Bladder cancer diagnosis in a new light

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BLADDER CANCER DIAGNOSIS IN A NEW LIGHT

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Bladder cancer diagnosis in a new light

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COLOFON

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Bladder cancer diagnosis in a new light

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- Voor mijn ouders -

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Introduction

CHAPTER 1

General introduction

Parts of this chapter have been published as:

Can we improve transurethral resection of the bladder tumour for non-muscle invasive bladder cancer?
EIML Liem, TM de Reijke
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Urologic Oncology, 2018 Jul;36(7)

BACKGROUND

Epidemiology and etiology

Bladder cancer is the 10th most common cancer worldwide [1,2]. In the United States 81.400 of newly diagnosed cases are estimated for 2020, making bladder cancer in men the fourth most common malignancy and in women the twelfth most common malignancy [1]. In 2018, in the Netherlands 6.583 new cases of bladder cancer were diagnosed [3]. The incidence in men is roughly 4 times higher than in women [2].

The most important risk factor is smoking of tobacco [4–6]. Other risk factors are genetic predisposition, exposure to pelvic radiotherapy, Schistosomiasis and to certain chemicals, mainly used in industrial settings, which process for example paint, dye, metal and petroleum products [4,7,8].

Pathology

The vast majority of bladder tumours are urothelial carcinomas. Other subtypes are squamous cell carcinoma, small cell carcinoma and adenocarcinoma [9]. For risk stratification and to determine prognosis, bladder cancer is classified in stage and grade, number of tumours and recurrence rate. The most frequently used system for staging bladder cancer is the 2009 TNM classification approved by the Union International Contre le Cancer (UICC) (Table 1) [10]. Approximately 75% of new cases of bladder cancer are non-muscle invasive bladder cancer (NMIBC), in which the disease is confined to the mucosa [11]. Grouped as NMIBC are stages Ta, T1 and Tis (carcinoma in situ [CIS]), whereas stages T2, T3 and T4 are grouped as muscle invasive bladder cancer (MIBC). For histological grading of NMIBC the 1973 World Health Organization (WHO) classification distinguishes well differentiated (Grade 1), moderately differentiated (Grade 2) and poorly differentiated (Grade 3) urothelial carcinoma. In 2004, the WHO and the International Society of Urological Pathology (ISUP) published a new grading classification. This grading system differentiates papillary urothelial carcinoma neoplasm of low malignant potential (PUNLMP), low-grade (LG) urothelial carcinoma and high-grade (HG) urothelial carcinoma (Table 2). The current guidelines of the European Urology Association (EAU) are still based on both grading classifications [12].

Table 1. UICC TNM 2009 classification for bladder cancer [10].

T – Primary tumour	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ: 'flat tumour'
T1	Tumour invades subepithelial connective tissue
T2	Tumour invades muscle T2a Tumour invades superficial muscle (inner half) T2b Tumour invades deep muscle (outer half)
T3	Tumour invades perivesical tissue T3a Microscopically T3b Macroscopically (extravesical mass)
T4	Tumour invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall T4a Tumour invades prostate stroma, seminal vesicles, uterus or vagina T4b Tumour 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
N2	Metastasis in multiple regional lymph nodes in the true pelvis
N3	Metastasis in common iliac lymph node(s)
M – Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis

Table 2. The WHO 1973 and 2004 grading systems.

1973 WHO grading	
Grade 1	Well differentiated
Grade 2	Moderately differentiated
Grade 3	Poorly differentiated
2004 WHO grading	
Papillary urothelial neoplasm of low malignant potential (PUNLMP)	
Low-grade (LG) papillary urothelial carcinoma	
High-grade (HG) papillary urothelial carcinoma	

Diagnosis

Cystoscopy is the gold standard in bladder cancer diagnosis. It enables direct visualisation of the bladder mucosa and identification of abnormalities of the urothelium (Figure 1). Most urothelial carcinomas appear as papillary lesions.

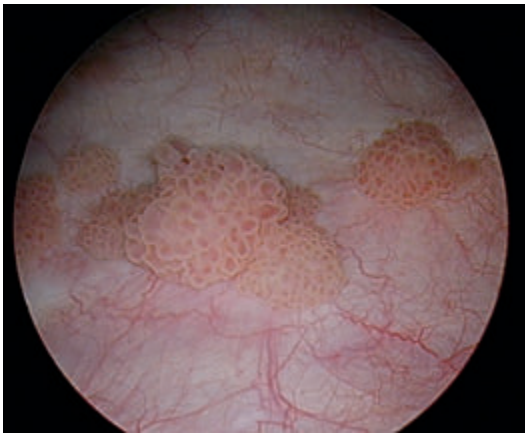

Figure 1. Multiple papillary tumours identified during cystoscopy.

However, CIS is a flat lesion and may appear as erythematous velvet-like lesions. During cystoscopy these lesions can be missed or misinterpreted as inflammatory lesions or reactive to intravesical instillations.

Over the years, several techniques have been introduced to enhance visualisation of tumours during cystoscopy. Photodynamic diagnosis (PDD) enhances visualisation of neoplastic tissue. Following administration of 5-aminolevulinic acid (5-ALA) or hexyl-aminolevulinate (HAL) protoporphyrin IX accumulates more in cancerous urothelium compared to normal tissue. When protoporphyrin IX is illuminated with blue light, it emits red fluorescence and can be used to visualize urothelial carcinoma. PDD has a higher detection rate for both papillary tumours and CIS compared to white light (Figure 2) [13–18].

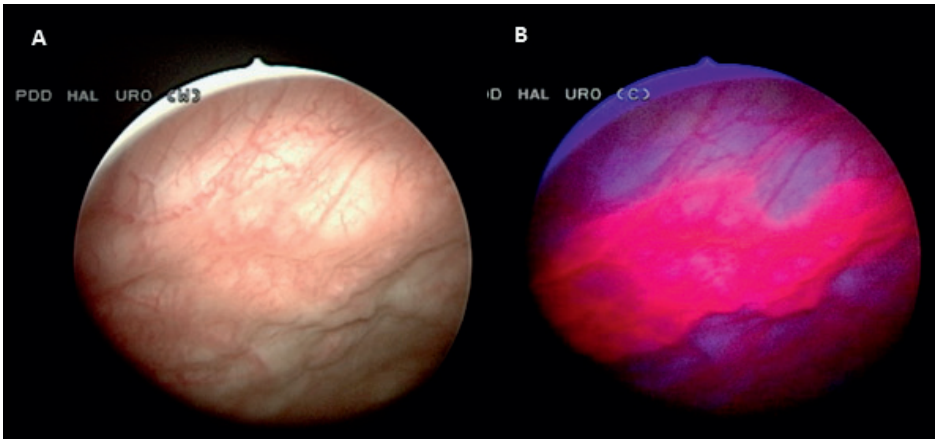

Figure 2. A) White light cystoscopy does not show any apparent tumour, B) The same location using PDD shows a suspicious lesion (reprinted with permission from Ipsen).

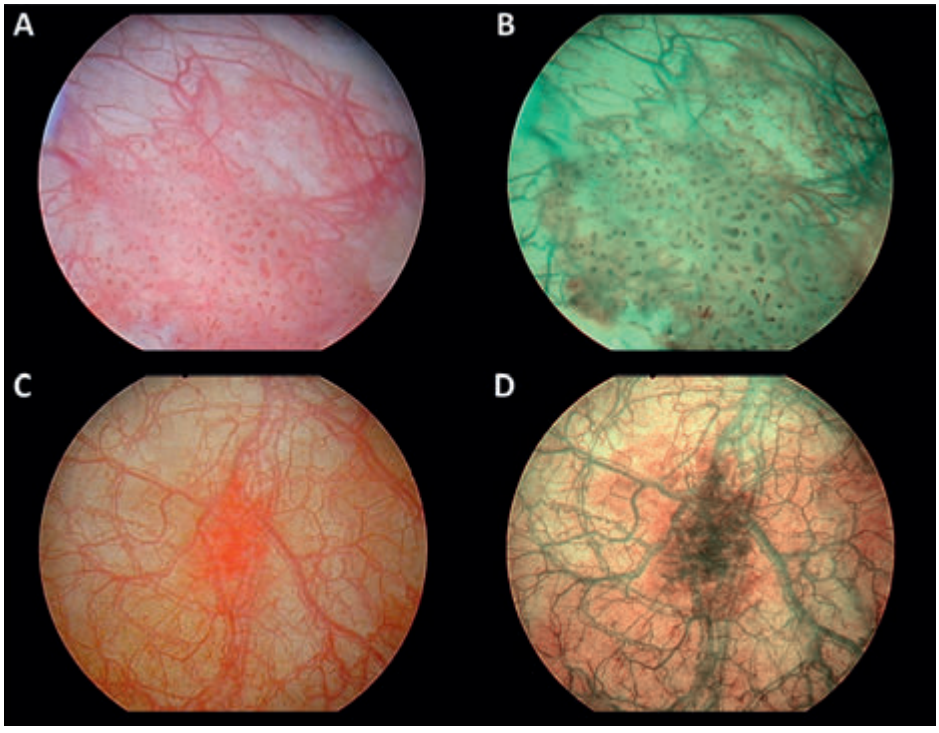

Figure 3. A) Bladder tumour with white light cystoscopy. B) The same tumour with NBI. C) Carcinoma in situ with white light cystostopcy. D) The same lesion with NBI.

Narrow band imaging (NBI) is an image enhancement technique without the need of fluorescent agents. It uses 2 specific wavelengths; blue (415 nm) and green (540 nm), which are both strongly absorbed by haemoglobin. This absorption by blood results in enhanced contrast between hypervascular tissue and normal mucosa. As tumours often are well vascularised this technique improves visualisation of urothelial carcinomas. NBI improves tumour detection and possibly reduces the recurrence rate due to better identification of the cancerous lesions (Figure 3) [19–25].

Although cystoscopy (with or without imaging techniques such as PDD or NBI) allows confirming the presence of a bladder tumour, it lacks histopathologic evaluation without resection. Subsequent histological evaluation of tissue is thus essential. Confocal laser endomicroscopy (CLE) is a probe-based optical imaging technique that produces real-time high-resolution microscopic images of tissue in vivo. Confocal microscopy detects mainly in-focus scattered light, which results in a high-resolution image (spatial resolution of 1 µm) in a single horizontal plane at an imaging depth of 65 µm. CLE imaging requires a fluorescent agent, for which fluorescein is most commonly used. Fluorescein is a non-specific contrast agent and stains the extracellular matrix.

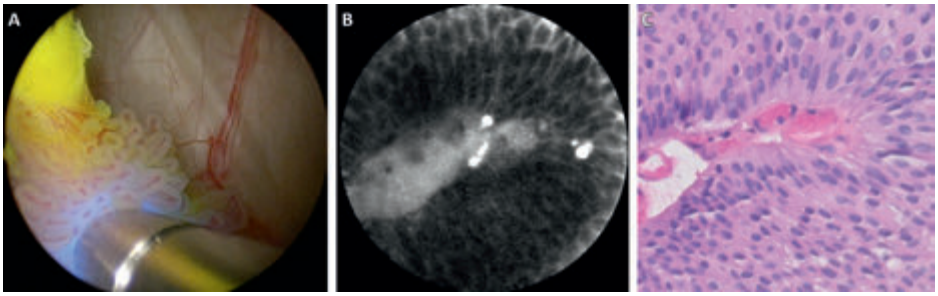

Figure 4. Confocal laser endomicroscopy. A) White light cystoscopy of a low-grade TaG2 papillary tumour. B) Corresponding CLE image. C) Corresponding hematoxylin and eosin-stained slide.

By the excitation of fluorescein by a 488 nm low-power laser light and the subsequent emission around 520 nm, cell structures can be visualised, which allows subcellular imaging. Initially, CLE imaging was introduced in gastroenterology for diagnosing Barrett's oesophagus [26,27]. Currently, other applications in gastroenterology, pulmonology, otolaryngology and urology are being explored [28–31]. Using CLE it is possible to image bladder cancer and identify malignant characteristics (Figure 4) [32,33].

Treatment of non-muscle invasive bladder cancer

Primary treatment for NMIBC is transurethral resection of the bladder tumour (TURB). The goal of TURB in NMIBC is both diagnostic and therapeutic to obtain adequate tissue samples to determine tumour stage and grade and to resect all visible abnormalities. Complete resection including a sample of the underlying muscularis propria (detrusor muscle) is recommended by the guidelines of the EAU and American Urological Association (AUA) [12,34]. An accurate histopathological evaluation by the pathologist depends on the quality of the resection and processing of the tumour specimen [35].

As recommended by the EAU guidelines, a TURB should be performed systematically, starting with bimanual examination of the bladder to assess disease extent and resectability [12]. The resectoscope should be inserted under visual control with inspection of the whole urethra, followed by inspection of the whole bladder urothelium. When indicated (e.g. high-risk disease), biopsies should be taken from the prostatic urethra and cold-cup random bladder biopsies before resection of the bladder tumour. In order to determine invasion depth, it is important to include the underlying bladder wall in the resected specimen. Small tumours (<1cm) can be resected en-bloc, whereas larger tumours need to be resected in fractions. To minimise tissue damage, cauterisation should be avoided as much as possible during TURB. Indication to take biopsies from normal looking urothelium is when cytology is positive and no papillary tumour is detected. Biopsies from the prostatic urethra are indicated when a tumour is present at the bladder neck or when CIS is present or suspected. A second TURB, 2–6 weeks following initial resection, should be performed in selected cases: 1) when initial TURB

is incomplete, 2) if no muscle is present in the specimen after initial resection (except in case of Ta LG/G1), 3) in all T1 tumours, and 4) in all Grade 3 or high-grade tumours [12].

TURB can be performed using monopolar or bipolar electrocautery. Traditionally, TURB was performed using monopolar electrocautery, although the last decade bipolar resection is increasingly being used. Main advantage of bipolar TURB is that normal saline can be used for irrigation, thus minimising the risk of TUR-syndrome, as was reported in transurethral resection of prostatic tissue (TURP) [36]. Both techniques are well tolerated and efficient to treat bladder tumours [37,38]. Comparative studies have not provided solid evidence that bipolar resection for bladder tumours is superior compared with monopolar resection in surgical outcome, complication risk or tumour recurrence rate [39,40].

Currently, en-bloc resection that allows resection of tumours >1 cm in one piece is emerging [41]. Several techniques have been described, though all these techniques have in common that first a circular incision is made in the mucosa around the tumour, and subsequently the tumour is dissected en-bloc including the muscle layer [42–45]. This method seems well-tolerated and feasible to use in selected cases. The specimen is of good quality and well oriented, which makes it easier for the pathologist to assess tumour stage [46]. In context of the seeding theory, en-bloc resection could potentially decrease recurrence rates. However, long-term studies are still lacking and RCTs should be performed to compare en-bloc resection with conventional TURB and evaluate the oncologic outcome.

Depending on the risk-group, patients with NMIBC should receive additional intravesical adjuvant treatment. Based on an individual patient data meta-analysis, the EAU guidelines recommend administration of a single instillation of chemotherapy immediately postoperatively in presumably low-, or intermediate-risk tumours with a low recurrence rate (less than one recurrence per year) [47]. However, another large prospective study including 2.243 patients was not able to identify any subgroup that does not benefit from a single postoperative instillation [48]. An immediate, single postoperative instillation prevents circulating tumour cells following TURB from implantation in the bladder wall and has an ablative effect at the resection site on residual tumour cells [49–52]. A single, immediate postoperative intravesical instillation of chemotherapy reduces the five-year recurrence rate from 59% to 45% in low-risk NMIBC [47]. The most commonly used chemotherapeutic agents for intravesical instillations are Mitomycin C (MMC) and Epirubicin. The instillation should be administered within 24 hours following TURB, preferably within the first two hours. However, in the case of a bladder perforation, extensive resection or bleeding that requires bladder irrigation, the instillation of chemotherapy should not be administered, because of the risks of local and systemic complications due to extravasation of the chemotherapy instillation [53].

For intermediate-, and high-risk NMIBC patients additional adjuvant instillations are recommended by the EAU and the AUA guidelines [12,34]. For intermediate-risk NMIBC intravesical chemotherapy or Bacillus Calmette-Guérin (BCG) immunotherapy is recommended. For high-risk NMIBC, intravesical BCG immunotherapy is recommended. BCG is the most effective intravesical therapy currently available [54,55]. BCG is a live attenuated strain of the Mycobacterium bovis and induces an immune response in the bladder, although the exact anti-tumour mechanism is still under investigation. Induction BCG therapy is empirically given as an induction course of six weekly intravesical instillations followed by a course of maintenance instillations. Although it is the most effective intravesical immunotherapeutic treatment currently available, it is also associated with more side effects compared to intravesical chemotherapy [56]. These side effects can lead to interruption or discontinuation of BCG instillations in up to 20% of patients [57]. In 63% of patients, local side effects such as irritative voiding symptoms, symptoms of cystitis and haematuria may occur. In 31% of patients, systemic side effects such as arthralgia, hypersensitivity reactions, persistent fever and BCG sepsis with possible fatal consequences may occur [58]. Additionally, BCG treatment fails in up to 40% of patients [59–61]. To minimize side effects, BCG instillations should be started within 14 days following TURB. BCG instillations should be postponed in case of macroscopic haematuria or bacterial cystitis [62]. For optimal efficacy a BCG maintenance schedule should be given. Though it is still not clear which BCG maintenance schedule is the most effective, BCG maintenance should be given for at least one year [63,64].

Follow-up of non-muscle invasive bladder cancer

In the follow-up of bladder cancer, cystoscopy is often combined with urinary cytology to determine if a recurrence is present. Urinary cytology is an examination based on pathologists' interpretation of morphological changes of exfoliated urothelial cells in voided urine or bladder washings. Urinary cytology has a high specificity (81–95%) for bladder cancer. For Grade 3 or HG bladder tumours urinary cytology has a high sensitivity (75–84%), however, for Grade 1 or LG tumours, sensitivity is limited (16–53%) [65–67]. Cytological evaluation can be influenced by recent intravesical instillations, urinary tract infections and the presence of stones in the bladder or by a limited number of cells present in the urine sample. Another limitation of urinary cytology is the high inter-, and intra-observer variability since the interpretation of cytology is highly investigator dependent. Recently, the Paris system was introduced for reporting urinary cytology. This system focuses on diagnosing HG urothelial carcinoma and provides well-defined morphological criteria, including the atypical urothelial cell groups. This system seems easy to apply, is well reproducible and seems to correlate well with histology [68,69].

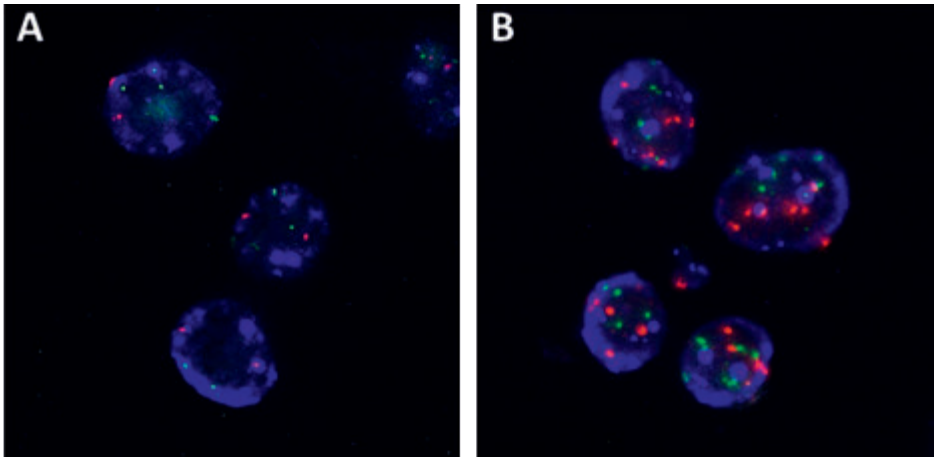

Figure 5. A) UroVysion® fluorescence in situ hybridization (FISH) in normal cells. B) UroVysion® FISH in abnormal cells.

To overcome the low sensitivity of urinary cytology, various urinary tests have been developed. Sensitivity of urinary markers is in general higher compared to urinary cytology, though at the cost of a lower specificity. Urinary markers are currently not widely accepted in daily practice nor in clinical guidelines. Currently, various biomarkers are commercially available, based on different biological properties; protein based (nuclear matrix proteins [NMP-22], bladder tumour antigen [BTA STAT, BTA TRAK]), cellular antigens (uCyt+), DNA-based (UroVysion), RNA-based (CxBladder detect) and DNA methylation-based (EpiCheck) [70,71]. One of these urinary markers is UroVysion®, which is a fluorescence in situ hybridization (FISH) test. This test is FDA approved for bladder cancer detection in patients with hematuria and for the follow-up of patients with NMIBC. FISH is a cytogenetic technique that enables detection, localisation and visualisation of specific DNA sequences. The UroVysion® bladder cancer FISH kit consists of 4 probes to detect genetic alterations most commonly associated with bladder cancer; increased copy number of chromosomes 3, 7, 17 and a total deletion of 9p21. Since this test is based on detecting genetic alterations, it will not be influenced by recent intravesical instillations, such as BCG (Figure 5) [72].

GOALS OF THIS THESIS

Histological staging and grading are important factors determining prognosis and treatment management of bladder cancer. Unfortunately, during cystoscopy histologic assessment of bladder tissue is not possible. The first goal of this thesis is to evaluate confocal laser endomicroscopy (CLE) for bladder cancer diagnosis. CLE has the ability to visualise tissue on a microscopic level in vivo and could, therefore, overcome this shortcoming during cystoscopy. In this thesis we present early clinical research for the

validation of CLE for urothelial cancer grading. The second goal of this thesis is to improve bladder cancer management for patients treated with Bacillus Calmette-Guérin (BCG). The use of fluorescence in situ hybridization (FISH) is evaluated to identify patients at risk for bladder tumour recurrence following BCG induction therapy. Thus, with our work we aim to find approaches to improve bladder cancer diagnosis and management.

OUTLINE OF THIS THESIS

Part II of this thesis focuses on confocal laser endomicroscopy (CLE) for bladder cancer diagnosis. In **Chapter 2** the research protocol is described that aims to evaluate the use of CLE in the diagnosis and grading of urothelial carcinoma in the bladder. In this chapter the protocol is reported together with a research protocol for the upper urinary tract, since both protocols share similar methodology. **Chapter 3** provides a comprehensive introduction into the principle of CLE and a detailed description on how to perform CLE-imaging in the bladder as well as in the upper urinary tract. **Chapter 4** presents a validation of earlier proposed CLE-features. Based on these CLE-features NMIBC can be identified and grading of bladder tumours is possible. In **Chapter 5** grading of NMIBC is performed using automated image analysis. Using a shallow neural network it is possible to perform automated image analysis of CLE-images of the bladder differentiating low-grade and high-grade lesions.

Part III of this thesis focuses on the application of fluorescence in situ hybridization (FISH) in bladder cancer patients for predicting tumour recurrence following Bacillus Calmette-Guérin (BCG) immunotherapy. **Chapter 6** presents a prospective, multicentre Dutch study that evaluates the value of FISH for predicting tumour recurrence at three different time points during BCG induction therapy. In **Chapter 7** an international meta-analysis based on individual patient data combines the results of four different studies for predicting tumour recurrence, in patients treated with BCG, using FISH.

Part IV finalises this thesis, providing a summary in **Chapter 8** and concluding remarks and future perspectives in **Chapter 9**.

LIST OF ABREVIATIONS

5-ALA	5-aminolevulinic acid
AUA	American Urological Association
BCG	Bacillus Calmette-Guérin
CIS	Carcinoma in situ
CLE	Confocal laser endomicroscopy
EAU	European Association of Urology
FISH	Fluorescence in situ hybridization
HAL	Hexyl-aminolevulinate
HG	High-grade
ISUP	International Society of Urological Pathology
LG	Low-grade
MMC	Mitomycin C
NBI	Narrow band imaging
NMIBC	Non-muscle invasive bladder cancer
PDD	Photodynamic diagnosis
PUNLMP	Papillary urothelial neoplasia of low malignant potential
TURB	Transurethral resection of bladder tumour
TURP	Transurethral resection of the prostate
UICC	Union International Contre le Cancer
WHO	World Health Organisation

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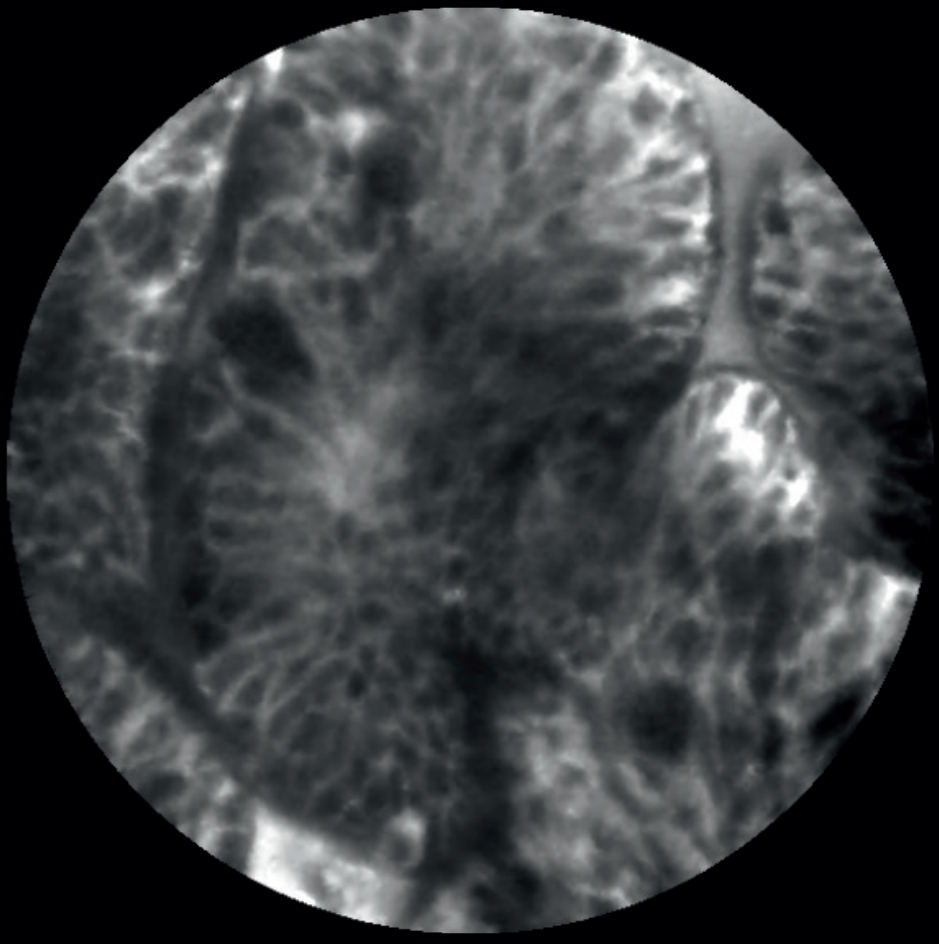
PART II

Confocal laser endomicroscopy for bladder cancer diagnosis



CHAPTER 2

Confocal laser endomicroscopy for the diagnosis of urothelial carcinoma in the bladder and the upper urinary tract: protocols for two prospective explorative studies

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ABSTRACT

Background: Visual confirmation of a suspicious lesion in the urinary tract is a major corner stone in diagnosing urothelial carcinoma. However, during cystoscopy (for bladder tumours) and ureterorenoscopy (for tumours of the upper urinary tract) no real-time histopathologic information can be obtained. Confocal laser endomicroscopy (CLE) is an optical imaging technique that allows for in vivo high-resolution imaging and may allow real-time tumour grading of urothelial lesions.

Objective: The primary objective of both studies is to develop descriptive criteria for in vivo CLE images of urothelial carcinoma (low-grade, high-grade, carcinoma in situ) and normal urothelium by comparing CLE images with corresponding histopathology.

Methods: In these two prospective clinical trials, CLE imaging will be performed of suspicious lesions and normal tissue in the urinary tract during surgery, prior to resection or biopsy. In the bladder study, CLE will be performed in 60 patients using the Cystoflex UHD-R probe. In the upper urinary tract study, CLE will be performed in 25 patients during ureterorenoscopy, who will undergo radical treatment (nephroureterectomy or segmental ureter resection) thereafter. All CLE images will be analysed frame by frame by three independent, blinded observers. Histopathology and CLE based diagnosis of the lesions will be evaluated. Both studies comply with the IDEAL stage 2b recommendations.

Results: Presently, recruitment of patients is ongoing in both studies. Results and outcomes are expected in 2018.

Discussion: For development of CLE based diagnosis of urothelial carcinoma in the bladder and the upper urinary tract, a structured conduct of research is required. This study will provide more insight in tissue-specific CLE criteria for real-time tumour grading of urothelial carcinoma.

Trial registration: The study protocol for Confocal Laser Endomicroscopy in the bladder was registered in the Dutch Central Committee on Research Involving Human Subjects with the number NL55537.018.15 on February 4, 2016 and, retrospectively, on Clinicaltrials.gov as registration number NCT03013894 on January 5, 2017. The study protocol for Confocal Laser Endomicroscopy in the upper urinary tract was registered in the Dutch Central Committee on Research Involving Human Subjects with the registration number NL52989.018.16 on May 25, 2016 and, retrospectively, on Clinicaltrials.gov as registration number NCT03013920 on January 5, 2017.

INTRODUCTION

Urothelial carcinoma is the most common malignancy of the urinary tract. The majority of these tumours are located in the bladder and only 5% are located in the upper urinary tract [1]. For bladder cancer, direct visualisation of the urothelium with white light cystoscopy (WLC) is the cornerstone for diagnosis and follow-up. Despite its effectiveness and established role, WLC has limitations, such as, its diagnostic accuracy, especially for carcinoma in situ (CIS) [2]. Histopathologic grading and staging of urothelial carcinoma are essential for diagnosis, prognosis and choice of therapy. However, real-time histopathologic assessment is lacking during cystoscopy in an outpatient setting as well as in the operating theatre. For upper urinary tract urothelial carcinoma (UTUC), ureterorenoscopy (URS) with endoscopic biopsies of suspicious areas is considered the diagnostic standard. Also, for UTUC, histopathologic evaluation is essential for prognosis and treatment selection as endoscopic treatment is reserved for low grade tumours only [3]. Similar to the diagnostics of bladder cancer, real-time histopathologic assessment is lacking during URS. The use of optical imaging techniques, such as Confocal Laser Endomicroscopy (CLE), may enable real-time optical biopsies to overcome these limitations for bladder cancer and UTUC evaluation.

CLE is a fibre optic probe-based imaging technique that enables real-time in vivo optical sectioning of tissue. The Cellvizio© CLE system uses a low-energy 488 nm laser source. The presence of a pinhole limits the detection to in-focus backscattered fluorescent light, enabling high resolution imaging in a single horizontal plane. CLE imaging requires the administration of a fluorescent contrast agent. The most commonly used fluorescent dye is fluorescein. Topical or intravenous application of fluorescein stains the extracellular matrix and enables visualisation of the cellular microarchitecture and morphology. Tissue types can be differentiated based on these specific cellular features. For in vivo endoscopic CLE imaging, various sized probes with different image properties are commercially available. CLE was initially applied for in vivo imaging in the gastrointestinal tract, and applications in pulmonology are explored [4–6]. In urology, CLE was first examined in the bladder. It seemed feasible to differentiate between normal mucosa and urothelial carcinoma using CLE imaging in a pilot study. Based on histopathology from resected bladder tumours, CLE criteria to differentiate between normal bladder tissue, low-grade and high-grade bladder tumours were proposed [7,8]. These criteria have also been suggested for the upper urinary tract as the histologic morphology and microarchitecture are alike. However, a CLE probe with different imaging properties is used in the upper urinary tract and only small patient cohorts have been evaluated [9,10].

The development of CLE towards real-time optical biopsies of urothelial carcinoma may lead to advances in diagnosis and prognosis and may affect the cost-benefit of the disease management. Currently, bladder cancer is the most expensive solid malignancy

per patient. The high recurrence rate of early stage tumours with long-term survival and adjuvant treatments with close follow-up results in high costs [11,12]. Even though laser fulguration of low-risk tumours has been performed in outpatient settings, it is not widely used due to the lack of histologic confirmation and therewith, the risk of inadequate treatment [13,14]. Immediate evaluation of tumour grade with CLE could potentially increase the application of laser fulguration and enable treatment of real-time confirmed low-grade tumours in an outpatient setting. Application of laser fulguration in an outpatient setting would lead to an increase in availability of treatment of low-grade bladder cancer and reduction of medical costs. Potentially, CLE may also allow for real-time evaluation of surgical radicality and, therewith, reduce recurrence rates in urothelial cancer. In the upper urinary tract, CLE has the potential to improve the diagnostic approach for UTUC.

Accurate staging and grading of UTUC remains challenging. Visual white light assessment of UTUC grade during URS is inaccurate in approximately 30% of the cases [15]. Moreover, the restricted anatomical space of the upper urinary tract and the subsequent miniaturisation of tissue-harvesting instruments limit the yield of ureterorenoscopic biopsies. The diagnostic yield and the diagnostic accuracy for tumour stage of endoscopic biopsies are limited [16,17]. However, tumour grade from endoscopic biopsies may be an indication for tumour stage [18,19]. As such, tumour grade from endoscopic biopsies is a major decisive factor for endoscopic treatment eligibility. Though, in 69–90% of endoscopic biopsies, tumour grade is in concordance with the histopathologic grade from radical resection [17,18,20,21]. Moreover, endoscopic biopsies hold a risk of complications.

CLE may overcome such diagnostic limitations for tumour grade assessment. Optical real-time assessment of tissue type and tumour grade could aid perioperative clinical decision-making. Histologic assessment without tissue biopsies could prevent possible impaired endoscopic vision after biopsies during URS. Additionally, the digital data from CLE imaging allows for real-time computer aided diagnosis with software, augmenting the practical and diagnostic value of optical imaging techniques. Further exploration of different optical imaging modalities for tumour diagnosis may lead to multimodal optical biopsies for real-time tumour grading and staging, possibly replacing tissue biopsies in future. However, a structured conduct of research is required to guide us towards optical biopsies. The aim of these two study protocols is to contribute to the development of essential knowledge for CLE-based diagnosis of urothelial carcinoma in the bladder and the upper urinary tract. In this paper, we describe two study protocols for CLE in the urinary tract together as both protocols share many methodological and disease-specific similarities.

METHODS

Study objectives

The primary objective of both studies is to develop descriptive criteria for in vivo CLE images of urothelial carcinoma (low-grade, high-grade, CIS) and normal urothelium by comparing CLE images with corresponding histopathology.

Secondary objectives are to develop a CLE image atlas of the urinary tract, to assess the technical feasibility and procedure-related adverse events, to assess CLE image quality, to qualitatively evaluate CLE images, to preliminarily assess the diagnostic yield and to establish an estimation of the diagnostic accuracy of CLE-based diagnosis in comparison with histopathology and to assess interobserver agreement.

Study design

Approval of the local medical ethical committee was obtained for both study protocols (registry number: NL55537.018.15 and NL52989.018.16). Both studies are prospective, single centre, in vivo, observational human studies to assess CLE features of normal urothelium and urothelial carcinoma (low-grade, high-grade or CIS) in the bladder and in the upper urinary tract. Both explorative studies are in agreement with the IDEAL stage 2b recommendations [22]. The two study protocols differ mainly in the location of urothelial carcinoma and subsequently, the surgical approach, the type of CLE probe, the administration of fluorescein and the reference standard. Differences in protocols are explained separately and listed in Table 1.

For both studies, CLE images are recorded with a fibre optic probe-based system (Cellvizio© 100 series, Mauna Kea Technologies, Paris, France). The Cystoflex UHD-R probe of 8.4 Fr (Mauna Kea Technologies, Paris, France) is used for CLE imaging in the bladder. The Uroflex-B probe of 2.7 Fr (Mauna Kea Technologies, Paris, France) is used in the upper urinary tract. The smaller Uroflex-B probe contains less optical fibres and, therewith, a lower image resolution compared to the Cystoflex UHD-R probe. Both forward-looking probes are illustrated in Table 1 and Figure 1.

CLE imaging requires the application of a fluorescent contrast agent. Fluorescein (fluoresceinedisodium, Fresenius Kabi, Zeist, The Netherlands) is a non-toxic and commonly used fluorescent dye for CLE imaging [23]. It stains the extracellular matrix and is administered topically prior to CLE imaging during the surgical procedure.

Table 1. Differences listed between the two protocols.

	CLE bladder study *(NL55537.018.15)*	**CLE upper urinary tract study *(NL52989.018.16*)**
Population	60 consecutive patients with primary or recurrent bladder tumour	25 patients with UTUC that will undergo a RNU or SU after diagnostic URS with CLE imaging
Inclusion criteria	- Bladder tumour or possible CIS - Scheduled for TURB - Signed informed consent	- Suspicion of UTUC - Scheduled for diagnostic URS - Signed informed consent
Exclusion criteria	- Allergy for fluorescein - Possible pregnancy or lactating women	- Allergy for fluorescein - Possible pregnancy or lactating women - Patients not eligible for RNU or SU
Urologic instruments at use	Karl Storz 22 Fr cystoscope with 0° optics for CLE imaging, and Karl Storz or Olympus 26 Fr resectoscope for transurethral resection	Karl Storz Flex Xc or Olympus V2 8.5 Fr flexible digital ureterorenoscope
Contrast agent	Topical application of 300–400 mL of 0.1% fluorescein via Foley catheter and left indwelling for 5 minutes	Topical application of 0.5 mL 2.5% fluorescein via working channel for immediate imaging
CLE probe	Cystoflex UHD-R - Diameter 2.6 mm - Lateral resolution 1 µm - Field of view 240 µm - Imaging depth 50–65 µm	Uroflex-B - Diameter 0.85 mm - Lateral resolution 3.5 µm - Field of view 320 µm - Imaging depth 40–70 µm
Histopathologic reference standard	En-bloc resected bladder tumour	RNU or SU

CLE = Confocal laser endomicroscopy, UTUC = Upper tract urothelial carcinoma, CIS = Carcinoma in situ, TURB = Transurethral resection bladder tumour, URS = Ureterorenoscopy, RNU = Radical nephroureterectomy, SU = Segmental ureter resection

In both studies, patients will undergo in-vivo CLE imaging during surgery, prior to resection or biopsy of suspicious areas for standard histopathologic assessment. The probes are introduced through the working channel of the standard endoscopes. In the bladder, a Karl Storz cystoscope of 22 Fr with 0° optics is used for CLE imaging. Transurethral resection is subsequently performed with a Karl Storz or Olympus resectoscope of 26 Fr. For CLE imaging of the upper urinary tract, a flexible digital ureterorenoscope of 8.5 Fr is used (Karl Storz Flex Xc or Olympus V2). After placing the probe in direct contact with the region of interest, image sequences of 8–12 frames per second of the real-time visualisation of the cellular microarchitecture are recorded (Supplemental Videos 1 and 2). In general, recordings are conducted in both protocols twice for 1 minute of the region of interest. In case of multiple regions of interest, multiple CLE recordings are performed. At a later stage, recorded CLE images will be analysed independently by three blinded observers and compared to the corresponding histopathology. For CLE imaging in the bladder, the reference standard for

comparison of histopathology will be the specimen of the en-bloc resected lesion. For the upper urinary tract, the reference standard will be the specimen of the radical resection (radical nephroureterectomy [RNU] or segmental ureter resection [SU]). Histopathology analysis is performed according to the standard clinical protocol and is performed by a specialised uropathologist (CDSH), who is blinded for the CLE images. A follow-up of 30 days is considered to register any adverse events following the study procedure.

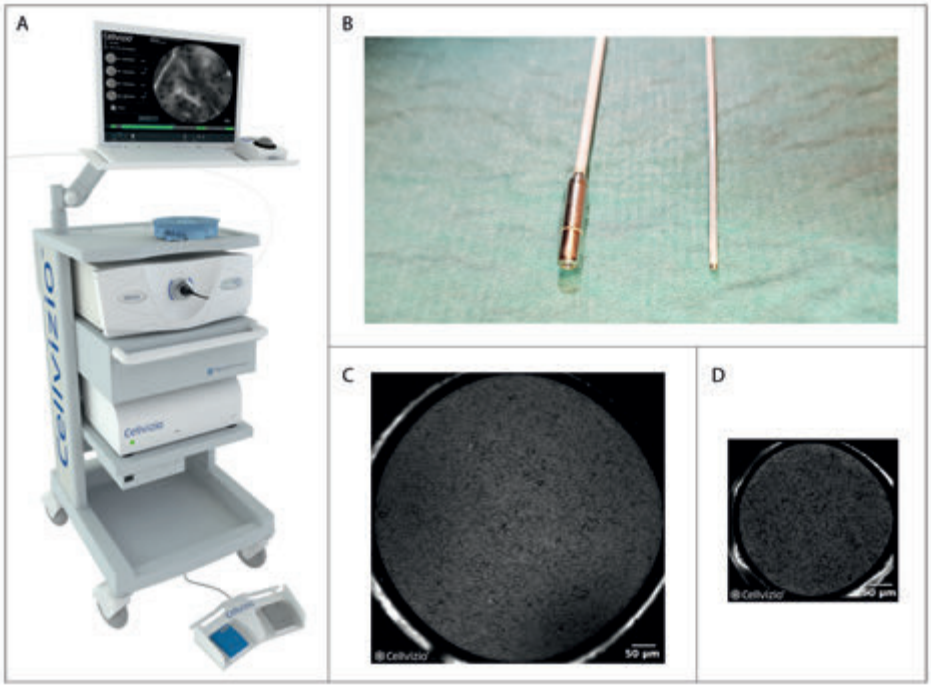

Figure 1. Cellvizio© CLE system and the probes used for the urinary tract. A) Cellvizio© CLE system. B) The two different probes used in the urinary tract. On the left the Cystoflex UHD-R probe with a diameter of 2.6 mm, which is used in the bladder. On the right the Uroflex-B probe with a diameter of 0.85 mm, which is used in the upper urinary tract. C) RAW image of the Cystoflex UHD-R probe displaying the single fibers. D) RAW image of the Uroflex-B probe displaying the single fibers.

Supplemental Video 1. A demonstration of CLE imaging of a bladder tumour. The CLE probe is in direct contact with the bladder tumour for CLE imaging.

Supplemental Video 2. A demonstration of CLE imaging of an upper urinary tract tumour. The CLE probe is direct contact with the tumour for CLE imaging.

Population and sample size

Patients eligible for either study are >18 years with a suspicious lesion in the urinary tract, scheduled for either transurethral resection (TURB) (lower urinary tract) or diagnostic URS (upper urinary tract). The main exclusion criteria are fluorescein allergy and pregnancy (Table 1). All patients will be recruited in the Amsterdam UMC Hospital (Amsterdam, The Netherlands), and all study procedures will be performed in this institution. Patients will be informed about the study in oral and written form. Patient inclusion is confirmed by signing written informed consent. Patients will only be included in one study at the time. A total of 60 consecutive evaluable patients with bladder tumours or suspicion of CIS will be included in the bladder cancer study. Based on prevalence, we expect to include 32 low-grade, 22 high-grade and 6 CIS lesions. For the upper urinary tract study, 25 patients with UTUC that will undergo a radical treatment (RNU or SU) following the diagnostic URS will be included. However, the indication for radical treatment is determined after diagnostic URS. In general, about one third of the UTUC patients are treated with radical surgery in our centre. Therefore, we expect to include 70 consecutive UTUC patients to reach the total number of 25 patients who will undergo radical treatment. Considering the possible selection bias for radical treatment, we expect to include 10 low-grade, 12 high-grade and 3 CIS lesions. These sample sizes are based on prior publications and comply with the IDEAL recommendations for explorative studies [7,9,22].

Study procedures

Protocol for Confocal Laser Endomicroscopy in the bladder

In the operating theatre prior to the TURB under general or regional anaesthesia, visual inspection with WLC and image enhancement modalities (narrow band imaging or Image1S) will be performed. At least one suspicious lesion (papillary or flat) and one region of normal appearing bladder tissue will be selected for CLE imaging. The regions of interest will be marked laterally with the cautery electrode for topographic matching. After marking the regions of interest, 300–400 mL of fluorescein (0.1% fluorescein diluted in saline) will be administered intravesically using an indwelling catheter. After instillation of the fluorescein for 5 minutes, the bladder will then be emptied, and excessive fluorescein will be rinsed out. The Cystoflex UHD-R probe will be introduced through the working channel of a 22Fr Karl Storz cystoscope with 0° optics. After placing the probe into direct contact and perpendicular to the tissue, CLE images will be recorded twice for approximately 1 minute of each marked region. After CLE imaging, the tumour will be resected en-bloc and a small chip of the marked normal urothelium will be resected for histopathologic matching. Transurethral resection is performed with a Karl Storz or Olympus resectoscope of 26 Fr.

Protocol for Confocal Laser Endomicroscopy in the upper urinary tract

The complete ureter and renal pelvis are inspected with white light and image enhancement modalities (narrow band imaging or Image1S) during standard flexible digital ureterorenoscopy under general anaesthesia to identify regions of interest. Only in case of visually confirmed upper tract tumours during URS, study-related activities

will be performed. If multiple regions of interest are identified, the region that is most easily accessible for endoscopic biopsies is selected for CLE imaging. Fluorescein (0.5 mL of 2.5% fluorescein diluted in saline) is administered through the working channel. The Uroflex-B probe is introduced via the working channel of the 8.5 Fr flexible digital ureterorenoscope (Karl Storz Flex Xc or Olympus V2) and placed into direct contact with the region of interest for immediate CLE imaging. Each region of interest is imaged twice for approximately 1 minute with CLE. After imaging, endoscopic biopsies for the standard diagnostic process are taken from the same location. Depending on the histopathologic diagnosis, the indication for a radical treatment will be determined.

Data analysis

The method of analysis is identical for both study protocols. Demographic-, and disease-specific characteristics of the study populations (eg. age, sex, medical history of urothelial carcinoma, tumour focality, tumour location and tumour size) will be collected. Three blinded observers, consisting of two researchers (EIMLL & JEF) and one uropathologist (CDSH), will independently analyse the CLE images frame by frame in an offline setting with the Cellvizio© Viewer software (Mauna Kea Technologies, Paris, France). Modified criteria for CLE image evaluation will be used for analysis (Table 2) [8].

Table 2. Modified CLE image characteristics and their variables for analysis.

CLE feature	Variables
Papillary aspect	Present | not present
Polarity of cells	Present | not present
Organisation of cells	Organised | disorganised
Cohesiveness of cells	Cohesive | discohesive
Cellular morphology	Monomorph | pleiomorph
Definition of cell borders	Distinct | indistinct
Vasculature	Capillary network | fibrovascular stalk | large vessels

CLE = Confocal laser endomicroscopy

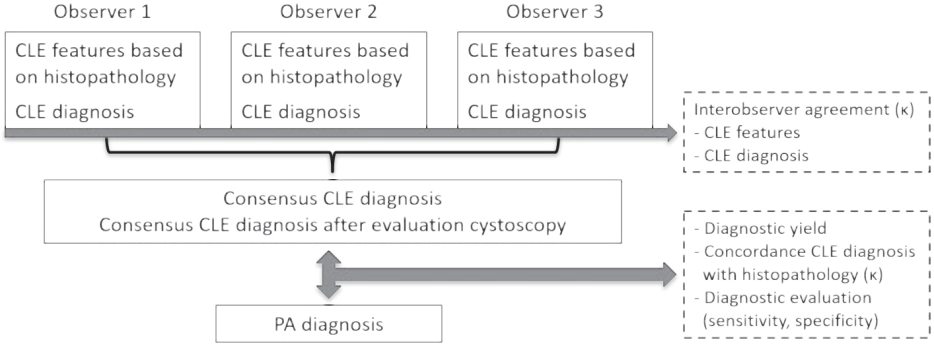

Figure 2. A schematic overview of the data analysis plan.

The CLE image quality per region of interest will be scored on a Likert scale as poor, fair or good. Image quality will be used for qualitative evaluation of the technique and for subgroup analysis. Based on histologic features, for each region of interest a CLE-based diagnosis will be made by each observer as benign urothelium or urothelial carcinoma. The diagnosis of urothelial carcinoma is subdivided in low-grade or high-grade urothelial carcinoma (WHO 2004 classification), CIS or as inconclusive. An inconclusive CLE diagnosis is defined as poor image quality where CLE features cannot be assessed. After individual analysis, a consensus will be reached for the CLE-based diagnosis by all three observers for each region of interest. The appropriateness of consensus of the CLE-based diagnosis is evaluated by viewing the endoscopic images. After determining CLE-based diagnosis and consensus, CLE images will be compared to the corresponding histopathology specimen (either en-bloc resected bladder tumour or RNU/SU specimen) for evaluation of the concordance of CLE based diagnosis with histopathologic diagnosis. Differences between diagnosed groups will be analysed with chi-square test. For an initial evaluation of the diagnostic value, sensitivity and specificity will be calculated based on a 2x2 table where CIS is classified as high-grade tumour. Proportions of specific agreement and Fleiss kappa analysis will be used for interobserver agreement of CLE-based diagnosis. A schematic overview of the data analysis is displayed in Figure 2.

Safety

The investigators will monitor patient safety. They can withdraw a patient from the study for medical reasons. In accordance to section 10, subsection 4, of the "Wet Medisch-Wetenschappelijk Onderzoek met Mensen" (medical research involving human subjects act in The Netherlands), the investigators will suspend the study if there is sufficient ground that continuation of the study will jeopardise patient health or safety. The investigators will notify the accredited Institutional Review Board if this is the case. In case of an adverse event or serious adverse event, the responsible authorities will be informed.

Risks and benefits

There are no direct benefits for patients participating in these two studies. In the future however, the results of these studies may be important for patients diagnosed with a tumour in the urinary tract. All patients participating in both studies are scheduled for standard treatment of tumour(s) of the urinary tract; either TURB or URS with endoscopic biopsies. CLE is a minimally invasive imaging technique that can be performed in conjunction with conventional endoscopic treatment. Previous studies of CLE combined with topical administration of fluorescein have proven to be safe [7,24]. Fluorescein is a commonly used fluorescent dye, and its safety is well established for its use in ophthalmological angiography [25]. In patients not at risk for a previously demonstrated allergic reaction, this dye is safe. Patients with a known allergic reaction to fluorescein cannot participate in this study.

RESULTS

Presently, recruitment of patients is ongoing in both studies. Results and outcomes are expected in 2018. Summarised raw data will be made available through publication in an international peer-reviewed medical journal.

DISCUSSION

CLE is an optical imaging technique that may enable real-time optical biopsies. The exploration of tissue specific CLE criteria is essential for the development towards real-time tumour grading of urothelial lesions. Both trials will provide more insight into CLE features of urothelial carcinoma in the bladder and the upper urinary tract and into its diagnostic value.

The design of the bladder protocol aims for topographic matching of CLE images with the resected specimen. The cauterisation marks placed laterally to the region of interest prior to CLE imaging ensures that imaging and resection is done of the exact topographic tissue. Nonetheless, it will be challenging to create an identical histopathological slide of the resected specimen in exactly the same plane as the imaged tissue. We assume that this approach is the closest approximation for topographic matching without interference in the standard clinical histopathologic process.

The study design of the upper urinary tract protocol will lead to a surplus of study measurements, considering that only about one-third of the UTUC patients will receive radical surgery as treatment. Since mainly patients with a high-grade or high-volume low-grade tumour will qualify for radical treatment, selection bias could be a risk. The data acquired of patients who are not suitable for radical treatment enables secondary analysis for the comparison of CLE images with the histology of endoscopic biopsies of the imaged regions of interest. In the current study design identical topographic matching of CLE images in the upper urinary tract with the specimen of the RNU is limited by the fact that in general the diagnostic URS with CLE imaging is not performed during the same procedure as the radical resection. However, topographic matching is approximated by tumour mapping during URS (mapping and annotation of location, size and appearance) for identification of lesions during the histopathologic assessment.

As with all new techniques, a learning curve for the handling and image interpretation may be expected for CLE. Besides potential intraobserver variability, the learning process might also influence the CLE image quality of the first cases. We aim to limit the number of users to a minimum number of experienced endourologists to minimise the potential effect of a learning curve.

Despite these limitations, we expect that the results of these trials will contribute to determining the role of CLE imaging for the diagnosis of bladder cancer and UTUC in clinical practice. In the light of the limitations of cystoscopy and URS, CLE holds the potential to enable real time tumour grading of urothelial carcinoma.

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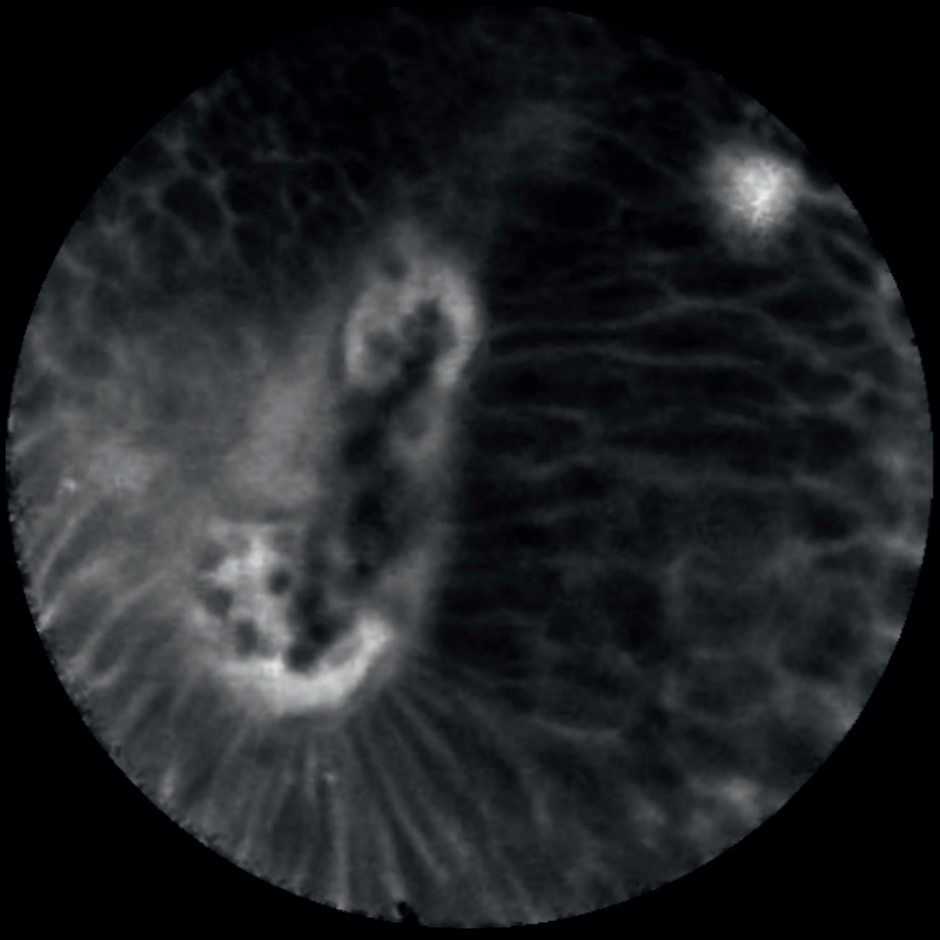

CHAPTER 3

Confocal laser endomicroscopy for the diagnosis of urothelial carcinoma in the bladder and the upper urinary tract

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Videourology, 2018 Jul;32(2)

ABSTRACT

Introduction: Endoscopic evaluation with postoperative histopathological assessment of suspicious tissue forms the cornerstone for the diagnosis of urothelial carcinomas of the bladder (UCB) and of the upper urinary tract (UTUC). However, the limitation of the current diagnostic pathways is the lack of real-time in vivo histologic assessment.

Confocal laser endomicroscopy (CLE) is an optical imaging technique that has the potential to overcome this limitation. This probe-based technique may enable real-time assessment of histological grade of urothelial carcinoma during cystoscopy and ureterorenoscopy [1–5].

In the bladder, real-time grade assessment could allow for immediate risk-stratification of small bladder tumours during cystoscopy and, therewith, enable laser fulguration of identified low-risk UCB in an outpatient setting. In the upper urinary tract, real-time grade assessment of UTUC could improve intra-operative risk stratification for the selection of endoscopic treatment. Moreover, CLE may have the potential to improve the limited diagnostic yield and accuracy from endoscopic tissue biopsies for UTUC diagnosis [6].

In this video, we introduce the principles of CLE, illustrate its use during cystoscopy and ureterorenoscopy, and present the evaluation of CLE images for the diagnosis of UCB and UTUC.

Materials and methods: We performed CLE imaging during rigid cystoscopy and flexible ureteroscopy in patients with suspicious lesions for UCB or UTUC within the scope of two ongoing clinical trials (Clinicaltrials.gov registration: NCT03013894 and NCT03013920). The aim of these trials is to evaluate/describe diagnostic criteria/features and to evaluate the diagnostic value of CLE for urothelial carcinoma.

For CLE imaging, the 8.4 Fr Cystoflex UHD-R probe (lateral image resolution of 1 µm) is used in the bladder, and the 2.7 Fr Uroflex-B probe (lateral resolution of 3.5 µm) is used in the upper urinary tract (both Mauna Kea Technologies, France). Both probes are used in conjunction with the 488 nm laser scanning unit (Cellvizio© 100 series, Mauna Kea Technologies, France). The CLE probes are introduced through the working channel of either the 26 Fr rigid cystoscope of Karl Storz with 0° optics or the flexible digital ureteroscope (Olympus V2 or Karl Storz Flex Xc, 8.5 Fr). A fluorescent dye (fluorescein disodium, Fresenius Kabi, Zeist, The Netherlands) is administered topically before CLE imaging to stain the extracellular matrix for visualization of the microarchitecture [7].

Results: Placing the CLE probe in contact with the tissue of interest enables real-time imaging at cellular resolution. Tumour diagnosis and grade differentiation are based

on visual evaluation of the cellular microarchitecture. After CLE imaging, the standard endoscopic procedure may be resumed.

Conclusions: CLE is an optical imaging technique that enables real-time in vivo visualisation of the cellular microarchitecture. This probe-based technique may be used during standard cystoscopy and ureterorenoscopy for intra-operative risk stratification of UCB and UTUC by histological grading. This could lead to an advancement in personalised care with improved intra-operative decision-making. The results of the ongoing studies to evaluate the diagnostic value of CLE are awaited.

Video 1. Confocal laser endomicroscopy for the diagnosis of urothelial carcinoma in the bladder and the upper urinary tract. *Videourology 2018*

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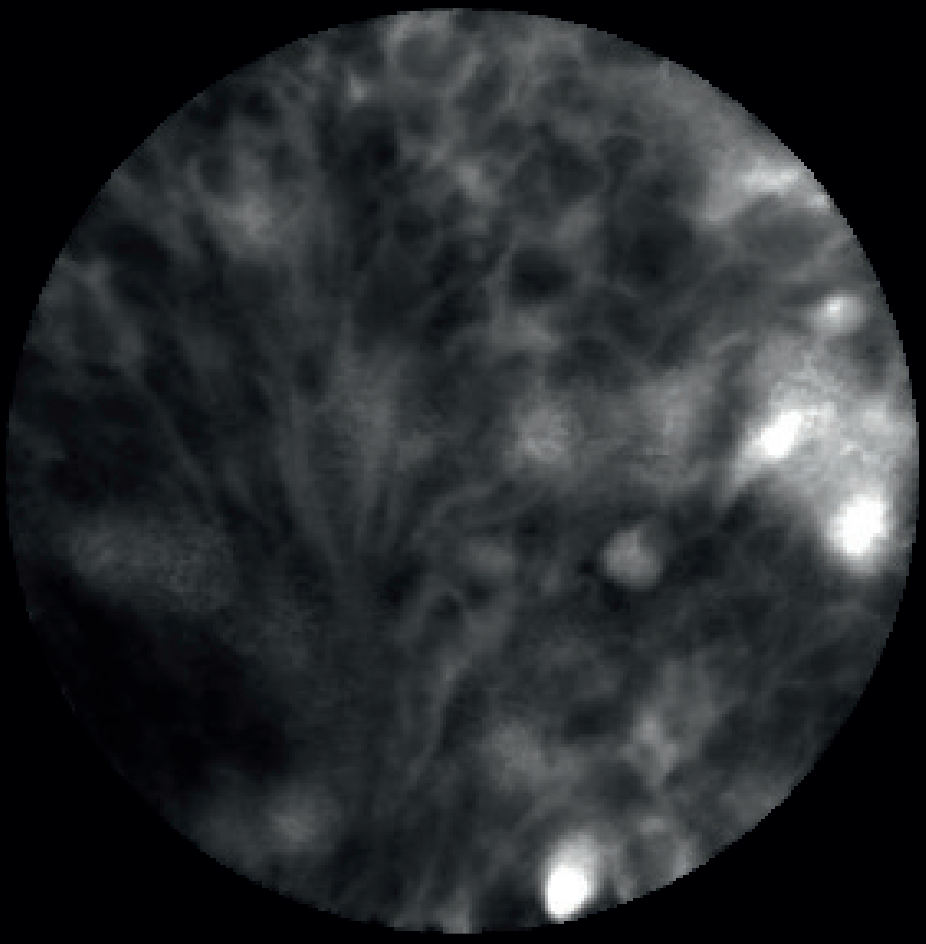

CHAPTER 4

Validation of confocal laser endomicroscopy features of bladder cancer: the next step towards real-time histologic grading

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European Urology Focus, 2020 Jan 15;6(1)

ABSTRACT

Background: Cystoscopy enables the visualization of suspicious bladder lesions but lacks the ability to provide real-time histopathologic information. Confocal laser endomicroscopy (CLE) is a probe-based optical technique that can provide real-time microscopic images. This high-resolution optical imaging technique may enable real-time tumour grading during cystoscopy.

Objective: To validate and adapt CLE criteria for bladder cancer diagnosis and grading.

Design, setting and participants: Prospectively, 73 patients scheduled for transurethral resection of bladder tumour(s) were included. CLE imaging was performed intraoperatively prior to en-bloc resection. Histopathology was the reference standard for comparison.

Intervention: Cystoscopic CLE imaging.

Outcome measurement and statistical analysis: Three independent observers evaluated the CLE images to classify tumours as low-, or high-grade urothelial carcinoma (UC) or benign. Interobserver agreement was calculated with Fleiss kappa analysis, and diagnostic accuracy with 2x2 tables.

Results: Histopathology of 66 lesions (53 patients) revealed 25 low-grade, 27 high-grade UCs and 14 benign lesions. For low-grade UC, most common features were papillary configuration (100%), distinct cell borders (81%), presence of fibrovascular stalks (79%), cohesiveness of cells (77%), organised cell pattern (76%) and monomorphic cells (67%). A concordance between CLE-based classification and histopathology was found in 19 cases (76%). For high-grade UC, pleomorphic cells (77%), indistinct cell borders (77%), papillary configuration (67%) and disorganised cell pattern (60%) were the most common features. A concordance with histopathology was found in 19 cases (70%). In benign lesions the most prevalent features were disorganised cell pattern (57%) and pleomorphic cells (52%), and a concordance with histopathology was found in four cases (29%).

Conclusion: The CLE criteria enable identification of UC. CLE features correlate to histopathologic features that may enable real-time tumour grading. However, flat lesions remain difficult to classify.

Patient summary: Confocal laser endomicroscopy may enable real-time cancer differentiation during cystoscopy, which is important for prognosis and disease management.

INTRODUCTION

Bladder cancer is the most common malignancy of the urinary tract in both men and women [1]. Currently, cystoscopy is the cornerstone for the diagnosis and follow-up of bladder cancer, enabling the identification of abnormalities of the bladder mucosa. However, white light cystoscopy (WLC) lacks the ability to provide histopathologic information, which is essential for diagnosis and prognosis [2]. In recent years, optical imaging techniques have been developed which may overcome this limitation.

Confocal laser endomicroscopy (CLE) is a high-resolution optical imaging technique that allows for probe-based in vivo optical sectioning of tissue during endoscopy. The contrast is based on fluorescence that is excited by a laser. The contrast can be enhanced by administering a fluorescent label that binds to the cells, thereby allowing the visualisation of the cellular microarchitecture of the tissue. CLE imaging was first introduced in gastroenterology to diagnose Barrett's oesophagus [3–6]. Shortly thereafter, applications were explored in pulmonology, otolaryngology and urology [7–9]. By advancing a fibre-based probe through the working channel of a cystoscope, the bladder wall is visualised on a cellular level, providing 'optical biopsies' of the tissue. Sonn et al. [9,10] were the first to perform ex vivo and in vivo CLE imaging of the urinary tract. Nonetheless, translation of the images into a diagnosis is not straightforward. Diagnostic criteria for bladder cancer diagnosis were proposed, however, these criteria have not yet been validated [11,12].

Owing to the high recurrence rate, relatively long-term survival, adjuvant treatment modalities and stringent follow-up, bladder cancer is currently one of the most expensive malignancies per patient [13,14]. An improved cost benefit of disease management could become possible when direct histopathologic information during cystoscopy becomes available, as it could potentially lead to advances in diagnosis and treatment of bladder cancer. To achieve such developments, the present study primarily aims to validate and adapt the proposed CLE criteria for bladder cancer grading. Secondary objectives are to investigate a preliminary diagnostic accuracy of CLE-based grading and also in conjunction with WLC.

PATIENTS AND METHODS

Study design

The study protocol was approved by the institutional review board and was registered in the Dutch Central Committee on Research involving Human Subjects (NL55537.018.15) and on Clinicaltrials.gov (NCT03013894). The study was carried out according to the guidelines of good clinical practice. Written informed consent was obtained from all participants. This prospective clinical trial was in agreement with the IDEAL stage 2b recommendations and was carried out as described previously [15,16].

Patients

Patients were prospectively recruited in the Amsterdam UMC hospital (Amsterdam, The Netherlands). Adult patients, with a primary or recurrent bladder tumour or suspicion of carcinoma in situ (CIS), who were scheduled for transurethral resection of the bladder tumour (TURB), were eligible for the study. Main exclusion criteria were fluorescein allergy and pregnancy.

Study procedure

CLE imaging was performed during TURB using a low-power 488 nm laser system (Cellvizio© 100 series, Mauna Kea Technologies, Paris, France) in conjunction with the Cystoflex UHD-R probe (Mauna Kea Technologies) with a 2.6 mm outer diameter, a field of view of 240 μm, a 1 μm lateral resolution and an imaging depth of 50–65 μm.

CLE imaging was performed during TURB, prior to the resection of the suspect lesion. After cystoscopy, at least one suspicious lesion was marked using a cautery electrode. To stain the extracellular matrix of the bladder mucosa, ~300 mL fluorescein 0.1% was administered intravesically via a Foley catheter and left indwelling for 5 minutes [17]. The CLE probe was introduced through the working channel of 22 Fr rigid cystoscope with 0º optics. After placing the probe in direct perpendicular contact with the marked region of interest (ROI), images of the cellular microarchitecture were recorded (8–12 frames/second) (Supplemental Video 1)[18]. In general, two recordings of 1 minute were obtained per ROI. After CLE imaging, the imaged lesion was resected en-bloc. Histopathologic workup and analysis were performed according to standard clinical protocol by a uropathologist (CDSH), blinded to CLE images.

CLE image evaluation

Prior to the CLE image analysis, three observers (EIMLL, JEF and CDSH) were trained with a CLE training program of Chang et al [12]. The CLE images of the current study were analysed offline frame by frame with the Cellvizio© Viewer software (Mauna Kea Technologies) by the three observers, who were blinded to clinical information and histopathology. For the CLE image analysis, the presence of the proposed CLE features (papillary configuration, organisation of cells, cohesiveness of cells, cellular morphology, definition of cell borders, vasculature) by Chang et al. [12] and an additional feature, polarity of the cells, was assessed (Figure 1). Cellular polarity was defined as the relative orientation of cells and nuclei in the same direction.

Supplemental Video 1. A demonstration of CLE imaging of a bladder tumour. The CLE probe is in direct contact with the tissue for CLE imaging.

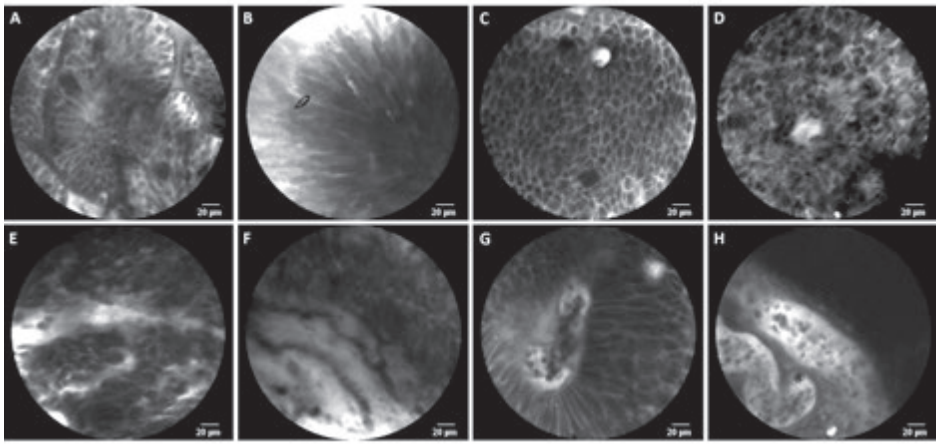

Figure 1. Examples of the different CLE features that were evaluated. A) presence of papillary configuration. B) Polarity of urothelial cells, that is, alignment and orientation in the same direction. C) Organised cell pattern, with cohesive and monomorphic cells, and distinct cell borders. D) Disorganised cell pattern, with pleomorphic cells, and indistinct cell borders. E) Disorganised cell pattern, with discohesive and pleomorphic cells, and indistinct cell borders. F) Capillary network. G) Fibrovascular stalk is visible. H) Large vessel.

Based on the identified CLE features, the observers classified the ROI according to the World Health Organisation (WHO) 2004 classification (low-grade urothelial carcinoma [UC], high-grade UC or benign lesion). After individual analysis, consensus was reached through a two-step process. First, consensus for classification based solely on CLE images was reached. Thereafter, corresponding WLC images were added to account for the potential additional value of endoscopic evaluation adjunct to CLE imaging. With the additional information of the WLC images, a second joined consensus for the CLE-based classification was formed. To determine the concordance of the CLE-based classification with histopathology, CLE images were compared to the corresponding histopathology of the en-bloc resected specimen (Figure 2).

Endoscopic tumour evaluation

During TURB, pictures and short videos of the CLE-imaged tumours were recorded. After a washout time of at least 4 weeks, these images were presented to three urologists (TMdR, JB and GMK), blinded to any clinical information, to predict the histologic grade of the lesions according to the WHO 2004 classification. After individual prediction, a joined consensus was reached.

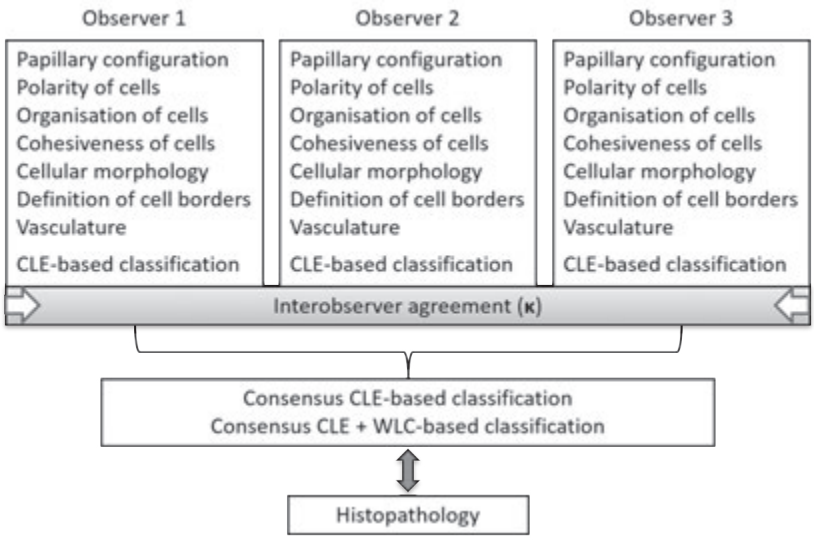

Figure 2. Schematic overview of the CLE image evaluation.

Sample size and statistical analysis

The sample size was based on prior publications and conform the IDEAL recommendations for explorative studies [16]. In 62 consecutive patients with a bladder tumour or suspicion of CIS, CLE imaging was performed.

Statistical analyses were performed using SPSS Statistics version 24 and Matlab R2017b. Descriptive statistics were used to determine demographic and disease-specific characteristics. For the primary objective, interobserver agreements with regard to the endoscopic evaluation, CLE features, and CLE-based classifications were determined using Fleiss kappa analysis. The diagnostic accuracy for CLE, WLC, and CLE and WLC combined, including sensitivity and specificity, was calculated with 2x2 tables.

RESULTS

Patient characteristics

Seventy-three consecutive patients were included in the study between March 2016 and September 2017. CLE imaging was performed in 62 patients, with a total of 82 suspicious lesions (Figure 3). Lesions of which more than half of the CLE feature assessments were non-diagnostic were excluded. In total, 66 suspicious lesions were included for final analysis, yielding a diagnostic rate of 86%. Histopathology of the 66 lesions revealed 25 low-grade UCs, 27 high-grade UCs (including 2 cases of CIS) and 14 benign lesions (2 normal, 8 reactive, 2 inflammatory lesions, 1 inverted papilloma, 1 urothelial proliferation of uncertain malignant potential). Patient and tumour characteristics are summarised in Table 1.

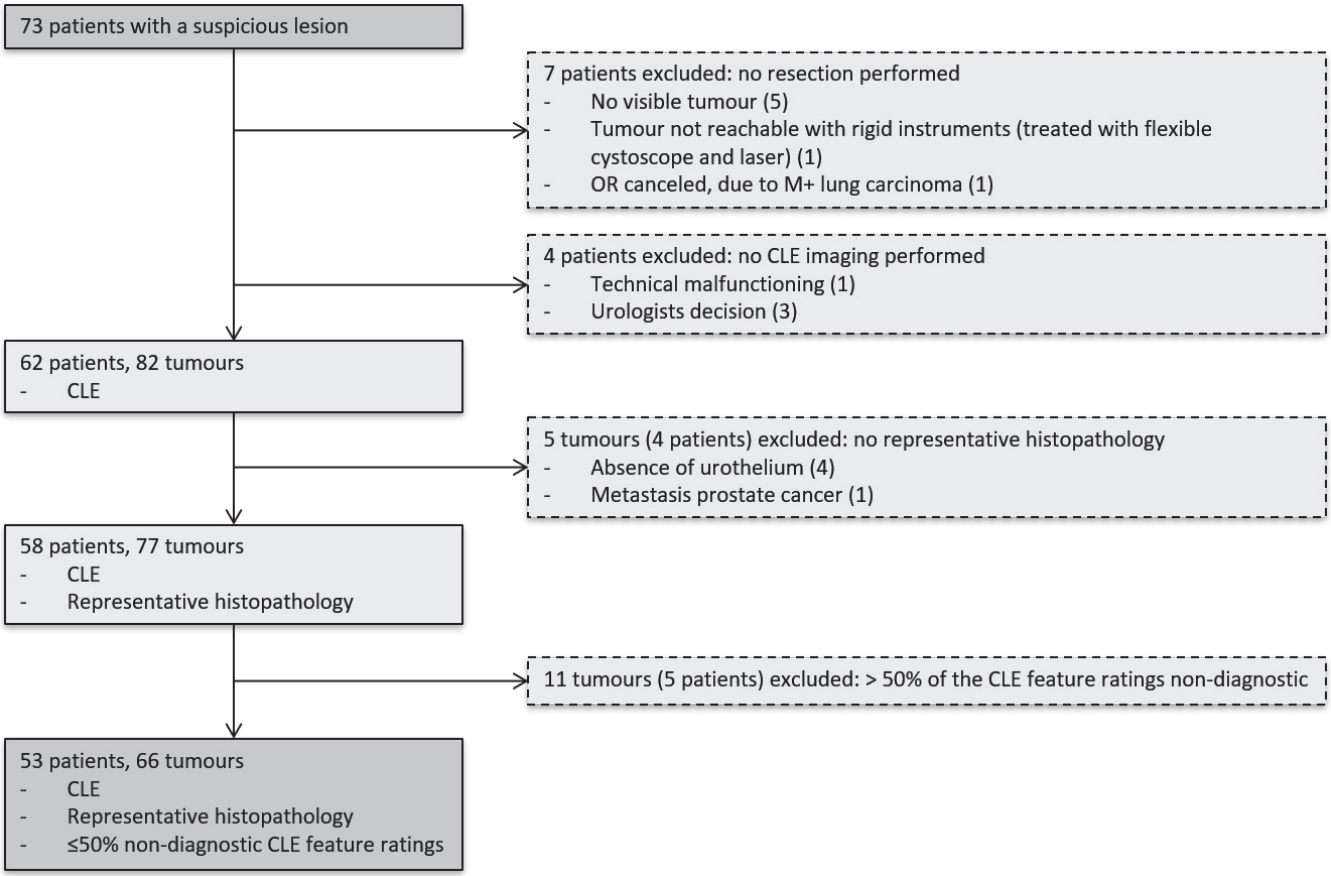

Figure 3. Flow diagram of the inclusion.

Differentiating CLE features

Percentages of the different CLE features specified per type of lesion are displayed in Supplemental Table 1. CLE features with a mean prevalence ≥60% for low-grade UC were: presence of papillary configuration (100%), distinct cell borders (81%), presence of fibrovascular stalks (79%), cohesiveness of cells (77%), organised cell pattern (76%), monomorphic cells (67%) and presence of polarity (61%). For high-grade UC prevalent CLE features were: pleomorphic cells (77%), indistinct cell borders (77%), presence of papillary configuration (67%) and disorganised cell pattern (60%). Benign lesions did not show any CLE features with a mean prevalence ≥60%.

Interobserver agreement

The interobserver agreement of the different CLE features varied between fair and substantial (Table 2), with moderate or substantial agreement for the features of papillary configuration, organisation of cells, cellular morphology and definition of cell borders. Interobserver agreement for CLE-based classification was substantial (κ=0.676, 95% confidence interval [CI]: 0.647–0.704).

Table 1. Patient and tumour characteristics.

A. Patient characteristics		**N=53**	
Age (years), *mean (sd)/med [iqr]*		70 (12)	70 [62 – 79]
Gender, *n (%)*	Male	39	(74)
	Female	14	(26)
History of bladder cancer, *n (%)*		29	(55)
Previous intravesical treatment, *n (%)*	No	32	(60)
	Yes	21	(40)
B. Tumour characteristics		**N=66**	
Tumour size, *n (%)*	< 3 cm	54	(82)
	> 3 cm	12	(18)
Tumour stage, *n (%)*~	T0	15	(23)
	CIS only	2	(3)
	Ta	40	(61)
	T1	5	(8)
	≥T2	3	(5)
Tumour grade WHO 1973, *n (%)*	Benign	15	(23)
	CIS only	2	(3)
	Grade 1	4	(6)
	Grade 2	32	(48)
	Grade 3	13	(20)
Tumour grade WHO 2004, *n (%)*	Benign	14	(21)
	Low-grade	25	(38)
	High-grade	27	(41)

Sd = standard deviation, med = median, iqr = interquartile range
~ Tumour stage of 1 patient could not be determined

Table 2. Modified CLE image characteristics and their variables for analysis. Interobserver agreement is displayed for the CLE features and CLE-based classification (low-grade UC, high-grade UC or benign lesion).

CLE feature	Variables	Fleiss κ	95% CI	Agreement
Papillary configuration	Present | not present	0.777	0.741 – 0.813	Substantial
Polarity of cells	Present | not present	0.382	0.356 – 0.408	Fair
Organisation of cells	Organised | disorganised	0.575	0.545 – 0.605	Moderate
Cohesiveness of cells	Cohesive | discohesive	0.337	0.307 – 0.367	Fair
Cellular morphology	Monomorphic | pleomorphic	0.430	0.398 – 0.462	Moderate
Definition of cell borders	Distinct | indistinct	0.666	0.632 – 0.701	Substantial
Vasculature	Capillary network | fibrovascular stalk | large vessels	0.574	0.551 – 0.598	Moderate
CLE classification		0.676	0.647 – 0.704	Substantial

CLE = Confocal laser endomicroscopy

CLE-based classification

The concordance with histopathology was higher with the consensus-based classification compared with individual assessment by three observers. The individual CLE-based classification of the three observers was in concordance with histopathology in 38–40 lesions (58.5–62.5%), whereas consensus for CLE-based classification was confirmed by histopathology in 42 of 66 lesions (63.6%). In 19 lesions (76%) of low-grade UC, the CLE-based classification was in concordance with histopathology (sensitivity 76%, specificity 76%). For high-grade UC, the CLE-based classification was in concordance with histopathology (sensitivity 70%, specificity 69%) in 19 lesions (70%). In 4 cases (29%) of benign lesions, the CLE-based classification was in concordance with histopathology (sensitivity 29%, specificity 96%) (Table 3).

WLC-based classification

In 38 lesions (58.5%), the WLC-based consensus classification was in accordance with histopathology. Sensitivity and specificity were 54% and 71% for low-grade UCs, 67% and 61% for high-grade UCs, and 50% and 100% for benign lesions, respectively (Table 3).

CLE-based classification after WLC evaluation

The CLE-based consensus classification after viewing WLC images showed an agreement with histopathology in 44 lesions (68.2%). Concordance with histopathology was found in 19 (79%), 18 (67%) and 7 (50%) for low-grade UC, high-grade UC and benign lesions, respectively. Sensitivity and specificity were 79% and 78% for low-grade UC, 67% and 79% for high-grade UC, and 50% and 92% for benign lesions, respectively (Table 3).

Table 3. Diagnostic accuracy for the differentiation between benign, low-grade or high-grade urothelial carcinoma. Sensitivity and specificity for CLE-based tumour evaluation, WLC-based tumour evaluation and CLE-based tumour evaluation after reviewing endoscopy images.

	CLE evaluation (n=66)		**WLC evaluation (n=65)***		**CLE + WLC evaluation (n=65)***	
	Sensitivity	*Specificity*	*Sensitivity*	*Specificity*	*Sensitivity*	*Specificity*
Low-grade						
Observer 1	72%˜	70%˜	42%	73%		
Observer 2	76%	76%	50%	83%		
Observer 3	76%≈	69%≈	71%	56%		
Consensus	76%	76%	54%	71%	79%	78%
High-grade						
Observer 1	62%˜	67%˜	70%	50%		
Observer 2	63%	67%	70%	53%		
Observer 3	67%≈	73%≈	44%	79%		
Consensus	70%	69%	67%	61%	67%	79%
Benign						
Observer 1	29%˜	96%˜	43%	100%		
Observer 2	21%	92%	57%	98%		
Observer 3	25%≈	96%≈	50%	94%		
Consensus	29%	96%	50%	100%	50%	92%

CLE = Confocal laser endomicroscopy, WLC = White light cystoscopy

***** Due to technical problems endoscopic images of one tumour were not recorded.

˜ It was not possible to determine CLE-based classification in 1 case.

≈It was not possible to determine CLE-based classification in 2 cases.

DISCUSSION

This study is the first validation of the previously proposed CLE features for bladder cancer diagnosis [11,12]. The CLE-based consensus classification with and without adjunct WLC image assessment was in concordance with histopathology in 68.2% and 63.6% of the lesions, respectively. Concordance of the purely WLC-based classification and histopathology was lower (58.5%), suggesting that CLE might be of additional value to cystoscopy for real-time bladder cancer assessment. In comparison to Herr et al. [19], the concordance rate of WLC-based classification with histopathology seems to be low. However, in their study, the observers were not blinded for additional clinical information. Furthermore, they limited their grading assessment to G1 and G3 (WHO 1937) of recurrent tumours, which may overestimate the concordance of WLC-based grading.

The diagnostic accuracy for CLE-based bladder cancer grading of this study is in line with the results of Chang et al [12]. Nevertheless, we found a higher sensitivity for low-grade UC, and a slightly higher specificity for high-grade UC.

Based on the interobserver agreement for the CLE analysis, we can conclude that assessment of the CLE features by independent observers yields comparable results. Evaluating the CLE images based on seven criteria can be laborious and time consuming. Considering that papillary aspect is a predominant CLE feature (≥60%) for both low- and high-grade UC, our results suggest that organisation of cells, cellular morphology and definition of cell borders are the most discriminating features for grade differentiation (Figure 4). Differentiation based on the presence of two or more of these three features yields a similar sensitivity (low-grade 75%, high-grade 80%) and specificity (low-grade 76%, high-grade 66%) (Table 4). Importantly, these three CLE features have a moderate to substantial interobserver agreement. Image assessment based on three CLE features would simplify the interpretation, and make it more accessible for clinicians, though this remains to be investigated prospectively.

Identifying differentiating CLE features for CIS was not possible since only 2 CIS lesions were included in the study. The higher discordance for benign lesions in comparison to low- and high-grade UC may be due to the heterogeneity of this group (2 normal, 8 reactive, 2 inflammatory lesions, 1 inverted papilloma, 1 urothelial proliferation of uncertain malignant potential). As a result, accurate differentiation of flat lesions remains challenging.

In this study, CLE imaging prolonged the TURB procedure for 10-15 minutes, including 5 minutes of fluorescein instillation time. To shorten the CLE procedure, the fluorescein could be administered directly onto the ROI, as applied in the upper urinary tract [15]. In daily practice, imaging time may be shorter because normal tissue does not have to be imaged, and it may not be necessary to obtain multiple recordings of multiple regions as in the extensive protocol in our study.

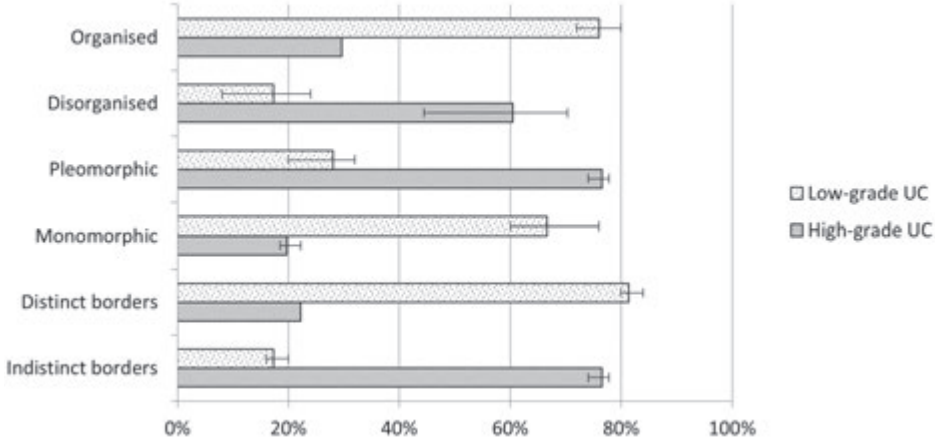

Figure 4. The most prominent features to differentiate between low- and high-grade UC based on CLE images. The error bars represent the range how often different features were recognised by the independent CLE observers. CLE = confocal laser endomicroscopy, UC = urothelial carcinoma.

Table 4. Diagnostic yield for macroscopic papillary lesions (n=58), classification based on 3 CLE features. One lesion was excluded because only 1 out 3 features was evaluable. A) 2x2 tables for low-grade UC versus other lesions (high-grade UC and benign). B) 2x2 tables for high-grade UC versus other lesions (low-grade UC and benign).

A. Low-grade UC			
	Histopathology		
CLE	Low-grade UC	Other	Total
Low-grade UC	18	8	26
Other	6	25	31
Total	24	33	57
Sensitivity = 75%, specificity = 76%			

B. High-grade UC			
	Histopathology		
CLE	High-grade UC	Other	Total
High-grade UC	20	11	31
Other	5	21	26
Total	25	32	57
Sensitivity = 80%, specificity = 66%			

CLE = Confocal laser endomicroscopy, UC = Urothelial carcinoma

The use of CLE in urology is still in an early stage, and possible applications in clinical practice are being explored. Histologic information during cystoscopy could improve the cost benefit of bladder cancer management in the long run, as it could lead to advances in diagnosis and treatment of bladder cancer. For example, laser fulguration has been performed in an outpatient setting as treatment of low-risk bladder tumours. However, this technique is not commonly used due to the lack of histopathologic certainty and potential undertreatment [20,21]. CLE may enable real-time grading prior to laser fulguration to assure treatment of low-grade tumours. The shift of treatment from the operating theatre to the outpatient clinic could lead to a decrease in medical costs and shortening of waiting time for surgery. Next, CLE would be of great additional diagnostic value if it enables the identification of CIS. CLE may also be used during TURB to confirm surgical radicality or the presence of detrusor muscle in the resected tissue. By reducing histopathologic uncertainty during cystoscopy, CLE might enable active surveillance in patients with low-risk UC when subsequent surgical treatment is not preferred. CLE may also be used for upper tract UC to assist in patient selection for kidney-sparing treatment [22,23]. In addition, the combination of CLE with other optical imaging techniques (e.g. photodynamic diagnosis, narrow band imaging, optical coherence tomography) for guided or multimodal optical assessment should be investigated [24].

A limitation of this study was the impossibility to identify discriminating CLE features for benign lesions and CIS, due to heterogeneity of benign lesions and the small number of both benign lesions and CIS. In addition, heterogeneity within bladder tumours may

be a limitation [25]. Considering the limited field of view of the probe (240 µm), only a fraction of the tumour surface is imaged. Therefore, the recorded image sequence may give a biased view with regard to the whole tumour and might be responsible for discrepancies between CLE-based classification and histopathology. Additionally, variability in CLE image quality could impede the CLE image evaluation. Specifically, at the start of this study there was a learning curve with regard to probe stabilisation. Movement artefacts could have contributed to the 14% non-diagnostic rate of CLE images. Lastly, despite a washout time of several weeks to months, a recall bias might still exist for the urologists who predicted the tumour grade based on the WLC images. However, this bias would have led to an overestimation; hence, the actual concordance of the WLC-based diagnoses with histopathology would be even lower.

In this study, we have extended the work of Chang et al. [12] and validated CLE features for bladder cancer classification. Before CLE imaging can be used routinely for bladder cancer diagnosis, there are still some hurdles to overcome. Multicentre collaborations for larger clinical trials are required to fine-tune the established CLE criteria, develop a diagnostic nomogram, and to further explore future applications. In addition, the digital data of CLE offer opportunities for automated image analysis and deep machine learning, which should be explored jointly to create big data.

CONCLUSION

This study is the first prospective validation of earlier published CLE features for bladder cancer diagnosis and grading. CLE images correlate to histopathologic features and may enable real-time differentiation between low- and high-grade UC. Our data demonstrates that the proposed CLE features suffice to identify and grade bladder tumours. Moreover, our data suggests that bladder cancer grading might be possible based on three CLE features. The differentiation of flat lesions remains to be investigated.

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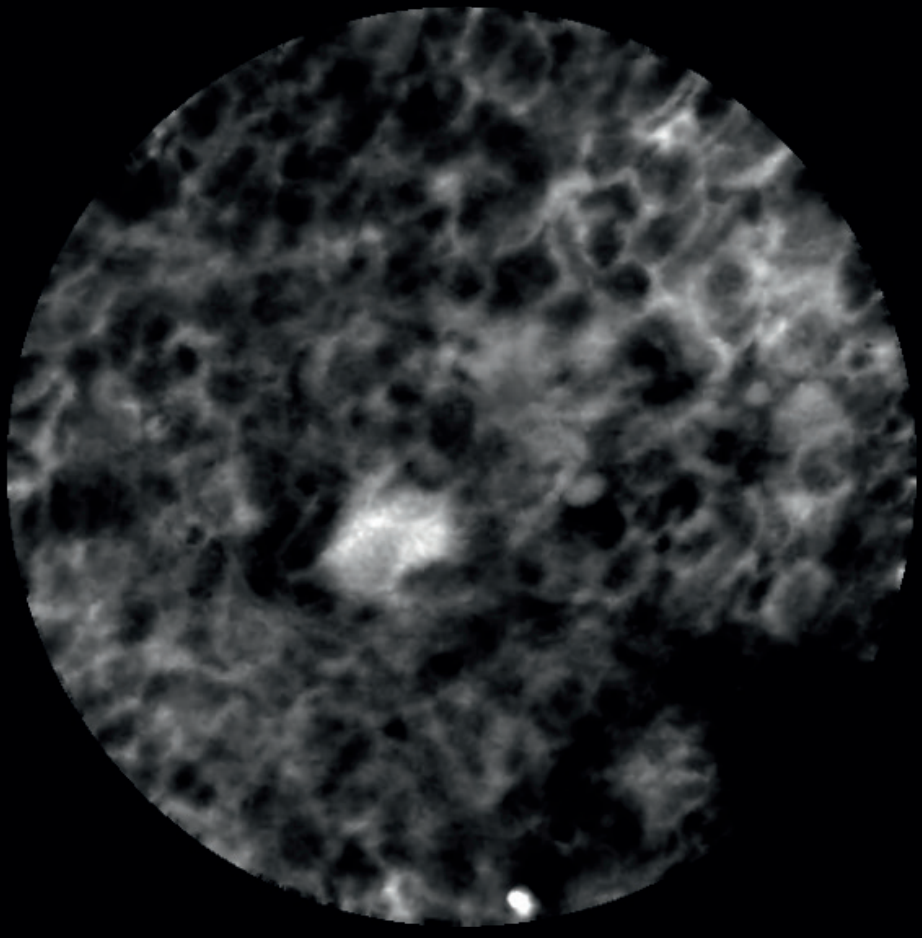
Supplemental Table 1. Prevalence of CLE features in percentages specified per histopathologic diagnosis.

	Low-grade UC				High-grade UC				Benign			
	Obs1 (%)	Obs2 (%)	Obs3 (%)	Mean (%)	Obs1 (%)	Obs2 (%)	Obs3 (%)	Mean (%)	Obs1 (%)	Obs2 (%)	Obs3 (%)	Mean (%)
Papillary aspect present	100	100	100	100	67	67	67	67	50	50	57	52
Papillary aspect not present	0	0	0	0	33	33	33	33	50	50	43	48
Polarity present	56	60	68	61	22	26	26	25	14	21	21	19
Polarity not present	32	36	4	24	59	59	37	52	57	64	29	50
ND Polarity	12	4	28	15	19	15	37	23	29	14	50	31
Organised cell pattern	76	72	80	76	30	30	30	30	36	36	36	36
Disorganised cell pattern	20	24	8	17	70	67	44	60	64	64	43	57
ND Organisation	4	4	12	7	0	4	26	10	0	0	21	7
Cohesive cells	80	80	72	77	26	37	44	36	50	50	50	50
Discohesiveness of cells	16	20	28	21	63	52	52	56	14	36	43	31
ND Cohesiveness	4	0	0	1	11	11	4	9	36	14	7	19
Monomorphic cells	60	64	76	67	19	19	22	20	36	43	43	40
Pleomorphic cells	32	32	20	28	74	78	78	77	64	57	36	52
ND Morphology	8	4	4	5	7	4	0	4	0	0	21	7
Distinct cell borders	80	84	80	81	22	22	22	22	36	50	57	48
Indistinct cell borders	20	16	16	17	74	78	78	77	64	50	36	50
ND Cell borders	0	0	4	1	4	0	0	1	0	0	7	2
Capillary network present	0	0	0	0	7	11	11	10	29	29	21	26
Fibrovascular stalk present	76	84	76	79	52	56	52	53	43	36	43	40
Large vessels present	4	8	16	9	15	7	26	16	7	7	7	7
No vasculature present	20	8	8	12	26	26	11	21	21	29	29	26

UC = Urothelial carcinoma, Obs = observer, ND = non-diagnostic, it was not possible for the observer to classify a feature



CHAPTER 5

Towards automated in vivo bladder tumour stratification using confocal laser endomicroscopy

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ABSTRACT

Purpose: Urothelial carcinoma of the bladder (UCB) is the most common urinary cancer. White light cystoscopy (WLC) forms the corner stone for the diagnosis of UCB. However, histopathological assessment is required for adjuvant treatment selection. Probe-based confocal laser endomicroscopy (CLE) enables the visualization of the microarchitecture of bladder lesions during WLC, which allows for real-time tissue differentiation and grading of UCB. To improve the diagnostic process of UCB, computer-aided classification of CLE videos of in vivo bladder lesions were evaluated in this study.

Materials and methods: We implemented pre-processing methods to optimize contrast and to reduce striping artefacts in each individual CLE frame. Subsequently, a semi-automatic frame selection was performed. The selected frames were used to train a feature extractor, based on pre-trained ImageNet networks. A recurrent neural network, in specific long-short term memory (LSTM), was used to predict the grade of the bladder lesions. Differentiation of lesions was performed at two levels, namely 1) healthy and benign versus malignant tissue and 2) low-grade versus high-grade papillary UCB. A total of 53 patients with 72 lesions were included in this study, resulting in ~140.000 CLE frames.

Results: The semi-automated frame selection reduced the number of frames to ~66.500 informative frames. The accuracy for the differentiation of 1) healthy and benign versus malignant urothelium was 79% and 2) high-grade and low-grade papillary UCB was 82%.

Conclusion: A feature extractor in combination with LSTM results in proper stratification of CLE videos of in vivo bladder lesions.

INTRODUCTION

Urothelial carcinoma of the bladder (UCB) is the most common malignancy of the urinary tract among both men and women. White light cystoscopy (WLC) is the corner stone for macroscopic visualisation of the urothelium and is therefore a valuable tool to localise the lesions in patients suspected of having bladder cancer. Yet, WLC does not provide the necessary information to differentiate between lesion subtypes since WLC by itself does not provide histopathologic information [1]. Histopathology-based differentiation of UCB is essential for treatment selection and prognosis.

Currently, suspicious bladder lesions are endoscopically resected for histopathological examination. In case of UCB, the tumour is graded by the pathologist based on the cellular microarchitecture and the nuclear appearance. Besides, for diagnostic purposes, endoscopic resection also serves as the primary treatment. Yet, adjuvant therapy may be required in case of intermediate- or high-grade UCB.

Probe-based confocal laser endomicroscopy (CLE) visualizes the cellular microarchitecture in real-time and has the potential to enable tissue differentiation during WLC. Real-time assessment potentially allows for minimally invasive laser ablation of low-grade tumours during outpatient cystoscopy [2–5]. The non-invasive CLE could potentially also enable watchful waiting in case of low-grade UCB in patients with severe comorbidities. CLE uses a fluorescent dye, introduced either topically or intravenously. By excitation of this dye with a 488 nm low-power laser, the contrast in the images obtained [6]. So far, seven criteria for the assessment of CLE images for UCB diagnosis and grading have been published [7–9]. However, the elaborate and subjective scoring method is subject to interobserver variation and an overall concordance with histopathological examination of 70% has been reported. In case of benign lesions, the concordance drops to 29% [9]. To circumvent the possible subjectivity of clinicians, a reproducible and reliable interpretation method of CLE images with good histopathological examination concordance is needed.

Due to striping artefacts coming from electrical noise, uneven distribution of fluorescent dye and motion artefacts, diagnostic features may not be visible in the CLE image sequence. Several approaches to alleviate non-informative frames have been described, nonetheless these methods suffer from low sensitivity and specificity [10–12]. Consequently, current (automated) classification studies still rely on a fully manual frame selection [10,13,14].

Previous approaches to automatically differentiate lesions using CLE have been implemented using, for example, neural, oral, oesophageal and (ex vivo) breast tissue [10–12, 15–17]. To our knowledge, automated tumour differentiation has not been applied on bladder tissue. In this work, we present semi-automatic frame optimisation and selection, followed by a fully automatic classification of the CLE video sequences of in vivo bladder lesions.

MATERIALS AND METHODS

Patient selection

For this prospective study, patients of the Amsterdam UMC Hospital (Amsterdam, The Netherlands) with a suspect primary or recurrent bladder tumour who were scheduled for transurethral endoscopic resection of the bladder tumour were recruited (Dutch Central Committee on Research Involving Human Subjects NL55537.018.15, ClinicalTrials.gov: NCT03013894) [18]. All patients signed a written informed consent. Before resection of the suspicious lesion, CLE video sequences of both suspicious lesions and macroscopically healthy appearing urothelium were obtained. The imaged regions were resected for histopathological examination after CLE. The histopathological grade was assessed according to the World Heatlth Organization (WHO) 2004 guidelines by one expert uropathologist and formed the reference standard in this study [1].

Data acquisition

CLE was used in combination with topical administration of fluorescein and stains the extracellular matrix of the bladder tissue [19]. The CLE data were acquired using the Cellvizio© 100 series (Mauna Kea Technologies, Paris, France), in combination with the CystoFlex UHD-R Confocal Miniprobe (Mauna Kea Technologies). This probe has a 2.6 mm outer diameter and was introduced through the working channel of a standard rigid cystoscope. The images obtained with the CystoFlex UHD-R Confocal Miniprobe have a field of view of 240 µm with a 1 µm lateral resolution and it images the tissue between 55 and 65 µm, resulting in a depth of field of 10 µm. Images were acquired at a rate between 8 and 12 frames per second to obtain a video sequence of the bladder mucosa. The probe was positioned in direct contact with the area of interest. In general, two video sequences [IQR: 2–3] of each suspected lesion and the healthy bladder mucosa, if present, were recorded.

Image pre-processing

The contrast of the individual frames was automatically stretched using the Cellvizio Viewer. An automatic notch filter was applied to all individual frames to minimize the (quasi-)periodic noise causing the stripe pattern. The contrast enhancement and denoising is illustrated in Figure 1.

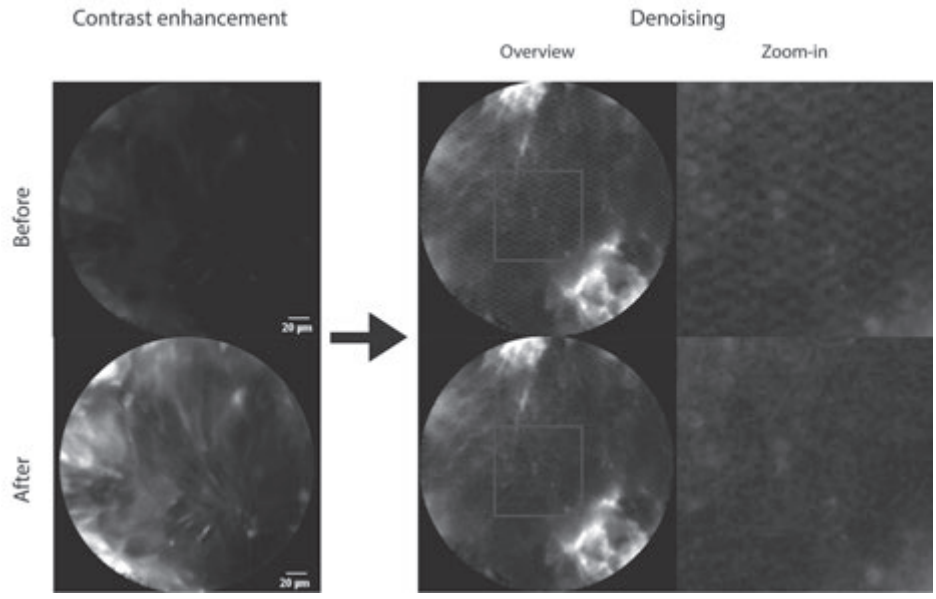

Figure 1. Overview of the pre-processing steps. on the left, the result of the contrast enhancement as embedded in the Cellvizio© Viewer is shown. On the right, the effect of the notch filter is shown. The zoom-in is located at the location of the grey square.

Frame selection

Due to artefacts in the image sequence, we decided to sort each frame automatically into one of three categories: (i) non-informative, (ii) in-doubt and (iii) informative frames. The category of the image content was determined by the number of contours present in each frame after Gaussian filtering and the range and spread of gray values throughout the image, as illustrated in Figure 2. The automatically sorted data was inspected by two expert observers (ML and EIMLL), who classified the 'in-doubt' frames as either informative or non-informative frames. A stricter selection of informative frames was made by EIMLL, for which at least 30% of the field-of-view should contain in-focus bladder mucosa as the papillary character of many bladder tumours resulted in a field of view that was only partially filled with tissue. An example of a CLE frame with a similar region of a histopathology section can be seen in Figure 3A, examples on informative and non-informative frames can be seen in Figure 3B.

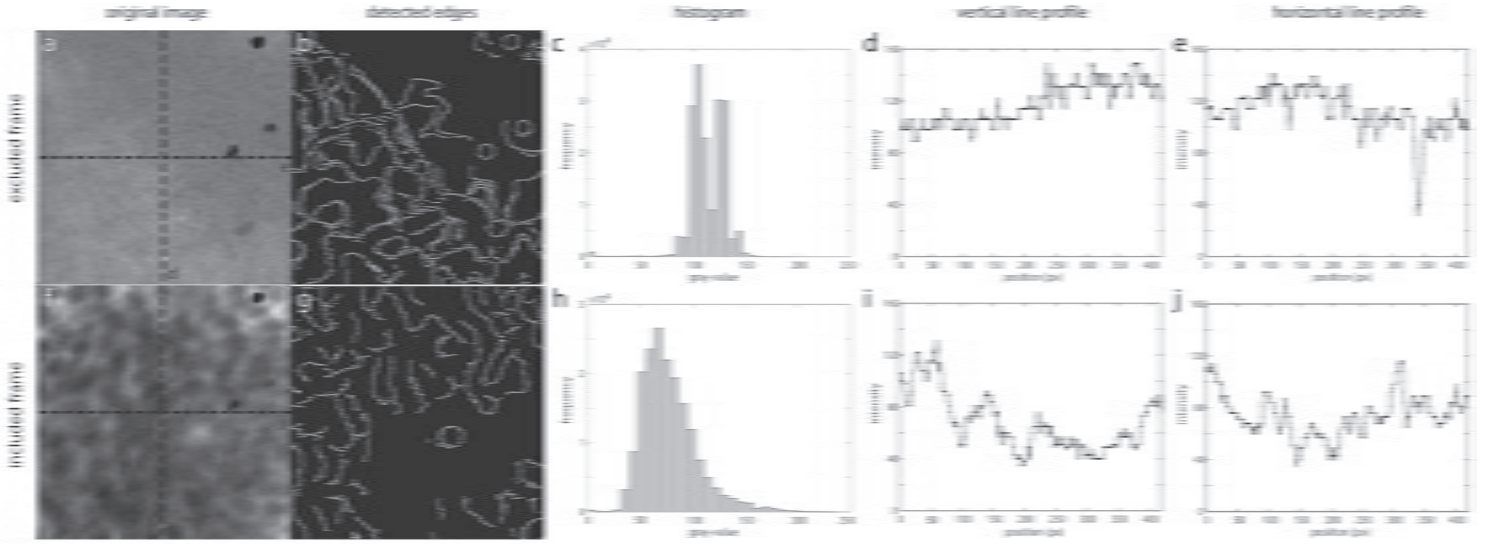

Figure 2. Overview of the analysis of the image content. The original images are shown on the left-hand side in A) and F). B and G) Contours present in the original images. The histograms in C) and H) represent the occurrence of grey values as present in the original images. The two columns on the right-hand side D, E, I and J) give an overview of the intensities at the horizontal and vertical dashed lines, as indicated in the original images in A) and F).

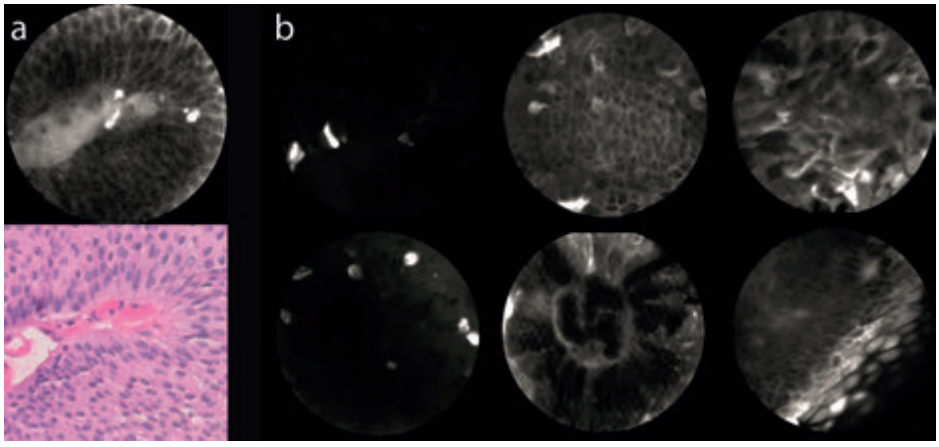

Figure 3. A) An example of the confocal laser endomicroscopy frame with a similar region of hematoxylin and eosin histopathology section. B) The examples in the left-hand side do not contain bladder mucosa and are considered non-informative frames. Images in the middle and right-hand side show a clear representation of the cellular microarchitecture of the bladder mucosa and are therefore informative frames.

Classification

All lesions were classified by a convolutional neural network followed by a recurrent neural network (RNN). The convolutional neural network was used to extract features from each individual frame. During the training phase of the convolutional neural network, image augmentation was applied to each frame. This included random rotation over the full range, as well as flipping of the frames along the horizontal and vertical axes.

In each network, roughly 20% of the frames were used for validation during the training phase and 10% was used to assess the performance of the network. The other frames, consisting of roughly 70% of the data, were used for training of the network. No overlap of patients could exist between those three sets. By using cross-validation, three different training, validation and testing sets were obtained with no overlap in the validation and testing sets in all batches.

Feature extraction

The performance of several convolutional neural network architectures to classify CLE sequences are compared. Three approaches are based on ImageNet pre-trained networks, that is, Inception V3, Xception and Inception ResNet V2, which were fine-tuned for the classification of each individual frame. In case of Inception V3, both mid-level and high-level features were fine-tuned, while in case of Xception and Inception ResNet V2 only high-level features were fine-tuned. This means that in all architectures, feature maps of the low-level convolutional blocks were fixed and only feature maps of the (mid- and) high-level convolutional block(s) were optimised for the classification of

CLE data. For eight epochs, the classification layers were fine-tuned for the CLE data. Afterwards, the last (four in case of Inception V3) convolutional block(s) together with the classification layers of each network were optimised for the CLE data, while fixing the ImageNet feature maps of the earlier layers. The network with the lowest cross-entropy was selected as the best performing network. The networks were trained on 299x299-pixel gray-scale images in the Keras framework (v2.1.6) using Tensorflow (v1.8.0) as the backend [20,21]. The classification result was binary, and therefore the sigmoid was used as activation function of the last fully connected layer. The optimizer used was Adam, with a learning rate of 0.0005 (β_1=0.99, β_2=0.999) [22].

Sequence analysis

The features of each CLE frame were subsequently combined into sequences, and a long short-term memory (LSTM) network was trained for classification of the time series. LSTM is a subset of RNNs that deals with vanishing or exploding gradients unlike conventional RNNs [23]. The sequences contain 10 frames each, which are equally spaced in time over the complete duration of the video sequence, resulting in multiple non-overlapping sequences per tissue sample. The majority vote of all sequences of a single video was set to be the final class label of the CLE video. To assess the diagnostic accuracy, this grade of each single video was compared with the classification given by histopathological examination of the imaged lesion.

A two-layer bidirectional LSTM was followed by a dense layer with two output nodes. The activation function was the sigmoid, and the dropout rate was set 0.5 in each layer. Adam was used as the optimizer, with a learning rate of 1^{-5} (β_1=0.99, β_2=0.999, decay=1^{-6}) [22].

The classification differentiates 1) benign urothelium (including both benign tumours and healthy urothelium) from malignant urothelium (both low-grade and high-grade papillary UCB and the aggressive flat carcinoma in situ [CIS]) and 2) low-grade papillary UCB from high-grade papillary UCB of papillary lesions identified with WLC.

RESULTS

Patient selection

A total of 73 patients were included for this study, resulting in 53 patients with 72 bladder lesions eligible for analysis (Figure 4). Twenty-two patients were diagnosed with high-grade UCB, comprising a total of 29 high-grade lesions. In two patients diagnosed with high-grade papillary UCB, a CIS lesion was also diagnosed. In one patient, 2 CIS lesions were found. Another 19 patients were diagnosed with low-grade UCB, with a total of 25 low-grade tumours. The remaining 11 patients, with 12 benign lesions, were not diagnosed with bladder cancer. In two patients with papillary UCB, also a benign bladder lesion was identified, making a total of 14 benign bladder lesions.

Figure 4. Flow chart of the included patients.

Frame selection

A total of ~140.000 frames were collected. After the semi-automatic 'loose selection', a median of 37% of the frames was disregarded (Q1–Q3: 21–54%). During the 'strict manual selection', a median of 12% of the loosely selected frames were disregarded (Q1–Q3: 4–36%), resulting in a median of 514 frames per lesion (Q1–Q3: 304–784 frames). This resulted in a total of 66.500 selected frames corresponding to ~6.500 sequences, in which roughly half of the frames contained a papillary lesion, from 116 different regions (including normal urothelium) derived from 53 patients.

Classification

Classification of benign and malignant tissue

All pre-trained ImageNet architectures were fine-tuned for the differentiation of benign (consisting of normal urothelium and benign tumours) and malignant tissue (comprising CIS, low-grade and high-grade papillary UCB). Each test set contained at least two videos of benign tumours (in the benign class) and two CIS videos (in the malignant class). In the first experiment, four CIS videos were included in the malignant class. The best average accuracy was obtained when using the Inception ResNet V2, resulting in 79% accuracy, with a sensitivity of 82% and specificity of 77%. The average area under the curve (AUC) was quite similar for all architectures. All results are listed in Table 1.

Table 1. Results of differentiation of healthy tissue and benign tumours from malignant tissue. The sensitivity, specificity, accuracy and area AUC are given for each architecture and for each experiment. Experiment 1 contained 11 benign and 11 malignant videos. In experiment 2, 8 benign and 13 malignant videos were included, and in experiment 3, a total of 9 benign and 14 malignant videos were present.

	Sensitivity (%)				**Specificity (%)**				**Accuracy (%)**				**AUC**			
	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean	#1	#2	#3	Mean
Inception V3	82	77	71	**77**	55	88	100	**81**	68	81	83	**77**	0.66	0.91	0.89	**0.82**
Inception ResNet V2	82	85	79	**82**	55	75	100	**77**	68	81	87	**79**	0.76	0.74	0.92	**0.81**
Xception	73	69	57	**66**	73	88	89	**89**	73	76	70	**70**	0.74	0.88	0.84	**0.82**

AUC = Area under the curve

Classification of papillary UCB

For differentiation of low-grade papillary UCB from high-grade papillary UCB, the fine-tuned ImageNet pre-trained Inception V3 network performs best for the CLE data. In all cases, the testing set contained 8 low-grade papillary lesions, and 4–6 high-grade papillary lesions. An overview of the results can be found in Table 2. On average, an accuracy of 82% was reached in the differentiation between low-grade and high-grade papillary UCB, with an area under the curve of 0.86.

Table 2. Results of the classification of low-grade and high-grade papillary urothelial carcinoma of the bladder.

Experiment (# of lesions)	Sens (%)	Spec (%)	Acc (%)	PPV (%)	NPV (%)	AUC
1 (4 HG 8 LG)	75	88	83	75	88	0.94
2 (4 HG 8 LG)	100	88	92	80	100	0.88
3 (6 HG 8 LG)	67	75	71	67	75	0.75
Average	81	84	82	74	88	0.86

Sens = sensitivity, Spec = specificity, Acc = accuracy, PPV = positive predictive value, NPV = negative predictive value, AUC = area under the curve, HG = high-grade, LG = low-grade

DISCUSSION

In this study, we have developed and investigated a computer-aided classification method for CLE imaging to enable automatic differentiation of bladder lesions and grading of papillary UCB during cystoscopy. Additionally, semi-automatic frame selection was implemented in the diagnostic approach to select frames with (perceptible) functional information. The automatic classification showed a high agreement with the histopathological grade for differentiation of low-grade and high-grade papillary lesions. The automatic differentiation of benign versus malignant bladder tissue resulted in a moderate to high agreement with histopathology.

Comparison with current literature

The histopathological grade of UCB is an important predictor of prognosis. Therefore, tumour grade is a key factor in treatment selection [24]. However, the diagnostic accuracy of WLC for UCB grade prediction is insufficient [25]. In the study of Mariappan et al. [26] sensitivity and specificity of 56% and 84%, respectively, were found for the differentiation between high-grade and low-grade papillary UCB based on WLC appearance. Our proposed automatic classification method yielded a similar specificity with a higher sensitivity.

To our knowledge, this is the first study to automatically classify CLE videos for the differentiation of bladder lesions. In other fields, CLE studies for differentiating tissues in other organs found similar accuracies between 80 and 90% [10–12,15–17]. In the present study, the differentiation between benign and malignant tissue was proven to be more

difficult, probably owing to the inclusion of reactive, benign urothelial lesions and CIS, which share a similar cellular microarchitecture. In particular, the differentiation between benign tumours from CIS would be a valuable addition to WLC as large difficulties are faced to visually differentiating those lesion subtypes [27,28].

Limitations

The acquired dataset is limited, especially cases with benign tissue and CIS are underrepresented in this study. Therefore, it was chosen to only differentate between benign and malignant tissue, as well as low-grade and high-grade papillary UCB. The dataset was limited in size and the use of deep networks could have resulted in overfitting. Therefore, the ImageNet weights of the pre-trained Inception V3, Xception and Inception ResNet V2 network were fine-tuned by only optimising the (mid- and) high-level features for CLE data. Furthermore, image augmentation techniques, such as flipping and rotation of the frames, were applied to increase the robustness of the classification performance.

Furthermore, in future studies it could be interesting to compare the videos obtained with different probes. In the current study, all video sequences were obtained using the CystoFlex UHD-R Confocal Miniprobe, which can only be introduced in the bladder using a rigid cystoscope. Therefore, it was difficult to obtain CLE videos of certain parts of the bladder. To facilitate implementation of the proposed automatic classification method in the clinical workflow in the outpatient clinic, it is important to test its performance on data obtained via the flexible cystoscope, for instance using the UroFlex-B Miniprobe [1,29]. However, this probe has a lower lateral resolution with a wider field of view (325 µm), and images the tissue between 40 and 70 µm from the probe head. The depth of field of this flexible probe is three times larger than that of the CystoFlex UHD-R Confocal Miniprobe, as used in this study, which results in a blurring of the images due to the fact that an increased number of cell layers is mapped onto the detector.

Future perspectives

UCB is a disease with frequent recurrence, therefore requiring intensive surveillance with WLC performed in the outpatient clinic. As CLE can be used to differentiate between low- and high-grade papillary UCB, low-grade papillary tumours may be treated with, for example, laser vaporization during outpatient cystoscopy [2–5]. Additionally, CLE could potentially be used to confirm the radicality of transurethral resection of UCB by scanning the resection bed. This might help to reduce the number of re-resections. In light of re-resections and the high recurrence rate of UCB, a reduction in the number of transurethral surgeries might also benefit health-care costs. Furthermore, the classification methods could also be applicable for CLE-based grading of upper urinary tract urothelial carcinoma [30]. Automatic real-time assessment of tumour grade during diagnostic endoscopy in the upper urinary tract could enable intra-operative treatment selection.

However, collaboration on existing datasets are required to externally validate the proposed methodology before implementation in the clinical workflow. If larger datasets become available for the proposed classification system, it may result in more generalisable and more accurate results. To further improve clinical utility of CLE, the semi-automated frame selection should be automated. The current strict manual selection may serve as the starting point to optimise the selection process even further. A convolutional neural network could be trained to differentiate between informative and non-informative frames [11]. This would open up the way to 'real-time' implementation of our classification algorithms within the clinical workflow. Given these preliminary results, such a real-time implementation seems feasible and enables agile clinical decision-making for a part of the patient population.

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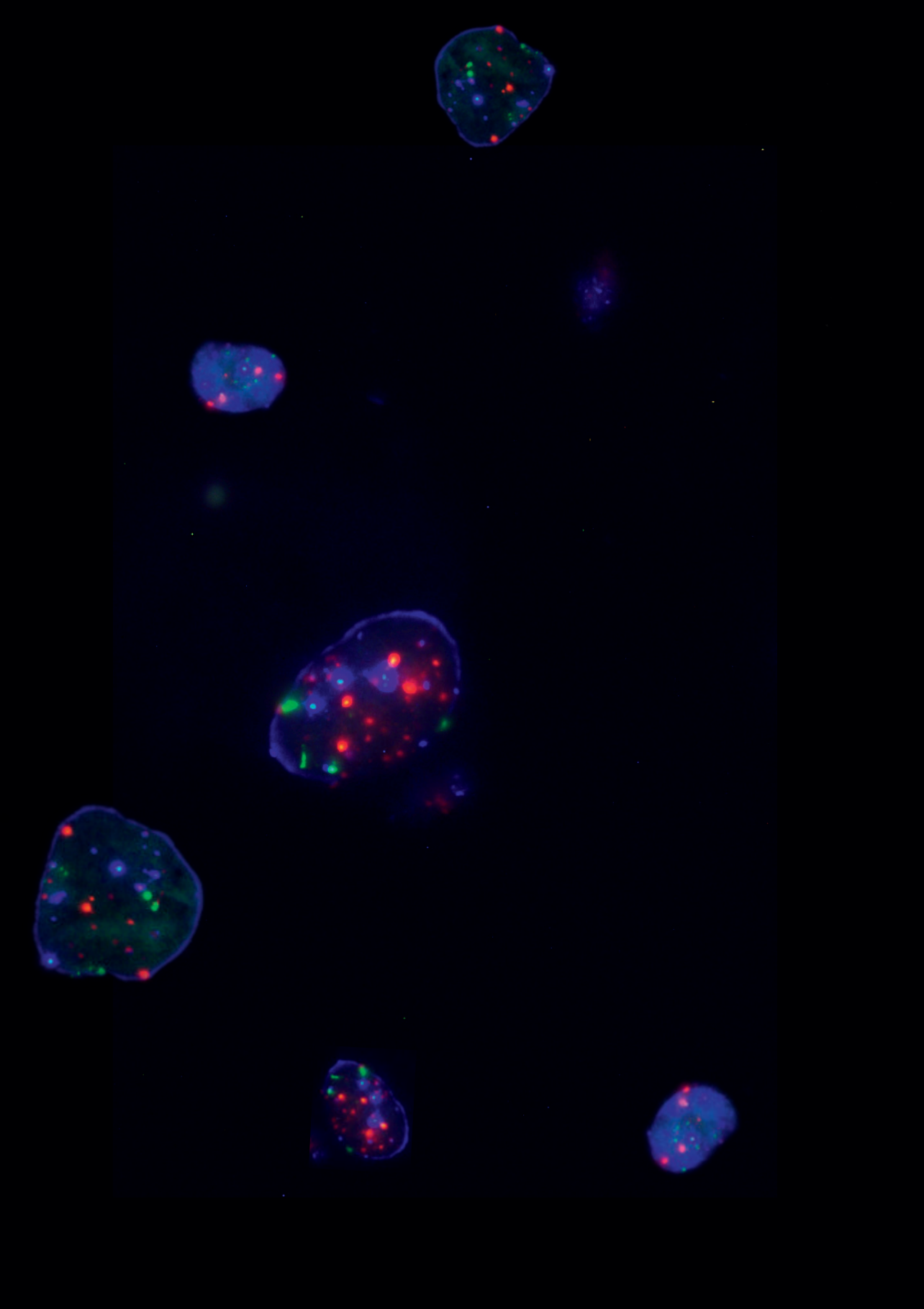
PART III

Fluorescence in situ hybridization for predicting recurrence after Bacillus Calmette-Guérin



CHAPTER 6

Fluorescence in situ hybridization as prognostic predictor of tumour recurrence during treatment with Bacillus Calmette-Guérin therapy for intermediate- and high-risk non-muscle invasive bladder cancer

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ABSTRACT

A significant number of patients with intermediate- or high-risk bladder cancer treated with intravesical Bacillus Calmette-Guérin (BCG) immunotherapy are non-responders to this treatment. Since we cannot predict in which patients BCG therapy will fail, markers for responders are needed. UroVysion® is a multitarget fluorescence in situ hybridization (FISH) test for bladder cancer detection. The aim of this study was to evaluate if FISH can be used to early identify recurrence during treatment with BCG. In a multicentre, prospective study, three bladder washouts at different time points during treatment (t_0=week 0, pre-BCG, t_1=6 weeks following transurethral resection of the bladder tumour [TURB], t_2=3 months following TURB) were collected for FISH from patients with bladder cancer treated with BCG between 2008 and 2013. Data on bladder cancer recurrence and duration of BCG maintenance therapy were recorded. Thirty-six (31.6%) out of 114 patients developed a recurrence after a median of 6 months (range 2–32). No significant association was found between a positive FISH test at t_0 or t_1 and risk of recurrence (p=0.79 and p=0.29). A positive t_2 FISH test was associated with a higher risk for recurrence (p=0.001). Patients with a positive FISH test 3 months following TURB had a 4.0–4.6 times greater risk for developing a recurrence compared to patients with a negative FISH. Patients with a positive FISH test 3 months following TURB and induction BCG therapy have a higher risk for developing tumour recurrence. FISH can therefore be a useful additional tool for physicians when determining a treatment strategy.

INTRODUCTION

Non-muscle invasive bladder cancer (NMIBC) is a heterogeneous histopathological condition with different prognoses. Based on risk factors, patients are classified into risk groups with low-, intermediate- and high-risk for recurrence and progression [1]. For intermediate- or high-risk NMIBC, adjuvant intravesical therapy with Bacillus Calmette-Guérin (BCG) is recommended in the guidelines of the European Association of Urology and the American Urological Association [1,2].

In spite of its effectiveness, intravesical BCG-therapy is not devoid of limitations [3]. BCG treatment may induce local side effects in 62.8% and systemic side effects in 30.6% of patients with possible fatal outcome [4]. This may lead to interruption or discontinuation of BCG treatment in up to 20% of patients [5]. Besides, in up to 40% of patients BCG treatment fails [6–8]. BCG failure can be divided into different types: BCG intolerant, refractory and relapsing. BCG intolerant patients have to stop due to side effects, whereas BCG refractory patients do not respond to BCG induction therapy and have persistent disease, while BCG relapsing patients initially do respond to BCG treatment, but after a disease-free period develop a recurrence [9,10]. Since BCG is mainly given to treat patients with a high risk for progression to muscle invasive disease, it is important to identify non-responding patients early. However, currently no diagnostic tool is available to discriminate between BCG responders and BCG non-responders. A predictive test is desirable and might be helpful in treatment decision.

UroVysion® (Abbott Molecular, Illinois, USA) fluorescence in situ hybridization (FISH) is able to detect genetic alterations most commonly associated with bladder cancer. The assay detects aneuploidy of chromosomes 3, 7 and 17 and a deletion of locus 9p21 [11]. Since FISH is based on detection of genetic alterations, results or interpretation of the test will not be influenced by the inflammatory response of the bladder to BCG, as opposed to cystoscopy and urine cytology [12].

If we can predict which patients are at risk for developing a recurrence during BCG treatment, it is possible to prevent under-treatment by timely changing from BCG therapy to other intravesical therapy or to radical therapy i.e. radical cystectomy. Furthermore, early identification of BCG non-responders will limit the associated risks of BCG therapy. The aim of this study is to determine the usefulness of FISH as predictor of tumour recurrence in patients with NMIBC treated with BCG instillations.

MATERIALS AND METHODS

Patient inclusion

From 2008 to 2013, five centres included patients with NMIBC treated with BCG instillations in a prospective study evaluating the accuracy of FISH in bladder washout (BWO). Informed consent was verbally obtained of all participants prior to inclusion. Patients had histologically confirmed primary or recurrent intermediate- or high-risk NMIBC (CIS, Ta, T1, all grades) and were scheduled for BCG induction therapy after complete transurethral resection of the bladder tumour(s) (TURB). Administration of a single immediate post-operative chemotherapy instillation or re-resection was left to the discretion of the treating urologist. Exclusion criteria included presence of muscle invasive disease, no histologic confirmation of bladder tumour and synchronous upper urinary tract urothelial carcinoma.

BCG instillation protocol

All patients were scheduled to receive at least induction BCG-therapy of six weekly instillations following TURB. Maintenance therapy was administered depending on hospitals' protocols. In general, maintenance therapy consisted of three weekly instillations during 1 to 3 years (at 3, 6, 12, 18, 24, 30, 36 months). Patients were followed by cystoscopy every 3 months during the first 2 years after inclusion or until a recurrence was diagnosed. Data on bladder cancer recurrence and duration of BCG maintenance therapy was recorded. A recurrence was defined as histopathologically proven NMIBC or muscle invasive disease (T ≥2). Tumour grade was assessed based on the 1973 World Health Organization (WHO) classification. Progression was defined as the histologic confirmation of muscle invasive disease (T ≥2).

Bladder washout protocol

BWOs for FISH evaluation were collected at three time points: before the first BCG instillation (t_0), before the last induction BCG instillation at 6 weeks (t_1) and at 3 months during first cystoscopy follow-up (t_2). BWOs at t_0 and t_1 were done via a catheter, and 50 cc 0.9% saline was used to flush the bladder. At t_2, the BWO was done at the end of the cystoscopy via the working channel of the cystoscope. Each BWO was preserved in carbowax (polyethylene glycol). Cytospins were made within 72 hours and stored in a –20ºC freezer until FISH test was performed.

FISH protocol

All BWOs were analysed using the multitarget UroVysion® bladder cancer kit. The FISH kit is composed of a mixture of four-target multicolour probes, three chromosome enumeration probes (CEP 3, CEP 7 and CEP 17) and one single locus-specific indicator probe (LSI 9p21). Cytospins were made of collected BWOs and fixed using Carnoy's solution (3:1 methanol/glacial acetic acid). Slides were pre-treated using the Vysis pre-treatment kit (Abbott Molecular, Illinois, USA), and FISH was performed according to the

manufacturer's instructions provided with the assay. In short, slides were denatured in 2x SSC at 73 ± 1ºC for 2 minutes and incubated in pepsin buffer at 37ºC for 10 minutes. After 5 minutes washing at room temperature with phosphate-buffered saline (PBS), the slides were fixed in 1% formaldehyde for 5 minutes. The slides were washed again in PBS at room temperature for 5 minutes and dehydrated in consecutively 70, 85 and 100% ethanol, for 1 minute each. For hybridization the multitarget UroVysion® probe mixture was added and incubated overnight at 73ºC (denaturation 2 minutes) and 37ºC (hybridization 8–16 hours) using the ThermoBrite system. Post-hybridization the slides were washed in 0.4 SSC at room temperature for 5 minutes, 0.4 SSC at 73ºC for maximum 2 minutes, and in 2x SSC at room temperature for 1 minute. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole). FISH assays were examined using a fluorescence microscope (Leica DM 5000B and Leica DM 5500) with the following filters: A4 (DAPI), TX2 (CEP 3, red), L5 (CEP 7, green), SAQ (CEP 17, aqua) and SGO (LSI 9p21, gold).

Data analysis

Slides were screened for 25 morphologically abnormal cells (large nuclear size, irregular nuclear shape, patchy DAPI staining or cell clusters), and considered positive if one of the following criteria were met: ≥4 cells have a gain of 2 or more chromosomes (3, 7 or 17) or ≥12 cells have a loss of both copies of LSI 9p21 [13]. During the course of the trial three designated researchers evaluated all slides. The researchers were instructed and trained by one of the manufacturer's cytogenetic consultants.

Statistics

Data were analysed using SPSS Statistics version 23. Descriptive statistics were used for patient characteristics. Patient and tumour characteristics of patients with a FISH result available at t_1 and t_2 were compared with the whole cohort with a FISH result available at t_0, to evaluate whether missing cases at t_1 and t_2 influenced the results. P-values were calculated by using one-sample test proportion. Kaplan-Meier method was used to estimate recurrence-free survival and progression-free survival based on positive or negative FISH test at the three time points (t_0, t_1 and t_2). The log-rank test was used for statistical significance. Hazard Ratios were calculated using Cox proportional regression analysis. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the test at each time point during evaluation were calculated using 2x2 tables.

Table 1. Patient characteristics.

	N	
Patients, *n*	114	
Male, *n (%)*	88	(77.2)
Female, *n (%)*	26	(22.8)
Mean age (years), [range]	70.7	[42-94]
Median follow-up (months), [range]	23	[2-32]
History of bladder cancer, *n (%)*	34	(29.8)
Previous intravesical treatment, *n (%)*	15	(13.2)
Mitomycin C	8	
BCG	7	

BCG = Bacillus Calmette-Guérin

Table 2. Tumour characteristics.

	N	
Tumour stage, *n (%)*		
CIS only	23	(20.2)
Ta	43	(37.7)
T1	48	(42.1)
Tumour grade, *n (%)*		
CIS only	23	(20.2)
G1	6	(5.3)
G2	7	(6.1)
G2 + CIS	4	(3.5)
G3	57	(50.0)
G3 + CIS	17	(14.9)
Intermediate-Risk, *n (%)*	7*	(6.2)
High-Risk, *n (%)*	105*	(93.8)
Single tumours, *n (%)*	42	(36.8)
Multifocal, *n (%)*	72	(63.2)

CIS = Carcinoma in situ
* 2 patients could not be classified, because information regarding tumour size was missing

Table 3. Recurrence during 24 months follow-up.

	CIS only	G1	G2	G3	G3 + CIS	Total
CIS only	9	0	0	0	0	9
Ta	0	5	5	3	2	15
T1	0	0	1	6	1	8
T2	0	0	0	3	1	4
Total	9	5	6	12	4	36

CIS = Carcinoma in situ

RESULTS

Patient characteristics and outcomes

In total 147 patients were enrolled during the study period with 114 patients finally being eligible for data evaluation. Patient and tumour characteristics are summarised in Tables 1 and 2. Sixty-six patients received BCG maintenance (4–25 months). Median follow-up for the whole cohort was 23 months (range 2–32). During follow-up 36 patients (31.6 %) developed a recurrence (Table 3) at a median time of 6 months (range 3–28). Disease progression to muscle invasive bladder cancer occurred in 4 of the 36 patients after a median time of 13 months (range 7–23). High-grade tumour recurrence occurred in 25 patients (Table 3). During follow-up, one patient developed a ureter tumour and 15 patients died; 3 patients as a result of metastatic bladder cancer, 6 patients due to non-urologic reasons and 6 patients with an unknown cause. Six patients were lost to follow-up, with no available data.

FISH results

Patients were considered suitable for analysis if at least two evaluable BWOs were available for FISH, with one sample being collected at t_0 and a second sample at either t_1 or t_2 (Figure 1). Of 58 patients (50.9%) FISH results at all three time points were available, and of 56 patients (49.1%) two FISH samples were available (n=48 for t_0 and t_1, n=8 for t_0 and t_2). FISH test was available at t_0 in 114 patients and was positive in 48 patients (42.1%). At t_1 FISH test was available in 106 patients. Thirty-six patients converted from pre-BCG positive FISH to post-BCG negative FISH at t_1. In total 16 patients (15.1%) had a positive FISH result at t_1. At first cystoscopic surveillance (t_2), 66 FISH results were available, of which 18 were positive (27.3%). Of these patients, 10 patients (15.2%) initially had a negative pre-BCG FISH result that converted to a positive FISH result at t_2.

Survival analysis

Kaplan-Meier curves for recurrence of the whole cohort and for the three time points in which FISH was performed are shown in Figure 2. No association between a positive FISH result and tumour recurrence was found at t_0 (p=0.79) and a non-significant correlation was observed at t_1 (p=0.29). At t_2 a positive FISH test was significantly associated with a higher risk of recurrence (p=0.001). Cox regression showed that patients with a positive FISH test at t_2 had a 4.6 times greater risk of tumour recurrence compared to patients with a negative FISH test at 3 months following TURB (95% confidence interval [CI]: 1.71–11.84). When corrected for an immediate postoperative chemotherapy instillation, repeat TURB and number of maintenance BCG instillations a positive FISH test at t_2 had a 4.0 greater risk (95% CI: 1.45–11.10) of tumour recurrence compared to patients with a negative FISH test.

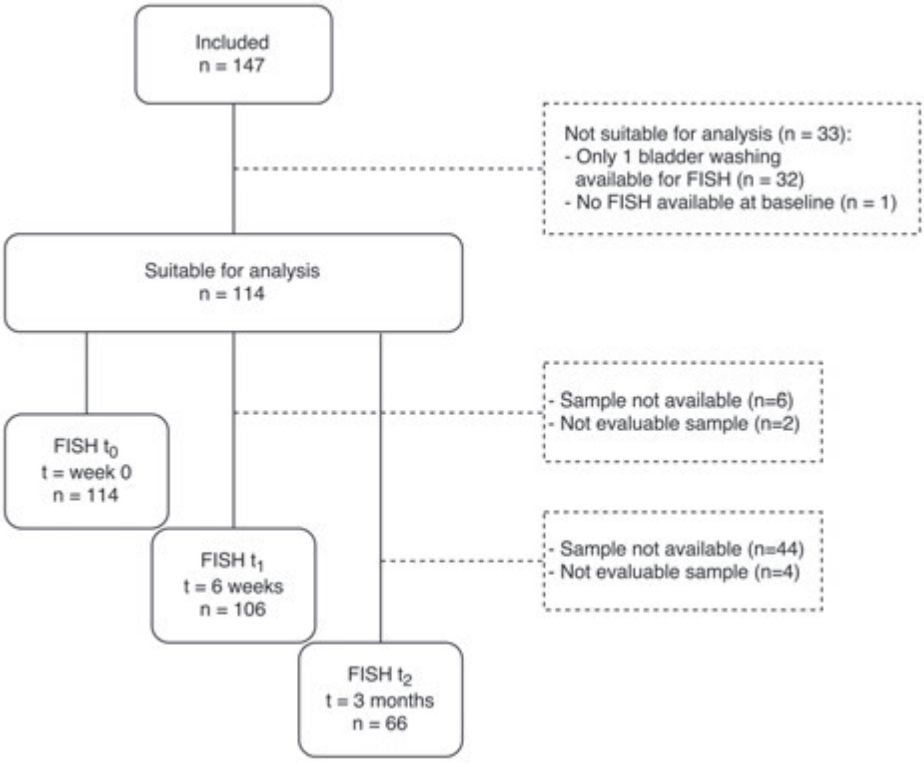

Figure 1. Flow chart showing patient inclusion and exclusion.

Due to the small number of progression events during the study period a separate Kaplan-Meier analysis for this outcome was not possible.

Diagnostic test evaluation

Sensitivity of FISH at t_0, t_1 and t_2 was 44, 21 and 59%, and specificity was 59, 88 and 84%, respectively. For the three different points in time PPV was 33, 44 and 56% and NPV was 70, 71 and 85%, respectively. Accuracy of the FISH test at t_0, t_1 and t_2 was 54, 67 and 77%, respectively (Table 4).

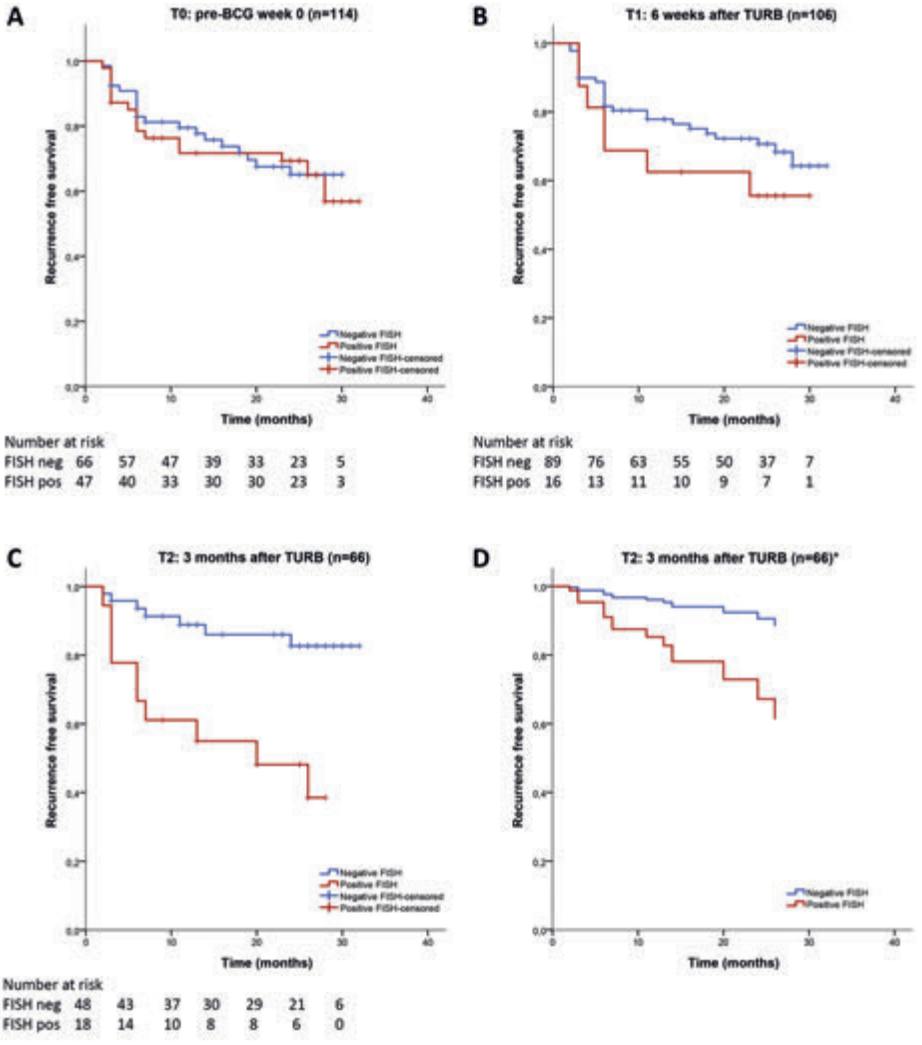

Figure 2. Recurrence-free survival curves of t_0, t_1 and t_2, and hazard curve of t_2, corrected for possible confounding. A) Kaplan-Meier curve of patients with a positive FISH test versus negative FISH test pre-BCG (t_0). B) Kaplan-Meier curve of patients with a positive FISH test versus negative FISH test post-BCG induction at 6 weeks (t_1). C) Kaplan-Meier curve of patients with a positive FISH test versus negative FISH post-BCG at 3 months (t_2). D) Hazard curve of t_2, corrected for immediate post-operative instillation, repeat TURB and number of BCG maintenance instillations.

Table 4. Evaluation of UroVysion® FISH at different time points. A) FISH evaluation at t_0. B) FISH evaluation at t_1. C) FISH evaluation at t_2.

A. T_0 – pre-BCG					
	Recurrence during FU			Sens	0.44
FISH	yes	no	Total	Spec	0.59
Positive	16	32	48	PPV	0.33
Negative	20	46	66	NPV	0.70
Total	36	78	114	Acc	0.54

B. T_1 – post-BCG at 6 weeks					
	Recurrence during FU			Sens	0.21
FISH	yes	no	Total	Spec	0.88
Positive	7	9	16	PPV	0.44
Negative	26	64	90	NPV	0.71
Total	33	73	106	Acc	0.67

C. T_2 – pre-BCG at 3 months					
	Recurrence during FU			Sens	0.59
FISH	yes	no	Total	Spec	0.84
Positive	10	8	18	PPV	0.56
Negative	7	41	48	NPV	0.85
Total	17	49	66	Acc	0.77

FU = Follow-up, Sens = sensitivity, Spec = specificity, PPV = positive predictive value, NPV = negative predictive value, Acc = accuracy

DISCUSSION

The results from this study confirm earlier data from smaller or single-centre studies, establishing the potential of FISH as part of a predictive diagnostic workup. This study demonstrates that the UroVysion® FISH test 3 months following TURB and BCG induction can be of value when considering disease management for patients with intermediate- or high-risk NMIBC. Patients with a positive FISH test at t_2 had a 4.0–4.6 times greater risk to develop a recurrence than patients with a negative FISH test. At t_2, sensitivity, specificity and accuracy of FISH was 59, 84 and 77%, respectively. On the contrary, despite a trend at t_1, the results of the FISH test at t_0 and t_1 were not significantly associated with the risk for tumour recurrence. Risk assessment for tumour progression using FISH could not be determined due to the small number of progression events.

The literature regarding the efficacy of UroVysion® for predicting recurrence risk following adjuvant instillations is scarce. Kipp et al. [14] and Whitson et al. [15] published results of patients who received bladder instillations using intravesical therapy including BCG, Mitomycin C and Thiotepa. Both groups reported that a positive FISH test following intravesical therapy was associated with a higher risk of recurrence. Additionally, a positive FISH test prior to intravesical treatment was associated with a higher risk of recurrence, and a positive FISH

test following intravesical treatment was associated with a higher risk of progression to muscle invasive disease [14]. Three other studies focused on risk assessment for tumour recurrence using FISH in patients treated with BCG instillations only [16–18]. These groups also reported that a positive FISH test following BCG therapy was associated with a higher risk of recurrence. Our results at t_0 and t_1 are in line with results reported by Mengual et al. [16] and Savic et al [17]. However, Kamat et al. [18] found a positive association for t_0. This discordance could be explained by the difference in patient cohorts. In the cohort evaluated by Kamat et al. [18], 89% of the patients had a previously treated bladder tumour and 48% had CIS as secondary finding, whereas in our cohort this was 30 and 18%, respectively.

Although not significant, the association between a positive FISH test at t_1 and the risk of recurrence indicates a positive trend. We hypothesize that patients with a false positive FISH at t_1 did not fully benefit from the BCG induction therapy yet, since BCG-induced delayed immune reaction may differ in each patient [19,20].

At first cystoscopic surveillance following TURB, 18 patients had a positive FISH test. However, some had a false positive FISH test. A follow-up of two years might be too short to detect progression and leads to underestimation of recurrent and progressive disease. Conversely, a negative FISH test 3 months following initial TURB does not exclude patients to develop a recurrence. In our study seven patients had a false negative FISH test at t_2 (15% of all patients with a negative FISH result at t_2) and did develop a recurrence bladder tumour during follow-up at a median of 7 months (range 2–24). Of these, two patients progressed to muscle invasive disease (Supplemental Table 1*)*. Although UroVysion® is designed to detect genetic changes associated with most bladder cancers, some bladder tumours have different genetic changes that will not be detected using this test [21–25].

A limitation of this study is the number of BWO samples not available or suitable for analysis. This reduces the power of the study. Secondly, the number of patients with an available FISH result at t_2 is limited. When comparing patients with available FISH results at t_2 and at t_0, patient and tumour characteristics were similar, except for tumour focality. This could imply that tumours of patients that had a FISH result available at t_2 were slightly more aggressive (Table 5 and 6). Furthermore, in this study BWOs were used for logistic reasons. Though UroVysion® is intended to be performed in voided urine samples, it has been demonstrated that the test is valid when performed in BWO samples [13]. Also, BWOs were processed over the course of seven years. It cannot be ruled out that during this period some samples were improperly handled or stored. However, six and a half years after collecting the urine samples, still good fluorescent signals were obtained. Lastly, the duration of BCG maintenance therapy is still a subject of debate. Patients received a 6-week induction course of BCG and in the majority of cases this was followed by BCG maintenance therapy depending on hospital protocol. This may have influenced the chance of developing a recurrence [26]. We could not assess the effect of the different maintenance protocols.

Based on our results, a positive UroVysion® FISH result alone is not sufficient to decide to switch from BCG to radical cystectomy at an early stage (3 months following TURB). There is a substantial risk of overtreatment if all patients with a positive FISH test at t_2 would undergo more aggressive treatment. A positive FISH test following BCG treatment (t_2) is, however, associated with a higher risk of developing a recurrence. A recent update of the guideline of the American Urological Association recommends the use of UroVysion® to assess response to intravesical BCG therapy (level of recommendation: expert opinion) [2]. We recommend for future clinical trials to incorporate FISH at later time points after induction therapy (≥3 months following initial TURB).

Table 5. Patient characteristics (t_0, t_1, t_2).

	T_0		**T_1**		**T_2**		**P (t_0 vs. t_2)**
Patients, *n*	114		106		66		
Male, *n (%)*	88	(77.2)	81	(76.4)	51	(77.3)	0.548
Female, *n (%)*	26	(22.8)	25	(23.6)	15	(22.7)	
Mean age (years), [range]	70.7	[42–94]	70.5	[42–94]	72.3	[50–94]	
Median follow-up (months), [range]	23	[2–32]	24	[2–32]	23	[2–32]	
History of bladder cancer, *n (%)*	34	(29.8)	32	(30.2)	19	(28.8)	0.467
Previous intravesical treatment, *n (%)*	15	(13.2)	13	(12.3)	10	(15.2)	
Mitomycin C	8		8		5		
BCG	7		5		5		

BCG = Bacillus Calmette-Guérin

Table 6. Tumour characteristics (t_0, t_1, t_2).

	T_0		**T_1**		**T_2**		**P (t_0 vs t_2)**
Tumour stage, *n (%)*							
CIS only	23	(20.2)	21	(19.8)	14	(21.2)	0.467
Ta	43	(37.7)	39	(36.8)	26	(39.4)	0.434
T1	48	(42.1)	46	(43.4)	26	(39.4)	0.377
Tumour grade, *n (%)*							
CIS only	23	(20.2)	21	(19.8)	14	(21.2)	0.467
G1	6	(5.3)	6	(5.7)	3	(4.5)	0.534
G2	7	(6.1)	7	(6.6)	3	(4.5)	0.422
G2 + CIS	4	(3.5)	4	(3.8)	2	(3.0)	0.592
G3	57	(50.0)	51	(48.1)	34	(51.6)	0.902
G3 + CIS	17	(14.9)	17	(16.0)	10	(15.2)	0.530
Intermediate-Risk, *n (%)*	7*	(6.2)	7*	(6.7)	2**	(3.1)	
High-Risk, *n (%)*	105*	(93.8)	97*	(93.3)	63**	(96.9)	0.224
Single tumours, *n (%)*	42	(36.8)	39	(36.8)	17	(25.8)	
Multifocal, *n (%)*	72	(63.2)	67	(63.2)	49	(74.2)	0.039

CIS = Carcinoma in situ
* 2 patients could not be classified because information regarding tumour size was missing
** 1 patient could not be classified because information regarding tumour size was missing

CONCLUSION

This study demonstrates that a positive UroVysion® test at 3 months following TURB and induction BCG therapy for intermediate- and high-risk urothelial carcinoma of the bladder is associated with a statistical significant higher risk of recurrence. Therefore, it can be a useful tool for urologists to assess which patients have a higher risk of developing a recurrence.

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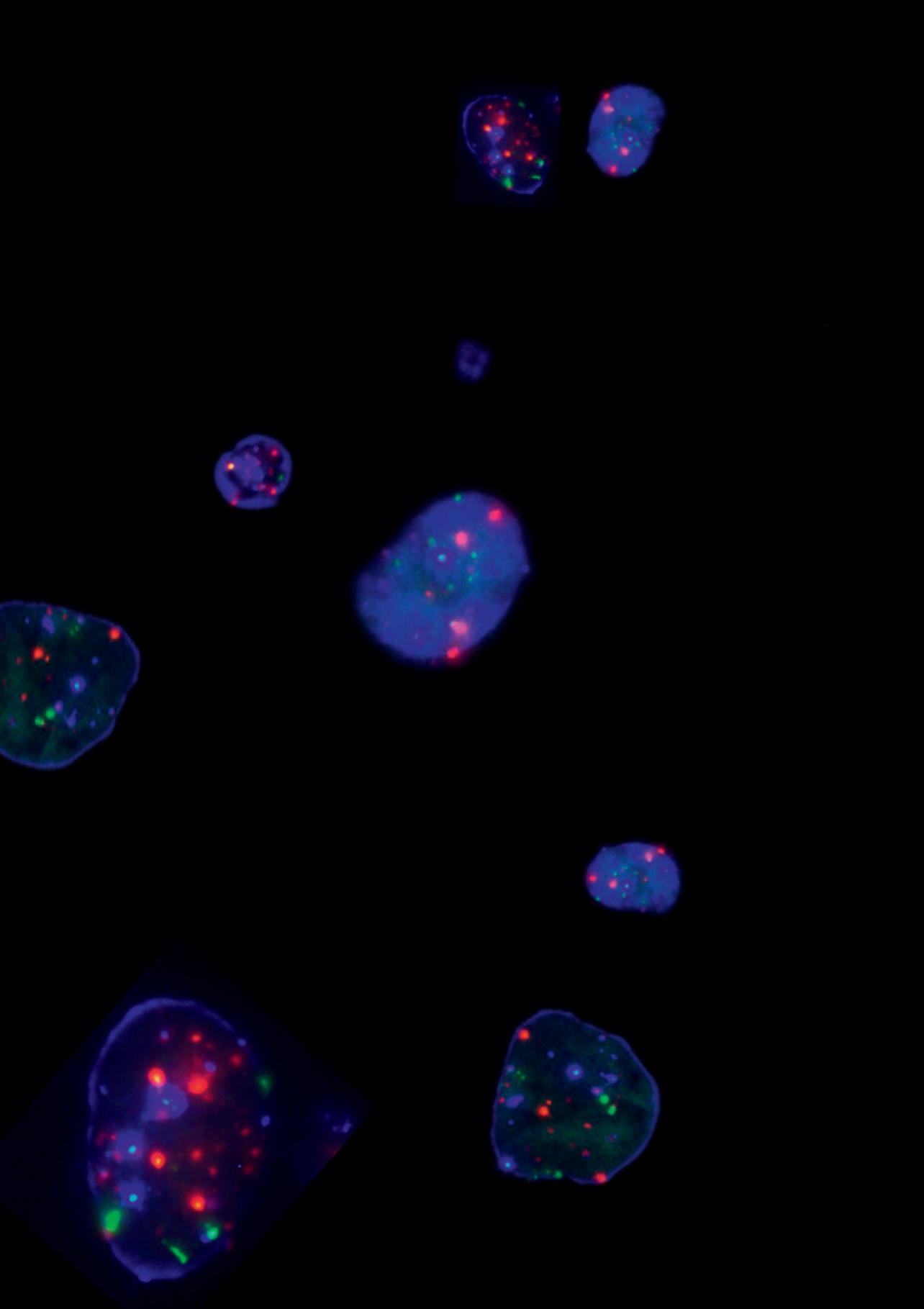

CHAPTER 7

The role of fluorescence in situ hybridization for predicting recurrence after adjuvant Bacillus Calmette-Guérin in patients with intermediate- and high-risk non-muscle invasive bladder cancer: a systematic review and meta-analysis of individual patient data

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ABSTRACT

Purpose: The objective of this study was to assess the value of fluorescence in situ hybridization (FISH) to predict early recurrence in patients with intermediate-, and high-risk non-muscle invasive bladder cancer treated with Bacillus Calmette-Guérin (BCG).

Materials and methods: We performed a systematic review was conducted using MEDLINE®, Embase® and the Cochrane library. Individual patient data (IPD) from prospective observational studies evaluating FISH in patients treated with BCG were included. A two-stage IPD meta-analysis was done to assess the value of FISH to predict tumour recurrence after BCG induction therapy.

Results: From a total of four studies we obtained IPD on 422 patients, of whom 408 with a median follow-up of 18.8 months were included in the final analysis. When FISH was positive, the hazard ratio (HR) for recurrence was 1.20 (95% CI: 0.81–1.79) pre-BCG (t_0), 2.23 (95% CI: 1.31–3.62) at 6 weeks at the end of BCG induction (t_1), 3.70 (95% CI: 2.34–5.83) at 3 months after transurethral resection of the bladder tumour (TURB) (t_2) and 23.44 (95% CI: 5.26–104.49) at 6 months after TURB (t_3).

Conclusion: A positive FISH test post-BCG correlated with higher risk of recurrent tumour. FISH could aid urologists in risk stratification and counselling patients. Based on the HR and the narrowest CI, the preferred timing of FISH is 3 months after transurethral resection of the bladder tumour.

INTRODUCTION

Intravesical Bacillus Calmette-Guérin (BCG) is recommended by international guidelines as treatment of intermediate-, and high-risk non-muscle invasive bladder cancer (NMIBC) [1–4]. However, recurrence develops in up to 40% of patients despite BCG therapy and they are exposed to the risk of progression as well as to therapy local and systemic side effects [5–9]. Early identification of recurrence could minimise these risks and other treatment options can be considered at an earlier stage.

Currently, follow-up of high-risk tumours is recommended with cystoscopy and urinary cytology. Cytology has high sensitivity for Grade 3 (high-grade) tumours, but low sensitivity in Grade 1 (low-grade) tumours. However, BCG can hamper cytological evaluation and, therefore, it is less reliable after BCG therapy [10,11].

The UroVysion® fluorescence in situ hybridization (FISH) test detects chromosomal aberrations, associated with bladder cancer, and is not influenced by the BCG-induced inflammatory response [12,13]. In patients who received BCG several small studies have described a positive role of FISH to predict recurrence following BCG instillations. To obtain more convincing evidence we performed a systematic review and meta-analysis of individual patient data (IPD) from available studies assessing the prognostic value of FISH after BCG instillations for NMIBC.

MATERIALS AND METHODS

Protocol and registration

We registered this systematic review and IPD meta-analysis in PROSPERO (International Prospective Register Of Systematic Reviews) (registration No. CRD42018077631). It is reported according to the PRISMA-IPD (Preferred Reporting Items for Systematic Reviews and Meta-analysis of Individual Participant Data) statement [14]. In all studies ethical approval was documented in the original publications. This study received Institutional Review Board approval (IRB No. W17 356).

Eligibility criteria and literature search

All prospective observational studies which evaluated FISH for tumour recurrence in patients with NMIBC treated with BCG therapy (induction with or without maintenance) were eligible for study inclusion. We performed a systematic literature search without restrictions using MEDLINE® via PubMed®, Embase® and the Cochrane library, including the CDSR (Cochrane Database of Systematic Reviews) and the CENTRAL (Cochrane Central Register of Controlled Trials). The search strategy was done on September 7, 2017 and updated on September 6, 2018 (Supplemental Table 1). The reference lists of the included studies were examined for additional studies.

Study selection and risk of bias

Two independent investigators (EIMLL and RWMV) screened all identified titles and abstracts. The full text of all candidate studies was retrieved. These studies were reviewed (EIMLL, RWMV) and disagreements about study inclusion were resolved by a third investigator (TMdR). The risk of bias was assessed according to the QUIPS (Quality In Prognosis Studies) tool (EIMLL, RWMV) [15].

Individual patient data collection and data integrity

IPD in all eligible clinical trials were requested for 1) baseline characteristics, including patient demographics and clinicopathological characteristics; 2) FISH tests timing and results; and 3) the clinical outcome, including time to and histopathology of recurrence. Before pooling the data into a single database, we carefully checked the data of all included trials. Any discrepancies were discussed and resolved with the authors.

Outcomes and effect measures

The value of FISH at different time points was evaluated to predict tumour recurrence in patients treated with BCG. Recurrence was defined as a histologically proven bladder tumour. The predictive value for progression to muscle invasive disease (stage ≥T2) was a secondary outcome. FISH tests were considered positive according to the definition in the individual studies. When assessing FISH tests, some investigators considered tetraploid cells to be normal and some considered them to be aberrant. In this study, tetraploid cells were considered aberrant cells. If the original study reported a negative FISH test despite the presence of tetraploid cells, the FISH test was considered positive in the current study when the definition of a positive FISH test was met due to the tetraploid cells.

Synthesis methods

IPD at baseline and during follow-up were collected from all participating studies. Patients lacking all cystoscopic follow-up data or missing all FISH evaluations were considered incomplete and were excluded from the analyses. Patient-, and disease-specific characteristics were explored across studies using descriptive statistics. Follow-up was considered time since the initial transurethral resection of the bladder tumour (TURB) to the date of histologically proven recurrence or last follow-up.

Fixed-effect two-stage IPD meta-analysis forest plots were calculated. Heterogeneity across studies was assessed by the Cochrane Q chi-squared test and Higgins I^2. We assumed no clinical heterogeneity among studies in population, intervention or outcome. Therefore, we performed fixed effect meta-analyses.

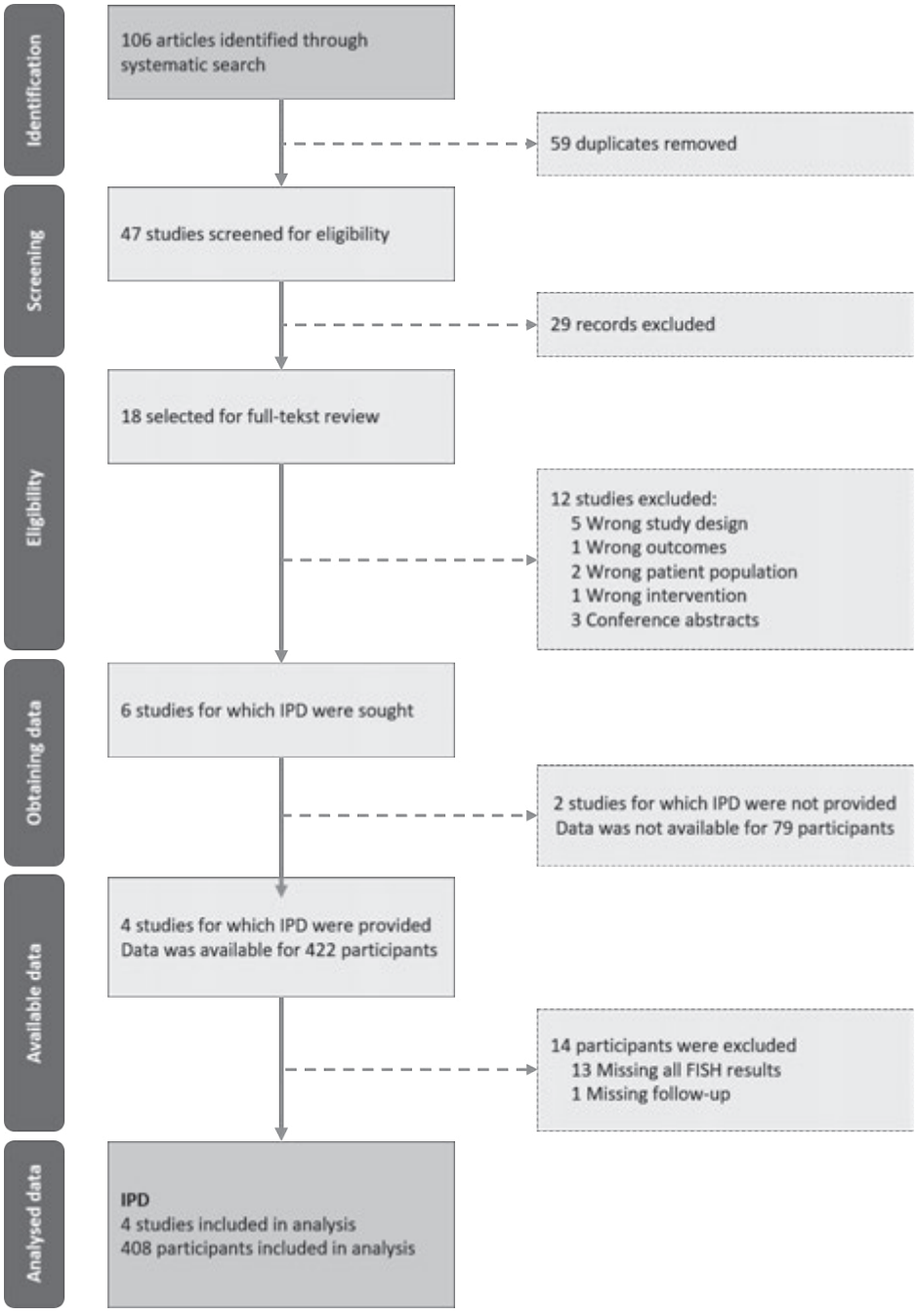


Figure 1. PRISMA flow diagram of the systematic search conducted on September 6, 2018.

Table 1. Risk of bias according to the Quality In Prognosis Studies (QUIPS) tool.

	Mengual et al. [17]	**Savic et al.** [19]	**Kamat et al.** [20]	**Liem et al.** [21]
1. Study participation				
1) Adequate participation in the study by eligible persons	Yes	Partial	Yes	Partial
2) Description of the source population or population of interest	Partial	Yes	Yes	Yes
3) Description of the baseline study sample	Yes	Yes	Yes	Yes
4) Adequate description of the sampling frame and recruitment	Yes	Yes	Yes	Yes
5) Adequate description of the period and place of recruitment	Yes	Yes	Yes	Yes
6) Adequate description of inclusion and exclusion criteria	Partial	No	Yes	Yes
The study sample adequately represents the population of interest	*Low risk of bias*	*Moderate risk of bias*	*Low risk of bias*	*Low risk of bias*
2. Study attrition				
1) Adequate response rate for study participants	Yes	Unsure	Yes	Unsure
2) Description of attempts to collect	No	No	No	Partial
3) Reasons for loss to follow-up are provided	No	No	No	Partial
4) Adequate description of participants lost to follow-up	No	No	No	Partial
5) There are no important differences between participants who completed the study and those who did not	No	No	No	Partial
The study data available adequately represent the study	*High risk of bias*	*High risk of bias*	*High risk of bias*	*High risk of bias*
3. Prognostic factor measurement				
1) A clear definition or description of the prognostic factor is provided	Yes	Yes	Yes	Yes
2) Method of prognostic factor measurement is adequately valid and reliable	Yes	Yes	Yes	Yes
3) Continuous variables are reported or appropriate or appropriate cut points are used	Yes	Yes	Yes	Yes
4) A clear definition of the outcome is provided	Yes	Yes	Yes	Yes
5) Method of outcome measurement used is adequately valid and reliable	Yes	Yes	Yes	Yes
6) Appropriate methods of imputation are used for missing prognostic factor data	Yes	Yes	Yes	Yes
The prognostic factor is measured in a similar way for all participants	*Low risk of bias*	*Low risk of bias*	*Low risk of bias*	*Low risk of bias*

Table 1. *Continued.*

	Mengual et al. [17]	**Savic et al.** [19]	**Kamat et al.** [20]	**Liem et al.** [21]
4. Outcome measurement				
1) A clear definition of the outcome is provided	Yes	Yes	Yes	Yes
2) Method of outcome measurement used is adequately valid and reliable	Yes	Yes	Yes	Yes
3) The method and setting of outcome measurement is the same for all study participants	Yes	Yes	Yes	Yes
The outcome of interest is measured in a similar way for all participants	*Low risk of bias*	*Low risk of bias*	*Low risk of bias*	*Low risk of bias*
5. Study confounding				
1) All important confounders are measured	No	No	Partial	Yes
2) Clear definitions of the important confounders measured are provided	No	No	Partial	Yes
3) Measurement of all important confounders is adequately valid and reliable	No	No	Partial	Yes
4) The method and setting of confounding measurement are the same for all study participants	No	No	Yes	Partial
5) Appropriate methods are used if imputation is used for missing confounder data	NA	NA	NA	NA
6) Important potential confounders are accounted for in the study design	No	No	Yes	No
7) Important potential confounders are accounted for in the analysis	No	No	Partial	Yes
Important potential confounding factors are appropriately accounted for	*High risk of bias*	*High risk of bias*	*Moderate risk of bias*	*Low risk of bias*
6. Statistical analysis and reporting				
1) Sufficient presentation of data to assess the adequacy of the analytic strategy	Yes	Yes	Yes	Yes
2) Strategy for model building is appropriate and is based on a conceptual framework or model	Yes	Yes	Yes	Yes
3) The selected statistical model is adequate for the design of the study	Yes	Yes	Yes	Yes
4) There is no selective reporting of results	Yes	Yes	Yes	Yes
The statistical analysis is appropriate, and all primary outcomes were reported	*Low risk of bias*	*Low risk of bias*	*Low risk of bias*	*Low risk of bias*

NA = Not applicable

The hazard ratios (HR) and 95% confidence intervals (CI) were calculated with Cox regression analysis. Positive predictive value (PPV) and negative predictive value (NPV) were calculated using 2x2 tables. For time to event outcomes, including time to recurrence and time to disease progression, the starting point was the date of the initial TURB. The two time to event outcomes were estimated by Kaplan-Meier analysis with recurrence or disease progression as the event. Patients who died of another cause prior to recurrence or progression were censored. Time to event distributions were compared using the log-rank test. For the meta-analysis forest plots and Kaplan-Meier analysis a landmark analysis was also performed which excluded patients with recurrence at or before the landmark. The different time points when FISH was done were considered the landmarks. Exploratory subgroup analyses were based on a-priori defined subgroups. All tests were two-sided with $p<0.05$ considered significant. All analyses were done with Stata/MP™, version 15.1.

RESULTS

Study selection and individual patient data availability

The systematic search identified 6 eligible studies (Figure 1) [16–21]. For 2 studies, the principal investigators no longer had access to IPD [16,18]. IPD were finally available for 4 cohort studies, resulting in a total of 422 patients [17,19–21].

Risk of bias in studies

In general risk of bias of all 4 studies was comparable, with low risks of bias except for study attrition and confounding. Table 1 lists the risk of bias assessment.

Study and patient characteristics

Table 2 summarises the main characteristics of the included studies. A total of 14 patients were excluded from all analyses, including 1 missing follow-up data and 13 missing all FISH results, resulting in a total of 408 patients included in the final analysis. Table 3 summarises baseline and tumour characteristics. Median follow-up was 18.8 months (interquartile range [IQR] 10.2–28.0 months).

Pooled analyses regarding recurrence

Recurrence developed during follow-up in 141 of the 408 patients (34.6%). Median time from initial TURB to recurrence was 9 months (IQR 5–16 months). In 5 patients recurrent tumour was reported in the original study, but based only on high-grade (Grade 3) cytology. In the current analysis these 5 patients were not scored as having a histologically proven tumour recurrence. Two studies considered tetraploid cells as normal cells and 2 considered tetraploid cells as aberrant cells. A total of 19 negative FISH tests reported in the original studies revealed tetraploid cells. For this analysis the tetraploid cells were considered aberrant, and the FISH test was considered positive when the definition of a positive FISH test was met (Table 2). Subsequently, 13 of these 19 FISH tests met the definition of a positive FISH test and were considered positive.

Table 2. Characteristics of the included study.

Study	Setting	N	Dates	FISH	Definition positive FISH	Definition recurrence
Mengual et al. [17]	1 Spanish centre	65	Sept 2003 – Oct 2004	- Pre-BCG - Post-BCG, 3m	100 cells scored, and one of the following criteria: • ≥5 cells aneuploidy of 2 or more chromosomes (chr. 3, 7, 17) • ≥20 cells with a total loss of 9p21	Histological proven bladder cancer
Savic et al. [19]	7 Swiss centres	68	Feb 2003 – Feb 2006	- Pre-BCG - Post-BCG, 3m	25 cells scored, and one of the following criteria: • ≥4 cells aneuploidy of 2 or more chromosomes (chr. 3, 7, 17) • ≥12 cells with a total loss of 9p21	G3 cytology or histological proven bladder cancer
Kamat et al. [20]	1 American centre	126	June 2005 – Feb 2012	- Pre-BCG - Post-BCG, 6w - Post-BCG, 3m - Post-BCG, 6m	25 cells scored, and one of the following criteria: • ≥4 cells aneuploidy of 2 or more chromosomes (chr. 3, 7, 17) • ≥12 cells with a total loss of 9p21	Histological proven bladder cancer
Liem et al. [21]	5 Dutch centres	114	Dec 2007 – Jan 2013	- Pre-BCG - Post-BCG, 6w - Post-BCG, 3m	25 cells scored, and one of the following criteria: • ≥4 cells aneuploidy of 2 or more chromosomes (chr. 3, 7, 17) • ≥12 cells with a total loss of 9p21	Histological proven bladder cancer

FISH = Fluorescence in situ hybridization, BCG = Bacillus Calmette-Guérin

Table 3. Baseline patient and tumour characteristics.

	Total N=408		**Mengual et al.** [17] N=65		**Savic et al.** [19] N=68		**Kamat et al.** [20] N=142		**Liem et al.** [21] N=133	
Age (years), med [IQR]	70	[62–77]	72	[64–78]	73	[63.5–79.5]	67	[58–74]	71	[64–78]
Gender, *n (%)*										
Male	32	(79.4)	57	(87.7)	60	(88.2)	107	(75.4)	100	(75.2)
Female	84	(20.6)	8	(12.3)	8	(11.8)	35	(24.6)	33	(24.8)
Follow-up (months), med [IQR]	18.8	[10.2–28.0]	14.1	[10.9–18.0]	17.9	[12.6–23.1]	26.4	[8.7–53.0]	23.3	[7.1–26.8]
History of bladder cancer, *n (%)*										
No	194	(47.5)	40	(61.5)	35	(51.5)	24	(16.9)	95	(71.4)
Yes	210	(51.5)	25	(38.5)	30	(44.1)	118	(83.1)	37	(27.8)
Missing	4	(1.0)	0		3	(4.4)	0		1	(0.8)
Prior BCG therapy, *n (%)*										
No	321	(78.7)	58	(89.2)	13	(19.1)	130	(91.6)	120	(90.2)
Yes	77	(18.9)	7	(10.8)	51	(75.0)	12	(8.4)	7	(5.3)
Missing	10	(2.4)	0		4	(5.9)	0		6	(4.5)
Stage, *n (%)*										
CIS only	72	(17.6)	11	(16.9)	31	(45.6)	7	(4.9)	23	(17.3)
Ta	159	(39.0)	21	(32.3)	21	(30.9)	67	(47.2)	50	(37.6)
T1	166	(40.7)	22	(33.8)	16	(23.5)	68	(47.9)	60	(45.1)
Missing	11	(2.7)	11	(17.0)	0		0		0	
Grade, *n (%)*										
CIS only	72	(17.7)	11	(16.9)	31	(45.6)	7	(4.9)	23	(17.3)
G1	12	(2.9)	4	(6.2)	0		2	(1.4)	6	(4.5)
G2	75	(18.4)	16	(24.6)	10	(14.7)	33	(23.3)	16	(12.0)
G3	247	(60.5)	32	(49.2)	27	(39.7)	100	(70.4)	88	(66.2)
Missing	2	(0.5)	2	(3.1)	0		0		0	
CIS, *n (%)*										
No	236	(57.8)	43	(66.2)	26	(38.2)	76	(53.5)	91	(68.4)
Yes	161	(39.5)	11	(16.9)	42	(61.8)	66	(46.5)	42	(31.6)
Missing	11	(2.7)	11	(16.9)	0		0		0	
Risk group, *n (%)*										
Low-/Intermediate-risk	53	(13.0)	10	(15.4)	8	(11.8)	23	(16.2)	12	(9.0)
High-risk	344	(84.3)	44	(67.7)	60	(88.2)	119	(83.8)	121	(91.0)
Missing	11	(2.7)	11	(16.9)	0		0		0	

BCG = Bacillus Calmette-Guérin, CIS = Carcinoma in situ

Table 4. Overview of number of recurrences and available FISH evaluations at different time points and their FISH result.

	Pre-BCG (t_0)		Post-BCG, 6 weeks (t_1)		Post-BCG, 3 months (t_2)		Post-BCG, 6 months (t_3)	
	FISH neg	*FISH pos*	*FISH neg*	*FISH pos*	*FISH neg*	*FISH pos*	*FISH neg*	*FISH pos*
Mengual et al. [17]	5/10	19/55	0	0	9/36	15/29	0	0
Savic et al. [19]	4/20	14/30	0	0	10/48	12/20	0	0
Kamat et al. [20]	9/39	39/95	16/72	33/64	9/54	31/49	4/49	15/22
Liem et al. [21]	25/73	18/52	28/96	7/17	7/48	10/19	0	0
Total	43/142	90/232	44/168	40/81	35/186	68/117	4/49	15/22

BCG = Bacillus Calmette-Guérin, FISH = Fluorescence in situ hybridization

Table 5. Overview of FISH results and occurred conversions.

	Patients	Recurrence
No conversion FISH	127	28
All negative	99	57
All positive		
Conversion FISH negative to FISH positive	34	19
t_0 to t_1	12	8
t_0 to t_2	9	6
t_1 to t_2	12	5
t_2 to t_3	1	0
Conversion FISH positive to FISH negative	126	29
t_0 to t_1	58	14
t_0 to t_2	49	14
t_1 to t_2	14	1
t_2 to t_3	5	0
Alternating FISH	22	8
Alternating FISH negative to positive to negative		
negative | positive | negative | missing	1	0
negative | positive | negative | negative	2	0
missing | negative | positive | negative	1	0
negative | negative | positive | negative	2	0
Alternating FISH positive to negative to positive		
positive | negative | positive | missing	5	4
positive | negative | positive | positive	3	1
positive | positive | negative | positive	2	1
Alternating FISH positive to negative to positive to negative	6	2

FISH = Fluorescence in situ hybridization

FISH results were collected at four time points, including t_0: pre-BCG; t_1: at the end of BCG induction at 6 weeks; t_2: at 3 months after initial TURB; t_3: at 6 months after initial TURB). However, not all studies provided data at each of these time points (Supplemental Figure 1). Tables 4 and 5 show FISH results and conversions. Bladder recurrences were evaluated by cystoscopy followed by histological confirmation.

Predictive value of FISH for recurrence

The predictive value of FISH was determined at the time points t_0, t_1, t_2 and t_3. Landmark analyses were performed at t_1, t_2 and t_3.

At t_0 FISH results were available for 374 patients. Recurrence developed in 133 patients (35.6%), of whom 43 (30.3%) had FISH negative and 90 (38.8%) had FISH positive findings. A positive FISH result at t_0 was not associated with a higher risk of recurrence (HR 1.20, 95% CI: 0.81–1.79) (Figure 2A). Fixed-effect meta-analysis showed no heterogeneity (I^2=28.0%, p=0.244). PPV was 67.7% and NPV was 69.7%.

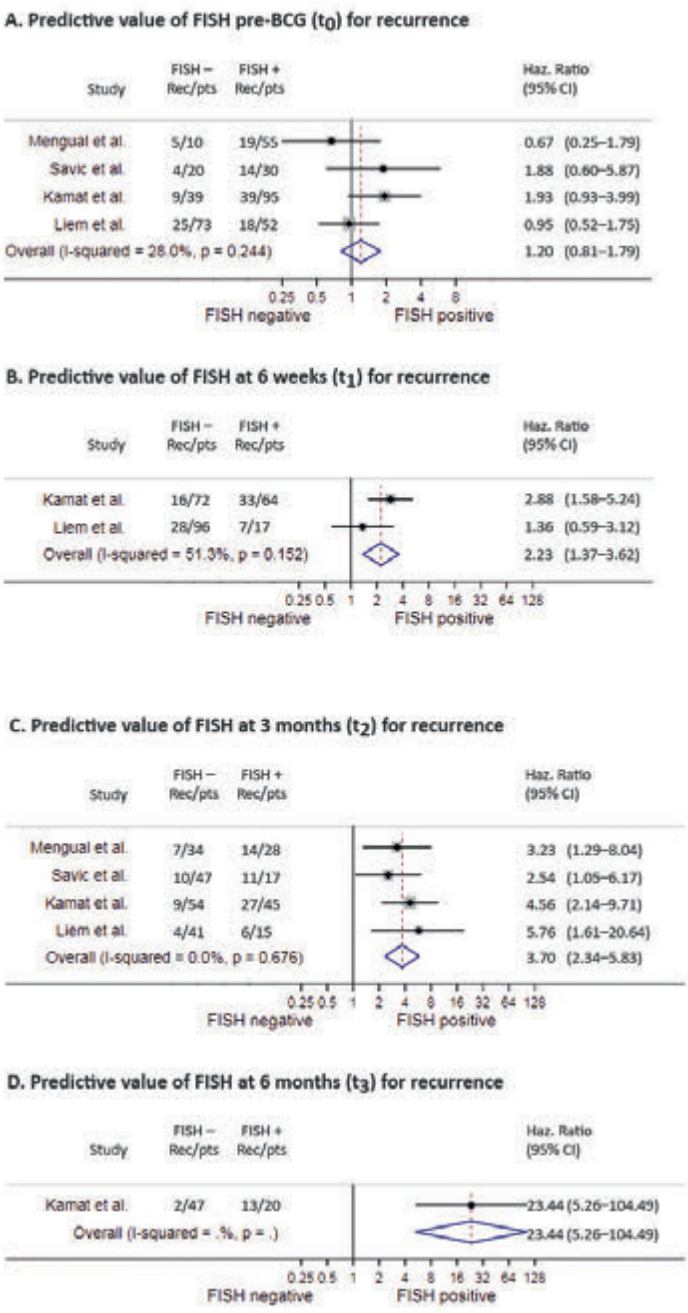


Figure 2. Pooled analysis for the predictive value of FISH for recurrence at different time moments. A) Fixed-effect meta-analysis for the predictive value of positive FISH pre-BCG (t_0). B) Fixed-effect meta-analysis, at landmark 6 weeks, for the predictive value of positive FISH at 6 weeks (t_1). C) Fixed-effect meta-analysis, at landmark three months, for the predictive value of positive FISH at 3 months (t_2). D) Fixed-effect meta-analysis, at landmark 6 months, for the predictive value of positive FISH at 6 months (t_3).

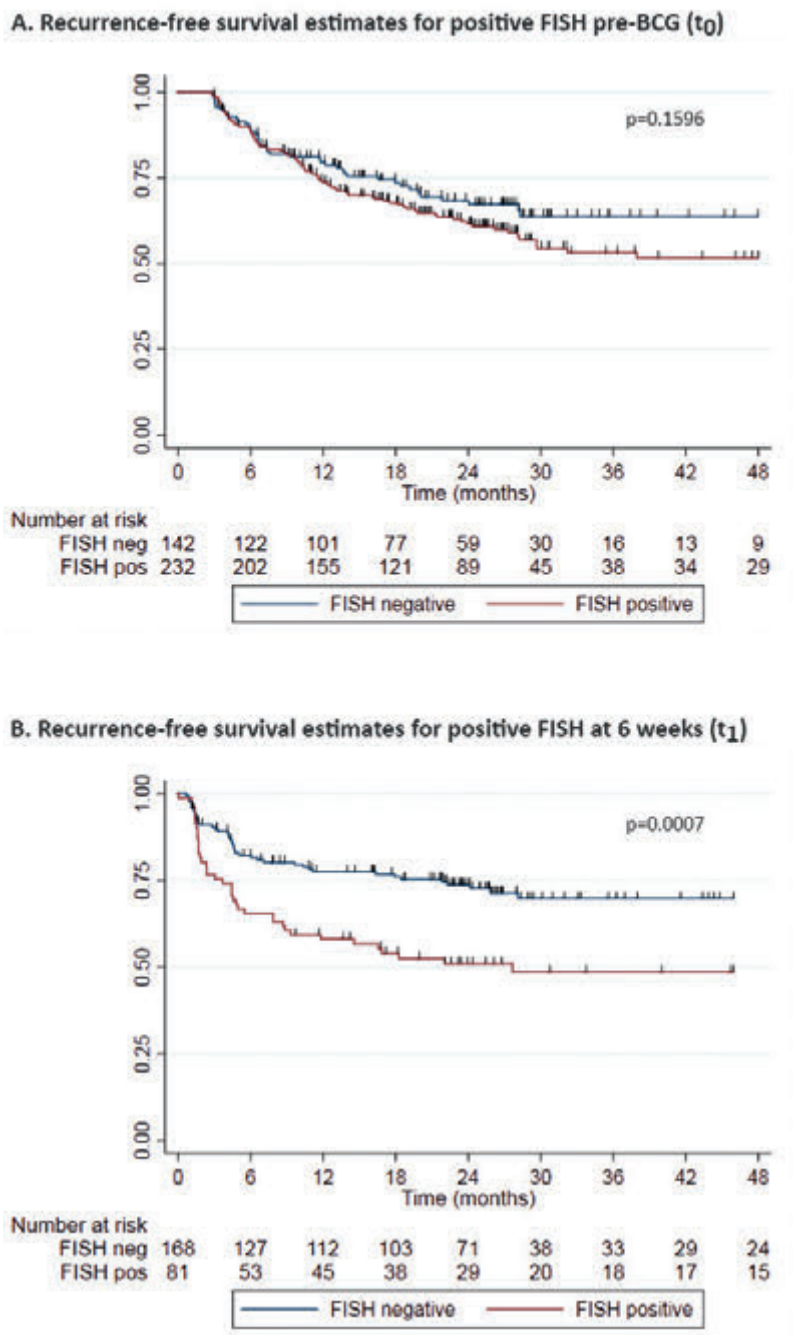
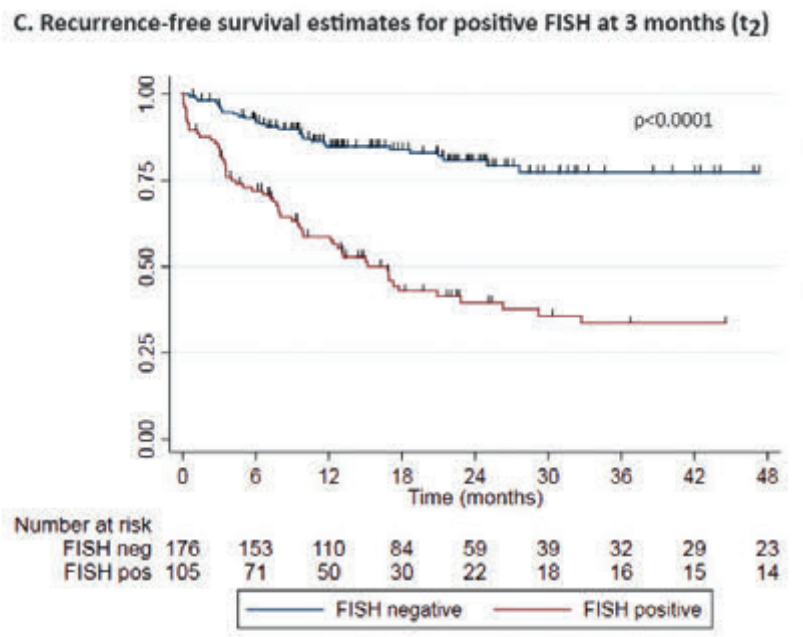


Figure 3. Kaplan-Meier curves for recurrence-free survival at different time points. A) Recurrence-free survival estimates for positive FISH pre-BCG (t_0). B) Recurrence-free survival estimates, at landmark 6 weeks, for positive FISH at 6 weeks (t_1). C) Recurrence-free survival estimates, at landmark 3 months, for positive FISH at 3 months (t_2). D) Recurrence-free survival estimates, at landmark 6 months, for positive FISH at 6 months (t_3).



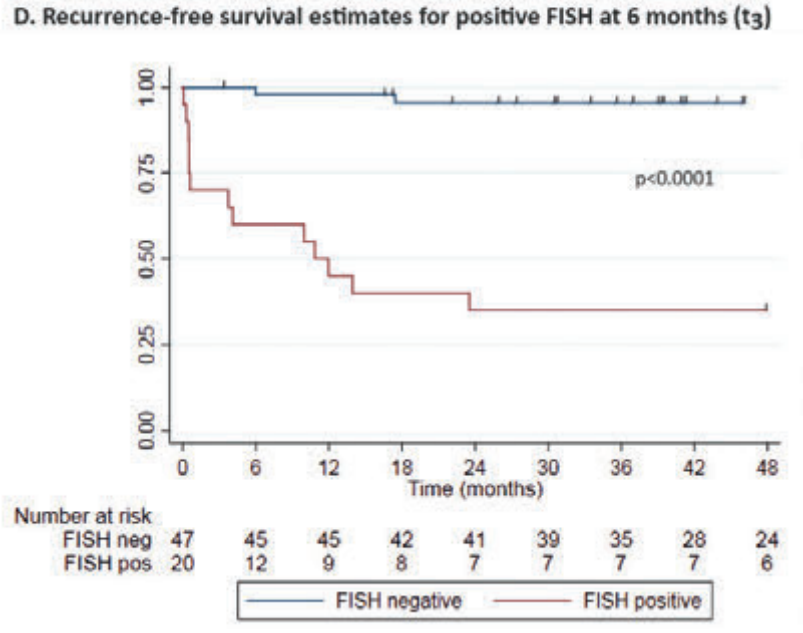


Figure 3. *Continued.*

A total of 249 FISH evaluations were available at t_1. In 84 patients (33.7%) tumour recurred during follow-up, which was FISH negative in 44 (26.2%) and FISH positive in 40 patients (49.4%). A positive FISH finding at t_1 was associated with a higher risk for recurrence (HR 2.23, 95% CI: 1.37–3.62) (Figure 2B). Meta-analysis revealed moderate heterogeneity (I^2=51.3%, p=0.152). PPV and NPV were 47.6% and 73.8%, respectively.

At total of 303 FISH evaluations from all studies were available at t_2. In 103 patients (34.0%) recurrence developed during further follow-up, which was FISH negative in 35 (18.8%) and FISH positive in 68 (58.1%). A positive FISH result at t_2 was associated with a higher risk for recurrence (HR 3.70, 95% CI: 2.34–5.83) (Figure 2C). Meta-analysis revealed no heterogeneity (I^2=0.0%, p=0.676). PPV and NPV were 66.0% and 81.2%, respectively.

A total of 71 patients with FISH evaluations t_3 were available in one trial and in 19 patients (26.8%) recurrence developed during further follow-up, which was FISH negative in 4 (8.2%) and FISH positive in 15 patients (68.2%). A positive FISH result at t_3 was associated with a higher risk of recurrence (HR 23.44, 95% CI: 5.26–104.49) (Figure 2D). However, this should be interpreted with caution given the wide 95% CI. PPV was 78.9% and NPV was 91.8%.

Recurrence-free survival analysis

Figure 3 shows the Kaplan-Meier curves of the different time points. Landmark analyses were performed at t_1, t_2 and t_3. The log-rank test did not demonstrate an association between a positive pre-BCG FISH test (t_0) and tumour recurrence (p=0.160). However, a positive FISH test after BCG induction therapy (t_1, t_2 or t_3) was associated with a higher risk of tumour recurrence (all p<0.005).

Predictive value of FISH for progression to muscle invasive disease (T ≥2)

Disease progression to muscle-invasive bladder cancer occurred in 17 patients (4.2%). Results were available in 374 patients at t_0, of whom 15 patients (4.0%) showed progression, which was FISH negative in 3 and FISH positive in 12 patients. Supplemental figure 2 shows forest plots at different time points. Due to insufficient numbers, no reliable conclusions could be drawn from this.

Subgroup analyses

Subgroup analyses were performed at t_0 and t_2. At these time points FISH tests were done in all studies. The predefined subgroups were patient age, gender, recurrent versus primary disease, presence of carcinoma in situ (CIS), prior BCG treatment, and BCG maintenance therapy versus BCG induction only. Patients with a positive FISH result at t_0 without CIS were at statistically significant higher risk for recurrence than patients with CIS and a positive FISH test (p=0.043, HR 1.32, 95% CI: 0.91–1.91). No significant differences were found in the other subgroups at t_0 or t_2 (Figure 4).

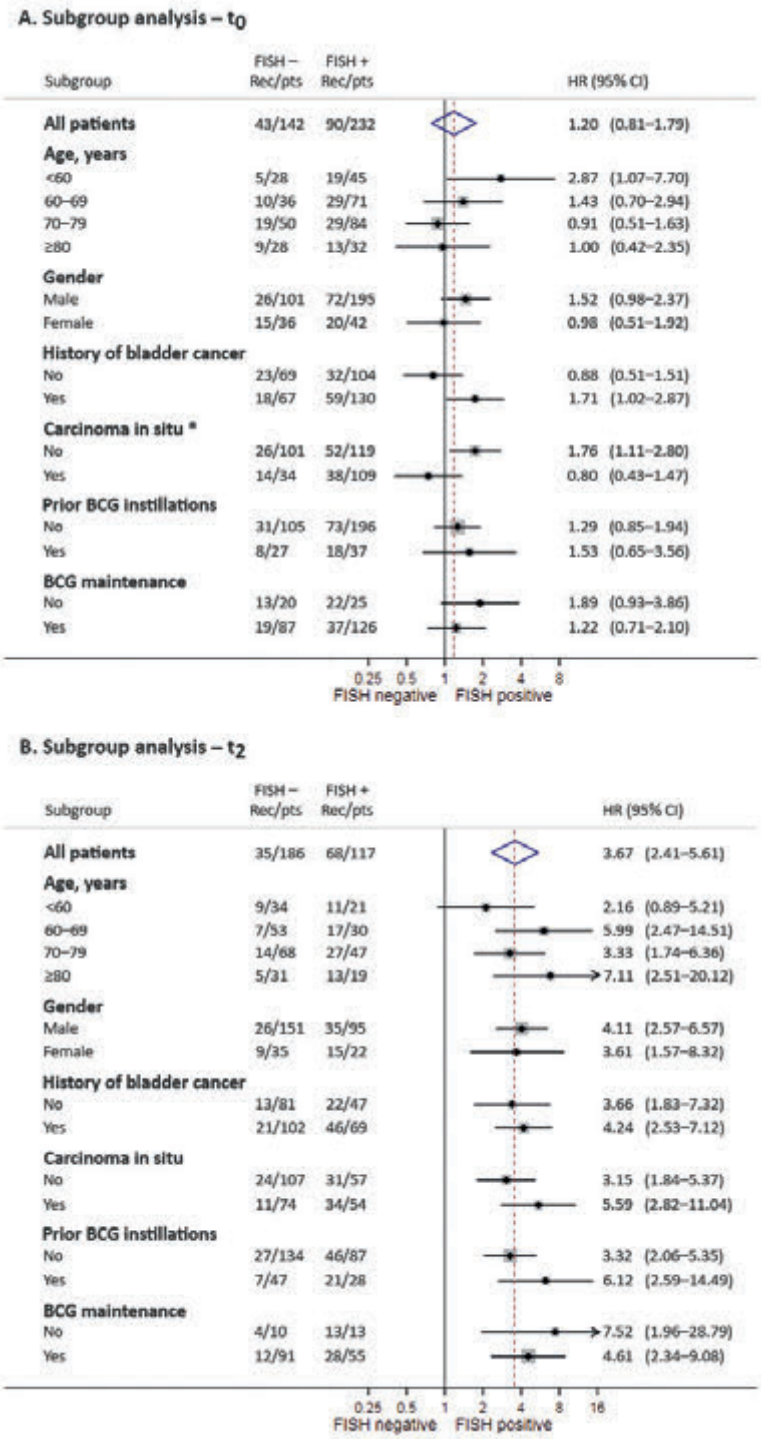


Figure 4. Subgroup analysis for A) t_0 – pre-BCG and B) t_2 – 3 months following initial TURB

DISCUSSION

To our knowledge this IPD meta-analysis is the largest study evaluating FISH for predicting tumour recurrence. It confirms that patients with a positive FISH test following BCG induction therapy are at higher risk for tumour recurrence during follow-up.

The pooled analysis at t_2 showed a HR of 3.70, which confirmed the conclusion of all 4 included studies. Two included studies evaluating the predictive value of FISH at t_1, i.e. the end of BCG induction therapy, described inconsistent results, possibly due to low number of patients [20,21].

In the current pooled analysis, a positive FISH test at 6 weeks was associated with a higher risk of recurrence during follow-up (HR 2.23). One included study [20] as well as one study for which IPD could not be obtained [16] reported that FISH had predictive value for recurrence when performed before BCG therapy began (t_0). However, this effect was not seen in the pooled analysis. In a recent study Lotan et al. [22] reported similar results at t_1 and t_2, but also a positive association at t_0, in contrast to the current pooled analysis. The HR of 23.44 at t_3 seems high and promising, but the wide 95% CI (5.26–104.49) makes this result unreliable. The FISH results to predict progression to muscle invasive disease should be interpreted with caution because of the limited number of events.

Subgroup analyses revealed a significant difference in the presence of CIS at t_0. In patients without CIS, positive FISH findings pre-BCG were associated with a higher risk of recurrence. In this patient group positive FISH following TURB might suggest residual tumour. However, at t_2 this difference was no longer seen. Positive FISH at t_2 may be related to an insufficient response to BCG.

After BCG induction therapy the overall false positive rate of FISH of 41% seems high (Table 4). However, it is possible that the median follow-up of 1.5 years was too short to identify all future recurrences. Most recurrences develop within 5 years of initial BCG induction therapy, although recurrence after 10 years is not unusual [23,24].

Kamat et al. [25] developed a CyPRIT (Cytokine Panel for Response to Intravesical Therapy) nomogram to predict the BCG response based on changes in levels of a combination of nine cytokines in urine samples. The FISH test and CyPRIT-nomogram identify a new group of patients with molecular or cytokine failure, but without a clinical tumour present yet. This may assist in risk stratification. It also introduces the opportunity to offer these patients other subsequent treatment strategies at an earlier stage which would hopefully result in a better outcome. Treating all patients who have a positive FISH test with radical cystectomy may be too rigorous, but discussing clinical trials or changing BCG maintenance therapy e.g. to chemohyperthermia, could be a viable option [26,27].

When FISH is performed in patients treated with BCG, we recommend performing FISH at 3 months, since this has the highest HR with the narrowest 95% CI.

A limitation of this IPD meta-analysis is that for two studies no IPD could be obtained on a total of 79 patients treated with BCG, mitomycin C or thiotepa. Also, the IPD of Lotan et al. [22] could not be included in the current study since that report was so recent. However, all studies revealed a higher risk of recurrent tumour when FISH was positive after BCG induction therapy. Another limitation of this analysis is that there was a slightly different definition of a positive FISH test in one study (Table 2). Although it is not likely that this would have changed the results of the current analysis, the more compliant FISH criteria could have led to overestimating positive FISH tests. There was no uniform BCG maintenance protocol across the studies, which could also have influenced the risk of recurrence [28]. Furthermore, the impact of concurrent cytology findings could not be evaluated since systematic cytology analyses were not available across the four trials. Considering the risk of bias across studies and statistical heterogeneity, our results should be interpreted with caution.

CONCLUSION

Patients with NMIBC and positive FISH findings after BCG induction are at higher risk for tumour recurrence. When FISH is performed 3 months after initial TURB, the predictive value of FISH is higher than that of FISH done immediately at the end of the induction course. FISH lacks predictive value for predicting recurrence pre-BCG. FISH could assist urologists in risk stratification and counselling patients prone to recurrence after BCG therapy, preferably within clinical trials.

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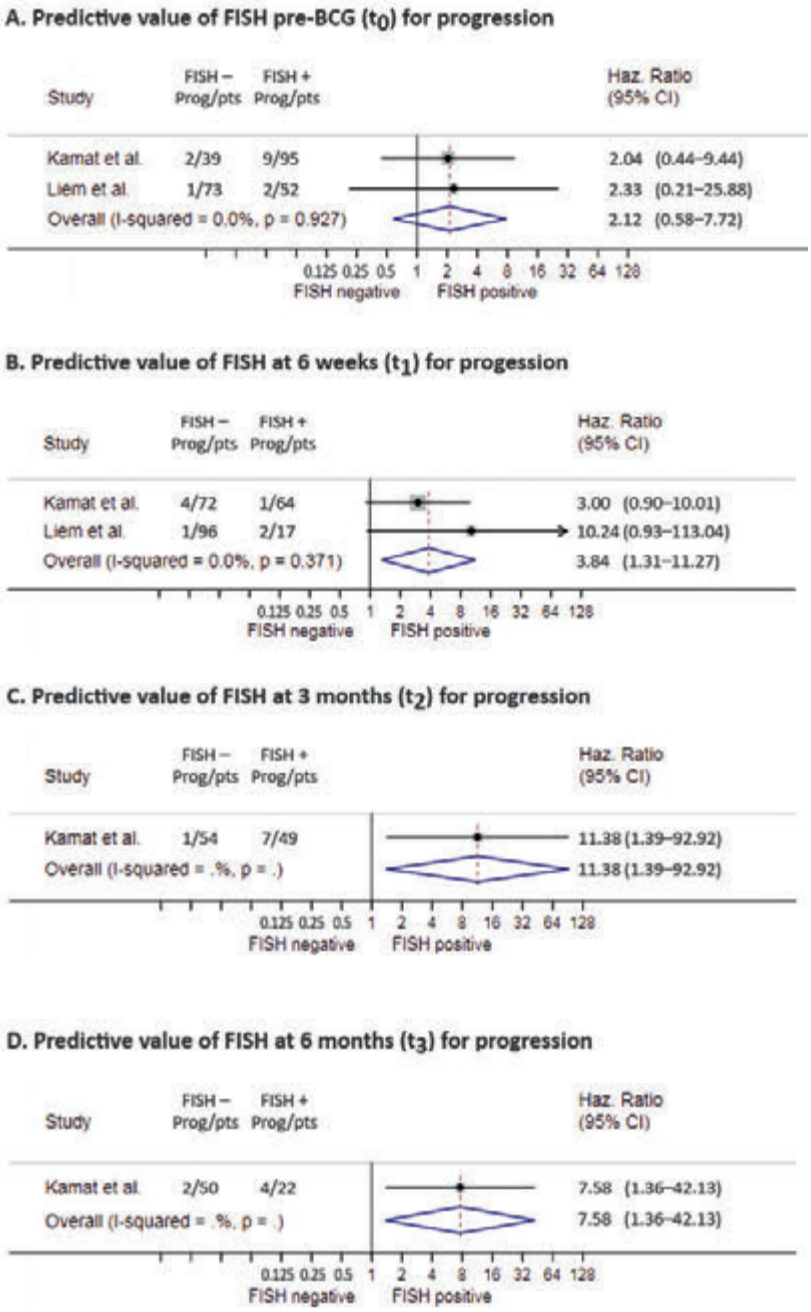
Supplemental Table 1. Search strategy conducted on September 7, 2017, updated on September 6, 2018.

MEDLINE (accessed via OVID Medline)	
#1	Urinary Bladder Neoplasms/
#2	(Bladder and (neoplas* or cancer or carcin* or tumour* or tumor* or metasta* or malig*)).ti,ab.
#3	Urinary Bladder/ AND (neoplas* OR cancer OR carcin* OR tumour* OR tumor* OR metasta* OR malig*)ti,ab.
#4	1 or 2 or 3
#5	In Situ Hybridization, Fluorescence/
#6	(fluorescence adj1 in adj1 situ adj1 hybridi*).ti,ab.
#7	FISH.ti,ab.
#8	5 or 6 or 7
#9	(bacillus adj1 calmette adj1 (guerin OR gu#rin)).ti,ab.
#10	BCG.ti,ab.
#11	9 or 10
#12	4 AND 8 AND 11
EMBASE (accessed via EMBASE)	
#1	'bladder tumor'/exp
#2	(Bladder and (neoplas* or cancer or carcin* or tumour* or tumor* or metasta* or malig*)):ti,ab
#3	'bladder'/exp AND (neoplas* OR cancer OR carcin* OR tumour* OR tumor* OR metasta* OR malig*):ti,ab
#4	#1 OR #2 OR #3
#5	'fluorescence in situ hybridization'/exp
#6	(fluorescence NEXT/1 in NEXT/1 situ NEXT/1 hybridi*):ti,ab
#7	FISH:ti,ab
#8	#5 OR #6 OR #7
#9	(bacillus NEXT/1 calmette NEXT/1 (guerin OR gu*rin)):ti,ab
#10	BCG:ti,ab
#11	#9 OR #10
#12	#4 AND #8 AND #11
Cochrane library (both CDSR and CENTRAL)	
#1	'bladder tumor'/exp
#2	(Bladder and (neoplas* or cancer or carcin* or tumour* or tumor* or metasta* or malig*))
#3	MeSH descriptor: [Urinary Bladder] explode all trees
#4	(neoplas* OR cancer OR carcin* OR tumour* OR tumor* OR metasta* OR malig*)
#5	#3 AND #4
#6	#1 OR #2 OR #5
#7	MeSH descriptor: [In Situ Hybridization, Fluorescence] explode all trees
#8	(fluorescence NEXT in NEXT situ NEXT hybridi*)
#9	FISH
#10	#7 OR #8 OR #9
#11	(bacillus NEXT calmette NEXT (guerin OR gu*rin))
#12	BCG
#13	#11 OR #12
#14	#6 AND #10 AND #13

Supplemental Figure 1. An overview of the number of patients at different time points



Supplemental Figure 2. Pooled analysis for the predictive value of FISH for progression at the different time points. A) Fixed-effect meta-analysis for the predictive value of positive FISH pre-BCG (t_0). B) Fixed-effect meta-analysis, at landmark 6 weeks, for the predictive value of positive FISH at 6 weeks (t_1). C) Fixed-effect meta-analysis, at landmark 3 months, for the predictive value of positive FISH at 3 months (t_2). D) Fixed-effect meta-analysis, at landmark 6 months, for the predictive value of positive FISH at 6 months (t_3).

PART IV

Discussion

CHAPTER 8

Summary
Samenvatting

ENGLISH SUMMARY

Chapter 1 sets the background for this thesis by describing the incidence, etiology, clinical aspects, diagnostic investigations and therapeutic options of non-muscle invasive urothelial carcinoma of the bladder. The general goal of the thesis is to improve bladder cancer diagnosis and management using optical imaging techniques, by advancing confocal laser endomicroscopy (CLE), a high-resolution imaging technique, for the diagnosis and grading of bladder cancer, and using fluorescence in situ hybridization (FISH) in patients treated with Bacillus Calmette-Guérin (BCG) to identify patients with a higher risk for developing tumour recurrence.

In ***Chapter 2*** the protocol is outlined of the prospective cohort study to describe CLE criteria for urothelial carcinoma. In 60 patients with a suspicious lesion in the bladder, CLE imaging will be performed, prior to tumour resection. By comparing the CLE images with the corresponding histopathology, CLE-features for low-grade urothelial carcinoma, high-grade urothelial carcinoma and carcinoma in situ will be identified. The protocol is reported together with a research protocol for CLE imaging in the upper urinary tract, since the methodology of the protocols are similar.

In ***Chapter 3*** a comprehensive introduction of the principle of CLE imaging is provided, and a detailed description of how to perform the imaging procedure. The procedure is demonstrated for in vivo CLE imaging in the bladder, as well as in the upper urinary tract. For image evaluation examples of normal urothelium, low-grade, and high-grade urothelial carcinoma are displayed and their CLE-features described.

Chapter 4 validates earlier proposed CLE-features. Sixty-six lesions were imaged using CLE before the tumour was resected en-bloc. All CLE images were reviewed by three independent observers, blinded for histopathology, and tumours were classified as low- or high-grade urothelial carcinoma, or benign lesions. The following CLE features were evaluated: papillary configuration, polarity of cells, organisation of cells, cohesiveness of cells, cellular morphology, definition of cell borders, and vasculature. Low-grade lesions presented different characteristics of the CLE-features than high-grade lesions. Classification of low-grade and high-grade urothelium carcinoma based on CLE-images was pretty good (low-grade 76%, high-grade 70%), although this was not the case for benign lesions (29%). Validation of CLE-features to diagnose and grade urothelial carcinoma is an important step for the advancement of CLE imaging for bladder cancer in clinical practice.

In ***Chapter 5*** the CLE-data was analysed using a recurrent neural network, to evaluate if computer aided classification of CLE images could improve the diagnostic process. Automated image analysis was performed after semi-automated frame selection. The accuracy for differentiation between healthy and benign versus malignant urothelium

was 79%. The accuracy for differentiation between low-grade and high-grade papillary urothelium carcinoma was 82%. Computer aided image analysis is a major advantage, since it is less elaborative and time consuming in comparison to subjective image analyses, and additionally it is not inter- or intra-observer dependent.

Chapter 6 reports a prospective multicentre study to evaluate the value of FISH for predicting tumour recurrence in bladder cancer patients treated with BCG. All 114 patients received BCG induction therapy following transurethral resection of the bladder tumour (TURB). FISH was performed at three different time points during treatment; t_0 – before BCG induction therapy (week 0), t_1 – at the end of BCG induction (6 weeks), t_2 – at 3 months following initial TURB. Patients with a positive FISH test at t_0 or t_1 did not have a higher risk for developing a tumour recurrence. However, patients with a positive FISH test at t_2 had a 4.0 – 4.6 times greater risk of developing a recurrence compared to patients with a negative FISH test.

In ***Chapter 7*** a systematic review and meta-analysis based on individual patient data (IPD) is reported, regarding the use of FISH for predicting tumour recurrence in bladder cancer patients treated with BCG. IPD from four studies were obtained. A two-stage IPD meta-analysis based on 408 patients was carried out. FISH was evaluated at four different time points; t_0 – pre-BCG, t_1 – at the end of BCG induction at 6 weeks, t_2 – at 3 months following initial TURB, t_3 – at 6 months following initial TURB. FISH lacks a predictive value for tumour recurrence pre-BCG. Patients with a positive FISH after BCG induction therapy have a higher risk for developing tumour recurrence. The predictive value of FISH is higher at 3 months following TURB, compared to FISH at 6 weeks. FISH could assist urologists in risk stratification and counselling patients who have a higher risk for developing a bladder tumour recurrence.

NEDERLANDSE SAMENVATTING

Hoofdstuk 1 schetst de achtergrond van dit proefschrift, en beschrijft de incidentie, etiologie, klinische aspecten, diagnostische onderzoeken en behandelopties voor het niet-spier invasief urotheel carcinoom van de blaas. Het algemene doel van dit proefschrift is het verbeteren van blaaskankerdiagnostiek en behandeling met behulp van optische beeldvormingstechnieken. Ten eerste door het gebruik van de confocale laser endomicroscopie (CLE), een beeldvormingstechniek met hoge resolutie, te evalueren voor het diagnosticeren en graderen van blaaskanker. Ten tweede door de toepassing van fluorescentie in situ hybridisatie (FISH) te evalueren bij patiënten die behandeld zijn met Bacillus Calmette-Guérin (BCG) voor het identificeren van patiënten die een hoog risico hebben op het ontwikkelen van een tumor recidief.

In ***Hoofdstuk 2*** is het protocol beschreven van een prospectieve cohort studie, met als doel het beschrijven van CLE criteria voor urotheel carcinoom. In 60 patiënten met een verdachte laesie in de blaas wordt CLE beeldvorming verricht, alvorens de tumor gereseceerd wordt. Door het vergelijken van CLE beelden met de corresponderende histopathologie, kunnen CLE-kenmerken voor laaggradig urotheel carcinoom, hooggradig urotheel carcinoom en carcinoma in situ geïdentificeerd worden. Het protocol is gezamenlijk beschreven met een onderzoeksprotocol voor CLE beeldvorming van de hoge urinewegen, aangezien de methodologie van de protocollen vergelijkbaar zijn.

In ***Hoofdstuk 3*** wordt een korte introductie gegeven over het principe van CLE beeldvorming, en een uitgebreide beschrijving over hoe de beeldvorming uitgevoerd moet worden. De procedure wordt getoond voor zowel *in vivo* CLE beeldvorming in de blaas, als ook in de hoge urinewegen. Voor evaluatie van de beelden worden voorbeelden getoond van normaal urotheel, laaggradig, en hooggradig urotheel carcinoom en hun bijbehorende CLE-kenmerken.

Hoofdstuk 4 valideert eerder voorgestelde CLE-kenmerken. Zesenzestig laesies zijn afgebeeld middels CLE, alvorens de tumor *en-bloc* gereseceerd is. Alle CLE beelden zijn door drie onafhankelijke beoordelaars, geblindeerd voor histopathologie, geëvalueerd en geclassificeerd als laag- of hooggradig urotheel carcinoom, of benigne laesie. De volgende CLE-kenmerken werden geëvalueerd: papillaire configuratie, polariteit van cellen, organisatie van cellen, cohesie van cellen, cellulaire morfologie, beschrijving van celranden, en vascularisatie. Laaggradige afwijkingen toonden andere eigenschappen van de CLE-kenmerken in vergelijking tot hooggradige laesies. Classificatie van laaggradig en hooggradig urotheel carcinoom op basis van CLE beelden was goed (laaggradig 76%, hooggradig 70%), echter dit was niet geval voor benigne laesies (29%). De validatie van CLE-kenmerken voor het diagnosticeren en graderen van urotheel carcinoom is een belangrijke stap voor het gebruik van CLE beeldvorming voor blaaskanker in de dagelijkse klinische praktijk.

In ***Hoofdstuk 5*** zijn de CLE-gegevens geanalyseerd door een terugkerend neuraal netwerk, om te beoordelen of een computergestuurde classificatie van CLE beelden het diagnostisch proces kan verbeteren. Een geautomatiseerde beeldanalyse is uitgevoerd na semi-automatische beeld selectie. De accuratesse voor het onderscheiden van gezond of benigne versus maligne urotheel was 79%. De accuratesse voor het onderscheiden van laaggradig en hooggradig papillair urotheel carcinoom was 82%. Computer gestuurde beeldanalyse is een groot voordeel, aangezien het minder bewerkelijk en tijdrovend is in vergelijking met subjectieve beeldanalyse, en is daarnaast niet inter- of intra-observer afhankelijk.

Hoofdstuk 6 beschrijft een prospectieve multicenter studie die de waarde van FISH onderzoekt voor het voorspellen van recidief bij blaaskanker patiënten die behandeld zijn met BCG. Alle 114 patiënten hebben BCG inductie therapie ondergaan, na transurethrale resectie van de blaastumor (TURB). Op drie verschillende tijdsmomenten is FISH afgenomen; t_0 – vòòr BCG inductie therapie (week 0), t_1 – aan het einde van BCG inductie (6 weken), t_2 – 3 maanden na initiële TURB. Patiënten met een positieve FISH test op t_0 of t_1 hadden geen hoger risico op het ontwikkelen van tumor recidief. Daarentegen hadden patiënten met een positieve FISH test op t_2 een 4.0–4.6 maal hoger risico op het ontwikkelen van een tumor recidief in vergelijking tot patiënten met een negatieve FISH test.

In ***Hoofdstuk 7*** is een systematische review en meta-analyse beschreven gebaseerd op individuele patiënten data (IPD), met betrekking tot het gebruik van FISH voor het voorspellen van tumor recidief in blaaskanker patiënten die behandeld zijn met BCG. Een twee-staps IPD meta-analyse is uitgevoerd op basis van 408 patiënten. FISH werd geëvalueerd op 4 verschillende tijdspunten; t_0 – vòòr BCG, t_1 – aan het eind van BCG inductie na 6 weken, t_2 – 3 maanden na initiële TURB, t_3 – 6 maanden na initiële TURB. Vòòr BCG ontbreekt de voorspellende waarde van FISH. Patiënten met een positieve FISH na BCG inductie therapie hebben een hoger risico op het ontwikkelen van een tumor recidief. De voorspellende waarde van FISH is hoger bij 3 maanden, in vergelijking tot FISH bij 6 weken. FISH kan de uroloog bijstaan bij risicostratificatie en begeleiding van patiënten die een hoger risico hebben op het ontwikkelen van een recidief blaastumor.

CHAPTER 9

Concluding remarks and future perspectives

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Confocal laser endomicroscopy

The goal of the first part of this thesis was to evaluate the use of confocal laser endomicroscopy (CLE) for bladder cancer diagnosis. Direct visualisation of bladder mucosa using cystoscopy is the gold standard in bladder cancer diagnosis and follow-up. Histological staging and grading are important parameters determining prognosis and treatment management. Currently however, histologic assessment of bladder tissue is not possible during cystoscopy. CLE has the ability to visualize tissue on a microscopic level in vivo and could therefore overcome this shortcoming during cystoscopy. The results presented in this thesis show promising results for CLE imaging of bladder tissue. It is a safe procedure to obtain in vivo imaging of bladder tissue on a cellular level, identifying CLE features for urothelial carcinoma. Based on these CLE features it is possible to diagnose and grade bladder cancer (Chapter 4). Currently, subjective interpretation of CLE images is time consuming. Also, subjective interpretation may not always be accurate, and the reproducibility of the subjective interpretation still needs to be explored. It would be a great advantage if the number of CLE features necessary to interpret CLE images could be reduced to three features as suggested in Chapter 4. A nomogram that could guide clinicians in grading bladder lesions based on CLE images would make CLE imaging easier to use. The use of CLE will be even more accessible for clinicians if subjective interpretation could be bypassed by real time computer-assisted automated image analysis. In Chapter 5 it is demonstrated that automated image analysis is feasible using a neural network. The automated image analyses were performed offline following a semi-automated image selection. For this technique to be successful in clinical practice, the semi-automated image selection should be fully automated, and the image analysis should be run in real-time, simultaneously with image acquisition. Additionally, the neural network should be trained based on a large, extensive image database for the image analysis to be reliable in clinical practice.

The research presented in this thesis is the beginning for the use of CLE in bladder cancer diagnosis, and further clinical applications for the use of CLE in bladder cancer diagnosis and management should be explored. For example, the use of CLE in the outpatient clinic could differentiate low-grade from high-grade urothelial carcinoma during cystoscopy. The ability of CLE to establish tumour grade without the need to collect tissue for histopathological assessment would allow treating low-grade tumours in the outpatient clinic by e.g. laser vaporisation [1,2]. Also, an 'active surveillance' monitoring, similar like in low-risk prostate cancer, could be considered for demonstrated low-risk urothelial carcinoma [3]. CLE could be used to monitor these tumours to determine if they remain low-grade. Another possible clinical application is the use of CLE for detecting carcinoma in situ (CIS). Detection of CIS is challenging, and often patients undergo random biopsies to detect CIS [4]. If CIS could be better recognised using CLE imaging, CLE could assist in patient selection and performing targeted biopsies, reducing

the number of unnecessary biopsies. CLE could potentially be performed in combination with other forms of enhanced imaging modalities e.g. photodynamic diagnosis (PDD). In general, PDD is performed with intravesical administered 5-ALA or HAL. Fortunately, PDD with oral administered 5-ALA has been demonstrated to be safe and effective [5], which would allow combining CLE with PDD. Using PDD, suspicious areas for urothelial carcinoma will be visualised, which could subsequently be imaged in detail on a cellular level using CLE. CLE could also have an added value during transurethral resection of the bladder tumour (TURB) to verify if the resection bed and margins are free of tumour. This application is currently evaluated in an ongoing study. CLE imaging, therefor, has the potential to provide histologic information in certain situations.

Fluorescence in situ hybridisation for predicting recurrence after BCG therapy

The goal of the second part of this thesis was to improve bladder cancer management for patients treated with Bacillus Calmette-Guérin (BCG). Therefore, we evaluated the use of fluorescence in situ hybridisation (FISH) to identify patients prone for tumour recurrence following adjuvant BCG therapy. The results reported in this thesis suggest that patients who have a positive FISH test 3 months following initial TURB and induction BCG therapy have a higher risk for developing tumour recurrence. By using FISH, it is possible to identify patients that are more likely to develop a recurrence in time at an earlier stage and before clinical signs are present. However, the high false-positive rate should be taken into account when FISH is used to change treatment strategy. Clinicians may discuss with their patients that have a higher risk for recurrence and/or progression whether they want to continue the planned BCG maintenance therapy or opt for a different treatment strategy such as for example radical cystectomy or chemohyperthermia. Radical cystectomy is an oncologically effective treatment, but is very invasive with substantial comorbidity and high impact on quality of life. Recently, chemohyperthermia with mitomycin C is also considered as a possible treatment when BCG therapy fails [6]. FISH could assist urologists in risk stratification and counselling patients that have a higher risk for developing tumour recurrence after BCG therapy.

Tailoring treatment management

In the past decades, clinical guidelines have been increasingly incorporated in clinical practice. These guidelines have led to unification of bladder cancer treatment and recommendation of treatments specific to tumour stage and grade (individualised treatment). An advantage is that these guidelines are often based on extensive scientific research, but the downside is that it generally takes time before new insights or scientific results are incorporated. Guidelines therefore lag behind the latest scientific results. The new insights presented in this thesis may be used as an extension in bladder cancer management and surveillance, besides traditional approaches. When CLE image analysis could be performed in real-time and is fully automated to reliably stage bladder tumours, CLE could potentially lead to better resection of non-muscle invasive tumours and speed up the diagnostic process. As mentioned before, several different applications

are conceivable to further personalise treatment, although these still remain to be explored and demonstrated in larger patient cohorts. In the case of high-risk bladder cancer patients treated with BCG, FISH could identify patients who have a higher risk for developing a recurrence. These patients may diverge from standard treatment and opt for a different treatment strategy at an earlier stage with hopefully a better outcome. FISH could be combined with current nomograms and other risk predictors such as the CyPRIT nomogram, to create a more precise prediction model [7]. Both CLE and FISH may contribute to tailor bladder cancer management. By enabling earlier diagnosis and improved risk assessment, treatment and management of bladder cancer can be individually tailored to optimise the best response and least burden for patients.

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APPENDICES

PhD portfolio
Author contributions
List of publications
About the author
Dankwoord

PHD PORTFOLIO

PhD candidate:	Esmeralda Ina Mei Liën Liem
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Co-supervisors:	Dr. D.M. de Bruijn Dr. J.R. Oddens

Graduate school courses	**Year**	**ECTS**
EndNote	2015	0.1
Searching for a systematic review	2015	0.1
Embase Medline via OVID	2015	0.1
Citation analysis and impact factors	2015	0.1
Oral presentation	2015	0.1
Practical biostatistics	2015	1.1
Basic Course Legislation and Organization for clinical Researchers (BROK)	2015	0.9
The AMC World of Science	2015	0.7
Scientific writing	2015	1.5
Clinical data management	2016	0.2
Clinical epidemiology – Evaluation of medical tests	2016	0.9
Advanced topics in biostatistics	2017	2.1

Seminars, workshops and masterclasses	**Year**	**ECTS**
Urology seminars AMC/VUMC	2015-2018	4.0
Onderzoekschool Oncologie Amsterdam 'Basic microscopy course'	2015	1.6
European School of Urology course 'Practical management of non-muscle invasive bladder cancer'	2016	0.1
European Association of Urology Review	2015-2018	1.0
American Association of Urology Review	2015-2018	1.0
European School of Urology course 'How to write: Results and discussion'	2017	0.1
European School of Urology course 'UTUC: Diagnosis and management'	2017	0.1

Attended (inter)national conferences	**Year**	**ECTS**
8th International symposium on Focal Therapy and Imaging (The Netherlands)	2015	0.25
Najaarsvergadering Nederlandse Vereniging voor Urologie (The Netherlands)	2015	0.25
31st European Association of Urology conference (Germany)	2016	0.25
6th International meeting Challenges in Endourology (France)	2016	0.25
Global congress on Bladder Cancer (Belgium)	2016	0.25
Najaarsvergadering Nederlandse Vereniging voor Urologie (The Netherlands)	2016	0.25
34th World Congress of Endourology (South Africa)	2016	0.25
32nd European Association of Urology conference (United Kingdom)	2017	0.25
Voorjaarsvergadering Nederlandse Vereniging voor Urologie (The Netherlands)	2017	0.25
112th American Urological Association conference (USA)	2017	0.25
7th International meeting Challenges in Endourology (France)	2017	0.25
37th Société Internationale d'Urologie conference (Portugal)	2017	0.25
Voorjaarsvergadering Nederlandse Vereniging voor Urologie (The Netherlands)	2018	0.25
33rd European Association of Urology conference (Denmark)	2018	0.25
Oncology Symposium (United Kingdom)	2018	0.25

Presentations	**Year**	**ECTS**
Video presentation '*Confocal laser endomicroscopy for bladder cancer diagnosis: how to do it*' International meeting Challenges In Endourology (Paris, France)	2016	0.5
Presentation '*Confocale laser endomicroscopie in de blaas: een nieuwe beeldvormingstechniek voor blaaskanker diagnostiek* ' Najaarsvergadering Nederlandse Vereniging voor Urologie (Nieuwegein, The Netherlands)	2016	0.5
Presentation '*Fluorescentie in situ hybridisatie voor het voorspellen van BCG non-responders bij BCG therapie voor blaascarcinoom'* Najaarsvergadering Nederlandse Vereniging voor Urologie, (Nieuwegein, The Netherlands)	2016	0.5
Poster presentation '*Fluorescence in situ hybridization as predictor of tumour recurrence for bladder cancer patients treated with Bacillus Calmette-Guérin*' World Congress of Endourology (Capetown, South Africa)	2016	0.5
Poster presentation '*Monopolar versus bipolar transurethral resection for primary non-muscle invasive bladder cancer'* European Association of Urology (London, United Kingdom)	2016	0.5
Presentation '*En-bloc resectie voor niet-spierinvasief blaascarcinoom: onze ervaringen'* Voorjaarsvergadering Nederlandse Vereniging voor Urologie ('s Hertogenbosch, The Netherlands)	2017	0.5
Presentation '*Monopolaire versus bipolaire transurethrale resectie van blaastumoren voor het niet-spierinvasief blaascarcinoom*' Voorjaarsvergadering Nederlandse Vereniging voor Urologie ('s Hertogenbosch, The Netherlands)	2017	0.5

Presentations *(continued)*	**Year**	**ECTS**
Video presentation '*Confocal laser endomicroscopy for bladder cancer diagnosis: how to do it and our preliminary results*' American Urology Association (Boston, USA)	2017	0.5
Poster '*En-bloc resection for primary non-muscle invasive bladder cancer: our initial experience*' International meeting Challenges In Endourology (Paris, France)	2017	0.5
Poster presentation '*Confocal laser endomicroscopy for bladder cancer diagnosis: our initial experience*' International meeting Challenges In Endourology (Paris, France)	2017	0.5
Poster '*Predicting tumour recurrence in patients treated with Bacillus Calmette-Guérin*' Société Internationale d'Urologie (Lisbon, Portugal)	2017	0.5
Poster '*En-bloc resection for primary non-muscle invasive bladder cancer: our initial experience*' Société Internationale d'Urologie (Lisbon, Portugal)	2017	0.5
Poster presentation '*Confocal laser endomicroscopy: a potential assisting tool in bladder cancer diagnosis*' European Association of Urology (Copenhagen, Denmark)	2018	0.5
Presentation '*Confocale laser endomicroscopie: blaaskanker diagnostiek in een nieuw licht*' Beeldvorming in de Urologie III ('s Hertogenbosch, The Netherlands)	2018	0.5
Presentation '*Hyperthermie en blaasspoelingen bij NMIBC: prime time?*' Tumorwerkgroep urologische tumoren Amsterdam IKNL (Amsterdam, The Netherlands)	2018	0.5
Presentation '*UroVysion FISH for the prediction of recurrence in bladder cancer patients treated with Bacillus Calmette-Guérin*' Oncology symposium (London, UK)	2018	0.5

Parameters of esteem	**Year**
Best poster presentation award 3rd price Challenges In Endourology	2017
Nomination for Vlietstra award Najaarsvergadering Nederlandse Vereniging voor Urologie	2017
Grants Cure for Cancer Subsidie Maurits & Anna de Kock stichting	2017

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L Bubendorf	LB	GM Kamphuis	GK	JR Oddens	JO
MTJ Bus	MB	MP Laguna Pes	MPL	TM de Reijke	TR
EEC Cauberg	ECC	TG van Leeuwen	TL	JJMCH de la Rosette	JR
ESY Chan	EC	R Li	RL	CD Savci-Heijink	CS
YD Choi	YC	JC Liao	JL	S Savic	SS
CP Dinney	CD	EIML Liem	EL	RJ Sylvester	RS
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Chapter 1

Can we improve transurethral resection of the bladder tumour for non-muscle invasive bladder cancer?

EIML Liem, TM de Reijke. Current opinion in Urology, 2017 Mar;27(2).

Conception and design	EL, TR
Data/literature acquisition	EL
Data/literature analysis and interpretation	EL, TR
Drafting of the manuscript	EL
Critical revision of the manuscript	TR
Supervision	TR

Monopolar versus bipolar transurethral resection for non-muscle invasive bladder carcinoma: a post-hoc analysis from a randomized controlled trial.

EIML Liem, M McCormack, ESY Chan, Y Matsui, P Geavlete, YD Choi, TM de Reijke, Y Farahat, BA Inman, JJMCH de la Rosette, S Naito. Urologic Oncology, 2018 Jul;36(7).

Conception and design	TR, JR, SN
Data/literature acquisition	EL, MM, EC, YM, PG, YC, TR, YF, BI, SN
Data/literature analysis and interpretation	EL, TR, JR
Drafting of the manuscript	EL
Critical revision of the manuscript	all authors
Supervision	TR, JR, SN

Chapter 2

Confocal laser endomicroscopy for the diagnosis of urothelial carcinoma in the bladder and the upper urinary tract: protocols for two prospective explorative studies.

EIML Liem, JE Freund, J Baard, DM de Bruin, MP Laguna Pes, CD Savci-Heijink, TG van Leeuwen, TM de Reijke, JJMCH de la Rosette. JMIR Research Protocols, 2018 Feb;7(2).

Conception and design	EL, JF, TR, JR, DB
Data/literature acquisition	EL, JF, JB, DB
Data/literature analysis and interpretation	EL, JF, DB, CS, TR
Drafting of the manuscript	EL, JF
Critical revision of the manuscript	JB, DB, ML, CS, TL, TR, JR
Supervision	DB, TR, JR

Chapter 3

Confocal laser endomicroscopy for the diagnosis of urothelial carcinoma in the bladder and the upper urinary tract.

JE Freund, EIML Liem, J Baard, GM Kamphuis, MP Laguna Pes, TM de Reijke, JJMCH de la Rosette, DM de Bruin. Videourology, 2018 jul;32(2).

Conception and design	EL, JF, TR, JR, DB
Data/literature acquisition	EL, JF, JB, GK
Data/literature analysis and interpretation	EL, JF, TR, DB
Drafting of the manuscript	EL, JF
Critical revision of the manuscript	JB, GK, ML, TR, JR, DB
Supervision	TR, JR, DB

Chapter 4

Validation of confocal laser endomicroscopy features of bladder cancer: the next step towards real-time histologic grading.

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Conception and design	EL, DB, TR
Data/literature acquisition	EL, JE, CS, GK, JB
Data/literature analysis and interpretation	EL, JE, CS, GK, JB, TR
Drafting of the manuscript	El, JF
Critical revision of the manuscript	CS, JR, JL, TL, TR, DB
Supervision	JR, TM, DB

Chapter 5

Towards automated in vivo bladder tumor stratification using confocal laser endomicroscopy.

M Lucas, EIML Liem, CD Savci-Heijink, JE Freund, HA Marquering, TG van Leeuwen, DM de Bruin. Journal of Endourology, 2019 Nov;33(11).

Conception and design	ML, EL, HM, TL, DB
Data/literature acquisition	EL, CS, JF
Data/literature analysis and interpretation	ML, EL, HM, DB
Drafting of the manuscript	ML, EL, JF
Critical revision of the manuscript	HM, TL, DB
Supervision	HM, DB

Chapter 6

Fluorescence in situ hybridization as prognostic predictor of tumor recurrence during treatment with Bacillus Calmette-Guérin therapy for intermediate- and high-risk non-muscle invasive bladder cancer.

EIML Liem, J Baard, ECC Cauberg, MTJ Bus, DM de Bruin, MP Laguna Pes, JJMCH de la Rosette, TM de Reijke. Medical Oncology, 2017 Sep 2;34(10).

Conception and design	ECC, TR
Data/literature acquisition	EL, JB, ECC, MB
Data/literature analysis and interpretation	EL, DB, TR
Drafting of the manuscript	EL, ECC
Critical revision of the manuscript	DB, ML, JR, TR
Supervision	TR

Chapter 7

The role of fluorescence in situ hybridization for predicting recurrence after adjuvant Bacillus Calmette-Guérin in patients with intermediate- and high-risk non-muscle invasive bladder cancer: a systematic review and meta-analysis of individual patient data.

EIML Liem, JR Oddens, RWM Vernooij, R Li, AM Kamat, CP Dinney, L Mengual, A Alcaraz, L Izquierdo, S Savic, GN Thalmann, L Bubendorf, RJ Sylvester, TM de Reijke. Journal of Urology, 2020 Feb;203(2).

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The role of fluorescence in situ hybridization for predicting recurrence after adjuvant Bacillus Calmette-Guérin in patients with intermediate- and high-risk non-muscle invasive bladder cancer: a systematic review and meta-analysis of individual patient data. EIML Liem, JR Oddens, RWM Vernooij, R Li, AM Kamat, CP Dinney, L Mengual, A Alcaraz, L Izquierdo, S Savic, GN Thalmann, L Bubendorf, RJ Sylvester, TM de Reijke. Journal of Urology, 2020 Feb;203(2)

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ABOUT THE AUTHOR

Esmée Liem was born in 1987 in Dordrecht, the Netherlands. She graduated in 2005 from the Johan de Witt Gymnasium in Dordrecht and started her medical education at the Erasmus University in Rotterdam. During her medical studies, Esmée obtained a Master of Science degree in Neuroscience at the Erasmus University in Rotterdam and completed a clinical internship abroad, at the Renji Hospital in Shanghai, China. After graduating from medical school in 2012, she started her medical career at the Snijdend Oncologische Groep at the Daniel den Hoed Kliniek, in Rotterdam and department of Urology at the Amsterdam UMC, in Amsterdam, where she shortly thereafter started her PhD. The PhD research presented in this thesis is the result of a close collaboration between the Department of Urology and the Department of Biomedical Engineering and Physics. In 2018 Esmée gained clinical experience at the Ophthalmology department in Gelre Ziekenhuizen in Apeldoorn and Zutphen. As of February 2019, she is working as a resident in training at the Eye Hospital Rotterdam. She is happily married to Theo Bettenhaussen and they are expecting their first child in April 2021.

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