)*	0.66	0.57 *(0.45-0.69)*	1.01 *(0.99-1.02)*	0.39	0.58 *(0.48-0.69)*
GFR at baseline	0.99 *(0.97-1.02)*	0.59	0.46 *(0.31-0.60)*	0.99 *(0.97-1.02)*	0.44	0.45 *(0.32-0.58)*
Neutrophil count at baseline	1.10 *(0.93-1.29)*	0.28	0.56 *(0.45-0.68)*	0.97 *(0.89-1.07)*	0.59	0.51 *(0.41-0.62)*
Leukocyte count at baseline	1.11 *(0.96- 1.28)*	0.18	0.57 *(0.46-0.68)*	0.99 *(0.91-1.07)*	0.72	0.52 *(0.41-0.62)*
Neutrophil percentage of WBC at baseline	1.01 *(0.67-1.05)*	0.77	0.55 *(0.44-0.67)*	1.00 *(0.96-1.04)*	0.90	0.52 *(0.42-0.63)*
dNLR at baseline, continuous	1.00 *(0.67-1.49)*	0.99	0.55 *(0.44-0.67)*	0.92 *(0.65-1.31)*	0.66	0.52 *(0.42-0.63)*
dNLR at baseline, dichotomized	2.70 *(1.15-6.38)*	0.02	0.62 *(0.51-0.73)*	1.98 *(0.92-4.26)*	0.08	0.58 *(0.48-0.69)*

CR, complete response; NR, non-response; PR, partial response; OR, odds ratio; AUC, area under the Receiver Operating Characteristic curve; dTUR, diagnostic transurethral resection; GFR, glomerular filtration rate; WBC, white blood cell count; dNLR, derived neutrophil-to-lymphocyte ratio

Table 2b. Univariable logistic regression analyses assessing the association between potential predictive variables and response to POC at cystectomy excluding N+ patients.

	CR (n= 27) vs. NR (n=37)			CR (n=27) + PR (n=7) vs. NR (n=37)		
	OR *(95%CI)*	***P* value**	**AUC** *(95% CI)*	**OR** *(95% CI)*	***P* value**	**AUC** *(95%CI)*
Age	1.02 *(0.96-1.08)*	0.50	0.58 *(0.42-0.74)*	1.04 *(0.99-1.10)*	0.15	0.63 *(0.49-0.76)*
Gender	1.21 *(0.40-3.68)*	0.74	0.52 *(0.38-0.66)*	1.23 *(0.44-3.44)*	0.69	0.52 *(0.39-0.66)*
Diagnostic imaging						
Extravesical extension	1.56 *(0.46-5.23)*	0.48	0.56 *(0.38-0.73)*	2.33 *(0.73-7.43)*	0.15	0.60 *(0.44-0.77)*
Hydronephrosis	1.59 *(0.46-5.50)*	0.47	0.55 *(0.38-0.73)*	1.07 *(0.34-3.33)*	0.91	0.51 *(0.35-0.67)*
Concomitant (<50%) non-urothelial cell type at dTUR	0.98 *(0.29-3.37)*	0.97	0.50 *(0.35-0.65)*	1.21 *(0.36-4.09)*	0.76	0.52 *(0.38-0.66)*
Concomitant *CIS* at dTUR	3.17 *(0.74-13.60)*	0.12	0.60 *(0.44-0.76)*	2.72 *(0.72-10.25)*	0.14	0.59 *(0.44-0.74)*
Hemoglobin at baseline	0.89 *(0.51-1.55)*	0.67	0.47 *(0.31-0.63)*	0.89 *(0.52-1.51)*	0.66	0.47 *(0.32-0.61)*
Creatinine at baseline	1.00 *(0.99-1.02)*	0.76	0.54 *(0.38-0.69)*	1.01 *(0.99-1.03)*	0.26	0.59 *(0.45-0.73)*
GFR at baseline	1.01 *(0.98-1.04)*	0.67	0.54 *(0.34-0.73)*	1.00 *(0.97-1.03)*	0.77	0.47 *(0.29-0.66)*
Neutrophil count at baseline	1.10 *(0.93-1.29)*	0.28	0.56 *(0.45-0.68)*	0.96 *(0.86-1.08)*	0.50	0.50 *(0.36-0.63)*
Leukocyte count at baseline	1.08 *(0.91- 1.29)*	0.38	0.55 *(0.40-0.69)*	0.99 *(0.91-1.07)*	0.72	0.52 *(0.41-0.62)*
Neutrophil percentage of WBC at baseline	1.03 *(0.85- 1.25)*	0.75	0.52 *(0.37-0.66)*	0.98 *(0.89-1.08)*	0.62	0.51 *(0.37-0.65)*
dNLR at baseline, continuous	0.79 *(0.50-1.25)*	0.31	0.47 *(0.33-0.62)*	0.82 *(0.54-1.24)*	0.35	0.50 *(0.36-0.63)*
dNLR at baseline, dichotomized	1.70 *(0.61-4.76)*	0.31	0.56 *(0.42-0.71)*	1.62 *(0.62-4.25)*	0.33	0.56 *(0.42-0.69)*

CR, complete response; NR, non-response; PR, partial response; OR, odds ratio; AUC, area under the Receiver Operating Characteristic curve; dTUR, diagnostic transurethral resection; GFR, glomerular filtration rate; WBC, white blood cell count; dNLR, derived neutrophil-to-lymphocyte ratio

Table 3. Multivariable logistic regression analyses assessing the association between predictive variables and the response to POC at cystectomy.

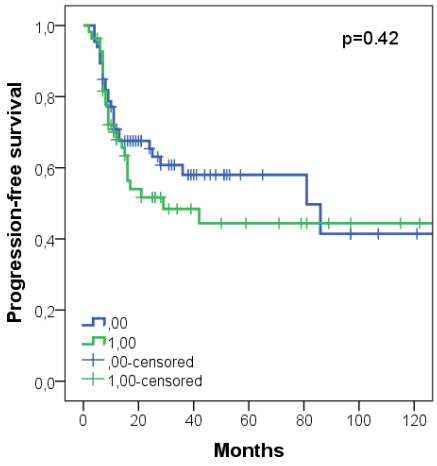
Multivariable model A **CR (n= 35) vs. NR (n=77)**	**OR *(95% CI)***	***P*-value**	**AUC *(95% CI)***	**Optimism-corrected AUC**
Step 1				
dNLR, dichotomized	2.08 *(0.69-6.33)*	0.20	0.78 *(0.68-0.88)*	
Hemoglobin at baseline	0.91 *(0.54-1.56)*	0.74		
Lymphadenopathy	5.21 *(1.69-16.11)*	<0.01		
Hydronephrosis	0.97 *(0.29-3.24)*	0.96		
Clinical T stage (T1 reference)	-	-		
T2	7.17 *(0.47-109.83)*	0.16		
T3	9.39 *(0.63-139.95)*	0.10		
T4	23.24 *(1.34-404.12)*	0.03		
POC regimen	0.75 *(0.23-2.40)*	0.63		
Final step				
Lymphadenopathy	5.49 *(1.85-16.33)*	<0.01	0.75 *(0.64-0.86)*	0.72
Clinical T stage (T1 reference)	-	-		
T2	7.97 *(0.56-114.00)*	0.13		
T3	11.11 *(0.82-150.25)*	0.07		
T4	30.30 *(1.94-473.87)*	0.02		
Multivariable model B **CR (n=35) + PR (n=11) vs. NR (n=77)**	**OR *(95% CI)***	***P*-value**	**AUC *(95% CI)***	**Optimism-corrected AUC**
Step 1				
dNLR, dichotomized	1.17 *(0.43-3.17)*	0.76	0.75 *(0.65-0.85)*	
Hemoglobin at baseline	0.81 *(0.49-1.34)*	0.41		
Lymphadenopathy	4.09 *(1.53-10.93)*	<0.01		
Extravesical extension	2.21 *(0.71-6.87)*	0.17		
Clinical T stage (T1 reference)	-	-		
T2	5.93 *(0.41-86.07)*	0.19		
T3	4.12 *(0.30-56.27)*	0.29		
T4	6.08 *(0.39-95.83)*	0.20		
POC regimen	0.65 *(0.23-1.88)*	0.43		
Final step				
Lymphadenopathy	3.81 *(1.52-9.57)*	<0.01	0.71 *(0.60-0.82)*	0.68
Extravesical extension	2.89 *(1.14-7.31)*	0.03		

CR, complete response; NR, non-response; OR, odds ratio; AUC, area under the Receiver Operating Characteristic curve; dNLR, derived neutrophil-to-lymphocyte ratio

Progression-free and overall survival

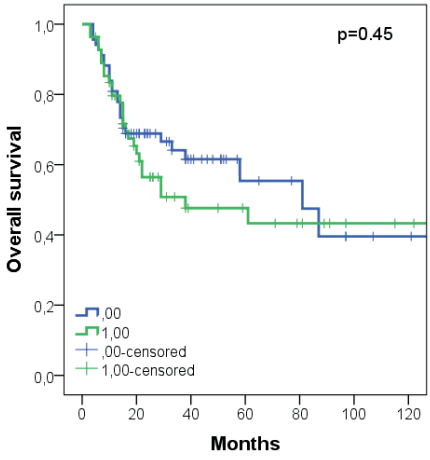
An elevated dNLR (>2.21) corresponded with an absolute shorter PFS and OS, although not significantly (median 15 vs 21 months, $p = 0.42$ and median 21 vs 24 months, $p = 0.45$, respectively) (fig. 1a and 1b). In the NAC-only cohort, the same trend was observed (median 26 vs 35 months, $p = 0.28$ and median 29 vs 35 months, $p = 0.29$, respectively) (fig. 2a and 2b). The corresponding Kaplan-Meier curves indicated that in the first 12 months after start of POC, both the PFS and OS rates of patients with low dNLR and high dNLR barely differed. However, after 12 months the survival rate lines diverged. To investigate if there were any significant differences, we compared all patients with a PFS and OS shorter than 12 months with patients with a PFS and OS of 12 months and longer. The group with a PFS <12 months had significantly higher clinical T stage, more frequent extravesical extension and lymphadenopathy on diagnostic imaging before cystectomy, less frequent cisplatin-based POC, and more often recurrence after cystectomy. There were also significantly more non-responders in both the shorter PFS and OS group (supplementary table 1 and 2). In the group with OS <12 months there was, besides more non-responders, also significantly more frequent lymphadenopathy at diagnostic imaging before cystectomy and a lower baseline hemoglobin count. The distribution of the type of POC regimen and clinical T stage was not significantly different between OS groups (supplementary table 2).

a



	N at risk									
Months	0	10	20	30	40	60	80	100	120	Median PFS
Low dNLR	66	51	35	25	18	8	7	3	2	21
High dNLR	55	37	23	15	12	9	7	4	3	15

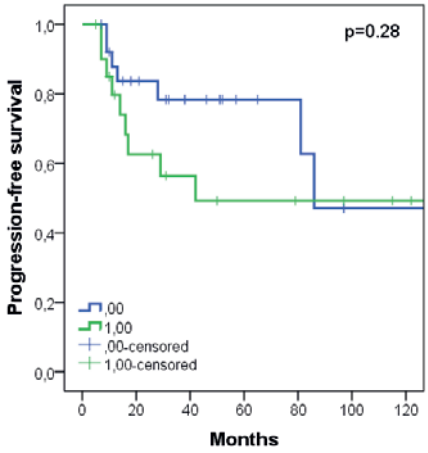
b



	N at risk									
Months	0	10	20	30	40	60	80	100	120	Median OS
Low dNLR	68	60	41	29	21	9	7	3	2	24
High dNLR	55	46	30	18	13	11	8	4	3	21

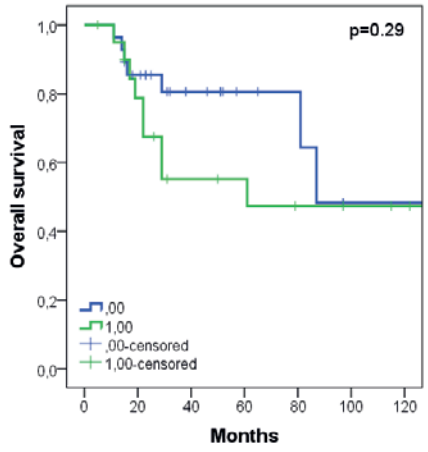
Figure 1. The progression-free survival (a) and overall survival (b) according to the dNLR. An elevated dNLR corresponded with an absolute shorter PFS (median 21 versus 15 months, p=0.42) or OS (median 24 versus 21 months, p=0.45), albeit not significantly.

a



	N at risk									
Months	0	10	20	30	40	60	80	100	120	Median PFS
Low dNLR	26	23	17	15	11	6	5	1	1	35
High dNLR	21	17	11	9	8	6	5	4	3	26

b



	N at risk									
Months	0	10	20	30	40	60	80	100	120	Median OS
Low dNLR	28	28	22	16	12	6	5	1	1	35
High dNLR	21	20	14	9	8	7	5	4	3	29

Figure 2. The progression-free survival (a) and overall survival (b) according to the dNLR , excluding node-positive patients. An elevated dNLR corresponded with an absolute shorter PFS (median 35 versus 26 months, p=0.28) or OS (median 35 versus 29 months, p=0.29), albeit not significantly.

DISCUSSION

There is an important clinical need for a marker that correlates with the response and eventual benefit by to the use of POC in patients with MIBC. Here, we show that an elevated NLR corresponded with non-response to POC at radical cystectomy. However, this correlation was nullified when corrected for positive lymph node status at diagnostic imaging and clinical T stage. Further, patients who had an elevated dNLR had a trend towards a shorter PFS and OS. This did not reach statistical significance, possibly due to the limited sample size. An elevated dNLR, did not show a statistically significant association with non-response, nor with prognosis, in a NAC-only cohort, possibly due to the limited sample size of this subgroup analysis. Our findings suggest that pre-chemotherapy dNLR, which is an easily available clinical marker, is insufficient to guide treatment decisions on POC in MIBC.

Although several studies have shown a relationship between NLR and adverse outcome in solid malignancies including MIBC[15-17], the literature on the association with response to NAC is scarce. Seah *et al*. could not find an association between pre-treatment NLR and the pathological response to NAC in MIBC patients (OR = 0.69, 95%-CI: 0.36–1.32, $p = 0.26$)[7]. Their definition of pathological response to NAC, however, differed from the definition we used; these authors defined a pathological response as T0/Tis, N0 disease together with down staging from T2/T4 to T1 and in N status (from N-positive to N0), whereas in our study a complete response to POC was defined as ypT0N0M0. More importantly, the sample size (26 patients) in the study by Seah *et al*. was too small to allow meaningful conclusions.

In esophageal cancer, the pre-treatment NLR was previously reported to be a significant predictive marker of pathologic response to NAC ($p < 0.01$)[19]. In metastatic advanced gastric cancer, as well, a low pre-treatment NLR was associated with a significantly higher chemotherapeutic disease control rate (90.0% vs. 80.4%; $p = 0.03$) and longer PFS and OS (186 vs. 146 days, $p < 0.01$ and 414 vs. 280 days, $p < 0.01$) [20].

Research of the tumor microenvironment has been intensive in recent years. The inflammatory microenvironment has been found to be an essential component of tumors[21]. The NLR is a marker of systemic inflammatory response that indicates the balance of the inflammatory system and immune system respectively[14]. The exact mechanisms underlying the prognostic implications of the NLR are unknown. However, it is known that cancer cells can induce neutrophils to form a microenvironment that

is beneficial to the malignant cells[22]. An elevated NLR is usually caused by neutrophilia together with lymphopenia and indicates a tumor-promoting environment[15]. This could explain why an elevated NLR is associated with an unfavorable outcome.

Our study has some limitations. First, this study has a retrospective design and a relatively small sample size. Data about factors that could influence the neutrophil and lymphocyte blood counts (e.g. medication) was not available for the majority of patients. Further, our cohort is a mixture of patients receiving neoadjuvant chemotherapy and induction chemotherapy. The presence of positive lymph nodes at time of start of chemotherapy and the clinical T stage had a major influence on non-response prediction. Our NAC-only cohort was probably too small to draw any significant conclusions on the predictive capability of the dNLR on therapy response. We believe this study again proves the difficulty of doing retrospective studies in such heterogeneous patient cohorts. Lastly, although the NLR might have potential as a prognostic biomarker, the use of the NLR as such a biomarker may be difficult to examine. Patients with a higher NLR had a worse outcome after cystectomy, but would perhaps have done even worse without POC. The results of this study should be re-examined in a large prospective cohort of patients with MIBC receiving POC before radical cystectomy. In addition, other molecular predictive markers like *ERBB2* and *ERCC2* mutation status may be combined with the dNLR and could potentially enhance the overall predictive capacity[10,11].

POC bears substantial short- and long-term toxicity, and hospital admission is necessary to administer cisplatin, making it a costly treatment as well. Therefore, an accurate prediction of the response to POC may improve patient selection and thus reduce patient burden by avoiding unnecessary treatment and reducing costs. The dNLR is an easily available biomarker at virtually no extra expense. However, the predictive and prognostic value of the dNLR seem insufficient in MIBC patients undergoing POC and radical cystectomy and should therefore not be used to guide treatment decisions.

CONCLUSIONS

Accurate prediction of the response to POC in MIBC may improve patient selection and thus reduce patient burden by avoiding unnecessary treatment and reduce cost. In the present study, we found that an elevated dNLR (>2.21) corresponded with poor outcome in terms of non-response to POC and shorter PFS and OS. However, this correlation was nullified when corrected for positive lymph node status and clinical T stage. Therefore, we conclude that the dNLR is insufficient to predict the response to POC in patients with MIBC undergoing radical cystectomy.

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Supplementary table 1. Univariable analysis of different potential predictor variables for patients with progression-free survival (PFS) of < 12 months or> 12 months.

Variables	**PFS <12 (n=45)**	**PFS >12 (n=69)**	***P*-value**
Age, years (median, range)	64 (41-76)	58 (34-77)	0.04
Gender			0.67
Male	34 (70.8%)	55 (75.3%)	
Female	14 (29.2%)	18 (24.7%)	
Non-Responder			<0.01
Yes	40 (83.3%)	35 (47.9%)	
No	8 (16.7%)	38 (52.1%)	
Smoking status			0.87
Never	4 (8.3%)	11 (15.1%)	
Former	10 (20.8%)	16 (21.9%)	
Current	7 (14.6%)	17 (23.3%)	
Unknown	*27 (56.3%)*	*29 (39.7%)*	
Charlson Comorbidity Index			0.10
3	-	2 (2.7%)	
4	2 (4.2%)	10 (13.7%)	
5	4 (8.3%)	6 (8.2%)	
6	4 (8.3%)	3 (4.1%)	
7	2 (4.2%)	-	
Unknown	*36 (75.0%)*	*52 (71.2%)*	
Karnofsky Performance Score			0.34
70	1 (2.1%)	1 (1.4%)	
80	3 (6.3%)	2 (2.7%)	
90	7 (14.6%)	11 (15.1%)	
100	1 (2.1%)	7 (9.6%)	
Unknown	36 (75.0%)	*52 (71.2%)*	
Tumor presentation			0.81
Primary	33 (68.8%)	57 (78.1%)	
Recurrence	9 (18.8%)	13 (17.8%)	
Unknown	*6(12.5%)*	*3 (4.1%)*	
Clinical tumor stage			<0.01
T1	-	4 (5.5%)	
T2	12 (25.0%)	14 (19.2%)	
T3	14 (29.2%)	40 (54.8%)	
T4	21 (43.8%)	15 (20.5%)	
Unknown	*1 (2.1%)*	-	
Extravesical extension at diagnostic imaging			0.01
Positive	23 (47.9%)	21 (28.8%)	
Negative	14 (29.2%)	38 (52.1%)	
Unknown	*11 (22.9%)*	*14 (19.2%)*	
Lymphadenopathy at diagnostic imaging			0.01
Positive	26 (54.2%)	26 (35.6%)	
Negative	12 (25.0%)	35 (47.9%)	
Unknown	*10 (20.8%)*	*12 (16.4%)*	

Variables	PFS <12 (n=45)	PFS >12 (n=69)	*P*-value
Hydronephrosis at diagnostic imaging			0.54
Positive	20 (41.7%)	26 (35.6%)	
Negative	19 (39.6%)	33 (45.2%)	
Unknown	*9 (18.8%)*	*14 (19.2%)*	
Hemoglobin level, mmol/L (median, range)	8.0 (5.5-9.5)	8.3 (5.5-10.3)	0.15
Creatinine level , µmol/L (median, range)	86 (53-209)	80 (46-202)	0.30
Glomerular Filtration Rate (GFR), ml/min (median, range)	74.5 (48-109)	76.5 (35-124)	0.65
POC regimen			0.03
Cisplatin based	28 (58.3%)	57 (78.1%)	
Carboplatin based	20 (41.7%)	16 (21.9%)	
Recurrence after cystectomy			<0.01
Yes	37 (77.1%)	20 (27.4%)	
No	11 (22.9%)	53 (72.6%)	
Last follow up status			<0.01
Alive, no evidence of disease	3 (6.3%)	50 (68.5%)	
Alive, with recurrent disease	5 (10.4%)	4 (5.5%)	
Dead of non-disease	6 (12.5%)	3 (4.1%)	
Dead of disease	30 (62.5%)	14 (19.2%)	
Lost to follow up	4 (8.3%)	2 (2.7%)	
Time to surgery, months (median, range)	5 (3-10)	5 (2-19)	0.75
Time from POC to surgery, months (median, range)	3 (1-6)	3 (2-9)	0.28

CR, complete response; NR, non-response; PR, partial response; POC, pre-operative chemotherapy

Supplementary table 2. Univariable analysis of different potential predictor variables for patients with an overall survival (OS) of < 12 months vs > 12 months.

Variables	OS <12 (n=29)	OS >12 (n=94)	*P*-value
Age, years (median, range)	63 (41-73)	60 (34-77)	0.44
Gender			0.48
Male	20 (69.0%)	71 (75.5%)	
Female	9 (31.0%)	23 (24.5%)	
Non-Responder			<0.01
Yes	25 (86.2%)	52 (55.3%)	
No	4 (13.8%)	42 (44.7%)	
Smoking status			0.93
Never	3 (10.3%)	12 (12.8%)	
Former	7 (24.1%)	21 (22.3%)	
Current	4 (13.8%)	20 (21.3%)	
Unknown	*15 (51.7%)*	*41 (43.6%)*	
Charlson Comorbidity Index			0.31
3	-	2 (2.1%)	
4	1 (3.4%)	12 (12.8%)	
5	2 (6.9%)	8 (8.5%)	
6	3 (10.3%)	5 (5.3%)	
7	1 (3.4%)	1 (1.1%)	
Unknown	*22 (75.9)*	*66 (70.2%)*	
Karnofsky Performance Score			0.54
70	-	2 (2.1%)	
80	2 (6.9%)	3 (3.2%)	
90	4 (13.8%)	14 (14.9%)	
100	1 (3.4%)	9 (9.6%)	
Unknown	*22 (75.9%)*	*66 (70.2%)*	
Tumor presentation			1.00
Primary	21 (72.4%)	70 (74.5%)	
Recurrence	5 (17.2%)	18 (19.1%)	
Unknown	*3 (10.3%)*	*6 (6.4%)*	
Clinical tumor stage			0.09
T1	-	4 (4.3%)	
T2	9 (31.0%)	18 (19.1%)	
T3	8 (27.6%)	46 48.9%)	
T4	12 (41.4%	25 (26.6%)	
Unknown	-	*1 (1.1%)*	
Extravesical extension at diagnostic imaging			0.81
Positive	11 (37.9%)	34 (36.2)	
Negative	11 (37.9%)	42 (44.7%)	
Unknown	*7 (24.1%)*	*18 (19.1%)*	
Lymphadenopathy at diagnostic imaging			<0.01
Positive	20 (69.0%)	32 (34.0%)	
Negative	3 (10.3%)	46 (48.9%)	
Unknown	*6 (20.7%)*	*16 (17.0%)*	

Variables	OS <12 (n=29)	OS >12 (n=94)	*P*-value
Hydronephrosis at diagnostic imaging			1.00
Positive	11 (37.9%)	35 (37.2%)	
Negative	13 (44.8%)	41 (43.6%)	
Unknown	*5 (17.2%)*	*18 (19.1%)*	
Hemoglobin level, mmol/L (median, range)	7.7 (5.5-9.5)	8.3 (5.5-10.3)	0.03
Creatinine level , µmol/L (median, range)	79 (53-138)	82 (46-209)	0.78
Glomerular Filtration Rate (GFR), ml/min (median, range)	74.5 (48-109)	78 (35-124)	0.68
POC regimen			0.49
Cisplatin based	19 (65.5%)	68 (72.3%)	
Carboplatin based	10 (34.5%	26 (27.7%)	
Recurrence after cystectomy			0.06
Yes	11 (37.9%)	39 (41.5%)	
No	18 (62.1%)	55 (58.5%)	
Last follow up status			<0.01
Alive, no evidence of disease	2 (6.9%)	53 (56.4%)	
Alive, with recurrent disease	-	9 (9.6%)	
Dead of non-disease	6 (20.7%)	3 (3.2%)	
Dead of disease	18 (62.1%)	26 (27.7%)	
Lost to follow up	3 (10.3%)	3 (3.2%)	
Time to surgery, months (median, range)	5 (3-7)	5 (2-19)	0.40
Time from POC to surgery, months (median, range)	3 (1-5)	3 (2-9)	0.68

CR, complete response; NR, non-response; PR, partial response; POC, pre-operative chemotherapy

A reported 20-gene expression signature to predict lymph node-positive disease at radical cystectomy for muscle-invasive bladder cancer is clinically not applicable

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ABSTRACT

Background*:* Neoadjuvant chemotherapy (NAC) for muscle-invasive bladder cancer (MIBC) provides a small but significant survival benefit. Nevertheless, controversies on applying NAC remain because the limited benefit must be weight against chemotherapy-related toxicity and the delay of definitive local treatment. Therefore, there is a clear clinical need for tools to guide treatment decisions on NAC in MIBC. Here, we aimed to validate a previously reported 20-gene expression signature that predicted lymph node-positive disease at radical cystectomy in clinically node-negative MIBC patients, which would be a justification for upfront chemotherapy.

Methods: We studied diagnostic transurethral resection of bladder tumors (dTURBT) of 150 MIBC patients (urothelial carcinoma) who were subsequently treated by radical cystectomy and pelvic lymph node dissection. RNA was isolated and the expression level of the 20 genes was determined on a qRT-PCR platform. Normalized Ct values were used to calculate a risk score to predict the presence of node-positive disease. The Cancer Genome Atlas (TCGA) RNA expression data was analyzed to subsequently validate the results.

Results*:* In a univariate regression analysis, none of the 20 genes significantly correlated with node-positive disease. The area under the curve of the risk score calculated by the 20-gene expression signature was 0.54 (95% Confidence Interval: 0.44-0.65) versus 0.67 for the model published by Smith *et al*. Node-negative patients had a significantly lower tumor grade at TURBT ($p = 0.03$), a lower pT stage ($p<0.01$) and less frequent lymphovascular invasion (13% versus 38%, $p<0.01$) at radical cystectomy than node-positive patients. In addition, in the TCGA data, none of the 20 genes was differentially expressed in node-negative versus node-positive patients.

Conclusions*:* We conclude that a 20-gene expression signature developed for nodal staging of MIBC at radical cystectomy could not be validated on a qRT-PCR platform in a large cohort of dTURBT specimens.

INTRODUCTION

Muscle-invasive bladder cancer (MIBC) comprises 20-25% of all newly diagnosed bladder cancers (urothelial carcinoma). Curative treatment options are few and survival is highly dependent on radical surgery and accompanying lymph node status. Patients having node-positive disease at radical cystectomy have a poor outcome. A large study on recurrence after cystectomy showed that around 80% of the cases with pathological node-positive disease (pN1-3) recurred versus only 20% of pathological node-negative organ-confined disease[1]. To eradicate micro metastases and thereby reducing the risk of node-positive disease at radical cystectomy, radical surgery can be preceded by cisplatin-based neoadjuvant chemotherapy (NAC). A clinical trial meta-analysis showed that about eight out of 100 patients with node-positive disease could benefit from upfront chemotherapy[2]. However, controversies on the use of NAC in MIBC remain because the survival benefit is small. A neoadjuvant regimen of cisplatin, methotrexate and vinblastine was shown to give a reduction in the risk of death (HR: 0.84) and an absolute increase in 10-year overall survival from 30% to 36% in a large multicenter randomized trial[3]. On the contrary, patients who do not respond to NAC experience a delay in time to curative surgery while being exposed to treatment-related toxicity. The limited survival benefit and the accompanying toxicities render many physicians reluctant to apply NAC for non-metastatic MIBC. As a result, the use of NAC in MIBC greatly varies across centers. Therefore, there is a clear clinical need for tools to guide treatment decisions on NAC in MIBC.

In other tumor types, several commercially available gene expression assays are used in clinical practice. In prostate cancer, Prolaris® and OncotypeDx Prostate® can be used for clinical decision making on surveillance of primary prostate cancer and Decipher® for clinical decisions on adjuvant therapy in surgically treated patients[4-6]. In breast cancer, assays such as OncotypeDx® and MammaPrint® have been clinically implemented[7]. In bladder cancer, a 12-gene expression signature to predict the outcome in non-muscle invasive bladder cancer was developed on a microarray platform and transferred to a qRT-PCR assay[8]. Although this 12-gene signature is still in need of further validation, it does suggest a potential role for gene expression assays in bladder cancer. Further, a 20-gene expression signature was developed to predict the presence of lymph node metastases at radical cystectomy in clinically node-negative MIBC patients. This signature might have the potential to be used to select patients for NAC[9]. Smith *et al* identified differentially expressed genes by microarray on 32 pairs of fresh frozen (FF) and formalin-fixed paraffin embedded (FFPE) tissues from three different cohorts of cystectomy specimens. Since NAC is administered before radical cystectomy, the selection for NAC would have to take place before cystectomy, i.e. after diagnostic

trans-urethral resection of the primary bladder tumor (dTURBT). Further, to clinically implement such a signature a less complex laboratory assay on paraffin-embedded tissue samples is mandatory. Therefore, we aimed to validate this 20-gene expression signature on a qRT-PCR platform in a large cohort of 150 FFPE dTURBT specimens of MIBC patients who subsequently underwent radical cystectomy and pelvic lymph node dissection.

MATERIALS AND METHODS

Patient selection and data collection

This study was approved by the Erasmus MC institutional review board (MEC-2014-641), samples were collected and analyzed according to the code of conduct for responsible use of left over materials[10]. As part of standard procedure, all patients were informed and offered an option to opt out. Patients that opted out, by written or verbal notification, were excluded from the study. In total, 201 patients who were diagnosed with MIBC (urothelial carcinoma) and who were treated by radical cystectomy and pelvic lymph node dissection were retrospectively collected for the present study. None of the patients had received NAC. In seven patients, the FFPE blocks of the dTURBT could not be retrieved. Of the remaining 194 patients, 30 patients were excluded because the tumor area did not fulfill the minimum demand of at least 70% tumor cells or the RNA quality was insufficient to complete the analyses. Another 14 patients had to be excluded because the lymph node status at time of cystectomy could not be retrieved from the pathology reports. Therefore, 150 patients were included in the qRT-PCR analyses. Based on reliability criteria (see RNA expression data analysis) another 11 patients were excluded from the statistical analysis leaving 139 patients for the final analyses (fig 1).

RNA expression data

The 20 genes included in the assay were: *TOX3, SLC11A2, FAM36A/COX20, LIMCH1, RAB15, AVL9, PCMTD2, PTHLH, DPP4, PCDHGA10, MT1E, MAP4K4, SLC16A1, BST2, MMP14, IFI27, NCLN, HLA G, RRBP1* and *ICAM1*. The Taqman assays were chosen based on the Affimetrix probes used by Smith *et al*[9] and were selected by best coverage and exon spanning. First, the qRT-PCR of the 20 genes was optimized using cell line RNA (TCCSUP) and pooled FFPE derived tumor RNA by dilution series and calibration lines per gene. Then, all FFPE tumor samples (H&E slides) were centrally reviewed to select areas that contained at least 70% tumor cells. Of these tumor cell areas, a 2.2-mm core biopsy was taken (Beecher Instruments®, Silver Spring, MD, USA). The core was deparaffinized

and RNA was isolated by High Pure FFPE RNA Micro Kit (Roche Applied Science®, Mannheim, Germany) according to the manufacturer's protocol. The RNA concentration was measured using the Qubit RNA Assay (Invitrogen, Ltd, Paisley UK). Next, total RNA was reverse transcribed and cDNA was synthesized using a pool of 22 Gene Expression Taqman assays (20 genes + 2 housekeeping genes). The assay was done in two replicates for all samples. Then, 2 µl of the cDNA was pre-amplified using Pooled Gene Expression TaqMan assays and TaqMan PreAmp Master Mix (Applied Biosystems, Foster City, USA). Amplification was done in 15 cycles of 15 seconds at 95°C and 4 minutes at 60°C each. Pre-amplification was then followed by denaturation of 10 minutes at 99.9°C. Quantitative qRT-PCR was done in duplicate using the 7500 FAST Real Time PCR System (Applied Biosystems, Foster City, USA) including the preAmp cDNA, TaqMan Universal Master MIX II and single Gene Expression TaqMan assays (both Applied Bio-systems, Foster City, USA). For normalization purposes, the housekeeping genes *HPRT*, *ACTB* and a plate control (T24 bladder cancer cell line RNA) was also included in the assay[11]. Two patient samples were run per plate, see for the plate design supplementary fig. 1. The qRT-PCR was done under the following conditions: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The threshold for determining the Ct value was set at 0.05. Since the amplification efficiency of the different assays was good, the comparative delta Ct method was used to quantify the gene expression levels.

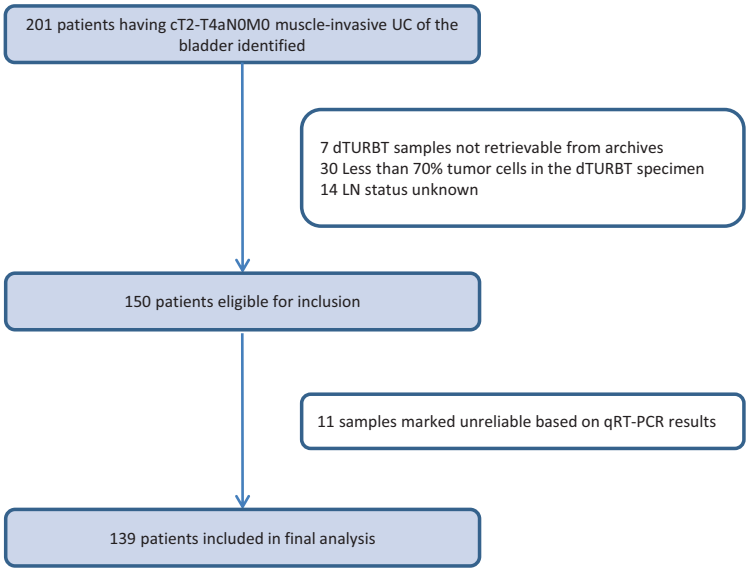

Figure 1. Flowchart of the selection of patients for the present study. UC: Urothelial Carcinoma, dTURBT: diagnostic transurethral resection of a bladder tumor, LN: lymph node.

RNA expression data analysis

The qPCR data were analyzed and visualized using the R/Bioconductor package HtqPCR version 1.26.0[12]. Sample data were split according to the qPCR plate design. A qPCR value was set to its replicated Ct value if the qPCR result was undetermined. However, if both replicated Ct values were undetermined both Ct values were set to 35 and were marked as undetermined. The qPCR data were set as unreliable if the Ct value was outside a confidence interval of 95% within each transcript. Samples were discarded if the housekeeping genes *ACTB* and *HPRT* had two or more data points that were undetermined or unreliable.

Correlation plots were generated using the Pearson's product-moment correlation coefficient values (*r*) and the subsequent hierarchical clustering was employed through complete linkage using the 1-*r* distances. Since *ACTB* expression analysis was the highest expressed gene and had the least missing values, all assays were normalized over the average *ACTB* Ct value resulting in a ΔCt value. Overall, the gene expression analysis was robust within each patient as depicted by the plots of the duplicate samples per patient (Supplementary fig. 2).

For each patient a risk score was calculated using the normalized Ct values based on the formula: the average ΔCt value of the genes downregulated in lymph node-positive (LN+) tumors minus the average ΔCt value of the genes upregulated in LN+ tumors according to Smith *et al*[9]. This formula was previously performed for validating microarray data using qPCR data[8]. Subsequently, using the risk score a logistic regression analysis was performed to predict the LN status. The area under the Receiver Operating Characteristic (ROC) curve was calculated and compared to the Area Under the Curve (AUC) reported by Smith *et al*[9].

The Cancer Genome Atlas (TCGA) clinical data and mRNA expression data was downloaded with CGDSR R package version 1.2.5 (https://github.com/cBioPortal/cgdsr)[13]. The lymph node-positive group was indicated by N1-3 and the lymph node-negative group by N0. The beeswarms were plotted with the R package beeswarm version 0.2.3 (https://github.com/aroneklund/beeswarm) and the Mann Whitney-*U*-test was applied to compare lymph node-positive with lymph node-negative patients. Statistical analyses were performed using SPSS for Windows (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp) and R statistical software version 3.3.1 (R Core Team, 2016), which was also used for plotting the data.

RESULTS

Patient and tumor characteristics

The clinical and histopathological characteristics of the 139 included patients are described in table 1. The mean age was 64 years and 111 (80%) patients were male. None of the patient characteristics significantly differed between node-positive and node-negative patients. Node-negative patients had a significantly lower tumor grade at TURBT ($p = 0.03$), a significantly lower pT stage disease at radical cystectomy ($p<0.01$) and less frequent lymphovascular invasion at radical cystectomy than node-positive patients (13% versus 38%, $p<0.01$).

The median RNA yield was similar for both node-negative and node-positive patients: 606 ng/µL (range 32-5360) versus 428 ng/µL (range 44-3000), $p = 0.81$.

20-gene expression signature validation

After normalization, the mean expression level differences per gene for node-positive versus node-negative patients were plotted in a bar plot (fig 2). The overall risk score per patient was calculated and a ROC curve was used to visualize the discriminatory value of the overall risk score. The predictive capacity of this risk score was minimal with an AUC of 0.54 (95% Confidence Interval (CI): 0.44-0.65, fig 3). Of the 20 genes included in the model, normalized *BST2* and *IF127* ΔCt values were the best performing individual predictors resulting in an odds ratio of 1.10 (95% CI: 0.96-1.25) and 1.11 (95% CI: 0.95-1.30) respectively (table 2). Multivariate analysis of the ΔCt values of these two genes combined resulted in an AUC of 0.59 (95% CI: 0.49-0.69).

Beeswarm plots of the different genes showed the distribution of ΔCt values for node-positive and node-negative samples per gene (*BST2* and *IF127* are shown in fig 4, whereas all genes are shown in supplementary fig. 3). Distribution of the ΔCt values in these plots showed major overlap in all genes with some outliers. Mean and median results of the ΔCt values showed similar trends in most genes (node-positive versus node-negative) with some exceptions (e.g. *TOX3*, median expression level higher in node-positive versus node-negative, mean expression level lower in node-positive versus node-negative).

Table 1. Patient and tumor characteristics of all included patients with lymph node-negative or lymph node-positive disease (*n*=139). The *P*-value indicates the (non)significant differences between both groups.

		LN negative (*n*=91)	LN positive (*n*=48)	*P*-value
Patient characteristics				
Age	Mean (range)	65 (32-83)	63 (37-81)	0.12
		N (%)	N (%)	
Gender	Male	74 (81)	37 (77)	0.35
	Female	17 (19)	11 (23)	
Smoking	Current	40 (44)	12 (25)	0.15
	Former	13 (14)	11 (23)	
	Never	12 (13)	7 (15)	
	Unknown	*26 (29)*	*18 (38)*	
dTURBT characteristics				
Clinical Stage	T2	91 (100)	47 (98)	0.35
	T3	-	1 (2)	
Grade (WHO 1973)	G2	8 (9)	2 (4)	0.03
	G3	74 (81)	46 (96)	
	Gx†	9 (10)	-	
Concomitant *CIS*	Yes	16 (18)	14 (29)	0.49
	No	63 (70)	33 (69)	
	Unknown	*3 (3)*	*1 (2)*	
Cystectomy characteristics				
Pathological Stage	T0	11 (12)	1 (2)	**<0.01**
	Tis	2 (2)	-	
	T1	4 (4)	-	
	T2	20 (22)	7 (15)	
	T3	21 (23)	17 (35)	
	T4	8 (9)	6 (13)	
	Tx†	25 (28)	17 (35)	
Grade (WHO 1973)	G2	6 (7)	1 (2)	0.70
	G3	47 (52)	29 (60)	
	Gx†	38 (42)	18 (38)	
Concomitant *CIS*	Yes	22 (24)	11 (23)	1.00
	No	11 (12)	5 (10)	
	Unknown	*58 (64)*	*32 (67)*	

		LN negative (*n*=91)	LN positive (*n*=48)	*P*-value
LVI	Yes	12 (13)	18 (38)	**<0.01**
	No	14 (15)	2 (4)	
	Unknown	*65 (72)*	*28 (58)*	
Total LN dissected	Mean (range)	16 (2-40)	15 (2-33)	0.94
Total LN positive	Mean (range)	-	2 (1-14)	**<0.01**
Sample characteristics				
RNA yield (ng/µl)	Median (range)	606 (32-5360)	428 (44-3000)	0.81

LN: lymph nodes, CIS: carcinoma-in-situ, LVI: lymphovascular invasion, †: stage and/or grade not specified

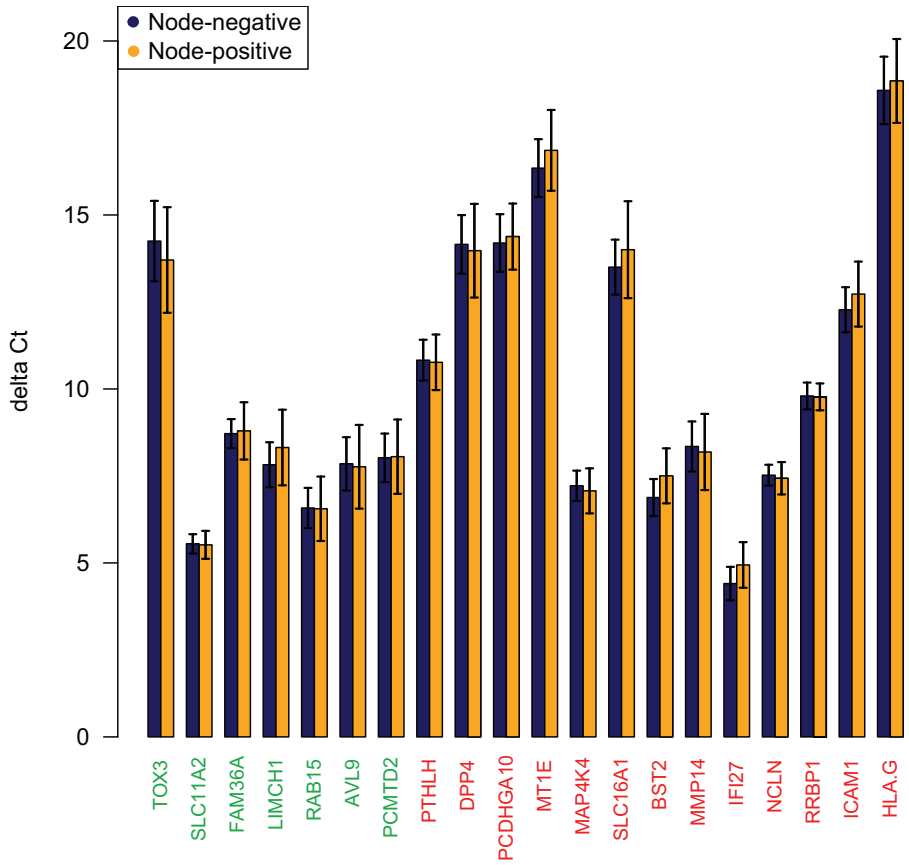

Figure 2. Risk score calculation, a barplot showing the mean ΔCt values per gene in node-negative versus node-positive patients. Gene names colored red indicate higher expression in node-positive patients and gene names colored green indicate lower gene expression in node-positive patients according to Smith *et al*[9].

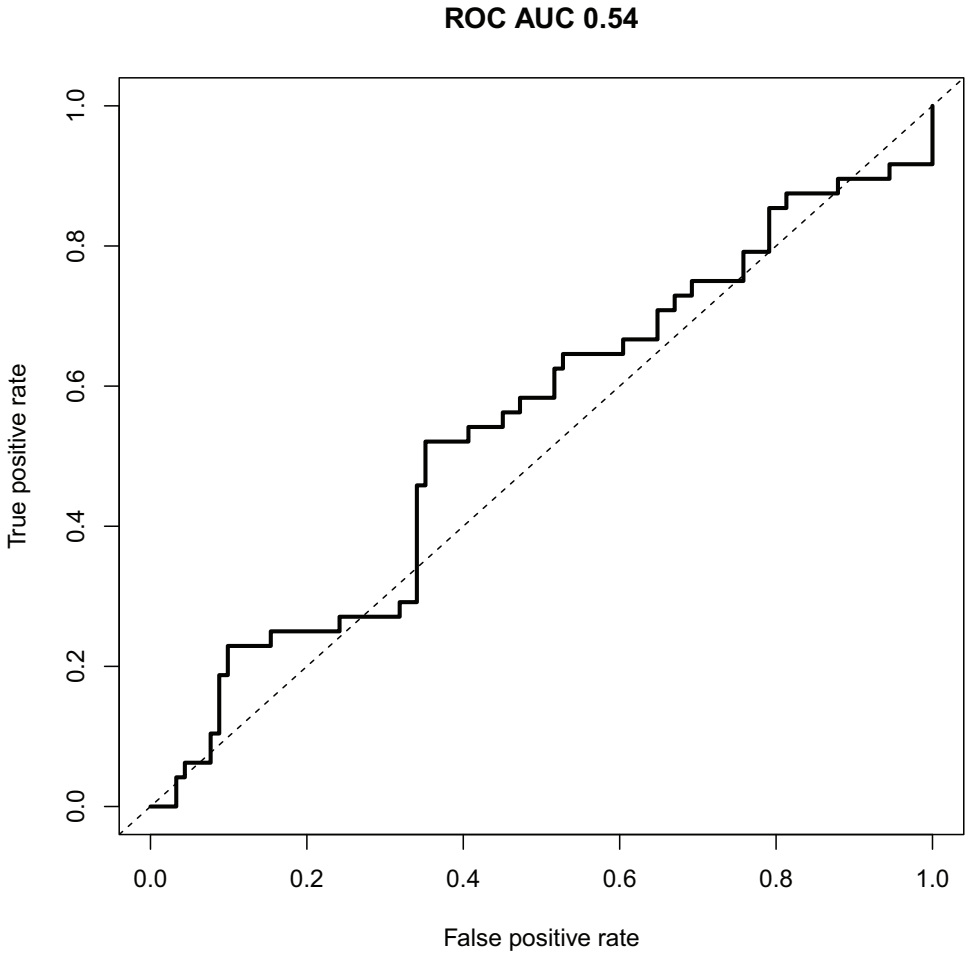

Figure 3. Model performance represented by the ROC curve. The predictive capacity of the 20-gene expression signature is represented by a ROC curve with an AUC of 0.54 (95% CI: 0.44-0.65).

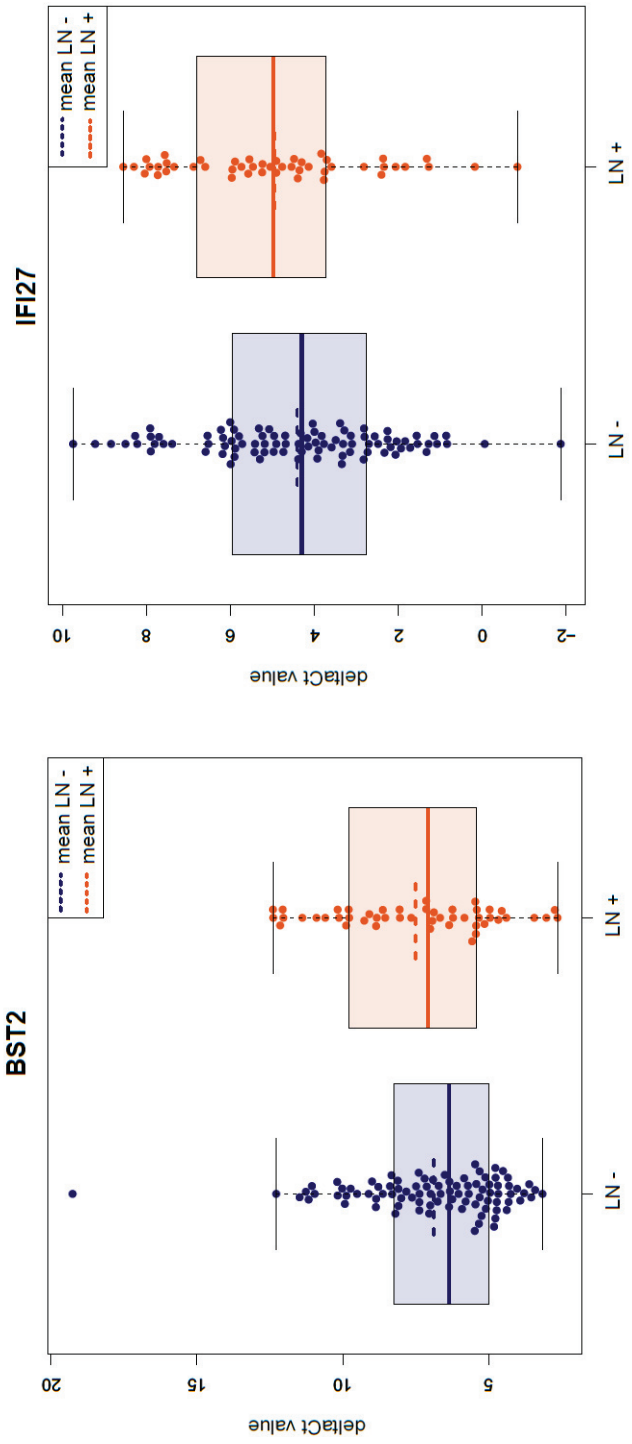

Figure 4. Beeswarm plots for *BST2* and *IF27* showing the distribution of ΔCt values for node-negative versus node-positive patient samples. The box indicates the upper and lower quartiles of distribution. The solid line indicates the median and the dotted line indicates the mean ΔCt value

Table 2. Univariate regression analysis of the Ct values of the 20 single genes predicting node-positive disease at radical cystectomy (*n* = 139).

Gene	OR (95% CI)	*P*-value
TOX3	0.98 (0.92-1.05)	0.57
SLC11A2	0.98 (0.76-1.28)	0.90
FAM36A	1.02 (0.87-1.18)	0.85
LIMCH1	1.05 (0.94-1.16)	0.40
RAB15	1.00 (0.88-1.13)	0.96
AVL9	0.99 (0.91-1.09)	0.90
PCMTD2	1.00 (0.91-1.11)	0.96
PTHLH	0.99 (0.87-1.13)	0.90
DPP4	0.99 (0.91-1.08)	0.81
PCDHGA10	1.01 (0.92-1.11)	0.78
MT1E	1.03 (0.95-1.13)	0.47
MAP4K4	0.97 (0.82-1.14)	0.70
SLC16A1	1.03 (0.95-1.12)	0.50
BST2	1.10 (0.96-1.25)	0.19
MMP14	0.99 (0.89-1.09)	0.80
IFI27	1.11 (0.95-1.30)	0.19
NCLN	0.96 (0.76-1.22)	0.74
HLA_G	1.01 (0.94-1.10)	0.73
RRBP1	0.99 (0.80-1.22)	0.93
ICAM1	1.05 (0.94-1.17)	0.42

Pearson correlation analysis showed significant positive correlation in a cluster of eight genes (fig 5). Of these eight genes, five were determined as upregulated (*AVL9, PCMTD2, FAM36A, LIMCH1, RAB15*) in node-positive disease and three were determined as downregulated (*NCLN, MAP4K4, MMP14*) in node-positive disease by Smith *et al*[9]

20-gene expression signature and validation in TCGA

The TCGA gene expression data by RNA sequencing from 408 chemo-naïve cystectomy specimens is publicly available[12] and was used as an additional external validation cohort. All 20 genes from the 20-gene expression signature and necessary clinical data were available for 365 MIBC samples. Beeswarm plots of the 20 genes of interest showed a similar overlap in data points (Supplementary fig. 4). Gene expression levels did not differ significantly between node-positive and node-negative patients for any of the genes (Mann Whitney-*U*-test, range p-values 0.07-0.93, Supplementary fig. 4). Pearson

correlation analysis did not convincingly substantiate the positive correlations in the eight genes found in our data (except *LIMCH1* and *RAB15*). It did show some correlation between a number of other genes (e.g. *ICAM, DPP4. TOX3*) (Supplementary fig. 5).

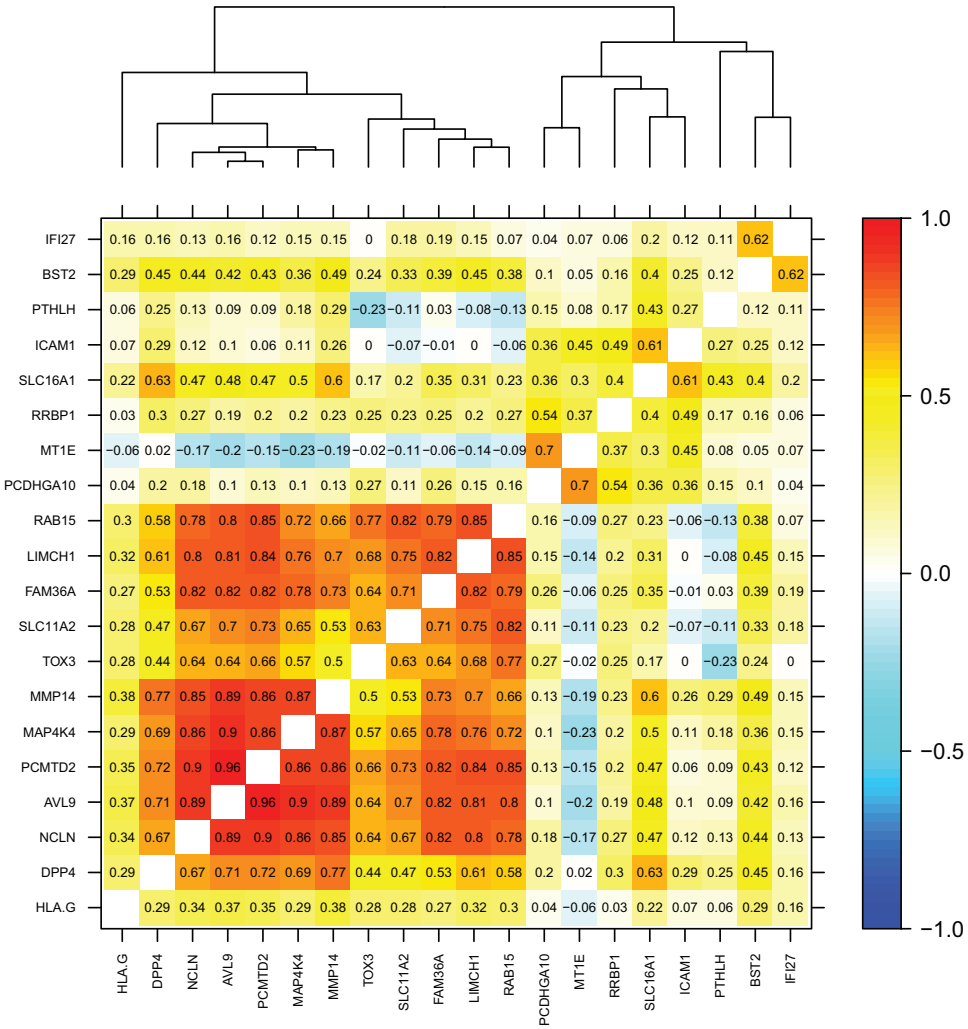

Figure 5. Pearsons'product moment correlation coefficient matrix showing a cluster of eight highly positively correlated genes (*AVL9, PCMTD2, FAM36A, LIMCH1, RAB15, NCLN, MAP4K4, MMP14*) (r = 0.66-0.96)

Technical evaluation

Since there were no major differences in patient and tumor characteristics between node-negative and node-positive patients, we checked the data for possible technical confounders that might have influenced the gene expression levels. We hypothesized that the originating hospital might have influenced the RNA quantity and quality of the samples, for instance by differences in fixation protocols. Since most samples originated from Erasmus MC (35%), we repeated the ROC analysis for Erasmus MC archival material only (n = 37 node-negative and n = 11 node-positive) as an attempt to rule out this bias. The predictive capacity was marginally poorer in this more homogeneous but significantly smaller sample set (AUC 0.52, 95% CI: 0.31-0.72).

DISCUSSION

Curative treatment options for MIBC are few and survival is highly dependent on radical surgery and lymph node status. The presence of micrometastasis in lymph nodes could be an additional argument to justify the administration of NAC before radical surgery. There is a clear clinical need to have tools that could aid in the decision whether or not to give NAC. In the present study, we aimed to validate a 20-gene expression signature, which previously was shown to predict the presence of lymph node metastases at cystectomy in clinically node-negative MIBC patients[9].

Since the dTURBT represents the starting point for treatment decisions in MIBC patients, we used RNA isolated from a large cohort of dTURBT and not radical cystectomy specimens. In addition, we aimed to validate the 20-gene expression signature using qRT-PCR, which is an easier to use technology than microarray analysis and is being used in many pathology laboratories around the world. Our data showed robust performance of the qRT-PCR per-formed: 1) the dilution series and calibration plots in the optimization of the qRT-PCR showed a good quality in the FFPE-derived RNA samples; 2) the gene plots of the duplicate samples per patient also showed a near optimal result as robust measurements in the RT-qPCR. Nevertheless, we were unable to validate the predictive capacity of the previously published 20-gene expression signature. Smith *et al* who obtained the 20-gene expression signature and accompanying cut-offs by optimization in two independent training cohorts of archival FFPE cystectomy specimens and one additional test cohort of 185 cystectomy specimens described an AUC of 0.67 (95% CI: 0.60-0.75)[9]. In contrast, our expression model only reached an AUC of 0.54 (95% CI: 0.44-0.65). In addition, analysis of the TCGA expression data in 365 MIBC patients also did not show a difference between node-positive and node-negative patients based on the suggested 20 genes.

Recently, Seiler *et al* published a study that attempted to design another gene expression signature to predict the presence of lymph node metastases at time of cystectomy[14]. Seiler *et al* used RNA isolated from cystectomy specimens for a whole transcriptome approach in order to include also non-coding transcripts. This approach resulted in a 51 K-nearest neighbor classifier (KNN51). Of the 51 genes included in this signature, none overlapped with the 20 genes from the 20-gene expression signature of Smith *et al*[9,14]. However, Seiler *et al* did attempt to validate the 20-gene expression signature. The 20-gene expression signature had an AUC of 0.46 and a non-significant OR of 1.39 versus an AUC of 0.82 and an OR of 2.65 ($p<0.01$) for the KNN51 model[14]. A number of important differences should be noted between the study of Seiler *et al* and our study. The platform used by Seiler *et al* was whole transcriptome expression analysis, a laborious technique that is not widely available for standard diagnostic procedures in contrast to the qRT-PCR technology we used. Further, Seiler *et al* like Smith *et al* used radical cystectomy for their analysis and not dTURBT specimens we used. As the authors stated in their conclusions, the KNN51 expression signature should also first be validated on dTURBT specimens before implementation in clinical practice is possible.

In general, the discordance between the study by Smith *et al* and our current study does not undermine the value of the primary study, given the different methodologies. However, we believe it does illustrate the practical limitations and the difficulties related to translation into the clinical setting. Several other hypotheses could be raised for the lack of validation of the predictive gene expression model in our study. In general, MIBC is known to be a heterogeneous cancer in terms of biological behavior. Even in similar histological subtypes, the biological profile of the tumor may differ greatly. Recent publications identified the presence of very distinct molecular subtypes in MIBC having different phenotypes and accompanying biological behavior[15,16]. These molecular subtypes might be differently distributed among the different datasets that were used to develop and validate the 20-gene expression signature. Secondly, the punches taken from the dTURBT samples might not have portrayed the overall tumor expression levels due to intra-tumor heterogeneity. Also, the punch taken from the FFPE TURBT material could have been contaminated by normal cells. We cannot fully exclude this possibility, even though we carefully selected areas of at least 70% tumor cells and we punched centrally in the tumor sections. Thirdly, from a statistical point of view, robustness of the predictive model would increase if gene expression could be defined as either `on' or `off'. The subtle differences in higher or lower expression of certain genes in node-positive versus node-negative samples probably led to part of the frailty of the model. Lastly, correlation between different genes in this expression signature could suggest a biological interaction, such as cross-communicating pathways. Even though we could not confirm this finding in the TCGA data and this data was not available for the

model presented by Smith *et al*. Multi-collinearity between different components of a predictive model risks overestimation of the predictive capacity of a model and should therefore be corrected for in the development process.

The present study showed the difficulty of validating gene expression data, not only in an independent patient cohort but also on a different platform. In line with the whole transcriptome approach by Seiler *et al* on 199 patients and the RNA sequencing data of 365 patients of TCGA, we were unable to validate the 20-gene signature on a qRT-PCR platform in 139 MIBC patients who underwent dTURBT. We therefore conclude that a previously developed 20-gene expression signature that predicted the presence of lymph node metastases at pelvic lymph node dissection and radical cystectomy in MIBC patients who were clinically node-negative is clinically not applicable and cannot serve as a tool to guide treatment decisions on NAC in MIBC patients.

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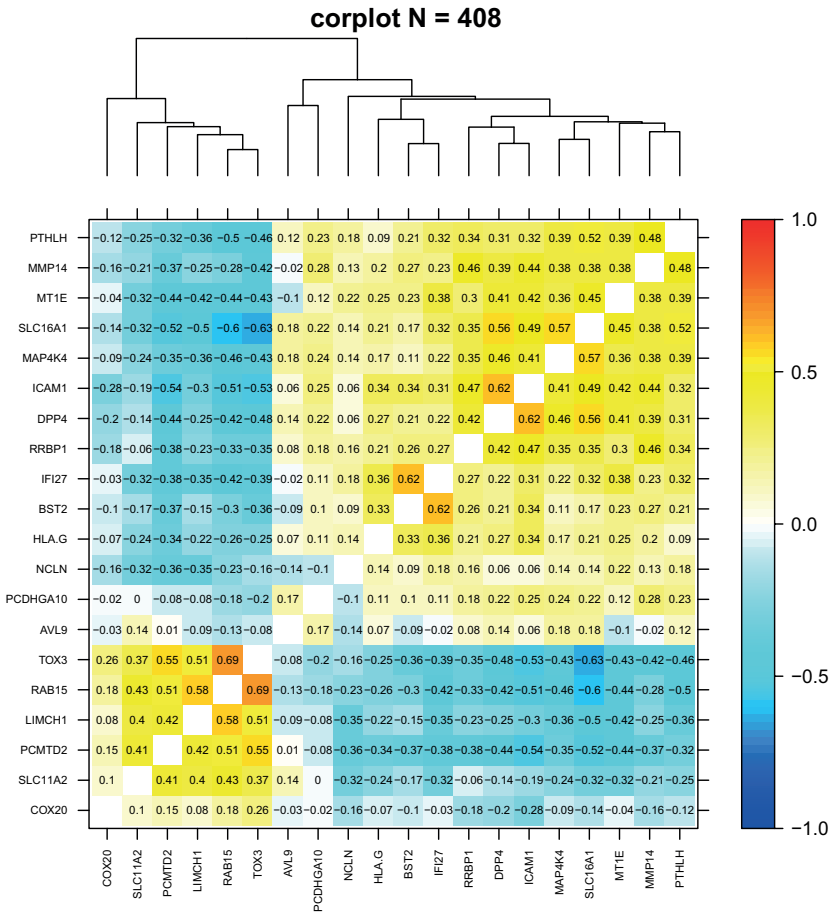
	1	2	3	4	5	6	7	8	9	10	11	12	sample
A	SLC11A2	COX20	TOX3	LIMCH1	RAB15	AVL9	PCMTD2	PTHLH	DPP4	PCDHGA10	MT1E	MAP4K4	1a
B	SLC16A1	BST2	MMP14	IFI27	NCLN	HLA-G	RRBP1	ICAM1	ACTB	HPRT			1a
C	SLC11A2	COX20	TOX3	LIMCH1	RAB15	AVL9	PCMTD2	PTHLH	DPP4	PCDHGA10	MT1E	MAP4K4	1b
D	SLC16A1	BST2	MMP14	IFI27	NCLN	HLA-G	RRBP1	ICAM1	ACTB	HPRT	T24 HPRT	nt HPRT	1b
E	SLC11A2	COX20	TOX3	LIMCH1	RAB15	AVL9	PCMTD2	PTHLH	DPP4	PCDHGA10	MT1E	MAP4K4	2a
F	SLC16A1	BST2	MMP14	IFI27	NCLN	HLA-G	RRBP1	ICAM1	ACTB	HPRT			2a
G	SLC11A2	COX20	TOX3	LIMCH1	RAB15	AVL9	PCMTD2	PTHLH	DPP4	PCDHGA10	MT1E	MAP4K4	2b
H	SLC16A1	BST2	MMP14	IFI27	NCLN	HLA-G	RRBP1	ICAM1	ACTB	HPRT	T24 HPRT	T24 HPRT	2b

Supplementary figure 1. Plate-design of the qRT-PCR assay validating the 20-gene expression signature. Two patients were run per plate in duplicate. For normalization purposes the housekeeping genes *HPRT, ACTB* and a plate control (T24 bladder cancer cell line RNA) was also included in the assay.

Supplementary figure 2. Plots of the Ct values for the 20 genes and household genes of the duplicate samples per patient. *Can be retrieved online at: http://journals.plos.org/plosone/*

Supplementary figure 3. Beeswarm plots of the 20 different genes and 2 household genes, which show the distribution of ΔCt values for node-positive and node-negative samples per gene (*n*=139). The box indicates the upper and lower quartiles of distribution, with the solid line indicating the median and the dotted line indicating the mean ΔCt value. *Can be retrieved online at: http://journals.plos.org/plosone/*

Supplementary figure 4. Beeswarm plots based on TCGA data (*n*=365) of the distribution of ΔCt values for the 20 different genes in node-positive and node-negative samples per gene. The box indicates the upper and lower quartiles of distribution, with the solid line indicating the median and the dotted line indicating the mean ΔCt value. *Can be retrieved online at: http://journals.plos.org/plosone/*



Supplementary figure 5. Pearson's product moment correlation coefficient matrix based on the TCGA RNA expression data of the 20 genes of interest (*n*=408).

Part III: Therapy & Surveillance

Targeted therapies in bladder cancer: an overview of *in vivo* research

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ABSTRACT

Survival of patients with muscle-invasive bladder cancer is poor and new therapies are needed. Currently, none of the targeted agents that are approved for cancer therapy have been approved for the treatment of bladder cancer and the few clinical trials that have been performed had limited success, often owing to a lack of efficacy and toxic effects. However, many other novel targeted agents have been investigated in animal models of bladder cancer. EGFR, FGFR3, VEGF, mTOR, STAT3, the androgen receptor and CD24 are molecular targets that could be efficiently inhibited, resulting in reduced tumor growth, and that have been investigated in multiple independent studies. Several other targets, e.g. COX-2, IL, Bcl-xL, Livin and Choline kinase α, have also been observed to inhibit tumor growth, but these findings have not been replicated to date. Limitations of several studies include the use of cell lines with mutations downstream of the target, providing resistance to the tested therapy. Furthermore, certain technologies, such as interfering RNAs, although effective *in vitro*, are not yet ready for clinical applications. Further preclinical research is needed to discover and evaluate other possible targets, but several validated targets are now available to be studied in clinical trials.

INTRODUCTION

Bladder tumors can be classified as either non-muscle-invasive bladder cancer (NMIBC) or muscle-invasive bladder cancer (MIBC). At diagnosis, 75% of patients will present with NMIBC (stage Ta, T*is* or T1) and 25% will present with MIBC (stage ≥T2).[1,2] In patients with NMIBC, although the 5-year survival is over 90%, the recurrence rate is high (>50%), which necessitates costly long-term surveillance with invasive cystoscopies.[1,3-5] By contrast, MIBC has a poor outcome: the 5-year overall survival after radical cystectomy and lymph node dissection ranges from 49% for T3-4N0 stage disease to 66% for T2N0 staged disease.[6] In patients with MIBC, cisplatin-based neoadjuvant chemotherapy preceding radical surgery results in an overall long-term survival benefit of 6%.[7] However, overall, no improvement in the survival of patients with MIBC has been accomplished over the past 20 years.[8,9] The high incidence and recurrence rate, together with the poor survival, make bladder cancer a serious public health problem.[10] Therefore, a clear clinical need for new effective therapies in both NMIBC and MIBC exists.

In the 1990s, targeted molecular therapy was introduced as a novel treatment strategy in oncology.[11] These therapies aim to interfere with cellular processes that are essential to cancer cell survival at the molecular level — for example, by blocking proteins involved in tumor cell proliferation or tumor cell metabolism, or by delivering toxic compounds to tumor cells. A number of targeted therapies are now well established for the treatment of different cancers: imatinib in leukemia and gastrointestinal stromal tumors, cetuximab in colorectal cancer and bevacizumab in kidney cancer. However, none of the registered targeted therapies have been approved for the treatment of bladder cancer and the small number of clinical trials that have been performed showed disappointing effects.[12] Patients included in these phase I and phase II clinical trials were mostly not selected based on their molecular tumor profile, which might explain the lack of treatment effect.[12] In addition, resistance to therapy occurs quickly, probably owing to molecular heterogeneity of the tumor.[13] This molecular heterogeneity contributes to the clonal evolution and selection of resistant subclones leading to therapy resistance.[14] Lastly, some trials investigating therapy with sunitinib or gefitinib were stopped early because of substantial toxic effects of treatment. [12,15,16]

This review provides a comprehensive overview of targeted therapies for bladder cancer that have been investigated in animal models, some of which have potential for clinical application. We provide insights into both the challenges and the promises of the preclinical development of the targeted agents, highlighting the most promising

studies (table 1), grouped by the mechanisms and pathways the investigated targets are involved in. Summaries of all studies included in this review are provided in the supplementary information.

OVERVIEW OF PRECLINICAL ANIMAL MODELS

In the development of novel targeted therapies, *in vitro* and *in vivo* studies precede clinical trials. Only few of the potential novel therapies will eventually be evaluated in clinical studies. In bladder cancer research, three types of animal models are typically used in *in vivo* research: syngeneic mouse or rat models, and heterotopic or orthotopic xenogeneic mouse or rat models (box 1).

Box 1 | Main types of animal models used in preclinical bladder cancer research

In vivo tumor models can be classified according to the genetic background of tumor and host and according to tumor location.

Genetic background
- Syngeneic model: the tumor arises from tissue of the host animal and has the same genetic background as the animal; examples include spontaneously developing cancers, BBN-induced tumors and mice that are genetically modified to develop tumors.
- Xenogeneic model: tissue or cells from another species are transplanted into the host animal

Tumor location
- Orthotopic model: the tumor develops in the same anatomical location as the source cell line or tissue, for example when bladder cancer cells are injected into the bladder wall.
- Heterotopic model: the tumor develops in a different anatomical location than the source cell line or tissue, for example when bladder cancer cells are subcutaneously injected into the flank, or results from metastasis.

Abbreviation: BBN, N-butyl-N-(4-hydroxybutyl) nitrosamine.

Syngeneic tumor models have the advantage of an intact tumor host environment including an active immune system.[17] However, obvious drawbacks of these models exist, as mouse tumors are intrinsically different from human tumors. In addition, only few tumor types are available.[17] Xenogeneic tumor models more closely resemble the tumors of bladder cancer patients; however, the stroma and vasculature is murine and the animals are immunodeficient to allow growth of human cancer cells.[17] Furthermore, tumors grown from injected cell lines no longer resemble the tumor from which the cell line originated, owing to years of cultivation, passaging and expansion. Xenografts

that are derived from fresh patient material represent a possibility to achieve better resemblance of the true heterogeneous tumor architecture. In 2015, the first study describing a method to create patient-derived xenografts for bladder cancer studies was published.[18]

In studies published since 2009, the heterotopic and orthotopic animal models were generated using many different bladder cancer cell lines and several of these have been genotyped, revealing specific oncogenic mutations (table 2). These mutations are important to consider when choosing which tumor model to use in *in vivo* studies, particularly when the target of the tested agent is upstream of these mutations in the signaling pathway, as downstream mutations might activate the signaling cascade despite inhibition of the upstream target. However, for correct interpretation of the treatment effect, awareness of all potential mutations – downstream or upstream of the target – that could influence the study result is required.

Furthermore, in 2013, a study showed that KU7 bladder cancer cells are actually HeLa cervical carcinoma cells, as the original KU7 cell line had been contaminated with HeLa cells at the source site at some point before 1984.[19] Consequently, KU7 cells should be considered cervical cancer cells and not bladder cancer cells. *In vivo* studies using KU7 animal models are listed in supplementary table 1, but owing to their nonurological origin they are not being discussed in this review.

RECEPTOR TYROSINE KINASES

Since 2009, many *in vivo* studies have investigated agents targeted at receptor tyrosine kinases (RTKs). An overview of these studies is given in supplementary table 2. RTKs, such as the epidermal growth factor receptor (EGFR), the fibroblast growth factor receptors (FGFRs) and the vascular endothelial growth factor receptors (VEGFRs), are essential for the communication between cells and their environment. Specific growth factors (for example, cytokines or hormones) bind to the extracellular domain of an RTK, thereby activating the intracellular kinase domain of the receptor. The activation results in the stimulation of downstream signaling cascades that affect cell proliferation and cell growth.[20] The RTK–Ras–MAPK pathway and the RTK–PI3K–Akt pathway are among the most frequently affected signaling pathways in cancer (figure 1, figure 2).[21,22] In bladder cancer, RTKs are often overexpressed or mutated, resulting in overstimulation of downstream signaling pathways.

EGFR

EGFR is overexpressed in many bladder tumors and EGFR overexpression correlates with a poor prognosis.[23,24] Examples of agents targeting EGFR are gefitinib, a small-molecule inhibitor used for the treatment of chemotherapy-refractory non-small-cell lung cancer, and cetuximab, an antibody used for the treatment of head and neck cancer and advanced colorectal cancer.[25,26] The effect of inhibiting EGFR in bladder cancer was investigated in five mouse studies (figure 2).[27-31] Cetuximab was used in three of these studies and researchers found that growth of MGH and T24 xenografts was inhibited by this antibody.[27,30,31] In two studies, cetuximab was combined with photodynamic therapy, which enhanced the inhibitory effect in one study and was synergistic in the other.[27,30] Similarly, combining afatinib (an inhibitor of EGFR and erbB-2) with cetuximab was more effective than cetuximab alone.[31] Addition of bevacizumab (an antibody against VEGFA) to cetuximab and photodynamic therapy resulted in regression of tumor vessels.[30]

Caution must be taken in interpreting the results of four of these studies, which used T24 cells[28,31] or MGH cells,[27,30] as these cell lines carry activating mutations downstream of the treatment target. Molecular profiling has shown that T24 cells have a mutation in *HRAS*, which activates the Ras-MAPK and P13K-Akt pathways downstream of EGFR.[32,33] One study in colorectal tumor samples demonstrated that activating mutations in another Ras protein (GTPase KRas, encoded by *KRAS*) render the tumor cells insensitive to cetuximab.[34] In one of the two studies using T24 cells, the authors describe a cetuximab resistance mechanism but the influence of the *HRAS* mutation in this resistance mechanism remains unknown.[31] In the other study using T24 cells, *LRIG1* was introduced into these cells that were then used in a xenograft model.[28]

The protein leucine-rich repeats and immunoglobulin-like domains protein 1, which is expressed from *LRIG1*, inhibits EGFR through direct interaction;[28] however, its effect on the Ras–MAPK and PI3K–Akt pathways is most likely nullified by the presence of the *HRAS* mutation in T24 cells. In addition, the cell line MGH, which was used in the two studies by Bhuvaneswari and colleagues,[27,30] has a mutation in *AKT1*; the corresponding protein PKB is an effector for the PI3K–Akt pathway.[32,33] MGH cells also carry an *FGFR3* mutation that might activate the Ras–MAPK pathway, independently of EGFR.[32,33] Although a beneficial effect of targeting EGFR was observed, the effect could have been more convincing if a different cell line would had been used to establish the treated mouse xenografts. Furthermore, the observation that EGFR was downregulated does not imply that the downstream signaling pathways were also downregulated.[27,30] By contrast, in the study of Rebouissou *et al.*,[29] in which EGFR was inhibited by erlotinib, resulting in tumor growth inhibition or delayed tumor detection by ultrasonography,

the results are convincing. The cell lines used (BFTC-905, JMSU1, KK47, L1207, UMUC6, VMCUB1) were selected using a 40-gene expression classifier, discriminating between high EGFR expression for a basal-like phenotype or low EGFR expression for non-basal-like phenotype, and thoroughly examined for downstream mutations (table 1).[29]

Several EGFR-targeted therapies have been evaluated in clinical trials and a number of clinical trials are ongoing.[12] For instance, erlotinib monotherapy was studied in the neoadjuvant setting in 20 patients with MIBC.[35] The authors reported a pathologic response rate with downstaging to <T2 in 35% of patients. Gefitinib and cetuximab combined with chemotherapy were studied in the metastatic setting.[36,37] Median overall survival times were 15.1 months and 14.3 months for gefitinib and cetuximab, respectively. Gefitinib was also studied as a single-agent second-line therapy in the metastatic setting: only one of 31 treated patients responded to treatment.[38] Nevertheless, we believe that treatments targeting the EGFR can still have a role as therapies for patients with bladder cancer in the future. The disappointing trial results and data from *in vivo* research clearly highlight the need to carefully select patients for therapy based on the molecular profile of their tumor.

In addition, agents targeting erbB-2 are being investigated in phase II trials in patients with bladder cancer but most results are still pending. Examples include a number of trials in which trastuzumab or lapatinib are being combined with chemotherapy or given as single agents.[39-44] Promising results are expected, particularly in the light of new data reported by The Cancer Genome Atlas (TCGA) Research Network[21] and Groenendijk and co-workers. [45] The TCGA study found that *ERBB2* was mutated or amplified in 9% of samples, demonstrating the importance of *ERBB2* mutations in the pathogenesis of bladder cancer.[21] Groenendijk *et al*.[45] found that *ERBB2* mutations were associated with a very good response to neoadjuvant chemotherapy: 24% of complete responders but none of the nonresponders had *ERBB2* mutations.[45]

Table 1. Promising *in vivo* research*

Target	Pathway	Tumor type (*n*)	Intervention	Study
EGFR	Ras–MAPK	Orthotopic (40) Heterotopic (144; 2 per mouse)	EGFR inhibitor (erlotinib)	29
FGFR-3	Ras–MAPK	Heterotopic (NR)	FGFR inhibitor (PD173074)	48
FGFR-3	Ras–MAPK	Heterotopic (80)	mAb against FGFR3 (R3Mab)	49
FGFR-3	Ras–MAPK	Orthotopic (128)	mAb against FGFR3(R3Mab)	50
FGFR-3	Ras–MAPK	Heterotopic (72)	FGFR inhibitor (PD173074)	51
FGFR-3 EGFR	Ras–MAPK	Experiment 1: Heterotopic (30) Experiment 2: NR	Experiment 1: FGFR inhibitor (PD173074) and/or EGFR inhibitor (gefitinib) Experiment 2: FGFR inhibitor (PD173074 and/or mAb against EGFR (cetuximab)	54
VEGFRs	Ras–MAPK	Metastatic (NR)	Tyrosine kinase receptor inhibitor (sunitinib) and/or chemotherapy (epirubicin)	149
VEGF-A VEGF-C VEGFR-3	Ras–MAPK	Heterotopic (22)	siRNA against VEGF-A, or siRNAs against VEGF-C and VEGFR-3	59
VEGF-A EphB4	Ras–MAPK	Heterotopic (32)	EphB4 inhibitor (sEphB4-HSA) and/or mAb against VEGF(bevacizumab)	150
mTOR	PI3K–Akt	Heterotopic (37)	Knockdown of TP53 and PTEN and mTOR inhibitor (rapamycin)	75
mTOR	PI3K–Akt	Heterotopic (40)	mTOR inhibitor (rapamycin) and/or chemotherapy (cisplatin)	75
mTOR	PI3K–Akt	Heterotopic (40)	mTOR inhibitor (rapamycin) and/or PI3K inhibitor (wortmannin)	76
STAT3 mTOR MAPK	Jak PI3K–Akt Ras-MAPK	Orthotopic (84)	mTOR inhibitor (rapamycin) and/or MAPK inhibitor (UO126) and/or STAT3 inhibitor (S3I201)	78
STAT3 Survivin	Jak Apoptosis	Heterotopic (35)	siRNAs against STAT3, survivin or both STAT3 and survivin	151
AR	Jak PI3K–Akt Ras–MAPK	Heterotopic (18)	siRNA against AR	94
AR	Jak PI3K–Akt Ras–MAPK	Heterotopic (10)	Androgen deprivation (surgical castration)	93
AR	Jak PI3K–MAPK Ras–Akt	Heterotopic (18)	siRNA against AR	95
CD24 AR	Metastasis Jak PI3K–Akt Ras–MAPK	Orthotopic (190) Heterotopic (20)	CD24-knockout mice or androgen deprivation (surgical castration)	99

Target	Pathway	Tumor type (*n*)	Intervention	Study
CD24	Metastasis	Heterotopic (16) Metastatic (44)	mAb against CD24 (ALB9)	96
CD24 HIF1α	Metastasis Angiogenesis	Heterotopic (40) Metastatic (32)	shRNA against CD24 or HIF1α	129

* Additional details can be found in the Supplementary Information online. Abbreviations: EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; EphB4, ephrin typeB receptor 4; mAb, monoclonal antibody; mTOR, mammalian target of rapamycin; STAT3, signal transducer and activator of transcription 3; MAPK, mitogen-activated protein kinase; AR, androgen receptor; CD24, Signal transducer CD24; HIF1α, hypoxia-inducible factor 1α; siRNA, small interfering RNA; VEGFRs, vascular endothelial growth factor receptors

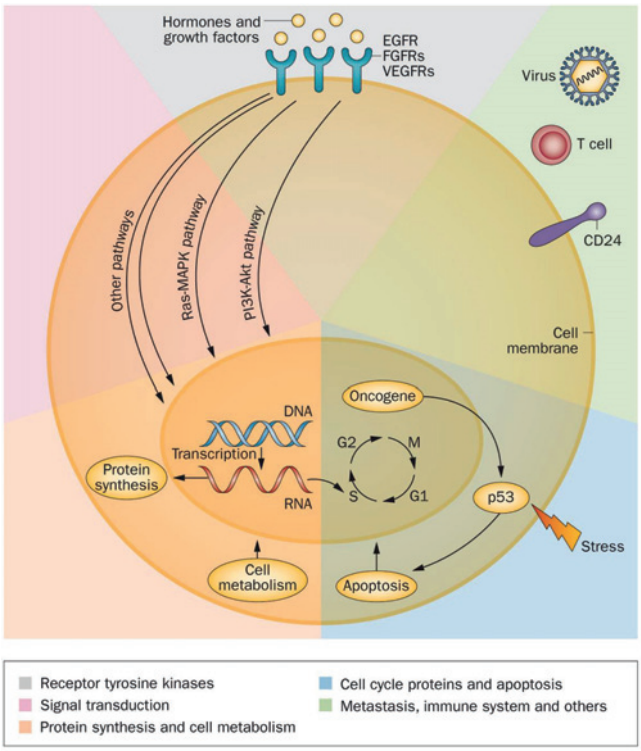

Figure 1. Cellular processes involved in cancer development. External stimuli, such as hormones and growth factors, stimulate receptors on the cellular membrane, leading to activation of downstream pathways. These pathways lead to activation of transcription factors and, thus, gene expression. RNA is either translated to functional proteins or can have regulatory functions on its own. In cancer cells, many processes eventually lead to increased cellular proliferation by amplifying cell growth, evading cell cycle arrest and evading apoptosis. Abbreviations: EGFR, epidermal growth factor receptor; FGFRs, fibroblast growth factor receptors; MAPK, mitogen-activated protein kinases; TP53, cellular tumor antigen p53; VEGFRs, vascular endothelial growth factor receptors.

FGFRs

In addition to EGFR overexpression, approximately two-thirds of all NMIBCs have activating *FGFR3* mutations.[46] In MIBC, <15% of tumors have *FGFR3* mutations, although >40% of MIBCs overexpress FGFR3.[47] Seven *in vivo* studies reported the effects of targeting FGFR3.[48-54] In most cases, the cell lines used either carried a mutant *FGFR3* gene or a *FGFR3* fusion gene, both leading to activation of downstream pathways. The FGFR-3 was inhibited by the small-molecule inhibitor PD173074 in four studies,[48,51,52,54] and by the monoclonal antibody R3Mab in two studies (Figure 2).[49,50] One study evaluated the effect of the pan-FGFR inhibitor BGJ-398.[53]

Five studies reported growth inhibition, reduced cell proliferation or both.[48-51,54] Induction of apoptosis — which demonstrates cytotoxic effects of the treatment —was only reported in one study.[48] The MGH cell line used in the study by Lamont *et al.*[52] has an *AKT1* mutation, leading to activation of the PI3K pathway regardless of inhibition of the FGFR; nevertheless, the investigators did observe a cytostatic response by treatment with PD173074. The cell line UMUC3 used in the study by Cheng *et al.*[53] has a *KRAS* mutation and a deletion in the *PTEN* gene, resulting in constitutive activation of both Ras–MAPK and PI3K–Akt pathways. In this study, blockage of tumor cell extravasation was reported but not growth inhibition. In another study, inhibition of FGFR3 and EGFR was combined in a mouse model using RT112 cells, which have no known downstream mutations in Ras–MAPK and PI3K–Akt pathways.[54] Combination therapy with the FGFR3 inhibitor PD173074 and cetuximab gave the best result regarding tumor growth inhibition, compared to treatment with either agent alone, and also led to sustained tumor growth control.[54] Overall, these mouse studies provided compelling evidence that targeting FGFR3 in human bladder tumors is potentially effective (table 1).

In clinical trials, however, the strategy of targeting FGFR3 has not yielded the expected positive results. The small-molecule inhibitor dovitinib was investigated as a single-agent, second-line treatment in patients with metastatic urothelial carcinoma.[55] The results were disappointing and the study was terminated owing to a lack of beneficial treatment effects. In a phase Ib study of dovitinib plus gemcitabine combined with cisplatin or carboplatin in patients with advanced solid tumors, which included two bladder cancer patients, no treatment effect was observed.[56] Furthermore, combined treatment was poorly tolerated owing to myelosuppression. A phase II trial of dovitinib in patients with NMIBC who did not respond to BCG treatment is ongoing and reports of the results are pending.[57]

Table 2. Bladder cancer cell lines and known oncogenic mutations and gene fusions relevant to this review[32]

Cell line [alternative name]	Origin	Oncogenic mutations and gene fusions*	*In vivo* studies‡
253J-BV [253JBV, 253J_B-V]	Human	Unknown	156
5637	Human	*ERBB2, TP53,RB1*	77, 111, 150
AY-27	Rat	Unknown	157-159
BFTC-905 [BFTC905]	Human	*NRAS, TP53*	29
BIU-87	Human	Unknown	160, 161
BTT739	Human	Unknown	59, 61, 91
EJ [MGH-U1]	Human	Unknown	92, 99, 131
HT-1197 [HT1197]	Human	*NRAS, PIK3CA*	162
HT-1376 [HT1376]	Human	*TP53, RB1*	162
J82	Human	*FGFR3, ERBB2, TP53*	51, 163, 164
JMSU1	Human	Unknown	29
KK47	Human	Unknown	29
KU-19-19 [KU1919]	Human	*NRAS, AKT1*	89
KU-7	Human	NA§	165-174
L1207	Human	Unknown	29
MB49	Mouse	Unknown	174-177
MBT-2 [MBT2]	Mouse	Unknown	60, 121, 124, 125, 130, 149, 178
MGH [MGH-U3, MGHU3, RN]	Human	*FGFR3, AKT1, CDKN2A*	27, 30, 48, 52
RT112 [RT-112]	Human	*TP53, FGFR3/TACC3* fusion	49, 50, 52, 54, 179, 180
RT4 [RT-4]	Human	*RHOA*	51, 74, 152, 181
SW780 [SW-780]	Human	*FGFR3/BIAIAP2L1* fusion	51, 52, 120, 182
T24 [T-24]	Human	*HRAS, TP53,*	28, 31, 94, 95, 100, 113, 114, 128, 151, 162, 177, 183-190
TSU-pr1	Human	NA⊠	143
UMUC1 [UM-UC-1]	Human	Unknown	50
UMUC3 [UM-UC-3]	Human	*PTEN, KRAS,TP53*	53, 75, 76, 88, 90, 93, 96, 99, 109, 112, 126, 129, 152, 191, 192, 193
UMUC6 [UM-UC-6]	Human	*FGFR3*	29
UMUC14 [UM-UC-14]	Human	*FGFR3*	48-50, 82, 120
VMCUB1 [VM-CUB-1]	Human	*TP53,ERBB2, PIK3CA CDKN2A*	29
YTS-1	Human	Unknown	144

* Only mutations registered in COSMIC databases are listed.[32] ‡ Only studies in which the cell lines have been used to create xenografts are listed. § Cervical carcinoma cell line. ⊠ TSU-pr1 cells have been derived from T24 cells. Abbrevation: NA, not applicable.

In summary, studies of the inhibition of RTKs in cell-line-derived animal models should be performed after molecular profiling of the chosen cell line to exclude the possibility of activating mutations downstream of the target RTK. In human bladder cancer tumors, mutations in the Ras family of genes are rare (for example, *HRAS* mutations exist in 5% of patients)[21] and mutations in the PI3K–Akt pathway occur in approximately 20% of bladder cancer tumors.[21] For these subgroups of patients, tumor models harboring mutations in these pathways will be useful in therapy evaluation. However, RTK inhibition therapy will probably lack effectivity and therapies not involving these pathways could be beneficial for this patient category.

The data reviewed here suggest that inhibition of RTKs after stratification of patients based on molecular analysis of the tumor does have potential in bladder cancer therapy for the majority of patients. In addition, combination of treatments might enhance treatment effects; however, some treatment combinations might also result in increased toxic effects.

VEGFRs and angiogenesis

As tumors become larger, hypoxia will occur unless the tumor is able to grow new blood vessels. Most tumors stimulate neoangiogenesis by secreting VEGF. VEGF binds to its cognate receptor on endothelial cells and stimulates the formation of new blood vessels.[58]

In one study, Feng and co-workers[59] treated mice that had heterotopic mouse bladder carcinomas with intratumoral injections of synthetic small interfering RNAs (siRNAs) against *Vegfa*, *Vegfc* and *Vegfr3*. The siRNAs specifically inhibited synthesis of the corresponding proteins (VEGFA, VEGFC and VEGFR3, respectively). VEGFA and VEGFC are two types of ligands that can bind to VEGFRs. Interestingly, all mice in the control group and in the group that received siRNAs against *Vegfc* and *Vegfr3* developed lung metastases but 37.5% of mice in the group treated with siRNA against *Vegfa* had no lung metastases. In addition, no animals in the group treated with siRNA against *Vegfa* had liver metastases compared with 50% and 25% of mice in the control group and the group receiving siRNAs against *Vegfc* and *Vegfr3*, respectively. These data suggest that inhibition of VEGF-A expression reduces the development of metastases.

Three of the studies on angiogenesis inhibition used technologies that cannot yet be implemented in clinical practice (for example, viral vehicles).[59-61] Two of these studies reported tumor growth inhibition [59,61], but one study did not find an effect on tumor growth.[60] All three studies reported a combination of either reduced angiogenesis, reduction in lymphatic vessels or metastasis.[59-61]

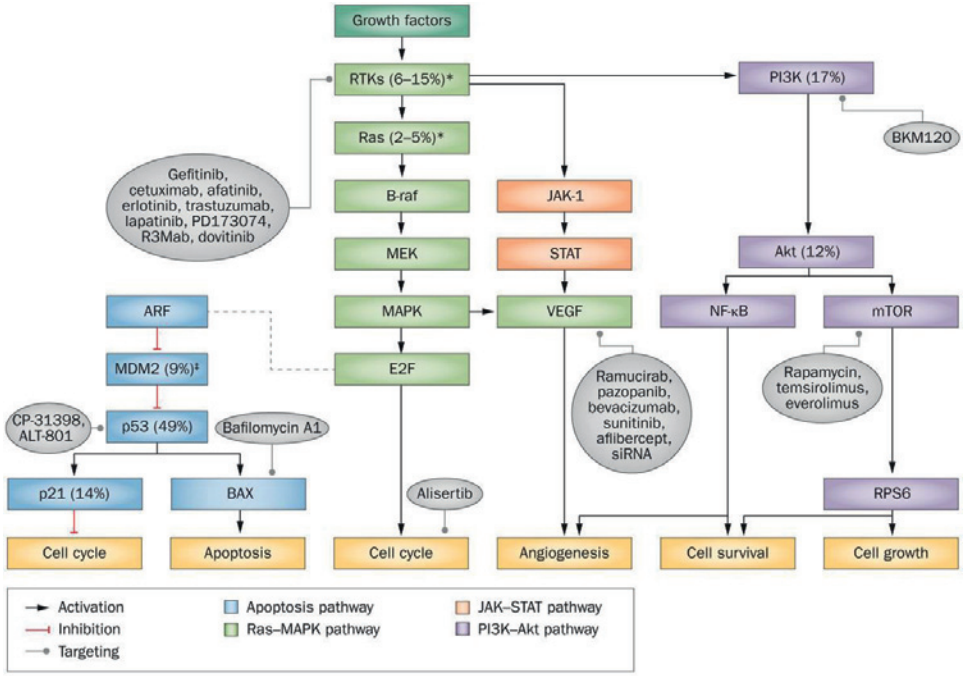

Figure 2. Cellular pathways affected in bladder cancer and targeting therapies. Multiple small-molecule inhibitors and antibodies against different receptors and pathways have been tested in preclinical and clinical studies. Agents targeting protein translation and cell metabolism affect the molecules at the end of signaling pathways and are not shown. Pathways and targets are located at various sites in and around the tumor cell (figure 1). The percentage of bladder cancer samples harboring mutations are shown for targets that have been screened by the TCGA.[21] *Percentage depends on the RTK analyzed. ‡Percentage harboring a copy number variation. Abbreviations: ARF, tumor suppressor ARF; Akt, serine/threonine-protein kinases; B-raf, serine/threonine-protein kinase B-raf; BAX, apoptosis regulator BAX; E2F, transcription factors of the E2F family; MAPK, mitogen-activated protein kinases; MDM2, E3 ubiquitin-protein ligase Mdm2; MEK, dual specificity MAPK kinases 1 and 2; NF-κB, nuclear factor κB; p21, cyclin-dependent kinase inhibitor 1; p53, cellular tumor antigen p53; RPS6, 40S ribosomal protein S6; RTK, receptor tyrosine kinase; STAT, signal transducer and activator of transcription 1 and 3; TCGA, The Cancer Genome Atlas Research Network; VEGF, vascular endothelial growth factor.

Many clinical trials of agents targeting VEGFs and VEGFRs in patients with bladder cancer have already been reported.[12] Four studies investigated the use of bevacizumab (targeting VEGF-A) or sunitinib (targeting VEGFRs) combined with chemotherapy in the neoadjuvant setting (figure 2).[62-65] Pathological response rates with downstaging to <T2 ranged from 22% to 53%. One study of sunitinib was terminated early owing to substantial toxic effects.[65] Combining bevacizumab with chemotherapy has also been studied as adjuvant treatment in two studies.[64,66] The reported median progression-free

survival was 6.5 months in one study[47] and 8.2 months in the other study.[66] In both studies, the aspired study goal of 50% improved progression-free survival was not met.[64,66]

Other studies have analyzed monotherapy of sunitinib, aflibercept (a fusion protein that binds VEGFs) or pazopanib (which targets VEGFRs) as second-line treatment strategies in phase II trials in patients with advanced urothelial cancer.[67-70] Two studies reported no beneficial effect of aflibercept and pazopanib,[68,69] and the other two studies reported a partial response in 7% and 17.1% of patients for sunitinib and pazopanib, respectively.[67,70] At present, three clinical trials investigating anti-VEGF therapies (bevacizumab, pazopanib and sunitinib) in patients with bladder cancer are ongoing.[71-73]

Overall, VEGF-targeted therapies show promise for clinical application. However, sunitinib treatment resulted in high toxicity in clinical trials[12,16] and a more selective VEGF inhibitor or antibody will be more likely to be well tolerated. Again, selection of patients based on the downstream mutations in their tumor will be necessary to achieve the highest benefits.

SIGNAL TRANSDUCTION

Several studies have identified potential new targets for bladder cancer therapy in different signal transduction pathways. Signals coming from cell membrane receptors must be communicated to downstream effector molecules. This signal transduction occurs through kinases, such as MAP kinases and PI3 kinases, or GTPase activity, for example of Ras proteins (figure 1, figure 2).

The signal transduction cascades offer many possible new therapeutic targets. As a consequence, inhibitors have not been generated for all potential targets. In cell culture and animal models, inhibition of a target by inhibiting translation of its mRNA by siRNAs usually gives the first insight into whether the target is suitable for further study. An overview of *in vivo* studies in which targeting of signal transduction pathways has been investigated is provided in supplementary table 3.

The protein mTOR acts in the PI3K–Akt pathway and is targeted by rapamycin. Several rapamycin analogues, such as temsirolimus and everolimus, have been developed and are in use for the treatment of advanced renal cell carcinoma. In three studies that used heterotopic xenograft models and one study that used an orthotopic mouse model, mTOR was targeted via intraperitoneal injection of rapamycin (figure 2).[74-76,78] All studies showed that rapamycin reduced tumor growth. In addition, Makhlin *et al.*[75] showed that

the addition of cisplatin to rapamycin treatment resulted in increased median survival and a lower proliferation index (decreased expression of the proliferation marker Ki67) in comparison with rapamycin alone. Similarly, Zhou *et al.*[78] demonstrated that combining rapamycin with inhibitors of MAPK and signal transducer and activator of transcription 3 (STAT3) significantly prolonged survival in a bladder cancer model in transgenic mice in comparison with rapamycin alone. Seront *et al.*[76] found that rapamycin was less effective in UMUC3 cells in comparison with UMUC14 cells (UMUC3 cells have a homozygous deletion of *PTEN*, whereas UMUC14 cells express wild-type *PTEN)*. The researchers also found that combining rapamycin with a PI3K inhibitor was required to inhibit UMUC3 cell tumor growth.[76] This finding, however, was not confirmed by Puzio-Kuter *et al.*[74] in a mouse model of conditional knockout of *PTEN* and Makhlin *et al.*[75] in a xenograft model using UMUC3 cells. In summary, *in vivo* studies investigating rapamycin treatment of bladder cancer have demonstrated that this inhibitor is promising, displaying effectiveness in human xenografted cell lines and models of mouse bladder cancer (table 1).

Examples of phase II clinical trials targeting mTOR and the PI3K – Akt pathway in patients with bladder cancer include several trials that combined temsirolimus or everolimus with chemotherapy.[79-81] The results of these studies are not yet available. Other trials have evaluated a single-agent strategy of temsirolimus or everolimus (figure 2).[82-84] One study was terminated because of futility.[83] Although most of the patients in these trials did not respond to the single-agent treatment, some patients had stable disease or partial response. For example, upon treatment with everolimus eight and two out of 37 patients in one study[82] and 12 and 2 out of 45 patients in another study[84] had stable disease or partial response, respectively, indicating a beneficial effect in selected groups of patients.

A single-agent strategy with the PI3K inhibitor BKM120 (also known as buparlisib) is being investigated in an ongoing trial but study results are still pending (figure 2).[85] BKM120 has previously shown partial responses in the treatment of patients with breast cancer.[86] Furthermore, one trial evaluating the effect of everolimus in patients with NMIBC is currently underway[86]; however, the tumors of the patients enrolled in this trial have not been molecularly characterized. Hence, the eventual treatment effect might differ between patients depending on the molecular profile of their tumor, which could result in an underestimation of the overall treatment effect. If enough patients are included, stratified analysis based on the molecular tumor profile might indicate which patients benefit more (or less) than others.

Other targets in signal transduction cascades include transcription factors (for example, STAT3, NF-κB, zinc finger protein 224 and the androgen receptor [AR]) or proteins that regulate transcription (for example, histone deacetylases).[78,88-97] Inhibition of these transcription factors resulted in tumor growth inhibition in all studies included in this review.[78,88-94,96,97]

Testosterone and the AR might have a role in the increased incidence of bladder cancer in men compared with women.[98] Hence, surgical castration was explored as therapy in two studies: Overdevest *et al.*[99] observed a reduction in tumor growth in orthotopic and heterotopic xenografted mice using this approach, whereas Shiota *et al.*[93] did not find a significant inhibition of tumor growth in their heterotopic xenograft model. In addition, two studies that investigated siRNAs targeting the AR in subcutaneous xenografts observed statistically significant tumor growth inhibition.[94,95] Considering these findings, inhibition of the AR might be a possible therapeutic strategy for men with bladder cancer (table 1); however, none of the possible transcription factor targets have been evaluated in clinical studies yet.

CELL CYCLE PROTEINS AND APOPTOSIS

Cell division occurs after the cell has gone through the four cell cycle phases G1, S, G2 and M (figure 1). Cell cycle checkpoint proteins, which regulate progression through the cell cycle, are often mutated or bypassed in malignant cells and are therefore considered potential targets for therapy. Studies that have investigated targeting cell cycle control and apoptosis pathways *in vivo* are listed in supplementary table 4.

In the M phase, the accurate formation of the mitotic spindle is controlled during a quality check. Aurora kinase A is a protein that is involved in mitotic spindle formation and function that was evaluated as a potential therapeutic target for bladder cancer by Zhou *et al.*[99] (figure 2). The researchers found that aurora kinase A expression was increased in clinical bladder cancer tumor samples and that inhibition of aurora kinase A with the inhibitor MLN8237 (also known as alisertib) in T24 bladder cancer xenografts in mice resulted in suppressed tumor growth. A phase II clinical trial evaluating alisertib combined with paclitaxel in advanced bladder cancer was registered in 2014.[100]

The apoptosis pathway can become activated during the cell cycle, for example when DNA damage occurs. In addition, apoptosis can be induced by activation of the Ras-MAPK pathway and subsequent activation of the tumor suppressor TP53, which is encoded by *TP53*, a gene that is often mutated in cancer cells. Mutations that change an amino acid in the DNA-binding domain of p53 inhibit its function as a transcription factor and,

as a consequence, expression of the proapoptotic apoptosis regulator BAX. Deletions of the gene likewise lead to loss of function.[101,102] In one study, CP31398, a compound that stabilizes the conformation of the DNA-binding domain of TP53 (figure 2), was administered to SV40T-transgenic mice.[103] The SV40 large T protein binds and inhibits both p53 and the retinoblastoma-associated protein and thereby induces bladder cancer.[104] Administration of CP31398 resulted in decreased tumor weight and inhibition of invasive tumor growth with an increase in apoptosis.[73] In clinical trials, p53-targeted therapy is being evaluated using ALT801, a fusion protein of IL2 and a T-cell receptor domain.[105] The efficacy of ALT801 is being investigated as a combination treatment with gemcitabine, with or without cisplatin, in patients with MIBC and NMIBC.[106,107] Patient recruitment is currently ongoing.

Bcl-xL (Bcl-2-like protein 1 expressed from *BCL2L1*) is an antiapoptotic protein of the Bcl2 family. One study investigated the clinicopathological significance of Bcl-xL expression in patients with upper urinary tract urothelial carcinoma (figure 2).[108] Using a subcutaneous UMUC3 xenograft model in mice, the researchers evaluated the therapeutic effect of the V-ATPase inhibitor bafilomycin A1 against Bcl-xL and observed reduced tumor growth with signs of apoptosis (confirmed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, which is used to detect DNA fragmentation caused by apoptosis). Another target in this category is livin, which belongs to the inhibitors of apoptosis proteins (IAP) family and was shown to predict disease relapse in bladder cancer.[109] Inhibiting livin in an *in vivo* heterotopic xenograft model led to tumor inhibition and increased apoptosis.[110].

Targeting and inducing the apoptosis pathway is an especially interesting strategy in cancer treatment, as the occurrence of apoptosis shifts the treatment effect from cytostatic to cytotoxic. This shift could, in theory, lead to tumor regression rather than only reduced cell proliferation. However, most of the targeted therapies that are currently used clinically are cytostatic rather than cytotoxic. With the exception of the study investigating the p53 stabilizer CP31398,[103] the number of animals used in experiments in this category of targets is small and none of the studies have been replicated to date.

PROTEIN SYNTHESIS AND CELL METABOLISM

Protein synthesis

During the G1 phase of the cell cycle, many proteins that are required for subsequent cell division are being synthesized (translation). As the step of protein translation is essential to cell proliferation, the participating proteins are potential targets for anticancer agents. Supplementary table 4 provides an overview of the relevant *in vivo* studies.

Eukaryotic translation initiation factor 3B (eIF3b) is a subunit of the translation initiation factor complex eIF3. Immunohistochemical assessment of 143 bladder cancer samples showed that eIF3b overexpression was correlated with a high tumor stage and short disease-specific survival in humans.[112] The researchers then used siRNAs to inhibit eIF3b formation in UMUC3 cells, which resulted in reduced tumor growth of subcutaneous xenografts in mice.[112]

Some bacterial toxins target the function of proteins during translation. One group used diphtheria toxin subunit A to inhibit translation of elongation factor 2.[112,113] Expression of the toxin was controlled by the promoter of the insulin-like growth factor 2 gene (*IGF2*) and/or the promoter of the long non-coding RNA gene *H19*. The products of *IGF2* and *H19* are highly expressed in many tumors but not in normal cells. In two studies using orthotopic T24 human bladder cancer xenograft models in mice, treatment inhibited tumor growth, resulting in tumors that were 86% and 83% smaller than in controls.[113,114]

Targeting the translational process has also been investigated in other types of cancer, for example by targeting eukaryotic translation initiation factor 4E (eIF4e). After multiple promising preclinical studies, a phase I trial (NCT00903708)[114] using an antisense oligonucleotide to block expression of eIF4e was completed in 2010.[115-118] Patients with stage 4 cancer for which no proven therapy existed were included, regardless of pathological diagnosis. Included cancer types were mesothelioma, malignant melanoma, non-small-cell lung carcinoma and several others. The expression levels of eIF4e in tumor biopsy samples taken after treatment were lower than those in pre-treatment samples. Unfortunately, the study did not show any clinical response, which again demonstrates the difficulty of translating preclinical results to the bedside.[118]

Cell metabolism

In cancer, cell metabolism is often unbalanced. Metabolism and catabolism are mediated and controlled by a multitude of different enzymes, which are therefore other potential targets for bladder cancer therapy. *In vivo* studies that investigated this strategy are listed in supplementary table 4. For example, stearoylCoA desaturase 1 is a rate-limiting

enzyme in fatty acid desaturation that was targeted by siRNA knockdown or by an oral small-molecule inhibitor, both combined with doxycycline therapy. Both strategies led to a significant reduction in tumor growth in subcutaneous bladder cancer xenografts in mice.[119]

Choline kinase α (ChoKα) is a metabolic enzyme responsible for the generation of phosphorylcholine (the hydrophilic polar head group of phospholipids).[120] Inhibition of this enzyme slowed tumor growth in both subcutaneously as well as orthotopic xenografts in mice and prolonged animal survival in the orthotopic mouse model from a median of 34 days to 42 days.[120] ChoKα is also overexpressed in lung, breast, prostate, colon, ovary and hematological malignancies.[121] In 2014, a phase I trial testing a ChoKα inhibitor in patients with solid tumors, including bladder cancer, finalized recruitment, but results have not been published yet.[122]

Two other studies have tested two other drugs that target metabolic pathways.[123,124] Treatment with a combination of αlipoic acid, which inhibits glycolysis, and hydroxycitrate, which inhibits lipid synthesis, was able to improve survival of mice with MBT-2 bladder tumor xenografts. Addition of octreotide or cisplatin boosted survival even further.[123,124]

Overall, targeting protein translation seems to be a promising strategy for the treatment of bladder cancer, even though the study targeting eIF4e showed the difficulty of reproducing favorable preclinical results in a phase I clinical trial.

METASTASIS, IMMUNE SYSTEM AND OTHERS

Other processes that involve potential molecular targets for the treatment of patients with bladder cancer include the formation of metastases and the immune response. In addition, novel treatment strategies, such as oncolytic viruses, are used to exploit tumor-cell-specific molecular mutations. A summary of studies investigating these strategies is provided in supplementary table 5.

Potential targets include proteins that interact with the extracellular matrix, for example urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor 1 (PAI1) and versican core protein. These targets are thought to be important in reducing the metastatic potential of bladder cancer cells and, hence, might be employed in preventive treatments.[125-127] Another promising target is the CD antigen CD24, a cell surface protein and cancer stem cell marker that is involved in metastatic progression in many cancers. In three preclinical studies, one group showed the relevance of

CD24 in bladder cancer development and metastasis.[95,98,128] Inhibition of CD24 with an antibody or siRNAs reduced tumor growth in mice with subcutaneous xenografts and reduced metastatic load in mice inoculated with tumor cells via their tail vein.[95,98,128] Also, fewer tumors developed in CD24-knockout animals upon administration of *N*-butyl-*N*-(4-hydroxybutyl)-nitrosamine, which induces bladder tumors.[98] Furthermore, the researchers found that androgens stimulated CD24 expression in tumor tissue and surgical castration of male mice inhibited tumor growth in the subcutaneous xenografts.[98] As inhibition of CD24 reduced tumor growth in multiple studies, we consider this strategy promising as a targeted therapy in bladder cancer (table 1).

Stimulation of the immune system is another way to target cancer cells. If the immune system is able to recognize tumor cells as foreign, the immune response might destroy the malignant cells.[129] One approach investigated the use of IL12 in the treatment of bladder cancer, using transfection of EJ bladder cancer cells with a plasmid encoding IL12 to force secretion of IL12.[130] The hypothesis was that secretion of IL12 would stimulate T lymphocytes and natural killer cells, which should result in increased tumor cell killing. Mice were injected subcutaneously with nontransfected EJ cells or transfected EJ cells.[130] In addition, some mice were treated with injections of pirarubicin, a doxorubicin analogue. Combination treatment resulted in significantly smaller tumors compared with single treatment or no treatment. Although these results are positive, the need to introduce a gene into a high percentage of tumor cells in a solid tumor makes this approach unlikely to be translated into clinical application.

Other bladder cancer treatment strategies targeting the immune system are currently under investigation in clinical trials.[131] These agents have so far not been analyzed in animal models of bladder cancer, but have proven their efficacy in clinical trials in other types of cancer. Immune checkpoint inhibitors, such as MPDL3280A (atezolizumab) pembrolizumab, ipilimumab and nivolumab are examples of such promising therapies targeting the immune system. MPDL3280A, an antibody against programmed cell death 1 ligand 1 (PDL1), was investigated as a single-agent, second-line therapy in a phase I trial in 68 patients with bladder cancer .[132] The objective response rate was 43% in patients with tumors positive for PDL1 expression upon immunohistochemical analysis. Reported overall toxicity was low. In 2014, the FDA granted MPDL3280A the breakthrough status for bladder cancer, which will expedite the development and review of the drug by the FDA.[133, 134] Many clinical trials of MPDL3280A are currently ongoing and results are pending.[135-142]

Finally, three *in vivo* studies employed oncolytic adenoviruses.[130,143,144] The viruses were genetically modified in such a way that they specifically attach to a tumor-associated

membrane protein or the extracellular matrix and then enter and lyse the tumor cells without damaging normal cells. Although this strategy leads to a reduction of tumor growth, the infection efficiency of the used viruses is probably not high enough for all tumor cells to be are targeted.[145] To achieve extensive effectiveness, infection should not only result in direct tumor cell killing, but also antigen release and immune stimulation.[146] Thus, since oncolytic viruses have shown suboptimal potency as monotherapies, combination treatments with, for example, immune check point inhibitors or immune-stimulatory molecules should be considered.[146,147] For example, in 2012, a phase I trial investigating the efficacy of intravesical administration of an oncolytic adenovirus expressing granulocyte-macrophage colony-stimulating factor demonstrated tolerable safety and a complete response rate of 48.6%.[148] However, at the moment, we do not consider treatment strategies using oncolytic viruses ready for implementation into the clinic.

CONCLUSIONS

Although targeted therapies have proven effective in other cancer types, none of the registered therapies have been approved for the treatment of bladder cancer. The small number of clinical trials that included patients with this disease had limited success.[12] With this review, which explores all animal studies investigating targeted therapeutic interventions for bladder cancer that were published since 2009, we aimed to provide a current, comprehensive overview of this exciting field of research.

We found several animal studies that reported tumor growth inhibition or reduced angiogenesis and were replicated by different groups or validated by the same group in separate studies (table 1). Inhibition of FGFR3 seemed very successful using the small-molecule inhibitor PD173074 [48,51,52,54], as well as via the monoclonal antibody R3Mab [49,50] and in combination with inhibition of EGFR.[54] These treatments resulted in tumor growth inhibition, reduced angiogenesis and increased apoptosis. Inhibition of EGFR alone using erlotinib was also successful, resulting in tumor growth inhibition in some heterotopic xenografts and prolonged survival of mice with orthotopic xenografts.[29] However, the results of clinical trials investigating therapeutic targeting of FGFR3 and EGFR have been disappointing,[12] most likely owing to the lack of patient stratification according to the downstream mutations in the pathways of these receptors.

Inhibition of angiogenesis through blocking VEGF or its receptor reduced tumor growth in animal models.[30,59-61,149,150] Similarly, inhibition of the cancer stem cell marker CD24 inhibited tumor growth [95,98,128], which was also the case for treatments targeting mTOR

and the PI3K–Akt pathway[75-77], STAT3 [77,90,151] and the AR.[92-95] Most experiments showed a cytostatic treatment effect, which suggests that tumor growth probably resumes when treatment is stopped. Combining targeted treatment with chemotherapy usually enhanced the therapeutic efficacy. In clinical trials, combination treatments have been attempted but has not demonstrated an improvement in overall survival and sometimes resulted in increased patient toxicity.[12]

EGFR inhibitors and antibodies have proven to be effective as targeted therapies in humans with non-small-cell lung cancer and colorectal cancer.[25,26] This work demonstrates that activating mutations in the Ras–MAPK and PI3K–Akt pathways downstream of EGFR render the tumor resistant to EGFR inhibition. We have identified four studies on EGFR inhibition in human bladder cancer cell line xenografts that had such mutations in one or both pathways.[27,28,30,31] In another study, the researchers carefully screened the EGFR downstream pathways of the cell lines before attempting to target EGFR.[29]

One limitation of the studies discussed in this review is the use of cell lines to create xenografts. Cell lines might not reflect the entire spectrum of tumor types, especially because relatively few bladder cancer cell lines are available. In addition, cell-line-derived tumors probably lack the tumor heterogeneity observed in most cancers. Thus, xenografts established directly from resected tumors might present a better model than cell lines. Such tumor models also have human stroma and engage murine stromal cells, which is likely to be important in cell–cell interactions and cell proliferation.[18] Furthermore, small-molecule inhibitors, inhibition of cell surface proteins by monoclonal antibodies and antibodies against ligands of cell surface receptors have proven their efficacy in the clinic. Inhibition or knock down of targets using small, modified RNAs (for example, siRNAs), transfection of genes and the use of viral vehicles are efficient *in vitro* but their effectiveness in delivery is still not good enough for systemic treatment of tumors and metastases.

Overall, our review shows that a multitude of different targets has been investigated and that most of the studies have not been replicated to date. Often, relatively few animals were used to register an effect. In addition, almost all studies reported inhibition of tumor growth but not increased apoptosis. Also, only five studies reported that the investigated treatment had no effect.[53,60,96,125,152] This small number suggests a possible publication bias. In some papers, the number of treated animals was not reported. In 2010, the ARRIVE (animals in research: reporting *in vivo* experiments) guidelines—

developed to improve the design, analysis and reporting of animal experiments—were published.[153,154] We suggest that scientists and scientific journals adhere to these guidelines in future studies.

Furthermore, bladder cancer research is consistently underfunded, considering the number of life years lost and the costs to health-care systems generated by patients with bladder cancer.[155] This lack of funding is the major reason why research on novel therapies in bladder cancer lags behind that in other tumor types. The community of bladder cancer researchers has to invest in reaching out to the different policy makers and grant organizations to improve funding.

In conclusion, EGFR, FGFR3, VEGF, mTOR, STAT3, the AR and CD24 represent promising and validated targets for the inhibition of bladder cancer growth in animal studies. Treatment strategies that combine different modalities and/or targets seem to be most promising and clinical studies should continue to pursue these findings. Careful patient selection in these clinical studies will be essential in order to demonstrate true benefit. Many of the other targets that are under preclinical investigation still need further validation.

KEY POINTS

- Over the past 20 years, survival of patients with muscle-invasive bladder cancer has not improved
- None of the targeted therapies that are approved for other cancers have been approved for the treatment of bladder cancer
- The small number of clinical trials that have been performed in patients with bladder cancer had limited success owing to several limitations
- Specific oncogenic mutations in different cell lines used in *in vivo* research might render these cell lines insensitive to the therapy that is being investigated
- Inhibition of EGFR, FGFR3, VEGF, mTOR, STAT3, the AR and CD24 resulted in inhibition of tumor growth in multiple *in vivo* studies
- Careful patient selection in clinical trials based on the molecular profile of the tumor will be essential in demonstrating benefit of new targeted therapies

REVIEW CRITERIA

The Embase, MEDLINE, OvidSP, Web of Science, PubMed publisher, Cochrane CENTRAL and Google Scholar databases were systematically searched (date of search: December 2014). Full search strategy as was conducted in Embase : ('molecularly targeted therapy'/de OR 'molecular therapy'/exp OR (target* NEAR/3 (molecul* OR therap* OR antibod* OR inhibit* OR mirna* OR microrna* OR treat* OR intervent* OR chemo*)):ab,ti) AND ('bladder tumor'/exp OR 'transitional cell carcinoma'/de OR ('carcinoma'/de AND 'bladder'/de) OR ((bladder OR urothel*) NEAR/3 (tumo* OR cancer* OR neoplas* OR carcin* OR metasta* OR transitional)):ab,ti) After the literature search was completed, all reviews, expert opinions, editorials, seminar articles, congress abstracts, articles published before 2009 and records in languages other than English or Dutch were excluded. In total, 93 published animal studies of targeting molecular targets in bladder cancer were eligible for review.

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Supplementary table 1. Receptor tyrosine kinase and angiogenesis

Target	N	Intervention	Conclusion	Ref
EGFR	40 total heterotopic	PDT and/or intraperitoneal injection of monoclonal antibody (cetuximab)	Combination therapy gave best result: tumor growth inhibition, increased apoptosis	1
EGFR	18 total heterotopic	Intratumoral injection of adenoviral vector-mediated transfer of tumor suppressor gene LRIG1	Tumor growth inhibition by inhibition of cell proliferation and angiogenesis	2
EGFR	72 mice (144 tumors) heterotopic 40 total orthotopic	Oral gavage of EGFR inhibitor (erlotinib) or vehicle	Heterotopic: tumor growth inhibition in three cell lines, one cell line was resistant and two insensitive Orthotopic: delayed tumor detection by echography and prolonged survival	3
EGFR VEGF	60 total heterotopic	PDT and/or intraperitoneal injection of VEGF monoclonal antibody (bevacizumab) and/or EGFR monoclonal antibody (cetuximab)	Combined therapy (PDT + one or both antibodies was comparable): Tumor regression due to regression of tumor vessels	4
EGFR ERBB2	40 total heterotopic (double-sided)	Oral EGFR and ERBB2 inhibitor (afatinib) and/or intraperitoneal injection of EGFR monoclonal antibody (cetuximab)	Combination therapy gave best result, tumor growth inhibition and overcome cetuximab resistance	5
ERBB2	Not available (heterotopic)	Gene gun delivered ERBB2 DNA vaccine with or without intraperitoneal injection of Ling Zhi-8	Tumor growth inhibition and induction of immune responses	6
FGFR-3	Unknown total heterotopic	Oral selective FGFR inhibitor (PD173074)	Tumor growth inhibition, apoptosis induction	7
FGFR-3	80 total heterotopic	Intraperitoneal injection of specific monoclonal antibody (R3Mab) or non-targeting human IgG	Tumor growth inhibition, promotes tumor cell lysis	8
FGFR-3	128 total orthotopic	Intraperitoneal injection of specific monoclonal antibody (R3Mab) or non-targeting human IgG	Antibody therapy gave dose dependent growth inhibition, reduced proliferation	9
FGFR-3	72 total heterotopic	Intraperitoneal injection of selective FGFR inhibitor (PD173074)	Dose dependent tumor growth inhibition in SW780 xenografts, but not in J82 xenografts. FGFR3 fusion proteins enhance sensitivity to the inhibitor	10
FGFR-1 FGFR-3	48 total heterotopic	Intraperitoneal injection of selective FGFR inhibitor (PD173074)	Delayed tumor growth, cell cycle arrest, no apoptosis	11

Target	N	Intervention	Conclusion	Ref
FGFR-1 FGFR-3	14 total orthotopic	Oral selective FGFR-1,2,3 inhibitor (BGJ-398)	No inhibitory effect on tumor growth, but blockage of tumor cell extravasation into the vasculature and metastasis	12
FGFR-3 EGFR	Experiment 1: 30 total heterotopic Experiment 2: Not available	Experiment 1: Intraperitoneal injection of FGFR inhibitor PD173074 and/or oral EGFR inhibitor gefitinib Experiment 2: Intraperitoneal injection of FGFR inhibitor PD173074 and/or cetuximab	Experiment 1: Combination therapy gave best result in tumor growth inhibition, but was poorly tolerated (weight loss) Experiment 2: Combination therapy gave best result in tumor growth inhibition and was well tolerated. On stopping treatment single-treated resumed growth, combination-treated sustained tumor control	13
VEGFR	Not available (metastatic)	Intraperitoneal injection of tyrosine kinase receptor inhibitor (sunitinib) and/or chemotherapy (epirubicin)	Combined therapy: Marginal reduction of tumor burden epirubicin alone gave best survival outcome	14
VEGF-A VEGF-C VEGFR-3	22 total heterotopic	Intratumoral injection of anti-VEGF-A or anti-VEGF-C and anti-VEGFR-3 siRNA	Tumor growth inhibition, reduced angiogenesis, lymph-angiogenesis and metastasis	15
VEGF-C VEGF-D TAM	18 total orthotopic	Intravenous injection of adenoviral vector carrying extracellular domain of VEGFR-3 or intraperitoneal injection of clodronate liposome	No influence on tumor growth, reduction of lymphatic vessels and lymphatic metastasis	16
VEGF EphB4	32 total heterotopic	Intraperitoneal injection of EphB4 inhibitor (sEphB4-HAS) and/or VEGF monoclonal antibody (bevacizumab)	Combined therapy gave the best result, complete tumor regression	17
Flk-1 (= VEGFR-2) C3d3	48 total heterotopic	Intramuscular injection of DNA vaccine (recombinant plasmid encoding Flk-1 and C3d3 fusion proteins or plasmid encoding Flk-1 alone or control)	Best result with fusion protein DNA vaccine; Tumor growth inhibition, prolonged survival and decreased angiogenesis (vessel density)	18
ADM	48 total heterotopic	Intratumoral injection of AMD-shRNA and/or intraperitoneal injection of cisplatin	Combined treatment gave best result in tumor growth inhibition	19

All compounds target the RAS-MAPK signaling pathway (figure 1). EGFR: epidermal growth factor receptor, PDT: photodynamic therapy, LRIG1: leucine-rich repeats and immunoglobin-like domains 1, VEGF(R): Vascular Endothelial Growth Factor (Receptor), ERBB2: V-ERB-B avian erythroblastic leukaemia viral oncogene homolog 2, Ling Zhi-8: fungal immunomodulatory protein, FGFR: fibroblast growth factor receptor, siRNA: small interfering RNA, TAM: tumor associated macrophages, EphB4: Ephrin type-B receptor 4, C3d3: a degradation product of the third complement component C3, ADM: adrenomedullin

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Supplementary table 2. Signal transduction

Target	Pathway	N	Intervention	Conclusion	Ref
mTORC1	PI3K	37 total renal graft model	Knockdown of *P53* and *PTEN* + intraperitoneal injection of mTORC1 inhibitor (rapamycin) or control	Tumor growth inhibition and reduced cell proliferation	1
mTOR	PI3K	40 total heterotopic	Intraperitoneal injection of mTOR inhibitor (rapamycin) and/or chemotherapy (cisplatin)	Combined therapy gave best result: inhibited cell proliferation and enhanced effectiveness of cisplatin, prolonged survival	2
mTOR	PI3K	40 total heterotopic	Intraperitoneal injection of mTOR inhibitor (rapamycin) and/or PI3K inhibitor (wortmannin)	In UM-UC3 xenografts, combined treatment inhibited tumor growth best. In UM-UC14 xenografts, both therapies led to tumor growth inhibition, but combination of both therapies did not enhance this effect.	3
COX-2	PI3K	18 total heterotopic	Oral COX-2 inhibitors (celecoxib and/or indomethacin) and/or anti-COX-2-cDNA	Tumor growth inhibition (cDNA single effect, but stronger effect when combined with inhibitor)	4
STAT3 EGFR	Jak Ras	34 total heterotopic	Intratumoral injection of STAT3 transcription factor decoy oligonucleotide and intraperitoneal injection of EGFR specific antibody (cetuximab)	Tumor growth inhibition and sensitization of resistant cells to cetuximab treatment	5
STAT3 mTOR MAPK	Jak PI3K Ras	*Therapy cohort* 24 total orthotopic *Survival cohort* 60 total orthotopic transgenic	Intraperitoneal injection of mTOR inhibitor (rapamycin) and/or MAPK inhibitor (UO126) and/or STAT3 inhibitor (S31-201)	Best result with triple therapy, tumor growth inhibition and prolonged survival	6
STAT3 Survivin	Jak Apoptosis	35 total heterotopic	Intratumoral injection of saline, culture medium, lipofectamine, vector control, STAT3-3 siRNA, Survivin-4 siRNA or STAT3-Survivin siRNA	STAT3 or Survivin silencing led to similar tumor growth inhibition, combining both treatments did not enhance this effect	7

Target	Pathway	N	Intervention	Conclusion	Ref
NF-κB	NF-κB	40 total heterotopic	Intraperitoneal injection of NF-κB inhibitor (DHMEQ)	Tumor growth inhibition, reduction in microvessel density, reduction in VEGF expression and induction of apoptosis	8
NF-κB	NF-κB	40 total heterotopic	Oral pathenolide analogue (DMAPT)	Dose dependent tumor growth inhibition	9
ILK	PI3K PPARγ	24 total heterotopic	Tumor cells transfected with a plasmid vector based siRNA to knockdown ILK were implanted heterotopically	Tumor growth inhibition, lower microvessel density and higher apoptosis rate	10
ILK	PI3K PPARγ	24 total heterotopic	Tumor cells transfected with a siRNA to knockdown ILK were implanted heterotopically	Tumor growth inhibition, reduced angiogenesis and inhibition of spontaneous metastasis	11
AR	Jak PI3K Ras	18 total heterotopic	Intratumoral injection of siRNA and electroporation to silence AR	Tumor growth inhibition, decreased proliferation and increased apoptosis	12
AR	Jak PI3K Ras	10 total heterotopic	Androgen deprivation (castration)	Tumor growth inhibition (not significant)	13
AR	Jak PI3K Ras	18 total heterotopic	Electroporation with negative-siRNA or AR-siRNA	Tumor growth inhibition and a reduced proliferation index	14
Jak2	Jak Angiogenesis	15 total heterotopic	Intragastric injection of methylsulfonylmethane (MSM) or MSM combined with a Jak2 inhibitor (AG490)	Combined treatment gave best result; tumor growth inhibition, increased necrosis and reduced metastatic load	15
Hsp70-2		12 total heterotopic	Intratumoral injection of HSP70-2 shRNA to knockdown HSP70-2	Tumor growth inhibition	16
Hsp-90 FGFR-3	Ras	32 total heterotopic	Intravenous injection of HSP90 inhibitor (ganetespib) and/or oral FGFR inhibitor (BGJ398)	Both treatments inhibited tumor growth similarly, combining treatment enhanced this effect.	17

Target	Pathway	N	Intervention	Conclusion	Ref
DEPDC1-ZNF224 complex		15 total heterotopic	Intratumoural injection of cell permeable peptide corresponding to the ZNF224-interacting domain in DEPDC1	Complex formation inhibition, tumor growth inhibition and increased apoptosis	18
Src	Ras	Unknown total heterotopic	Oral multi-targeted kinase inhibitor (dasatinib) and/or intraperitoneal injection of chemotherapy (cisplatin)	Combination therapy gave best result; Tumor growth inhibition, proliferation inhibition and increased apoptosis	19
PLCε	Ras	15 total heterotopic	Tumor cells transfected with shRNA to silence PLCε were implanted heterotopically	Tumor growth inhibition	20
CXCR4		18 total BNN-induced orthotopic	Subcutaneous injection of CXCR4 agonist (TF14016)	Lower BC prevalence. Therapeutic effect not clarified	21
ALB1		16 total heterotopic	Intratumoral injection of vehicle delivered siRNA or siRNA alone to knockdown ALB1	Tumor growth inhibition. Down regulation of PI3K-Akt pathway	22
NO		32 total heterotopic + orthotopic 20 total metastatic	No treatment vs NO synthase inhibitor L-NAME in drinking water	Tumor growth inhibition in both heterotopic and orthotopic mouse models. Reduced angiogenesis and metastasis.	23
RHAMM		12 total heterotopic	Xenografted with BC cells with or without prior lentiviral knockdown of RHAMM	Reduced tumor growth and decreased proliferation	24
HDAC-1	Transcription	105 total BNN-induced orthotopic	Intraperitoneal histone deacetylase inhibitor (valproic acid)	Delayed incidence of bladder tumors when given as prevention, no therapeutic effect	25

mTOR: mammalian target of rapamycin, COX-2: cyclooxygenase-2, STAT3: signal transducer and activator of transcription 3, MAPK: mitogen-activated protein kinase, NF-κB: nuclear factor-κB, ILK: integrin-linked kinase, AR: androgen receptor, siRNA: small interfering RNA, Hsp70/90: heat shock protein 70/90, DEPDC1: DEP domain containing 1 gene, ZNF224: zinc finger transcription factor 224, Src: tyrosine kinase Src (pronounced "sarc"), PLCε: phospholipase C ε, CXRC4: C-X-C chemokine receptor 4, ALB1: amplified in breast cancer 1, NO: Nitric Oxid, RHAMM: Receptor of Hyaluronan-Mediated Motility, HDAC-1: histone deacetylase type 1

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Supplementary table 3. Cell cycle, apoptosis, translation and metabolism

Target	Pathway	N	Intervention	Conclusion	Ref
Livin	Apoptosis	Not available heterotopic	Intratumoral injection of Livin antisense (ASODN) oligonucleotides or Livin missence oligonucleotides (MSODN)	Tumor growth inhibition and increased apoptosis	1
AATBC	Apoptosis	8 total heterotopic	Tumour cells with lentiviral-mediated AATBC knockdown were implanted heterotopically	Tumor growth inhibition	2
miRNA-449a	Cell cycle	8 total heterotopic	Intratumoral injection of liposome-encapsulated miRNA-449a	Tumor growth inhibition due to cell cycle arrest and cell proliferation suppression	3
Chk1	Cell cycle	28 total heterotopic	Intraperitoneal injection of chemotherapy (gemcitabine) and/or Chk1 inhibitor (PF477736)	Tumor growth inhibition, combined therapy gave best result	4
P53	P53	84 total orthotopic transgenic	Oral P53-stabilizing agent CP-31398	Tumor growth inhibition, tumor invasion inhibition, increased apoptosis	5
GLIPR1	P53	Unknown total heterotopic	Intratumoral injection of adenoviral vector-mediated GLIPR1 gene therapy and/or radiotherapy	Combination therapy gave best results; tumor growth inhibition and prolonged survival	6
Bcl-xL	NF-κB	16 total heterotopic	Intraperitoneal injection of a specific Bcl-xL inhibitor (bafilomycin A1)	Tumor growth inhibition and increased apoptosis	7
Aurora kinase A	Cell cycle	16 total heterotopic (double-sided)	Oral selective Aurora kinase A inhibitor (MLN8237 = millennium)	Tumor growth arrest and reduced proliferation index	8
PIM kinase	Cell cycle Apoptosis Translation	24 total heterotopic	Oral PIM kinase inhibitor TP-3654 or vehicle	Tumor growth inhibition	9
eEF2	Translation	24 total heterotopic 16 total orthotopic	Intratumoral injection or intravesical instillation with a double promotor DTA expressing vector (H19-DTA-P4-DTA)	Tumor growth inhibition in both tumor models	10
eEF2	Translation	24 total heterotopic 16 total orthotopic	Intratumoural injection or intravesical instillation with a double promotor DTA expressing vector (P4-DTA-P3-DTA, DTA = diphtheria toxin A fragment)	Tumor growth inhibition in both tumor models	11

Target	Pathway	N	Intervention	Conclusion	Ref
Ribosome	Translation	10 total heterotopic	Intravenous injection of anti-Fn14 monoclonal antibody conjugated to recombinant gelonin (rGel)	Tumor growth inhibition, increased apoptosis	12
eIF3b	Translation	10 total heterotopic 20 total metastatic	Transfection with siRNA to deplete eIF3b, injection of transfected tumor cells	Fewer tumors, tumor growth inhibition, reduced lung colonization	13
Arginine Folate	Urea cycle	44 total heterotopic	Intraperitoneal injection of ADI-PEG20 and/or Pemetrexed (PEM)	PEM alone had no effect. ADI-PEG20 treatment resulted in reduced tumor growth. Combining ADI-PEG20 with PEM increased this effect.	14
ChoKα	Phosphoryl-choline metabolism	40 total heterotopic 37 total orthotopic	Intraperitoneal injection of a specific ChoKα inhibitor (MN58b)	Tumor growth inhibition and prolonged survival	15
PDHK1 ATP citrate lyase IGF1 IGFBP	Glucose metabolism	30 total heterotopic	Intraperitoneal injection of hydroxycitrate (HCA) and/ or α-lipoic acid (ALA) and/ or octreotide (OCT) vs chemotherapy (cisplatin)	Combination of three drugs gave best results, tumor growth inhibition and prolonged survival	16
PDHK1 ATP citrate lyase	Glucose metabolism	36 total heterotopic	Intraperitoneal injection of hydroxycitrate (HCA) and/ or α-lipoic acid (ALA) and/or chemotherapy (cisplatin)	Combination of three drugs gave best results, tumor growth inhibition (NS) and prolonged survival	17
SRC-3	Glycolysis	14 total heterotopic	Tumor cells transfected with or without shRNA to silence SRC-3 were implanted heterotopically	Tumor growth inhibition in SRC-3 knockdown tumors	18
SCD1	Fatty acid metabolism	76 total heterotopic	Doxycycline-inducible knockdown of SCD1 or oral SCD1 small molecule inhibitor (A37062)	Tumor growth inhibition, increased apoptosis	19

Livin: inhibitor of apoptosis protein (IAP family), AATCB: Apoptosis-Associated Transcript in Bladder Cancer, Chk1: Checkpoint kinase 1, GLIPR1: glioma pathogenesis-related protein 1, Bcl-xl: B-cell lymphoma-extra-large, PIM kinase: proviral intergration site for moloney murime leukemia virus kinase, eEF2: elongation factor-2, DTA: diphtheria toxin A fragment, eIF3b: eukaryotic initiation factor 3b, ChoKα: choline kinase-α, PDHK1: pyruvate dehydrogenase kinase 1, ATP: adenosine triphosphate, IGF1: insulin growth factor 1, IGFBP: insulin growth factor binding protein, SRC-3: Steroid receptor coactivator-3, SCD1: stearoyl CoA desaturase 1

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Supplementary table 4. Metastasis, immune system and miscellaneous

Target	Process or pathway	N	Intervention	Conclusion	Ref
CD24	Metastasis	16 total heterotopic 44 total metastatic	Intravenous CD-24 monoclonal antibody (ALB9)	Tumor growth inhibition, reduction of metastatic load and prolonged survival	1
CD24 HIF-1α	Metastasis Angiogenesis	40 total heterotopic 32 total metastatic	Tumor cells transfected with shRNA to silence CD24 or HIF-1α were implanted heterotopically + intravenously injected (metastatic model)	Tumor growth inhibition and reduction in metastasis (CD24 downstream of HIF-1α)	2
CD24 AR	Metastasis Jak PI3K Ras	190 total orthotopic 20 total heterotopic	CD24 knockout mice or androgen deprivation (castration)	Less tumor development (CD24 knockout), tumor growth inhibition (castration)	3
Integrin α3β1	Metastasis	30 total heterotopic 72 total orthotopic	Heterotopic (orthotopic): Intraperitoneal injection (intravesical instillation) of monoclonal integrin α3β1 antibody (BCMab1) or monoclonal integrin α3β1 antibody conjugated with immunotoxin Ra (BCMab1-Ra) or mIgG	BCMab1 led to tumor growth inhibition, BCMab1-Ra led to complete tumor regression	4
Integrin α_v	Metastasis	20 total intraosseous metastatic 40 total intracardiac metastatic	Intraosseous inoculation of either UMUC3 cells or UMUC3 integrin αv knockdown cells Preventive protocol: daily intraperitoneal injections of either vector or GLPG187 during 28 days, after 1 day a single intracardiac inoculation of UMUC3 cells Curative protocol: inoculation of UMUC3 cells 3 weeks prior to daily intraperitoneal injection of either vector or GLPG187 during 15 days	Intraosseous inoculation of Integrin αv knockdown led to lower bone and total tumor burden (NS) Preventive protocol: significantly decreased number of metastasis and total tumor burden Curative protocol: No significant effects	5

Target	Process or pathway	N	Intervention	Conclusion	Ref
TM NF-κB	Metastasis	40 total orthotopic 40 total heterotopic	Tumor cells transfected with or without shRNA to silence TM were instilled intravesically Tumor cells transfected with or without shRNA to silence TM were instilled intravesically and treated with intraperitoneal injection of NF-κB inhibitor CAPE Tumor cells transfected with or without shRNA to silence TM were injected heterotopically Tumor cells transfected with or without shRNA to silence TM were injected heterotopically and treated with intraperitoneal injection of NF-κB inhibitor CAPE	Orthotopic: TM silencing led to a higher tumor take and larger tumor size, CAPE treatment reduced tumor development Heterotopic: TM silencing resulted increased tumor growth, CAPE treatment significantly reduced tumor growth	6
IL-12	Immune system	40 total heterotopic	Intraperitoneal injection of IL-12 gene plasmid and/or chemotherapy (pirarubicin = THP)	Best result with combined treatment, tumor growth inhibition	7
CD40 ligand	Immune system	30 total heterotopic	Intratumoral (it) or intravenous (iv) injection of either vvdd or vvdd-hCD40L	Tumor growth inhibition of both viruses, increased anti-tumor activity by CD40 expressing virus via intratumoral injection, no difference between viruses via intravenous injection.	8
T cells	Immune system	16 total heterotopic	Intratumoral injection of an oncolytic adenovirus (PPE3-SEA)	Tumor growth inhibition, increased presence of infiltrating CD3+ T cells	9
TRIAL	Immune system	32 total heterotopic	Intratumoral injection of oncolytic adenovirus encoding TRAIL (ZD55-TRAIL) and/or chemotherapy (gemcitabine)	Combined therapy gave best result, increased tumor growth reduction and increased apoptosis	10

Target	Process or pathway	N	Intervention	Conclusion	Ref
uPA	Extracellular matrix	30 total orthotopic	Intravesical PAI-1	Tumor growth inhibition, reduced invasion depth and decreased angiogenesis (less microvessels)	11
PAI-1	Extracellular matrix	30 total heterotopic	Oral gavage of small molecule inhibitor tiplaxtinin or vehicle	Treatment with tiplaxtinin resulted in tumor growth inhibition, angiogenesis inhibition, reduced proliferation index and increased apoptosis (no major differences between dosage)	12
PSCA	Cell signaling	Unknown total heterotopic	Doxycycline regulated shRNA against PSCA, mice were fed sucrose plus doxycycline	Tumor growth inhibition	13
Malat 1 Suz 12	TGF-β	9 total metastatic 27 total heterotopic	Injected or xenografted with BC cells with or without prior knockdown of Malat1 or Suz12	Malat1 and Suz12 inhibition led to a lower metastatic load then in controls (less metastasis and smaller metastasis, both gave comparable effects)	14
Versican CCL2 CCR2	Extracellular matrix Immune system	80 total metastatic	Intravenous injection of tumor cells transfected with a siRNA against Versican. CCL2/CCR2 knockout mouse or intraperitoneal injection of CCL2 antibody	Reduced metastatic load (in both experiments)	15
IDO	Immune system	Not available heterotopic	Skin delivery of IDO-specific siRNA to dendritic cells	Tumor growth inhibition and prolonged survival	16
IgG	Immune system	25 total heterotopic	Intratumoral injection of polyclonal antihuman goat IgG antibody with/without intraperitoneal injection of chemotherapy (mitomycin C = MMC)	Combination therapy gave best result, tumor growth inhibition and increased apoptosis.	17
Unknown		Unknown total orthotopic rat	Oral Allyl isothiocyanate (AITC)-rich mustard seed powder (MSP-1)	Tumor growth inhibition and inhibition of muscle invasion	18

Target	Process or pathway	N	Intervention	Conclusion	Ref
Unknown		38 total orthotopic rat	Oral N-acetylcysteine conjugate NAC-AITC	Tumor growth inhibition and inhibition of muscle invasion	19
Cells without P53 and Rb		30 total orthotopic	Single intravesical instillation of an oncolytic adenovirus; AxCAZ3-F/RGD, AxdAdB-3 or AxdAdB3-F/RGD	AxdAdB3-F/RGD gave best result in tumor growth suppression, bladder weight reduction and survival	20

CD24: cluster of differentiation 24, HIF-1α: hypoxia inducible factor - 1α, AR: androgen receptor, BNN: N-butyl-N-(4—hydroxybutyl)-nitrosamine, TM: thrombomedulin, CAPE: caffeic acid phenethyl ester, IL-12/24: interleukin 12/24, TRIAL: tumor necrosis factor (TNF)-related apoptosis-inducing ligand, uPA: urinary plasminogen activator, PAI-1: plasminogen activator inhibitor -1, PSCA: prostate stem cell antigen, Malat1: metastasis associated lung adenocarcinoma transcript 1, Suz1: suppressor of zeste 12 homolog (Drosophila), CCL2: CC chemokine ligand 2, CCR2: CC chemokine receptor 2, siRNA: small interfering RNA, IDO: indoleamine 2,3-dioxygenase

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Supplementary table 5. Studies using KU-7 animal models

Target	Pathway	Biological mechanism	N	Intervention	Conclusion	Ref
EGFR PPARγ	RAS	RTK	40 total heterotopic	Oral EGFR inhibitor (gefitinib) and/or PPARγ agonist (DIM-C)	Growth inhibition, combination therapy can overcome EGFR therapy resistance	1
Met-AP2	RAS	RTK	11 total orthotopic	Oral Met-AP2 inhibitor (nitroxoline)	Tumor growth inhibition	2
COX-2 CK2α	PI3K	Signal transduction	12 total orthotopic	Oral COX-2 inhibitor (meloxicam)	Tumor growth inhibition and reduced invasion depth	3
Hsp27 tubulin		Signal transduction	28 total orthotopic	Intravesical antisense oligonucleotides targeting Hsp27 (OGX427) and/or intravesical hemiasterlin synthetic analogue (HTI-286)	Combination therapy inhibited tumor growth	4
NGFI-Bα		Signal transduction	10 total heterotopic	Oral activator of NGFI-Bα (DIM-C-pPhOCH3)	Tumor growth inhibition and induction of apoptosis	5
c-MYC	Ras Wnt	Signal transduction	10 total orthotopic	Intravesical administration of c-MYC small molecule inhibitor (KSI-3716) or control	Tumor growth inhibition	6
Eg5	Cell cylce	Cell cycle and apoptosis	40 total heterotopic *N* metastatic model not available	Intraperitoneal injection of Eg5 inhibitors (KPYC10728, S(MeO)TLC and STLC)	S(MeO)TLC (high dose) performed best; tumor growth inhibition. Prolonged survival and metastasis regression in the metastatic model	7

Target	Pathway	Biological mechanism	N	Intervention	Conclusion	Ref
Survivin PLK1	Apoptosis	Cell cycle and apoptosis	16 total orthotopic	Intravesical instillation of siRNA to silence survivin and PLK1	Tumor growth inhibition (PLK1 siRNA > survivin siRNA)	8
Survivin	Apoptosis	Cell cycle and apoptosis	12 total orthotopic	Intravesical instillation of oncolytic adenovirus adenovirus using the surviving promoter (Ad5E1-apsurvivinE4) or vehicle	Tumor growth inhibition	9
Syndecan-1	Adhesion, cytoskeletal organization, cell-matrix interactions and others	Miscellaneous	19 total orthotopic	Intravesical siRNA to silence Syndecan-1 (CD138)	Tumor growth inhibition and increased apoptosis	10

EGFR: epidermal growth factor receptor, PPARγ: peroxisome proliferator-activated receptor gamma, Met-AP2: type 2 methionine aminopeptidase, COX-2: cyclooxygenase-2, CK2α: casein kinase 2α, Hsp27: heat shock protein 27, NGFI-Bα: nerve growth factor-induced Bα, c-MYC: transcription factor c-MYC, Eg5: kinesin-related motor protein EG5 (also known as kinesin family member 11 or kinesin-like protein KIF11), Survivin: inhibitor of apoptosis protein (IAP family), siRNA: small interfering RNA, PLK1: polo-like kinase 1

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FGFR3 mutation analysis in voided urine samples to decrease cystoscopies and cost in non muscle invasive bladder cancer surveillance: a comparison of 3 strategies

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ABSTRACT

Purpose. We determined whether *FGFR3* mutation analysis of voided urine samples would be cost-effective to partly replace cystoscopy in the surveillance of patients treated for non muscle invasive urothelial carcinoma.

Materials and methods. In this decision analytical study we analyzed data on 70 Dutch patients with *FGFR3* positive primary tumors and a median follow-up of 8.8 years. Surveillance strategies were compared in a Markov model. Modified surveillance consisted of *FGFR3* mutation analysis of voided urine samples every 3 months, and cystoscopy at 3, 12 and 24 months. Standard surveillance was defined as cystoscopy every 3 months and minimal surveillance was defined as cystoscopy at 3, 12 and 24 months. Analysis was stratified for 3 risk profiles, including surveillance after 1) the primary tumor, 2) the first to third recurrence and 3) the fourth recurrence or more. Sensitivity analysis was performed to evaluate the impact of variations in cost, sensitivity and specificity.

Results. The probability of no recurrence after 2 years of surveillance after a primary tumor was higher for modified surveillance than for standard and minimal surveillance, e.g. after primary tumors (95.7% vs 95.0% and 93.9%, respectively). The total cost of surveillance after the primary tumor was lower for minimal and modified surveillance (€2,254 and €2,558, respectively) than for standard surveillance (€5,861). Results were robust to changing inputs over plausible ranges and consistent for each of the 3 risk profiles.

Conclusions. Surveillance in which cystoscopy is partly replaced by *FGFR3* mutation analysis of urine seems a safe, effective and cost-effective surveillance strategy. Further validation in larger cohorts is required.

INTRODUCTION

Although the 5-year survival rate of NMIBC is high at 80% to 90%,[1] surveillance for recurrent disease places a major burden on patients and the health care system in general. More than half of these patients have 1 or more recurrences and must be monitored for the rest of their lives to detect recurrence and progression to muscle invasive cancer (10% to 20%).[2] Progressive disease leads to bladder loss via cystectomy and possibly to death. Therefore, an intensive surveillance protocol is generally followed.

Surveillance relies on regular cystoscopy, which is an invasive procedure associated with anxiety, dysuria, urinary tract infection and financial costs.[3–6] Moreover, cystoscopy can only detect recurrence inside the bladder and not in the renal pelvis or ureters, where these UCs may also recur. Thus, the diagnosis of such upper urinary tract recurrences may be delayed since they are only detected if symptoms occur. Therefore, substantial efforts have been made to design surveillance strategies that could decrease the frequency of cystoscopy in patients with NMIBC without compromising early detection.

Approximately two-thirds of NMIBCs are *FGFR3* mutant. These mutant tumors grow less aggressively and only few progress to muscle invasive disease.[7–10] A decrease in cystoscopy frequency in these patients is an attractive option with cystoscopy replaced by *FGFR3* mutation analysis of voided urine samples. We determined the cost-effectiveness and safety of such a surveillance strategy compared to those of standard surveillance and a minimal surveillance variant.

MATERIALS AND METHODS

Patient data

We studied a consecutive series of 70 patients with primary nonmuscle invasive UC (pTa or pT1) treated at our medical center in The Netherlands, as previously described in detail.[11] All patients had a *FGFR3* positive primary tumor and were retrospectively examined for recurrence and progression between 1983 and 2007 (see table 1). Recurrence was defined as a tumor removed at TUR with histopathological confirmation and without muscle invasion. Progression was defined as muscle invasive UC, i.e. pT2 or greater. All tumors were staged and graded according to the 1997 TNM[12] and 1973 WHO[13] classifications.

Table 1. Characteristics of 70 patients

No. gender (%)	
Male	46 (65.7)
Female	24 (34.3)
Median age at primary tumor (range)	66 (32–84)
No. primary tumor stage (%)	
Ta	61 (87.1)
T1	9 (12.9)
No. primary tumor grade (%)	
G1	36 (51.4)
G2	30 (42.9)
G3	4 (5.7)
No. *CIS* (%)	
Yes	2 (2.9)
No	68 (97.1)
Median months follow-up (range)	106.1 (42.7–270.9)
Recurrence	
No.	207
No. pts without (%)	19 (27.1)
No. pts with (%)	51 (72.9)
Median No./pt (range)	3 (1–18)
No. progression	4
No. death (%)	
Yes	21 (30)
No	49 (70)
Disease	8 (11.4)
Other cause	13 (18.6)
No. cystectomy (%)	
Yes	4 (5.7)
No	66 (94.3)

Decision model

We adapted a Markov decision model from a previously published model used to evaluate the cost-effectiveness of partly replacing cystoscopy by microsatellite analysis in the follow-up of patients with NMIBC.[14] Patients started in the well health state and eventually died (dead state). Other states related to recurrence and progression in the

bladder or UUT (fig. 1). The Markov model had a time horizon of 2 years with 3 monthly cycles. Patients were modeled by tumor, that is for each detected recurrence a new surveillance course began. Markov cohort analysis of 1,000 cases was performed to compare the surveillance strategies.

Model outcomes included the fraction of patients per health state, including a well state for those without recurrence, progression or a bladder, and cumulative financial costs for up to 2 years.

Surveillance strategies

We modeled 3 strategies (fig 2). 1) A standard surveillance arm included cystoscopy every 3 months. 2) A minimal surveillance arm was defined as a lower end reference and included cystoscopy at 3, 12 and 24 months. 3) A modified surveillance arm consisted of *FGFR3* mutation analysis of voided urine samples every 3 months. Follow-up cystoscopy was scheduled at 3, 12 and 24 months. Additional cystoscopy was performed when *FGFR3* mutation analysis had a positive outcome.

If recurrence was found at cystoscopy, it was assumed to be removed by TUR. This patient then restarted surveillance. In cases of progression to muscle invasive bladder cancer, the bladder was removed via cystectomy. If the outcome of *FGFR3* mutation analysis was positive but follow-up cystoscopy remained negative for 2 subsequent visits, CT of the abdomen was performed to rule out any UUT recurrence. When a UUT tumor was detected, unilateral nephroureterectomy was performed.

Transition probabilities and cost data

We determined the 3-month probability of recurrence and progression using data on 70 patients in a Dutch cohort by Cox proportional hazards regression analysis with SPSS®, version 15. We analyzed 207 recurrences and 4 progressions that occurred during a median follow-up of 8.8 years. The 3-month probability of progression was subsequently estimated, conditional on recurrence. Three sets of transition probabilities for recurrence and progression were analyzed, i.e. after 1) the primary tumor, 2) the first to third recurrence and 3) the fourth recurrence or more.

Sensitivity and specificity of the included tests were obtained from the literature, as were all remaining transition probabilities (supplementary table 1.[1,14,15] The specificity of *FGFR3* mutation analysis was set at 100% because mutations do not occur in the urine samples of nonpatients.

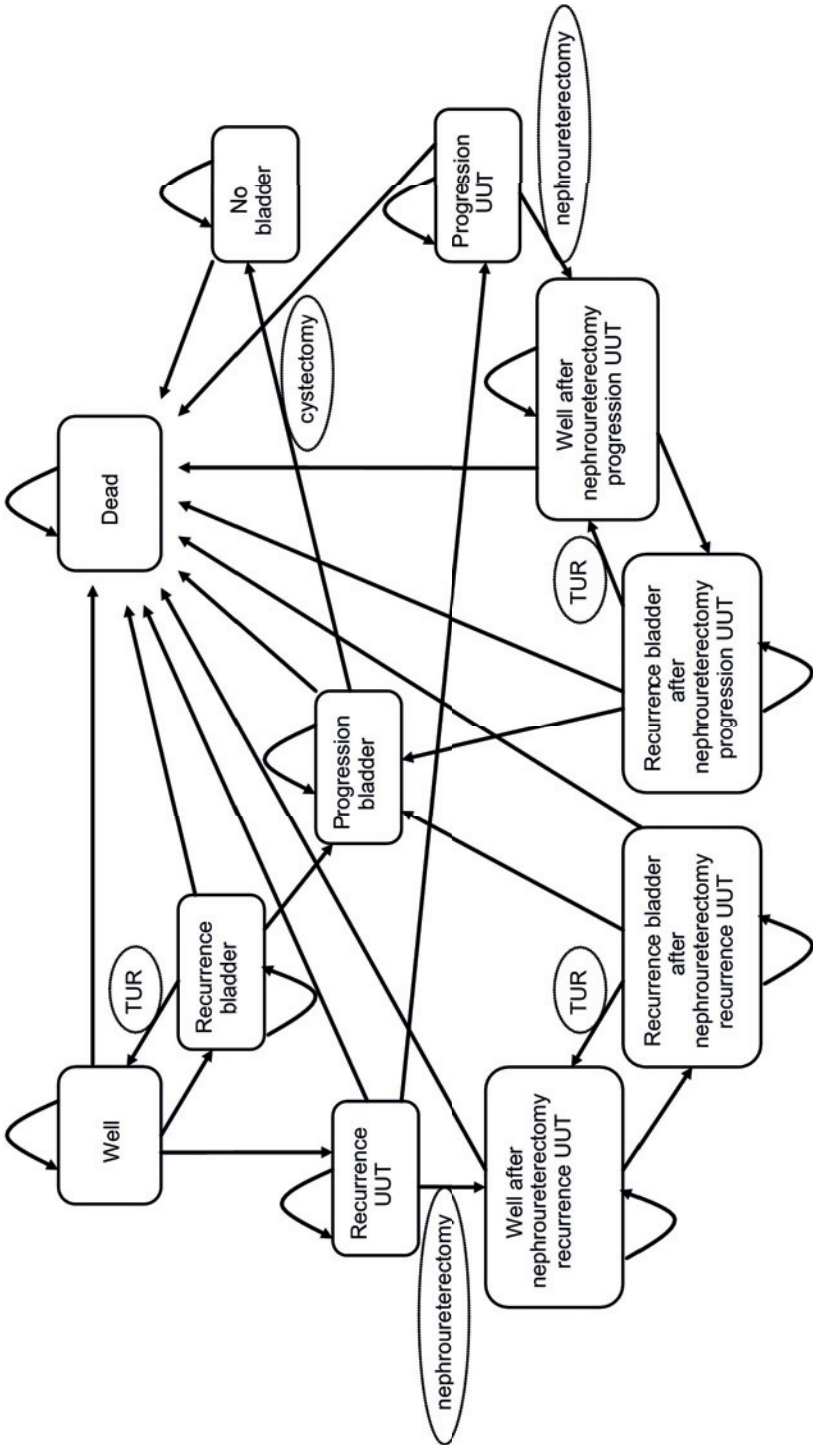

Figure 1. Decision model for bladder cancer surveillance[14]

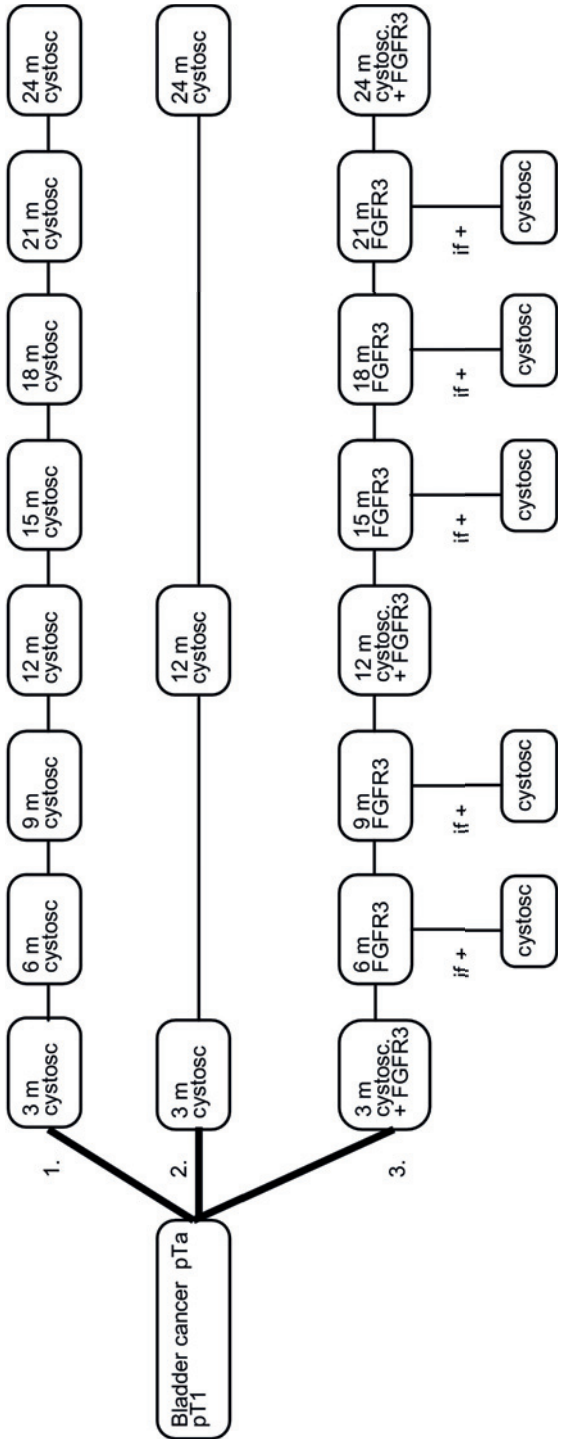

Figure 2. Three surveillance strategies for *FGFR3* positive nonmuscle invasive UC. *m*, months. 1, standard. 2, minimal. 3, modified. *cystosc*, cytoscopy. Plus sign indicates positive test for *FGFR3* mutation in voided urine.

Cost data were based on Dutch sources (supplementary table 1). Costs were determined using the micro-costing method using detailed hospital sources.[16] These bottom-up cost analyses included the costs of employment, material, equipment and overhead. For *FGFR3* mutation analysis we assumed that the assays were done in a volume of 48 analyses at the same time. The cost of the *FGFR3* tissue test on the primary tumor was included in the model. This test was done once in all patients in the modified surveillance arm (supplementary tables 1 and 2).

Model assumptions

We made certain structural model assumptions. 1) Only 1 recurrence developed per surveillance cycle. 2) Progression occurred after recurrence. 3) Only patients with tumor progression at a certain moment were at higher risk for death than the background mortality rate. 4) Cystoscopy never revealed recurrence or progression that originated in the UUT and invaded the bladder. 5) In case of progression to muscle invasive UC, the next step was cystectomy. 6) After cystectomy, surveillance stopped. 7) In case of positive CT of the abdomen, the next step was nephroureterectomy. 8) CT of the abdomen had 100% specificity and false-positive results were not possible. 9) The health state recurrence in the UUT after nephroureterectomy did not exist, preventing a second possible nephroureterectomy in the model.

Sensitivity analysis

One-way sensitivity analyses were performed to evaluate the effect of varying costs, sensitivity and specificity of the *FGFR3* mutation test and cystoscopy. Cost estimates were halved or doubled, and *FGFR3* mutation test and cystoscopy sensitivity and specificity were increased and decreased based on the literature to determine a plausible range (supplementary table 1).[1,9,14,15,17,18]

RESULTS

Input data

The 3-month risk of recurrence during the first 2 years of surveillance ranged from 1% to 3% after a primary tumor (supplementary table 3Patients at risk after a fourth recurrence were at 2.5 times higher 3-month risk (supplementary table 3). The risk of progression was substantially lower, e.g. less than 0.3% per 3 months, after the primary tumor.

Surveillance strategy comparison

After a primary tumor and 2 years of modified surveillance, 95.7% of patients were expected to be in the well state. This fraction was lower after 1 to 3 and 4 or more recurrences (95% and 93.6%, respectively). Standard and minimal surveillance led to lower results in 95% and 93.9% of patients, respectively, in the well state and on surveillance after a primary tumor (supplementary table 4The expected mortality rate was less than 3% for any strategy in each risk group, reflecting death from nonbladder cancer causes.

Minimal surveillance after the primary tumor led to more patients in the recurrence health state (bladder plus UUT) than standard or modified surveillance (3.1% vs 2.0% and 1.3%, respectively) and only 0.01% to 0.02% in the progression state (supplementary table 4The probability of achieving the no bladder health state (cystectomy after progression) was higher for modified and minimal surveillance (minimal 0.06% to 0.34%, standard 0.04% to 0.22% and modified 0.05% to 0.29%). Standard surveillance led to a slightly better detection rate of bladder recurrence during 2 years. However, with modified surveillance UUT recurrences were also detected that would have been missed by minimal or standard surveillance (UUT recurrence health state, supplementary table 4).

Costs

The cumulative diagnostic plus therapeutic costs during 2 years of surveillance were lowest for minimal surveillance after the primary tumor (€2,254) or after recurrences 1 to 3 and 4 (€2,287 and €2,363, respectively, supplementary tables 1 and 2). The costs of modified surveillance were similar. Therapeutic costs were higher than diagnostic costs for all surveillance strategies and all 3 risk profiles (supplementary table 2).

Surveillance burden

An average of almost 8 cystoscopies per patient was expected on standard surveillance, in contrast to approximately 3 and 4 for minimal and modified surveillance, respectively (supplementary table 5). For modified surveillance this would be an average of 1 cystoscopy in addition to the planned control cystoscopies at 3, 12 and 24 months. Patients in the modified surveillance arm would undergo almost another 8 urine tests.

Sensitivity analysis

Changing the model parameter values over plausible ranges did not make modified surveillance less effective than standard or minimal surveillance. The difference in the percentage of patients ending in the well health state after 2 years of surveillance varied from 0.14% to 3.0% for modified and standard surveillance, and from 0.84% to 8.1%

for modified and minimal surveillance, respectively. Differences in cumulative costs between standard and modified surveillance varied from €1,440 to €5,458 during 2 years of surveillance and from €217 to €1,345 between modified and minimal surveillance (data not shown).

DISCUSSION

The modified surveillance strategy showed promising results for *FGFR3* positive UC, in which cystoscopy is partly replaced by the *FGFR3* mutation test in voided urine. We considered 3 risk profiles, including surveillance after the primary tumor, after the first to third recurrence and after the fourth or higher recurrence. The effectiveness of modified surveillance was similar to that of standard surveillance and greater than the effectiveness of minimal surveillance. The costs of modified surveillance were substantially lower than those of standard surveillance and similar to those of minimal surveillance.

Studies have shown good potential for urine marker tests during surveillance for NMIBC.[19,20] However, those groups looked at urine tests in general. Many marker tests have been developed for this purpose.[21] We specifically tested the potential of the FGFR3 urine test.

We previously found that cross-sectional sensitivity of the *FGFR3* test was 58% using cystoscopy as the gold standard.[1] This relatively low sensitivity may possibly be explained by the imperfect sensitivity of cystoscopy. Urine markers may detect recurrence earlier than cystoscopy.[1] In addition, awareness of a positive urine test significantly improves the recurrence detection rate using cystoscopy.[22] This anticipatory effect was not included in the current model. Consequently, a combination of urine tests and subsequent cystoscopy, as represented by modified surveillance, may significantly improve recurrence detection. It may even lead to stage regression when recurrence is detected earlier, as observed for hexaminolevulinate cystoscopy.[23]

A strength of our study was the relatively long follow-up of the patients enrolled, which was needed to reliably estimate the transition probabilities of the different risk profiles. Long follow-up is especially needed to detect some progressions since the progression rate is low in *FGFR3* positive cases. This explains why simply decreasing the number of cystoscopies, as in the minimal surveillance strategy, had only minor implications for overall effectiveness. However, decreasing cystoscopies and partly replacing them with

urine tests has a benefit in effectiveness. It also results in a substantial cost reduction compared to standard surveillance and has only a slightly higher cost than minimal surveillance.

We counted the average number of tests that would be performed per patient as the burden of a surveillance strategy. The burden of cystoscopy is mainly discomfort from introducing the scope. The burden of the urine test is mainly the time spent waiting for results.[4,21] In the modified surveillance strategy we halved the number of cystoscopies but increased the total number of tests performed. How-ever, since we consider cystoscopy a safety net, it is still required in a modified surveillance strategy using *FGFR3* mutation analysis. Perhaps logistics and patient perception of urine tests can be improved, such that the burden of waiting time for test results can be decreased.

A number of limitations must be considered. Transition rates were based on relatively few patients and events, and on retrospective data. How-ever, others have confirmed the low recurrence and progression rates.*7,8*

We only modeled 2 years of follow-up, while surveillance may be required for the entire patient life-time. A longer time horizon might provide a stronger comparison among alternative strategies.

We also only modeled a limited number of potential surveillance strategies. Alternative strategies may consider whether a tumor recurs in the first year of surveillance. If not, it might be reasonable to reduce the intensity of surveillance in such patients. In that way the first year of surveillance would act as a stratification year to an adjusted surveillance strategy per patient.

Although false-positive results of *FGFR3* analysis are highly unlikely, the assumption of 100% specificity of the test may be an overestimation. Theoretically, false-positive findings may occur due to laboratory contamination.

We calculated the costs of *FGFR3* analysis based on an efficient practice of genetic testing in a non-profit laboratory in Europe. Commercial testing may be done at quite higher prices. Also, *FGFR3* tissue analysis of all primary tumors implies an increase of approximately €14 for all patients with NMIBC.

We analyzed only 1 specific urine test. Other tests have been developed, e.g. microsatellite analysis, ImmunoCyt™, CYFRA 21.1, cytokeratin 20 and UroVysion®.[21] A combination of different tests might show even more potential.

Modeling implies a simplification of clinical practice and the natural disease course. The assumptions made to make modeling possible are themselves a limitation. However, this study is meant as guidance for further clinical research.

Overall, our study shows the promising possibilities of urine tests in the surveillance of patients treated for NMIBC. *FGFR3* positive tumors are exemplary for the detection of tumor specific markers in voided urine samples. Further research is needed to continue the development of urine tests that perform well, especially for more aggressive tumor types.

CONCLUSIONS

For *FGFR3* positive tumors a modified surveillance strategy in which cystoscopy is partly replaced by *FGFR3* mutation analysis of voided urine samples seems safe, effective and cost-effective. However, further validation of these results is required to support implementation in clinical practice.

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Supplementary table 1. Input data for the Markov decision model to evaluate the cost-effectiveness of *FGFR3* mutation analysis on voided urine samples.

Sensitivity and specificity				
Test	**Location**	**Sens / Spec**	**Estimate (%) [plausible range]**	**Source**
FGFR3	Bladder	Sens	58 [28 – 86]	1
		Spec	100 [75-100]	1, expert opinion
	UUT	Sens	58 [28 – 86]	1
		Spec	100 [75-100]	1, expert opinion
Cystoscopy	Bladder	Sens	78 [46 – 86]	15
		Spec	65 [31 – 93]	15
	UUT	Sens	0	14
		Spec	100	14
CT	Abdomen	Sens	89	24
		Spec	100	Expert opinion

Transition probabilities			
Description		**3-month probability (%)**	**Source**
Recurrence probability		See table 2	Individual patient data
Recurrence probability UUT *		10	14
Recurrence probability bladder after nefro-ureterectomy		12	25
Progression after recurrence probability		See table 2	Individual patient data
Dead after progression probability	Year 1	12	14
	Year 2	8.5	14

Costs		
Description	**Costs (€) [plausible range]**	**Source**
FGFR3 urine analysis ^	€ 23 [12 – 46]	Dept. of Pathology, Erasmus MC (NL)
FGFR3 tissue analysis †	€ 14	Dept. of Pathology, Erasmus MC (NL)
Cystoscopy ^	€ 273 [137 – 546]	Dept. of Finance, Erasmus MC (NL)
CT abdomen ^	€ 185	Dept. of Urology, Erasmus MC (NL)
TUR #	€ 1,324	Dept. of Finance, Erasmus MC (NL)
Cystectomy #	€ 2,451	Dept. of Finance, Erasmus MC (NL)
Nephroureterectomy #	€2,328	Dept. of Finance, Erasmus MC (NL)

* given there is a recurrence, ^ diagnostic costs, # therapeutic costs, † included in modified surveillance

Supplementary table 2. Transition probabilities for recurrence and progression based on Cox regression analysis in 70 patients with primary non muscle invasive UC who had a *FGFR3* positive primary tumor

			3-month probability (%)		
Description	**Cycle**	**Month**	**after primary tumor**	**after recurrence 1 – 3**	**after recurrence 4+**
Recurrence probability	1	3	0.90	1.30	2.20
	2	6	2.80	4.10	7.00
	3	9	3.50	5.10	9.00
	4	12	3.10	4.50	7.80
	5	15	1.80	2.60	4.50
	6	18	1.10	1.60	2.70
	7	21	1.70	2.40	4.20
	8	24	1.70	2.40	4.20
Progression probability after recurrence	1	3	0.08	0.11	0.19
	2	6	0.24	0.35	0.61
	3	9	0.30	0.44	0.77
	4	12	0.27	0.39	0.67
	5	15	0.16	0.23	0.39
	6	18	0.09	0.13	0.23
	7	21	0.15	0.21	0.36
	8	24	0.14	0.21	0.36

Supplementary table 3a-c. Expected average health outcomes in percentages (%) over two years of follow-up with minimal surveillance, standard surveillance, or modified surveillance based on the Markov decision model and Markov cohort analysis with 1,000 fictional patients.

A. Primary tumor

Health state	Standard surveillance	Minimal surveillance	Modified surveillance
Well	95.04	93.92	95.69
Recurrence*	1.98	3.07	1.33
Bladder	0.41	1.53	0.67
UUT	1.58	1.54	0.66
Progression*	0.01	0.02	0.01
No bladder	0.04	0.06	0.05
Dead	2.93	2.93	2.93

*(Bladder + UUT)

B. 1st – 3rd recurrence

Health state	Standard surveillance	Minimal surveillance	Modified surveillance
Well	94.14	92.58	95.08
Recurrence*	2.83	4.33	1.88
Bladder	0.57	2.15	0.94
UUT	2.26	2.18	0.94
Progression*	0.02	0.04	0.01
No bladder	0.07	0.12	0.10
Dead	2.98	2.95	2.93

*(Bladder + UUT)

C. Recurrence 4+

Health state	Standard surveillance	Minimal surveillance	Modified surveillance
Well	91.96	89.45	93.59
Recurrence*	4.81	7.11	3.14
Bladder	0.97	3.53	1.58
UUT	3.83	3.58	1.56
Progression*	0.07	0.10	0.03
No bladder	0.22	0.34	0.29
Dead	2.94	2.99	2.95

*(Bladder + UUT)

Supplementary table 4. Diagnostic, therapeutic and cumulative costs (€) per patient over two years of follow-up with standard, minimal or modified surveillance based on the Markov decision model.

Surveillance strategy	Costs (€)	Primary tumor	1st – 3rd recurrence	recurrence 4+
Standard	Diagnostic	2,148	2,147	2,145
	Therapeutic	3,713	3,742	3,810
	Cumulative	5,861	5,889	5,955
Minimal	Diagnostic	806	806	805
	Therapeutic	1,448	1,482	1,558
	Cumulative	2,254	2,287	2,363
Modified	Diagnostic	1,057	1,072	1,105
	Therapeutic	1,514	1,578	1,724
	Cumulative	2,572	2,650	2,829

Supplementary table 5. Average number of cystoscopies and *FGFR3* urine tests per patient over two years of follow-up with standard surveillance, minimal surveillance or modified surveillance based on the Markov decision model.

Surveillance strategy	Risk profile	Average no. Cystoscopies	Average no. *FGFR3* urine tests
Standard	Primary tumor	7.87	0
	1st – 3rd recurrence	7.86	0
	recurrence 4+	7.86	0
Minimal	Primary tumor	2.95	0
	1st – 3rd recurrence	2.95	0
	recurrence 4+	2.95	0
Modified	Primary tumor	4.05	7.87
	1st – 3rd recurrence	4.08	7.86
	recurrence 4+	4.15	7.86

Part IV: General discussion and Summary

General discussion

The incidence of bladder cancer (BC) in the Netherlands has increased over 1.5-fold since the 1990s, with currently more than 7,000 new cases each year [1, 2]. Around two thirds of new patients are diagnosed with NMIBC, the remaining one third of patients are diagnosed with MIBC. The 5-year survival of NMIBC patients is very good (>90%), however recurrence rates are high (>50%) and progression to MIBC may occur [3-5]. Consequently, NMIBC necessitates long and costly surveillance. On the other hand, the prognosis of MIBC is very poor; the 5-year survival rate is around 60% for stage cT2 and only 6% for stage cT4 disease [6]. A total of 1,304 patients died of BC in 2016 in the Netherlands [2]. Treatment options for MIBC are limited and the survival rates have not improved much over the past 20 years, although recently introduced immune therapies present promising results.

The high incidence and recurrence rate of NMIBC, together with the poor survival rate of MIBC and the immense costs make BC a serious public health problem. Still, BC is understudied and awareness in the general population is shortcoming. A lack of funding opportunities is a major cause of this problem [7].

The general aim of this thesis was to evaluate and validate the use of (epi)genetic biomarkers in personalized BC management; including diagnosis of primary disease, prognosis of disease, tools for treatment allocation and their use in individualized surveillance regimens. In **part I** of this thesis, molecular urine markers are described that can detect BC in a population of hematuria patients. The proposed diagnostic urine assay was successfully validated in two independent patient cohorts. **Part II** of this thesis contains research on prognostic and predictive biomarkers: i) for the risk of progression of NMIBC to MIBC; ii) for the response to preoperative chemotherapy in MIBC patients undergoing radical surgery; and iii) for the presence of lymph node-positive disease at radical cystectomy. We were able to identify two genes that stratified EAU high-risk patients into three different risk groups for progression; good, moderate and poor. In addition, we investigated the neutrophil-to-lymphocyte ratio and a 20-gene expression signature as prognostic biomarkers in MIBC patients. In **part III,** an overview is given of *in vivo* research describing new molecular targets for therapy. We attempted to deduce reasons for a lack of efficacy of new therapies for BC in human studies. Also, a cost-effectiveness study was done to illustrate the clinical effects and monetary impact of implementation of urinary biomarker assays in a BC surveillance setting.

TOWARDS A REDUCTION OF CYSTOSCOPIES?

The vast majority of patients with a bladder tumor present with asymptomatic gross hematuria although bladder tumors can sometimes also cause other symptoms mimicking urinary tract infections, such as dysuria, frequency and urgency. Patients with hematuria represent an important burden in urology clinics (20%) and no definite consensus exists in current clinical guidelines on what the diagnostic work-up of patients with hematuria should be [8-14]. Mostly, it is recommended to do a visual inspection of the bladder by cystoscopy to rule out a bladder tumor. The prevalence of BC is estimated to range from 3% in microscopic hematuria cases to 28% in gross hematuria cases [9]. Consequently, most patients presenting with hematuria will not have BC, however, almost all of these patients undergo an invasive diagnostic cystoscopy [9].

In the primary diagnostic setting, a molecular urine assay could be employed as a rule-out assay for cystoscopy. The low prevalence rate in patients presenting with microhematuria necessitates a high NPV in order to not subject patients to unnecessary invasive procedures. In Sweden, none of the microhematuria patients undergo routine cystoscopies, however, we have shown that a small proportion of patients presenting with microhematuria can still harbor high risk tumors [14, 15]. Therefore, employing urine analysis as a rule-in assay is feasible when a highly sensitive biomarker assay is available. In addition, a molecular urine assay could also be employed in the primary diagnostic setting especially in women. A possible diagnostic delay in women is reported more frequently because UTIs are thought to be the cause of hematuria [16].

We developed a urine assay that combines methylation of the genes *ONECUT2*, *OTX1* and *TWIST1*, together with mutation markers in *FGFR3*, *TERT* and *HRAS,* and the clinical variable age [17]. In a retrospective discovery series, the assay performed well with a sensitivity of 97% and 83% specificity. The NPV proved 99.6-99.9% for an estimated 5-10% prevalence of BC in the investigated population [17]. The assay was validated in an independent retrospective case-enriched series, resulting in 93% sensitivity and 86% specificity [18]. Again, the NPV proved very high (99%). We estimated that the implementation of this assay could lead to a 77% reduction of cystoscopies in a primary diagnostics setting [18]. Since both development and validation cohorts consisted of retrospective, case-enriched series, we continued to validate the assay in a prospective natural cohort (n=1003) [15]. This was important since most patients presenting with hematuria will eventually not be diagnosed with BC. This prospective cohort was collected over a 1.5-year period in the Rotterdam region. All patients with microscopic or gross hematuria that would undergo a diagnostic cystoscopy were eligible for inclusion. The assay performed very well, with a sensitivity of 93%, a specificity of 81%

and a NPV of 99%. The predictive capacity of the assay was 95% [15]. We strongly believe that this study provides the evidence to convince clinicians to embrace implementation of the assay in clinical practice.

The assay will provide valuable information for clinical decision making; firstly, the performance of the cystoscopy will be enhanced with additional information provided by the urine assay. We have previously reported that the sensitivity of cystoscopy increases if the urologist is aware of a positive urine test [19]. Secondly, cystoscopy is used to inspect the bladder and urethra but cannot diagnose tumors in the upper urinary tract. A major advantage of a urine assay is the ability to detect both upper and lower urinary tract localizations of a urothelial tumor. A positive urine test combined with a negative cystoscopy, will encourage accelerated upper tract imaging leading to shorter delay in upper tract tumor diagnoses. Moreover, a negative urine assay can potentially reduce non-informative cystoscopies. A non-invasive ultrasound could be employed as an alternative in the search for other causes of hematuria.

Several other urine biomarker assays have been suggested in the literature over the years (eg NMP22®, Immunocyt™, Aura Tek FDP Test™, BTA stat®, Urovysion™ and Cxbladder™) [20-32]. Most assays did not prove to be sufficiently sensitive and/or specific to be able to replace cystoscopy [33]. As a result, none have been incorporated into clinical guidelines. In 2014, the prospective evaluation of the accuracy of the NMP22® test in a cohort of patients referred for hematuria evaluation was published [34]. In 381 patients, of whom 6% were diagnosed with BC, the predictive accuracy of the test was 80.2%. Unfortunately, this study maintained strict exclusion criteria; urinary tract infections, urinary retention or stone disease, kidney failure, ureteral stents, nephrostomy tubes, bowel interposition or recent genitourinary instrumentation. In contrast, in our studies the only exclusion criteria were; younger than 18 years of age and/or a prior history of BC. This closely resembles the true population of hematuria patients and is therefore more suitable for implementation into clinical practice. Furthermore, very different laboratory techniques are at the bases of these different urine assays. For instance, the NMP22® test is a protein-based assay, BTA stat® is an immunochromatographic assay, Urovysion™ is a FISH test and Cxbladder™ is a mulitiplex evaluation of a RNA gene expression signature [31]. Comparing all assays in a surveillance setting, Cxbladder™ performed best with an overall sensitivity of 91% and NPV of 96% [30].

The performance of most urine assays is based on comparison with the gold standard: cystoscopy. Importantly, sensitivity (68% to 84%) and specificity (31% to 91%) of standard white light cystoscopy are not perfect [35]. Blue-light cystoscopy is thought to improve this performance, however, most clinics do not have both techniques available

[36]. Therefore, the performance of most urine assays is probably underestimated. Further, cystoscopy is a point-of-care test. On the contrary, urine assays take several days to produce a test result. Therefore, application of a urine assay in acute macroscopic hematuria cases will, for now, not be feasible. However, there are patients who would prefer to have a urine test first and a cystoscopy only in case the urine test is positive. On the other hand, it is questionable how urgently a patient with macrohematuria should be evaluated by cystoscopy. Given the lead time of bladder tumors a diagnostic delay of 4 days seems acceptable. Furthermore, in our prospective validation study, we showed that the assay only missed very low risk TaG1 tumors in the microscopic hematuria patients. Therefore, we are convinced that performing urine analysis in microhematuria patients is very safe. Lastly, technological advances could overcome the time-to-result issue in the future, if urine biomarker assays become available as 'over the counter' tests, much like a urine dipstick analysis.

Implementation of urine assays could even be extended beyond the Urology-practice into the General Practitioners' office. Offering urine biomarker diagnostics in the General Practitioners' office may reduce the diagnostic delay of BC by increasing early referral. Before a urinary tract infection is treated with a course of antibiotics, the urine could be evaluated to see if a malignancy is causing the e.g. dysuria and microhematuria [16]. Ideally, this would shorten the time to diagnosis, potentially leading to downstaging of BC at time of diagnosis and eventually an increase in overall survival rate. Would technology allow for urine diagnostics to be an over-the-counter assay; this would revolutionize clinical practice. One could furthermore imagine urine diagnostics to be employed in a national screening program for BC [37]. A high risk population would be most eligible for inclusion in a national screening program, e.g. a population aged 50-70 years, tobacco smokers or chemical industry employees. Screening could entail analyzing a voided urine sample every 2-5 years. Again, a shorter time to diagnosis and downstaging would be the ultimate goal of such a screening program, thereby eventually increasing overall survival rate and reducing the need for invasive local treatment.

Overall, urine assays show great promise for clinical use. Ideally, the urine assays will be included in the guidelines for hematuria management. The assays are non-invasive, cheap and have the ability to detect upper tract malignancies. Combining urine diagnostics with established diagnostics, such as cystoscopy and ultrasound will probably be the best way to move the field forward in convincing clinicians of their added value to the clinic. A study on cost-effectiveness would be especially interesting. For instance, a registration study on microhematuria patients in the General Practitioner's office could

be performed, leading to better allocation of imaging diagnostics or cystoscopies. Another interesting option, as mentioned previously, would be a screening study in a high-risk population.

CAN MOLECULAR BIOMARKERS IMPROVE RISK STRATIFICATION FOR PROGRESSION OF NMIBC?

The prognosis of patients with NMIBC is mostly dependent on progression to MIBC, since survival of MIBC is far worse than NMIBC (5-year survival rate 60% for stage T2, 6% stage T4 disease versus >90% for NMIBC) [6]. The prognosis of NMIBC can be estimated by the EORTC risk tables and the EAU risk stratification [3, 38]. Several clinicopathological parameters are included in this risk score; the size, stage, grade of the tumor, the presence of concomitant *carcinoma-in-situ* (CIS), whether the tumor is primary or recurrent, and the prior recurrence-rate. The EAU guidelines advocate different adjuvant treatment protocols and surveillance schedules according to the risk stratification [38]. Patients can be appointed to either a low, intermediate or high-risk category. Even though molecular insight in bladder tumors has increased dramatically over the past years, no molecular markers have been added to this clinical risk stratification. We analyzed the added effect of several molecular markers to the known EAU risk stratification [39]. Interestingly, based on mutation status of *FGFR3* and methylation status of *GATA2*, the EAU high-risk patients could be stratified into three distinct risk categories; good, moderate and poor. The moderate risk group represented the same overall risk of progression to MIBC as the original EAU high-risk group [39]. The good sub-class had a much lower risk of progression and overtreatment could be reduced in this group. A lesser invasive surveillance regimen could be applied to this cohort of patients. Importantly, a group of patients with a very high risk of progression could be distinguished, the poor sub-class, with a 1.8 times higher risk of progression than the original EAU high-risk patient group [39]. This finding suggests that these patients should receive more intensive surveillance and additional treatments or should even be considered for early cystectomy. This sub-classification increases the accuracy of the EAU risk stratification, potentially leading to better treatment allocation and a reduction in overtreatment.

Another study, based on the same patient cohort, proposed a progression score based on a 12-gene expression signature, which was shown to add prognostic information beyond the EORTC risk score [3, 40]. This progression score performed particularly well in patients with an EORTC risk score > 6 points. Within this group of patients a high and a low-risk of progression sub-group could be appointed with a significantly different progression-free survival (p=0.035 or p=0.041, depending on the cutoff used) [40]. In 2010,

van Rhijn *et al*, compared a molecular grade with the EORTC risk scores [41]. The suggested molecular grade was based on *FGFR3* mutation status and MIB-1 expression. In a cohort of 230 primary NMIBC patients, both the EORTC risk score and the molecular grade showed independent predictive capacity for progression to MIBC. The EORTC risk score prediction improved by 6.8% to 81.7% predictive capacity when combined with the molecular grade [41]. Biomarkers to predict progression of NMIBC to MIBC independent of the EORTC or EAU risk scores have been suggested as well. Beukers *et al* presented a stratification tool based on methylation of *TBX2* and *TBX3* [42]. The three presented molecular grades differentiated patients with Ta-tumors into three different risk groups for progression (with a 5-year progression rate of 8, 29 and 63% respectively) [42].

While interpreting the results of the earlier EORTC progression score, the methylation-mutation progression sub-classification, the 12-gene progression score and different molecular grades, several important differences between the studies should be noted. First of all, the patient cohort on which the EORTC progression score was based originates from the years 1979-1989 [3]. At that time, treatment and follow-up of NMIBC patients differed a lot from the current practice. The first EAU NMIBC guideline was published in the year 2000 and BCG instillations were only FDA (U.S. Food and Drug Administration) approved in 1990. Thus, BCG instillations and the mandatory re-TUR for high-risk tumors were not common practice at that time. The recent prospective cohort on which the methylation-mutation sub-classification and the 12-gene risk signature were developed showed a much lower overall progression rate that the cohort described for EORTC risk score development. It is apparent, that the different treatment and follow-up of NMIBC has improved patient outcome considerably over the past decades. The low progression rate (approximately 5%) reported in the current study will complicate prospective replication studies because larger numbers of patients need to be included to obtain sufficient numbers of progressing cases. Further, the EORTC and EAU risk classification depend on pathological stage and grade of the initial tumor. In many studies, pathology reports are revised by a reference pathologist in order to reduce the considerable inter-observer variability inherent to pathological staging and grading. However, when we try to analyze an outcome that has been influenced by treatment or follow-up, such as progression-free survival, this may not be a good strategy because treatment and follow-up were based on the original pathology report. Revising pathology may stratify patients to other risk groups. It is our opinion that, as long as there is no expert pathology review up front, one should use the original pathology classification. Lastly, the 12-gene risk score was based on a qRT-PCR analyses and the methylation-mutation sub-classification and suggested molecular grades on a Snapshot analysis [39, 40]. In general, Snapshot analysis is less laborious and less costly than qRT-PCR analysis, although both techniques can be performed in a molecular pathology

laboratory. Overall, we can conclude that both the 12-gene progression score as the methylation-mutation sub-classification show great promise to improve the precision of the EAU and EORTC risk scores.

Ideally, our suggested risk stratification should be prospectively validated. Performing a randomized controlled trial will probably not be feasible, due to the low number of progressing cases and the duration of required follow-up. An alternative approach would be to perform a large retrospective study in progressing cases versus non-progressing NMIBC cases. Alternatively, a prospective registration study could be commenced. In such a study, molecular analyses could be performed on all EAU high-risk tumors. In this way, a sufficient number of progressing cases could be collected over the years to proof the principle of molecular sub-classification into good, moderate and poor subclasses. Eventually treatment allocation and surveillance intensity could be adjusted to these enhanced EAU risk profiles.

CAN MOLECULAR BIOMARKERS AID IN THE PREDICTION OF RESPONSE TO CHEMOTHERAPY AND THE RISK OF DISEASE PROGRESSION?

The prognosis of MIBC patients is highly dependent on the extent of the disease (local and distant) and the outcome of treatment interventions. Depending on clinical and pathological characteristics, standard treatment for non-metastatic MIBC consists of neoadjuvant chemotherapy followed by radical cystectomy and pelvic lymph node dissection. Bladder sparing treatment options are chemo-radiation or external beam radiotherapy. Unfavorable clinical and pathological characteristics are presence of hydroureteronephrosis, cT3b-T4a disease, and/or lymhovascular invasion, neuroendocrine or micropapillary aberrant histology on dTURBT [43]. Still, even patients with tumors that harbor favorable cliniclopathological features, almost 50% will be upstaged on final pathology [43]. Further, CT-imaging is known to be unreliable as a staging tool for BC and pathology reports are highly dependent on the experience of the pathologist [44]. This shows the difficulty of using clinical and pathological parameters as prognostic tools for MIBC.

Surprisingly, reports show that only 12-37% of patients actually receive NAC [45, 46]. The controversies on applying NAC can be appointed to the only modest 6% increase in 10-year survival [47]. Response rates to NAC treatment differ highly between patients. Approximately 30% of patients do not respond, but are exposed to treatment-related toxicity and may even be subjected to upstaging of disease or positive nodal disease

at time of cystectomy [48-50]. On the other hand, 25% of patients receiving NAC will have a complete response with no detectable tumor left at time of cystectomy. These complete responders have a 5-year survival rate of 80%, which is much better than the overall survival-rate [51, 52]. Further, only applying NAC in a subgroup of MIBC patients may also reduce cost of treatment, since hospital admission is necessary to administer chemotherapy. Up to now, no decision tools are available to select patients upfront for NAC that will respond or select patients that will not respond in order to withhold NAC. We evaluated the usefulness of the neutrophil-to-lymphocyte ratio, as a measure to predict poor outcome after pre-operative chemotherapy. The inflammatory microenvironment is an essential component of tumors and their behavior [53]. The NLR is a marker of systemic inflammatory response that indicates the balance of the inflammatory system and immune system respectively [54]. It is known that cancer cells can induce neutrophils to form a microenvironment that is beneficial to the malignant cells [55]. Therefore, an elevated NLR would be an indicator of potentially poor outcome. Although we found an association between a high pre-treatment NLR and poor outcome in terms of non-response and shorter PFS and OS, this result was nullified when corrected for lymph node status and clinical T stage [56]. Therefore, we had to conclude that the NLR is probably insufficient to predict response to NAC in MIC patients. A study from 2015 also showed no association between the NLR and pathological response to NAC in MIBC patients (OR = 0.69, 95%-CI: 0.36–1.32, p = 0.26) [50]. The authors of this publication, however, did use a different definition of response to NAC. The authors defined a pathological response as T0 or Tis and N0 disease together with down staging to T1 and N-positive to N0, whereas in our study a complete response to pre-operative chemotherapy (POC) was only defined as ypT0N0M0. Probably, the sample size of only 26 patients was too small to draw any meaningful conclusions. In our study of 123 patients, the sample size was perhaps still too small. In other malignancies, such as esophageal cancer, the NLR did prove a significant predictor for response to NAC ($p<0.01$) [57]. Perhaps other molecular predictive markers like *ERBB2* and *ERCC2* mutation status could be combined with the NLR and thereby potentially enhance the overall predictive capacity in MIBC patients [58, 59].

Another approach to allocate NAC is to select patients based on the probability of node-positive disease at time of cystectomy. In these patients, NAC could be employed to eradicate micrometastases and thereby reduce the risk of node-positive disease at radical cystectomy. Previously, a 20-gene expression signature was developed to predict the presence of lymph node metastases at radical cystectomy in clinically node-negative MIBC patients [60]. Smith *et al* identified differentially expressed genes by microarray from three different cohorts of cystectomy specimens. Since the TURBT represents the starting point for treatment decisions in MIBC patients, in our validation

study we used RNA isolated from a large cohort of dTURBT and not radical cystectomy specimens. In addition, we aimed to validate the 20-gene expression signature on a qRT-PCR platform, which is an easier to use technology than microarray analysis. Unfortunately, for this approach we also had to conclude that the 20-gene expression signature was clinically not applicable and cannot serve as a tool to guide treatment decisions on NAC in MIBC patients [61]. A different gene expression signature to predict the presence of lymph node metastases at time of cystectomy was developed in 2016 [62]. Seiler *et al* used RNA isolated from cystectomy specimens for a whole transcriptome approach. This resulted in a 51 K-nearest neighbor classifier (KNN51). Of the 51 genes included in this signature, none overlapped with the 20 genes from the earlier 20-gene expression signature of Smith *et al*. However, Seiler *et al* did attempt to validate the 20-gene expression signature. The 20-gene expression signature had an AUC of 0.46 and a non-significant OR of 1.39 versus an AUC of 0.82 and an OR of 2.65 ($p<0.01$) for the KNN51 model [62]. An important difference between our study and the study by Seiler *et al*, was the platform used for analyses. The whole transcriptome expression analysis described by Seiler *et al* is somewhat laborious and not widely available for standard diagnostic procedures in contrast to the qRT-PCR technology we used. Further, Seiler *et al* like Smith *et al* used radical cystectomy for their analysis and not dTURBT specimens we used. As the authors stated in their conclusions, the KNN51 expression signature should also first be validated on dTURBT specimens before implementation in clinical practice is possible. Even though the validation of the KNN51 signature is yet to be performed, we can conclude that the 20-gene expression signature is unfortunately not sufficient enough to predict the presence of nodal metastases in clinically node-negative MIBC patients.

As described in the introduction, chapter 1 of this thesis, clustering of MIBC tumors based on their gene expression profile revealed different gene expression subclasses. Several research groups have published different and partly-overlapping suggestions for classification [63-68]. In a meeting in 2016 several researchers attempted to facilitate progress and stimulate collaboration between the different research groups, with the ideal of eventually reaching agreement on a consensus taxonomy [69]. In 2017, Seiler *et al* described differences in response to NAC based on these different molecular subclasses [70]. Further, a single-patient genomic subtyping classifier was developed to predict subtypes with the highest clinical impact in the context of NAC. Albeit not yet prospectively validated, this publication demonstrates the promising impact of the discovery of the various molecular subclasses [70].

WHAT NEW POTENTIAL TREATMENTS FOR MIBC LIE ON THE HORIZON?

In other cancer types, targeted therapies have been long established. These therapies aim to interfere with cellular processes that are essential to cancer cell survival at the molecular level — for example, by blocking proteins involved in tumor cell proliferation or tumor cell metabolism, or by delivering toxic compounds to tumor cells. Well-known targeted therapies include gefitinib, a small-molecule EGFR inhibitor used for the treatment of chemotherapy-refractory non-small-cell lung cancer, and cetuximab, an EGFR antibody used for the treatment of head and neck cancer and advanced colorectal cancer [71, 72]. Other well-known targeted therapies are VEGF inhibitors (e.g. sunitinib) or VEGF antibodies (e.g. bevacizumab) that have been widely used to treat cervical, colorectal, lung, ovarian and renal cancer [73-77]. However, up to 2016 none of the known targeted therapies were approved for the treatment of BC. Perhaps, the magnitude of synchronous somatic mutations renders BCs more prone to targeted therapy resistance [78].

As of 2016, rapid developments in the field of immunotherapies have led to the accelerated approval by the FDA of five new immunotherapies for the treatment of BC, namely; atezolizumab, nivolumab, durvalumab, avelumab and pembrolizumab [79]. All immunotherapies are approved for the treatment of locally advanced or metastatic urothelial carcinoma patients with disease progression after chemotherapy. Pembrolizumab is the only drug that is additionally approved for patients with locally advanced or metastatic BC who are ineligible for second line cisplatin-containing chemotherapy. All above-mentioned compounds are immune checkpoint blockade therapies involved in the interaction between the programmed death-ligand 1 (PD-L1) and the programmed-death 1 receptor (PD-1). Avelumab, atezolizumab and durvalumab are PD-L1 antibodies, opposed to nivolumab and pembrolizumab, which are PD-1 antibodies. PD-1 is expressed on T cells and pro-B cells and can bind two ligands, PD-L1 and PD-L2. PD-L1 is found on tumor cells, PD-L1/PD-1 interactions result in an immunosuppressive effect beneficial for a pro-tumor environment [80]. Phase 2 studies have shown promising effectiveness of all immunotherapies, with overall response rates varying between 13.3% and 28.6%. Importantly, many patients suffered only mild side effects, although 40-50% of patients reported grade 3/4 side effects. In a successful phase 3 study, pembrolizumab showed a significantly longer overall survival of approximately 3 months compared to investigator's choice of chemotherapy as a second-line treatment for platinum-refractory MIBC [81]. Furthermore, significantly fewer severe side-effects of treatment were registered compared to chemotherapy (15% vs 49%) [81]. In May 2017, Roche (pharmaceutical company) reported unexpected

disappointing results from a phase 3 study on atezolizumab [82]. The phase 3 study was intended to confirm the results from the earlier phase 2 study, which resulted in the accelerated FDA approval of atezolizumab. However, the phase 3 study failed to reach its primary endpoint of improving overall survival in MIBC patients with high PD-L1 expression [83]. Perhaps an indication to restrain the enthusiasm somewhat and wait further confirmation from other studies.

In this thesis, we tried to elucidate reasons for the relative lack of targeted therapies in BC. Apart from immunotherapies, the small number of clinical trials in BC had limited success. To investigate whether promising new targets for therapy were being developed, we evaluated the results from animal studies published since 2009. It appeared that many potential targets had been investigated. In case of receptor inhibition or blockade, we concluded that the disappointing results from clinical trials were most likely due to the lack of patient stratification according to the downstream mutations in the pathways of the targeted receptors. For instance, activating mutations in the Ras–MAPK and PI3K–Akt pathways downstream of EGFR will render the tumor resistant to EGFR inhibition. We identified four studies on EGFR inhibition in human BC cell line xenografts that had such mutations in one or both pathways. In addition, in some cases a cytostatic effect was observed, which suggests that tumor growth probably resumes when treatment is stopped. Combining targeted treatment with chemotherapy usually enhanced the therapeutic efficacy in the mouse models. In clinical trials, combination treatments have been attempted but did not demonstrate an improvement in overall survival and sometimes resulted in increased toxicity in patients [84-86]. Furthermore, all xenografts included in our review were created by use of cell lines. Cell lines might not reflect the entire spectrum of tumor types, especially because relatively few BC cell lines are available. Also, there is a real risk of contamination amongst these cell lines [87]. In addition, cell-line-derived tumors probably lack the tumor heterogeneity observed in most cancers. A recent publication showed that N-butyl-N-(4-hydroxybutyl) nitrosamine (BNN)-induced BCs might still be a good option to use in animal studies. Although BNN-induced BCs are in fact mouse BCs, they do represent certain molecular characteristics similar to human MIBC [88]. Alternatively, xenografts established directly from resected tumors might present a better model than cell lines. Such tumor models also have human stroma and engage murine stromal cells, which is likely to be important in cell–cell interactions and cell proliferation [89]. Since 2015, several studies were published that describe the establishment of patient-derived xenografts for BC research [90]. This development will surely improve pre-clinical studies on new targeted therapies.

With the increasing knowledge of the biology of bladder tumors, biomarkers will continue to play an important role in identifying certain subgroups of patients. As was

initiated by the TCGA, combining different laboratory techniques to interpret overall tumor architecture is the next level of science [91]. For the future, it is to be expected that collaboration between different (inter)national laboratories will gain importance as the origin of tumor heterogeneity becomes clearer. Ideally, biomarker assays will combine different molecular test modalities. For instance, copy-number-variations combined with mutation data, to more precisely identify certain subgroups. Also, in the future more complex clinical studies will reveal the ideal momentum of administering certain treatments. Should immunotherapy be reserved for end-stage disease? Or should we start each patient on immunotherapy, with chemotherapy as a second option? Perhaps tumors can be sensitized to certain treatments, by for instance hyperthermia or sensitizing agents. Eventually, a continuous switchover strategy might be the ultimate solution.

IS IMPLEMENTATION OF URINE ANALYSIS FOR THE SURVEILLANCE OF NMIBC PATIENTS FEASIBLY?

Patients remain a minimal of five years under surveillance after treatment for BC, however, since BC recurs often, many patients will be kept under surveillance lifelong. Surveillance of patients treated for MIBC includes regular CT-scans and laboratory work-up, whereas surveillance after treatment for NMIBC entails regular cystoscopies [92]. Since, cystoscopies are time-consuming, expensive and an invasive procedure, urine diagnostics has great potential to reduce the burden of surveillance for NMIBC. In a simulated follow-up study we evaluated the safety and monetary impact of implementation of urine biomarkers assays in clinical practice [93]. Implementation of urine analyses proved safe as well as cost-effective. It is suggested that in the detection of recurrent BC a lower sensitivity and specificity of urine tests are achieved compared to primary BC detection [94]. A complete urine-based surveillance scheme seems therefore unfeasible. However, follow-up of patients treated for very low risk tumors seem good candidates for a surveillance scheme in which part of the cystoscopies are replaced by urine analysis. Further, analysis of multiple urines from one patient might be a manner to increase sensitivity and specificity [95]. Furthermore, one could argue that recurrences of low-risk tumors tend to be very small and low-risk as well. Missing such a recurrence at one surveillance time-point might not be too troublesome. Finally, in higher risk tumors urine biomarkers assays could enhance the accuracy of surveillance. Definitely, by implementing urine analysis in low-risk categories, a major cost-reduction could be achieved.

GENERAL CONSIDERATIONS AND CONCLUDING REMARKS

Several challenges remain for the research in NMIBC patients. Firstly, the content of urine is influenced by both fluid intake, as well as the coincidence of tumors shedding cells. Secondly, with well disseminated guidelines and improved treatments resulting in very low progression rates, prospective research on the risk of progression of NMIBC will be nearly impossible, due to the magnitude of the number of required study subjects. Finally, with differences in recurrence rates, prior treatments may vary between study subjects, resulting in a very heterogeneous patient population. Likewise, challenges remain while doing research in MIBC patients. Of all BCs, MIBC is particularly known to be a heterogeneous cancer in terms of biological behavior. Even in similar histological subtypes, the biological profile and behavior of the tumor may differ greatly. Even within one patient tumors at different time-points and locations (metastasis) show substantial heterogeneity [96]. In general, published studies are aimed at developing new hypotheses resulting in a lack of high quality validation studies. Further, as described in chapter 8, there is a superiority of positive findings in the literature, suggesting that publication bias does play a significant role.

The field of BC research is propelling forward with exciting new discoveries in recent years and no doubt more discoveries are on the horizon. Patients with BC and their treating physicians will have more diagnostic and treatment options in the future, improving clinical practice. Although new discoveries are essential in the ongoing research field, the lack of validation studies is a concern. Research funding is mostly available for studies aiming at new, out-of-the-box hypotheses. Still, to offer patients high-quality assays and treatments, we need to (independently) validate our results accordingly.

The BC research field is small compared to other tumor types. It is promising to see many publications in recent years coming from major international collaborations. The launch of collaboration-stimulating consortia, such as the International Bladder Cancer Network (IBCN) shows the great intentions of researchers interested in the field of BC. When the field continues to join forces, we will unquestionably be able to significantly enhance BC management in the end.

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SUMMARY

The general **aim of this thesis** was to evaluate the value of genetic and epigenetic biomarkers in the management of BC patients.

In **chapter 1**, a short introduction about the structure and function of the urinary tract is given and background information on BC is discussed. It covers the epidemiology, histological classification, diagnostic procedures, prognostic stratification, treatment regimens, and surveillance schedules of BC. BC is a very prevalent disease, which occurs three times more often in males than females and cigarette smoking is the most important known risk factor. However, awareness of the disease among the general population is still scant. Bladder cancer can either occur as NMIBC or MIBC, which results in very different treatments and survival outcomes. Although the survival of NMIBC is very good, the clinical challenge lies within the high recurrence rates and in identifying patients who are at risk of progression to invasive disease. Therefore, strict regimens for surveillance are mandatory, making BC the most costly cancer to manage. In contrast, the survival of MIBC patients is poor and new decision tools to allocate treatment, as well as new treatments are desperately needed.

In the first part of the thesis, several studies are described that comprise the discovery and the validation of a diagnostic urinary biomarker assay to select patients for cystoscopy. First, in **chapter 2**, the discovery of a methylation-mutation urine assay is described in a case-enriched series of 154 patients presenting with hematuria. The assay contained the variable age, methylation of the genes *OTX1*, *ONECUT2* and *TWIST1*, together with mutations of the *FGFR3*, *TERT* and *HRAS* genes. The assay was able to distinguish benign causes of hematuria and urothelial carcinoma as the cause of hematuria with 97% sensitivity and 83% specificity, resulting in a 99.6-99.9% NPV, with an estimated BC prevalence of 5-10%. In **chapter 3**, this assay was validated in an independent international case-enriched series of 200 hematuria samples. Again, the assay proved to be able to select patients with urothelial carcinoma as the cause of the hematuria with 95% accuracy as represented by the optimism-corrected AUC. Furthermore, we described the potential of a 77% reduction in the need for diagnostic cystoscopies when implementing such a urine-based selection tool in hematuria patients. We then proceeded in **chapter 4**, to further validate the rule-in urine assay for cystoscopy in a large prospectively collected cohort of 1003 patients presenting with either macroscopic or microscopic hematuria to the urology clinic. The previously described urine assay performed very well, with a sensitivity of 93% and a specificity of 81%. The assay had a NPV of 99% and all upper tract tumors were identified.

In part two of this thesis several studies are described in which the value of biomarkers in determining the prognosis of BC patients is evaluated. In **chapter 5**, the added value of several previously discovered methylation and mutation markers to the known clinical EAU risk classification were determined. In this study, a total of 1,239 patients in follow-up for NMIBC were prospectively included in hospitals in Denmark, Germany, Serbia, Spain, Sweden, and The Netherlands. The primary endpoint was progression to MIBC. With a median follow-up of 27 months, 4.6% patients had disease progression and we found that the EAU risk classification for patients at high-risk for progression was significantly improved by adding *FGFR3* mutation and *GATA2* methylation status. Our study resulted in a re-classification of the EAU risk classification into different high-risk sub-classes, with large differences in incidence rates for progression, namely 0.86 versus 4.32 versus 7.66. We conclude that implementation of these biomarkers into the EAU risk classification will increase its predictive capacity and may enable more personalized treatment. In **chapter 6,** the prognostic value of the derived neutrophil-to-lymphocyte ratio (dNLR) in MIBC was investigated in a retrospective cohort of 123 patients treated with platinum-based pre-operative chemotherapy (POC) and radical cystectomy. Analogue to what has been described in other tumors, an elevated dNLR corresponded with poor outcome in terms of survival and non-response to POC. However, after correction for well-known prognostic factors, such as positive lymph node status at diagnostic imaging and clinical T stage, the correlation for the dNLR was nullified. Therefore, we concluded that the dNLR is insufficient to predict response to POC in MIBC. In **chapter 7**, the clinical applicability of a previously reported 20-gene expression signature that might predict lymph node-positive disease in clinically node-negative MIBC patients was determined. This 20-gene expression signature could have potential as a decision tool to allocate NAC, since the presence of micro-metastasis would be reason to give NAC. We included 150 dTURBT samples of MIBC patients who were subsequently treated by radical cystectomy and pelvic lymph node dissection. In a univariate regression analysis, the expression level of none of the 20 genes significantly correlated with node-positive disease. The overall 20-gene expression signature resulted in an non-significant AUC of 0.54. Therefore, we concluded that this 20-gene expression signature is clinically not applicable and cannot serve as a tool to guide treatment decisions on NAC in MIBC patients.

The third part of this thesis entails studies in which the use of biomarkers in therapy and surveillance of BC patients is discussed. Up to January 2017, none of the targeted agents that were approved for cancer therapy were approved for the treatment of BC and the few clinical trials that have been performed had limited success, often owing to a lack of efficacy and toxic effects. In **chapter 8**, a comprehensive overview is given of targeted therapies for BC that have been investigated in animal models, some of which

have potential for clinical application. In this chapter, we provided insight into both the challenges and the promises of the preclinical development of targeted agents. We concluded that EGFR, FGFR3, VEGF, mTOR, STAT3, the androgen receptor and CD24 are molecular targets that can be efficiently inhibited, resulting in reduced tumor growth, and that these have been investigated in multiple independent studies. Several other targets, e.g. COX-2, IL, Bcl-xL, Livin and ChoKα, have also been observed to inhibit tumor growth, but these findings have not been replicated to date. Limitations of several studies included the use of cell lines with mutations downstream of the target, providing resistance to the tested therapy. Furthermore, certain technologies, such as interfering RNAs, although effective *in vitro*, are not considered ready for clinical applications. In **chapter 9**, we performed a cost-effectiveness analysis of the potential implementation of *FGFR3* mutation analysis on voided urine samples in the follow-up of NMIBC patients. A Markov decision model was created to analyze three different surveillance strategies; standard, minimal, and modified surveillance. Since risk of recurrence increases when the prior recurrence rate increases, we stratified the analysis of surveillance into three different risk profiles; after the primary tumor, after the first to third recurrence, and after the fourth recurrence or more. The probability of being without recurrence is higher for modified surveillance that for standard or minimal surveillance (surveillance after the primary tumor 95.7% versus 95.0% and 93.9%, respectively). The total two-year cost of surveillance is lower for minimal and modified surveillance compared to standard surveillance (€2,254 and €2,558, compared to €5,861). We conclude that partly replacing cystoscopies by *FGFR3* mutation analysis is not only safe, but also a cost-effective strategy.

Part four of this thesis, contains the general discussion and elaboration on future perspectives. In **chapter 10**, the results that are presented in the thesis are discussed and placed in the context of the global BC research field. Limitations of the different studies are elucidated and potential clinical implications of this thesis are summarized. Finally, challenges and aspirations for future studies are mentioned. In general, the field of BC research is steps behind other fields of cancer research (e.g. colon, breast, lung, and prostate). Also, BC is a very heterogeneous disease, complicating study design and clinical protocols. As awareness of BC increases, more funding will become available, aiding in the discovery of new targets and biomarkers for BC management. Over the past four years, major discoveries have propelled the field forward. Most important milestones include; the discovery of different molecular profiles of MIBC, and the development and approval of the new checkpoint inhibitors for metastatic BC. Now it is important to keep the momentum going, resulting is an ever brighter future for BC research and management.

SAMENVATTING

De algemene **doelstelling van dit proefschrift** is het evalueren van de waarde van genetische en epigenetische biomarkers in de aanpak van blaaskanker.

In **hoofdstuk 1**, wordt een korte introductie gegeven over de opbouw en functie van de urinewegen. Tevens wordt er achtergrond informatie gegeven over blaaskanker. Het omvat de epidemiologie, histologisch sub-classificatie, diagnostiek, prognostische stratificatie, behandel strategieën, en follow-up schema's van blaaskanker. Blaaskanker is een erg prevalente ziekte, welke drie keer vaker voorkomt bij mannen dan vrouwen. Het roken van sigaretten is de meest bekende risicofactor. Desondanks is de bekendheid van de ziekte onder de bevolking schaars. Blaaskanker komt voor als niet-spierinvasieve blaaskanker (NSIBK) of als spierinvasieve blaaskanker (SIBK), welke beide een heel andere behandeling en overleving hebben. Alhoewel de overleving van NSIBK erg goed is, ligt er een grote klinische uitdaging bij de hoge recidiefkansen en het identificeren van patiënten welke een verhoogd risico hebben op progressie naar spier-invasie. Daarom zijn strikte follow-up schema's noodzakelijk, deze maken de aanpak van blaaskanker tot meest kostbaar van alle kankertypes. De overleving van SIBK patiënten is daarentegen matig en nieuwe beslismodellen om de juiste behandeling te kiezen, als ook nieuwe behandel strategieën zijn hoog noodzakelijk.

In het eerste deel van dit proefschrift worden verschillende studies beschreven die gaan over de ontwikkeling en validatie van een diagnostische urine biomarker test voor de selectie van patiënten voor het ondergaan van een cystoscopie. Als eerste, in **hoofdstuk 2**, wordt een studie beschreven over de ontwikkeling van een methylatie-mutatie urinetest in een casus-verrijkte serie van 154 patiënten welke zich presenteren met hematurie. De test bevat de variabelen leeftijd, methylatie van de genen *OTX1*, *ONECUT2* en *TWIST1*, samen met de mutaties van *FGFR3*, *TERT* en *HRAS* genen. De test was in staat om onderscheid te maken tussen benigne oorzaken van hematurie en urotheelcelcarcinoom als oorzaak van de hematurie met een sensitiviteit van 97% en specificiteit van 83%, welke resulteerde in een 99.6-99.9% negatief voorspellende waarde, bij een geschatte blaaskanker prevalentie van 5-10%. In **hoofdstuk 3**, werd deze test gevalideerd in een onafhankelijk internationaal casus-verrijkte serie van 200 hematurie patiënten. Opnieuw bleek de test in staat om patiënten aan te wijzen welke urotheelcelcarcinoom als oorzaak van hun hematurie hadden met een 95% zorgvuldigheid zoals weergegeven door de optimisme-gecorrigeerde oppervlakte onder de curve. Verder beschrijven we de mogelijkheid om een 77% reductie van diagnostische cystoscopieën te bereiken wanneer deze op urine gebaseerde selectie wordt geïmplementeerd in hematurie patiënten. Dan gaat dit deel van het proefschrift

verder in **hoofdstuk 4**, waar de urinetest verder wordt gevalideerd als selectie-test voor het ondergaan van een cystoscopie. De testeigenschappen worden geanalyseerd in een groot prospectief verzameld cohort van 1003 patiënten welke verwezen zijn naar de uroloog met micoscopische of macroscopische hematurie. De eerder beschreven urine test gaf goede resultaten, met een sensitiviteit van 93% en een specificiteit van 81%. De test had een negatief voorspellende waarde van 99% en alle tumoren in de hogere urinewegen werden gedetecteerd.

In deel twee van dit proefschrift worden verschillende studies beschreven welke de waarde van biomarkers voor het bepalen van de prognose van blaaskanker patiënten evalueren. In **hoofdstuk 5**, wordt de toegevoegde waarde van eerder ontdekte methylatie en mutatie markers aan de bekende klinische EAU risico classificatie onderzocht. In deze studie werden in totaal 1239 patiënten in follow-up voor NSIBK prospectief geïncludeerd in ziekenhuizen in Denemarken, Duitsland, Servië, Spanje, Zweden en Nederland. De belangrijkste uitkomst van de studie was ziekteprogressie naar spier-invasie. Bij een mediane follow-up van 27 maanden, had 4.6% progressie van ziekte naar spier-invasie en we ontdekte dat de EAU risico classificatie voor patiënten met hoog risico voor progressie sterk kon worden geoptimaliseerd door de toevoeging van *FGFR3* mutatie en *GATA2* methylatie status. Onze studie resulteerde in een re-classificatie van de EAU hoog-risicoclassificatie in verschillende hoog-risico sub-classes, met zeer verschillende incidentie cijfers en progressie cijfers, namelijk 0.86 versus 4.32, versus 7.66. We concluderen dat de implementatie van deze biomarkers in de EAU risicoclassificatie de precisie zal doen toenemen en dat dit kan leiden tot meer gepersonaliseerde behandeling. In **hoofdstuk 6**, wordt de prognostische waarden van de afgeleide neutrofiel-lymfocyt ratio (aNLR) in SIBK onderzocht in een retrospectief cohort van 123 patiënten behandeld met platinum-houdende pre-operatieve chemotherapie (POC) en radicale cystectomie. Zoals dit ook eerder al beschreven was in andere soorten tumoren, correspondeerde een verhoogde aNLR met een slechte uitkomst wanneer gekeken werd naar overleving en achterblijven van respons op POC. Echter, na correctie voor bekende prognostische factoren zoals positieve lymfklierstatus op diagnostische beeldvorming en klinisch T stadium, verdween de correlatie met de aNLR. Daarom concluderen we dat de aNLR onvoldoende functioneert om de kans op respons op POC in SIBK te voorspellen. In **hoofdstuk 7** wordt de klinische toepasbaarheid onderzocht van een eerder beschreven 20-genen expressie panel welke de aanwezigheid van positieve lymfklieren in klinische lymfklier-negatieve SIBK patiënten zou kunnen voorspellen. Dit 20-genen expressie panel zou eventueel als beslishulp kunnen dienen in de afweging om wel of geen neoadjuvante chemotherapie (NAC) te geven, aangezien de aanwezigheid van micrometastasen reden zou zijn voor NAC. We includeerden 150 diagnostische transurethrale resectie monsters van

blaastumoren van SIBK patiënten welke daaropvolgend werden behandeld middels radicale cystectomie en pelviene lymfklierdissectie. In een univariate regressie analyse bleek het expressie niveau van géén van de 20 genen significant gecorreleerd met de aanwezigheid van positieve lymfklieren. Het totale 20-genen expressie panel resulteerde in een niet-significante oppervlakte onder de curve van 0.54. Daarom concludeerden we dat dit 20-genen expressie panel klinisch niet toepasbaar is en dat het daarom niet kan dienen als beslishulp voor het al dan niet toedienen van NAC in SIBK patiënten.

Het derde deel van dit proefschrift bevat studies welke het gebruik van biomarkers in de behandeling en follow-up van blaaskanker patiënten beschrijven. Tot januari 2017 waren geen van de gerichte therapieën welke goedgekeurd waren voor behandeling van andere kankerpatiënten goedgekeurd voor de behandeling van blaaskanker patiënten. De spaarzame klinische studies die waren uitgevoerd waren weinig succesvol, vaak ten gevolge van een gebrek aan effectiviteit en de aanwezigheid van schadelijke bijeffecten. In **hoofdstuk 8** wordt een uitgebreid overzicht gegeven van gerichte therapieën voor blaaskanker welke onderzocht zijn in diermodellen, waarvan sommigen de potentie hebben om klinisch toepasbaar te zijn. In dit hoofdstuk geven we een overzicht van zowel de uitdagingen als de potentie van de preklinische ontwikkeling van gerichte middelen. We concludeerden dat EGFR, FGFR3, VEGF, mTOR, STAT3, de androgeen receptor en CD24 moleculaire aangrijpingspunten zijn welke effectief kunnen worden afgeremd, wat resulteert in een afname van tumor groei. Bovendien waren deze moleculen onderzocht in meerdere studies. Verschillende andere aangrijpingspunten, zoals COX-2, IL, Bcl-xL, Livin en ChoKα, hebben ook een afname van tumorgroei laten zien, echter deze bevindingen zijn nog niet herhaald in andere studies. Enkele tekortkomingen van verschillende studies waren onder andere het gebruik van cellijnen welke mutaties hadden in moleculen verderop in de communicatie cascade, leidend tot resistentie van de onderzochte therapie. Daarnaast zijn verschillende onderzochte technologieën, zoals het aangrijpen op bepaalde types RNAs, alhoewel effectief in vitro, nog niet klaar voor klinische toepassingen. In **hoofdstuk 9** hebben we een kosten-effectiviteitsstudie uitgevoerd waarin we de potentiele implementatie van een *FGFR3* mutatie analyse op uitgeplaste urinemonsters in de follow-up van NSIBK patiënten hebben onderzocht. Een Markov beslismodel werd gebruikt om drie verschillende follow-up strategieën met elkaar te vergelijken: een standaard, minimaal en aangepaste follow-up strategie. Aangezien het risico op een recidief tumor toeneemt wanneer het voorgaande recidiefcijfer hoger wordt hebben we de analyses gestratificeerd voor drie verschillende risicoprofielen: na de primaire tumor, na de eerste tot derde recidief, na het vierde recidief of meer. De waarschijnlijkheid om zonder tumor te zijn is hoger voor de aangepaste follow-up strategie dan voor de standaard of de minimale follow-up strategie (95.7% na de primaire tumor versus 95.0% en 93.9%). De totale tweejaars

kosten van de follow-up is lager voor de minimale en aangepaste follow-up strategie in vergelijking met de standaard follow-up strategie (€2,254 en €2,558, in vergelijking tot €5,861). We concluderen dat het deels vervangen van cystoscopieën door *FGFR3* mutatie analyse niet alleen veilig is, maar ook een kosten-effectieve strategie.

Deel vier van dit proefschrift bevat de algemene discussie en een uiteenzetting van ontwikkelingen in de toekomst. In **hoofdstuk 10** bediscussiëren we de resultaten die beschreven staan in dit proefschrift en plaatsen we die resultaten in de context van het wereldwijde blaaskanker onderzoek. Tekortkomingen van de verschillende studies worden toegelicht en potentiele klinische implicatie van dit proefschrift worden samengevat. Tot slot benoemen we de uitdagingen en de na te streven toekomstige studies. In het algemeen loopt het onderzoek naar blaaskanker achter op andere takken van kankeronderzoek (zoals darm, borst, long en prostaat). Daarnaast is blaaskanker een erg heterogene ziekte, welke het bestuderen ervan bemoeilijkt en het ontwikkelen van klinische protocollen ingewikkeld maakt. Wanneer de bewustwording van blaaskanker toeneemt, zal er meer onderzoekssubsidie beschikbaar komen, wat zal bijdragen aan de ontdekking van nieuwe aangrijpingspunten voor therapie en biomarkers voor de aanpak van blaaskanker. Gedurende de laatste vier jaar hebben verschillende grote ontwikkelingen in het onderzoek naar blaaskanker het hele onderzoeksveld stappen vooruit gebracht. De meest belangrijke mijlpalen zijn de ontdekking van verschillende moleculaire profielen van SIBK en de ontwikkeling en goedkeuring van verschillende immunotherapieën voor gemetastaseerd blaaskanker. Het is nu vooral belangrijk om deze impuls vast te houden, wat ongetwijfeld zal leiden tot een steeds zonnigere toekomst voor blaaskanker onderzoek en de aanpak van blaaskanker.

Part V: Appendices

LIST OF ABBREVIATIONS

Akt	serine/threonine-protein kinase
ACR	American College of Radiology
AI	Allelic Imbalance
AR	Androgen Receptor
ARF	tumor suppressor ARF
AUA	American Urological Association
AUC	Area Under the Curve
BAX	apoptosis regulator BAX
BC	Bladder Cancer
BCG	Bacillus Calmette-Guerin
Bcl-xL	Bcl-2-like protein 1 expressed from BCL2L1
BNN	N-butyl-N-(4-hydroxybutyl) nitrosamine
B-raf	serine/threonine-protein kinas B-raf
CD24	signal transducer CD24
cDNA	complementary DNA
CI	Confidence Interval
CIS	Carcinoma In Situ
CMV	Cisplatin, Methotrexate and Vinblastine
CNA	Copy Number Alterations
CNV	Copy Number Variations
CR	Complete Response
CT	Computed Tomography
ddNTP	Dideoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
dNLR	derived Neutrophil-to-Lymphocyte Ratio
dTURBT	diagnostic Transurethral Resection of a Bladder Tumor
E2F	transcription factor of the E2F family
EAU	European Association of Urology
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
eIF	Eukaryotic translation Initiation Factor
EORTC	European Organisation for Research and Treatment of Cancer
EphB4	Ephrin type-B Receptor 4
EPI	Epirubicin
FDA	United States Food and Drug Administration
FFPE	Formalin-fixed Paraffin-embedded
FF	Fresh Frozen
FGFR	Fibroblast Growth Factor Receptor
GDP	Guanosine Diphosphate

GFR	Glomerular Filtration Rate
GTP	Guanosine Triphosphate
HB	Hemobglobin
HIF-1α	Hypoxia-Inducible Factor 1α
HR	Hazard Ratio
HRAS	Harvey rat sarcoma viral oncogene homolog
IAP	Inhibitors of Apoptosis Proteins
IGF	Insulin-like Growth Factor
IL	Interleukin
IR	Incidence Rate
KRAS	Kirsten rat sarcoma viral oncogene homolog
LN	Lymph Node
LOH	Loss of Heterozygosity
LVI	Lymphovascular Invasion
Mab	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MDM2	E3 ubiquitin-protein ligase Mdm2
MEK	dual specificity MAPK kinases 1 and 2
MIBC	Muscle Invasive Bladder Cancer
MMC	Mitomycin C
MSP	Methylation Specific PCR
mTOR	mammalian Target of Rapamycin
MVAC	Methotrexate, Vinblastine, Doxorubicin and Cisplatin
NA	Not Applicable
NAC	Neoadjuvant Chemotherapy
NF-κB	Nuclear Factor κB
NLR	Neutrophil-to-Lymphocyte Ratio
NMIBC	Non-Muscle Invasive Bladder Cancer
NPV	Negative Predictive Value
NR	Non-response
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
OR	Odds Ratio
OS	Overall Survival
P21	cyclin-dependent kinase inhibitor 1
P53	cellular tumor antigen p53
PAI	Plasminogen Activator Inhibitor
PCR	Polymerase Chain Reaction
PD-L1	Programmed cell Death 1 ligand 1
PCR	Polymerase Chain Reaction
PFS	Progression Free Survival
PI3K	Phosphatidylinositol 3 Kinase

PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
POC	Pre-operative Chemotherapy
PPV	Positive Predictive Value
PR	Partial Response
PUNLMP	Papillary Urothelial Neoplasm of Low Malignant Potential
qRT-PCR	quantitative real-time PCR
ROC	Receiver Operating Characteristic
RPS6	40S ribosomal protein S6
RTK	Receptor Tyrosine Kinase
SD	Standard Deviation
shRNA	small hairpin RNA
siRNA	small interfering RNA
STAT	Signal Transducer and Activator of Transcription
TCGA	The Cancer Genome Atlas
TERT	Telomerase Reverse Transcriptase
TNM	Tumor Nodes Metastasis
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TUR	TransUrethral resection
TURB	Transurethral Resection Bladder
TURBT	Transurethral Resection of a Bladder Tumor
UBC	Urinary Bladder Cancer
uPA	Urokinase-type Plasminogen Activator
VEGFR	Vascular Endothelial Growth Factor Receptor
VUC	Voided Urinary Cytology
WBC	White Blood cell Count

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Research School	Molecular Medicine (MolMed)
PhD period	2013-2018
Promotor	Prof. dr. Ellen C. Zwarthoff
Co-promotor	Dr. Joost L. Boormans

	Year	ECTS
Courses and workshops		
Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers (BROK)	2016	1.0
Leadership in Cancer Research: Essential Strategies and Skills for Success, AACR annual meeting 2016 – New Orleans, United States of America	2016	0.1
How to proceed with Hematuria, 31st Annual Congress of the EAU – Munich, Germany	2016	0.1
Course on R	2015	1.4
SNP Course XI: SNPs and Human Diseases	2014	2.0
Course on Molecular Diagnostics IX	2014	1.0
Workshop on InDesign CS6	2014	0.3
A broad spectrum of NGS applications in Molecular Medicine	2014	2.0
Research Integrity	2014	0.3
Biomedical English Writing	2014	2.0
Workshop on Photoshop and Illustrator CS5	2013	0.3
Basic Human Genetics	2013	0.5
Biomedical Research Techniques	2013	1.5
Scientific conferences		
Dutch Association of Urology – spring meeting, Nijmegen, The Netherlands[1]	2018	0.3
AUA Annual meeting 2018, American Urological Association, San Francisco, United States of America[1]	2018	1.2
33rd Annual EAU Congress, European Association of Urology – Copenhagen, Denmark[2]	2018	1.2
International Bladder Cancer Network (IBCN) annual meeting – Lisbon, Portugal	2017	0.5
AACR Annual meeting 2017, American Association for Cancer Research – Washington, United States of America	2017	1.2
32nd Annual EAU Congress, European Association of Urology – London, United Kingdom[2]	2017	1.2
Dutch Association of Urology – fall meeting, Nieuwegein, The Netherlands	2016	0.3

	Year	ECTS
International Bladder Cancer Network (IBCN) annual meeting – Bochum, Germany[1]	2016	0.5
24th Annual meeting of the EAU Section of Urological Research (ESUR), European Association of Urology – Parma, Italy[2]	2016	1.0
95th Annual Meeting of the South Central Section of the AUA, American Urological Association – Colorado Springs, United States of America[2]	2016	1.2
4th Daniel den Hoed Day – Rotterdam, The Netherlands[1]	2016	0.3
AACR Annual meeting 2016, American Association for Cancer Research – New Orleans, United States of America[2]	2016	1.2
31st Annual EAU Congress, European Association of Urology – Munich, Germany[2]	2016	1.2
16th Annual meeting of the Society of Urologic Oncology (SUO), Society of Urologic Oncology – Washington, United States of America[2]	2015	1.0
Annual meeting of CGC.nl (CancerGenomics) – Amsterdam, The Netherlands	2015	0.5
23rd Annual meeting of the EAU Section of Urological Research (ESUR), European Association of Urology – Nijmegen, The Netherlands[1,2]	2015	1.0
AACR Annual meeting 2015, American Association for Cancer Research – Philadelphia, United States of America[2]	2015	1.2
30th Annual EAU Congress, European Association of Urology – Madrid, Spain[2]	2015	1.2
Daniel100 Symposium - Cancer Research and Clinical Care: The Next 100 Years! – Rotterdam, The Netherlands	2014	0.1
TransBioBC Meeting, Biomedical Research Foundation of the Academy of Athens (BRFAA) – Athens, Greece[1]	2014	0.3
22nd Annual meeting of the EAU Section of Urological Research (ESUR), European Association of Urology – Glasgow, United Kingdom[2]	2014	1.0
Dutch Association of Urology, spring meeting – Rotterdam, The Netherlands	2014	0.3
29th Annual EAU Congress, European Association of Urology – Stockholm, Sweden[2]	2014	1.2
Dutch Association of Urology, fall meeting – Nieuwegein, The Netherlands[1]	2013	0.3

[1] oral presentation; [2] poster presentation

Teaching		
Tutoring first year medical students	2015-2017	3.0
Course on Molecular Diagnostics X, MolMed	2016	0.3
Supervising microscopy practical 'the male reproductive organs', third year medical students	2016	0.5
Course on Molecular Diagnostics VIII, MolMed	2014	0.3
Supervising MSc and DSc student projects		
- Florence E.C. Voets	2016	2.0
- Lorraine M. de Haan	2015	2.0
- Naeromy Y.C. Welvaart	2014	2.0

	Year	ECTS
Miscellaneous		
Lecturing		
Regional urology meeting 'refereeravond urologie regio Rotterdam'	2018	0.1
Research colloquium JNI	2017	0.1
Research colloquium JNI	2017	0.1
Research colloquium JNI	2016	0.1
Regional urology meeting 'refereeravond urologie regio Rotterdam'	2015	0.1
Pathology Laboratory Meeting, PALM	2014	0.1
JNI research meeting, visiting professorship D. Theodorescu	2014	0.1
Pathology Laboratory Meeting, PALM	2014	0.1
Research colloquium JNI	2014	0.1
Meetings		
Journal club (monthly)	2013-2017	1.4
Refer evenings Urology	2013-2018	2.0
Urology education (weekly)	2013-2017	5.0
Research colloquium JNI (weekly)	2013-2017	5.0
Pathology Laboratory Meeting (PALM) (every two weeks)	2013-2017	2.5
JNI oncology lectures (every two months)	2013-2017	0.7
Peer review		
- Urologic Oncology: Seminars and Original Investigations - Bladder Cancer - Scientific Reports	2016-2018 2016-2018 2018	0.3 0.3 0.1
Organization Bladder Cancer Research Day, visiting professorship Prof. Theodorescu, Erasmus MC, Rotterdam, the Netherlands	2015	2.0
Awards		
Best Poster Award Poster presentation: 20-gene expression signature to predict lymph node positive disease at radical cystectomy for muscle-invasive bladder cancer: Not validated, 32nd Annual EAU Congress, London, United Kingdom	2017	
Best Poster Award Poster presentation: Elevated neutrophil-to-lymphocyte ratio correlates with non-response to neo-adjuvant chemotherapy in muscle-invasive bladder cancer, 31st Annual EAU Congress, Munich, Germany	2016	
Best Poster Award Poster presentation: Improved diagnostic urine assay to select patients for initial cystoscopy, 30th Annual EAU Congress, Madrid, Spain	2015	

DANKWOORD

Dit proefschrift had nooit tot stand kunnen komen zonder de vrijwillige bijdrage van alle deelnemende patiënten. Ik besef mij dat u allen, totaal onbaatzuchtig, bereid bent geweest uw klinische gegevens, urine en weefsels te delen ten bate van toekomstige blaaskanker patiënten. Onze dank is groot.

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Absoluut onmisbaar, was de bijdrage van de dames-analisten op het lab. Irene, Kirstin en Angelique; jullie hebben een enorme inzet getoond. Ook al kwamen we soms om in de urine-potjes, TMAs en dozen DNAs, jullie doorzettingsvermogen was fantastisch.

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Lieve Esther, ik ben echt super trots op jou. Je bent een echte inspiratiebron, super sportief, mega down to earth en een ontzettende stoere bikkel. Jouw doorzettingsvermogen is indrukwekkend. Ik ben dol op onze culturele, manicure-bevattende, lekkere wijn-overgoten dinertjes en high tea parties.

Dames, met jullie steun, moet die promotie helemaal goed gaan komen. Ik ben trots met zulke power vrouwen aan mijn zijde!

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ABOUT THE AUTHOR

Kim van Kessel was born on February 2nd, 1985 in a small country town called Maarheeze in the south-eastern part of the Netherlands. She attended high school at the S.C. Augustinianum in Eindhoven and graduated in 2003. Then, she moved to Utrecht to study Biomedical Sciences for one year at the University of Utrecht. After which she passed her entry exam for Medical School at the Erasmus University in Rotterdam. After her second year of medical training she devoted one year to the Medical Student Association Rotterdam as a full-time board member. During this year she was responsible for sponsorship revenues as well as actively involved in the organization of a multitude of activities for medical students. In 2007 she continued her medical training and became a member of the Student Council to advocate student interests within the medical faculty. During this year she was also invited to complete a second masters in Clinical Epidemiology (chair: Prof. dr. A. Hofman) before starting her clinical rotations. As part of this program, she attended several courses at the Harvard School of Public Health in 2009 (supervisor: Prof. E.W. Steyerberg). All throughout the remainder of her medical training, she continued to represent medical students and medical interns in numerous councils and meetings. In 2012, she obtained her medical degree and started working in the IJsselland Ziekenhuis as a medical doctor in General Surgery. Hereafter, she gained clinical experience in the Urology Department of the Erasmus Medical Center. In September 2013, she started her full-time PhD position in the laboratory of Prof. dr. E.C. Zwarthoff at the Department of Pathology. During these years she tutored two groups of first year medical students, guided microscopy practicals to third year medical students and taught molecular diagnostics to Molecular Medicine master students. In July 2017 she returned to the IJsselland Ziekenhuis to start her General Surgery training as part of her residency in Urology. Kim currently lives in Rotterdam and continues to be actively involved in bladder cancer research.