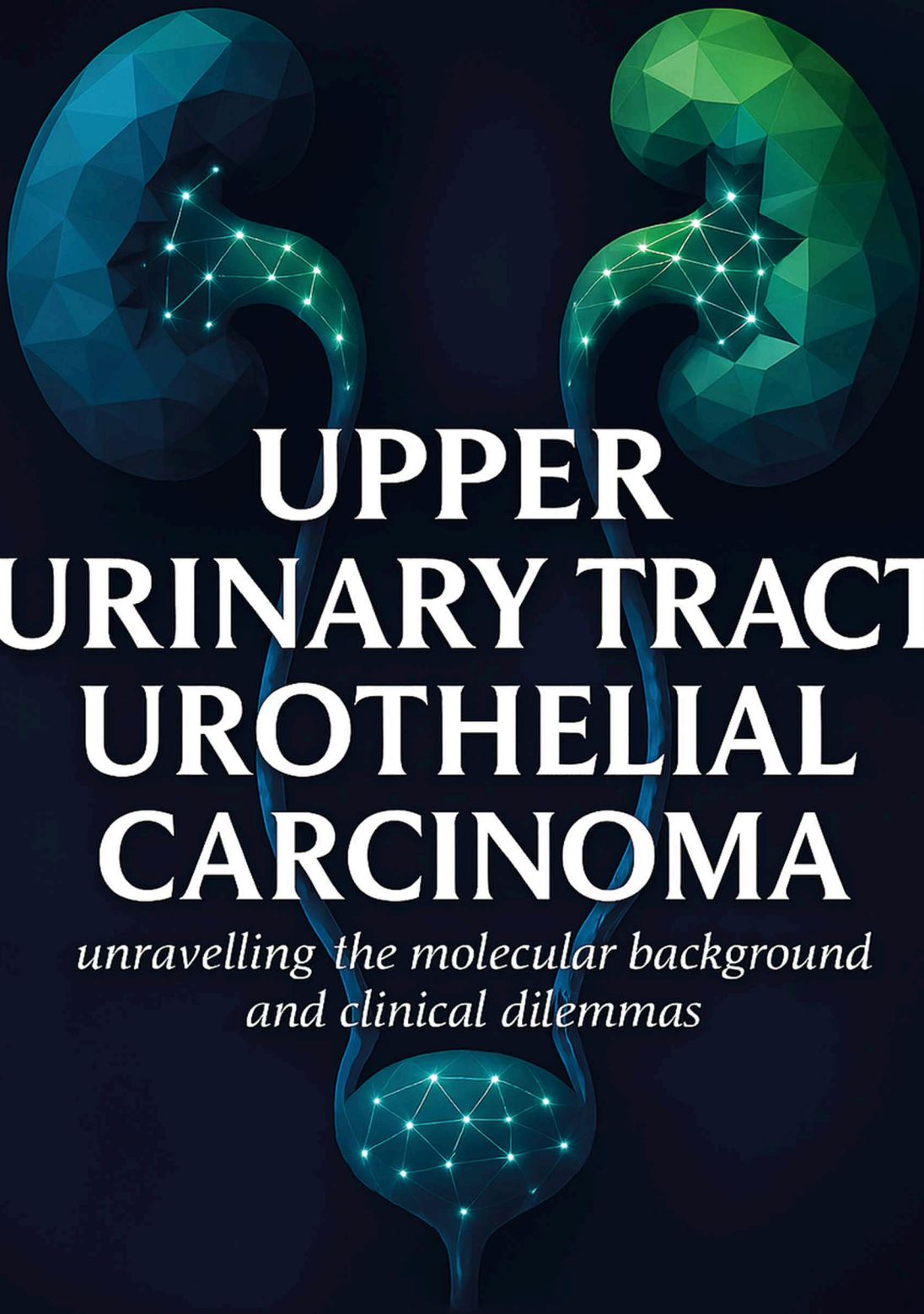

UPPER URINARY TRACT UROTHELIAL CARCINOMA

unravelling the molecular background and clinical dilemmas

THOMAS VAN DOEVEREN

Upper Urinary Tract Urothelial Carcinoma
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Thomas van Doeveren

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Upper Urinary Tract Urothelial Carcinoma

Unravelling the molecular background and clinical dilemmas

Het urotheelcarcinoom van de hoge urinewegen

Het ontrafelen van de moleculaire achtergrond en dilemma's in de klinische praktijk.

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“Now I’ve got a woman at home, she treats me well”

Ben Howard

“Prima, maar je weet je einddoel”

Familie van Doeveren

CONTENTS


1

GENERAL INTRODUCTION

Upper urinary tract urothelial carcinomas (UTUC) are relatively uncommon in comparison to carcinomas affecting the lower urinary tract, predominantly manifested as urothelial bladder carcinoma (UBC) [1]. Consequently, a paucity of common knowledge and literature regarding UTUC exists. Historically, it was deemed acceptable to extrapolate research findings considering UBC to UTUC. However, it is increasingly evident that, while UTUC and UBC do indeed share certain biological and molecular characteristics, noteworthy distinctions in genetics, therapeutic approaches, disease progression, and outcomes exists [2-4]. Fortunately, there is an expanding body of literature dedicated to comprehensively understanding UTUC as a distinct entity where this thesis contributes to the exploration of the latest developments on UTUC.

Upper urinary tract

UTUCs are neoplasms originating in the renal pelvis (the expanded funnel-shaped area in the kidneys collecting the urine) or ureter; i.e. the upper urinary tract. The majority of these tumors arise from the urothelium cell layer which covers the entire urinary tract and are therefore classified as urothelial carcinomas. The urothelium cell layer lines the interior of the urinary tract and serves as a natural barrier to prevent leakage of urine and solutes during periods of expansion or contraction. A muscle layer, underneath the urothelium, facilitates these contractions which are needed for peristalsis to transport the urine to the bladder. This muscle layer is less pronounced in the upper urinary tract compared to the well-developed muscle layer of the bladder (detrusor muscle). This difference can be attributed primarily to the distinct functions of the bladder, which are storage and voiding. In determining the stage of carcinoma development within the urinary tract, the muscle layer plays a pivotal role.

Upper urinary tract urothelial carcinoma

The majority of UTUC patients present with symptoms like hematuria (58%) and/or flank pain (19%); but one-third of the patients are diagnosed incidentally following imaging procedures performed for unrelated clinical reasons [5,6]. This is an important factor underlying the common delay in diagnosing UTUC and contributes to the fact that over 50% of UTUC patients present with invasive

disease [7-9]. The work-up in the diagnosis of UTUC encompasses imaging, preferably using CT-urography, along with a cystoscopy (to rule out bladder carcinoma), and (selective) cytology (examination of a single cell type of clusters of cells in fluid i.e. cancer cells). To confirm a histological diagnosis, the European Association for Urology (EAU) recommends the performance of a ureterorenoscopy (URS) to visualize a suspected UTUC and, if feasible, to incorporate a biopsy [1]. After diagnosis of UTUC, the disease stage is determined by the Tumor, Node, and Metastasis classification system for UTUC, described in **Table 1** [10]. Despite extensive diagnostics preoperative risk stratification of patients remains a challenge, due to the limited accuracy of CT-urography in detecting non-muscle invasive UTUC and the high risk of understaging regarding biopsies taken during URS [11,12].

Management strategies for localized UTUC

A radical nephroureterectomy (RNU) represents the standard treatment for patients with non-metastatic UTUC (**Figure 1**) [1]. This surgical procedure entails the *en bloc* removal of the kidney and ipsilateral ureter, including the bladder cuff. RNU can be performed via open surgery or less invasive techniques such as laparoscopic or robot-assisted. A critical component of RNU involves excising the distal ureter along with a 1 cm margin around the ureteric orifice; the bladder cuff [13,14]. In highly selected cases, involving low-risk UTUC-patients (unifocal disease, tumor size <2cm, cytology negative for high-grade tumor, low-grade biopsy, and no invasive aspect on CT-scan), kidney-sparing surgery (endoscopic removal or distal ureterectomy) may be considered. In patients with non-metastatic UTUC, it is recommended to contemplate a lymph node dissection (LND) following a specific template, as it may enhance survival for those with invasive UTUC [15].

Table 1. TNM classification of upper tract urothelial carcinoma.

	T – Primary Tumor
Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Ta	Non-invasive papillary carcinoma
T*is*	Carcinoma *in situ*
T1	Tumor invades subepithelial connective tissue
T2	Tumor invades muscularis
T3	Renal pelvis: Tumor invades beyond muscularis into peripelvic fat or renal parenchyma
	Ureter: Tumor invades beyond muscularis into periureteric fat
T4	Tumor invades adjacent organs or through the kidney into perinephric fat
	N – Regional lymph nodes
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node 2 cm or less in the greatest dimension
N2	Metastasis in a single lymph node more than 2 cm, or multiple lymph nodes
	M – Distant metastasis
M0	No distant metastasis
M1	Distant metastasis

For high-risk locally advanced UTUC, chemotherapy might be considered. Neo-adjuvant chemotherapy (NAC) is preferred since RNU can impair the post-surgical kidney function which is essential for receiving chemotherapy. However, as previously mentioned, preoperative risk stratification for UTUC and patient-selection for administering NAC remains challenging. Recently, the POUT trial examined the effectiveness of adjuvant chemotherapy initiated within 90 days following RNU and showed a significantly improved disease-free survival compared to RNU alone [16]. Subgroup analyses demonstrated a benefit for patients without lymph node involvement, negative surgical margins, and pT-stage T3/T4. The standard follow-up protocol for all UTUC patient consists of regular CT scans, cystoscopies, and urine cytology

Intravesical recurrences

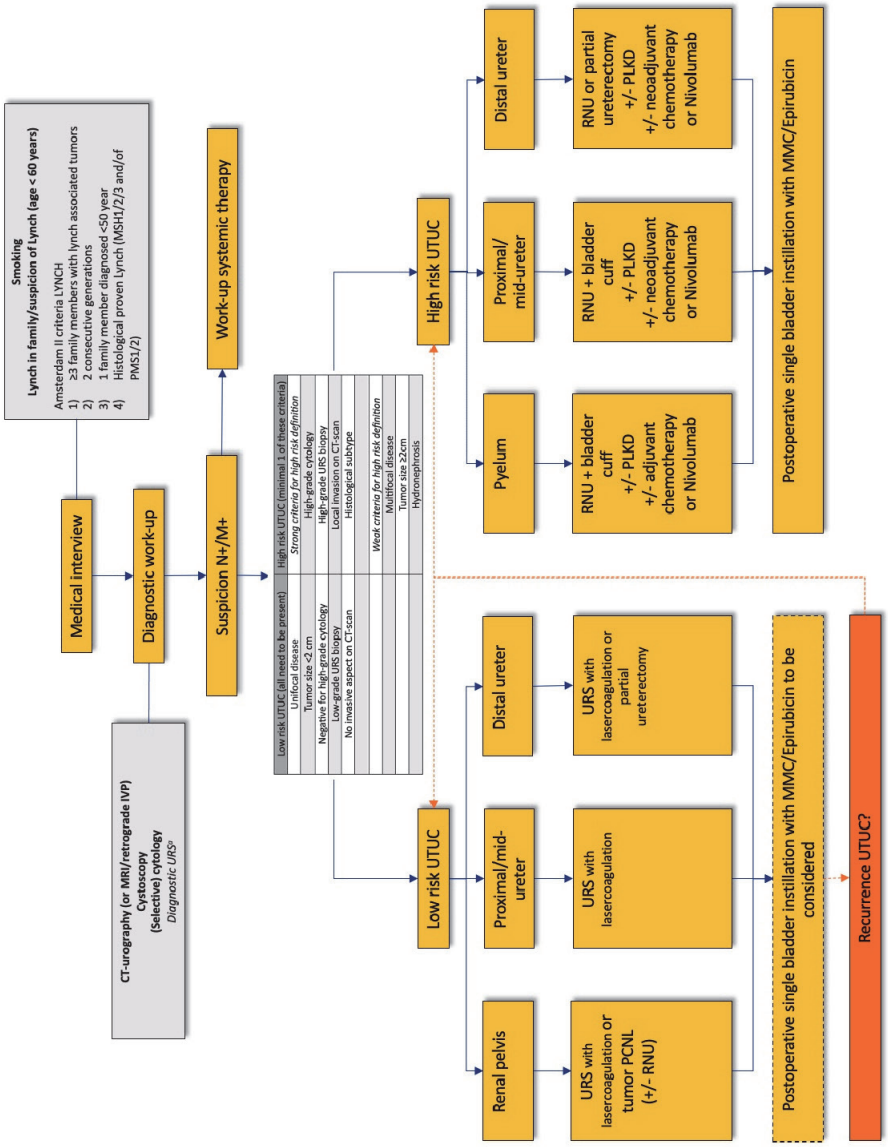
After surgical treatment for UTUC 22-47% of patients will be diagnosed with intravesical recurrences (IVR), of which 70% of cases is diagnosed within one year following surgery [13,17,18]. Two hypotheses have been proposed to explain the

development of IVR. The first hypothesis states that carcinogenic hits, such as smoking, affect the entire urinary tract, leading to the development of multiple, independent tumors [19]. The second hypothesis posits that 'seeds' of tumor cells originating from the primary UTUC implant in the bladder, resulting in IVR. A meta-analysis has identified multiple significant clinical predictors for developing IVR, some of which further validate the theory of seeding [20,21]. These predictors include a history of previous bladder cancer, positive preoperative urinary cytology, tumor location in the distal ureter, tumor multifocality, invasive pT-stage, tumor necrosis, laparoscopic surgery, and positive surgical margins [22]. There is level 1 evidence indicating that a single post-operative dose of intravesical chemotherapy reduces the risk of IVR, with an absolute risk reduction of 13% [18,23,24]. However, concerns regarding the risk of adverse events resulting from chemotherapy extravasation remain, leading to a reluctance to administer chemotherapy instillation. Extravasation of highly concentrated MMC (40mg) can result in long-lasting tissue injury, including fat necrosis and delayed healing.

Scope of this thesis

This thesis explores the molecular background, clinical challenges, and advancements in the treatment of UTUC and subsequent IVR. First, the incidence and survival rates of UTUC in the Netherlands was examined (Chapter 2). Then the focus was on deepening our understanding of the biological and molecular mechanisms underlying the development of IVR subsequent to UTUC surgery (Chapter 3, 4, and 5). Next, strategies were investigated to reduce IVR following UTUC surgery by evaluating the efficacy of a preoperative single intravesical instillation of chemotherapy in the REBACARE trial, instead of the conventional postoperative approach (Chapter 6 and 7). Finally, this thesis concludes with an assessment of health-related quality of life outcomes in patients undergoing radical surgery for UTUC (Chapter 8).

Figure 1. Flow chart for the management of UTUC. Based on the EAU Guideline 'Upper Urinary Tract Urothelial Cell Carcinoma 2024'.



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PART I

EPIDEMIOLOGY OF UPPER URINARY TRACT UROTHELIAL CARCINOMA


2

RISING INCIDENCE RATES AND UNALTERED SURVIVAL RATES FOR PRIMARY UPPER URINARY TRACT UROTHELIAL CARCINOMA: A DUTCH POPULATION-BASED STUDY FROM 1993 TO 2017

Thomas van Doeveren, Marianne van der Mark, Pim J. van Leeuwen, Joost L. Boormans, Katja K.H. Aben

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ABSTRACT

Aim: To assess trends in incidence, disease management and survival of upper urinary tract urothelial carcinoma (UTUC) in the Netherlands.

Materials and methods: Patients diagnosed with primary UTUC in the Netherlands between 1993 and 2017 were identified through the population-based Netherlands Cancer Registry (NCR). Patient- and tumor characteristics as well as information on treatment and vital status were retrieved from the NCR. Age-standardized incidence rates were calculated stratified by age, gender, calendar-period and disease stage. Relative survival served as approximation for cancer-specific survival.

Results: We identified 13,314 patients with primary UTUC. The age-standardized incidence rate increased from 2.0 in 1993 to 3.2 per 100,000 person-years in 2017, without change in gender distribution. The increase in incidence holds for all disease stages except organ-confined (T1-T2) disease. The most prominent increase was in superficial (Tis/Ta) and metastatic (M+) UTUC; from 0.6 to 1.2 and 0.1 to 0.4 per 100,000 person-years, respectively. The 5-year relative survival did not change over time; 57.0% (95% Confidence Interval: 55.9-58.1). Applied treatments were largely the same over the study period, although fewer radical nephroureterectomies and more kidney-sparing surgeries were performed in the most recent years. The use of perioperative intravesical chemotherapy had modestly increased.

Conclusion: Between 1993 and 2017, the age-standardized incidence of primary UTUC in the Netherlands has increased by more than 50%, but the relative survival of UTUC patients remained unchanged. Preventive measures against exposure to risk factors, early detection of disease, and more efficacious treatment modalities are needed to improve outcomes of patients with UTUC.

Keywords: upper urinary tract, urothelial carcinoma, incidence, survival, treatment, epidemiology

INTRODUCTION

Upper urinary tract urothelial carcinoma (UTUC) is a rare entity, with an incidence of 1-2 cases per 100,000 person years in Western countries [1]. It is less common than urothelial carcinoma of the bladder (UBC); only 5 to 10% of all urothelial carcinomas are located in the upper urinary tract (UUT).

The principal environmental risk factor for developing UTUC is tobacco use [2]. Genetic factors also play a role as UTUC is the second most common diagnosed extra-colonic cancer within the spectrum of Lynch syndrome [3]. Hematuria and flank pain are the most frequent presenting symptoms, although many patients present without symptoms [3-5]. A Computed Tomography (CT) Urography is recommended as the standard diagnostic and staging modality, which has replaced Intravenous Pyelography (IVP) [6, 7]. To obtain a histological diagnosis and more definite risk stratification, the *European Association of Urology* (EAU) recommends a diagnostic ureterorenoscopy (URS) with biopsy of the tumor [1]. Although URS techniques have improved, accurate tumor staging by diagnostic biopsies carries a high risk of understaging. Rojas *et al*. reviewed 137 biopsies obtained by URS in 81 patients and showed that the radical nephroureterectomy (RNU) specimen was discordant for tumor stage in 57% of cases [8]. Hence, preoperative risk stratification of patients with suspected UTUC remains a challenge.

RNU with bladder cuff excision is the recommended treatment for patients with non-metastatic UTUC. For low-risk UTUC, however, kidney-sparing surgery (KSS) seems to be a feasible alternative [9]. Following RNU, 22-47% of the patients develop a UBC within the first two years after surgery. A single postoperative intravesical instillation with chemotherapy significantly reduces the risk of a future UBC and is therefore recommended in current clinical guidelines [1]. Conversely to UBC, (neo)adjuvant chemotherapy is rarely applied in UTUC patients, although the improved survival by adjuvant chemotherapy following RNU from the recently reported POUT-trial might change that in the future [10].

To uncover any progress in the clinical management and outcome of patients with UTUC, we performed a population-based study and evaluated trends in incidence, disease management and survival of patients diagnosed with UTUC in the Netherlands from 1993 to 2017.

MATERIAL AND METHODS

Patients diagnosed with primary UTUC between 1993-2017 were identified through the Netherlands Cancer Registry (NCR). The NCR is a nationwide population-based registry held by the Netherlands Comprehensive Cancer Organization since 1989. The NCR receives notifications of newly diagnosed cancers from the nationwide network and registry of histo- and cytopathology (PALGA). An annual linkage to the national hospital discharge registry is performed to identify non-histologically confirmed cancers. Patient, tumor, and treatment information is retrieved from patients' electronic patient files by well-trained data managers. The vital status of patients is updated each year by linkage to the Personal Records Database, which keeps information on vital status of all Dutch residents.

Information on patient and tumor characteristics, as well as applied therapies, was extracted from the NCR. The diagnosis of UTUC was defined as International Classification of Disease for Oncology (ICD-O-3); C65.9 (renal pelvis) and C66.9 (ureter) [11]. Tumor stage was defined according to the 7th edition of the International Union Against Cancer Tumour-Node-Metastasis (TNM) classification, as this classification has not changed between 1993 and 2017 [12]. Patients having histology other than UCC were excluded (n = 327). For patients diagnosed with bilateral metachronous UTUC, only the primary tumor was included in the analysis.

Included patients were categorized into six disease stage groups, based on pathological TNM stage, supplemented with clinical TNM stage if histological confirmation of the primary tumor or metastasis could not be retrieved: i) superficial (TisTaN0M0), ii) organ-confined (T1-T2N0M0), iii) non-organ confined (T3-T4N0M0), iv) nodal metastatic (N+), v) distant metastatic (M+), and vi) unknown (TxNxMx).

Five calendar periods were defined based on the date of diagnosis; 1993-1997, 1998-2002, 2003-2007, 2008-2012, and 2013-2017. Treatment modalities were identified and grouped; RNU, KSS, surgery not otherwise specified (NOS), radiotherapy only, chemotherapy only, palliative chemotherapy plus radiotherapy, immunotherapy, instillation topical therapy UUT only, other therapy and no therapy. The group 'no therapy' consisted of patients who received active surveillance or best supportive care. As information on applied therapies was not recorded until 2005, analyses involving applied therapies were limited to patients diagnosed from 2005 onwards. Please note that during the period 2005-2008 a transition from general terminology for treatment to more specified terminology took place.

Statistical analysis

Age-standardized incidence rates using the 1976 European standard population expressed as the number of new cases per 100,000 person years (ESR), were calculated and analyzed according to year of diagnosis, gender, age at diagnosis, and stage of disease. Trends in incidence were presented by 3-years moving averages. The Estimated Annual Percentage of Change (EAPC) was calculated to evaluate changes over time.

Follow-up was defined as time from the date of primary diagnosis until date of death, emigration or last follow up. Relative survival was calculated as an approximation of disease-specific survival and was defined as the ratio of observed and expected survival [13]. Expected survival was calculated by the Ederer II method, using age, sex and calendar year specific life tables of the Dutch general population [14]. Relative survival rates were age-standardized by the International Cancer Survival Standard [15].

To evaluate trends in survival over time, relative survival was modelled using a generalized linear model assuming a Poisson distribution for the observed number of deaths. Significance of linear trends was obtained with p_{trend}-values from a likelihood ratio test comparing a model including the midpoint of the five calendar periods and a model without calendar periods. Statistical analyses were performed using SAS version 9.4 and STATA version 16.1.

RESULTS

Patient and tumor characteristics

We identified 13,314 patients with diagnosed primary UTUC. The median age at diagnosis had increased from 70 to 72 years over the 1993-2017 period (**Table 1**). Gender distribution had remained unchanged with a 2:1 male to female ratio across all five-time periods. Histological proof of the primary UTUC had been obtained in 94.8% of the 1,823 patients diagnosed during the 1993-1997 period, versus 83.9% of the 3,876 patients diagnosed during the 2013-2017 period. The proportion of histologically proven metastases had increased from 0.6% to 4.7%. Overall, the decrease of histologically proven primary or metastatic UTUC was 6%. The distribution of low-, intermediate- and high-grade UTUC changed over time, with more high grade/CIS in recent years

Incidence

The ESR of UTUC had increased from 2.0 in 1993 to 3.2 per 100,000 person years in 2017, equivalent to an EAPC of 1.8% ($p<0.01$). See **Figure 1** for the incidence rates of UTUC from 1993 to 2017 in the Netherlands, visualized as 3-year moving averages. In absolute numbers, this increase corresponded with a doubling of UTUC diagnoses – from approximately 400 in 1993 to 800 in 2017. This trend was irrespective of gender. The increase in incidence was most prominent in patients with a urothelial carcinoma of the ureter, i.e. EAPC ureter 2.4% ($p<0.01$) vs. renal pelvis 1.5% ($p<0.01$) (**Suppl. Fig. S1**). The incidence of UTUC in patients aged younger than 60 years had remained stable over time but had increased in the older age groups (**Suppl. Fig. S2**). Stage-stratified analyses showed a statistically significant increase in incidence across all tumor stages, except for organ-confined disease (**Figure 2**). The increase of the ESR was most prominent for metastatic UTUC: from 0.1 to 0.4 (EAPC 5.5%; $p<0.01$). For superficial UTUC, the ESR increased from 0.6 in 1993 to 1.2 (EAPC 2.7%; $p<0.01$) in 2017, with a steep increase from 2004 onwards. The age-standardized incidence rates based on the more recent 2013 European standard population, second edition, are visualized in the supplementary (**Figure S3-S6**).

Treatment

Between 2005 and 2017, RNU remained the most applied treatment modality (**Table 2**), although the proportion of patients who received RNU decreased from 72.3% of the 2,181 patients (2005-2008) to 62.9% of the 3,876 patients (2013-2017). The proportion of patients who received KSS more than doubled from 2005; from 6.0% to 13.6%. The number of lymph node dissections performed in combination with RNU remained limited; 9.3% (2005-2008) versus 11.8% (2013-2017). (Neo)adjuvant chemotherapy was hardly applied, but the use of postoperative intravesical instillations with chemotherapy had considerably increased; from 2.2% (2005-2008) to 9.7% (2013-2017).

Survival

The 5-year relative survival was 57.0% (95% confidence interval (CI) 55.9-58.1) and had not changed over time (p_{trend} = 0.05) (**Figure 3**). Tumor stage-specific analysis showed no improvement in survival of patients diagnosed with superficial (p_{trend} = 0.96) or organ-confined (p_{trend} = 0.82) disease from 1993 to 2017; with a 5-year relative survival of 85.7% (95% CI 83.9-87.3) and 69.6% (95% CI 67.6-71.6), respectively. For patients diagnosed with non-organ confined UTUC, the 5-year survival had modestly improved from 35.6% (CI 29.8-41.4) to 43.6% (CI 37.7-49.3) (p_{trend} = 0.05). The 1-year and 3-year survival for patients diagnosed with nodal metastatic UTUC had increased from 36.3% (95% CI 26.5-46.3) to 57.8% (95% CI 52.3-62.9) (p_{trend} = 0.03) and 16.5% (95% CI 9.2-25.6) to 31.9% (95% CI 24.7-39.2) (p_{trend} < 0.01), respectively. For distant metastatic disease, the 1-year relative survival had increased from 11.3% (95% CI 5.9-18.6) to 24.3% (95% CI 19.4-29.4) (p_{trend} = 0.29). Tumor grade-specific analysis for superficial UTUC showed a difference in the 5-yearr survival, as seen in figure 4.

DISCUSSION

In this nationwide, population-based study on 13,314 primary UTUC patients in the Netherlands, we found a significant increase in age-standardized incidence; i.e., from 2.0 to 3.2 cases per 100,000 person years from 1993 to 2017. The literature on the incidence of UTUC is scarce, studies are often not population-based and mostly reflect different time periods, which hampers adequate comparison

with the results of this study. An Australian study reported a stable age-standardized incidence rate between 2001-2011 [16]. Another study from Australia confirmed this observation and did also not find an increase in incidence for the period 1977-2003 [17]. Using the Surveillance, Epidemiology, and End Results (SEER)-database, *Raman et al.,* reported a slight increase from 1.88 in 1973 to 2.06 cases per 100,000 person years in 2005 in the USA [18]. Based on this SEER-database, a more recent study covering the period from 2004 to 2016, showed a decrease from 1.3 to 1.1 cases per 100,000 person years. However, pTa and pTis UTUC were not included [19]. Two other population-based studies, one conducted in the UK and one in Denmark, describing the periods 1985-2009 and 1944-2003, respectively, also found an increase in UTUC incidence [20, 21]. The most recent publication on trends in the incidence of UTUC was based on the Norwegian cancer registry, which reported a similar trend over time as we found in our study; an increase in incidence from 3.21 to 4.71 per 100,000 person years during the period 1999 to 2018 [22].

Although the ageing of the population contributes to the increase in the absolute number of patients diagnosed with UTUC, ageing does not explain the increase in the age-adjusted incidence. As smoking is the most important risk factor for both UTUC and UBC and smoking habits declined over the last decades, one could have expected a decrease in the trends in incidence for UTUC, as described for UBC [23]. However, an explanation for the discrepancy in trends in incidence between UBC and UTUC might be that UTUC develops slower than UBC, as the UUT has no storage function whereas the bladder has. Consequently, the urothelium of the UUT is less intensely exposed to carcinogenic toxins and incidence rates may lag behind on those of UBC.
The most important factor affecting the rising incidence of UTUC is the more extensive use of cross-sectional imaging in clinical practice. As approximately one third of UTUC are incidental findings, the degree of abdominal imaging in clinical practice directly impacts incidence numbers [24]. In addition, the sensitivity of CT-urography for the detection of UTUC has shown to be superior to conventional IVP (96% versus 50-61%) [25-27]. In 2011, the EAU recommended CT-imaging as the preferable diagnostic modality for UTUC instead of IVP [28]. The release of the first EAU guidelines on UTUC in 2004 had probably already

raised awareness on this disease [29]. Hence, growing awareness, improved imaging techniques and consensus in the diagnostic work-up for UTUC might have contributed to the increase in incidence.

The better diagnostic accuracy of CT-imaging of the UUT, in combination with enhanced quality of flexible diagnostic URS and selective urinary cytology, might also be an important contributor to the stage migration from organ-confined towards a higher proportion of diagnosed superficial UTUC from ~2005 onwards [7, 30]. After the introduction of Multidetector CT (MDCT) urography, correct staging of UTUC improved from 59.5% to 87.5% [31, 32]. The increase in the incidences of nodal and metastatic disease, also reported by *Ruvolo et al.,* might also be attributed to better diagnostic accuracy of CT-imaging, as recommended by the EAU since 2011 [19, 28, 29]. For detecting lymph node involvement, MDCT has a reported sensitivity of 87.5% and specificity of 98% [33]. For FDG-PET/CT, the sensitivity rate of 50% as reported for MDCT for detecting distant metastases even improved to 85% [34]. The observed 'grade'-migration towards a higher proportion of patients diagnosed with TaG3/Tis tumors might be explained by a better awareness among pathologists and urologists on tumor grade as prognostic factor for this stage category [35]. With the applicability of kidney sparing surgery in recent years, it has become more important to find concordance on tumor grade for superficial tumors prior to treatment. For carcinoma in situ, however, detection by imaging and ureterorenoscopy remains challenging and a 'paradigm shift' is needed [36].

The 5-year relative survival had not improved over the 25-year time period in the Netherlands. This is in line with reported findings in other countries. An Australian population-based study including 722 patients described a stable 5-year relative survival of 30% (2001-2006) and 36% (2007-2011) [16]. A nationwide study from the UK, which included patients diagnosed between 1985 and 2010, showed a decline in the 5-year relative survival from 60% to 48% [20]. *Eylert et al.* speculated that this might be explained by a sharp rise in incidence for patients > 80 years, and that likely more deaths were attributed to UTUC since more cross-sectional imaging was used. *Adibi et al*. also described a stable 5-

year cancer specific survival from 1983 to 2007 in patients all treated by RNU[37]. A Canadian study reported a similar relative 5-year survival as we observed (i.e. 57% in both studies) in 830 UTUC patients between 1995-2004 [38]. Contrary, the 5-year cancer-specific survival in Norway improved between 1999 and 2018 from 57.4% to 65.4% [22]. Although information on adjuvant treatment regarding UTUC was limited, the authors stated that this improvement might be explained by the increased use of perioperative chemotherapy and the introduction of immunotherapy in recent years. A conclusion that cannot be confirmed within our cohort.

One might expect that the observed shift towards superficial UTUC should have improved the survival for the entire cohort in our study. However, the concomitant increase of patients diagnosed with advanced UTUC and increased number of high grade superficial UTUC seemingly has compensated this expected gain. The increased incidence in the older age groups might also have contributed to this lack of improvement in survival as with increasing age the survival decreases. Older patients are less likely to undergo surgical treatment, and are often not eligible for chemotherapy. Noteworthy, due to an increase in absolute number of patients diagnosed with UTUC, an increasing number of deaths is attributed to UTUC annually. The improved stage-specific survival we found for non-organ confined, nodal and distant metastatic UTUC patients is probably the result of stage-migration [39]. As imaging techniques have become more sensitive for the detection of small metastases before they become clinically apparent, both (micro)metastatic and non-metastatic patients are staged more accurately. This stage-shift eventually leads to an improved survival in all three stage-groups.

Our finding that treatment approaches had remained largely the same, is in line with the unchanged survival. The shift to more KSS in recent years can probably be ascribed to the discrimination of low- and high-risk UTUC recommended by the EAU guidelines since 2011, in combination with improved equipment and techniques to perform KSS [28]. In combination with the improved detection of superficial, low-grade tumors, promising recent techniques, such as chemoablation and laservaporisation of UTUC, most likely will increases the use of KSS

[40, 41]. The significant increase we found in the use of perioperative intravesical chemotherapy in recent years, is in line with the 2015 EAU guidelines on UTUC, which recommended a post-operative bladder installation of chemotherapy to reduce the risk of a future intravesical recurrence [42-44].

Limitations

The Netherlands Cancer Registry allows us to evaluate trends over time in incidence, treatment and survival of a rare entity as UTUC. Data in the NCR is collected by well-trained data managers applying (inter)national coding rules leading to a high quality and uniform registration. However, information on causes of death is not available in the NCR. Thus, we could not calculate cancer-specific survival rates, and had to resort to the relative survival as an approximation of the cancer specific survival. As smoking is an important risk factor for the development of UTUC, the relative survival might slightly be overestimated as background mortality due to smoking is underestimated. On the other hand, UTUC as cause of death might have been wrongfully scored as death due to kidney cancer in death certificates, which also would have affected survival rates. We had to limit the analyses concerning changes in treatment to the period from 2005 onwards as specific treatment information in the NCR was only available from that time point. At last, within the NCR tumors with predominantly UCC are registered as UCC, regardless of the presence of a minor component of aberrant histology component. Therefore, this would have had a negligible influence on survival.

CONCLUSION

In conclusion, the age-standardized incidence of UTUC in the Netherlands has increased with more than 50% over the past decades. A stage shift towards superficial UTUC has occurred. A concomitant increase was seen in the proportion of patients with advanced disease as well. Improved quality and increased utilization of imaging techniques for the upper urinary tract might have contributed to these observed trends. The relative survival has not improved, which corresponds to the overall lack of changes in therapies, although more patients received KSS and perioperative intravesical chemotherapy in recent years. Effective prevention

strategies, earlier detection and new, more effective treatment modalities are required to achieve progress in the care for UTUC patients.

FUNDING

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DECLARATION OF INTEREST STATEMENT

J.L. Boormans reports on consultancy work for MSD, Janssen, Ambu, Ismar health care, during the conduct of the study; and received a research grant from Decipher Biosciences. All other authors report no conflict of interest.

ETHICAL STATEMENT

This study was approved by the Privacy Review Board of the NCR (K17.177 IKNL).

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FIGURE LEGENDS

1. **Figure 1.** European standardized rates and absolute number of patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 (3-year moving average)
2. **Figure 2.** European standardized rates of patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 stratified by disease stage (3-year moving average)
3. **Figure 3.** The 1-yr, 3-yr and 5-yr relative survival, including 95% confidence intervals (CI), of patients diagnosed with primary UTUC stratified by time period (panel A) and stratified by disease stage; B) superficial (Tis-Ta) disease; C) organ-confined (T1-T2) disease; D) non-organ confined (T3-T4) disease; E) nodal metastatic (N+) disease; F) distant metastatic (M+) disease
4. **Figure 4.** Relative survival, including 95% confidence intervals (CI), of patients diagnosed with primary superficial UTUC stratified by tumor grade WHO1973; TaG1, TaG2, TaG3/Tis and unknown.

SUPPLEMENTARY

1. **Figure S1.** European standardized rates of 13,314 patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 stratified by tumor location; renal pelvis versus ureter (3-year moving average)
2. **Figure S2.** European standardized rates of 13,314 patients diagnosed with UTUC in the Netherlands from 1993 till 2017 stratified by age (3-year moving average)
3. **Figure S3-S6.** Incidence rates of patients diagnosed with primary UTUC in the Netherlands form 1993 till 2017 based on the European Standard Population (ESP) 2013, second edition

Table 1: Patient and tumor characteristics of 13,314 patients diagnosed with UTUC between 1993 and 2017 in the Netherlands.

Variable	1993-1997 N = 1823	1998-2002 N = 1985	2003-2007 N = 2419	2008-2012 N = 3211	2013-2017 N = 3876
Age (years), median (IQR)	70 (63-76)	70 (62-77)	71 (63-77)	72 (64-79)	72 (65-78)
Gender, %					
Male	66.8	67.5	66.4	67.6	67.2
Female	33.2	32.5	33.6	32.4	32.8
Diagnose based on, %					
Histologically proved UTUC	94.8	93.2	89.7	87.0	83.9
Histologically proved metastases	0.6	0.8	1.5	2.9	4.7
Urinary cytology	2.8	3.4	4.9	5.1	5.9
Clinical assessment	1.9	2.7	3.9	5.0	5.5
Location of UTUC, %					
Renal pelvis	57.1	58.6	56.9	56.9	54.2
Ureter	42.9	41.4	43.1	43.1	45.8
Disease stage, %					
Superficial (Tis-Ta)	30.6	30.5	28.9	33.0	36.1
TaG1	34.4	26.9	22.9	26.8	27.5
TaG2	44.3	48.1	52.2	50.4	47.2
TaG3/Tis	6.5	11.1	12.3	13.9	16.8
Unknown	14.8	13.9	12.6	8.9	8.5
Organ-confined (T1-T2)	32.4	28.6	29.3	25.3	19.9
Non-organ confined (T3-T4)	19.7	21.2	20.4	18.7	18.6
Nodal metastases (Tany N+ M0)	5.6	7.9	8.1	9.0	8.3
Distant metastases (Tany Nany M+)	5.3	7.2	9.3	9.2	11.8
Unknown (TxNxMx)	6.4	4.6	4.0	4.8	5.2

UTUC = upper urinary tract urothelial carcinoma

Table 2: Distribution of applied therapies by calendar period in patients diagnosed with primary UTUC in the Netherlands between 2005-2017

Variable	2005-2008 N = 2181		2009-2012 N = 2584		2013-2017 N = 3876	
	N	%	N	%	N	%
Radical nephroureterectomy	**1576**	**72.3**	**1884**	**72.9**	**2439**	**62.9**
Plus neoadjuvant chemotherapy	12	0.8	22	1.2	48	2.0
Plus adjuvant chemotherapy	43	2.7	31	1.6	43	1.8
Plus intravesical chemotherapy	34	2.2	46	2.4	236	9.7
Plus lymph node dissection	146	9.3	171	9.1	287	11.8
Kidney Sparing Surgery	**131**	**6.0**	**183**	**7.1**	**529**	**13.6**
Surgery, not otherwise specified	**108**	**5.0**	**9**	**0.3**	**19**	**0.5**
Radiotherapy only	**31**	**1.4**	**49**	**1.9**	**66**	**1.7**
Chemotherapy only	**44**	**2.0**	**88**	**3.4**	**170**	**4.4**
Palliative chemotherapy + radio-therapy	**17**	**0.8**	**17**	**0.7**	**33**	**0.9**
Immunotherapy	**-**	**-**	**-**	**-**	**4**	**0.1**
Instillation topical therapy UUT only	**13**	**0.6**	**13**	**0.5**	**30**	**0.8**
Other therapy	**12**	**0.5**	**30**	**1.2**	**70**	**1.8**
No therapy	**249**	**11.4**	**311**	**12.0**	**516**	**13.3**

UUT = upper urinary tract

Figure 1. European standardized rates and absolute number of patients diagnosed with primary upper urinary tract urothelial carcinoma (UTUC) in the Netherlands from 1993 till 2017 (3-year moving average. EAPC, estimated annual percentage of change; ESR, European standardized rate.

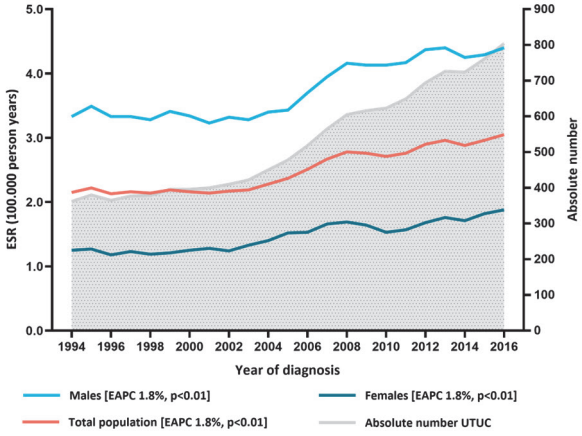


Figure 2. European standardized rates of patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 stratified by disease stage (3-year moving average).

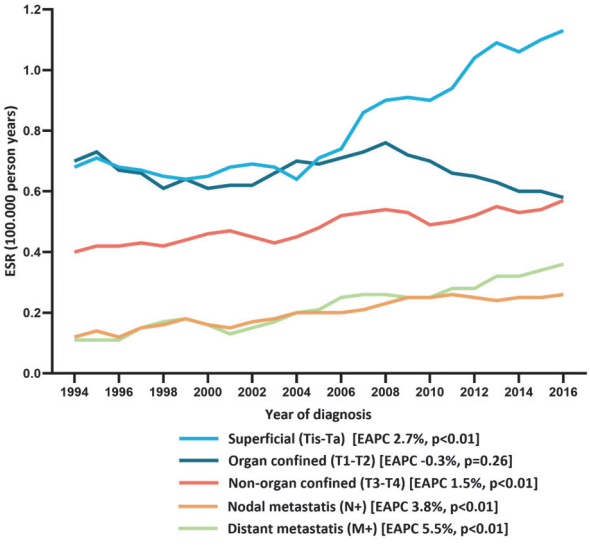


Figure 3. The 1-yr, 3-yr and 5-yr relative survival, including 95% confidence intervals (CI), of patients diagnosed with primary UTUC stratified by time period (panel A) and stratified by disease stage; B) superficial (Tis-Ta) disease; C) organ-confined (T1-T2) disease; D) non-organ confined (T3-T4) disease; E) nodal metastatic (N+) disease; F) distant metastatic (M+) disease.

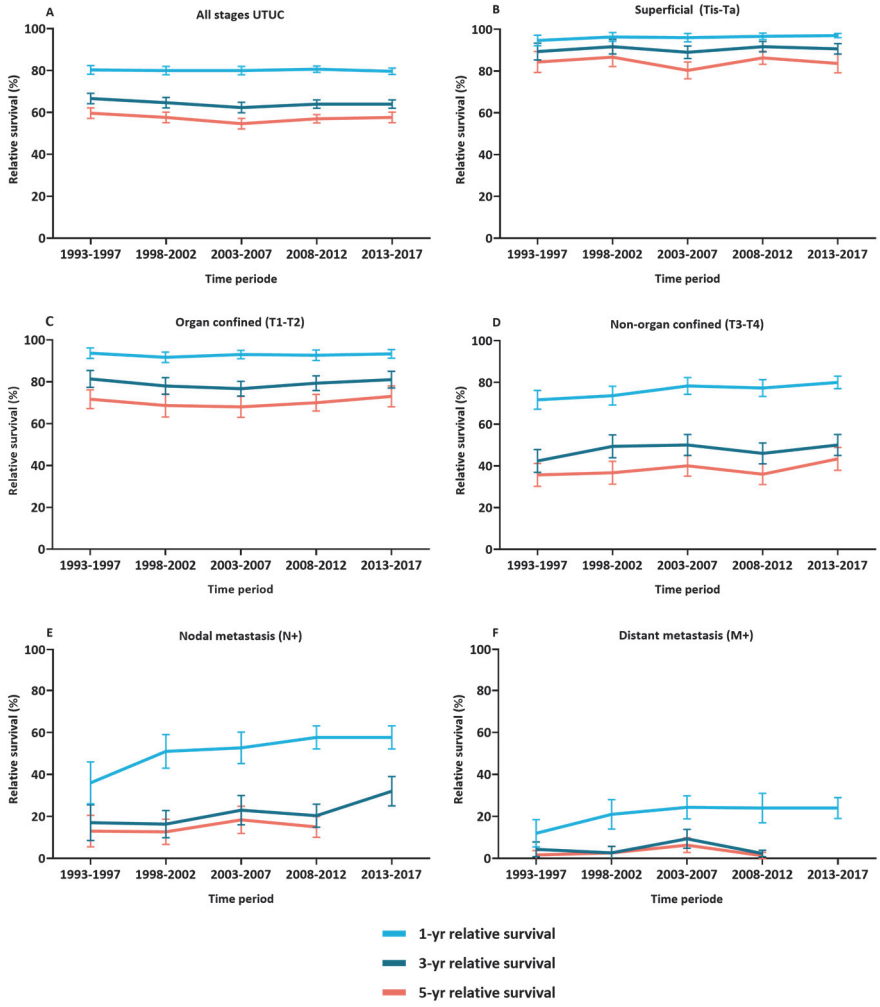


Figure 4. Relative survival, including 95% confidence intervals (CI), of patients diagnosed with primary superficial UTUC stratified by tumor grade WHO1973; TaG1, TaG2, TaG3/Tis and unknown.

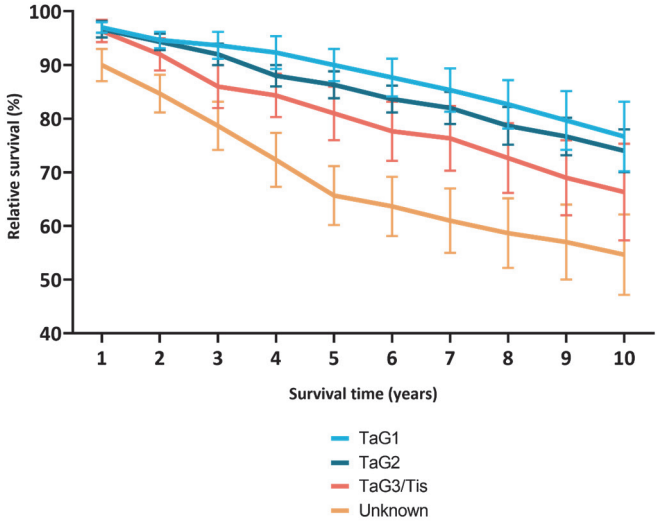


Figure S1. European standardized rates of 13,314 patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 stratified by tumor location; renal pelvis versus ureter (3-year moving average)

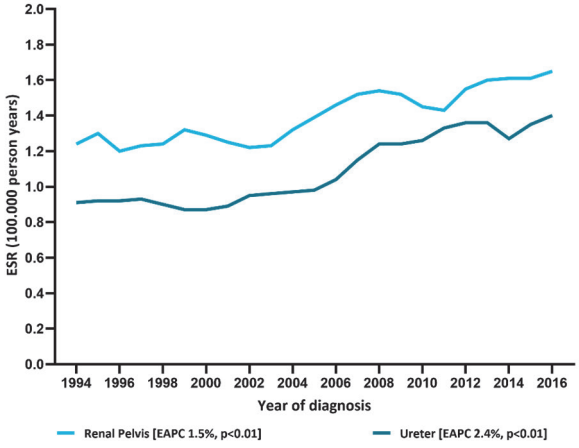


Figure S2. European standardized rates of 13,314 patients diagnosed with UTUC in the Netherlands from 1993 till 2017 stratified by age (3-year moving average)

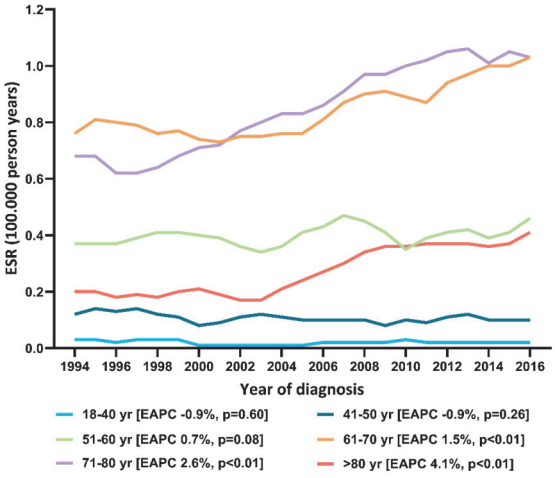


Figure S3: European standardized rates and absolute number of patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 (3-year moving average).

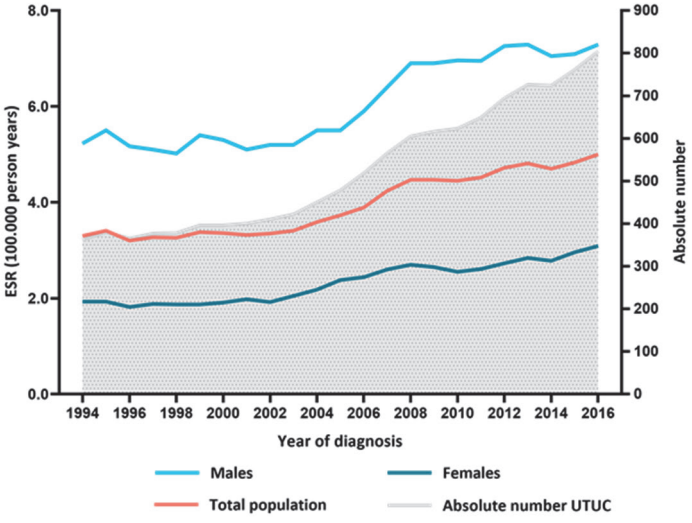


Figure S4: European standardized rates of patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 stratified for stadium (3-year moving average).

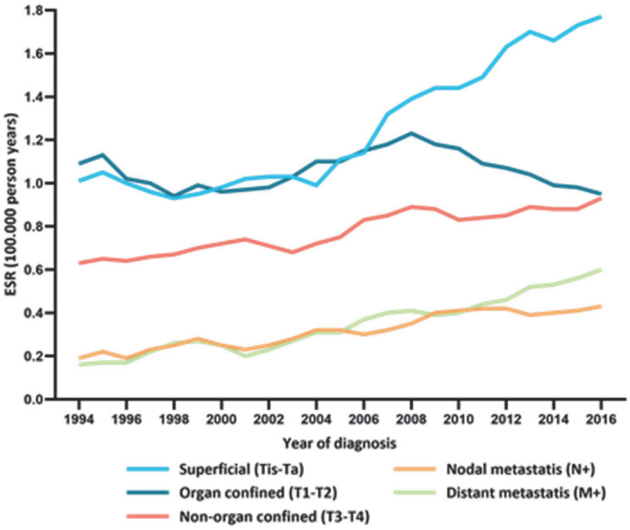


Figure S5: European standardized rates of patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 stratified by tumor location; renal pelvis versus ureter (3-year moving average).

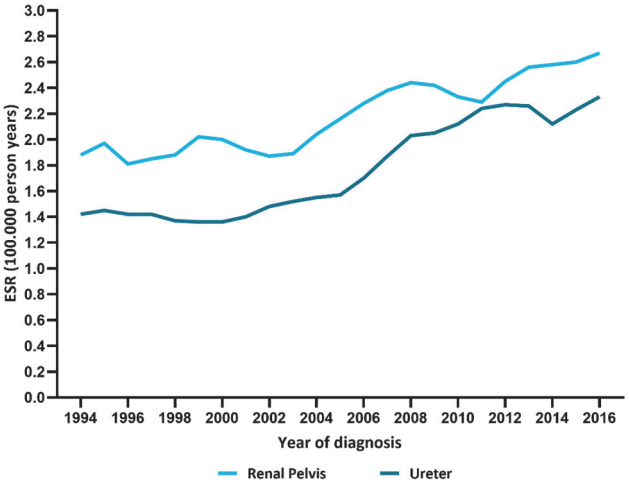
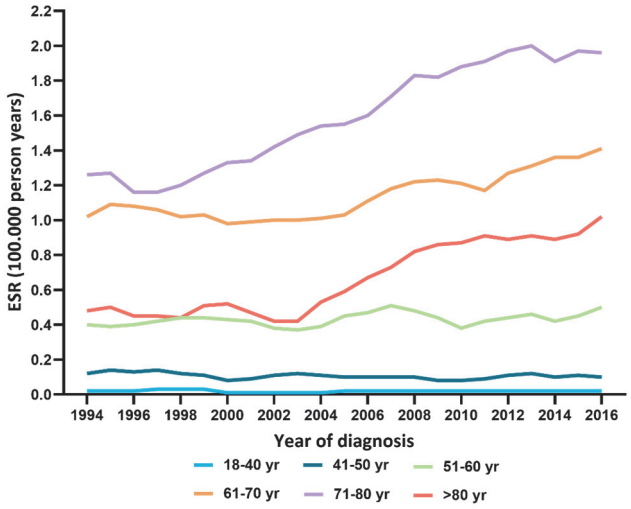


Figure S6: European standardized rates of patients diagnosed with primary UTUC in the Netherlands from 1993 till 2017 stratified by tumor location; renal pelvis versus ureter (3-year moving average).



PART II

MOLECULAR CHARACTERISTICS OF UPPER URINARY TRACT UROTHELIAL CARCINOMA AND INTRAVESICAL RECURRENCES


3

SYNCHRONOUS AND METACHRONOUS URTHELIAL CARCINOMA OF THE UPPER URINARY TRACT AND THE BLADDER: ARE THEY CLONALLY RELATED? A SYSTEMATIC REVIEW

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ABSTRACT

Purpose: Following radical nephroureterectomy for upper urinary tract urothelial carcinoma (UTUC), intravesical recurrence (IVR) is found in 22-47% of patients. Patients with a primary urothelial carcinoma of the bladder (UBC) have an increased risk of a future UTUC (1-5%). Paired UTUC and UBC might represent clonally related tumors due to intraluminal seeding of tumor cells or might be separate entities of urothelial carcinoma caused by field cancerization. We systematically reviewed all the relevant literature to address the possible clonal relation of UTUC and paired UBC.

Materials and methods: MEDLINE, EMBASE, and COCHRANE databases were systematically searched for relevant citations published between January 2000 and July 2019. This study was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines. Of 5038 citations identified, eighty-six full papers were screened, and nine studies met the inclusion criteria.

Results: The populations studied and the molecular techniques used to assess clonality of UTUC and paired UBC differed largely over time. Eight studies reported on primary UTUC and meta- or synchronous IVR without a history of UBC. A total of 118 tumors (55 UTUC and 63 IVR) from 49 patients were included, of which 94% seemed to be clonally related. Five studies reported on primary UBC and subsequent UTUC with a total of 61 tumors (30 UBC and 31 UTUC) from 14 patients; a possible clonal origin was identified for 85% of the tumors.

Conclusion: Taking into account the limitations of microsatellite technology in comparison to *Next Generation Sequencing* (NGS) and currently accepted concepts of tumor heterogeneity and evolution, this systematic review shows that most, if not all, UTUC and paired UBC likely are clonally related.

1. INTRODUCTION

Urothelial carcinomas can arise throughout the entire urinary tract, but the urinary bladder is the predominant side of origin. The incidence of upper urinary tract urothelial carcinoma (UTUC) is 1-2 per 100,000 persons/year in Western Europe, and UTUC accounts for 5-10% of all urothelial carcinomas [1]. UTUC and urothelial bladder cancer (UBC) are considered similar entities. Accordingly, results of studies on UBC are often extrapolated to UTUC. Although UBC and UTUC share certain histopathological characteristics and have several risk factors in common, with tobacco use as the most imperative one, important clinical and molecular differences exist between the two entities [2]. At diagnosis, 60% of UTUC patients have an invasive tumor versus 20-25% of UBC patients [1,3]. Hence, the prognosis of UTUC is poor with a 5-year overall survival (OS) of approximately 70%; for invasive disease the 5 year OS is less than 40%, which is lower than reported for UBC patients treated with radical cystectomy [4,5]. Recent genomic characterization of UTUC revealed different molecular alterations in comparison to UBC and, in contrast to UBC, UTUC seemed to be associated with Lynch syndrome (LS) [6-8].

Following radical nephroureterectomy (RNU), which is the recommended treatment for non-metastatic UTUC, intravesical recurrence (IVR) within the first two years following surgery is found in 22-47% of the patients [1,9,10]. Clinical risk factors for the development of an IVR following RNU are: a history of UBC, tumor multiplicity, tumor location (distal ureter), advanced tumor stage, and the operative modality [11]. Guidelines recommend administration of a single dose of intravesical chemotherapy within 10 days after RNU to reduce the risk of a future IVR [1,12,13]. A neoadjuvant regimen of intravesical Mytomicin C is being evaluated in an ongoing multicenter study [14].

UTUC patients also have an increased risk of developing a tumor in the contralateral upper urinary tract; 2-6% develop a recurrence in the contralateral upper urinary tract following RNU [15]. Moreover, the incidence of concomitant UBC at the time of diagnosis of primary UTUC is 17% [16], whereas the risk of developing an UTUC following the diagnosis of a primary UBC is much

lower. In a cohort of 1,529 patients with primary non-muscle invasive UBC, the incidence of a subsequent UTUC was only 2.6%, although the proportion was higher in multifocal and high-risk tumors [17]. In summary, urothelial carcinoma is an important risk factor for developing a subsequent tumor throughout the entire urinary tract; patients with a primary UTUC have the highest risk of developing a recurrence in the bladder.

Two hypotheses have been proposed for the increased risk of recurrence in the urinary tract following a primary diagnosis of urothelial carcinoma. One hypothesis is that the entire urinary tract is affected by carcinogenic hits [18], which results in multifocal tumors that develop independently from one another. These tumors are therefore thought not to share the same progenitor cell. However, this would not explain the difference in incidence of UTUC and UBC in general, nor the difference in incidence of tumors in the contralateral urinary tract vs. the bladder after a primary diagnosis of UTUC. The second hypothesis states that by intraluminal seeding or intraepithelial spread, tumor cells located in the upper urinary tract implant in the bladder and give rise to a recurrence [19,20]. In the latter, IVR will be of monoclonal origin as it arises from the antecedent UTUC. This hypothesis seems plausible taking into account the low incidence of UTUC and hence the chance that a patient would develop two or more tumors that derive in different parts of the urinary tract is very low [21,22]. In 2002, a review concluded that the majority of the studies investigating the clonal relationship of multiple urothelial carcinomas of the urinary tract revealed tumors to be of monoclonal origin [23].

We present the results of a systematic review of all the relevant and recent literature addressing whether synchronous and metachronous urothelial carcinoma of the upper urinary tract and bladder are clonally related.

2. MATERIALS AND METHODS

The electronic databases Medline (Ovid) and Embase, the Cochrane Central Register of Controlled Trials, and the Cochrane Database of Systematic Reviews were searched for citations published between January 2000 and July

2019. The review was performed according to the Preferred Reported Items for Systematic Reviews and Meta-analysis statement [24] and the protocol has been published in the PROSPERO database (CRD42018105617).

Original studies that performed a genomic characterization of UTUC and paired IVR (i.e. both tumors diagnosed in the same patient) were included, whereas studies that reported on a molecular analysis of UTUC and UBC samples not derived from the same patient were excluded (see **Figure 1**). Keywords arranged in variable combinations included "upper urinary tract urothelial carcinoma," "intravesical recurrences," "ureter," "renal pelvis," bladder urothelial carcinoma," "clonality," and "molecular genetics" (see **Supplementary Materials** for details of the search strategy). The search was complemented by cross-referencing of the studies included. Two reviewers (T.v.D. and J.L.B.) independently screened all abstracts and full-text articles. Disagreement was resolved by discussion, and if no agreement was reached, a third independent party acted as arbiter (E.C.Z.).

2.1 In- and exclusion criteria

Studies with UTUC patients who developed a subsequent IVR and studies with UBC patients who developed a subsequent UTUC were included. Studies that reported on patients who had recurrences limited to either the upper or lower urinary tract were excluded. At least one genomic alteration had to be present in one of the two paired tumors of a patient in order to be included in the final analysis.

2.3 Definition of a clonal relationship between UTUC and paired UBC

Monoclonal origin: Tumors were considered to be of clonal origin when both the UTUC and paired UBC shared synonymous/non-synonymous or noncoding somatic mutations, microsatellite instability (MSI), methylation and Loss of Heterozygosity (LOH). These molecular alterations had to be identical in expansion or deletion. An interface of 100% between the alterations of the two tumors was not considered mandatory since subclones derived from the primary tumor can expand in the number of alterations independently over time. A single concordant alteration, pattern of methylation, or LOH between two paired tumors, as

assessed by *Next Generation Sequencing (NGS), bisulfate sequencing, or Whole Exome Sequencing (WES)*, was considered determinative for a clonal relationship or a shared progenitor cell, as these techniques permit to approach the exact gene position of an alteration. The possibility that a shared molecular alteration alters on the exact same gene position in two analyzed tumors of the same patient was considered to be negligible, especially in 'passenger genes' [25].

Undefined clonal origin: In case of absence of concordant molecular alterations, we marked the paired tumors as 'undefined' and not of 'oligoclonal origin'. We chose to do so as for the analysis we were dependent on the (sometimes limited) number of markers/loci analyzed in the studies included. Theoretically, it could be possible that both tumors did share a progenitor cell and were clonally related but that the specific examined marker(s) did not cover that specific alteration. In those cases, it was not possible to exclude clonality and, as such, tumors were classified as 'undefined'.

It is important to stress that the determination of clonal relatedness by the aforementioned definitions in some cases differed from the original authors' conclusions, which discrepancy might lead to a different assessment of clonally related tumors.

2.4 Definition of synchronous UTUC and UBC

A synchronous recurrence was defined if both tumors, either UTUC or UBC, were diagnosed within three months following the diagnosis of the primary tumor.

2.5 Calculation of proportion of clonally related tumors

The large variety of techniques used to analyze clonality of UTUC and paired UBC precluded a formal meta-analysis. All patients were considered to share equal weight in the final analysis, i.e. a patient with multiple recurrences should have the same contribution to the analysis as a patient with only one recurrence. To do so, the contribution of a patient for the final analysis was calculated as follows:

$$patients\ contribution = \frac{1}{n} \cdot\ n_c$$

In which *n* is the number of recurrences and n_c is the number of clonally related recurrences.

For example: the contribution to the final analysis of a patient who had five recurrences (n=5), of which four (n_c=4) were clonally related to the primary tumor, was considered 0.8.

$$patients\ contribution = \frac{1}{5}\ x\ 4 = 0.8$$

The final percentage of clonally related tumors per study was calculated with the formula:

$$percentage\ clonally\ related\ tumors = \frac{\sum nc}{\mathrm{N}} \cdot 100\%$$

In which *N* is the total number of patients from a study and $\sum n_c$ is the sum of all clonal contributions of all included patients of that study.

3. RESULTS

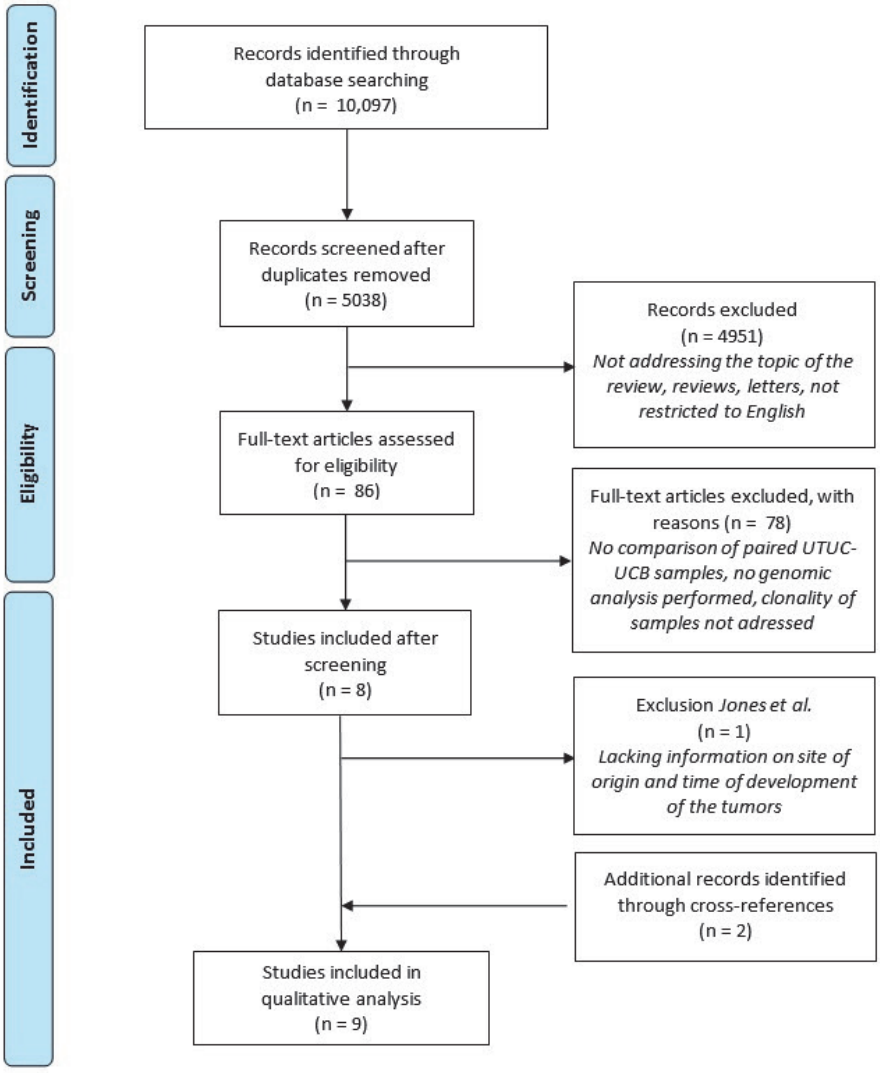
After removal of duplicates, titles and abstracts of 5038 records identified in the initial search were screened for relevance. In total, 4951 abstracts were excluded because the inclusion criteria were not met. Eventually, 86 full-text papers were evaluated and nine studies met the inclusion criteria (see **Figure 1**). Forty-six of the 78 studies that were excluded performed a genomic characterization of either UBC or UTUC without a comparison between the two entities; seven studies focused on prognostic molecular markers; 11 records were reviews; 11 studies did not include any genomic analysis; and three studies analyzed unpaired cohorts of UBC and UTUC. Furthermore, a publication by *Jones et al.* was excluded from the analysis because information on the site of origin in the urinary tract and the timing of tumor development was lacking [26]. See **Table 1** for an overview of the nine studies included in this review.

Table 2: Study characteristics of the series that analyzed a possible clonal relationship of UTUC and IVR/UBC (n = 9).

	Year	Study design	Number of patients	Paired UTUC-UBC samples	Number of tumors
Takahashi et al. [63]	2000	Case report	1	Yes	1 UTUC, 1 UBC
Dalbagni et al. [64]	2001	Retrospective	13	Yes	11 UTUC, 39 UBC
Hafner et al. [65]	2001	Retrospective	19	Yes	6 UTUC, 16 UBC *72 UC
Takahashi et al. [66]	2001	Pro- and retrospective	15	Yes	16 UTUC, 18 UBC
Catto et al. [67]	2006	Prospective	9	Yes	12 UTUC, 20 UBC
Warrick et al. [68]	2010	Retrospective	1	Yes	3 UTUC, 2 UBC
Wang et al. [69]	2013	Retrospective	5	Yes	6 UTUC, 6 UBC
Du et al. [70]	2017	Retrospective	3	Yes	10 UTUC, 4 UBC
Audenet et al. [71]	2018	Prospective	29	Yes	29 UTUC, 29 UBC

**Location in urinary tract not specified. UC: urothelial carcinoma*

Figure 3: PRISMA flow diagram of the study.



3.1 Primary UTUC and subsequent UBC

Eight of the nine studies included patients who had a primary UTUC and a meta- or synchronous IVR without a history of UBC (see **Table 2**). Since some patients developed more than one UTUC and/or IVR, a total of 118 (55 UTUC, 63 IVR) tumors from 49 patients were included in the analysis (Supplementary information **Figure S1**). The paired tumors had been analyzed for clonality by various techniques, which had changed over time. In total, 93.5% of the patients had concordant patterns of molecular alterations, indicating that a large proportion of IVR and UTUC were of monoclonal origin [27-31,33-35].

Takahashi et al. (2000) analyzed one case of primary UTUC and IVR for microsatellite shifts and LOH of chromosomes 2, 4, 8, 9, 11, and 17 using 21 markers [27]. Each tumor showed LOH of chromosomes 9q and 17p, and the IVR had additional LOH of chromosomes 2q, 4p, and 11p (**Fig. S1, patient #1**). These tumors were considered to be of clonal origin.

Dalbagni et al. assessed mutations of the *TP53* gene in four patients who had 16 tumors (5 UTUC, 11 IVR) [28]. All tumors showed identical mutations of *TP53* and thus the paired tumors were considered to be of monoclonal origin (**Fig. S1, patient # 2 - #5**). *Hafner et al.* assessed mutations in *TP53* exons 5 to 9, LOH of chromosome 9, MSI at six loci, and protein expressions of hMLH1 and hMSH2 in 15 patients [29]. This study only reported data on a patient considered not to have clonally related paired tumors; consisting of one UTUC followed by three IVRs. The UTUC had loss of the short allele of D9S113, whereas the IVRs had loss of the longer allele of the same marker. The three IVRs also had identical alterations of *TP53*, which were not present in the UTUC. Therefore, we could not consider these tumors as clonally related and were therefore scored as 'undefined' (**Fig. S1, patient #6**).

Catto et al. combined MSI analysis using 17 markers together with methylation of seven promoter regions [31]. MSI analysis was performed in 210 patients; only nine patients had a UTUC and an IVR showing MSI. Five of these nine patients had a primary UTUC followed by one or multiple IVR(s). All paired tumors of these five patients shared at least one identical alteration of the methylation

markers or had a similar pattern of MSI indicating a clonal relationship (**Fig. S1, patient #20 - #24**).

In a second study by *Takahashi et al.* (2001), which used identical markers as in the 2000 study, a total of 14 UTUC patients who developed 16 IVRs were analyzed [30] (**Fig. S1, patient #7 - #19**). Only one patient seemed to have discordant molecular alterations; the primary UTUC showed no alterations in the analyzed markers, while the two IVRs had LOH of a marker on chromosome 11p (**Fig. S1, patient #17**). Therefore, we considered the clonal relationship of the paired tumors as 'undefined'.

Wang et al. analyzed paired tumors of five patients by three markers for LOH of chromosome 9 and exons 5-8 of the *TP53* gene [33] (**Fig. S1, patient #25 - #29**). All five paired samples showed identical patterns of chromosomal loss or *TP53* mutations. One patient, however, had an identical pattern of *TP53* and D9S303, with a complete loss of D9S171 in the UTUC. Conversely, the IVR only had a loss of the shorter allele (**Fig. S1, patient #26**). It is possible that tumor cells of the primary tumor had seeded or migrated to the bladder in the possession of LOH of the shorter allele of D9S171. Due to evolution, the UTUC might have lost the other allele, contributing to a discordant pattern of this marker between the two tumors. However, an identical LOH pattern is considered an indication of clonality [59], whereas identical point mutations in the *TP53* gene are considered even a stronger indication because many possible point mutations exist that lead to inactivation of *TP53*. Hence, we concluded that all tumors were clonally related (**Fig. S1, patient #25 - #29**).

Du et al. analyzed by *whole exome sequencing* (WES) three cases: one female patient had three synchronous UTUCs and two IVRs; the other two patients each had one UTUC and one IVR [70] (**Fig. S1, patient #30 - #32**). The three UTUCs and one IVR shared the same alterations in *TP53, BRAF*, and *APC* genes. The other IVR had a mutation in *MTOR* and shared no alterations with the other tumors, so there was no proof of clonality (**Fig. S1, patient #31**). One of the two other patients showed a clonal relationship of both tumors (**Fig. S1, patient #32**). The other patient showed no shared alterations and, since *Du et al.*

used WES, this is a strong indication that these tumors were not clonally related (**Fig. S1, patient #30**).

Audenet et al. applied NGS with the targeted 230 to 468-gene MSK-IMPACT oncopanel to analyze primary UTUCs of 17 patients who subsequently developed an IVR [35,36]. All paired tumors had identical point mutations, which is a strong indicator of monoclonal origin as the chance that identical point mutations develop independently is highly unlikely. Comparing the somatic mutations between the initial UTUC and the subsequent IVR revealed that 86% of the mutations were present in both tumors. Hence, the additional mutations of the IVR were presumably caused by ongoing tumor evolution (**Fig. S1, patient #33 - #49**).

Table 2: Overview of the studies that analyzed patients diagnosed with a primary UTUC and who subsequently developed a UBC, the molecular techniques used, and the proportion of clonally related tumors (n = 49 patients).

	Patients (n)	Number of tumors (n)	Median months to recurrence (range)	Target/Technique	Number of patients with clonally related tumors	Percentage clonally related (%)
Takahashi et al., 2000[63]	1	1 UTUC, 1 IVR	8	LOH (MSI markers): chromosome 9p, 9q, 11p, 17p, 4p, 4q, 2q, 8p;	1/1	100%
Dalbagni et al., 2001[64]	4	5 UTUC, 11 IVR	14.5 (1-38)	*TP53* (exons 5-8).	4/4	100%
Hafner et al., 2001[65]	1	1 UTUC, 3 IVR	NA	LOH (chrom 9); p53 (exons 5-9); MSI (six loci); IHC (MLH1, MSH2).	0/1	0%
Takahashi et al., 2001[66]	13	14 UTUC, 16 IVR	9.0 (0-28)	LOH (MSI markers): chromosome 9p, 9q, 11p, 17p, 4p, 4q, 2q, 8p;	12/13	92.3%
Catto et al., 2006[67]	5	7 UTUC, 6 IVR	23.4 (0-47)	MSI-H; Methylation promoter regions: *hMLH1, p16, p14, E-cadherin, RARB, RASSF1A, MINT31.*	5/5	100%
Wang et al., 2013[69]	5	5 UTUC, 5 UBC	NA	LOH (9q21, 9q32, 9q22); *TP53* (17p13).	5/5	100%
Du et al., 2017[70]	3	5 UTUC, 4 IVR	0	WES; Somatic variants; Copy number; Mutational signature.	1.8*/3	60.0%
Audenet et al., 2018[71]	17	17 UTUC, 17 IVR	22.1 (3-87.8)	MSK-IMPACT (NGS).	17/17	100%

LOH: Loss of Heterozygosity, MSI: Microsatellite Instability (H = high), WES: Whole Exome Sequencing, MSK-IMPACT: Memorial Sloan Kettering Cancer Center integrated mutation profiling of actionable cancer targets (275-468 genes).

4 of the 5 (80%) tumors from one patient showed a clonal origin.

3.2 Primary UBC and subsequent UTUC

Five of the studies included evaluated the possible clonal relationship in patients diagnosed with primary UBC who subsequently developed a recurrence in the upper urinary tract. Since some patients developed more than one UBC and/or UTUC following the primary diagnosis of UBC, a total of 14 patients having 30 UBCs followed by 31 UTUCs (see **table 3**) were included. A total of 85.1% of the tumors were considered to be of monoclonal origin [29-32,35].

In four of the studies, which included 11 patients with 19 UBCs and 15 UTUC recurrences, all tumors originating from one patient had identical alterations, indicating a monoclonal origin [30-32,35]. The studies by *Catto et al., Takahashi (2001) et al.* and *Audenet et al.* are discussed in section 3.1 above [30,31,35]. The techniques used to analyze a clonal relationship did not differ for patients having a primary UBC and a subsequent UTUC. These three studies showed all paired tumors to be of monoclonal origin (**Fig. S2, patient #4 - #6 and #8 - #14**).

Warrick et al. included one patient having one UBC and three UTUC and found with the use of NGS identical mutations in the genes *HRAS, FLT4, MLL2, NTRK3*, and *PIK3CA*[32]. Copy number analysis and LOH revealed a compatible pattern of gain and loss between the paired tumors (**Fig. S2, patient #7**).

Hafner et al. included patients having one or multiple UBC(s) followed by one or more UTUC [29]. Two patients had one UBC with one subsequent UTUC and both tumors could not be defined as clonally related (**Fig. S2, patient #1 and #2**). The other patient had multiple urothelial carcinomas, i.e., nine UBCs with three subsequent UTUCs (**Fig. S2, patient #3**). Clustering, based on the reported molecular markers, showed that multiple UTUC and UBC shared common alterations and these were therefore marked as clonally related (**Figure S3**). One UBC had a distinct pattern of alterations, however, and was marked as ‘undefined’.

Table 3: Overview of the studies that analyzed patients diagnosed with a primary UBC and who subsequently developed a UTUC, the molecular techniques used, and the proportion of clonally related tumors (n = 14 patients).

	Patients (n)	Number of tumors (n)	Median months to recurrence (range)	Target/Technique	Number of patients with clonally related tumors	Percentage clonally related (%)
Hafner et al., 2001[65]	3	11 UBC, 5 UTUC	24 (0-43.0)	LOH (chrom 9) TP53 (exons 5-9) MSI (six loci) IHC (MLH1, MSH2)	0.92*/3	30%
Takahashi et al., 2001[66]	1	5 UBC, 3 UTUC	*NI*	LOH (MSI markers): chromosome 9p, 9q, 11p, 17p, 4p, 4q, 2q, 8p; Subchromosomal breakpoints.	1/1	100%
Catto et al., 2006[67]	2	6 UBC, 2 UTUC	17.0 (0-31.0)	MSI-H; Methylation: *hMLH1, p16, p14, E-cadherin, RARB, RASSF1A, MINT31.*	2/2	100%
Warrick et al., 2015[68]	1	1 UBC, 4 UTUC	0	NGS (409 genes); LOH; CNV.	1/1	100%
Audenet et al., 2018[71]	7	7 UBC, 7 UTUC	7.3 (3.9-21.7)	MSK-IMPACT (NGS).	7/7	100%

IHC: Immunohistochemistry, NGS: Next Generation Sequencing, CNV: Copy Number Variations, NI: not informative

**12 of the 13 (92%) tumors from one patient showed a clonal origin.*

4. DISCUSSION

We conducted a systematic review of the relevant literature on the possible clonal relation of synchronous and metachronous urothelial carcinoma of the upper urinary tract and bladder. Based on the available literature, we concluded that the majority of UTUC and paired UBC had a clonal relation. Literature on this matter, however, was scarce and the techniques used differed significantly between series and over time. Some of the techniques used are nowadays considered less accurate to address a possible clonal relation of two tumor entities. Conversely, currently available large-scale sequencing techniques such as WES or *Whole Genome Sequencing* (WGS) can much better provide profound evidence whether paired UTUC and UBC samples are of monoclonal origin, as the probability that point mutations occur multiple times independently from another is negligible. Hence, the more recent studies included in this review provide more conclusive evidence on clonally related UTUC and paired UBC.

The order of clinical detection of multiple tumors in visceral organs is not always in line with the molecular development of the tumors. This characteristic has previously been proposed for multiple metachronous UBC by *van Tilborg et al.* [37]. Moreover, clones that derive from a primary tumor of the upper urinary tract could evolve over time and develop additional genomic alterations. An IVR derived from such a clone, however, could be diagnosed prior to the primary UTUC and molecular analysis of both entities will, in such cases, reveal more genomic alterations of the IVR in addition to overlapping mutations. This 'tumor evolution' could also apply to the primary UTUC. Therefore, not all alterations will necessarily be shared by two paired tumors due to evolution of tumors, although a large proportion will. Consequently, a 100% overlap of alterations is rarely present in clonally related UTUC and paired UBC, as *Audenet et al.* demonstrated with an 86% overlap [35]. Therefore, when analyzing recurrences in the urinary tract and when interpreting a clonal or a non-clonal relationship of both entities, one should be aware that the clinical order is not necessarily the molecular order of tumor development [31,37].

The proportion of patients diagnosed with a primary UBC who later developed a UTUC recurrence might be overestimated in the studies included. Twelve of the 29 (41.3%) patients analyzed by *Audenet et al.* had a primary UBC followed by a diagnosis of UTUC, which is a higher proportion than that reported in the literature (1-5%) [1,17]. Four of these twelve patients, however, showed a clonal relationship compatible with a previously developed UTUC instead of a primary UBC, as the UBCs showed a surplus of alterations compared to the UTUCs. Therefore, it is possible that the UTUCs originated first and the UBCs were clones or subclones of the UTUC with an accumulation of molecular alterations, and had developed later than the UTUCs.

Whether IVR are formed by seeding/migration of tumor cells originating from the upper urinary tract or by field cancerization remains subject of debate [18-20]. The majority of patients develop an IVR within two years following RNU, possibly due to manipulation of the tumor during surgery [11]. This hypothesis of distributing tumor cells by manipulation is further supported by the fact that a diagnostic ureterorenoscopy prior to RNU increases the risk of an IVR [38]. In addition, a systematic review showed that instability of the UTUC, defined by presence of necrosis and positive preoperative urinary cytology, correlated with the risk of IVR [11]. As we found in the present review that 94% of the primary UTUC and IVRs were clonally related, we assume that in primary UTUC patients the most important mechanism of developing an IVR is seeding or migration of tumor cells. However, it is not excluded that field cancerization could contribute to the development of separate entities of urothelial carcinoma in the upper and lower urinary tract. Analyzing a cohort of 512 UTUCs, *Xylinas et al.* showed that smoking was significantly associated with the risk of an IVR [39]. *Du et al.* addressed exposure to the Aristolochic Acid (AA) [34], a widely used herb in Chinese medicine, in a Chinese patient cohort and found that all tumors had predominant T to A transversions in the 5'-CpTpG-3'motif, which is a mutational signature caused by AA [40]. The mutagenic aspect of this herb might contribute to field cancerization in patients and hence to the development of non-clonally related urothelial tumors. Patients with Lynch syndrome (LS) have a higher risk of developing urothelial carcinoma, mainly UTUC [8]. LS is a hereditary cancer syndrome characterized by mutations in mismatch repair genes leading to mismatch repair deficiency and MSI. Possibly,

LS could lead to the independent development of UTUC and UBC, but literature is lacking on this matter. One LS patient analyzed by *Audenet et al.* showed a clonal relation of paired UTUC and IVR *(personal communication F. Audenet)*.

Clonality of primary tumors and metachronous or synchronous intracaval recurrences have been analyzed in malignancies originating from other hollow visceral organs than the urinary tract, such as the lung, colon, and oral cavity. LOH analysis and mutational status of *EGFR, TP53,* and *KRAS* in multifocal lung cancer (n = 115) revealed that 64-79% of multiple synchronous intrapulmonary, mostly non-small cell carcinomas (NSCLCs), were clonally related 41-43]. For tumors of the oral cavity, however, it was not clear whether multiple tumors resulted from field cancerization or intraluminal spread [44]. With the use of microarray-based SNP and copy-number genotyping of 104 paired synchronous colorectal cancers, a clonal relationship was found in 36% [45]. Patients with oligoclonal NSCLSCs seemed to have a better outcome than patients with NSCLCs of monoclonal origin, which has also been reported for patients with oligoclonal colorectal tumors [43,45]. These data show that clonality of paired tumors originating from the same hollow visceral organ might correspond with clinical outcome. Therefore, it is of importance to investigate this phenomenon in the urinary tract by larger, prospective studies.

In case UTUC and IVR are clonally related, the way is paved for the identification of patient-specific genomic alterations that can be used to develop non-invasive urine-based assays for the diagnostic surveillance following RNU. Cystoscopy, which is invasive and causes discomfort to the patient, might be replaced by this alternative urine-based strategy [46]. Large-scale genomic characterization of UTUC and paired bladder recurrences could also identify new biomarkers that correlate with the risk of a future urinary tract recurrence or clinical outcome and possibly new actionable molecular alterations. With an accuracy of only 62-69% of two previous designed predictive tools for the risk of IVR development after RNU, addition of biomarkers might provide a better prediction of recurrences [47,48].

5. CONCLUSION

Patients diagnosed with a urothelial carcinoma of the urinary tract are at increased risk of developing a subsequent tumor throughout the entire urinary tract. Patients with a primary UTUC have the highest risk of developing a future UBC. We systematically reviewed all the relevant literature to address whether UTUC and paired UBC derive from the same progenitor cell or whether they develop independently as a result of field cancerization. The populations studied and the molecular techniques used to assess clonality differed largely between the studies and over time. Taking into account the limitations of microsatellite instability technology versus NGS and the currently accepted concepts of tumor heterogeneity and evolution, we conclude that it is highly likely that UTUC and paired UBC of one patient are clonally related and most likely are formed by seeding of tumor cells.

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LEGENDS TO (SUPPLEMENTARY) FIGURES

- **Figure 1**: PRISMA flow diagram of the study.

- **Figure S1**: Results of the studies that analyzed clonal relation of UTUC and IVR

- **Figure S2:** Results of the studies that analyzed possible clonal relation of UBC and UTUC

- **Figure S3:** Clustering of tumors analyzed by *Hafner et al.* within one patient. The molecular order of the tumors (right) differed from the order of clinical detection of the tumors (left).

STRATEGY SUPPLEMENTARY

Update April 19th 2018: 4520 – Sept 20th 2018: +219 – June 14th 2019: +297

Results

Database	*Number of refs*	*Refs after deduplication*
Embase.com	3045	2983
Medline Epub (Ovid)	3298	603
Cochrane Central	91	66
Web of Science	3463	1307
Google Scholar	200	77
Total	***10097***	***5036***

Deduplicated: 5061

Embase.com (Embase incl. Medline): 3045

('upper urinary tract urothelial carcinoma'/de/**mj** OR 'upper urinary tract transitional cell carcinoma'/de**/mj** *OR* 'ureter tumor'/exp**/mj** OR 'kidney pelvis cancer'/de**/mj** OR 'kidney pelvis carcinoma'/de**/mj** OR 'kidney pelvis tumor'/de**/mj** OR 'transitional cell carcinoma'/de**/mj** OR ((('upper urinary tract' OR 'kidney pelvic' OR 'kidney pelvis' OR ureter* OR 'transitional cell' *OR urothelial**) NEAR/6 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*))):ab,ti**) AND (**'bladder tumor'/exp**/mj** OR 'transitional cell carcinoma'/de**/mj** OR (((bladder* OR 'transitional cell' OR urothelial*) NEAR/6 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*))):ab,ti**) AND (**'molecular genetics'/exp**/mj** OR 'molecular pathology'/de**/mj** *OR 'gene expression profiling'/de* OR (genom* OR genetic* OR *((gene OR genes OR MicroRNA OR 'Micro RNA' OR MiRNA) NEAR/4 (expressi* OR profil* OR alterati* OR mutat* OR mutant* OR loss* OR deletion*))* OR molecular*):ab,ti**) NOT (**'Conference Abstract' OR Editorial**)/it NOT (**[animals]/lim NOT [humans]/lim**)**

Medline Epub: 3298

(*Urologic Neoplasms/ OR *Urethral Neoplasms/ OR *Ureteral Neoplasms/ OR (exp *Kidney pelvis/ AND exp *Neoplasms**/**) OR *Carcinoma, Transitional Cell/ OR

(((upper urinary tract OR kidney pelvic OR kidney pelvis OR ureter* OR transitional cell *OR urothelial**) ADJ6 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*))).ab,ti.**) AND (***Urinary Bladder Neoplasms/ OR *Carcinoma, Transitional Cell/ OR (((bladder* OR transitional cell OR urothelial*) ADJ6 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*))).ab,ti.**) AND (***Molecular Biology/ OR *Pathology, Molecular/ *OR exp Gene Expression Profiling/* OR (genom* OR genetic* OR *((gene OR genes OR MicroRNA OR Micro RNA OR MiRNA) ADJ4 (expressi* OR profil* OR alterati* OR mutat* OR mutant* OR loss* OR deletion*))* OR molecular*).ab,ti.**) NOT (**congresses OR editorial**).**pt. **NOT (**exp animals/ NOT humans/**)**

Cochrane Central: 91

(((("upper urinary tract" OR "kidney pelvic" OR "kidney pelvis" OR ureter* OR "transitional cell" *OR urothelial**) NEAR/6 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*))):ab,ti**) AND (**(((bladder* OR "transitional cell" OR urothelial*) NEAR/6 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*))):ab,ti**) AND (**(genom* OR genetic* OR *((gene OR genes OR MicroRNA OR "Micro RNA" OR MiRNA) NEAR/4 (expressi* OR profil* OR alterati* OR mutat* OR mutant* OR loss* OR deletion*))* OR molecular*):ab,ti**)**

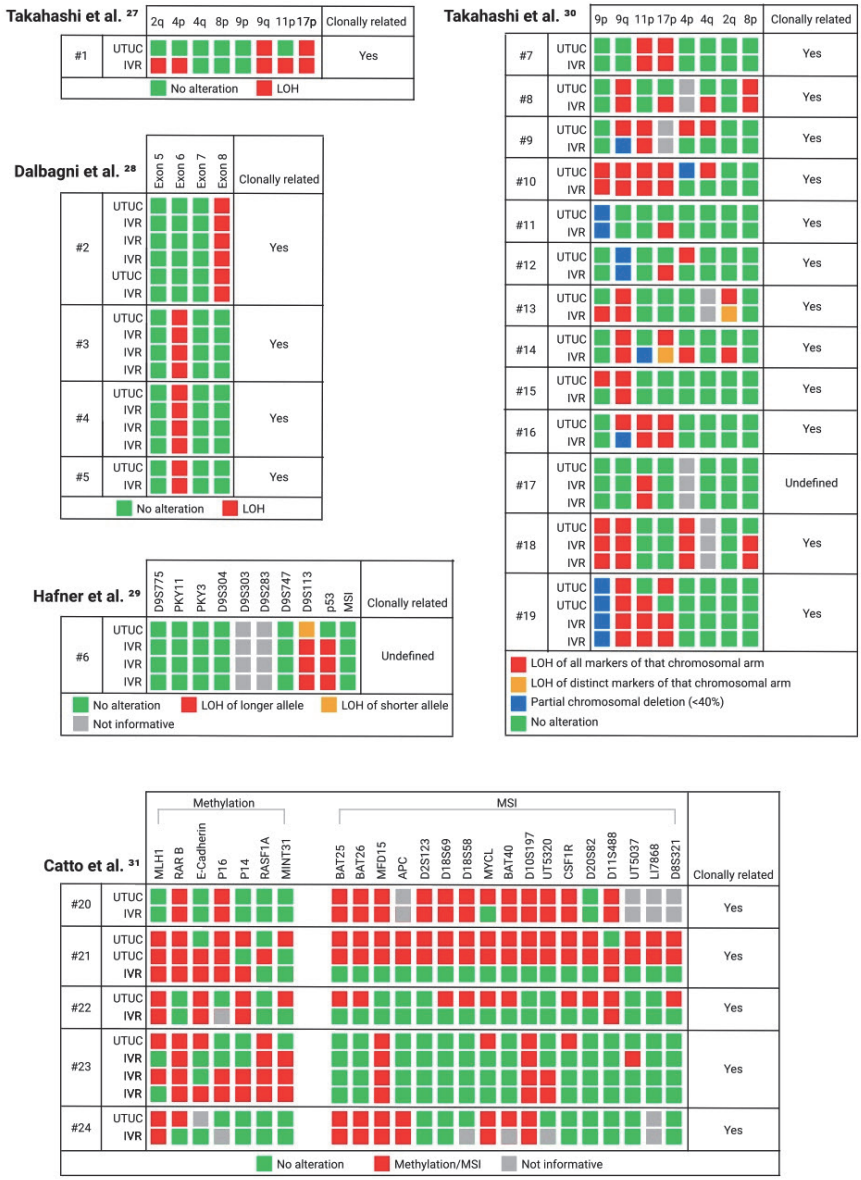
Web of Science: 3463

TS=((((("upper urinary tract" OR "kidney pelvic" OR "kidney pelvis" OR ureter* OR "transitional cell" *OR urothelial**) NEAR/5 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*)))**) AND (**(((bladder* OR "transitional cell" OR urothelial*) NEAR/5 (*neoplas* OR* cancer* OR carcinoma* OR tumor* OR tumour*)))**) AND (**(genom* OR genetic* OR *((gene OR genes OR MicroRNA OR "Micro RNA" OR MiRNA) NEAR/4 (expressi* OR profil* OR alterati* OR mutat* OR mutant* OR loss* OR deletion*))* OR molecular*)**) NOT (**(animal* OR mouse OR mice OR rat OR rats) NOT (human* OR patient*)**)) AND** DT=Article

Google Scholar: 200 *(top relevant refs)*

"upper urinary tract"|"kidney pelvic"|ureter|"transitional cell"|*urothelial* bladder|"transitional cell"|urothelial *neoplasms*|cancer|carcinoma|tumor genomics|genetic|*gene*|*genes*|*MicroRNA expression*|*profile*|*alteration*|*mutation*|*deletion*|molecular

Figure S1. Results of the studies that analyzed clonality of primary UTUC and IVR (n = 9)



Wang et al. [32]

		D9S171	D9S177	D9S303	TP53	Clonally related
#25	UTUC					Yes
	IVR					
#26	UTUC					Yes
	IVR					
#27	UTUC					Yes
	IVR					
#28	UTUC					Yes
	IVR					
#29	UTUC					Yes
	IVR					

Complete loss of allele
LOH of shorter allele
No alteration
Not informative

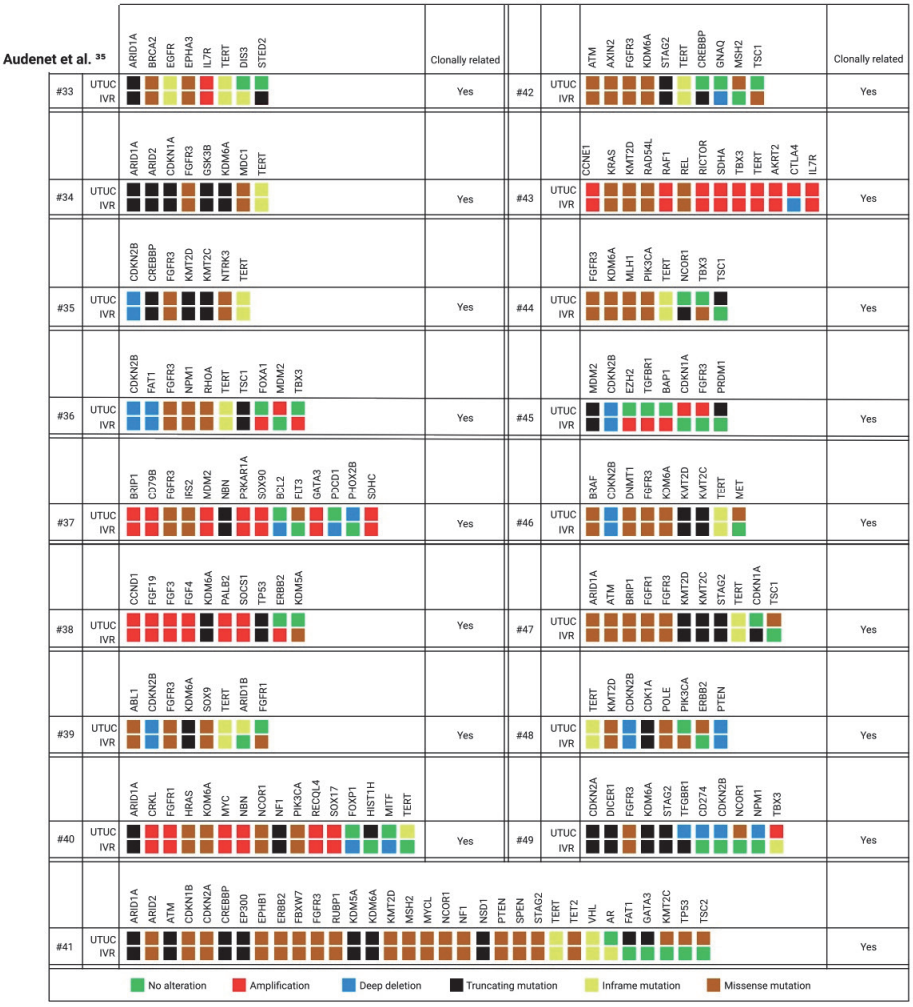
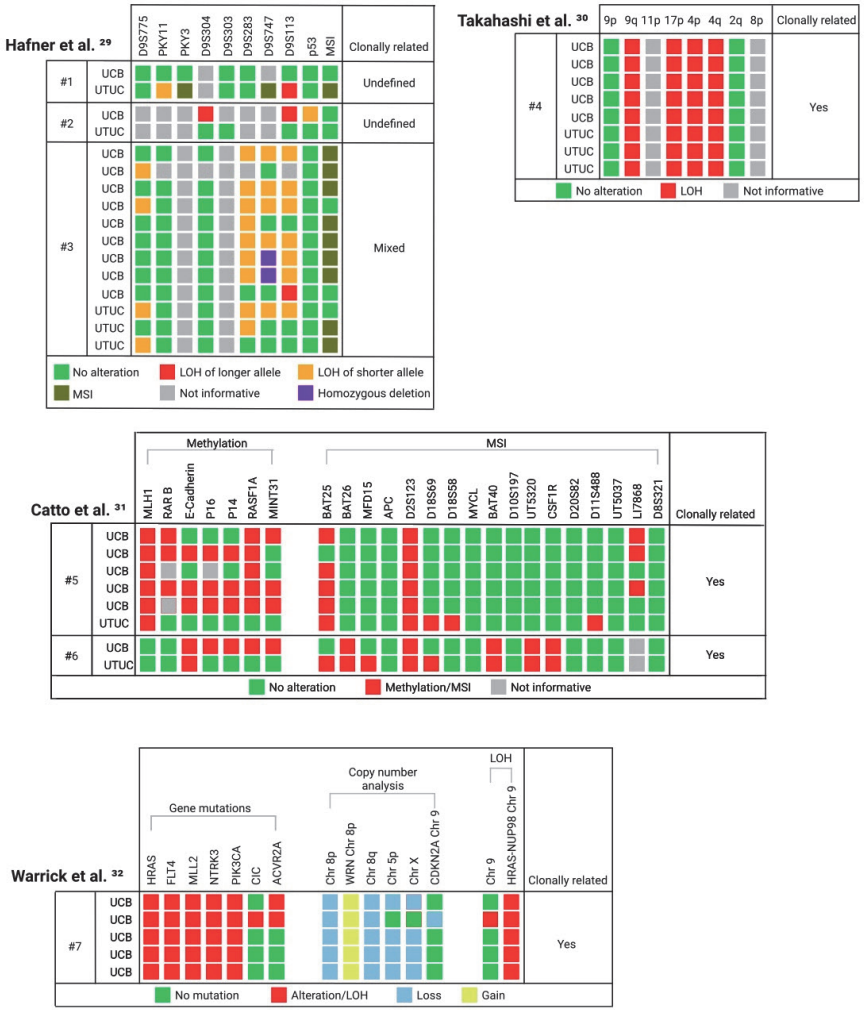

Audenet et al. [35]
#33
UTUC
IVR
ARID1A
BRCA2
EGFR
EPHA3
IL7R
TERT
DIS3
STED2
Clonally related
Yes
#34
ARID1A
ARID2
CDKN1A
FGFR3
GSK3B
KDM6A
MDC1
TERT
Yes
#35
CDKN2B
CREBBP
FGFR3
KMT2D
KMT2C
NTRK3
TERT
Yes
#36
CDKN2B
FAT1
FGFR3
NPM1
RHOA
TERT
TSC1
FOXA1
MDM2
TBX3
Yes
#37
BRIP1
CD79B
FGFR3
IRS2
MDM2
NBN
PRKAR1A
SOX90
BCL2
FLT3
GATA3
PJCD1
P-HOX2B
SDHC
Yes
#38
CCND1
FGF19
FGF3
FGF4
KDM6A
PALB2
SOCS1
TP53
ERBB2
KDM5A
Yes
#39
ABL1
CDKN2B
FGFR3
KDM6A
SOX9
TERT
ARID1B
FGFR1
Yes
#40
ARID1A
CRKL
FGFR1
HRAS
KDM6A
MYC
NBN
NCOR1
NF1
PIK3CA
RECQL4
SOX17
FOXP1
HIST1H
MITF
TERT
Yes
#41
ARID1A
ARID2
ATM
CDKN1B
CDKN2A
CREBBP
EP300
EPHB1
ERBB2
FBXW7
FGFR3
RUBP1
KDM5A
KDM6A
KMT2D
MSH2
MYCL
NCOR1
NF1
NSD1
PTEN
SPEN
STAG2
TERT
TET2
VHL
AR
FAT1
GATA3
KMT2C
TP53
TSC2
Yes
#42
ATM
AXIN2
FGFR3
KDM6A
STAG2
TERT
CREBBP
GNAQ
MSH2
TSC1
Yes
#43
CCNE1
KRAS
KMT2D
RAD54L
RAF1
REL
RICTOR
SDHA
TBX3
TERT
AKRT2
CTLA4
IL7R
Yes
#44
FGFR3
KDM6A
MLH1
PIK3CA
TERT
NCOR1
TBX3
TSC1
Yes
#45
MDM2
CDKN2B
EZH2
TGFBR1
BAP1
CDKN1A
FGFR3
PRDM1
Yes
#46
B3AF
CDKN2B
DNMT1
FGFR3
KDM6A
KMT2D
KMT2C
TERT
MET
Yes
#47
ARID1A
ATM
BRIP1
FGFR1
FGFR3
KMT2D
KMT2C
STAG2
TERT
CDKN1A
TSC1
Yes
#48
TERT
KMT2D
CDKN2B
CDK1A
POLE
PIK3CA
ERBB2
PTEN
Yes
#49
CDKN2A
DICER1
FGFR3
KDM6A
STAG2
TFGBR1
CD274
CDKN2B
NCOR1
NPM1
TBX3
Yes
No alteration
Amplification
Deep deletion
Truncating mutation
Inframe mutation
Missense mutation

Figure S2. Results of the studies that analyzed clonality of primary UCB and subsequent UTUC (n = 5)



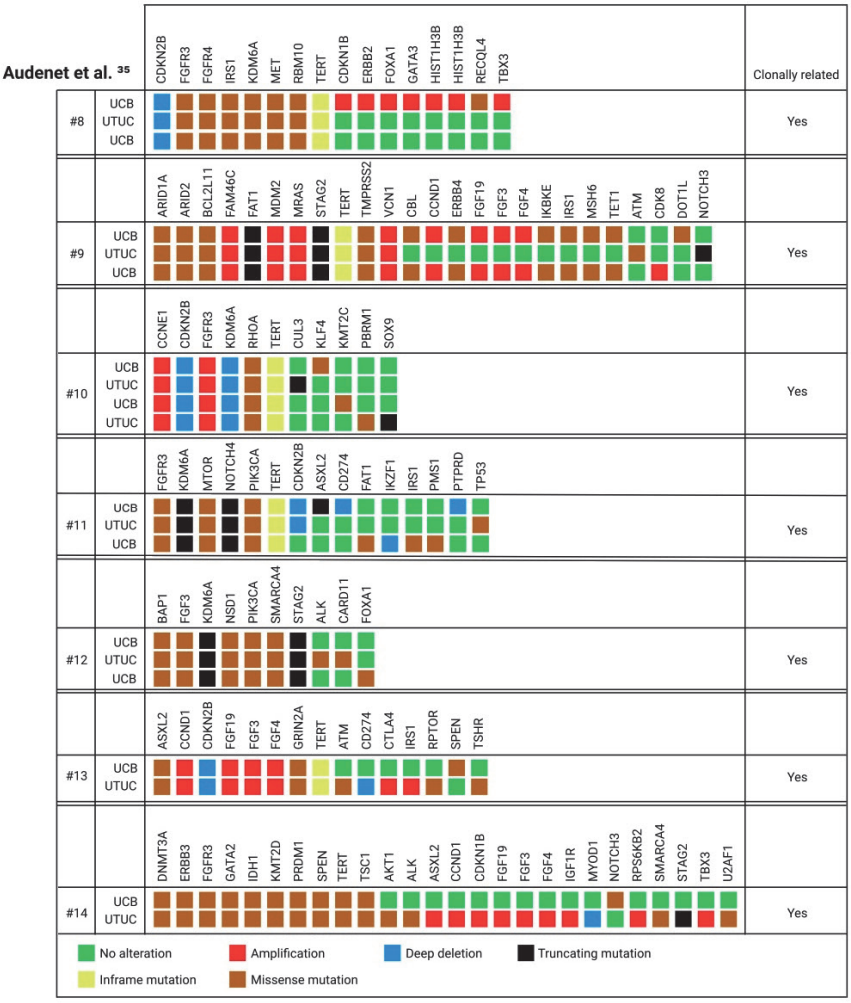
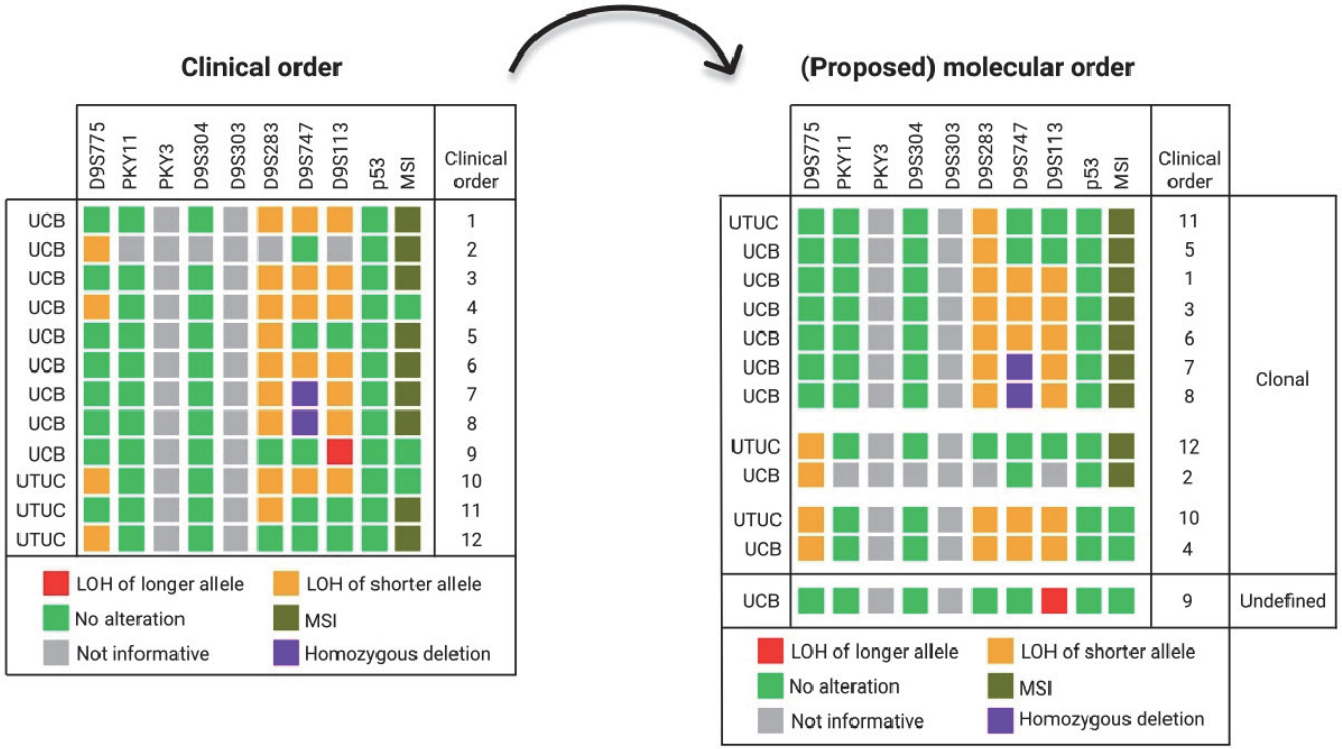

Audenet et al. [35]
CDKN2B FGFR3 FGFR4 IRS1 KDM6A MET RBM10 TERT CDKN1B ERBB2 FOXA1 GATA3 HIST1H3B HIST1H3B RECQL4 TBX3
Clonally related
#8 UCB UTUC UCB
Yes
ARID1A ARID2 BCL2L11 FAM46C FAT1 MDM2 MRAS STAG2 TERT TMPRSS2 VCN1 CBL CCND1 ERBB4 FGF19 FGF3 FGF4 IKBKE IRS1 MSH6 TET1 ATM CDK8 DOT1L NOTCH3
#9 UCB UTUC UCB
Yes
CCNE1 CDKN2B FGFR3 KDM6A RHOA TERT CUL3 KLF4 KMT2C PBRM1 SOX9
#10 UCB UTUC UCB UTUC
Yes
FGFR3 KDM6A MTOR NOTCH4 PIK3CA TERT CDKN2B ASXL2 CD274 FAT1 IKZF1 IRS1 PMS1 PTPRD TP53
#11 UCB UTUC UCB
Yes
BAP1 FGF3 KDM6A NSD1 PIK3CA SMARCA4 STAG2 ALK CARD11 FOXA1
#12 UCB UTUC UCB
Yes
ASXL2 CCND1 CDKN2B FGF19 FGF3 FGF4 GRIN2A TERT ATM CD274 CTLA4 IRS1 RPTOR SPEN TSHR
#13 UCB UTUC
Yes
DNMT3A ERBB3 FGFR3 GATA2 IDH1 KMT2D PRDM1 SPEN TERT TSC1 AKT1 ALK ASXL2 CCND1 CDKN1B FGF19 FGF3 FGF4 IGF1R MYOD1 NOTCH3 RPS6KB2 SMARCA4 STAG2 TBX3 U2AF1
#14 UCB UTUC
Yes
No alteration
Amplification
Deep deletion
Truncating mutation
Inframe mutation
Missense mutation

Figure S3. Clustering of tumors analyzed by Hafner et al [29] within one patient. The molecular order (right) differed from the order of clinical diagnosis (left).



4

THE CLONAL RELATION OF PRIMARY UPPER URINARY TRACT UROTHELIAL CARCINOMA AND PAIRED UROTHELIAL CARCINOMA OF THE BLADDER

Thomas van Doeveren, Jose A. Nakauma-Gonzalez, Andrew S. Mason, Geert J.L.H. van Leenders, Tahlita C.M. Zuiverloon, Ellen C. Zwarthoff, Isabelle C. Meijssen, Angelique C. van der Made, Antoine G. van der Heijden, Kees Hendricksen, Bas W.G. van Rhijn, Charlotte S. Voskuilen, Job van Riet, Winand N.M. Dinjens, Hendrikus J. Dubbink, Harmen J.G. van de Werken, Joost L. Boormans

International Journal of Cancer, 2020

Novelty and impact: In this study, we assessed the possible clonal relationship of upper urinary tract cancer and subsequent bladder carcinoma using targeted DNA sequencing. Since almost 75% of the patients had tumors which were clonally related this strongly suggest that seeding of tumor cells represents the most important mechanism of bladder carcinoma development following a radical nephroureterectomy. This result underscore the rationale to minimalize the risk of seeding during surgery, carefully consider the need of a diagnostic ureterorenoscopy plus biopsy per patient, and to apply peri-operative intravesical instillations with chemotherapy.

ABSTRACT

The risk of developing urothelial carcinoma of the bladder (UBC) in patients treated by radical nephroureterectomy (RNU) for an upper urinary tract urothelial carcinoma (UTUC) is 22-47% in the two years after surgery. Subject of debate remains whether UTUC and the subsequent UBC are clonally related or represent separate origins. To investigate the clonal relationship between both entities, we performed targeted DNA sequencing of a panel of 41 genes on matched normal and tumor tissue of 15 primary UTUC patients treated by RNU who later developed 19 UBCs. Based on the detected tumor-specific DNA aberrations, the paired UTUC and UBC(s) of 11 patients (73.3%) showed a clonal relation, whereas in four patients the molecular results did not indicate a clear clonal relationship. Our results support the hypothesis that UBCs following a primary surgically resected UTUC are predominantly clonally-derived recurrences and not separate entities.

INTRODUCTION

Patients undergoing radical nephroureterectomy (RNU) for upper urinary tract urothelial carcinoma (UTUC) have a 22-47% risk of developing a subsequent urothelial carcinoma of the bladder (UBC) within two years[1]. Two hypotheses have been proposed for this increased risk. Firstly, the entire urinary tract of patients with urothelial carcinoma undergoes a "field change", priming the tissue for independent transformations[2]. Upper and lower tract tumors therefore develop independently from one another and are not clonally related. Secondly, by intraluminal seeding or intraepithelial spread, cancer cells from the primary UTUC implant in the bladder wall and develop into a UBC resulting in clonally related tumors[3]. Recently, we performed a systematic review of the literature on the clonal relationship between UTUC and paired UBC and found that 94% of the cases originated from the same progenitor cell[4]. However, the molecular techniques used differed largely over time and research groups, plus only a limited number of studies used comprehensive large-scale DNA sequencing techniques, which enables more conclusive assessment of a clonal relation between these two entities.

In this study we used targeted DNA Next Generation Sequencing to analyze the clonal relationship of primary UTUC and subsequent UBC in patients treated with an RNU based on shared genomic alterations.

MATERIALS AND METHODS

DNA extraction

Tumor Hematoxylin and Eosin-slides were reviewed by an expert genitourinary pathologist (GvL) and regions containing ≥50% tumor cells were selected for DNA isolation (**Suppl. Table 2**). Tumor and corresponding normal tissue sections were manually microdissected in 5% Chelex 100 Resin (Bio-Rad, Hercules, CA, USA) Cell lysis solution (Promega, Madison, WI, USA). DNA was extracted by proteinase K (Roche, Mannheim, Germany) digestion at 56°C. Proteinase K was inactivated for 10 minutes at 95°C after which the samples were centrifuged for 5 minutes at 14000 rpm to collect cell debris and chelexresin.

Finally, DNA was collected into new tubes and the concentration was measured by Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), as described by the manufacturer.

Next Generation Targeted Sequencing

For targeted next-generation sequencing (NGS), a custom-made cancer panel was designed using the AmpliSeq designer (Thermo Fisher Scientific, Waltham, MA, USA). This panel comprised 330 amplicons covering 41 genes, multiple hotspot regions in various cancer-related genes, and 154 single nucleotide polymorphisms in multiple tumor suppressor regions to detect copy number variations (**Table 2 and Suppl. Table 3**)[5-7]. NGS was performed with the Ion Torrent platform using supplier's materials and protocols (Thermo Fisher Scientific). Median coverage depths were 1994x for UTUC, 1712x for UBC and 1914x for the adjacent normal tissue. Libraries were made using the Ion AmpliSeq Library Kit plus–384 LV, template was prepared with the Ion 510/520/530 Chef kit, and sequencing was performed on a 530-chip using the Ion S5 system. Data was analyzed using SeqPilot (JSI medical systems). To correct for potential germline mutations, NGS was also performed on DNA isolated from matched non-malignant kidney tissue. The final tumor cell percentage was calculated based on the DNA quality and quantity and the results of the NGS.

Genomic alterations

A visual inspection by an experienced technician (ICM) and clinical scientist (HJD) in molecular pathology making use of Torrent Variant Caller and SNPitty was carried out to identify the genomic alterations[6]. These genomic alterations were stored in VCF format[6,8]. **Figure 1** summarizes all detected genomic alterations; SNVs, Indels, Allelic Imbalance (AI), amplifications and homozygous deletions. For AI analysis, single nucleotide polymorphisms with a total coverage of >100 reads were included. For any informative SNP without AI a variant allele frequency (VAF) of 0.5 was expected. With a VAF of <0.5 (relative loss of variant allele) or a VAF of >0.5 (relative loss of reference allele) AI was indicated[9].

Clonality assessment

A possible clonal relationship between UTUC and subsequent UBC(s) was assessed by interrogating all single nucleotide variants (SNVs; including synonymous mutations), amplifications, Indels and supportive information on AI. To identify if a mutation that was reported in one sample but not in the paired other sample because of insufficient quality reads or absence of that mutation, the following steps were undertaken. A list of all mutations reported in one patient (UTUC and UBCs) was gathered. For every specific position, reads for normal and tumor samples (Phred quality score above ≥15) were subtracted from the BAM files using the bam2R function from the deepSNV (v1.30.0) R package[10]. Only sites where all samples (tumor and normal) reported a minimum total reads of 30x were included for clonality analysis. The total number of reads was the sum of reference reads plus alternative reads. The VAF from normal tissue samples (VAF_N) was used as reference to determine SNVs. SNVs and Indels were identified when $VAF_N < 0.10$ and $VAF_T > 0.10$. Three samples, the UBC's from patient II, V and VI, showed some degree of DNA degradation and the VAF_T threshold value was increased to 0.30 to discard most of the false positives with very low VAF_T.

The probability of a clonal relationship between UTUC and UBC samples from the same patient was evaluated following the clonality test approach developed by *Ostrovnaya et al.*[11]. The test was performed on all SNVs and Indels. As described by *Mauguen et al*., the clonality test based on SNVs and Indels was performed using the mutation reference data set for bladder cancer from the *TCGA* study[12]. More specifically, frequencies of specific SNVs are assumed to be known. The frequency $f = x / n$, where x is the number of tumors with a specific SNV and n is the total number of tumors based on $n = 411$ bladder cancer tumors from the *TCGA* cohort. Note that hotspot mutations would have high frequencies and rare mutations would have very low frequencies. When mutations have not been reported in the *TCGA* data set (in case of Indels and rare SNVs), the frequency of these mutations was estimated as $f = m / (n + m)$, where m is the number of patients carrying that specific SNV or Indel. The frequency of hotspot mutations in *TERT* promoter (*pTERT*) have not been included in the *TCGA* data set. We completed the data set by adding reported

frequencies of *pTERT* C228T (64%) and C250T (13%) mutations from a study by *Allory et al.*[13]. Based on the marginal frequency of all SNVs and Indels, the Likelihood Ratio test was applied to estimate the probability of a clonal origin of the paired UTUC and UBC[11]. P-values were adjusted with the Benjamin & Hochberg method and adjusted p-values < 0.05 were considered significant.

RESULTS

In total, 15 patients with primary UTUC, treated by RNU, who subsequently developed 19 UBCs, treated by transurethral resection of the bladder, were included. Patient, treatment, and tumor characteristics of the study population are listed in **Table 1** and **Suppl. Table 1**. Shared genomic variants revealed that UTUC and paired UBC(s) were clonally related in 11/15 patients (73.3%) (**Figure 1**). No significance (pAdj = 0.086) was found for the single shared *TERT* (C250T) mutation in patient IV, however comparable AI-patterns supported clonal origin. Patient XIII, diagnosed with Lynch Syndrome (LS), only shared a *Fibroblast Growth Factor Receptor* (FGFR)-3 mutation (p.R248C; c.742C>T) between both tumors. However, as this mutation only occurs in less than 1% of urothelial carcinoma, a clonal relationship remained statistically significant (pAdj = 0.025). Patients II and XV also exhibited only a single shared mutation between both tumors, but as these alterations are common hotspot mutations in urothelial carcinoma, the presence in both entities did not unambiguously reflect a clonal relation. In patients I and VI, we did not observe any shared somatic mutations, so could not support a clonal relationship.

DISCUSSION

Studies which used large-scale sequencing techniques to assess the clonality of UTUC and paired UBC are scarce. In 2017, *Du et al.* analyzed five patients with synchronous UTUCs (n=9) and UBCs (n=4) by whole exome sequencing[14]. Tumors were clonally related in only two patients; a lower proportion than we found in the present study. Exposure to aristocholic acid was linked to tumor development in all five patients, which possibly affected the entire urothelium leading to field cancerization. *Audenet et al.* reported on a cohort of 29 patients with paired UTUC and UBC, and found all tumors to be clonally

related, although this cohort also included patients with a history of primary UBC and some exhibited synchronous tumors[15]. In the present study, we only included patients with primary UTUC and metachronous UBC(s); an approach which more accurately reflects the natural course of surgically treated UTUC patients.

The observed differences in cohort clonality may reflect patient idiosyncrasies, but also highlight remaining technical challenges. Targeted panels do not to cover all genomic aberrations, so clonality might have been underestimated in this study. Shared alterations could have been missed due to the extent of this panel, which increases the likelihood that the UBCs, which were found not to be clonally related, could have been clonally-derived recurrences. Reductions in sequencing cost, and the application of whole genome or exome RNA-DNA sequencing, offer opportunities to expand the search for clonal markers. Tumor heterogeneity may be an alternative explanation for the ~25% of paired tumors we analyzed which did not appear clonally related: it cannot be unambiguously excluded that clonality was masked for these tumors. Furthermore, as a relatively rare cancer, there is limited data on UTUC-specific mutation frequencies. Pertinently, recent work proposed enrichment of the FGFR3 p.R248C amino-acid substitution in LS-linked UTUC, and so it is debatable whether this shared alteration alone indicates a clonal relationship in patient XIII. Particularly when LS-patients may exhibit a higher probability of developing multiple urinary tract tumors[16]. Notwithstanding these limitations, our observation that almost 75% of the paired tumors were clonally related strongly suggests that seeding of tumor cells from the upper urinary tract to the bladder represents the most important mechanism of UBC development following RNU. Importantly, three patients in our cohort developed multiple subsequent UBCs, and all tumors were clonally related to the primary UTUC, which further supports the mechanism of seeding of tumor cells.

CONCLUSION

The results of this study underscore the rationale to: **i)** minimalize the risk of seeding of tumor-cells during RNU; **ii)** carefully consider the need for diagnostic

work-up by ureterorenoscopy and biopsy, which can dissociate cancer cells, and **iii)** apply peri-operative intravesical instillations with chemotherapy to kill cancer cells floating in urine. Large-scale genomic characterization of a properly selected cohort of UTUC and paired UBC using unbiased sequencing techniques will overcome the aforementioned limitations and will further clarify clonal relationships between in-patient upper and lower tract urothelial carcinomas.

Conflict of interest: J.L. Boormans reports on consultancy work for MSD, Janssen, Ambu, Health care and Ismar, during the conduct of the study; and received a research grant from Decipher Biosciences. All other authors report no conflict of interest.

Data Availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement: No approval was required for the this study.

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Table 1. Patient, treatment and tumor characteristics of 15 patients diagnosed with a primary upper urinary tract urothelial carcinoma and a subsequent urothelial carcinoma of the bladder.

Variable		Variable	
Patient characteristics, n = 15			
Male sex – no. (%)	8 (53.3%)	Smoking status	
Age, yrs., median (IQR)	67 (12.5)	Never	3 (20.0%)
		Former	9 (60.0%)
		Current	3 (20.0%)
Treatment characteristics, n = 15			
Preoperative URS – no. (%)	10 (66.7%)	Hospital that performed RNU – no. (%)	
Bladder cuff removal – no. (%)	10 (66.7%)	Erasmus Medical Center, Rotterdam	7 (46.7%)
Perioperative systemic chemotherapy – no. (%)	0 (0.0%)	Netherlands Cancer Institute, Amsterdam	7 (46.7%)
Perioperative intravesical instillation with chemotherapy – no. (%)	2 (13.3%)	Radboud University Center, Nijmegen	1 (6.6%)
UTUC characteristics, n = 15			
Lateralization – no. (%)			
Left	7 (46.7%)		
Right	8 (53.3%)		
Localization – no. (%)			
Renal pelvis	9 (60.0%)		
Ureter	6 (40.0%)		
Pathological T-stage – no. (%)			
pTa	9 (60.0%)		
pT1	1 (6.7%)		
pT2	3 (20.0%)		
pT3	2 (13.3%)		
Tumor grade (WHO 1973) – no. (%)			
Grade 1	1 (6.7%)		
Grade 2	9 (60.0%)		
Grade 3	5 (33.3%)		

Table 1 (continued). Patient, treatment and tumor characteristics of 15 patients diagnosed with a primary upper urinary tract urothelial carcinoma and a subsequent urothelial carcinoma of the bladder.

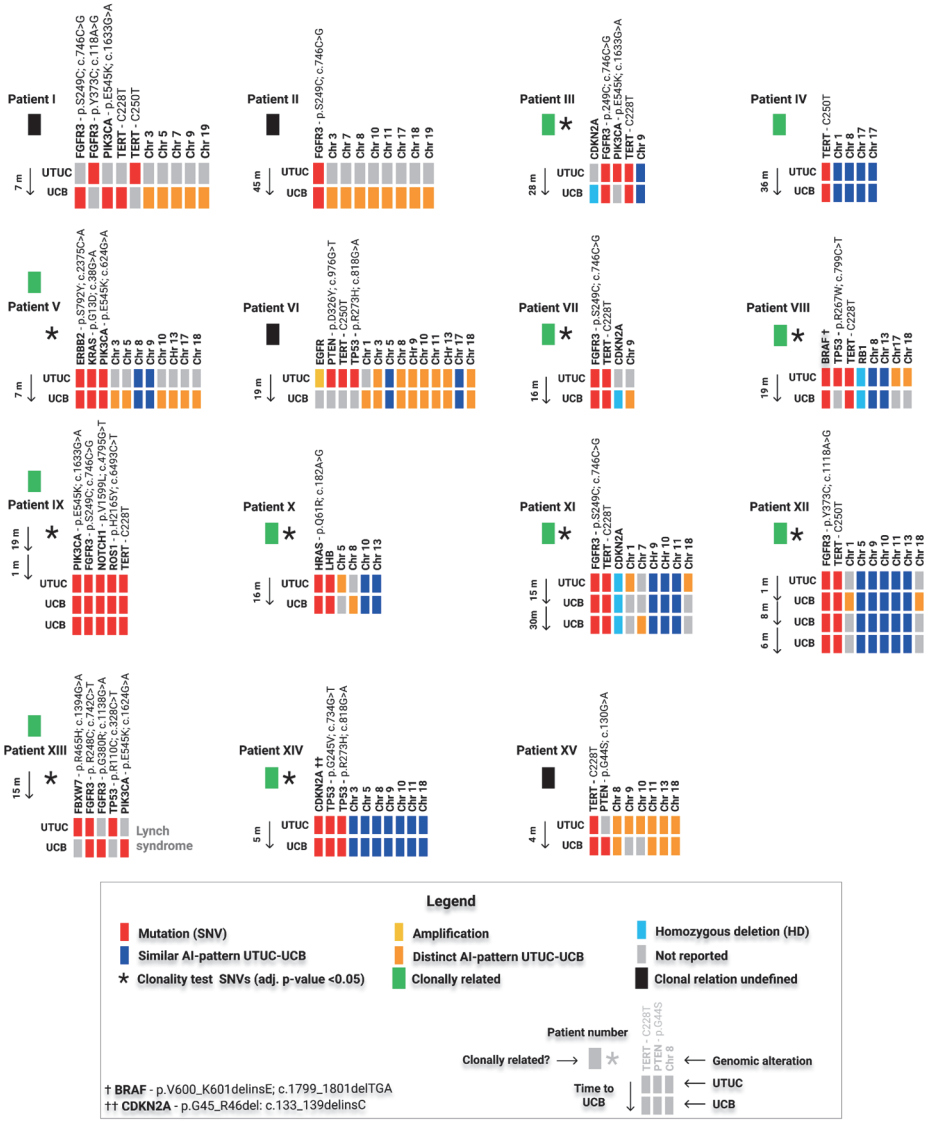
Tumor grade (WHO 2004/2016) – no. (%)			
Low grade	5 (33.3%)		
High grade	10 (66.7%)		
Pathological N-stage – no. (%)			
pNx	14 (93.3%)		
pN0	1 (6.7%)		
UBC characteristics, n = 19		**Time to UBC (months), median (IQR)**	**16.0 (11.5)**
Pathological T-stage – no. (%)			
pTis	2 (10.5%)		
pTa	15 (79.0%)		
pT1	2 (10.5%)		
Tumor grade (WHO 1973) – no. (%)			
Grade 1	2 (10.5%)		
Grade 2	10 (52.6%)		
Grade 3	7 (36.8%)		
Tumor grade (WHO 2004/2016) – no. (%)			
Low grade	8 (42.1%)		
High grade	11 (57.9%)		

QR = Inter quartile range; RNU = Radical Nephroureterectomy; UTUC = Upper urinary tract urothelial carcinoma; UBC = urothelial carcinoma of the bladder; URS = ureterorenoscopy; TNM-stage based on 7th TNM classification of malignant tumours

Table 2. Genes included in the *Next Generation DNA targeted sequencing* panel

Diagnostic V5.1 Next Generation Sequencing panel			
Erasmus Medical Center, Rotterdam			
Gene	**Exons covered**	**Gene or region**	**Numbers of SNPs included**
CDKN2A		Chr1p	11 SNPs
PTEN		Chr8p	9 SNPs
TP53		Chr7	9 SNPs
AKT1	Exon 3	Chr19q	9 SNPs
ALK	Exon 20, 22-25	*APC*	9 SNPs
Amel_X	Not applicable	*ARID1A*	8 SNPS
Amel_Y	Not applicable	*ATM*	9 SNPs
APC	Exon 14	*BRCA1*	9 SNPs
ARAF	Exon 7	*BRCA2*	9 SNPs
BRAF	Exon 11, 15	*CDKN2A*	9 SNPs
CHEK2	Exon 4, 5, 12, 13	*FHIT*	9 SNPs
CTNNB1	Exon 3, 7, 8	*PTEN*	9 SNPs
EGFR	Exon 18-21	*RB1*	9 SNPs
ERBB2	Exon 19-21	*SMAD4*	9 SNPs
EXH2	Exon 16	*STK11*	9 SNPs
FBXW7	Exon 9, 10	*TP53*	9 SNPs
FGFR1	Exon 7, 9	*VHL*	9 SNPs
FGFR2	Exon 7, 9		
FGFR3	Exon 7, 9		
FOXL2	Exon 3		
GNA11	Exon 4,5	**Total number of amplicons**	
GNAS	Exon 8, 9	330	
HRAS	Exon 2-4		
IDH1	Exon 4		
IDH2	Exon 4		
KIT	Exon 8, 9, 11, 13, 14, 17		
KRAS	Exon 2-4		
MAP2K1	Exon 2, 3		
MET	Exon 2, 14, 19		
MYD88	Exon 5		
NOTCH1	Exon 26, 27		
NRAS	Exon 2-4		
PDGFRa	Exon 12, 14, 18		
PIK3CA	Exon 10, 21		
POLD1	Exon 12		
POLE	Exon 9, 13		
RAF1	Exon 7		
RET	Exon 11, 16		
RNF43	Exon 3, 4, 9		
SMAD4	Exon 3, 9, 12		
STK11	Exon 4, 5, 8		
TERT promoter	Promoter region		

Figure 1 Assessment of the clonal relation of 15 primary UTUC and 19 subsequent UBCs based on (non)shared tumor-specific genomic alterations between both entities detected by Next Generation Sequencing. Additional transcriptomic profiling based on mRNAseq data is included for patient X, XI, XII and XIV (NU = normal ureteric tissue; UTUC = Upper urinary tract urothelial carcinoma; UBC = Urothelial carcinoma of the bladder)



Supplementary Table 1. Pathological T-stage, WHO grade 1973 and 2004/2016, and time to recurrence from RNU in months of 15 UTUC and 19 paired UBC

	UTUC	UBC I (months*)	UBC II (months)	UBC III (months)
Patient I	TaG1-low grade	TaG2-low grade (7)		
Patient II	TaG2-low grade	TaG1-low grade (45)		
Patient III	TaG2-low grade	TaG2-low grade (28)		
Patient IV	TaG2-high grade	TaG3-high grade (36)		
Patient V	TaG2-low grade	TaG2-low grade (7)		
Patient VI	T2G3-high grade	TisG3-high grade (19)		
Patient VII	T3G2-low grade	T1G2-low grade (16)		
Patient VIII	TaG2-high grade	TaG2-high grade (19)		
Patient IX	TaG2-high grade	TaG2-high grade (19)	TaG2-high grade (20)	
Patient X	T3G3-high grade	TaG3-high grade (16)		
Patient XI	TaG2-high grade	TaG1–low grade (15)	TaG3-high grade (45)	
Patient XII	T1G3-high grade	TaG3-high grade (1)	TaG2-low grade (9)	TaG2-low grade (15)
Patient XIII	TaG3-high grade	TaG2-high grade (15)		
Patient XIV	T2G2-high grade	T1G3-high grade (6)		
Patient XV	T2G3-high grade	TisG3-high grade (4)		

*months to recurrence from RNU; UTUC = upper urinary tract urothelial carcinoma; UBC = urothelial carcinoma of the bladder

Supplementary Table 2. The actual tumor cell percentage based on the Next Generation DNA targeted sequencing data of 15 UTUC and 19 paired UBC

Tumor cell percentage				
	UTUC	**UBC I**	**UBC II**	**UBC III**
Patient I	70%	60%		
Patient II	70%	70%		
Patient III	70%	70%		
Patient IV	70%	70%		
Patient V	80%	50%		
Patient VI	60%	50%		
Patient VII	90%	90%		
Patient VIII	90%	90%		
Patient IX	80%	80%	80%	
Patient X	70%	80%		
Patient XI	80%	80%	80%	
Patient XII	80%	70%	70%	70%
Patient XIII	80%	70%		
Patient XIV	90%	80%		
Patient XV	80%	50%		

UTUC = upper urinary tract urothelial carcinoma; UBC = urothelial carcinoma of the bladder

Supplementary Table 3. Reference ID's of the 154 SNPs and corresponding chromosome number used in the Next Generation Sequencing panel

Reference SNP ID numbers							
Chrom.	Reference ID	Chrom.	Reference ID	Chrom.	Reference ID	Chrom.	Reference ID
1	Rs72901775	5	Rs163454	10	Rs2248456	17	Rs11658073
1	Rs7546616	5	Rs2019720	10	Rs1426618	17	Rs1905338
1	Rs12567277	5	Rs2431512	10	Rs2274312	17	Rs3760386
1	Rs9439532	5	Rs448475	10	Rs10887863	17	Rs9915489
1	Rs9434673	5	Rs13190040	11	Rs11212118	17	Rs8070179
1	Rs622662	5	Rs7727449	11	Rs10431058	17	Rs799905
1	Rs6673156	5	Rs6871811	11	Rs11212459	17	Rs11655135
1	Rs7663	7	Rs190	11	Rs228589	17	Rs231478
1	Rs1127818	7	Rs2028209	11	Rs645485	17	Rs7217858
1	Rs10794522	7	Rs983613	11	Rs227093	18	Rs7241428
1	Rs2445635	7	Rs2072453	11	Rs949286	18	Rs1893489
1	Rs383913	7	Rs730437	11	Rs2162156	18	Rs2427777
1	Rs11247593	7	Rs6950826	11	Rs625040	18	Rs620898
1	Rs11247594	7	Rs2037588	13	Rs9602144	18	Rs17736674
1	Rs7504	7	Rs2057932	13	Rs9548593	18	Rs7244552
1	Rs3813795	7	Rs6957957	13	Rs7334588	18	Rs2445441
1	Rs2504786	8	Rs1564480	13	Rs2126043	18	Rs9951319
1	Rs6564	8	Rs9644053	13	Rs9534262	18	Rs12606702
1	Rs157208	8	Rs900779	13	Rs693963	19	Rs2312104
3	Rs2600160	8	Rs68063696	13	Rs7993153	19	Rs55913760
3	Rs2542381	8	Rs2249102	13	Rs450789	19	Rs1683561
3	Rs7613920	8	Rs7013223	13	Rs5011113	19	Rs7253869

Supplementary Table 3 (continued). Reference ID's of the 154 SNPs and corresponding chromosome number used in the Next Generation Sequencing panel

3	Rs263411	8	Rs10112431	13	Rs1572871	19	Rs4995472
3	Rs696219	8	Rs13276054	13	Rs1326466	19	Rs4807072
3	Rs33269	8	Rs6557686	13	Rs7338119	19	Rs12460075
3	Rs892607	9	Rs10964823	13	Rs7994141	19	Rs1061828
3	Rs2697161	9	Rs614647	13	Rs9568036	19	Rs2074552
3	Rs1062246	9	Rs10757225	13	Rs9535032	19	Rs7283
3	Rs12491197	9	Rs10757261	13	Rs9595961	19	Rs33841
3	Rs13317082	9	Rs3814960	13	Rs7987258	19	Rs1291
3	Rs28597374	9	Rs10965215	13	Rs2407610	19	Rs166539
3	Rs67777617	9	Rs828582	17	Rs4796561	19	Rs10113
3	Rs7646323	9	Rs10511705	17	Rs90951	19	Rs6521
3	Rs11130822	9	Rs7852081	17	Rs4796409	19	Rs193040
3	Rs9816766	10	Rs10749542	17	Rs1050541	19	Rs10217
3	Rs11708109	10	Rs3802665	17	Rs7141	19	Rs10448
3	Rs7647357	10	Rs187893001	17	Rs62062589		
5	Rs9784704	10	Rs4933453	17	Rs8073033		
5	Rs30341	10	Rs7076964	17	Rs6503098		

Chrom. = Chromosome

5

A DEEP-LEARNING WORKFLOW TO PREDICT IF UPPER TRACT UROTHELIAL CANCER PROTEIN-BASED SUBTYPES CAN SUPPORT THE IDENTIFICATION OF PATIENTS BENEFITTING FROM MOLECULAR TESTING

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ABSTRACT

Upper tract urothelial carcinoma (UTUC) is a rare and aggressive, yet understudied, urothelial carcinoma (UC). The more frequent UC of the bladder comprises several molecular subtypes, associated with different targeted therapies and overlapping with protein-based subtypes. However, if and how these findings extend to UTUC remains unclear. Artificial intelligence-based approaches could help elucidate UTUC's biology and extend access to targeted treatments to a wider patient audience. Here, UTUC protein-based subtypes were identified, and a deep-learning (DL) workflow was developed to predict them directly from routine histopathological H&E slides. Protein-based subtypes in a retrospective cohort of 163 invasive tumors were assigned by hierarchical clustering of the immunohistochemical expression of three luminal (FOXA1, GATA3, and CK20) and three basal (CD44, CK5, and CK14) markers. Cluster analysis identified distinctive luminal ($N = 80$) and basal ($N = 42$) subtypes. The luminal subtype mostly included pushing, papillary tumors, whereas the basal subtype diffusely infiltrating, non-papillary tumors. DL model building relied on a transfer-learning approach by fine-tuning a pretrained ResNet50. Classification performance was measured via three-fold repeated cross-validation. A mean area under the receiver operating characteristic curve of 0.83 (95% CI: 0.67–0.99), 0.8 (95% CI: 0.62–0.99), and 0.81 (95% CI: 0.65–0.96) was reached in the three repetitions. High-confidence DL-based predicted subtypes showed significant associations ($p < 0.001$) with morphological features, i.e. tumor type, histological subtypes, and infiltration type. Furthermore, a significant association was found with programmed cell death ligand 1 (PD-L1) combined positive score ($p < 0.001$) and *FGFR3* mutational status ($p = 0.002$), with high-confidence basal predictions containing a higher proportion of PD-L1 positive samples and high-confidence luminal predictions a higher proportion of *FGFR3*-mutated samples. Testing of the DL model on an independent cohort highlighted the importance to accommodate histological subtypes. Taken together, our DL workflow can predict protein-based UTUC subtypes, associated with the presence of targetable alterations, directly from H&E slides.

INTRODUCTION

Urothelial carcinomas (UCs) are malignant epithelial neoplasms arising from the urothelial lining of the urinary tract [1]. The rare upper tract UC (UTUC) represents 5–10% of all UCs, whereas the remaining 90–95% are urothelial bladder cancer (UBC). UTUC is frequently associated with poor prognosis, with two-thirds of patients being diagnosed at an invasive tumor stage [2]. Owing to the histopathological similarity between UTUC and UBC [3,4], and the preponderance of the latter, UTUC is an understudied disease. However, a better understanding of UTUC biology could allow the identification of distinctive molecular traits with potential strong impact on patient stratification and treatment [3–5].

In recent years, transcriptome-based subtyping allowed an improved stratification of several cancer entities into subgroups of patients sharing similar molecular features [6]. In muscle-invasive BC (MIBC), different molecular subtypes have been proposed [7–12], and in 2020 a consensus classification identified luminal and basal as the two distinctive subtypes [1,13]. These subtypes offer valuable support for guiding targeted therapy options. Indeed, the luminal subtype appears associated with higher responsiveness to fibroblast growth factor receptor 3 (*FGFR3*)-targeted therapies, and the basal subtype to immunotherapies such as programmed cell death ligand 1 (PD-L1) and programmed cell death protein 1 (PD-1) inhibitors [13,14]. For UTUC, only few studies have so far investigated its genomic and transcriptomics landscape [15–17]. Additionally, no consensus subtypes have been identified yet [2,3].

Assessment of molecular subtypes via high-throughput sequencing is neither ubiquitously available nor cost-effective. Thus, alternative subtyping approaches should be considered. In MIBC, studies showed a substantial overlap between molecular and protein-based subtypes [18,19], identifiable via more widespread, routinely applicable immunohistochemistry (IHC) analyses. Moreover, AI-based approaches have recently emerged as novel research tools able to provide automated and accurate pathological diagnoses, leveraging the information residing into whole slide images (WSIs) [20]. Here, we identify UTUC protein-based subtypes via a set of markers able to well characterize the

luminal/basal differentiation of the urothelium in both the upper and lower urinary tract. These protein markers have been used in previous studies to stratify UTUC and UBC patients [21–24] and have shown to correlate well with RNA expression [18,19]. In addition, we propose a deep-learning (DL) approach that can predict the identified protein-based subtypes relying only on digitized hematoxylin and eosin (H&E) slides.

MATERIALS AND METHODS

The 'German cohort' served as training cohort. It comprised formalin-fixed paraffin-embedded (FFPE) material of N = 163 retrospectively analyzed patients diagnosed with UTUC between 1995 and 2012 at University Hospital Erlangen-Nürnberg (Erlangen, Germany) and University Hospital Gießen and Marburg (Marburg, Germany). The 'Dutch cohort' served as independent test cohort. It comprised N = 55 samples diagnosed with UTUC between 2017 and 2020 as part of a multicenter, phase II, prospective trial conducted at University Medical Center Rotterdam (Rotterdam, The Netherlands) [25]. All patients underwent radical nephroureterectomy or partial ureterectomy, without any treatment before surgical tissue collection. All samples were invasive (i.e. with tumor stage ≥ pT1) and for both cohorts one WSI per patient was used, namely the one showing the most representative invasive part of the tumor. All cases were systematically reviewed by two uropathologists (VB and AH) according to the tumor, node, metastasis (TNM) classification (2017) and the WHO classification of genitourinary tumors [26]. Clinicopathological characteristics of the two cohorts are summarized in Table 1. The uropathologists also evaluated slides in terms of histological subtype, infiltration type, and tumor type. Approval for this study was obtained from the Ethics Committee of the Friedrich-Alexander University Erlangen-Nürnberg (No. 329_16B) and the Erasmus Medical Centre Rotterdam (METC 2017-227 NL60919.078.17). All patients gave informed consent. The study was carried out in accordance with the Declaration of Helsinki and to the principles of Good Clinical Practice (GCP).

H&E staining and tissue microarray (TMA) analysis were performed for the two cohorts in the respective pathology centers. For each patient a total of four

representative tissue cores (1 mm of diameter), two covering the tumor centrum and two covering the invasion front, were punched from the associated paraffin block and transferred to distinct recipient blocks using the TMA Grand Master (3DHistech, Budapest, Hungary).

IHC analysis

All IHC analyses were performed at the Institute of Pathology, University Hospital Erlangen (Erlangen, Germany) on a Ventana BenchMark Ultra (Ventana, Tucson, AZ, USA) autostainer accredited by the German Accreditation Office (DAKKs) according to DIN EN ISO/IEC 17020. For protein-based subtyping, we relied on a set of three basal (i.e. CK14, CK5, and CD44) and three luminal (i.e. CK20, FOXA1, and GATA3) markers that characterize the luminal/basal differentiation of the urothelium in both the upper and lower urinary tract. More specifically, our marker choice was based on the following considerations:

1. the urothelium of both the upper and lower urinary tract can be stratified into three major epithelial cell layers based on their localization and cell type [27]:
    - the basal layer sits on the basement membrane. It is a proliferative cell layer containing stem cells and expressing the basal cytokeratins (CK) 5/6 and CK14, as well as the hyaluronic acid receptor (CD44);
    - the intermediate layer contains moderately differentiated cells with variable expression of CD44, reduced expression of CK5/6, and high expression of CK18;
    - the superficial layer contains the so-called umbrella cells, which are fully differentiated and express uroplakin proteins as well as CK20.

2. Starting from the differentiation of normal urothelium, urothelial neoplasms develop via two distinct oncogenic pathways [27,28]:
    - the luminal pathway is driven by the main transcription factors (TFs) GATA3, FOXA1, and PPARG. Cancer cells express markers characteristic of the superficial cell layer;
    - the basal pathway is driven by the TFs p63, STAT2, and EGFR. Resulting cancer cells express markers characteristic of the basal layer.

A subset of the protein markers we utilized have also been used by other groups to stratify UTUC and UBC patients [21–24]. In addition, the six-marker set (i.e. CK5, CK14, CD44, FOXA1, GATA3, and CK20) has been extensively used and validated by our group, who showed that the IHC expression of the chosen markers correlates well with RNA expression [18,19].

IHC staining was performed on 2–3 µm TMA sections from each block using the following antibodies: CK14 (clone SP53, Cell Marque, Rocklin, CA, USA), CK5 (clone XM26, Diagnostic BioSystems, Pleasanton, CA, USA), CD44 (clone DF1485, Dako, Santa Clara, CA, USA), CK20 (clone Ks 20.8, Dako), FOXA1 (polyclonal, Abcam, Waltham, MA, USA), and GATA3 (clone L50-823, DCS Innovative Diagnostic Systems, Hamburg, Germany). The expression of these markers was histologically quantified (VB and PV) using the histoscore (*H*-score), which converts immunoreactivity into a semiquantitative range [0–300] proportional to both staining intensity and percentage of positively stained cells [29]. To validate model predictions, immunohistochemical evaluations at whole slide level were performed using the same markers for selected cases.

PD-L1 expression on immune and tumor cells was assessed on TMAs using the PD-L1 assay (clone SP263, Ventana) as previously described [30]. Quantification was performed by a pathologist (VB) using both immune cells (IC) score and combined positive score (CPS). The IC score was calculated as the percentage of the area occupied by PD-L1-positive IC relative to the total tumor area, whereas the CPS was calculated as the number of immune and tumor cells positive for PD-L1 out of the total number of tumor cells. Only samples with IC score ≥5% or CPS ≥10 were considered positive for PD-L1 [31,32].

DNA isolation and *FGFR3* SNaPshot analysis

Tumor DNA was isolated using the Maxwell 16 LEV Blood DNA Kit (Promega, Mannheim, Germany) according to the manufacturer's instructions as previously described [33]. *FGFR3* mutational analysis was performed using the SNaPshot method, which simultaneously detects nine hot-spot mutations, as previously described [34].

Clustering-based protein-based subtype identification and statistical analyses

Hierarchical clustering and statistical analyses were performed within the R environment v.4.0.3 [35]. For protein-based subtyping, the expression of each luminal/basal marker in each patient was taken equal to the median *H*-score across the four TMA cores. Unsupervised hierarchical clustering was then performed on the standardized marker expression.

Association between categorical variables was assessed using Fisher's exact test. To compare the distribution of continuous variables, the Wilcoxon rank- sum test for independent samples (two groups), the Kruskal–Wallis test (more than two groups), or the one-tailed Wilcoxon signed-rank test (paired samples) were used. Analyses of overall survival (OS) and disease-specific survival (DSS) were performed using the Kaplan–Meier estimator, and statistical differences were assessed through the log-rank test. p values <0.05 were considered statistically significant. Further details are provided in Supplementary materials and methods.

WSI annotation and preprocessing

Slides belonging to the two cohorts were digitized in the respective pathology centers using a Panoramic P250 scanner (3DHistech) at different resolution levels. For each WSI, tumor tissue was manually annotated in QuPath [36] (v.0.2.3) by a trained observer (MA) under expert supervision (VB) (see supplementary material, Figure S1). An automated Python-based pipeline (https://github.com/MiriamAng/TilGenPro) was implemented to tessellate the identified tumor areas into nonoverlapping tiles of 512 x 512 pixel edge length, perform quality filtering, and stain-normalization (see supplementary material, Figure S2). Further details are provided in Supplementary materials and methods.

DL algorithm and its validation

Our DL framework relied on a transfer-learning approach by fine-tuning a ResNet50 [37] initialized with weights pre-trained on the ImageNet database [38]. A repeated three-fold cross-validation was used to estimate the model's generalization accuracy and error. Here, to ensure independence between training and validation folds, random splitting was performed at patient level (see

supplementary material, Figure S3). To account for class imbalance, the number of tiles belonging to each class within the training set was equalized. The DL model's prediction for single tiles of the validation set was averaged to obtain a WSI-level subtype prediction. For each repetition, the area under the receiver operating characteristic (AUROC) curve, accuracy, precision, recall, and F1-score were assessed as mean and 95% confidence interval (CI) across the three validation folds relying on Student's *t*-distribution. Confusion matrices for a given repetition were obtained by concatenating the predictions for the three validation folds. The predicted class in the independent test cohort was taken equal to the class with the highest average prediction value across the three models from the best-performing repetition. Further details are provided in Supplementary materials and methods.

RESULTS

Hierarchical clustering of protein marker expression identifies luminal, basal, and indifferent UTUC subtypes

To characterize protein-based UTUC subtypes, the expression of three basal (CK5, CK14, and CD44) and three luminal (CK20, FOXA1, and GATA3) differentiation markers of the urothelium was assessed in a cohort of 163 invasive samples, referred to as the 'German cohort'. Hierarchical clustering of protein marker expression identified a 'luminal' cluster (80 samples, 49.1%), a 'basal' cluster (42 samples, 25.8%), and an 'indifferent' cluster (41 samples, 25.1%) with low expression of both basal and luminal markers (Figure 1A). Only 2 of the 41 indifferent samples had marker expression equal to zero, while for the others weak expression could be detected.

The basal samples exhibited shorter OS and DSS than the luminal and indifferent samples (Figure 1B). Tumor stages differed across the three subtypes (p = 0.01), with almost half of pT4 samples in the basal group (see supplementary material, Table S1). Both infiltration (p = 0.02) and tumor type (p = 0.02) differed between basal and indifferent cases (Figure 1C,D). In contrast, no significant differences were found when comparing luminal and indifferent subtypes. Indeed, basal samples showed a clear prevalence of diffusely

infiltrative and non-papillary tumors, whereas the other two subtypes showed a higher pro- portion of pushing and papillary tumors.

Collectively, hierarchical clustering based on the IHC expression of six differentiation markers of the urothelium identified three distinctive protein-based UTUC subtypes, with high histopathological similarity between the indifferent and the luminal subtype.

DL successfully predicts luminal and basal protein- based subtypes from H&E slides and identifies candidate heterogeneous slides

We hypothesized that a DL model could predict the identified protein-based subtypes using only the information contained in the digitized H&E slides. With this aim, slides were annotated in QuPath (see supplementary material, Figure S1) and preprocessed via an automated Python-based pipeline (https://github.com/MiriamAng/TilGenPro; see supplementary material, Figure S2). A DL model was then learned on the 163 German samples in a weakly super- vised way. Notably, each tile inherited as true class label the protein-based subtype (i.e. luminal, basal, and indifferent) assigned to the parent slide by hierarchical clustering of the expression of the chosen markers. A total of 100,178 luminal, 66,770 basal, and 57,874 indifferent tiles were used to train and validate a DL-based classifier in a three-time repeated three-fold cross-validation set- ting (see supplementary material, Figure S3). While most basal (AUROC = 0.77; 95% CI: 0.67–0.86; repetition three) and luminal (AUROC = 0.71; 95% CI: 0.44–0.99; repetition two) samples were correctly predicted, more than 55% of samples labeled as indifferent on the basis of protein expression were predicted luminal by our DL model on the basis of digitized H&E slides alone (see supplementary mate- rial, Figure S4 and Table S2). This difficulty of the DL model in predicting the indifferent subtype was consistent with the observed histomorphological similarity with the luminal subtype. Therefore, we decided to train a new DL model focusing on those samples assigned, on the basis of protein expression, to the most distinctive luminal and basal subtypes (Figure 2A). Again, we relied on a repeated cross-validation setting. Our classifier achieved a mean AUROC of at least 0.8 (AUROC = 0.83; 95% CI: 0.67–0.99; repetition one) and mean accuracy of ≥0.75 (mean accuracy = 0.79; 95% CI: 0.75–0.84; repetition two) (see Figure 2B and supplementary material, Table S3). For further

analyses, we focused on the results of repetition 2, as it showed the best accuracy and most consistent performance metrics across the three hold-out folds (see supplementary material, Table S3). First, the so called 'high-confidence' slides were identified, i.e. those slides whose prediction score for the luminal/basal class, given as output by the model, was at least 0.7 (see supplementary material, Table S4). These slides were selected exclusively on the basis of the DL model prediction scores, irrespective of their protein-based subtype assigned via clustering of protein marker expression. The true positive rates in the high-confidence luminal and basal slides were respectively 86.2% (50/58) and 87.5% (14/16). Visual inspection of tile-level prediction maps of the top high-confidence slides confirmed the pathological description of these subtypes at histo- pathological level, i.e. dense nuclei with small stroma bridges as distinctive feature of the luminal subtype (Figure 3A) and dense stroma and keratinization for the basal subtype (Figure 3B) [1]. In the top scoring luminal slide, 99.9% of tiles were predicted luminal. In the top basal, 90% of tiles were predicted basal. These predictions were confirmed by whole slide level staining with the six luminal and basal markers (see Figure 3A,B and supplementary material, Figure S5A,B). Taken together, these results show that the DL model was able to successfully predict the most distinctive luminal/basal protein-based subtypes on the basis of digitized H&E slides alone.

Next, we focused on the 22 'low-confidence' slides, i.e. those slides whose prediction score for the luminal/basal class, given as output by the model, was between 0.4 and 0.6 (see supplementary material, Table S5). As for the high-confidence slides, the low- confidence slides were chosen exclusively on the basis of the DL model prediction scores, irrespective of their protein-based subtype. These slides showed no significant difference in the distribution of luminal and basal marker expression ($p = 0.43$). Tile-level prediction maps allowed categorization of these slides into 'heterogeneous slides', with distinguishable clusters of predicted luminal and basal tiles, and slides without any visible luminal/basal structured patterns. Visual inspection of a selected candidate heterogeneous slide supported the predictions, with basal and luminal tiles showing the characteristic histopathological features (Figure 3C). Furthermore, whole slide IHC validation showed positivity of the entire tumor area for the three luminal markers

and the basal marker CK14 (see supplementary material, Figure S5C). Notably, in the luminal-predicted area, the CK14 basal marker appeared expressed only in the outer cellular layer, whereas in the basal-predicted area it appeared in all cell layers (Figure 3C). Taken together, the DL model was able to identify candidate heterogeneous slides showing co-presence of luminal and basal areas.

High-confidence predicted slides show the expected histopathological features and are significantly associated with PD-L1 and *FGFR3* status

To further characterize the high-confidence predictions, we also examined their marker expression and morphological characteristics. High-confidence predicted luminal and basal slides showed higher expression of luminal ($p = 6.62 \times 10^{-9}$) or basal markers ($p = 0.00241$) respectively (Figure 4A). High-confidence luminal predictions were mainly characterized by papillary tumors, with not otherwise specified (NOS) histological subtype, and with pushing infiltration type. Instead, high-confidence basal predictions were mainly non-papillary tumors, either squamous or with subtype histology and with diffuse infiltration (Figure 4B). Of note, five of the eight wrongly predicted luminal slides were samples characterized by papillary growth of the tumor with NOS histology. Instead, both wrongly predicted basal slides were diffusely infiltrating, non-papillary tumors with subtype histology. These results show that high-confidence predictions showed morphologic features consistent with the DL-predicted subtype.

Next, we investigated the association of high- confidence slides with clinically relevant biomarkers (Figure 4C). The proportion of PD-L1-positive samples was higher in high-confidence predicted basal slides, both using IC score ($p = 0.01$) and CPS ($p < 0.001$). Vice versa, the proportion of *FGFR3*-mutated samples was higher in high-confidence predicted luminal slides ($p = 0.002$). Interestingly, one wrongly predicted basal slide was actually PD-L1 positive, whereas four wrongly predicted luminal slides were *FGFR3* mutated.

External validation of the DL model highlights the importance of subtype histology

To test the generalization ability of the DL model, an external cohort of 55 invasive UTUC patients, referred to as the 'Dutch cohort', was used. Hierarchical clustering

identified also in this cohort luminal (31 samples, 56.3%), basal (4 samples, 7.3%), and indifferent (20 samples, 36.4%) subtypes, with expression profiles matching those in the German cohort (see supplementary material, Figure S6A). WSI-level predictions on the Dutch cohort were obtained employing an ensemble of the three DL models trained on repetition 2. The DL model correctly classified all luminal samples, with an average prediction score of 0.89, but not the four basal samples (see supplementary material, Figure S6B). Three of these samples showed histological subtypes with glandular, squamous, and sarcomatoid features; one sample was instead predominantly characterized by papillary growth of the tumor with NOS histology. However, in this fourth sample, basal features could be observed at the invasion front, where two out of four TMA cores were punched. Interestingly, the tile-level prediction map highlighted a small tumor area predicted basal in correspondence to the invasion front, whereas the remaining papillary area was mainly predicted luminal (see supplementary material, Figure S6C).

Thus, although a satisfying performance of the DL model was reached in the prediction of the luminal samples, the presence of histological subtypes might have made prediction of the basal samples difficult.

DISCUSSION

We identified three protein-based UTUC subtypes via a set of markers that are able to characterize the luminal/basal differentiation of the urothelium in both the upper and lower urinary tract. The three subtypes were identified in a completely unsupervised way, using hierarchical clustering of the protein marker expression. The samples belonging to the indifferent subtype had very low protein expression of both luminal and basal markers, thus clearly differing from both luminal and basal samples. However, our characterization in terms of infiltration type and tumor type highlighted the histopathological similarity between the indifferent and luminal subtypes. This similarity was reflected in the performance of a three-class DL model, which, utilizing only the digitized H&E slides, predicted as luminal a large number of samples assigned to the indifferent protein-based subtype. Future studies with molecular data might help to elucidate the molecular-level differences between the indifferent and the luminal protein-based subtypes.

Instead, a two-class DL model trained on only the samples assigned to the luminal and basal protein- based subtypes could predict with high accuracy these most distinctive luminal/basal subtypes relying only on digitized H&E slides. Furthermore, the DL model identified candidate heterogeneous slides for which whole slide IHC validation confirmed the co-presence of luminal and basal areas closely matching the tile- level predictions.

At the histopathology level, invasive UCs present different morphological appearances [1]. As previously observed, MIBC histological subtypes are strong indicators of mRNA-/protein-based subtypes [18]. Notably, the high-confidence predictions by our model, even including those slides where the DL-predicted subtype did not match the protein-based subtype, showed morphological features consistent with the DL-predicted subtype. For example, we saw that five out of the eight WSIs labeled 'basal' on the basis of the protein expression, but predicted luminal by our DL model, showed a NOS histology, which is characteristic of the luminal subtype. Moreover, high- confidence predicted slides were significantly associated with *FGFR3* mutation and PD-L1 expression. Indeed, basal predictions contained a higher proportion of PD-L1-positive samples and luminal predictions a higher proportion of *FGFR3*-mutated samples. Because of the implementation of anti-PD- L1 and PD-1 therapies, and specific *FGFR3* inhibitors [26,39] in UCs, histopathology laboratories have been facing increased requests for PD-L1 assessment and *FGFR3* alteration testing. Our DL model predictions, based exclusively on the information contained in digitized H&E slides, could thus offer valuable support to pathologists for the prioritization of UTUC patients who should undergo *FGFR3*/PD-L1 testing. This would also contribute to extending patient access to targeted therapies and improve their management and care in clinical practice. Yet, a fully digital diagnostics workflow would be required to implement such prioritization support in daily practice. In addition, testing on even larger UTUC cohorts would be needed.
Several challenges were encountered during our study. Hierarchical clustering in the independent Dutch cohort highlighted the same biological tendency observed in the German cohort, which is even more remarkable considering the prospective nature of the former. This strongly supported the existence of three distinct UTUC protein-based subtypes. However, the cluster membership of single samples might

vary with varying samples being clustered. This uncertainty in the training sample labels might negatively affect the DL model performance. An additional level of uncertainty in training labels was due to the assessment of marker expression in selected TMA cores. It is common practice to stain several tumors at once, while also taking into account tumor heterogeneity. Yet, expression in TMA cores might not be representative of whole slide level expression, as clearly seen for the predominantly papillary case in the Dutch cohort with a basal-like morphology at the invasion front. RNA-sequencing analyses might offer more robust subtype assignment, thanks to genome-wide profiling, yet might still fail to correctly characterize heterogeneous samples. Another challenge was linked to histological subtypes. Given the rarity of UTUC, we had decided not to exclude them, as had been done in previous UBC studies [19]. However, as the results on the Dutch cohort showed, this might have impaired model performance. Histological subtypes have gained increasing importance, given their impact on pathological and clinical outcomes [40]. Thus, it would be very important to develop machine learning approaches able to accommodate the prediction of histological subtypes from H&E slides. This might be achievable with the future availability of an even more extensive UTUC cohort, with a sufficient number of samples for all histological subtypes to ensure robust training of a DL model.

Furthermore, in the future it would be very interesting to investigate the extension of our DL framework to biopsy samples. Here, challenges will be related to whether an intrinsically small and superficial biopsy sample provides enough information to predict sub- types and ultimately offers support in deciding on the best treatment strategy. Finally, additional steps could be integrated into the workflow to facilitate the use of the developed DL model in a fully digital pathology laboratory. First, an upstream tumor segmentation step could be implemented to avoid the manual annotation of new samples. In addition, it would be useful to develop a downstream postprocessing tool for the automatic detection of candidate heterogeneous slides based on luminal/basal patterns analysis of tile-level prediction maps.

Collectively, our results show that the most distinctive protein-based UTUC subtypes can be predicted directly from H&E slides and are associated with the presence of targetable alterations. Thus, our study lays the foundation for

an AI-based tool to support UTUC diagnosis and extend patient access to targeted treatments.

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AUTHOR CONTRIBUTIONS STATEMENT

MA, FF and VB conceived and designed the study. MA, TvD, PV, JS, RS, CIG, HH, SW, HT, DS, BW, GJLHvL, ME, JLB, FF and VB acquired the data. MA, SL, FF and VB analyzed and interpreted the data. MA and FF performed the statistical analyses. MA, FF and VB drafted the manuscript. ME, AH, JLB and FF obtained funding. SF, CM and VZ pro- vided resources. All authors critically revised the manuscript for important intellectual content, read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

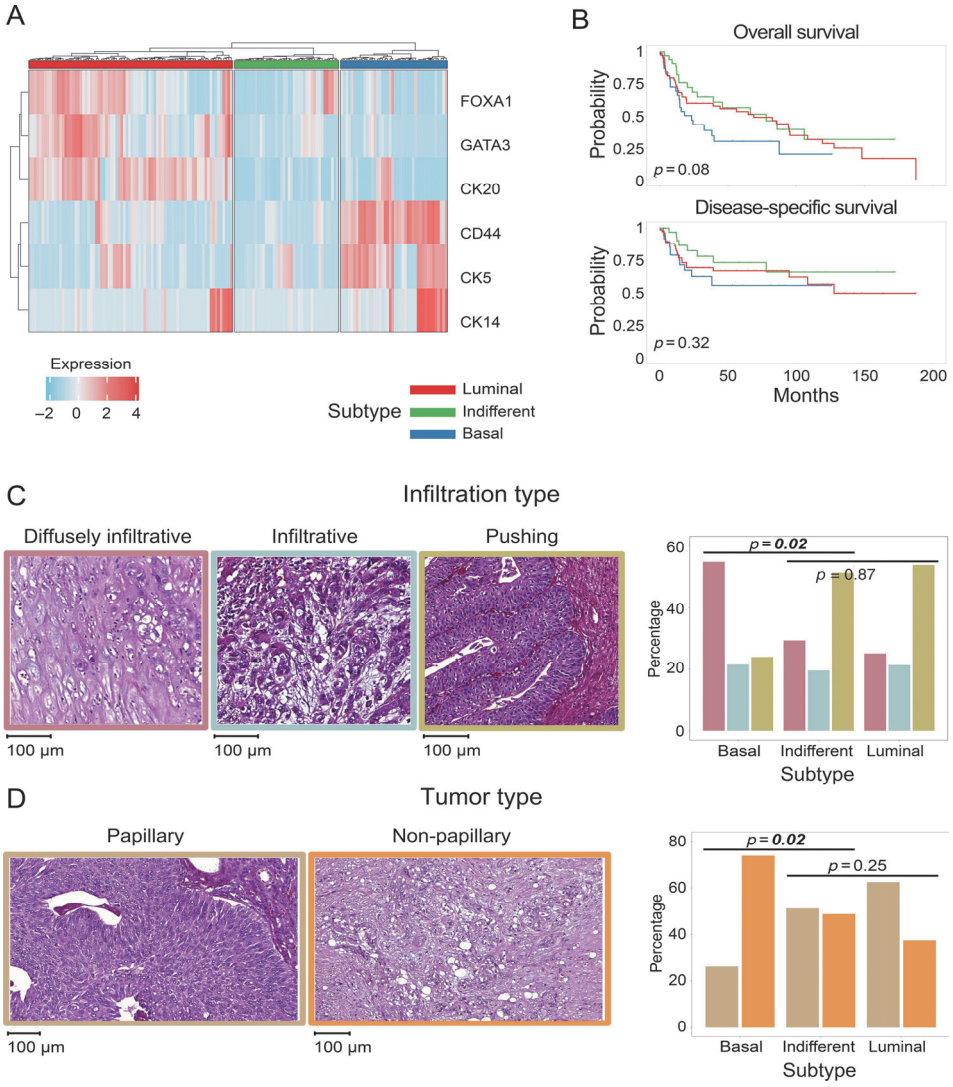
The data supporting the findings of this study are available from the corresponding authors upon reason- able request.

Table 1. Clinicopathological variables characterizing the German and the Dutch cohorts.

Clinicopathological variable	German cohort	Dutch cohort
Age at diagnosis (years), median (min–max)	73 (47–94)	71 (52–85)
Gender, *n* (%)		
Female	52 (31.9)	21 (38.2)
Male	111 (68.1)	34 (61.8)
Tumor grade (WHO 1973), *n* (%)		
G1	0 (0)	0 (0)
G2	52 (31.9)	12 (21.8)
G3	111 (68.1)	36 (65.5)
Missing	0 (0)	7 (12.7)
Tumor grade (WHO 2004), *n* (%)	–	
High		54 (98.2)
Low		0 (0)
Missing		1 (1.8)
Tumor grade (WHO 2016), *n* (%)		–
High	142 (87.1)	
Low	21 (12.9)	
Primary tumor, *n* (%)		
pT1	33 (20.2)	17 (30.9)
pT2	28 (17.2)	13 (23.6)
pT3	81 (49.7)	25 (45.5)
pT4	21 (12.9)	0 (0)
Regional lymph nodes, *n* (%)		
pN0	54 (33.1)	22 (40)
pN1	16 (9.8)	3 (5.5)
pN2	14 (8.6)	2 (3.6)
Missing	79 (48.5)	28 (50.9)
Distant metastasis, *n* (%)		
cM0	79 (48.5)	54 (98.2)
cM1	8 (4.9)	0 (0)
Missing	76 (46.6)	1 (1.8)
Anatomic origin, *n* (%)		
Renal pelvis	98 (60.1)	31 (56.4)
Ureter	47 (28.8)	24 (43.6)
Both	18 (11.1)	0 (0)

Estimates are given as median (minimum, maximum) or frequency (percentage) with respect to the total number of analyzed samples (N = 163 for the German cohort and N = 55 for the Dutch cohort). A dash (-) is used to indicate information not available within a given cohort.

Figure 1 (see next page). Protein-based UTUC subtypes and their histopathological characterization. (A) Heatmap visualization of the hierarchical clustering analysis performed on the expression of the three basal (CD44, CK5, and CK14) and three luminal (FOXA1, GATA3, and CK20) markers in our UTUC German cohort (N = 163 invasive samples). Heatmap colors represent marker expression (quantified via the standardized H-score, i.e. in terms of standard deviation differences with respect to the average H-score of the marker across all samples; white: equal to the average expression; red: higher than the average expression; blue: lower than the average expression). The color ribbon at the top of the heatmap indicates the three protein-based subtypes: luminal (red), indifferent (green), basal (blue). (B) Kaplan–Meier overall survival (top) and disease-specific survival (bottom) curves for the identified subtypes. p values from log-rank test. (C and D) Characterization of the identified subtypes in terms of infiltration type (C) and tumor type (D). Representative histopa thology images are shown on the left and bar plot distributions on the right. The p values shown above the bar plots refer to two different Fisher's exact tests: one test to compare the distribution of histopathology features in basal versus indifferent subtypes (left) and one test to compare the distribution of histopathology features in luminal versus indifferent subtypes (right). The histopathology images are framed in the respective color used in the bar plot to the right ($p < 0.05$ in italic bold).


A
FOXA1
GATA3
CK20
CD44
CK5
CK14
Expression
−2 0 2 4
Subtype
Luminal
Indifferent
Basal
B
Overall survival
Probability
1
0.75
0.5
0.25
0
p = 0.08
Disease-specific survival
p = 0.32
0 50 100 150 200
Months
C
Infiltration type
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Infiltrative
Pushing
100 μm
100 μm
100 μm
p = 0.02
p = 0.87
Percentage
60
40
20
0
Basal
Indifferent
Luminal
Subtype
D
Tumor type
Papillary
Non-papillary
100 μm
100 μm
p = 0.02
p = 0.25
80
60
40
20
0
Basal
Indifferent
Luminal
Subtype

Figure 2. DL-based prediction of luminal and basal protein-based subtypes. (A) Steps of the DL framework: (1) Starting from 80 luminal and 42 basal WSIs, a library made up of 100,178 luminal (red) and 66,770 basal (blue) stain-normalized tiles is generated using an automated, custom-developed, pre-processing pipeline. (2) The tiles library is used for training the network using three-fold cross-validation (CV) (gray: training folds, yellow: validation fold). Tiles of the trained set are balanced between the two classes. The CV is repeated three times. (3) For each training/validation set combination, a DL model is trained using a transfer-learning approach. (4) For each tile, the model outputs a prediction value for the luminal (pluminal) and for the basal (pbasal) class. WSI-level predictions for the luminal (pWSI luminal) and basal (pWSI basal) subtypes are calculated by averaging the tile-level predictions for each class. The subtype associated with the highest prediction is assigned to the entire slide. In the schematization, color intensity is proportional to the prediction score. (B) AUROC for the three repetitions (with basal subtype as positive class). The mean AUROC ± standard deviation across folds is reported for each repetition. AUROC, area under the receiver operating characteristic; NB, number of basal slides; NL, number of luminal slides

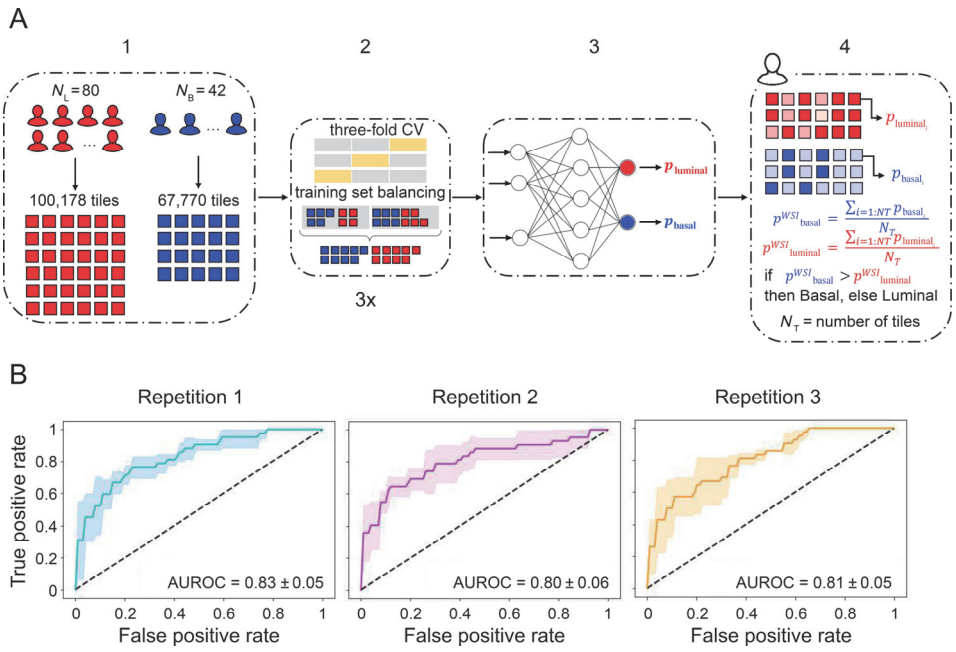


Figure 3. Whole-slide IHC validation of deep-learning predictions. (A) Slide predicted with the highest pWSI luminal, (B) slide predicted with the highest pWSI basal, and (C) candidate heterogeneous slide. For all three slides, from left to right: digitized whole slide with annotated tumor areas (red); tile-level prediction map (red: luminal; blue: basal; intensity dependent on prediction score); selected areas; and corresponding areas of the whole-slide IHC validation using CK20 as representative luminal marker and CK14 as representative basal marker. Whole-slide IHC validation with all six luminal/basal markers is provided in supplementary material, Figure S5.

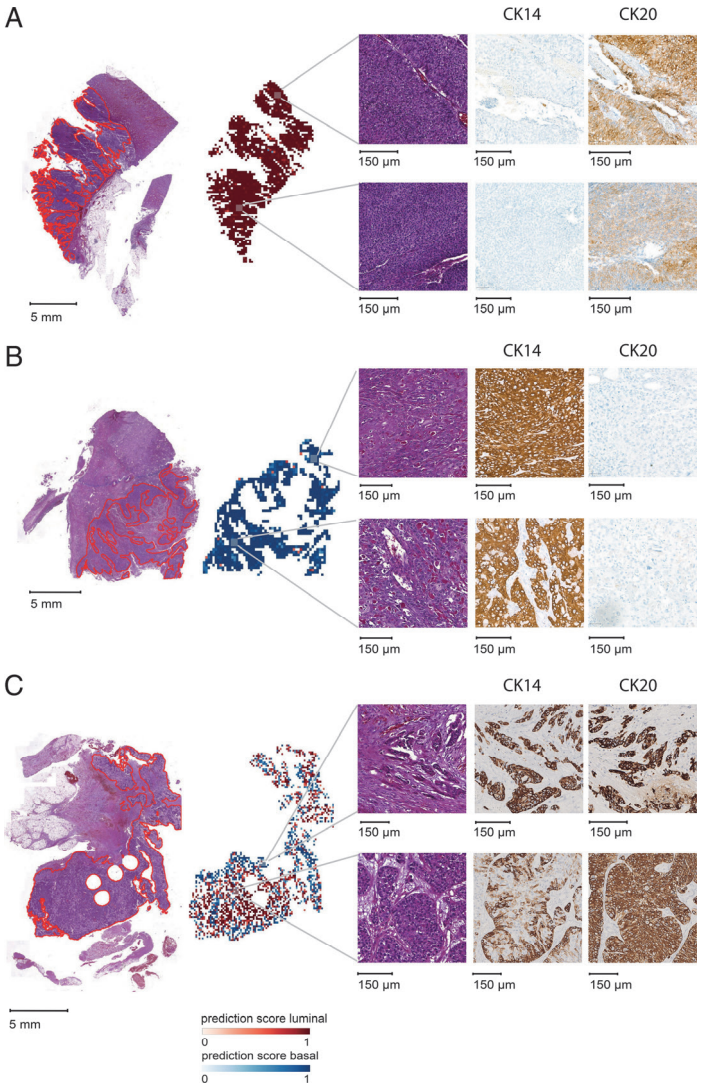
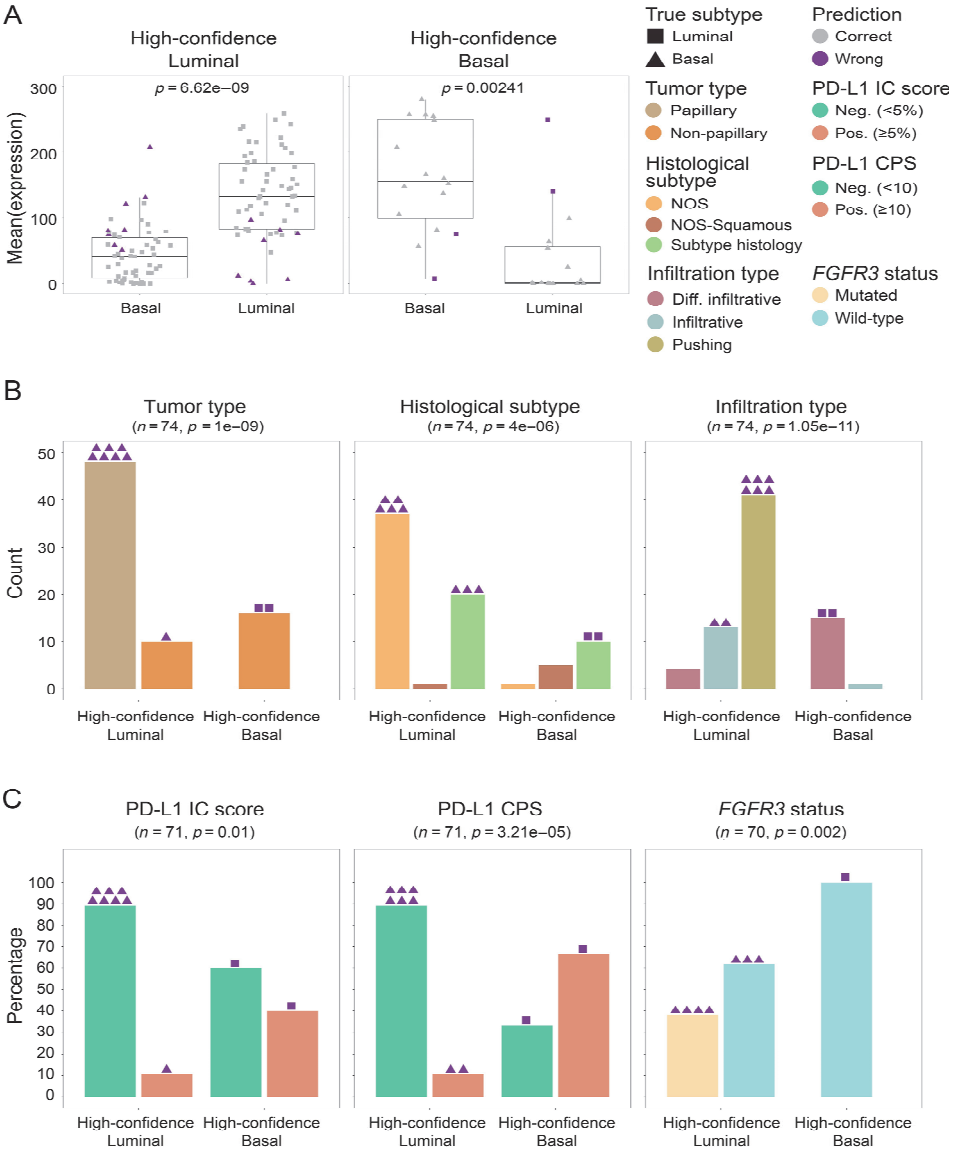


Figure 4 (see next page). Characterization of the high-confidence predicted luminal and basal slides. (A) Boxplot distributions of the mean expression values for the basal (CK14, CK5, and CD44) and luminal (CK20, FOXA1, and GATA3) markers in the high-confidence predicted luminal (left) and basal (right) slides. Shape represents the true slide label (triangle: basal; square: luminal) and color represents the prediction accuracy (gray: correct predictions; violet: incorrect predictions). p values from one-tailed Wilcoxon signed-rank test. (B) Characterization of the high-confidence predicted slides in terms of tumor type (left), histological subtypes (middle), and infiltration type (right). An overview of the morphological features for the wrongly predicted slides is provided through the colored symbols above the bars. n: number of samples; p values from two-tailed Fisher's exact test. (C) Characterization of the high-confidence predicted slides in terms of clinically relevant biomarkers: PD-L1 status (measured as CPS = combined positive score; IC score = immune cells score) and FGFR3 mutational status. An overview of PD-L1 and FGFR3 status of the wrongly predicted slides is provided through the colored symbols above the bars. n: number of samples; p values from one-tailed Fisher's exact test.


A
High-confidence Luminal
High-confidence Basal
p = 6.62e−09
p = 0.00241
Mean(expression)
300
200
100
0
Basal
Luminal
True subtype
Luminal
Basal
Prediction
Correct
Wrong
Tumor type
Papillary
Non-papillary
PD-L1 IC score
Neg. (<5%)
Pos. (≥5%)
Histological subtype
NOS
NOS-Squamous
Subtype histology
PD-L1 CPS
Neg. (<10)
Pos. (≥10)
Infiltration type
Diff. infiltrative
Infiltrative
Pushing
FGFR3 status
Mutated
Wild-type
B
Tumor type
(n = 74, p = 1e−09)
Histological subtype
(n = 74, p = 4e−06)
Infiltration type
(n = 74, p = 1.05e−11)
Count
50
40
30
20
10
0
High-confidence Luminal
High-confidence Basal
C
PD-L1 IC score
(n = 71, p = 0.01)
PD-L1 CPS
(n = 71, p = 3.21e−05)
FGFR3 status
(n = 70, p = 0.002)
Percentage
100
90
80
70
60
50
40
30
20
10
0

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SUPPLEMENTARY MATERIALS AND METHODS

Clustering-based protein-based subtypes identification and statistical analyses Hierarchical clustering and statistical analyses were performed within the R environment v.4.0.3 [35]. To identify protein-based subtypes, the expression of each marker in each patient was taken equal to the median H-score across the four TMA cores. Unsupervised hierarchical clustering was performed on the standardized marker expression (i.e. scaled to a mean of zero and a standard deviation of one) using Ward's clustering method [41]. Ward's algorithm was implemented relying on the R function *hclust* using as input the dissimilarity matrix computed through the Euclidean distance and ward.D2 as argument for the agglomeration method [42].

Heatmap visualization of the hierarchical clustering analysis was performed relying on the function *Heatmap* from the R-package ComplexHeatmap v.2.4.3.

Association analysis between categorical variables was performed using Fisher's exact test. To compare the distribution of continuous variables, the Wilcoxon rank-sum test for independent samples (two groups) or the Kruskal-Wallis test (more than two groups) were used. Differences in the distribution of luminal and basal marker expression were evaluated using the one-tailed Wilcoxon signed-rank test for paired samples.

Analyses of overall survival (OS) and disease-specific survival (DSS) were performed using the Kaplan-Meier estimator relying on the R-packages *survminer*

v.0.4.9 and *survival* v.3.2-13. The statistical difference between survival curves was assessed through the log-rank test. P-values (p) < 0.05 were considered statistically significant.

Slides digitization and WSI annotation

Slides belonging to the two cohorts were digitized in the respective pathology centers using a Panoramic P250 scanner (3DHistech, Budapest, Hungary).

Glass slides from the German cohort were scanned at 20-fold magnification with a resolution of 0.389 microns per pixel (mpp) whereas those from the Dutch cohort had three different resolution levels, i.e. 0.1214 mpp at 40-fold magnification, 0.2428 mpp at 20-fold magnification and 0.2484 mpp at 40-fold magnification. To facilitate the analysis and pre-processing of WSIs, digitized slides from the two cohorts were organized into two distinct QuPath [36] (v.0.2.3) projects and stored as .qpproj files. Within each project, for each WSI the tumor tissue was manually annotated by a trained observer (MA) under the supervision of an expert uropathologist (VB).

Manual annotation consisted in drawing a region of interest (ROI) around the tumor area and leaving out healthy tissue. Annotations were made at high levels of magnification using the brush and wand tools available in QuPath to exclude as much as possible non-tumor tissue including necrosis, bleeding/blood vessels, peri- tumoral lymphocytes and scanning artifacts (Figure S1).

WSI pre-processing pipeline

WSIs pre-processing as well as deep-learning (DL) analyses were performed in Python v.3.7.12 and run in a dedicated conda environment on a remote server based on Ubuntu's 20.04.5 long-term support (LTS) operating system with NVIDIA Tesla V100-PCIE-32GB graphics processing unit (GPU).

An automated Python-based pipeline (https://github.com/MiriamAng/TilGen Pro) was implemented for the pre-processing of groovy script is run to tessellate the annotated tumor areas into smaller non- overlapping square patches, a.k.a. tiles, of 512x512 pixel edge length. Subsequently, the generated tiles undergo a quality-filtering step. Namely, for a given WSI the median pixel intensity value across the RGB channels is calculated for each of the belonging tiles and a log10-transformed median pixel intensities distribution is obtained. A lower/upper percentile-based threshold can thus be set on the obtained distribution to filter-out tiles with a log10 median intensity lower or equal than the lower threshold (this corresponds to tiles characterized by darker regions) and/or greater or equal than the upper threshold (this corresponds to tiles with a high amount of

white pixels). The pipeline was run using as values for the lower/upper thresholds the 5th and 90th percentiles respectively. Finally, to reduce stain variation between training and test set, the tiles passing the quality-filtering step are stain-normalized according to the Macenko method [43] (Figure S2). Macenko stain-normalization was implemented by assigning to the input parameters α and β the values of 1 and 0.15 respectively, as recommended by the authors [43], and using as reference H&E optical density (OD) matrix the one provided by Mitko Veta's 'Staining unmixing and normalization' code (https://github.com/mitkovetta/staining-normalization).

For the Dutch cohort, non-overlapping tiles of 512x512 pixel edge length were generated with the same resolution level as the German cohort (i.e. 0.389 mpp). A total of 341,906 (mean: 2,098; range: 89-7,514; 146,611 luminal; 109,681 basal; 85,614 indifferent) and 112,562 (mean: 2,047; range: 58-4,964; 57,319 luminal; 10,258 basal; 44,985 indifferent) tiles were generated for the German and the Dutch cohort respectively. To control, in training dataset composition, the over- representation of tiles from WSIs characterized by larger tumor areas, and instead maximize the representativeness of tiles associated with smaller tumor areas, a maximum number of 2,000 tiles was randomly subsampled from the WSIs of the German cohort during training.

Deep-learning algorithm and its validation

For protein-based UTUC subtypes prediction a 'classical weakly supervised' [44] DL framework was employed. Namely, all tiles originating from a given WSI inherited the corresponding patient-level label assigned by hierarchical clustering [44, 45].

Although in real life tumor homogeneity cannot be assumed, weakly supervised learning approaches are particularly suited to the computational pathology field, whenever DL algorithms aim at predicting labels that cannot be directly annotated on digitized H&E slides [19, 46, 47]. Indeed, clinically relevant labels (e.g. mutational status or subtypes) are known only at patient level, while DL model training is performed on smaller image tiles generated from the WSI. The goal of the developed DL model is to make a prediction of the

protein-based subtypes identified via hierarchical clustering using only the information contained in the digitized H&E slides. In order to achieve this, the DL model must 'learn'. During model learning, the dataset with known class labels (in our case, the German cohort) is split into two portions: a training portion and a validation portion. The training portion is passed to the model together with the known class labels. The known class labels are necessary to quantify how much a given prediction deviates from its true class and this information is used to make the model learn via parameters tuning. The validation portion is instead used to provide an early estimate on how well the model learned and tune the model's hyper-parameters [48]. Often a cross-validation framework is utilized, where a dataset is divided into n folds (portions) and n DL models are learnt, each time using the i-th fold (i=1 … n) as validation portion, a.k.a. hold-out fold, and the remaining (n-1) folds as training portion.

To predict protein-based subtypes in UTUC we chose a ResNet50 [37]. The ResNet50, initialized with weights pre-trained for the visual recognition challenge on the ImageNet database [38], was then fine-tuned for the specific task relying on a transfer-learning approach. For model's implementation the DL library *fastai* v.1.0.61 [49], which is built on top of the open source PyTorch machine-learning framework [50], was employed. The pre-trained ResNet50 was retrieved from the *vision.learner* fastai module. This module, through the cnn_learner method, allows to easily retrieve a pre-trained model with a head suitable for the specific classification task.

To adapt the pre-trained model to the classification of protein-based subtypes, only the model's head was fine-tuned while keeping the layers of the backbone frozen. Model fitting was performed relying on the 1-cycle training policy [51] through the fastai function *fit_one_cycle*, by setting a maximum number of 30 epochs, a maximum learning rate of 10-5, and a weight decay of 0.1. In addition to weight decay, other regularization techniques were implemented to avoid overfitting, including data augmentation and early stopping. Data augmentation was performed relying on the fastai *get transforms* function using the default random transformations (i.e. horizontal flipping, rotation, zooming, warping, and lighting). To introduce rotational invariance also

vertical flipping was adopted by setting to true the argument flip vert. For early stopping implementation validation accuracy was chosen as quantity to be monitored throughout the whole training process. Notably, the fastai early stopping callback was implemented to terminate training after a patience time of three epochs with no improvement (min_delta = 0.01) of the monitored metric. The fastai save model callback was then used to save the model at the best epoch, i.e. the best model.

A three-time repeated three-fold cross-validation was used to estimate the model's generalization accuracy and error. Here, to ensure independence between training and validation sets, the random splitting into the three folds was performed at patient level. Further, the splitting was performed in a stratified manner, i.e. preserving the percentage of samples for a given class within each partition (Figure S3). To this aim, we relied on the module *StratifiedKFold* from the scikit-learn package v.0.24.1 using a different value of the random state argument for each repetition and setting to true the shuffle parameter. At each round of the cross- validation, the DL model was trained on two folds out of three and evaluated on the hold-out fold. Run times to fully train the DL model in a three-time repeated three-fold cross-validation setting were around 1.5 day. To account for class imbalance, a tiles balancing procedure was implemented to equalize the number of tiles belonging to each class within the training set. During inference, a prediction value per subtype was assigned to each image tile. For each WSI, tile-level predictions were then averaged, class-wise, and the subtype predicted with the highest average prediction was assigned to the WSI.

Performance metrics to evaluate model's performance were calculated relying on the sklearn.metrics module. The area under the receiver operating characteristic (AUROC), accuracy, precision, recall, and F1-Score were assessed for each repetition as mean across the three hold-out folds and 95% confidence interval (CI) relying on Student's t-distribution. Confusion matrices for a given repetition were instead obtained using the concatenated model's predictions on the associated hold- out folds.

As final model for the independent test cohort, a cross-validation ensemble was used. Namely, for each WSI of the Dutch cohort WSI-level predictions were obtained using each of the three models from the best-performing repetition on the German cohort. Then, the final WSI-prediction was taken as the class (luminal/basal) with the highest average prediction value across the three models.

SUPPLEMENTARY FIGURES

Figure S1. Example of tumor annotation. (left) Overview of a digitized whole slide image (WSI) with tumor tissue annotation borders in red; (right) zoomed-in tumor area representative of the employed annotation criteria: necrosis (1), bleeding/blood vessels (2), peri-tumoral lymphocytes (3) and scanning artifacts such as blurring and poor fixation (4) were excluded from the annotated area.

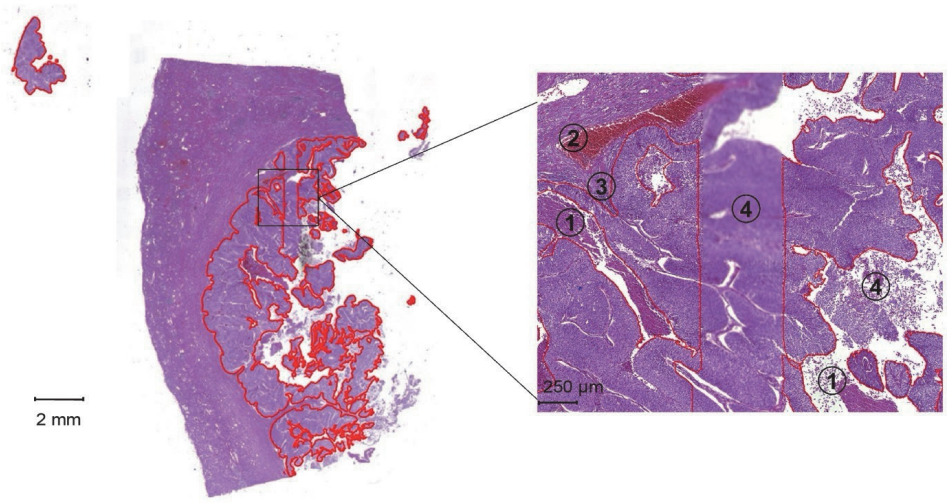


Figure S2. Overview of the WSI pre-processing workflow. Graphical representation of the main steps performed by the automated Python-based pre- processing pipeline. A QuPath project (Project.qpproj) or a set of WSIs to process (provided via a csv file, or specified individually as a list of one or more WSI names) can be used as input for the pipeline. The pipeline performs the following steps: (1) the annotated tumor area (red) within each slide is tessellated into smaller tiles; (2) tiles undergo a quality-filtering step based on lower/upper thresholds identified on the log10-transformed median intensity values distribution; (3) tiles are stain-normalized according to the Macenko method.

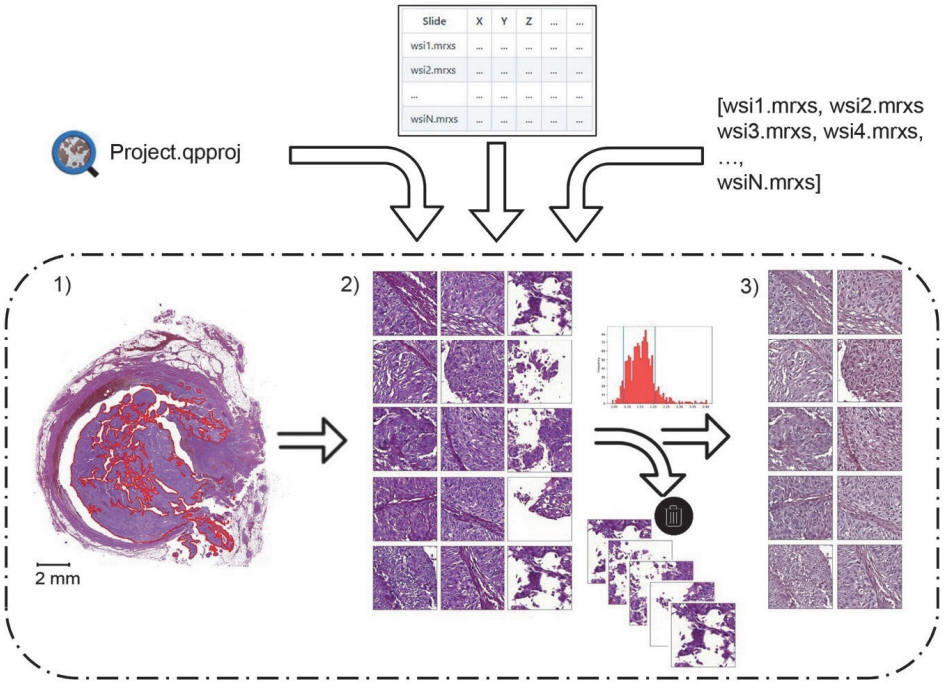


Figure S3. Overview of the training set generation procedure in a three-fold cross-validation setting. In the example, each icon represents a patient (in our case corresponding to a slide, as only one slide per patient was used) of the analyzed cohort and the three colors (red, blue and green) represent three different classes of patients. The patients' cohort is randomly split in three stratified partitions, i.e. with the same class distribution as in the whole cohort. Two partitions out of three are used, in turn, to train the deep-learning model while the correspondent hold-out folds are used for validation. Tiles belonging to the two training partitions are pooled together and class-balanced. In the example tiles are represented by the square symbol and inherit the same color (i.e. class) of the parent patient.

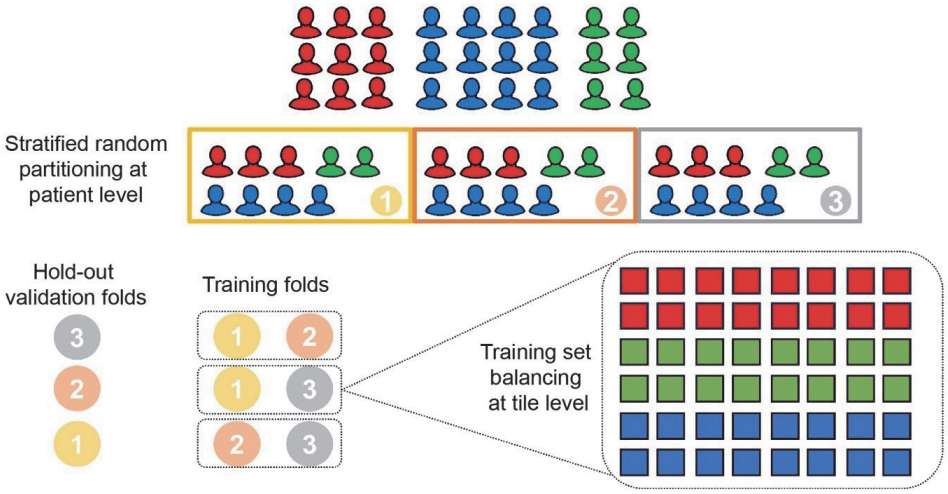


Figure S4. Deep-learning model performance in the prediction of the luminal, basal, and indifferent subtypes. (left) AUROC curves and (right) confusion matrices for (A) repetition 1, (B) repetition 2 and (C) repetition 3 of the three-fold cross-validation. AUROC curves are shown for each subtype (blue: basal, green: indifferent, red: luminal). The mean AUROC ± standard deviation (sd) is reported for each repetition. Confusion matrices are normalizedover the true class (row). AUROC: area under the receiver operating characteristic.

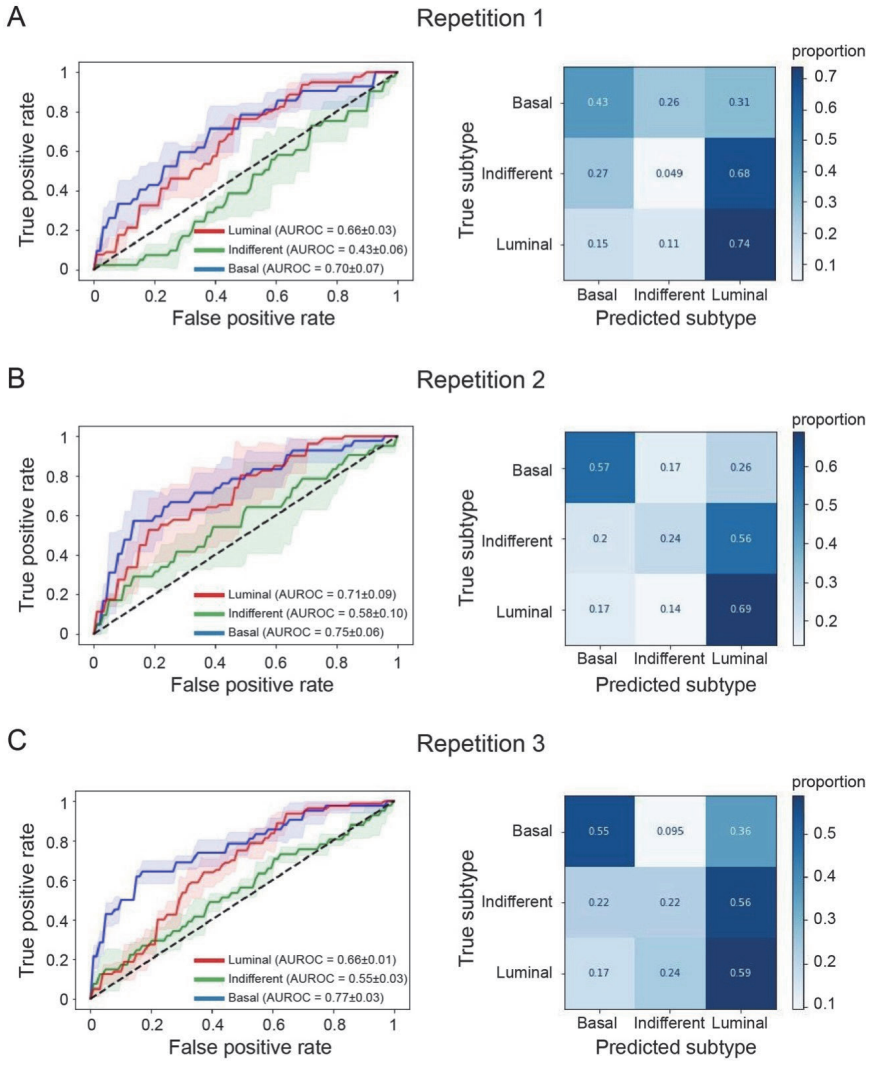


Figure S5. Whole-slide IHC validation with the entire marker set of the samples shown in Figure 3. Whole-slide IHC-validation with the three basal (CK14, CK5, CD44) and three luminal (CK20, FOXA1, GATA3) markers for (A) the top high- confidence predicted luminal slide, (B) the top high-confidence predicted basal slide, and (C) a candidate heterogeneous slide.

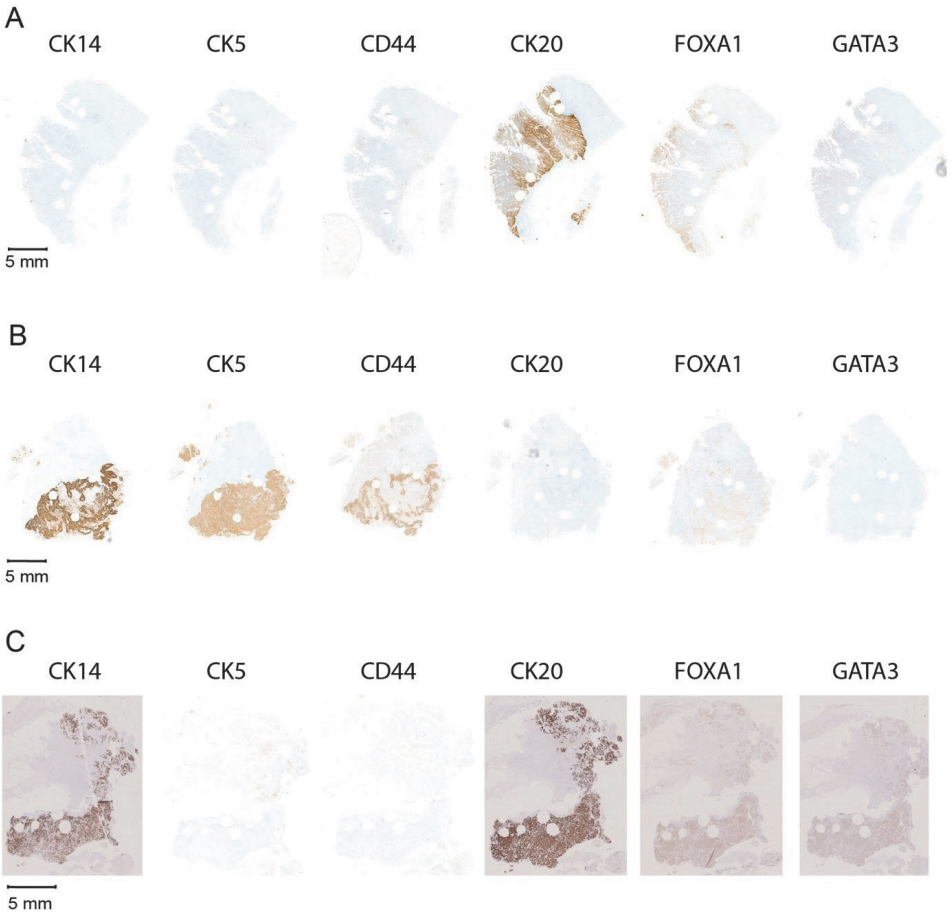
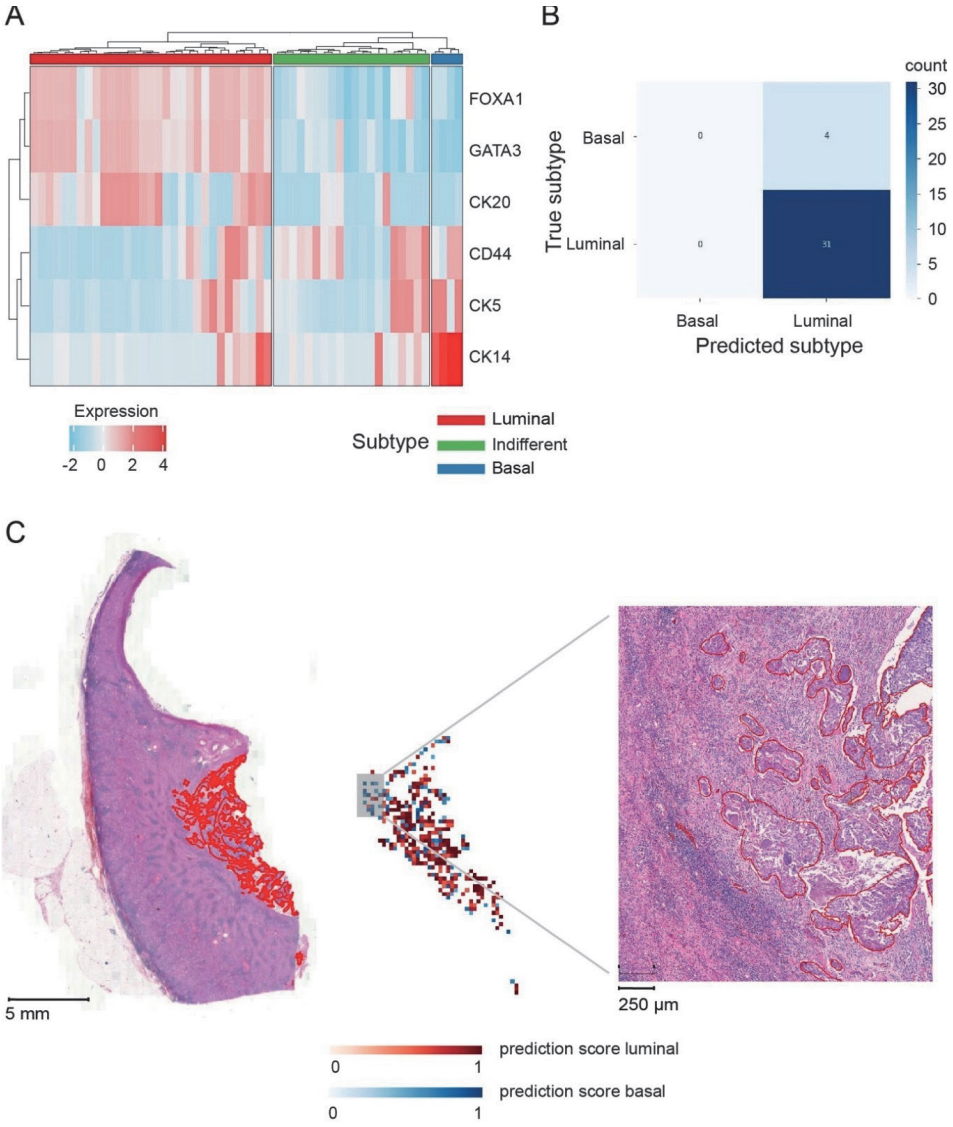


Figure S6 (see next page). Validation of the deep-learning model on the Dutch cohort. (A) Heatmap visualization of the hierarchical clustering analysis performed on the expression of the three basal (CD44, CK5, CK14) and three luminal (FOXA1, GATA3, CK20) markers in the independent UTUC cohort from the Netherlands (N = 55 invasive samples). Heatmap colors represent marker expression (quantified via the standardized H-score, i.e. in terms of standard deviation differences with respect to the average H-score of the marker across all samples; white: equal to the average expression; red: higher than the average expression, blue: lower than the average expression). The color ribbon at the top of the heatmap indicates the three protein- based subtypes: luminal (red), indifferent (green), basal (blue). (B) Confusion matrix with a summary of model's generalization accuracy. (C) Selected basal slide predicted luminal by the model. From left to right: digitized whole-slide image (WSI) with annotated tumor areas (red); tile-level prediction map (red: luminal, blue: basal; intensity dependent on prediction score); zoomed area for a better visualization of the basal morphological features of the invasion front.


A
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Subtype
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True subtype
Basal
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0
4
0
51
Basal
Luminal
Predicted subtype
C
5 mm
250 µm
prediction score luminal
0
1
prediction score basal
0
1

Table S1. Association analysis of the identified subtypes with the main clinicopathological variables. Estimates are given as median (minimum, maximum) or frequency (percentage) calculated on the total available samples. Kruskal-Wallis and Fisher's exact tests were used respectively for continuous and categorical variables.

Clinico-pathological variable	Luminal	Indifferent	Basal	p-value
Age at diagnosis (yrs), median (min-max)	72 (47-94)	70 (48-85)	77 (51-87)	0.02
Gender, n (%)				0.07
F	24 (30)	9 (22)	19 (45.2)	
M	56 (70)	32 (78)	23 (54.8)	
Tumor Grade (WHO 2016), n (%)				0.18
low	12 (15)	7 (17.1)	2 (4.8)	
high	68 (85)	34 (82.9)	40 (95.2)	
Primary Tumor, n (%)				0.01
pT1	20 (25)	10 (24.4)	3 (7.1)	
pT2	19 (23.8)	4 (9.8)	5 (11.9)	
pT3	36 (45)	21 (51.2)	24 (57.2)	
pT4	5 (6.2)	6 (14.6)	10 (23.8)	
Regional Lymph Nodes, n (%)				0.11
N0	31 (77.5)	11 (47.8)	12 (57.1)	
N1	4 (10)	6 (26.1)	6 (28.6)	
N2	5 (12.5)	6 (26.1)	3 (14.3)	
Distant Metastasis, n (%)				0.57
M0	36 (87.8)	23 (95.8)	20 (90.9)	
M1	5 (12.2)	1 (4.2)	2 (9.1)	

F: female; M: male.

Table S2. Performance metrics of the deep-learning classifier in the prediction of the luminal, basal, and indifferent protein-based subtypes for the three repetitions. For each repetition a measure of precision, recall, F1-Score, and AUROC is reported separately for the luminal, basal, and indifferent subtype together with the overall accuracy across the three subtypes. All performance metrics are reported as mean [95% CI] across the three hold-out folds, i.e. the data portions used, in turn, as validation set in the three-fold cross-validation setting.

		Basal	**Luminal**	**Indifferent**
Repetition 1	**Precision**	0.43 [0.28-0.58]	0.59 [0.43-0.75]	0.11 [0-0.43]
	Recall	0.43 [0.12-0.74]	0.74 [0.56-0.91]	0.05 [0-0.15]
	F1-Score	0.43 [0.2-0.66]	0.65 [0.5-0.81]	0.06 [0-0.21]
	AUROC	0.7 [0.5-0.9]	0.66 [0.58-0.74]	0.43 [0.25-0.61]
	Accuracy	0.48 [0.32-0.65]		
Repetition 2	**Precision**	0.51[0.38-0.64]	0.63 [0.44-0.82]	0.35 [0.15-0.56]
	Recall	0.57 [0.1-1]	0.69 [0.57-0.8]	0.24 [0.05-0.43]
	F1-Score	0.54 [0.24-0.83]	0.65 [0.59-0.71]	0.29 [0.1-0.48]
	AUROC	0.75 [0.56-0.94]	0.71 [0.44-0.99]	0.58 [0.27-0.88]
	Accuracy	0.55 [0.45-0.65]		
Repetition 3	**Precision**	0.51 [0.37-0.64]	0.55 [0.5-0.6]	0.33 [0-0.7]
	Recall	0.55 [0.45-0.65]	0.59 [0.02-1]	0.22 [0-0.57]
	F1-Score	0.52 [0.46-0.59]	0.55 [0.23-0.88]	0.24 [0.06-0.41]
	AUROC	0.77 [0.67-0.86]	0.66 [0.62-0.71]	0.55 [0.45-0.65]
	Accuracy	0.48 [0.3-0.67]		

AUROC: area under the receiver operating characteristic curve; CI: confidence interval.

Table S3. Performance metrics of the deep-learning classifier in the prediction of the luminal and basal protein-based subtypes for the three repetitions. For each repetition a measure of accuracy, AUROC, sensitivity, specificity, precision, and F1-Score is reported for the basal subtype (here taken as the 'positive' class) as mean [95% CI] across the three hold-out folds, i.e. the data portions used, in turn, as validation set in the three-fold cross-validation setting.

	Repetition 1	**Repetition 2**	**Repetition 3**
Accuracy	0.75 [0.52-0.99]	0.79 [0.75-0.84]	0.75 [0.48-1]
AUROC	0.83 [0.67-0.99]	0.8 [0.62-0.99]	0.81 [0.65-0.96]
Sensitivity (Recall)	0.67 [0.46-0.87]	0.6 [0.49-0.7]	0.6 [0.32-0.87]
Specificity	0.8 [0.42-1]	0.9 [0.84-0.96]	0.83 [0.55-1]
Precision	0.67 [0.25-1]	0.76 [0.67-0.85]	0.66 [0.2-1]
F1-Score	0.66 [0.42-0.9]	0.67 [0.6-0.73]	0.62 [0.27-0.98]

AUROC: area under the receiver operating characteristic curve; CI: confidence interval.

PART III

CLINICAL MANAGEMENT OF UTUC AND INTRAVESICAL RECURRENCES


6

REDUCE BLADDER CANCER RECURRENCE IN PATIENTS TREATED FOR UPPER URINARY TRACT UROTHELIAL CARCINOMA: THE REBACARE-TRIAL

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Contemporary Clinical Trials Communications, 2018

ABSTRACT

Background: Following radical nephro-ureterectomy for urothelial carcinoma of the upper urinary tract (UUT), the reported bladder recurrence rate of urothelial carcinoma is 22-47%. A single intravesical instillation of chemotherapy within 10 days following nephro-ureterectomy has the potential to decrease the risk of a bladder recurrence significantly. Despite recommendation by the European Association of Urology guideline to administer a single instillation postoperatively, the compliance rate is low because the risk of extravasation of chemotherapy.

Aim: To reduce the risk of bladder cancer recurrence by a single intravesical instillation of Mitomycin immediately (within 3 hours) before radical nephro-ureterectomy or partial ureterectomy.

Methods: Adult patients (age ≥ 18 years) with a (suspicion of a) urothelial carcinoma of the UUT undergoing radical nephro-ureterectomy or partial ureterectomy will be eligible and will receive a single intravesical instillation of Mitomycin within 3 hours before surgery. In total, 170 patients will be included in this prospective, observational study. Follow-up will be according to current guidelines.

Results: The primary endpoint is the bladder cancer recurrence rate up to two years after surgery. Secondary endpoints are: a) the compliance rate; b) oncological outcome; c) possible side-effects; d) the quality of life; e) the calculation of costs of a single neoadjuvant instillation with Mitomycin and f) molecular characterization of UUT tumors and intravesical recurrences.

Conclusions: A single intravesical instillation of Mitomycin before radical nephro-ureterectomy or partial ureterectomy may reduce the risk of a bladder recurrence in patients treated for UUT urothelial carcinoma and will circumvent the disadvantages of current therapy.

1. INTRODUCTION

Urothelial carcinoma of the upper urinary tract (UUT) is a relatively rare disease with an incidence of 2 per 100.000 person/year in Europe [1]. At diagnosis 60% of UUT tumors are invasive versus only 15-25% for urothelial carcinoma of the bladder [2,3]. The outcome of UUT urothelial cancer is rather poor: the 5-year survival rate following radical nephro-ureterectomy (RNU) varies from less than 50% for pathological stage pT2 or pT3 disease versus less than 10% for pT4 disease [3]. The characteristics of high-risk UUT disease are: high-grade tumor at biopsy, multifocalitiy, positive urinary cytology, transmural disease, hydro-nephrosis on imaging and a tumor size ≥ 1 cm [4-6]. For high-risk urothelial carcinoma of the UUT, RNU with excision of the ipsilateral bladder cuff is the treatment of choice, either by open or minimally invasive surgery [2].

Despite this radical surgical procedure, the bladder recurrence rate at two years following RNU for UUT urothelial carcinoma varies from 22 to 47% [4,7-9]. A recent study showed that 70% of these recurrences occurred in the first year following RNU [10]. Risk factors for a bladder recurrence following RNU are previous bladder cancer, tumor multiplicity, tumor location, tumor stage, and the operative modality [7,11]. For the prediction of intravesical recurrences following RNU, two studies designed predictive tools with an accuracy of 62% to 69%. This indicates the difficulties in predicting which patients will develop subsequent bladder recurrences [11,12].

Recently, two randomized controlled trials have shown that a postoperative intravesical instillation of chemotherapy reduced the risk of a bladder recurrence following RNU [8,9]. A meta-analysis of these two studies showed that an intravesical instillation with chemotherapy within 10 days following RNU decreased the risk of bladder recurrence with 52%; the absolute risk reduction was 13% [10]. Despite the fact that the European Association of Urology (EAU) guideline recommends a single postoperative intravesical instillation with chemotherapy based on the result of these two studies [2], the compliance rate in clinical practice to this additional treatment is low. A survey among

Dutch urologists showed that only 10% actually administers a postoperative instillation [10]. This reluctance is mainly due to the fact that a fresh wound is present in the bladder, which could lead to extravesical leakage of chemotherapy and with that potential life-threatening sequelae [13].

Here, we present the REBACARE study, in which patients receive a single intravesical instillation with chemotherapy just before RNU or partial ureterectomy for an UUT urothelial carcinoma. As subsequent bladder recurrences probably result from intraluminal seeding and the implantation of cancer cells [14,15], a preoperative instillation with chemotherapy could eradicate possible seeding of cancer cells in the bladder. This neoadjuvant strategy has previously been shown to be effective in the treatment of non-muscle invasive bladder cancer using device-assisted instillations of Mitomycin [16]. The approach of a single instillation with chemotherapy before surgery has the following advantages: **i)** it will circumvent the possibility of extravesical leakage of chemotherapy; **ii)** it will spare the patient an invasive diagnostic procedure (cystogram); and **iii)** it could result in a better compliance of urologists.

2. STUDY DESIGN

2.1 Study management

The REBACARE study is designed as a multicenter, prospective, non-randomized cohort study in a clinical setting. Inclusion of patients will take place from September 2017 till December 2019. The estimated end of the study is December 2021, two years following RNU or partial ureterectomy of the last included patient. The follow-up will be in accordance with the 'EAU guideline for the treatment of upper urinary tract urothelial carcinoma' in which the surveillance regimen consists of cystoscopy, urinary cytology, and CT urography scans [2]. Only bladder recurrences (urothelial carcinoma) within two years following surgery will be counted for study purposes. In case a bladder recurrence is suspected, a diagnostic biopsy is warranted to histologically confirm a urothelial carcinoma of the bladder (Appendix A for the flow-chart of the trial).

The relapse rate in the study cohort will be compared with the relapse rate of a matched historical cohort. This historical cohort will consist of patients older than 18 years who underwent a RNU or partial ureterectomy for urothelial carcinoma of the UUT, performed between 2001-2015 in the participating centers, received no perioperative intravesical instillation of chemotherapy and who had no previous history of a urothelial carcinoma of the bladder.

2.2 Population

Adult patients (age ≥18 years) who undergo a RNU or partial ureterectomy (open or laparoscopic) for a primary urothelial carcinoma of the UUT will be eligible. These patients will be selected from participating centers in the Netherlands. Approximately 150 RNU's for urothelial carcinoma of the UUT were performed yearly in the Netherlands between 2006 and 2010.

No exact information is available for the total number of partial ureterectomy procedures performed in the Netherlands for UUT urothelial carcinoma. However, probably, these numbers are increasing due to the growing elderly population who are diagnosed with UUT but are too frail to undergo a RNU or have impaired renal function. Moreover, evidence is emerging that partial ureterectomy is feasible not only for an imperative indication, such as patients having a solitary kidney [17]. Given this increase in the number of partial ureterectomies performed, it is estimated that at least 90 patients per year can be included in the present study, whereby this number includes both patients undergoing RNU or partial ureterectomy.

See Appendix B for full inclusion and exclusion criteria of the study.

2.3 Study objectives

2.3.1 Primary objective

To demonstrate that a single intravesical instillation of chemotherapy immediately (within 3 hours) before RNU or partial ureterectomy for urothelial carcinoma of the UUT reduces the risk of a subsequent urothelial bladder cancer recurrence up to two years after surgery with 40% (from 22-47% to 13.2-28.2%) compared to a matched historical cohort who received no perioperative intravesical instillation.

2.3.1.1 Index objective: Risk reduction

A trial by O'Brien *et al*. randomized 144 patients to receive Mitomycin 40 mg at the time of urethral catheter removal following RNU (median time 7 days) and 140 patients to receive standard of care [9]. In the Mitomycin arm, 105 of 144 patients (73%) and 115 of 140 patients (82%) in the standard of care arm received their allocated treatment. Thirteen of 105 patients who received Mitomycin and 20 of 115 patients allocated to standard of care treatment did not complete follow up. By modified intention-to-treat analysis, 21 of 120 patients (17%) in the Mitomycin arm developed a bladder recurrence in the first year versus 32 of 119 patients (27%) in the standard of care arm (p=0.055). By treatment as per protocol analysis, 17 of 105 patients (16%) in the Mitomycin arm and 31 of 115 patients (27%) in the standard treatment arm developed a bladder recurrence (p=0.03). This resulted in a relative risk reduction in the recurrence rate in the first year following RNU of almost 40%; the absolute risk reduction was 11%. Ito *et al*. evaluated the efficacy of a single early intravesical instillation of Pirarubicin within 48 hours following RNU in the prevention of bladder recurrence [8]. In this smaller study, 36 patients were included in both the intervention and control arm. Significantly fewer patients in the Pirarubicin group compared to the control group had a bladder recurrence at 2 years following surgery (16.9% in the intervention *vs.* 42.2% in the control group). Consequently, this resulted in a considerable higher relative risk reduction as shown by O'Brien *et al*. (Ito *et al*. Odds ratio (OR) 0.280; 95% Confidence Interval (CI): 0.093-0.831, p=0.023 *vs*. O'Brien *et al*. OR 0.577; 95% CI 0.310-1.073, p=0.82) [10]. It's possible that they achieved a higher reduction in recurrence due to the administration of chemotherapy within 48 hours instead of within 10 days after surgery.

In addition a single instillation of chemotherapy following transurethral resection of bladder tumors (TURBT) for low- and intermediate-risk urothelial carcinoma of the bladder (UBC) induces a relative risk reduction of 40% to prevent a subsequent bladder tumor recurrence [18-20]. To prevent the implantation of tumor cells, the instillation should be given as soon as possible following TURB. In all studies which examined the effectiveness of a single, immediate, post-operative, intravesical instillation of chemotherapy following

TURB, the instillation was given within 24 hours following surgery [21]. This postoperative instillation following TURBT is most effective when administered within few hours of surgery [22].

2.3.2. Secondary objectives

a) To show a ≥80% compliance rate and accurate and consistent protocol performance of a single neoadjuvant instillation with MMC three hours before RNU or partial ureterectomy for a urothelial carcinoma of the UUT.
b) To assess the 2-year overall, cancer-specific and recurrence-free survival of a single neoadjuvant instillation with MMC before RNU or partial ureterectomy for UUT urothelial carcinoma compared with no perioperative intravesical instillations.
c) The toxicity of the regime as assessed by the CTCAE.
d) The impact on the quality of life of the subjects when receiving a neoadjuvant instillation with Mitomycin.
e) Costs from a societal perspective using a time horizon of two years and incremental cost-effectiveness ratios.
f) A molecular characterization of the UUT urothelial carcinoma and subsequent (recurrent) urothelial carcinoma of the bladder (side-study).

2.3.2.1. Index objective: Compliance rate

Despite level I evidence showing that a postoperative instillation with chemotherapy following RNU decreases the risk of a subsequent bladder recurrence, which is also recommended by the EAU guideline (Level B evidence) [2], the compliance rate is low in current clinical practice. A Dutch survey showed a compliance rate of less than 10% [10]. Therefore, by conducting this trial, we aim to show not only that a neoadjuvant instillation of chemotherapy is equally effective as a postoperative instillation in reducing the risk of a subsequent bladder cancer recurrence, but it must also lead to a much higher compliance rate of clinicians to this neoadjuvant strategy because it lacks the potential risk of extravesical extravasation of chemotherapy.

2.3.2.2 Index objective: Survival rates

At the time of diagnosis, 60% of all urothelial carcinomas of the UUT are invasive resulting in overall poor survival rates for patients with urothelial carcinoma of the UUT. In a large retrospective study by Adibi *et al.,* the 5-year survival rates among 1462 patients who underwent RNU were less than 50% for stage pT2 or pT3 disease and less than 10% for pT4 disease [23].

Several studies have assessed individual patient risk factors for oncologic outcomes [5,7,12,24,25]. Lughezzani *et al.* and Mathieu *et al.* identified tumor stage and grade to be the most significant factors in oncological outcome [5,26]. Moreover, with respect to surgery, cancer-free surgical margins and the method of bladder cuff resection (trans- or extravesically) had the most significant impact on cancer-specific survival and overall survival. The most significant risk factors for intravesical recurrence of a urothelial cell carcinoma post RNU were a previous history of a urothelial cell carcinoma of the bladder and multifocalitiy of the UUT tumor [25].

In the present study, the 2-years survival rates post RNU and partial ureterectomy will be assessed and stratified by individual patient characteristics. The technique of bladder cuff resection is mandatory, including a trans- or extravesical approach, and uniformly performed in all study participants. A secondary aim of this study is to develop a novel predictive model for clinical outcome (bladder cancer recurrence and survival) following RNU for urothelial carcinoma of the UUT. Predictive nomograms are used widely in urology to help patients counseling and complex decision-making regarding treatment, but none of these to date have been developed based on prospective data and none have achieved widespread routine use, due to low level of evidence and lack of external validation. In a meta-analysis by Mbeutcha *et al*. on predictive models for the treatment of urothelial carcinoma of the UUT [24], a positive predictive value of 89% was achieved when combining hydronephrosis, ureteroscopic grade and urinary cytology for prediction of advanced-stage of UUT urothelial carcinoma [6]. If all three were negative, the negative predictive value was 100%. Xylinas *et al*. acquired an accuracy of 69% for postoperative prediction of intravesical recurrence risk at 2 years [11]. They combined age,

gender, history of bladder cancer, tumor location, clinical stage, concomitant *carcinoma in situ* (*CIS*), lymph node metastasis, bladder-cuff excision and surgical approach.

2.3.2.3. Index objective: Toxicity

Moriarity *et al*. reported on the safety of an intravesical instillation with MMC or Adriamycin that was administered during RNU in 51 patients. Through a two-way catheter, inserted at the beginning of the procedure, MMC (40 mg) or Adriamycin (40 mg) was instilled. The catheter was clamped for one to two hours (median time 60 min, range 45-120 min). Just before the bladder was opened for the resection of the ureteric orifice, the chemotherapy was drained passively and the bladder was occasionally irrigated with saline. In total 31 of the 51 RNU's were performed by an extravesical excision of the bladder cuff. The other techniques consisted of intravesical excision of the bladder cuff or intramural ureterectomy. Nine out of 51 patients underwent a distal ureterectomy only. The intra- and postoperative complications were monitored up to 90 days following surgery. No adverse events were reported that were attributable to MMC or Adriamycin instillation [27]. Furthermore, in the studies on the efficacy of a single instillation of post-operative intravesical chemotherapy by O'Brien *et al*. and Ito *et al*. only non-serious adverse events were reported [8,9].

Although the reported toxicity is acceptable it is important to recognize and monitor possible side-effects attributable to the MMC instillation. To manifest possible adverse events the toxicity will be assessed in the present study until 3 months following surgery by the National Cancer Institute Common Toxicity Criteria (CTCAE) version 4.0. These toxicity criteria consist of standardized definitions for adverse events that describe the severity of organ toxicity for patients receiving cancer therapy.

2.3.2.4. Index objective: Quality of life

It is hypothesized that a neoadjuvant instillation with Mitomycin will not have a negative impact on the quality of life. To address this hypothesis, all patients will have to complete two questionnaires at inclusion (T0), before surgery

(with neo-adjuvant treatment), and at two weeks (T2) and three months (T3) following surgery. The EQ5D-5L, a standardized patient-reported instrument to measure general health, and the EORTC QLQ-C30, a questionnaire to assess the quality of life of cancer patients will be used. Both are validated questionnaires for measuring the quality of life within patients suffering from cancer. All time points (T0, T2 and T3) coincide with regular visits to the outpatient department in order to limit the extra burden for participating patients. To be able to adequately address the quality of life end point of the study, the drop-out rate for completed questionnaires must be less than 10%.

2.3.2.5. Index objective: Costs

The costs consist of direct costs (e.g., single gift of Mitomycin, personnel costs of health professionals involved, disorder related medication, disorder related innervations, time duration of hospital, informal care) and indirect costs (productivity loss) associated with each regimen. The economic evaluation will be a cost-utility analysis and a cost-effectiveness analysis performed from a societal perspective and will only be applicable to the Dutch healthcare system.

2.3.2.6. Index objective: molecular characterization (side-study)

Due to the rarity of the disease, little is known about molecular aberrations related to urothelial carcinoma of the UUT and the prognostic profile of molecular alterations that correspond with or even might predict bladder recurrences. In a time that cancers are increasingly stratified by their molecular alterations and treatment decisions can be based upon these alterations, it is important to investigate the genetic profiles of urothelial carcinoma of the UUT. Sfakianos *et al*. compared the genetic profile of 59 high-grade urothelial carcinomas of the UUT with another cohort of 102 high-grade UBC by targeted sequencing [28]. The spectrum of genes mutated in tumors of the UUT and UBC was similar, but the frequency of mutations in *FGFR3, HRAS, TP53* and *RB1* was not. In high-grade urothelial carcinoma of the UUT *FGFR3* and *HRAS* were more frequently mutated, whereas mutations in *TP53* and *RB1* were less prevalent compared to high-grade UBC. Most of the disparity in clinical manifestation between urothelial carcinoma of the UUT and UBC may result from anatomical differences because of the thinner smooth muscle

coffering the UUT, but Sfakianos *et al*. showed that there are also genomic differences that might contribute to this phenomenon [28]. These observations provide evidence that urothelial carcinoma of the UUT and the bladder have distinct biological behaviors despite their histopathological similarities and therefore might require individualized treatment recommendations.

In both retrospective and prospective studies, a high proliferation index as assessed by *Ki-67* expression was associated with disease recurrence and cancer-specific survival in urothelial carcinoma of the UUT [29-33]. Furthermore, alterations in the *mTOR-pathway*, and the genes *HER2, BCAT1, CDCA5* and *p53* might play a role in the prognosis of high grade urothelial carcinoma of the UUT, but the impact of these biomarkers hasn't been sufficiently validated because of the small portion of samples in single-institution cohorts [24].

Currently two hypotheses for the development of a bladder recurrence following RNU for urothelial carcinoma of the UUT are postulated: a) intraluminal seeding and implantation of cancer cells [14,15]; multifocal tumors are descendants of a single transformed cell, which proliferates and spreads by intraluminal seeding or intraepithelial spread or b) in field cancerization [34], where it is assumed that multiple cells become initiated or partially transformed as a result of carcinogenic hits. In order to address these hypotheses it is important to compare the genomic profile of the primary urothelial carcinoma of the UUT and the subsequent bladder recurrence within the same patients.

Therefore, at inclusion, patients will be asked to provide separate consent for the use of their tumor tissue for molecular analysis. DNA will be isolated from the primary urothelial carcinoma of the UUT, the bladder recurrence and a buccal swap or non-malignant kidney tissue. Genomic sequencing will be performed to investigate tumor-specific somatic mutations and copy number variations to compare the molecular profile of the primary urothelial carcinoma of the UUT and a subsequent carcinoma of the bladder.

2.4 Sample size calculation

The estimated recurrence of urothelial carcinoma of the bladder following RNU for a UUT urothelial carcinoma is based on the literature. It has been shown that in patients not treated with adjuvant intravesical therapy following RNU, the bladder recurrence rate at two years was between 10-50% (mean 33.2%, total number of patients reported 995, range 36-223) [10]. We hypothesize a reduction in the risk of a bladder recurrence of 40% after RNU or partial ureterectomy by the neoadjuvant regimen of a single instillation with chemotherapy within three hours before surgery. Consequently, this translates into a 19.9% estimated bladder recurrence rate for this study. Therefore, it is calculated that a sample size of 170 patients is needed to show a 40% difference two years following surgery with a power of 80% using a two-sided p-value of 0.05.

2.5 Ethical approval

The study abides by the principles of the Declaration of Helsinki. Ethical approval for this study was obtained from the institutional review board of Erasmus University Medical Center Rotterdam (METC 2017-227, NL60919.078.17). Also the board of directors of all participating hospitals have given permission for execution of this particular trial.

3. STATISTICAL ANALYSIS

All analyses are based on the intention-to-treat principle, i.e. all eligible patients will be included in the analysis independently of whether they received treatment or not. Data characterized by normal distribution will be expressed as mean ± standard deviation. Parameters not normally distributed will be expressed as median (range).

3.1 Primary study parameter

The bladder relapse rate at two years following surgery is the primary endpoint of the study. The relapse rate will be compared with the relapse rate of a matched historical cohort on a 1:2 basis (1 intervention cohort:2 historical cohort). The historical cases will be selected by the following criteria: age ≥ 18

years, treated by RNU or partial ureterectomy for a histologically proven UUT urothelial carcinoma (cT1-T4 with or without *CIS*), no lymph node or distant metastasis at the moment of diagnosis as assessed by CT thorax-abdomen (cN0M0), a minimum of two years of follow-up following surgery, no perioperative systemic chemotherapy administered, and no history of urothelial carcinoma of the urinary tract before diagnosis of the UUT urothelial carcinoma. The difference in relapse rate between the intervention cohort and the matched historical cohort will be assessed using a multivariable Cox regression analysis and stratified by the following confounders: age, type of surgery (RNU versus partial ureterectomy), pathological stage, tumor grade, tumor size, tumor location, tumor multiplicity, concomitant *CIS*, medical center of treatment and surgical techniques (open versus laparoscopic).

3.2 Secondary study parameters

The difference in overall, cancer-specific and recurrence-free survival between the intervention cohort and matched historical cohort will be estimated using a multivariable Cox regression analysis. The toxicity of the treatment at different time points will be tested using a repeated measurements analysis. The quality of life at baseline, at 2 weeks and at 3 months following surgery will be compared using a repeated measurements analysis. Furthermore, potential risk factors will be identified using multivariable Cox proportional hazards. Co-variables included in the analysis are: type of surgery (partial ureterectomy or RNU (laparoscopic or open)), result of pre-operative urine cytology, histological stage and grade of the tumor, tumor location, concomitant *CIS* and lymph node involvement.

The primary economic analysis will be a cost-utility analysis performed according to the Dutch guideline to determine whether neoadjuvant intravesical instillations with Mitomycin before organ-sparing surgery or RNU are a cost-effective alternative to the standard of care (historical cohort) [35]. Additionally, a cost-effectiveness analysis will be conducted to determine the costs per prevented bladder recurrence. The time horizon will be from start of therapy (t=0) till 24 months follow-up to take all relevant costs and effects regarding the MMC and standard of care strategy into account. The costs are defined as

direct and indirect costs associated with procedures performed within each regimen. The costs will be estimated by multiplying resource utilization with the cost per unit of resource (market prices, guideline prices or self-determined prices based on costing methods, i.e. full costing) [35]. The incremental cost-effectiveness ratio (ICER) of MMC will be calculated (i.e., the difference in costs of MMC versus standard of care divided by the average change in QALYs and bladder recurrence rate, respectively). The sensitivity of various costs per unit of resource will be tested in sensitivity analyses.

All statistical analyses will be performed using statistics software (SPSS version 21.0 for Windows, Chicago, IL, USA). A two-tailed *p*-value of <0.05 is considered significant.

4. STUDY PROCEDURES

A flow diagram of the REBACARE trial is presented in Appendix A. The following procedures are performed for research purposes at a different time point or in addition to the standardized care.

4.1 Treatment

After consent is obtained for both the primary study and the side-study, patients will be asked to provide a buccal swab for the collection of germline DNA. On the day of surgery, MMC is administered intravesically in all patients within 3 hours before surgery. The MMC is given directly into the bladder by an indwelling catheter. The indwelling catheter is inserted through the urethra and after instillation of the MMC the catheter is clamped, which allows the medication to remain in the bladder. The doses will be a suspension of 40 mg MMC in 50 ml sterile saline (NaCl 0.9%) and must remain in the bladder for a period of at least 1 hour with a maximum of 2 hours, if possible. The patient is then transported to the operating room. Once the bladder is emptied by the indwelling catheter, the bladder will be continuously rinsed with NaCl 0.9% to remove all remains of the MMC and possible floating tumor cells. The indwelling catheter will remain inside the bladder during surgery and the rinsing will be stopped at the moment the treating surgeon is about to incise the bladder

wall for excision of the ureteric orifice. The indwelling catheter will remain for some days after surgery until the patient has recovered. The exact number of days the indwelling catheter will remain in the bladder following surgery is at the discretion of the treating physician. From a pilot experiment it is known that neoadjuvantly administered intravesical MMC can no longer be detected on the surgical equipment or inside the operating room once the bladder is appropriately rinsed with 2 x 50mL of NaCl 0,9% (*Dr. A.G.M. van der Heijden, personal communication*). Therefore, medical personnel who will treat the study participants will not be exposed to MMC.

The surgical procedure is not performed for research purposes, however the participating physicians will be asked to carry out the procedures in a standardized matter; i.e. both the RNU or partial ureterectomy must start with clipping of the ureter distal of the tumour before manipulation of the ureter can take place. For a RNU, the ureteric orifice must be circumcised and resected *'en block'* attached to the ureter (bladder cuff). The pathology report must describe the presence of the ureteral clip and, for the latter, the presence of a bladder cuff including the ureteric orifice. The administration of antibiotic prophylaxis is advocated and the antibiotic regimen (orally or intravenously) should be in accordance with the local guidelines of the participating hospitals or based on a urinary culture.

4.2 Follow up

The follow up will be in line with the standardized care and will not include additional investigations. Cystoscopy plus urine cytology will be performed at 3, 6, 12, 18 and 24 months. The follow-up also includes CT-urography at 6, 12, 18, 24 months. In case of an invasive tumor, follow-up will include a CT-thorax at 6 and 12 months. All patients will complete two questionnaires at three moments during the study (Appendix A) to examine the quality of life following this treatment. To demonstrate side effects, patients will fill in a side-effects form 4 times. In case a bladder recurrence is suspected, it is warranted to take a diagnostic biopsy to histologically confirm and classify urothelial carcinoma of the bladder.

4.3 Side study: molecular analysis

Both a buccal swap and a biopsy of the tumor will be collected from participants who provide separate informed consent. DNA will be isolated from the primary UUT tumor, the subsequent intravesical recurrence and non-malignant kidney tissue or a buccal swab. Genomic sequencing will be performed to investigate tumor-specific somatic mutations and copy number variations to compare the molecular profile of the primary UUT tumor and subsequent bladder tumor.

5. DISCUSSION

5.1 Exposition of protocol

Following RNU for urothelial carcinoma of the UUT, the reported recurrence rate of urothelial carcinoma in the bladder is 22-47% (4-7). Intraluminal seeding [14,15] or in field cancerization [34] are thought to be the two hypotheses of this high recurrence rate. Based on the assumption that intraluminal seeding has most of the impact on this recurrence rate, a single postoperative instillation of chemotherapy following RNU has been introduced, and has shown to decrease the risk of bladder recurrence by 52% (relative risk reduction) [10]. Given the fact that many treating physicians waive the addition of a postoperative instillation with chemotherapy following RNU or partial ureterectomy, despite recommendation by the EAU guideline [2], could be an indication that another (neo)adjuvant treatment is desirable. To avoid the limitations in current treatment protocols of urothelial carcinoma of the UUT, the REBACARE trial will be the first prospective trial that could change current management. The fact that intravesical instillation will be administered before surgery makes this study unique and plausible positive for the compliance rate.

The REBACARE trial has additional aims besides the reduction of a subsequent bladder recurrence and a safer toxicity profile in comparison with a postoperative instillation, since the study also aims to explore the genetic profile of urothelial carcinoma of the UUT in more detail within a side-study performed during the REBACARE-trial. This is of specific interest, in a time in which the decision of certain treatment modalities of various tumors does not only rely

on clinicopathological characteristics, but can possibly also rely on tumor-specific molecular alterations. Furthermore, molecular characterization may reveal tumor-specific alterations that are targets for new anticancer therapies. More important, by performing a molecular comparison of the primary UUT tumor and a subsequent bladder recurrence, the hypothesis that intraluminal seeding is responsible for developing bladder recurrences can be explored. If this is the case, tumors of both the UUT and the subsequent bladder tumor should have identical molecular alterations, i.e. tumor-specific mutations. On the other hand Sfakianos *et al*. showed differences in the genetic profiles and mutational status between urothelial carcinoma of the UUT and UBC [28]. Although these alterations were not examined within the same patient, their findings support the hypothesis that subsequent bladder tumors are *de novo* primary tumors with specific molecular alterations. Lastly, a molecular comparison of the primary UUT of the patients who develop a recurrence versus those who do not might reveal important aberrations that could explain the risk of the development of a bladder recurrence or might even be predictive of a bladder cancer recurrence. The REBACARE trial is one of the first studies in which DNA from the primary tumor as well as DNA from the bladder recurrence of the same patient will be compared and this will result in unique information.

5.2 Study limitations

There are several limitations associated with the design of the study. Theoretically, the REBACARE trial could be designed as a prospective randomized controlled trial generating level 1 evidence. However, a randomized controlled trial would not be feasible due to the large number of study participants in relation to the relative low number of patients that will be diagnosed and treated for urothelial carcinoma of the UUT. In addition, due to the low compliance rate for a postoperative instillation with chemotherapy, inclusion of patients in the post-operative instillation arm (standard of care) will take very long. Furthermore, the recommendation in the EAU guideline for a postoperative instillation of chemotherapy following RNU makes it not ethical to conduct a study in which any form of intravesical instillation with chemotherapy is withholded in the control arm.

The exclusion criteria of a previous UBC will limit the inclusion rate of the REBACARE trial as the majority of patients with urothelial carcinoma of the UUT are known to have had one or more episodes of UBC in their history. However, this was necessary, because it is known that these patients are at much higher risk to develop a subsequent bladder recurrence following surgery for urothelial carcinoma of the UUT [11,24,36]. Including these patients will potentially jeopardize the outcome of this trial because it will have an impact on the primary endpoint of this trial.

CONCLUSION

Approximately 40% of patients will develop a intravesical recurrence following a RNU for urothelial carcinoma of the UUT. A single postoperative instillation with chemotherapy reduces the risk of a bladder cancer recurrence significantly. Nevertheless, the compliance rate of clinicians with a single postoperative instillation is low due to the potential risk of extravasation of chemotherapy. The REBACARE trial is the first prospective trial that aims to determine the effect of a single preoperative intravesical instillation with chemotherapy on the risk of a bladder cancer recurrence following RNU for urothelial carcinoma of the UUT. This change in order of treatment may prove to be equally effective as a postoperative instillation but with a safer profile and has the potential to change clinical practice in a definitive way.

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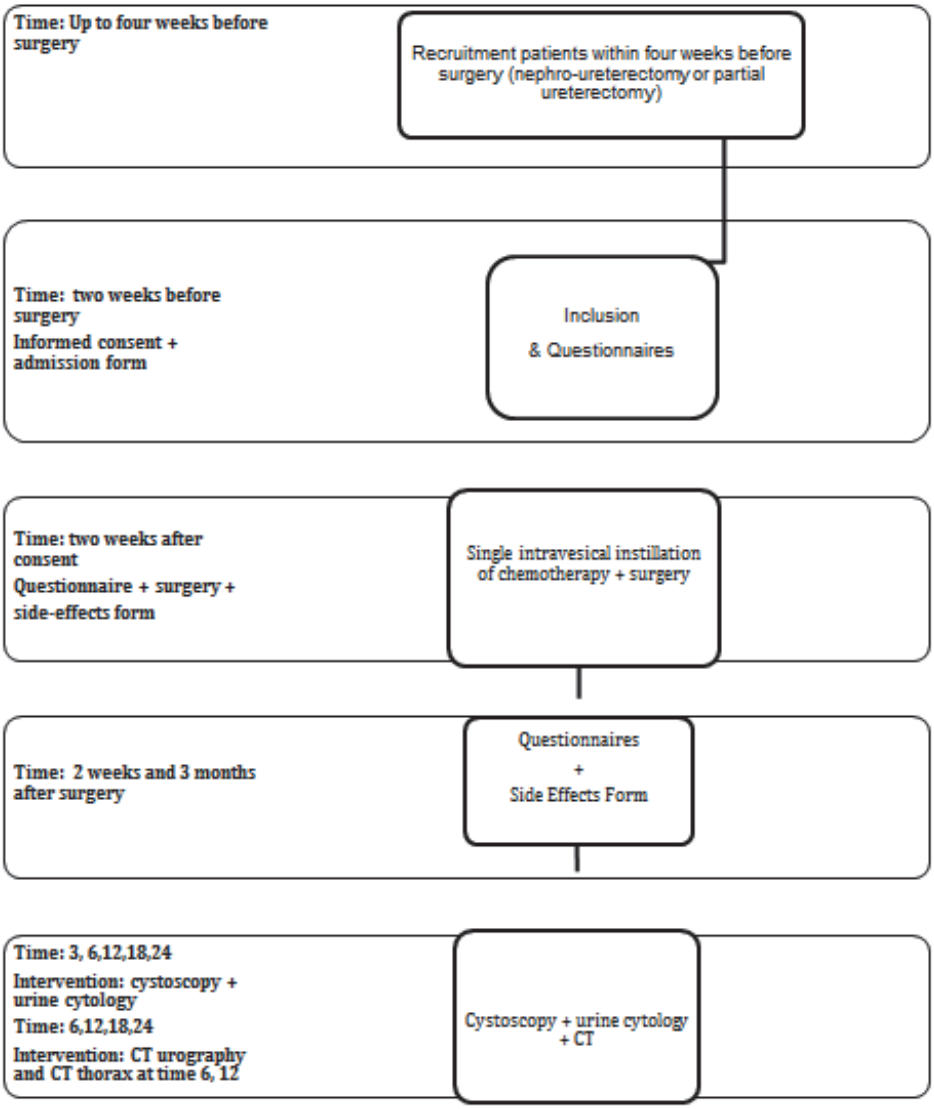
Appendix A. Flow-chart of the REBACARE trial



Appendix B. In- and exclusion criteria of the REBACARE trial

Inclusion criteria	1.	Histologically proven urothelial carcinoma of the UUT with or without concurrent *carcinoma in situ* (*CIS* only is also allowed) or patients with a suspicion of a urothelial carcinoma of the UUT on CT-scan plus a urinary cytology sample showing high-grade urothelial carcinoma;
	2.	Patients planned to be treated either by partial ureterectomy or by a radical nephro-ureterectomy (open or laparoscopic) including a bladder cuff;
	3.	Age ≥ 18 years;
	4.	WHO performance status 0, 1 or 2;
	5.	Negative pregnancy test in woman with childbearing potential;
	6.	Written informed consent.
Exclusion criteria	1.	If pre-operative histology by biopsy: aberrant histology of the UUT tumor of >50% (adenocarcinoma, small cell carcinoma, squamous cell carcinoma).
	2.	History or presence of a malignant tumor or *carcinoma in situ* of the bladder.
	3.	History of UUT urothelial carcinoma on the contralateral side or presence of bilateral UUT urothelial carcinoma.
	4.	Known allergy against Mitomycin.
	5.	Anticipated adjuvant intravesical treatment with chemo- or immunotherapy.
	6.	Acute urinary tract infection at the time of inclusion as assessed by urinary culturing.
	7.	Lymphadenopathy or distant metastases as assessed by preoperative CT-scan of thorax and abdomen.
	8.	Any other concurrent severe or uncontrolled disease preventing the safe administration of intravesical Mitomycin.
	9.	Breastfeeding women.

7

INTRAVESICAL INSTILLATION WITH CHEMOTHERAPY BEFORE RADICAL SURGERY FOR UPPER URINARY TRACT UROTHELIAL CARCINOMA: THE REBACARE TRIAL

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European Urology, 2025

ABSTRACT

Background and Objective

Intravesical instillation with chemotherapy after radical surgery for upper urinary tract urothelial carcinoma (UTUC) reduces the risk of intravesical recurrence (IVR). However, compliance is low due to possible extravesical leakage after bladder cuff excision. This study aimed to enhance compliance and reduce IVR risk by evaluating the efficacy of a preoperative intravesical instillation with chemotherapy.

Methods

In this prospective, single-arm, multi-institutional, phase II clinical trial, 190 chemo-naïve, primary UTUC patients without prior, or concurrent bladder cancer, received a single intravesical MMC instillation for 1-2 hours within 3 hours before surgery. The primary endpoint was the 2-year histologically confirmed IVR rate, with a target reduction of >40%; from 33.2% (literature-reported) to <20%. A historical cohort of 247 UTUC patients who did not receive perioperative intravesical chemotherapy served as reference. Secondary endpoints included compliance, toxicity, and IVR-free survival, analyzed by multivariable Cox regression, stratified by previous diagnostic ureteroscopy (d-URS).

Key Findings and Limitations

The 2-year IVR rate was 24% (95% CI 18-31%) in the intention-to-treat and 23% (95% CI 13-32%) in the per-protocol analysis. Multivariable analysis showed d-URS to be associated with increased IVR risk. In the REBACARE cohort, patients without d-URS had a threefold lower IVR risk (HR 0.33, 95% CI 0.12-0.87) than in the reference cohort. Compliance with preoperative instillation was 96% and no grade >2 toxicity occurred.

Conclusions and Clinical Implications

A preoperative intravesical instillation with MMC was feasible, well-tolerated, and significantly reduces IVR risk in patients without d-URS. These findings suggest that a preoperative instillation seems a viable strategy for this subset of UTUC patients and that d-URS should be performed judiciously.

Patient summary

This clinical trial showed that a single bladder instillation with chemotherapy before surgery for upper urinary tract urothelial carcinoma was safe and feasible, with a 96% compliance rate. It reduced the 2-year risk of bladder cancer recurrence in patients who had not undergone a diagnostic ureteroscopy.

1. INTRODUCTION

The incidence of upper urinary tract urothelial carcinoma (UTUC) in Western Europe is 2-3 cases per 100,000 individuals annually, constituting 5-10% of urothelial carcinoma diagnoses, with a notable increase in incidence in recent decades [1-4]. Patients with non-metastatic UTUC undergo radical nephroureterectomy (RNU) with excision of an ipsilateral bladder cuff, with or without lymph node dissection [2]. Adjuvant platinum-based systemic chemotherapy is recommended in patients with locally advanced UTUC to improve disease-free survival [2,5]. In selected patients with low-risk UTUC, kidney-sparing surgery is an alternative treatment option [2]

A significant challenge in the clinical management of UTUC patients is the high risk of intravesical recurrences (IVR) after RNU. The reported IVR rate is 22-47% within two years in patients who did not receive a perioperative intravesical instillation [6,7]. It is hypothesized that IVR derives from seeding of cancer cells from the upper urinary tract to the bladder, which might occur prior to clinical diagnosis [8-10]. Diagnostic ureteroscopy (d-URS), used in the diagnostic workup for UTUC confirmation, is reported to be associated with an increased IVR risk [11,12].

Current guidelines recommend a single postoperative intravesical chemotherapy to mitigate IVR risk [2,13,14]. However, its adoption in clinical practice is hampered, mainly due to concerns over extravesical chemotherapy leakage after bladder cuff excision, which can lead to severe morbidity and even mortality [15-20].

We hypothesized that an intravesical instillation of chemotherapy immediately before radical surgery for UTUC may offer comparable IVR risk reduction without the risk of extravesical leakage, potentially enhancing physician compliance. Therefore, we initiated the REduce BlAdder Cancer After Radical nEphroureterectomy (*REBACARE*) study, which assessed the efficacy, safety, and compliance rate of a preoperative Mitomycin C (MMC) instillation in patients with primary localized non-metastatic UTUC undergoing radical surgery.

2. MATERIALS (PATIENTS) AND METHODS

2.1 Study population

Eligible participants were adults diagnosed with primary UTUC, stage cTanyN0-1M0, scheduled for radical surgery. The UTUC diagnosis relied on a biopsy during diagnostic d-URS and/or CT-urography together with urine cytology suspicious of high-grade urothelial cancer cells. Surgery comprised open or laparoscopic (conventional or robotic) RNU or partial ureterectomy with ipsilateral bladder cuff excision. Exclusion criteria included prior or synchronous bladder carcinoma, contralateral UTUC, prior intravesical chemotherapy, >50% aberrant histology on preoperative biopsy, MMC allergy, acute urinary tract infection, or pregnancy. Patients with post-operative histology showing absence of cancer or >50% aberrant histology were also excluded, as well as patients who received a postoperative instillation with chemotherapy.

The study, approved by the institutional review board of the Erasmus Medical Center, obtained enforceability permission for all sites (METC 2017-227 NL60919.078.17). It adhered to the Declaration of Helsinki and Good Clinical Practice and was registered at clinicaltrialsregister.eu (EudraCT number: 2017-000949-53). All patients provided written informed consent before study inclusion. The informed consent procedure has been described in detail before [21].

2.2 Study design

The *REBACARE* trial, a phase II, single-arm study, was conducted across 18 Dutch hospitals. Patients were included from November 2017 until August 2020. Patients received a single intravesical instillation of MMC (40mg in 50ml sterile saline) within three hours before radical surgery. MMC was administered via an indwelling catheter and had to remain in the bladder for 1-2 hours, providing tolerance of the patient. Thereafter, the bladder was continuously rinsed with NaCl 0.9% to remove MMC remnants and possible floating tumor cells. Bladder irrigation continued during radical surgery and was stopped at the initiation of bladder cuff excision. Surgery had to commence within three hours after the removal of MMC. RNU or partial ureterectomy was performed following local guidelines

of the participating centers, but the distal ureter had to be clipped at the beginning of the procedure after the first identification of the ureter. Also, an *en-bloc* bladder cuff excision was mandatory when possible. Performance of a lymph node dissection was to the treating physician. Follow-up adhered to the *European Association of Urology* (EAU) guidelines on UTUC, encompassing cytology and cystoscopy every 3 months and CT imaging semi-annually for two years. If a cystoscopy was suspicious of a bladder tumor, a transurethral biopsy or resection of the tumor was mandatory. Toxicity and adverse events were assessed using the National Cancer Institute Common Toxicity Criteria (CTC) version 4.0 and the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 from inclusion up to six months following surgery and reported using the Clavien-Dindo classification [22].

2.3 Endpoints and Sample Size Calculation

The primary endpoint was the histologically confirmed 2-year IVR-rate. The literature-reported proportion of IVR 2-year post-RNU, when no postoperative instillation is administered, is assumed to be 33.2% [7,13,14,23,24]. By assuming a >40% reduction in IVR risk (from 33.2% to 19.9%) due to the beneficial effect of the preoperative instillation with chemotherapy, a total number of 170 included patients in the REBACARE trial was required (two-sided superiority test for comparing two independent proportions; power of 80%, two-sided *p*-value of 0.05). Inclusion was increased to 190 due to a larger than expected number of drop-outs (MEC-2017-227, amendment 10, 19th March 2020, NL60919.078.17, v11).

Secondary endpoints included compliance rate and toxicity of the preoperative instillation, and IVR-free, metastasis-free, cancer-specific, and overall survival. For calculating metastasis-free survival, patients were censored on the date of last performed CT-imaging. For cancer- and overall survival, patients were censored on last date to be alive.

2. 4 Reference cohort

A historical cohort, including patients with primary pTanyN0-1M0 UTUC treated with radical surgery and who had not received any form of intravesi-

cal chemotherapy (not post- nor preoperatively) and who were without a history of bladder cancer served as a reference cohort. It comprised data from four Dutch hospitals (2000-2018) and a coexisting international, retrospective cohort (2005-2020) from 18 institutions across Europe, Asia, and the United States [25,26]. The goal of the REBACARE study was to assess the efficacy of a preoperative intravesical instillation with chemotherapy in reducing the risk of IVR in comparison with this historical cohort of UTUC patients.

2.5 Statistical analysis

Descriptive analyses were performed to characterize the patient, tumor, and treatment features of the *REBACARE* and reference cohorts. Time-to-event endpoints were assessed using Kaplan-Meier and Cox Proportional Hazard regression and were calculated from time of inclusion (T0). The primary end point was evaluated by *intention-to-treat* and *per-protocol* analyses. The Multivariable Cox Proportional Hazard Regression, adjusted for various factors, including d-URS (yes/no) as an *interaction term* to assess whether a d-URS had different effects per cohort. This model, including the interaction between d-URS and cohort, showed a statistically better fit on the data than a model with main effects alone, p = 0.019. For a detailed description of the statistical analyses, see the supplementary materials. Analyses were performed using SPSS version 28.0.1. (IBM) and R version 4.3.1. (R core team, 2023).

3. RESULTS

3.1 Patient Characteristics and Compliance rate

Between November 2017 and August 2020, 190 patients were enrolled in the *REBACARE* trial (**Figure 1**). Twelve patients were excluded: 8 due to absence of cancer or >50% aberrant histology in the surgical resection specimens, and 4 for not meeting the inclusion criteria (acute UTI: n = 2, lymphadenopathy >cN1 and/or distant metastasis on preoperative CT-scan: n = 2) (**Supplementary Table 1**). Most patients were male (69%) with a median age of 70 years (IQR 63-75) (**Table 1**). A d-URS was performed in 104 (59%) patients, with biopsy in 78 (44%). The vast majority (n = 169, 95%) underwent RNU, with lymphadenectomy in 32 (18%) patients. Pathological T-stage was ≥pT2 in 79 (42%) and 6 (7.6%) patients were staged as pN+.

Clinicopathological characteristics of the *REBACARE* and the reference cohort were largely similar, except for distribution of a previous d-URS (59% vs 45%), clipping of the ureter (69% vs 25%), proportion of pT3 (28% vs 37%) and pTa stage (35% vs 23%), and multifocality (13% vs 20%).

One hundred seventy-one patients (96%) received the preoperative MMC instillation (**Supplementary Table 2**). The median instillation duration was 75 minutes (IQR 60-105), with surgery starting at a median of 105 minutes after removal of the MMC instillation. Eighty-four patients (44%) were not treated according to the study protocol for the following reasons: no bladder cuff excision (n = 20), no ureter clipping (n = 32), duration of instillation <60 minutes (n = 14), and initiation of radical surgery >3 hours post-instillation (n = 24).

De median follow-up of those without IVR or death in the REBACARE trial was 24 months (IQR 24-24) and in the historical cohort 24 months (IQR 20-24).

3.2 Intravesical Recurrence Rate

By *intention-to-treat*, the 2-year IVR rate was 24% (95% CI 18-31%), not reaching the predetermined efficient risk reduction of >40% (IVR rate <20%). The median time to IVR was 7.5 months (IQR 5.0-14.0) and the IVR-free survival at 1 year was 83% (95% CI 78-89%). In the reference cohort, the 2-year IVR rate was 26% (95% CI 20-31%). The 2-year IVR-free survival was 75% (95% CI 69-82) in the *REBACARE* cohort versus 70% (95% CI 64-77) in the reference cohort (**Figures 2A and 2B**). *Per-protocol* analysis demonstrated a 2-year IVR rate of 23% (95% CI 13-32%). As the IVR-rate in the *per-protocol* was similar to the *intention-to-treat* analysis, the following analyses were limited to the latter.

In the subgroup of patients from the REBACARE cohort who did not undergo a d-URS (n= 73, 41%), multivariable analysis demonstrated a threefold lower risk of IVR (HR 0.33, 95% CI 0.12-0.87, p = 0.025) than patients without a diagnostic URS in the reference cohort (**Table 2**, **Figure 3 and Supplementary Table 3 for baseline characteristics**). In the subgroup of patients from the REBACARE cohort in whom a d-URS was performed in the diagnostic workup of UTUC, the risk of IVR was significantly higher (HR 1.83, 95% CI 1.08-3.10, p =

0.025). The subgroup of patients from the reference cohort with a history of d-URS had a higher IVR risk than those without d-URS, however, no significance was reached. Lastly, UTUC located in the mid- or distal ureter was associated with an increased risk of IVR for both cohorts.

To assess whether there is an association between the date of radical surgery and the risk of IVR in the reference cohort, a new model including 'year of surgery' was developed using the predicters from the model described above. This new model showed that year of surgery was not a statistical predictor for IVR (HR = 1.04, 95% CI 0.98-1.11) and no adjustments for secular trends over time were necessary.

3.3 Survival Analysis (REBACARE cohort)

The 2-year metastasis-free survival was 77% (95% CI 71-83%) (**Supplementary Figure 1**). The 2-year cancer-specific survival and 2-year overall survival was 90% (95% CI 85-94) and 86% (95% CI 81-91), respectively.

3.4 Toxicity and Adverse Events

Twenty-seven (15%) patients experienced surgical complications (hemorrhage, urine leakage, wound infection, and bowel motion) within 30 days post-surgery, with only 2 (7.4%) grade >II (Clavien-Dindo) complications (both hemorrhage). Grade III or worse adverse events within six months post-surgery were reported in 48 patients (27%), none being related to MMC. A total of 23 treatment related complications were reported, most frequently bladder spasms (n = 13, 56%), for which medication was prescribed in 11 cases. Second most frequent complication was hematuria (n = 6, 26%) for which 2 patients received a temporary indwelling catheter for continuous rinsing.

DISCUSSION

The *REBACARE* trial is the first prospective study to assess the efficacy and feasibility of an intravesical instillation with MMC before radical surgery for primary UTUC. Although our study did not reach the predetermined reduction threshold of >40% in the 2-year IVR rate, a significant reduction was observed

among patients who had not undergone a d-URS in the diagnostic workup for UTUC. Therefore, a single preoperative MMC instillation could be a viable strategy for this subgroup of patients. A preoperative instillation exhibited an excellent safety profile and achieved almost 100% compliance. Although a d-URS still may contribute to the diagnostic workup of UTUC, i.e. patient-selection for radical or kidney-sparing surgery, it warrants careful consideration per-patient due to its strong association with the risk of IVR.

The guideline-recommended postoperative instillation with chemotherapy following radical surgery for UTUC is based on the outcomes from the *THP Monotherapy Study Group Trial* and the *ODMIT-C trial* [13,14]. These trials were initiated based on observed benefits in reducing the risk of IVR following transurethral resection of the bladder [27,28]. The theory of seeding of cancer cells, supported by molecular studies [8-10], posits that IVR may arise from intraluminal seeding or the spread of cancer cells during surgery for UTUC. Hence, a postoperative instillation with chemotherapy aims to mitigate the IVR risk by killing residual cancer cells in the bladder. Although it has been reported that a postoperative instillation after RNU is safe, the potential risk of extravesical leakage remains a concern, contributing to reported compliance rates of less than 50% in real-world clinical practice [17-20,29]. In contrast, a preoperative instillation bypasses these risks, and within the REBACARE study excellent safety and compliance rates were demonstrated. It is hypothesized that a preoperative instillation of MMC may prevent tumor cells form implanting in the urothelium as this neoadjuvant strategy has been shown to be effective in non-muscle invasive bladder cancer using device-assisted instillations of MMC before TURBT [30]. Continuous bladder irrigation with a saline solution might also inhibit tumor cell implantation.

Despite not achieving the predetermined IVR reduction threshold of >40%, the observed IVR rate of 24% was lower than the literature-reported mean IVR rate of 33.2% derived from study populations who did not receive a pre- or postoperative instillation [7,13,14,23,24,31]. This suggests the potential benefit of a preoperative instillation. Furthermore, it is noteworthy that the 2-year IVR-rate of the reference cohort (26%) was also much lower than the reported

mean IVR rate in the literature. This might be due to more favorable characteristics of the reference cohort, such as stage <pT2 UTUC and a lower proportion of patients with a history of a d-URS (45% versus 59%). In the REBACARE cohort, 38 out of 43 patients who developed an IVR had a d-URS (88%) in their history. In addition, in 30 of these 38 (79%) patients the UTUC was biopsied during d-URS. In addition, being a retrospective cohort, potentially underreporting of IVR might have occurred in the reference cohort

The 2-year IVR rate of 24% observed in the REBACARE trial may be deemed inadequate when compared to the IVR rates reported in the two trials that evaluated a postoperative instillation with chemotherapy. The study by *Ito et al*. randomized patients to receive a single dose of pirarubicin within 48 hours after RNU (n = 36) versus observation (n = 36) [13]. They reported a 1- and 2-year IVR rate of 17% versus 32% and 17% versus 42%, respectively, favoring the intervention arm. The ODMIT-C trial, which randomized patients to receive a single dose of MMC after RNU prior to catheter removal (<10 days after surgery) versus observation, was limited by a follow-up duration of 1 year. The reported IVR rate was 16% versus 27% in favor of the intervention arm in the *per-protocol* analysis (88% of included patients, p=0.03) [14]. The *intention-to-treat* analysis showed no statistically significant difference between the treatment arms. Importantly, histological confirmation of IVR was not mandatory, potentially leading to underreporting of the IVR rate. It is important to consider whether the outcomes of these two trials accurately reflect real-world daily clinical practice, as a substantial proportion of patients do not receive a postoperative instillation due to concerns about extravesical leakage of chemotherapy.

Almost 60% of patients in our study underwent d-URS during the diagnostic workup of UTUC, potentially contributing to the risk of IVR, as these patients exhibited a fivefold higher risk of developing IVR (HR 0.33 vs. 1.83) than patients without a history of d-URS. No benefit from preoperative instillation was observed in this subgroup, indicating that a d-URS was an important confounder. Given the time lapse between d-URS and radical surgery, which allows tumor cells to implant in the urothelium of the bladder, might be an explanation why a perioperative instillation is of limited benefit in these patients.

The necessity to perform a d-URS in the diagnostic workup of UTUC is debatable. While offering advantages, such as histopathological diagnosis and upper urinary tract inspection, it also has limitations, including the risk of understaging when a biopsy is performed, ureter perforation, urinary tract infection, a delay in time to definitive treatment, and an increased risk of IVR, which might be higher when a biopsy is performed [11,32,33]. Hence, the outcomes of the present study underscore the need to carefully consider d-URS on a per-patient basis, adhering to the EAU guidelines on UTUC recommending its use only when other diagnostic modalities are inconclusive. Future studies should explore alternative strategies, such as peri-URS bladder instillations with chemotherapy, to optimize IVR prevention in UTUC patients [34].

The limitations of the present study largely relate to its single-arm design. Due to the rarity of UTUC, a randomized controlled trial to compare a preoperative versus postoperative instillation was not considered feasible. Furthermore, because the recommendation for a postoperative instillation was only included in the guidelines just before the REBACARE trial started, it was not possible to compile a reference cohort with a representative number of cases who had received a postoperative instillation. Additionally, less than 50% of the included patients were treated according to the study protocol: ureteral clipping, bladder cuff excision, instillation for 1-2 hours, and less than 3 hours between instillation and surgery. However, the per-protocol analysis demonstrated similar results to the intention-to-treat analysis, suggesting that deviations from the protocol may not have significantly influenced the outcomes. Moreover, since repeated cystoscopy was not mandatory between d-URS and radical surgery, small IVR could have developed before radical surgery. At last, more prognostic risk factors are known for developing IVR but due to the number of participants and expected events we were constrained in the number of variables without risking underpowering of overfitting the analyses. The most significant risk factors based on current literature were included [35].

In conclusion, the *REBACARE* trial demonstrated that a single preoperative instillation with MMC before radical surgery for primary UTUC was safe, feasible, and significantly reduced the risk of IVR in patients without a history of d-URS.

Therefore, a preoperative instillation with MMC seems a viable strategy for a subset of UTUC patients. Since a d-URS was strongly associated with increased risk of IVR, it should be performed judiciously and restricted to patients in whom imaging and/or urine cytology are inconclusive.

DISCLOSURES OF INTERESTS

J.L. Boormans reports consultancy work for MSD, Janssen, BMS, AstraZeneca, Merck AG/Pfizer, and Bayer, book writing for Astellas and research collaborations with Merk AG/Pfizer, MSD, Janssen, and VitroScan during the conduct of the study. All other authors report no conflict of interest.

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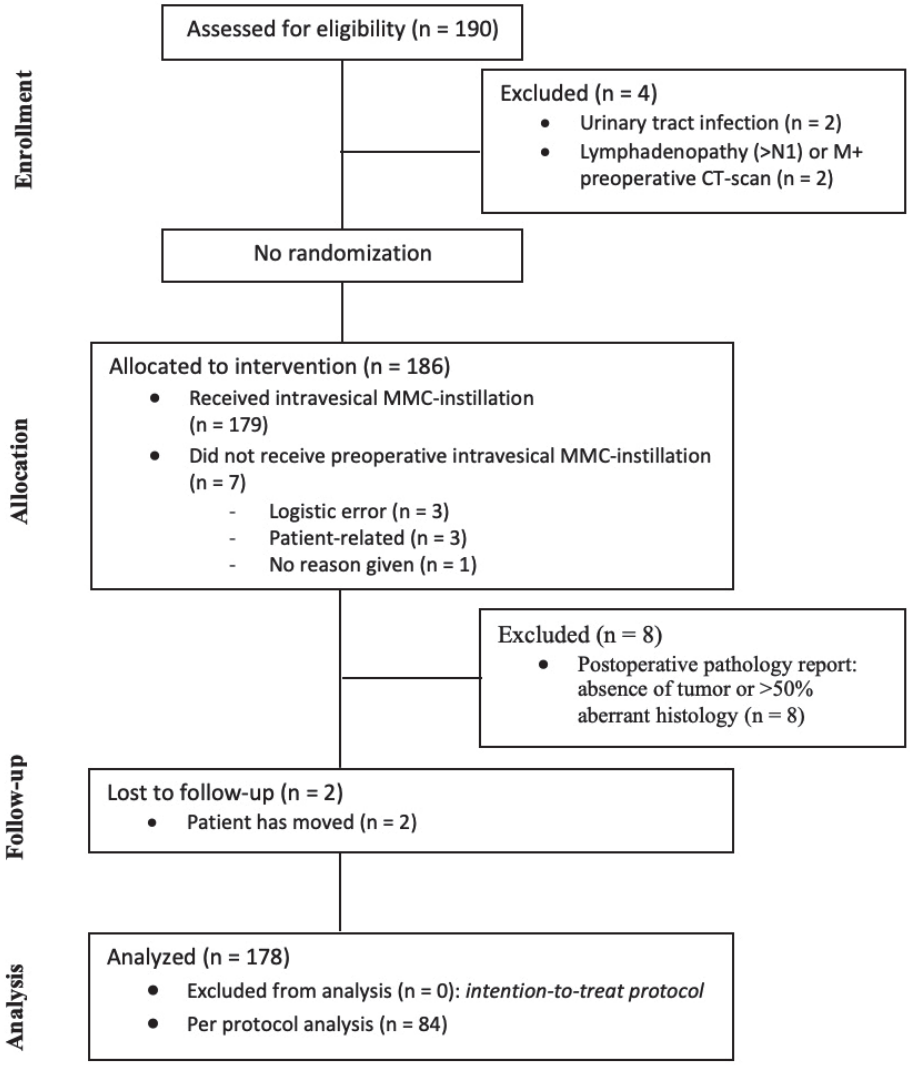
Figure 1. Study diagram of the REBACARE trial, according to CONSORT guidelines. Of the 190 patients with primary upper tract urothelial carcinoma included, 186 met the inclusion criteria. No randomization was performed due to the single-arm study design. Eventually, 179 patients received the intravesical MMC-instillation. Post-surgery, 8 patients were excluded due to absence of tumor or >50% aberrant histology leaving a total of 178 patients to be included in the *intention-to-treat* analysis.



CT = computer tomography, MMC = Mitomycin C

Figure 2A and 2B. The intravesical recurrence-free survival of patients with primary non-metastatic upper urinary tract urothelial cell carcinoma who received a preoperative instillation with Mitomycin C (A: REBACARE cohort) versus patients who had not received a perioperative instillation with chemotherapy (B: Reference cohort). Dashed line marks the 2-year free survival.

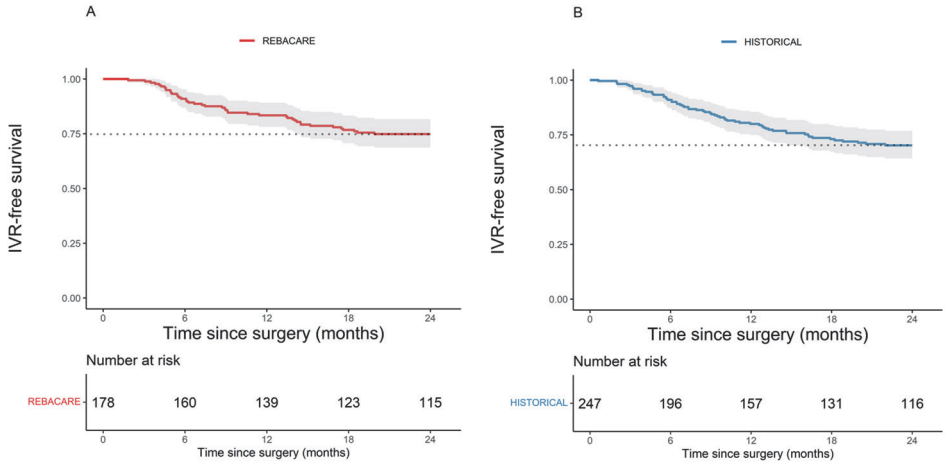


Figure 3. Predicted* intravesical recurrence-free survival of four hypothetical patients included in the REBACARE cohort versus the reference cohort, stratified by diagnostic ureterorenoscopy in the diagnostic work up for upper tract urothelial carcinoma, by Multivariable Cox Proportional Hazard Regression analysis. *pT-stage = T1-T2, sex = male, preoperative cytology = benign or atypia, age, tumor location = mid- or distal ureter, concomitant carcinoma in situ (CIS) = no, multifocality = no.

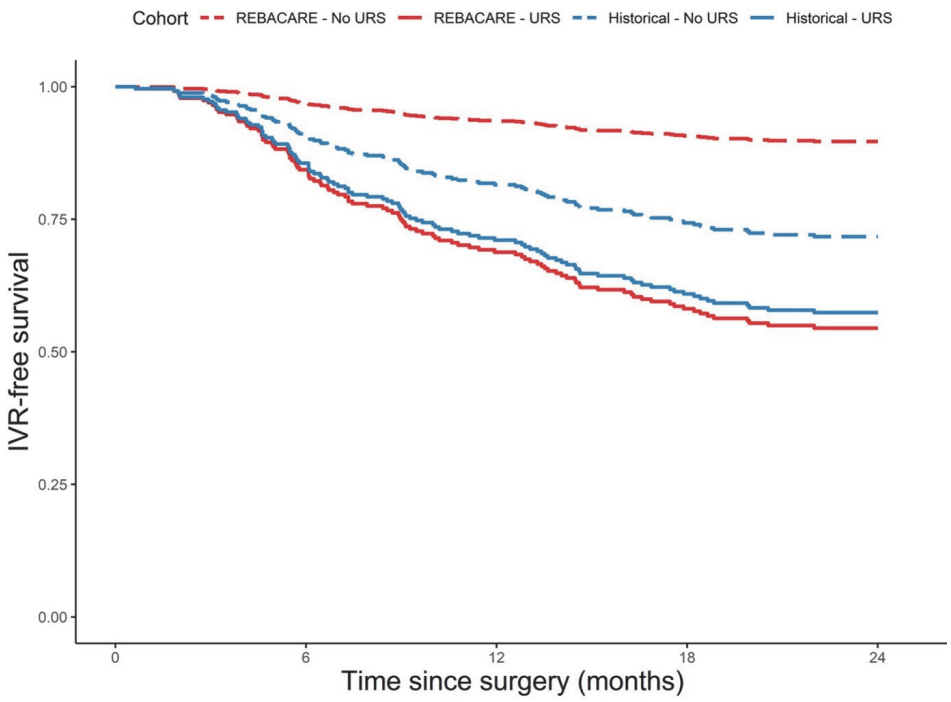


Table 1. Baseline characteristics of patients with primary upper urinary tract urothelial carcinoma, stage cTanyN0N1M0 included in the REBACARE trial and the reference cohort.

Characteristic	REBACARE-trial N = 178	Reference cohort N = 247
Sex, *n (%)*		
Female	55 (31)	83 (34)
Age (yr)		
<50	9 (5.1)	10 (4.0)
50-59	20 (11)	50 (20)
60-69	53 (30)	67 (27)
70-79	85 (48)	90 (36)
>80	11 (6.2)	30 (12)
Median (IQR)	70 (63-75)	69 (60-76)
Urinary cytology, preoperative		
Not done	26 (15)	83 (34)
Benign	54 (30)	68 (28)
Atypia/inconclusive	39 (22)	4 (2)
High grade, malignant	57 (32)	38 (15)
Missing data	2 (1.1)	54 (22)
Diagnostic URS		
No	73 (41)	128 (52)
Yes, without biopsy	26 (15)	41 (17)
Yes, with biopsy	78 (44)	70 (28)
Missing data	1 (0.6)	8 (3)
Type of surgery, *n (%)*		
RNU, open	22 (13)	43 (17)
RNU, laparoscopic/Robot	147 (83)	195 (79)
Partial ureterectomy, open	5 (2.8)	8 (3.2)
Partial ureterectomy, laparoscopic/Robot	4 (2.2)	0 (0)
Lymph Node Dissection, *n (%)*		
Yes	32 (18)	32 (13)
Missing data	4 (2.3)	0 (0)
Pathological tumor stage, *n (%)*		
Tis	4 (2.3)	28 (11)
Ta	63 (35)	57 (23)
T1	31 (17)	36 (15)
T2	25 (14)	8 (3.2)

Table 1 (continued). Baseline characteristics of patients with primary upper urinary tract urothelial carcinoma, stage cTanyN0N1M0 included in the REBACARE trial and the reference cohort.

T3	49 (28)	91 (37)
T4	5 (2.8)	27 (11)
pTx	1 (0.6)	0 (0)
Tumor grade (WHO 1973), *n (%)*		
Grade 1	19 (11)	8 (3.2)
Grade 2	70 (39)	39 (16)
Grade 3	81 (45)	51 (21)
Missing data	8 (4.5)	149 (60)
Lymph node involvement, *n (%)*		
N0	25 (14)	39 (16)
N1	6 (3.4)	7 (2.8)
N2	1 (0.6)	0 (0)
Nx	146 (82)	201 (81)
Concomitant CIS		
Yes	17 (10)	41 (17)
Missing data	2 (1.1)	4 (1.6)
Primary tumor location		
Renal pelvis or proximal ureter	122 (69)	173 (70)
Mid- or distal ureter	56 (32)	74 (30)
Multifocality		
Yes	23 (13)	50 (20)

CIS: *carcinoma in* situ; IQR: interquartile range; RNU: radical nephroureterectomy; URS: ureterorenoscopy.

Table 2. Multivariable Cox Proportional Regression analysis with risk factors associated with the risk of intravesical recurrence with diagnostic ureterorenoscopy included as interaction term in patients with primary upper urinary tract urothelial carcinoma.

	Hazard ratio	95% CI	p-value
pT-stage			
Tis-Ta-Tx	Ref.	Ref.	
T1-T2	0.80	0.43-1.37	0.4
T3-T4	0.85	0.54-1.33	0.5
Sex			
Male	Ref.	Ref.	
Female	0.66	0.42-1.04	0.077
Preoperative cytology			
Not done or unknown	Ref.	Ref.	
Benign or atypia	0.77	0.48-1.28	0.3
High grade	0.88	0.50-1.53	0.6
Age (per 10 year)	0.92	0.76-1.11	0.4
Tumor location			
Renal pelvis or proximal ureter	Ref.	Ref.	
Mid- or distal ureter	1.83	1.23-2.73	**0.003**
Concomitant CIS			
No	Ref.	Ref.	
Yes	1.04	0.58-1.86	0.9
Multifocality			
No	Ref.	Ref.	
Yes	1.11	0.66-1.87	0.7
Groups			
Historical and no d-URS	Ref.		
Historical and d-URS performed	1.67	0.98-2.83	0.057
REBACARE and no d-URS	0.33	0.12-0.87	**0.025**
REBACARE and d-URS performed	1.83	1.08-3.10	**0.025**

CIS = *carcinoma in situ*; CI = Confidence Interval; d-URS = diagnostic ureterorenoscopy. *P*-values of <0.05 were considered statistically significant.

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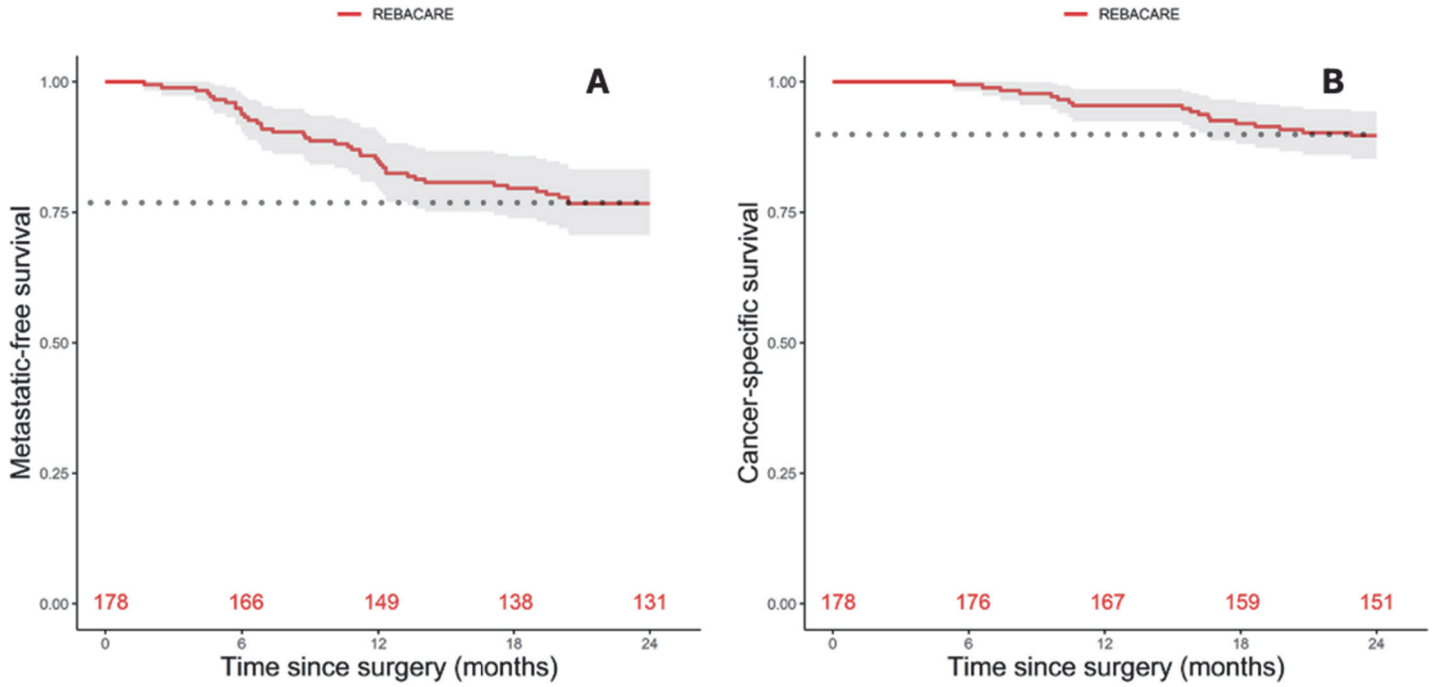
Supplementary figure 1. The metastasis-free survival (A) and cancer-specific survival (B) of 178 patients with upper tract urothelial carcinoma included in the REBACARE study. Dashes line marks the 2-year free survival.



Supplementary Table 2. Characteristics of the preoperative instillation with Mitomycin C in 178 patients with primary upper urinary tract urothelial carcinoma, stage TanyN0N1M0 treated by radical surgery

	REBACARE-trial N = 178 (IQR)
Preoperative instillation with MMC	
No	7 (4.0%)
Yes	171 (96%)
Median time of instillation, minutes (IQR)	75.0 (60.0-105.0)
Median time between instillation and surgery, minutes (IQR)	105.5 (79.0-152.8)
Treated *per-protocol*	
No	94 (53)
Yes	84 (47)

IRQ: interquartile range

Supplementary table 3. Baseline characteristics of patients with primary upper urinary tract urothelial carcinoma, stage cTanyN0N1M0 included in the REBACARE trial and the reference cohort stratified by history of diagnostic URS (yes or no).

	REBACARE No d-URS N = 73	REBACARE URS N = 104	Historical No d-URS N = 128	Historical URS N = 111
pT-stage				
Tis-Ta-Tx	23 (32)	45 (43)	45 (35)	35 (32)
T1-T2	23 (32)	32 (31)	25 (20)	16 (14)
T3-T4	27 (37)	27 (26)	58 (45)	60 (54)
Sex				
Male	47 (64)	75 (72)	82 (64)	74 (67)
Female	26 (36)	29 (28)	46 (36)	37 (33)
Preoperative cytology				
Not done or unknown	11 (15)	17 (16)	64 (50)	65 (59)
Benign or atypia	31 (42)	61 (59)	39 (30)	33 (30)
High grade	31 (42)	26 (25)	25 (20)	13 (12)
Age (median, IQR)	71 (64-75)	70 (63-75)		
Tumor location				
Renal pelvis or proximal ureter	56 (77)	66 (63)	93 (73)	77 (69)
Mid- or distal ureter	17 (23)	38 (37)	35 (27)	34 (31)
Concomitant CIS				
No	62 (85)	96 (92)	107 (84)	88 (79)
Yes	10 (14)	7 (6.7)	18 (14)	22 (20)
Unknown	1 (1.4)	-	3 (2.3)	1 (0.1)
Multifocality				
No	66 (90)	88 (85)	110 (86)	79 (71)
Yes	7 (10)	16 (15)	18 (14)	32 (29)

CIS = *carcinoma in situ*; CI = Confidence Interval; URS = ureterorenoscopy.

SUPPLEMENTARY: STATISTICAL ANALYSIS

Descriptive analyses were performed to describe the patient, tumor, and treatment characteristics of the *REBACARE* and reference cohort. Categorical characteristics were described using frequencies (*n*) and percentages (%), and continuous variables were described using medians and interquartile ranges.

Time-to-event endpoints were analyzed by the Kaplan-Meier method and multivariable Cox Proportional Hazard regression.

The 2-year IVR rate was evaluated according to the *intention-to-treat* en *per-protocol* analysis and compared to the predefined predicted reduction of >40% (20% IVR rate). Secondly, the 2-year IVR rate was compared to the 2-year IVR rate of the reference cohort.

Multivariable Cox Proportion Hazard regression was adjusted for age at diagnosis, sex, pT-stage (I: CIS-pTa, II: pT1-pT2, III: pT3-pT4), preoperative cytology (I: unknown, II: benign or atypia, III: high-grade urothelial cancer cells), tumor location (I: renal pelvis and proximal ureter, II: mid- or distal ureter), concomitant *carcinoma in situ* (CIS), and multifocality. As literature showed diagnostic URS to be an important effect modifier in developing an IVR […], the interaction between "cohort x performance of diagnostic URS (yes/no)" was included in the model as interaction term to assess whether cohort and performance of a diagnostic URS had different effects per cohort. Low-frequency categories were merged. Using this model, we calculated the predicted 2-year intravesical recurrence free-survival by entering values of the included variables of hypothetical UTUC patients.

Analyses are based on the locked of data taken on Dec 15, 2022. All statistical analyses were performed using SPSS version 28.0.1.0 (SPSS Inc., Chigaco, IL, USA) and R version 4.3.1. (R Foundation for Statistical Analysis, Vienna, Austria)

8

SHORT-TERM CHANGES IN HEALTH-RELATED QUALITY OF LIFE OF PATIENTS UNDERGOING RADICAL SURGERY FOR UPPER URINARY TRACT UROTHELIAL CARCINOMA: RESULTS FROM A PROSPECTIVE PHASE II CLINICAL TRIAL

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ABSTRACT

Background: The possible negative impact of radical surgery on patients' Health-Related Quality of Life (HRQoL) plays an important role in preoperative counseling. Here, we analyzed HRQoL of patients treated for Upper Urinary Tract Urothelial Carcinoma (UTUC) in the context of a single-arm phase II multicenter study, in which the safety and efficacy of a single preoperative intravesical instillation with Mitomycin C was investigated.

Objective: To investigate early changes in HRQoL in patients undergoing radical surgery for UTUC and identify factors associated with these outcomes.

Design, setting, and participants: Patients with pTanyN0-1M0 UTUC were prospectively included. HRQoL was assessed using the EORTC QLQ-C30 questionnaire at baseline, and at one- and three months post-surgery.

Outcome measurements and statistical analysis: A linear mixed model was used to evaluate changes in HRQoL over time and identify variables associated with these outcomes. The clinical effect size (CES) was used to assess the clinical impact and level of perceptibility of HRQoL changes for clinicians and/or patients based on given thresholds.

Results and limitations: Between 2017 and 2020, 186 patients were included. At baseline, and one- and three-months post-surgery, response rates were 91%, 84% and 78%, respectively. One month after surgery, a statistically significant and clinically relevant deterioration was observed in physical-, role-, and social functioning, and for the included symptom scales: constipation, fatigue, and pain. An improvement in emotional functioning was observed. At three months, HRQoL returned to baseline levels, except emotional functioning, which improved at one month and persisted to be better than before surgery. Age >70 years was associated with worse physical functioning, but better social- and emotional functioning. Male patients reported better emotional functioning than females. Postoperative complications were negatively associated with social functioning.

Conclusion: UTUC patients treated with radical surgery experienced a significant, albeit temporary decline in HRQoL. Three months following surgery HRQoL outcomes returned to baseline levels. This information can be used to counsel UTUC patients before undergoing radical surgery and contextualize recovery post-surgery.

Patient summary: We investigated changes in quality of life as reported by patients who underwent surgery for upper tract urothelial carcinoma (UTUC). We found that patients experienced a decline in quality of life one month after surgery, but this was temporarily with full recovery of quality of life three months after surgery. These findings can help doctors and other medical staff in counseling UTUC patients before undergoing radical surgery.

INTRODUCTION

Urothelial carcinoma predominantly originates in the urinary bladder, but in 5-10% of patients the upper urinary tract, i.e., ureter or renal pelvis, is the primary site of origin [1]. The incidence of upper tract urothelial carcinoma (UTUC) is on the rise in multiple countries [2-4]. In the Netherlands, the age-standardized incidence rate increased from two cases per 100,000 persons per year in 1993 to over three per 100,000 persons per year in 2017 [4]. Although kidney-sparing surgery is a treatment option in selected low-risk UTUC patients, the *European Association of Urology* (EAU) UTUC guideline recommends radical nephroureterectomy (RNU) with ipsilateral bladder-cuff excision for localized UTUC [1]. Following RNU, patients undergo close surveillance, although adjuvant chemotherapy can be considered for locally advanced UTUC (pathological stage T3/T4) [5,6]. So, surgery and subsequent treatment trajectory can significantly impact patients' quality of life.

The POUT (Peri-Operative chemotherapy versus sUrveillance in upper Tract urothelial cancer) trial is the only study to have reported on the Health-Related Quality of Life (HRQoL) for UTUC patients treated with RNU. In this trial, UTUC patients received RNU with or without adjuvant chemotherapy [5]. Mean global health status scores as measured by the *European Organisation for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire (QLQ)-C30* were reported at baseline (i.e. shortly after the surgery) and after 3, 6, 12, and 24 months. In UTUC patients treated by RNU, but without adjuvant chemotherapy, no clear changes were observed in the time period between RNU and three months later. In UTUC patients receiving adjuvant chemotherapy, the mean global health score significantly deteriorated during and after chemotherapy up to six months after baseline. No results were reported on the impact of surgery on other scales of the EORTC QLQ-C30 (social-, cognitive-, physical-, role-, and emotional functioning), nor were factors evaluated that may affect global health status.

Given the evident lack of literature considering the impact of radical surgery on HRQoL outcomes subsequent to RNU for UTUC, we aimed to assess the impact

of surgery on HRQoL and identify factors associated with changes in HRQoL outcomes in UTUC patients.

METHODS

Study design

Patients diagnosed with UTUC between 2017 and 2020 and treated with radical surgery were included in the REBACARE trial, a single-arm multicenter study (EU Clinical Trials Register; EudraCT Number 2017-000949-53). Study details are previously published [16]. Adults (age ≥18 years) diagnosed with primary cTanyN0-1M0 UTUC without receiving neoadjuvant chemotherapy and without (a history of) bladder cancer were enrolled between November 2017 and July 2020 in 18 hospitals in The Netherlands. The majority of patients received a single pre-operative intravesical instillation with mitomycin-C (intention-to-treat protocol) within 3 hours before RNU or partial ureterectomy with bladder cuff excision instead of a postoperative intravesical instillation, which is standard of care [7]. Patient, tumor, and treatment characteristics were prospectively collected. The primary endpoint was the proportion of histologically proven intravesical recurrences two years after surgery. The secondary endpoint was the assessment of HRQoL by the EORTC QLQ-C30 questionnaire at three points in time: at the time of inclusion (baseline; following diagnosis of UTUC but prior to surgery), one month, and three months after surgery. Hard-copy questionnaires were used at baseline. Online questionnaires were used at one and three months after surgery for which patients were invited by email. A varying time window of two weeks was allowed for each measurement. Completed questionnaires (hard copy and online questionnaires) were processed and linked to the corresponding patient by data managers of the Netherlands Comprehensive Cancer Organisation (IKNL). The study was approved by the institutional review board of the Erasmus Medical Center and received enforceability permission for all participating sites (METC 2017-227 NL60919.078.17). The REBACARE trial was undertaken according to the principles of Good Clinical Practice (GCP), and sponsored by the Dutch Cancer Society (KWF; project number 10319).

EORTC QLQ-C30

The validated EORTC QLQ-C30 version 3.0 was used to assess HRQoL [8,9]. EORTC QLQ-C30 is a tool widely used to assess HRQoL in cancer patients, with 30 items covering different QoL scales; one scale assesses the global health status and five functional scales measure physical, role, emotional, cognitive, and social functioning. Three symptom scales measure the burden of fatigue, pain, and nausea/vomiting. In addition, six single items assess cancer-related symptoms, such as dyspnea, sleeping problems, appetite loss, constipation, diarrhea, and financial difficulties. All items are scored on a four-point scale, ranging from 'not at all' to 'very much', except for the global health score, which has a 7-point scale, ranging from 'very poor' to 'excellent'. All scores are linearly transformed to a 0-100 scale. For the global health score and functional scales, a higher score indicates better functioning, whereas for the symptom scales a higher score indicates a higher symptom burden. Missing data were imputed according to the EORTC guidelines, provided that at least half of the items in that specific scale were completed [18].

Based on expert opinion and the expected minimal effect of the surgical intervention on dyspnea, sleep problems, appetite loss, nausea/vomiting, diarrhea, and financial difficulties these symptom scales were not evaluated in the current study.

Statistical analyses

Descriptive analyses provide insight into the patient, tumor, and treatment characteristics. Data are separately presented for all included patients of the REBACARE trial, full responders, and responders who completed two of the three questionnaires. Categorical characteristics were described using frequencies (n) and percentages (%) and continuous variables were described using means and standard deviations (SD) or medians and interquartile range (IQR). All statistical analyses were performed using SPSS version 28.0.1.0 and R version 4.2.1.

Longitudinal linear mixed model analyses assessed HRQoL changes over time for all five functional scales, the global health scale and the three included

symptom scales. All analyses were adjusted for pre-defined confounders, including sex (male vs female), age at diagnosis (reference 70 years), age-adjusted Charlson Comorbidity Index (CCI), lymph node dissection (yes/no), pathological tumor stage (pT stage <pT2 vs ≥pT2), type of surgery (open vs laparoscopic/robot-assisted) and surgical complications (yes (any degree of the Clavien-Dindo Classification)/no) [10]. Since all patients are measured at the same time points, time was used as a categorical variable [11]. The model included baseline scores, as well as the scores at one month and three months. To adjust for clustering within patients, each individual patient was included as a random intercept. Longitudinal linear mixed model analysis corrects for data missing at random [11]. The effect size on HRQoL for all pre-defined confounders is presented separately, including the beta-coefficient, the 95% Confidence Intervals, and *p* values. Additionally, the interaction between 'time (as a categorical variable) x sex', and 'time x age', were included in the model as interaction terms to assess whether sex and/or age had different effects on HRQoL at different points in time (effect modification) [11].

To assess clinical relevance, clinical effect size (CES) was used to evaluate the impact of (statistically significant) differences. CES is calculated by the change in mean score for the functional, global health, and symptom scales between baseline and one, and three months and is categorized as trivial, small, medium, and large improvement/deterioration. The outcomes of CES are based on the thresholds suggested by the 'Guidelines of interpretation of longitudinal QOL differences' by *Cocks et al.* [12]. This approach considers whether the impact on each HRQoL scale is perceptible for patients and/or clinicians apart from solely statistical significance [12,13].

RESULTS

In total, 190 patients diagnosed with primary non-metastatic UTUC were enrolled in the REBACARE trial. Of these, 186 patients underwent radical surgery and 171 (92%) eventually received a preoperative intravesical instillation with MMC as part of the trial. The baseline questionnaire was completed by 170 (91%) patients. See **Figure 1** for the flow chart of the REBACARE trial, including

response rates. **Table 1** presents the baseline characteristics of all surgically treated patients in the REBACARE trial, responders who completed all questionnaires (N = 133), and responders who completed at least two questionnaires (N=157). There were no significant differences observed in patient, tumor, and treatment characteristics between the three different groups, except for one: full responders were more likely to be diagnosed with a pT3 tumor (33% vs 28-29%) and less likely with pT1 tumors (14% vs 17-18%).

Changes over time in health-related quality of life

At one month following surgery, the global health status and cognitive functioning did not statistically differ from the average baseline score: -4.0 points (95% CI -9.3 to 1.3; CES small) and -3.8 points (95% CI -9.1 to 1.5; CES small). However, physical (-16.5 points, 95% CI -21.4 to -11.7, p<0.001), role (-28.8 points, 95% CI -37.7 to -20.0, p<0.001), and social functioning (-12.5 points, 95% CI -18.8 to -6.2, p<0.001) significantly deteriorated compared to baseline (**Table 2**) and for these scales' medium to large CES were noted. Additionally, patients reported higher emotional functioning scores at one month compared to baseline; an improvement of 6.8 points (95% 1.2 to 12.4, p = 0.017) that was considered of small clinical relevance. Symptom scales showed that fatigue, pain, and constipation scores were higher at one month compared to baseline (p>0.001, medium to large CES). At three months post-surgery, all functioning and symptom scales had returned to baseline levels except for the improvement in emotional functioning, which persisted (9.5 points, 95% CI 3.9 to 15.2, p = 0.001) and was of medium clinical relevance.

Patient, tumor, and treatment-related factors and HRQoL

The results of the longitudinal linear mixed model analyses, excluding the time variable (interaction terms), are presented in **Table 3**. Age was found to be associated with better social- and emotional functioning, but worse physical functioning. Men reported better emotional functioning, while a surgical complication (any degree vs no surgical complication) had a negative impact on social functioning. No significant associations were observed for pathological T-stage (<pT2 vs ≥pT2), Charlson Comorbidity Index (≤4 vs >4), lymph node dissection (yes vs no), and type of surgery (open vs laparoscopic). Furthermore,

no significant associations were found for patient, tumor, and treatment-related factors with the different symptom scales.

Sex was the only observed effect modifier, as females experienced significantly greater improvement in emotional functioning from baseline to one month following surgery compared to men (**Figure 2**). No effect modification for sex was noted in other HRQoL scales.

DISCUSSION

This study showed that patients with UTUC who underwent radical surgery, preceded by an intravesical instillation with MMC for most patients, experienced a temporary decline in physical, role, and social functioning, but all scores returned to pre-treatment levels at three months after surgery. Similar results were found for fatigue, pain, and constipation. However, for emotional functioning, an improvement was observed at one month which persisted at three months after surgery. Older patients experienced better social- and emotional functioning but worse physical functioning. Male sex was associated with greater emotional well-being and surgical complications compromised social functioning.

A comparison between the current REBACARE trial and the POUT trial is challenging due to the difference in the timing of the baseline assessment of the QLQ-C30. In the POUT trial, the assessment was carried out shortly after surgery, while in the REBACARE trial, it was conducted prior to surgery. Nevertheless, for the group of patients who received RNU only, no clear changes during the period shortly after and up to three months following RNU were noted for the global health status, which is consistent with the trend found in our study [5]. It is worth noting that the global health status deteriorated again after three months in the POUT trial. This could be explained by the fact that the POUT trial included only patients with advanced UTUC who had a high risk of disease progression. Previous studies have shown that an advanced disease stage has a negative impact on HRQoL [14-16]. High dropout rates over time and disease progression may have contributed to the observed deterioration

in global health status. However, it is crucial to be aware of a potential decline in HRQoL for patients included in the REBACARE trial, or surgically treated UTUC patients in general, more than 3 months after surgery. On the other hand, the observed rapid recovery in patient QoL in both the POUT trial and the REBACARE trial could be considered a possible confirmation of the feasibility of adjuvant chemotherapy, currently recommended by the EAU for a subgroup of UTUC patients [1]. Patients included in our study showed a statistically significant and clinically relevant improvement in emotional functioning over time. This contrasts with the results concerning the other functional scales for which only temporary effects within the study period were observed. The improvement in emotional functioning might reflect reduced anxiety due to surgical eradication of the tumor, as described in previous studies evaluating oncological surgery [17,18]. The timing of the first assessment, conducted shortly after diagnosis, may have amplified this effect, as the initial diagnosis of UTUC could have caused an immediate deterioration in emotional functioning.

We found that females scored lower on emotional functioning at baseline and at one- and three months post-surgery compared to men. This difference is consistent with other cancer populations [19-21]. Varying results have been reported on gender disparities with regard to coping and anxiety or depression post-surgery for multiple malignancies [22,23]. For most cancers, female patients tend to experience more anxiety or depression following diagnosis and treatment [24]. Notably, in our study, females tend to experience a greater improvement in emotional functioning during the period pre-surgery to one-month post-surgery compared to men. Although the exact reason for this observation remains unknown, it is important to further investigate this finding. It may have implications for counseling female UTUC patients on the possibility of significant emotional recovery following surgery.

Patients who experienced a surgical complication within the first month following radical surgery for UTUC showed a decline in their social functioning. This is consistent with a study by *Brown et al*. on patients with colorectal cancer who underwent surgery, which reported a negative impact of surgical complications

on social functioning [25]. In that study, patients with complications had significantly lower social functioning scores at three months after surgery, which persisted up to 36 months. The reasons for this effect may include longer hospital stay, additional interventions or medication, slower recovery, and psychological or physical consequences. Clinicians should take note of these potential long-term effects of surgical complications on the HRQoL of surgically treated patients.

This study is, to our knowledge, the first to report on the impact of radical surgery for UTUC on multiple scales of HRQoL and potential confounders associated with these outcomes. As the incidence of UTUC and consequently the number of radical surgeries increases, understanding the patient-reported quality of life after surgery becomes essential to enhance shared decision-making and monitor UTUC patients in daily clinical practice [26,27]. This understanding can be used to inform patients before undergoing surgery and to contextualize their recovery post-surgery. Moreover, it may help to align the expectations of patients and surgeons as they often have differing assumptions regarding the impact of surgery on HRQoL [28,29].

The present study has several limitations that should be acknowledged. Firstly, due to the design of the REBACARE trial, patients with node-positive (>pN1) or distant metastasis UTUC were excluded. Therefore, the outcomes of our study cannot be generalized to UTUC patients with metastatic disease. Secondly, although the compliance rate for completed questionnaires at baseline was high (91%), only 72% of the patients completed the questionnaires at all three assessment points. As the reasons for non-response are largely unknown, it is possible that patients selectively dropped out, which could introduce bias in our results. Finally, we did not differentiate the degree of surgical complications within the linear mixed model analysis, making it unclear how much the effect on HRQoL is attributable to patients with more severe (higher Clavien Dindo grade) surgical complications.

CONCLUSION

Patients undergoing radical surgery for UTUC experience a temporary deterioration in most HRQoL scales shortly after surgery, with full recovery observed at three months post-surgery. An improvement was observed in emotional well-being. These findings can help clinicians counsel patients about the expected impact of radical surgery for UTUC on HRQoL and identify patients at risk for impaired recovery of their quality of life. Considering the EAU's recommendation for adjuvant treatment following surgery, this study suggests that, for the majority of eligible patients, HRQoL will be satisfactory with this treatment approach.

DISCLOSURE OF INTEREST

All authors report no conflict of interest.

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TAKE HOME MESSAGE

A temporary decline in patient-reported Health-Related Quality of Life can be expected following radical surgery for UTUC with a full recovery to pre-surgery levels three months after surgery.

Figure 1: Flow diagram of the REBACARE HRQoL study. 'Responders' answered all the questions.

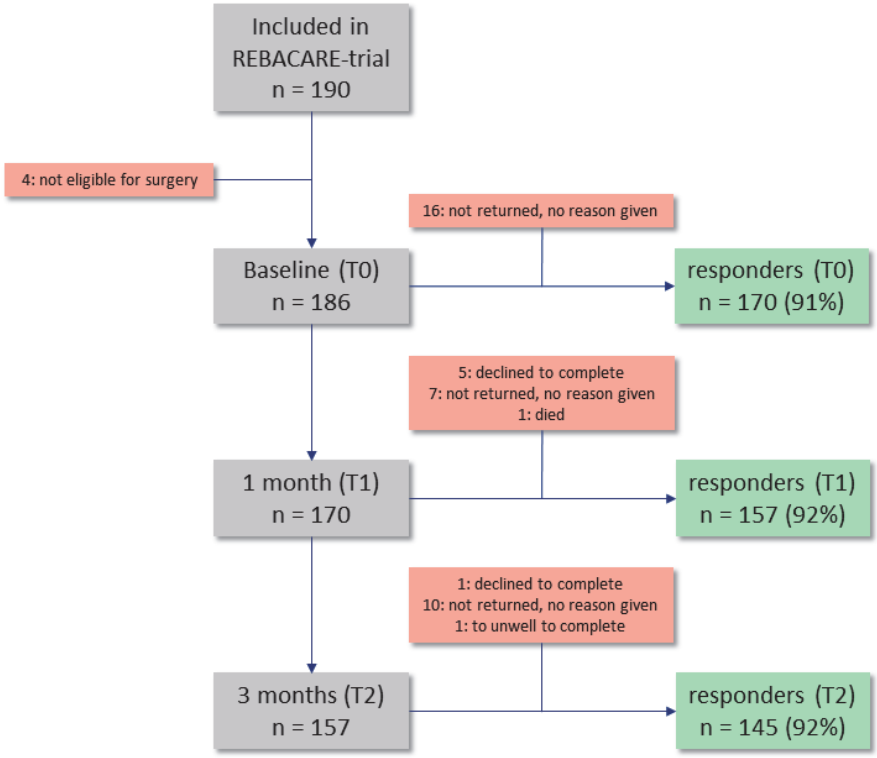

Figure 2: Mean scores at baseline, and at one and three months after radical surgery for all functional scales of the *European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire*-C30 for male versus female UTUC patients. Scores were adjusted for age, pT-stage, age-adjusted CCI, surgical complication, type of surgery, and lymph node dissection using a linear mixed model analysis.

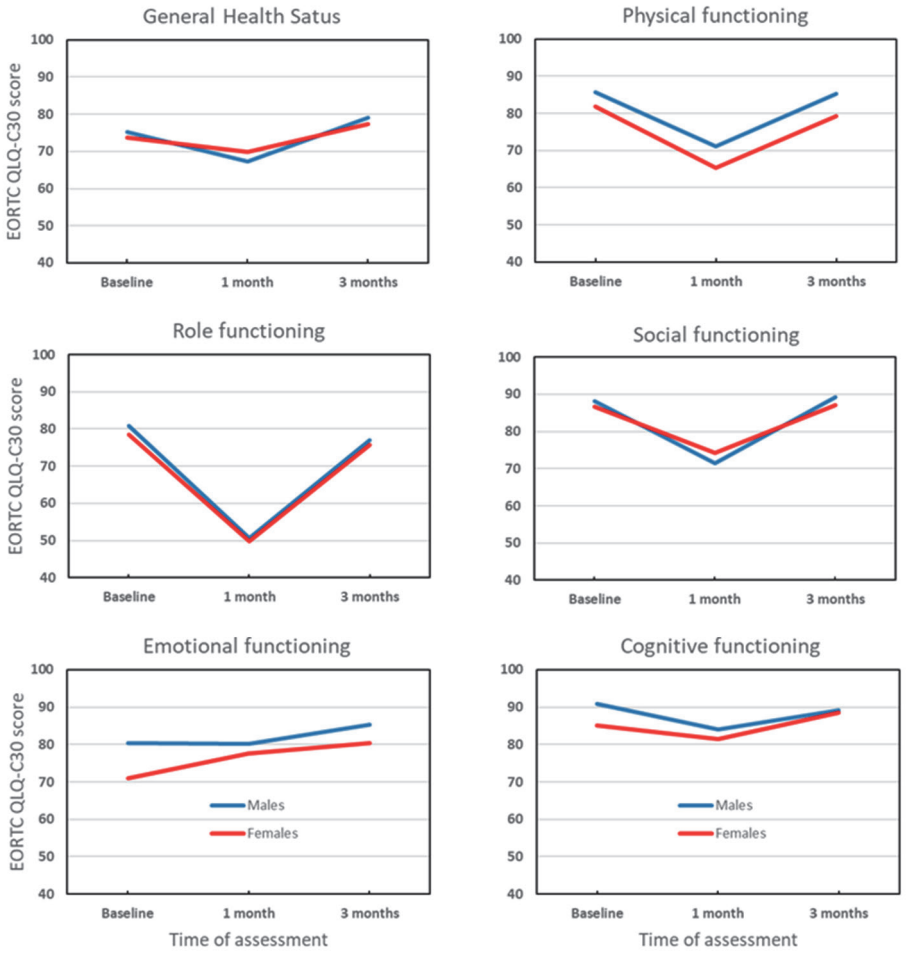

Table 1: Baseline characteristics of all surgically treated patients included in the REBACARE trial, the full responders, and the responders who filled in at least two of the three *European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire*-C30 questionnaires.

Characteristic	REBACARE-trial† N = 186	Responders completed all three questionnaires N = 133	Responders with at least two questionnaires N = 157
Sex, *n (%)*			
Female	60 (32)	41 (31)	48 (31)
Age (yr)			
Mean (SD)	68.3 (9.1)	68.9 (8.6)	68.4 (9.0)
WHO performance status, *n (%)*			
0	145 (78)	108 (81)	127 (81)
1	34 (18)	20 (15)	24 (15)
2	3 (1.6)	3 (2.3)	3 (1.9)
Unknown	4 (2.2)	2 (1.5)	3 (1.9)
Charlson Comorbidity Index, *n (%)*			
Median (IQR)	6.0 (4-7)	6.0 (4-7)	6.0 (4-7)
≤4	57 (31)	41 (31)	50 (32)
>4	129 (69)	92 (69)	107 (68)
Type of surgery, *n (%)*			
RNU, open	23 (13)	17 (13)	19 (12)
RNU, laparoscopic/Robot	153 (82)	108 (81)	130 (83)
Distal ureterectomy, open	6 (3.2)	4 (3.0)	4 (2.5)
Distal ureterectomy, laparoscopic/Robot	4 (2.2)	4 (3.0)	4 (2.5)
Preoperative intravesical instillation with MMC, *n (%)*	171 (92)	124 (93)	144 (92)
Days of hospitalization, median (min-max)	9 (1-17)	8 (1-16)	9 (1-17)
Lymph Node Dissection, *n (%)*			
Yes	35 (19)	27 (20)	29 (19)
No	146 (78)	105 (79)	125 (80)
Unknown	5 (2.7)	1 (0.8)	3 (1.9)
Pathological tumor stage, *n (%)*			
Tis	4 (2.2)	3 (2.3)	4 (2.5)
Ta	63 (34)	43 (32)	52 (33)

Table 1 (continued): Baseline characteristics of all surgically treated patients included in the REBACARE trial, the full responders, and the responders who filled in at least two of the three *European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire*-C30 questionnaires.

T1	34 (18)	19 (14)	26 (17)
T2	25 (13)	20 (15)	24 (15)
T3	52 (28)	44 (33)	46 (29)
T4	5 (2.7)	3 (2.3)	4 (2.5)
pTx	3 (1.6)	1 (0.8)	1 (0.6)
Tumor grade (WHO 1973), *n (%)*			
Grade 1	19 (10)	13 (10)	16 (10)
Grade 2	72 (39)	51 (38)	60 (38)
Grade 3	84 (45)	61 (46)	72 (46)
Unknown	11 (5.9)	8 (6.0)	9 (5.7)
Lymph node involvement, *n (%)*			
No	27 (15)	21 (16)	22 (14)
Yes	8 (4.3)	6 (4.6)	7 (4.7)
pNx	151 (82)	106 (80)	128 (82)
Patients with a surgical complication (<30 days)*, *n (%)*	59 (32)	42 (31)	52 (33)
Grade I	54 (29)	44 (33)	52 (33)
Grade II	12 (6.5)	5 (3.8)	9 (5.7)
Grade III	14 (7.5)	10 (7.5)	11 (7.0)
Grade IV	1 (0.5)	1 (0.8)	1 (0.6)
Grade V	1 (0.5)	-	-
Readmission rate post-surgery (<30 days), n (%)	18 (10)	12 (9)	16 (10)

Abbreviations: BMI = Body Mass Index, IQR = Inter Quartile Range, RNU = Radical Nephroureterectomy,
SD = Standard Deviation, WHO = World Health Organization. †Surgically treated. *Some patients had multiple surgical complications

Table 2: Changes in the functioning scales of the *European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire*-C30 from baseline to one and three months after radical surgery for UTUC. The clinical effect size, shown as trivial, small, medium, or large based on the thresholds as indicated by *Cocks et al.* [12]. Scores were adjusted for age, pT-stage, age-adjusted CCI, surgical complication, type of surgery, and lymph node dissection using a linear mixed model analysis.

EORTC QLQ-C30	Mean	Change†	95% CI	*p* value	CES
Global health status					
Baseline	73.8				
After 1 month	69.8	-4.0	[-9.3, 1.3]	0.14	Small
After 3 months	77.4	3.6	[-1.8, 9.0]	0.19	Trivial
Physical functioning					
Baseline	81.8				
After 1 month	65.3	-16.5	[-21.4, -11.7]	**<0.001**	Medium
After 3 months	79.2	-2.8	[-7.5, 2.3]	0.30	Trivial
Role functioning					
Baseline	78.5				
After 1 month	49.7	-28.8	[-37.7, -20.0]	**<0.001**	Large
After 3 months	75.8	-2.8	[-11.8, 6.2]	0.5	Trivial
Social functioning					
Baseline	86.7				
After 1 month	74.2	-12.5	[-18.8, -6.2]	**<0.001**	Medium
After 3 months	87.0	0.3	[-6.1, 6.7]	>0.9	Trivial
Emotional functioning					
Baseline	70.9				
After 1 month	77.7	6.8	[1.2, 12.4]	**0.017**	Small
After 3 months	80.4	9.5	[3.9, 15.2]	**0.001**	Medium
Cognitive functioning					
Baseline	85.2				
After 1 month	81.4	-3.8	[-9.1, 1.5]	0.16	Small
After 3 months	88.5	3.3	[-2.1, 8.7]	0.22	Small
Fatigue					
Baseline	27.1				
After 1 month	44.5	17.4	[10.9, 24.0]	**<0.001**	Medium
After 3 months	25.3	-1.8	[-8.5, 4.9]	0.6	Trivial
Pain					
Baseline	19.5				
After 1 month	38.3	18.9	[11.4, 26.4]	**<0.001**	Large
After 3 months	19.9	0.5	[-7.2, 8.1]	0.9	Trivial
Constipation					
Baseline	6.4				
After 1 month	22.5	16.1	[7.7, 24.6]	**<0.001**	Medium
After 3 months	8.3	1.9	[-6.7, 10.5]	0.7	Trivial

Abbreviations: CES = Clinical Effect Size [117]; *p* values of <0.05 are considered significant; HRQoL subscales range from 0 to 100; †Difference between mean baseline score and mean defined time point.

Table 3: Associations between patient, treatment, and tumor characteristics and the functional scales of the *European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire*-C30 during 3 months following radical surgery for UTUC using a linear mixed model analysis.

EORTC QLQ-C30	Global health status			Physical functioning			Role functioning			Social functioning			Emotional functioning			Cognitive functioning		
Functional scales	B	95% CI	*p*	B	95% CI	*p*	B	95% CI	*p*	B	95% CI	*p*	B	95% CI	*p*	B	95% CI	*p*
Age (reference 70 yr)	0.1	[-0.2, 0.5]	0.4	-0.3	[-0.6, -0.02]	**0.035**	0.1	[-0.3, 0.6]	0.6	0.5	[0.1, 0.9]	**0.017**	0.5	[0.1, 0.9]	**0.012**	0.2	[-0.1, 0.5]	0.22
Sex																		
Female		Reference			Reference			Reference			Reference			Reference			Reference	
Male	1.5	[-4.6, 7.5]	0.6	3.9	[-1.6, 9.4]	0.17	2.3	[-6.4, 10.9]	0.6	2.2	[-4.9, 9.2]	0.6	9.3	[2.6, 16.0]	**0.007**	5.7	[-0.02, 11.5]	0.052
pT-stage																		
<pT2		Reference			Reference			Reference			Reference			Reference			Reference	
≥pT2	-2.3	[-7.0, 2.3]	0.3	-1.2	[-5.4, 3.0]	0.6	-1.3	[-7.4, 4.8]	0.6	-2.5	[-7.9, 2.9]	0.4	-2.5	[-7.7, 2.8]	0.4	1.6	[-2.7, 5.9]	0.5
CCI																		
≤4		Reference			Reference			Reference			Reference			Reference			Reference	
>4	-0.03	[-5.4, 5.4]	>0.9	-0.6	[-5.5, 4.2]	0.8	0.2	[-6.8, 7.2]	>0.9	0.4	[-5.8, 6.7]	>0.9	0.5	[-5.6, 6.6]	0.9	-0.5	[-5.5, 4.5]	0.9
Type of surgery																		
Open		Reference			Reference			Reference			Reference			Reference			Reference	
Laparo-scopic/Robot	2.0	[-4.5, 8.5]	0.6	1.7	[-4.2, 7.6]	0.6	3.7	[-4.8, 12.1]	0.4	3.4	[-4.1, 10.9]	0.4	2.7	[-4.7, 10.0]	0.5	-1.0	[-7.1, 5.0]	0.7
Lymph node dissec-tion																		
No		Reference			Reference			Reference			Reference			Reference			Reference	
Yes	-2.0	[-7.3, 3.3]	0.5	0.1	[-4.7, 4.9]	>0.9	0.9	[-7.1, 8.9]	0.8	1.1	[-5.1, 7.3]	0.7	-2.1	[-7.7, 3.5]	0.5	-3.2	[-8.4, 1.9]	0.22
Surgical complica-tion																		
No		Reference			Reference			Reference			Reference			Reference			Reference	
Yes	-2.7	[-7.2, 1.9]	0.25	-2.5	[-6.6, 1.6]	0.24	-3.2	[-10.1, 3.7]	0.4	-6.3	[-11.6, -0.9]	**0.022**	0.7	[-4.2, 5.5]	0.8	-1.0	[-5.4, 3.4]	0.7

Abbreviations: CCI = Charlson Comorbidity Index; CI = Confidence Interval; *p* values of <0.05 were considered statistically significant;

Supplementary table 1: Scores at baseline, one, and three months after radical surgery for UTUC and the associations between patient, tumor, and treatment characteristics of the symptom scales constipation, fatigue, and pain of the *European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire*-C30. The clinical effect size, shown as trivial, small, medium, or large based on the thresholds as indicated by *Cocks et al.* [12]. Scores were adjusted for age, pT-stage, age-adjusted CCI, surgical complication, type of surgery, and lymph node dissection using a linear mixed model analysis.

EORTC QLQ-C30	**Constipation**				**Fatigue**				**Pain**			
Symptom scales	Score	B	95% CI	*p*	Score	B	95% CI	*P*	Score	B	95% CI	*P*
Baseline	6.4				27.1				19.5			
After 1 month	22.3	16.1	[7.7, 24.6]	**<0.001**	44.5	17.4	[10.9, 24.0]	**<0.001**	38.4	18.9	[11.4, 26.4]	**<0.001**
After 3 months	8.3	1.9	[-6.7, 10.5]	0.7	25.3	-1.8	[-8.5, 4.9]	0.6	20.0	0.5	[-7.2, 8.1]	0.9
Age (reference 70 yr)		-0.1	[-0.5, 0.4]	0.8		0.01	[-0.4, 0.4]	>0.9		-0.2	[-0.7, 0.2]	0.3
Sex												
Female			Reference				Reference				Reference	
Male		-3.5	[-11.4, 4.4]	0.4		-6.5	[-14.0, 1.1]	0.09		-2.5	[-10.3, 5.4]	0.5
pT-stage												
<pT2			Reference				Reference				Reference	
≥pT2		2.8	[-2.5, 8.2]	0.3		2.3	[-3.6, 8.2]	0.4		-0.8	[-6.6, 5.0]	0.8
CCI												
≤4			Reference				Reference				Reference	
>4		1.6	[-4.6, 7.9]	0.6		1.2	[-5.6, 8.0]	0.7		1.7	[-5.0, 8.4]	0.6
Type of surgery												
Open			Reference				Reference				Reference	
Laparoscopic/Robot		1.4	[-6.1, 8.8]	0.7		-4.8	[12.9, 3.4]	0.3		-3.9	[-11.9, 4.1]	0.3
Lymph node dissection												
No			Reference				Reference				Reference	
Yes		-1.0	[-8.4, 6.4]	0.8		-0.3	[-6.8, 6.3]	0.9		-2.9	[-10.0, 4.3]	0.4
Surgical complication												
No			Reference				Reference				Reference	
Yes		3.9	[-2.4, 10.3]	0.23		5.7	[0.02, 11.3]	0.050		5.8	[-0.4, 11.9]	0.066

CCI = Charlson Comorbidity Index; CI = Confidence Interval; *p* values of <0.05 are considered significant

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PART IV

GENERAL DISCUSSION

9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Although the incidence of upper urinary tract urothelial carcinoma (UTUC) has shown to be increasing [1-3], UTUC remains a rare cancer since less than <1000 patients are diagnosed with UTUC yearly in the Netherlands. Over the past 25 years, treatment modalities for UTUC have seen little change, and no significant improvements in survival have been achieved [3]. The introduction of the European Association of Urology (EAU) guideline on UTUC in 2004 has raised more awareness of this disease and brought some standardization to diagnostic tools and therapeutic options. This guideline is updated annually to incorporate the latest significant advancements in UTUC treatment; however, uncertainties are still present in diagnostics, treatment modalities, and follow-up of this disease [4].

Risk stratification remains a crucial yet challenging aspect of UTUC management, especially with the advent of kidney-sparing surgery (laser ablation via ureterorenoscopy) and neoadjuvant chemotherapy [4]. Accurate patient selection for these treatment modalities is essential for optimizing oncological outcomes. However, preoperative CT imaging is notoriously unreliable in predicting pT-stage and identifying low-risk UTUC, while MRI imaging is even less specific and sensitive [5-7]. Urine cytology, the last widely available minimally invasive diagnostic tool, also performs poorly predicting high-grade or invasive disease [8].

As a result, many UTUC patients undergo diagnostic ureterorenoscopy (URS) – a technically demanding procedure that, despite its value, has limitations [4]. It is prone to underestimating pT-stage and tumor grade due to biopsy constraints [9-11] and carries risks such as ureteral perforation, subsequent stenosis, and an increased risk of intravesical recurrence (IVR) [12-14]. Even after radical surgery, in the absence of postoperative intravesical instillation with chemotherapy, 22-47% of the patients develop such an IVR [15,16]. However, little progress has been achieved in preventing these recurrences. The psychological burden associated with the potential diagnosis of IVR is substantial, compounded by the need for frequent invasive cystoscopies to enable early detection.

In the context of this very high recurrence rate in this patient population despite introduced therapies, there is an urgent need to better understand and address IVR in daily clinical practice. Therefore, the aim of this thesis was to provide deeper insight into the mechanisms underlying IVR development and prevention, ultimately contributing to new guidelines and directions for future research in this niche of UTUC management.

'Seeding' as major driver of developing intravesical recurrences in UTUC

Since such a large proportion of primary UTUC patients who are treated with radical surgery will eventually develop an intravesical recurrence, intra-luminal seeding, a mechanism of tumor cell spread observed in various malignancies, is one of the major hypotheses as the primary driver of the development of these recurrences [17-19]. By analyzing both the primary UTUC and the corresponding IVR in the same patient at the molecular level, researchers can identify a shared origin, a concept referred to as "clonality'. This approach, widely utilized in the study of many malignancies, has increasingly been applied to UTUC and IVR. However, there was this ongoing debate if clonality was indeed the reason of these IVRs, as molecular and biochemistry analyses were very limited and not yet 'in depth' [20-27]. As molecular techniques continue to advance, more detailed and comprehensive analyses have now become possible and evidence for this hypothesize became much stronger [28]. Our studies described in **Chapter 4**, together with other recent studies, showed that the vast majority (>70%) of primary UTUC and IVR within the same patients are clonally related [18,28,29]. In these studies, next-generation sequencing was used, which enables more conclusive assessment of a clonal relation between the two entities based on shared genomic alterations. Since large genomic atlases (such as the TCGA) are publicly available, the probability of a certain mutation found in the primary UTUC and IVR can be calculated [30,31]. By adding all these probabilities together, a robust statement can be made about the probability of clonality, which is a significant improvement over older techniques . With upcoming publications using even larger next-genaration sequencing panels [32], or in the future even whole-genome sequencing, the evidence for clonality becomes more and more solid and the debate on the underlying discussion can be closed. Hence, by today's

knowledge, this high percentage of clonality strongly indicates that IVR arises mostly due to the seeding of tumor cells from the primary UTUC rather than a pan-urothelial disease of the urinary tract leading to the development of multiple, independent, primary tumors.

If 'seeding' is assumed, a number of observations from daily practice can be explained. it is known that, for example, patients with positive preoperative cytology (free-floating tumor cells) are at higher risk for developing IVR [15]. It can also explain the fact that patients with a primary UTUC have a significantly higher risk of developing an IVR, compared to the risk of developing a UTUC subsequent to the diagnosis of bladder carcinoma [15,33-35]. Tumor cells seed and implant 'downstream' in the bladder, whereas in a pan-urothelial defect, carcinoma development should not be confined to a specific location in the urinary tract.

Also, increasing evidence suggests that diagnostic ureteroscopy (URS) before UTUC surgery significantly raises the risk of IVR, with again, tumor cell seeding as the key mechanism [13]. During URS the primary UTUC is manipulated and disrupted, especially when a biopsy is carried out, realizing these free-floating cancer cells [13,36]. Additionally, fluid irrigation, repeated ureteroscope introduction, and use of a guide-wire use possible further facilitate this spread of tumor cells to the bladder. A postoperative instillation with chemotherapy directly following URS to prevent implantation of these tumor cells might mitigate this risk, and this strategy is currently assessed in the Dutch SINCERE trial [37]. As cancer spread due to free floating tumor cells is such an important mechanism, this highlights the potential role of urinary markers not only in the diagnosis of UTUC but also in the surveillance of patients during follow-up after treatment of the primary UTUC (kidney-sparing or radical surgery) and in the diagnosis or surveillance considering IVR. These markers may encompass urine assays to detect the most prevalent molecular mutations associated with primary UTUC [38]. Furthermore, the development of patient-specific urine assays tailored to the unique molecular profile of the primary tumor could significantly enhance the precision and efficacy of post-treatment monitoring. The initial findings linking mutations in *FGFR3, KDM6A, CCND1*, and

TP53 to an increased risk of IVR are a promising step toward identifying potential urinary biomarkers [28]. However, further research is needed to validate this concept .

A postoperative instillation with chemotherapy to lower the risk of intravesical recurrence following radical surgery for UTUC might not be as effective as opposed in real-world clinical practice.

Several precautions are taken during surgical treatment to prevent the seeding of tumor cells of the primary UTUC. One key precaution involves clipping the distal ureter below the primary UTUC location to prevent cancer cells from seeding into the bladder [4]. Additionally, a bladder cuff is resected *en bloc* as literature identifies this area as a common site for intravesical recurrences [16,39]. Despite these measures, up to 47% of UTUC patients still experience IVR within the first two years post-surgery [15,16].

To mitigate this risk, the EAU guideline on UTUC recommends a single postoperative instillation of chemotherapy since 2015 [40]. This recommendation is based on the findings of two significant prospective studies: the THP Monotherapy Study Group Trial by *Ito et al.* (Japan) and the ODMIT-C trial by *O'Brien* et al . (United Kingdom) [41,42]. In the THP Monotherapy Study, 77 patients were randomly assigned to receive or not receive pirarubicin within 48 hours after radical surgery [42]. The 2-year IVR rate was significantly lower in the intervention group (17%) compared to the control group (42%).

Similarly, the ODMIT-C trial involved 284 randomized patients who received a single dose of Mitomycin C (MMC) postoperatively during catheter removal (up to 10 days following surgery) [41]. The intention-to-treat analysis showed a 1-year IVR rate of 17% in the intervention group versus 27% in the control group ($p = 0.055$), and the per-protocol analysis
showed IVR rates of 16% versus 27% ($p = 0.03$), respectively.

Despite these promising results, both studies had significant limitations. The THP Monotherapy Study was underpowered, with a recurrence rate assumption

of 9% versus 42%, and there was an imbalance in disease grade, a factor associated with IVR development. The ODMIT-C trial, despite its large sample size, was based on an optimistic relative reduction of over 50% in IVR rate and lacked histological proof, leading to potential detection bias. Additionally, the study was unblinded and only reached significance in the per-protocol analysis. These factors suggest that the outcomes may not fully reflect real-world clinical practice.

Furthermore, there is no consensus on the optimal timing for chemotherapy instillation, and its effectiveness is highly dependent on strict adherence to the protocol. It is also crucial that the instillation in daily clinical practice is performed only when there is no risk of leakage of the chemotherapy. This potential risk of leakage is the major concern that often prevents clinicians in the use of a postoperative chemotherapy instillation, although this bladder area is closed with sutures, and tested for leakage during surgery. Additionally, many clinicians perform a costly and invasive cystogram to assess whether there is still an incomplete closure of the bladder and chemotherapy cannot be given. Although complications due to leakage are rare, they can be severe and even fatal [43-47]. Reported complications include acute and chronic pain, ureteral obstruction, lower urinary tract symptoms, cellulitis, persistent leaks, intestinal obstruction, and death following a single postoperative chemotherapy instillation. With regard to the timing of the instillation, as early as possible following surgery appears to be most effective, what is already known from the treatment of non-muscle invasive bladder carcinoma (NMIBC) [48].

The current evidence supporting postoperative chemotherapy instillation is thus questionable and may not accurately represent the realities of everyday clinical practice. Moreover, a significant number of patients likely miss out on this treatment due to concerns about extravesical leakage [43-47]. This leaves both clinicians and UTUC patients in need of a safer and more effective alternative – one that not only reduces the risk of IVR, but also improves compliance by clinicians.

A preoperative instillation with chemotherapy; the future for UTUC patients. A promising solution could be a preoperative instillation, administered while the anatomy remains intact, eliminating the risk of leakage [49]. Also, patients

do receive all treatment at the same day, potentially reducing stress for patients possible resulting in a better quality of life during this period. Additionally, this approach eliminates the need for costly and invasive cystograms, and may enhance clinician compliance compared to postoperative instillation protocols.

The REBACARE trial was designed to evaluate the impact of a preoperative mitomycin C (MMC) instillation on the risk of intravesical recurrence (IVR) following radical surgery for UTUC [49]. Similar to the THP Monotherapy Study Group, mentioned earlier, the REBACARE trial focused on the first two years post-surgery, the period in which the risk of IVR is highest; ranging from 22-47% without perioperative instillation. Despite a recurrence rate of 24% over the two-year period in the REBACARE trial, no significant benefit of preoperative chemotherapy instillation was observed in the total patient population [50]. A comparable reference cohort, which did not receive any instillation, showed a similar recurrence rate of 26%.

Previous literature has already tentatively suggested that diagnostic URS performed during the workup for UTUC may be a significant risk factor for developing IVR [13]. The REBACARE trial confirmed this, demonstrating that patients who underwent diagnostic URS had a significantly higher risk of IVR after surgery, regardless of preoperative instillation or not [50]. Importantly, the study revealed that when diagnostic URS was not performed, patients who received preoperative chemotherapy instillation had a significantly lower risk of IVR.

Several key conclusions can be drawn from these findings:

1. A diagnostic URS is a major risk factor for IVR, likely due to the manipulation of the primary UTUC and dissemination of cancer cells in the urinary tract during this procedure;
2. A preoperative instillation with chemotherapy, but possible also the current recommended postoperative instillation, may be inefficient in preventing IVR caused by a diagnostic URS. This is potentially due to the delay between URS and radical surgery, allowing tumor cells to implant

fully and making chemotherapy less efficient as ablative measure.

3. Patients who do not undergo a diagnostic URS benefit from a preoperative instillation, with a significantly lower IVR rate (<10%) during the first two years post-surgery. This is important, as these patients will almost certain be able to receive this instillation as the anatomy of the bladder is still intact and will receive the full therapy (instillation + radical surgery) at the same day. For this group of patients, a preoperative instillation should be considered in the future and might be a meaningful improvement over current clinical practice.
4. When a diagnostic URS is performed, clinicians and guidelines might recommend a postoperative instillation (based on macroscopic imaging and if there has been a biopsy performed during URS) to irradicate the free-floating tumor cells following this intervention. By administering this instillation immediately after URS, it could prevent the implant of tumor cells in the bladder during the waiting period before radical surgery.

Future Perspectives

Looking ahead to the future of UTUC patients care, several findings from this thesis warrant further investigation and implementation to improve treatment outcomes and optimize the care pathway for patients undergoing surgery for UTUC.

A key development is the rising incidence of UTUC in the Netherlands over recent decades, largely due to the increased use of cross-sectional imaging [3]. This has led to a stage shift, with more cases of both superficial and advanced UTUC –each requiring distinct treatment strategies [4]. However, from 1993 to 2017, no significant survival improvement was observed, highlighting an urgent need for change. Encouragingly, kidney-sparing surgery (KSS) has gained traction, particularly with the earlier detection of UTUC resulting in more primarily diagnosed superficial UTUC [51-53]. Previously, radical nephroureterectomy was the only option, but KSS is now an essential alternative in highly selected patients and will play an increasingly important role in treating an aging population with rising comorbidities [4]. Preserving renal function in these patients is

critical and RNU can have significant negative effects. Our findings show, for example, that Health-Related Quality of Life (HRQoL) declines significantly after surgery, taking an average of three months to recover [54]. However, older patients may experience slower or incomplete recovery, and long-term HRQoL outcomes remain unclear. The POUT trial suggests a decline in HRQoL at three months, but its relatively young, fit (chemotherapy-eligible) cohort may not reflect outcomes in elderly patients [55]. In select cases of low-grade UTUC, even a 'watch-and-wait' approach, supported by shared decision-making, could be considered in elderly, vulnerable patients to ensure quality of life for as long as possible. Significant progress has also been made in the past decade in treating advanced UTUC by introducing (neo)adjuvant chemotherapy and immunotherapy [56-58]. Given the increasing use of KSS and these advancements in systemic therapies following our studied period of 1993 to 2017, survival outcomes for UTUC patients may already be improving.

However, as mentioned before, risk stratification is highly important before it can even be decided what the appropriate therapy should be with this particular patient. The necessity of diagnostic URS within this diagnostic work-up of UTUC warrants re-evaluation or at least a well-considered decision at patient-level. While diagnostic URS offers valuable insights, such as histopathological diagnosis and upper urinary tract inspection, it has been associated with an increased risk of intravesical recurrence due to the potential for tumor cell seeding. Also, it is not without risks, including the risk of understaging when a biopsy is performed given the small size of biopsies that can be taken using a ureterorenoscopy, ureter perforation, urinary tract infection, and a delay in time to definitive treatment [9-11] [9-14]. The guideline on UTUC of the EAU is quite clear and URS should only be performed when other diagnostic modalities (CT-scan and cytology) are inconclusive [4]. Future advancements in CT imaging may improve risk stratification by better distinguishing low-risk tumors and assessing invasiveness. Artificial Intelligence (AI) also holds promise for preoperative risk assessment by enhancing the predictive value of cytology and improving diagnostic accuracy during URS. Early AI applications have shown higher accuracy in diagnosing UTUC and predicting IVR compared to cytology alone [59]. During URS, AI could assist in tumor localization, differentiation between benign and malignant lesions, and pT-stage or tumor grade prediction –

potentially reducing the risk for biopsy. AI-driven segmentation of the ureter's lumen, for example, have already shown to further aid in UTUC detection and improve navigation through the upper tract, minimizing collateral damage [60,61]. These advancements could enhance diagnostic precision and optimize patient outcomes.

And when d-URS is unavoidable, the potential benefits of implementing post-URS chemotherapy instillation should be considered in future research and guidelines; which strategy is currently under review in the SINCERE trial (Netherlands) [37]. Administering an instillation with chemotherapy immediately after URS could prevent tumor cells from implanting in the bladder during the waiting period before radical surgery. The macroscopic imaging confirming a UTUC, or the performance of a biopsy, should give the decision in performing such a post-URS instillation. This strategy could help mitigate the increased recurrence risk observed in patients who undergo d-URS. The advantages of a preoperative instillation before radical surgery (REBACARE) do not apply to diagnostic URS, as no defect is made in the bladder and it is precisely the URS that will show whether or not a UTUC is present. Namely, by adhering the guideline, a diagnostic URS will only be performed in case of doubt. Ideally, a future study to assess this hypothesis should be an international RCT (due to the relative rare appearance of UTUC) including a group of patients treated with URS (diagnostic or treatment) followed by RNU (in case of a diagnostic URS) treated with standard of care (no instillation after URS) and a group of patients treated with an instillation of MMC/epirubicin post-URS (at the same of day of URS; as timing is crucial). Follow up should be focused on the rate of IVR (2-yrs) with histological prove.

And, when clinicians decide not to perform a diagnostic URS for that particular patient, a preoperative instillation before radical surgery for UTUC should be recommended [50]. This strategy lowers the risk of intravesical recurrences significantly, the majority of patients can receive this instillation due to an intact anatomy, the instillation and surgery can be performed on the same day, the fear of leakage of the chemotherapy due to the defect in the bladder is irradicated, and all these advantages will potentially lead to a higher compliance rate by clinicians and therefore lowering the incidence of intravesical recurrences.

In any case, intravesical recurrences will be an important factor in patient follow up and cystoscopies will be necessary. Integrating urine-based biomarkers in future follow-up protocols could, however, significantly reduce the need for frequent cystoscopies, which are invasive and burdensome for patients [38]. As >70% of the intravesical recurrences are related to the primary UTUC, molecular characteristics of the primary tumor can be used in developing patient-specific urine-tests to early detect an intravesical recurrence [28,29,62]. By identifying these recurrences through non-invasive urine markers, clinicians could offer a more patient-friendly follow up approach, enhancing the quality of life while maintaining close monitoring of disease status. At this time, urinary markers are on the verge of breaking through in non-muscle invasive bladder cancer, however, high sensitivities are particularly reached for high-grade disease [63]. It is expected that if this can be successfully be introduced for non-muscle invasive bladder cancer, UTUC will likely follow quickly. The upcoming results of the MOluculair ChARacterization of Upper Urinary Tract Carcinoma (MOLCARUTUC) consortium showing *TERT, FGFR3* and *HRAS* as protentional markers are promising [32]. Future research is, however, forced to focus on these molecular markers and the implementation of these noninvasive urine tests, with the current rising of cost and need for sustainability in medical care.

Also, recent advancements in devices for intravesical chemotherapy delivery have the potential to change the management of intravesical recurrences. One such device is the TAR-210, designed to locally deliver erdafinitib (FGFR-inhibitor) directly into the bladder. This device has demonstrated significant promise in preventing the development of intravesical recurrences following non-muscle invasive bladder cancer. Since current literature identifies *FGFR* as a key driver of intravesical recurrences following surgery for primary UTUC, erdafinitib represents a compelling candidate for future therapeutic strategies [28,32]. Moreover, these devices are versatile and can be adapted to deliver other treatments, such as gemcitabin, broadening their clinical utility.
Finally, patient-tailored treatments and predictive markers are essential for advancing cancer care. This approach is particularly relevant in the context of (neo)adjuvant chemotherapy and immunotherapy, as demonstrated in the treatment of muscle-invasive bladder cancer, where markers like PD-L1 and

FGFR mutation status are used to guide therapy [64]. An important development in bladder cancer treatment is the characterization of the primary tumor to identify mutational expression profiles, which can predict prognosis and response, thus reducing the risk of overtreatment.

For upper tract urothelial carcinoma (UTUC), however, the use of molecular markers for additional therapies is still limited. Recent findings suggest that UTUC may be classified into five molecular subtypes based on mutations, including active *FGFR3* mutations, *RAS* gene mutations, *TP53* pathway inactivation via *TP53* mutations or *MDM2* amplification, high mutation levels, and the absence of *FGFR3, RAS,* or *TP53* pathway defects (referred to as triple-negative) [65]. Emerging data from the MOLcular ChAracterization of Upper Urinary Tract Urothelial Carcinoma (MOLCARUTUC) consortium support these distinct subtypes, though the clinical implications remain to be fully explored. However, promising results are anticipated, particularly for treatments like *FGFR* inhibitors [32].

A crucial step toward integrating this into clinical practice has already been made in our study, where we developed a workflow that allows pathologists to identify specific UTUC subtypes using routine histopathological H&E slides [66]. Since these slides are routinely used in clinical settings, this approach could be easily implemented, helping to distinguish patients who require further molecular characterization. This would optimize therapeutic strategies and guide decisions about the need for additional therapies.

Together, these strategies hold the potential to improve both short- and long-term outcomes in UTUC patients, making treatment and follow-up more efficient, less invasive, and better targeted to individual risk profiles.

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10

PERFECTIONISM: PROPOSAL FOR A FUTURE STUDY FOLLOWING THE REBACARE-TRIAL

BASED ON 'REPLY TO 'BLADDER CANCER RECURRENCE FOLLOWING MANAGEMENT OF UPPER TRACT UROTHELIAL CARCINOMA: BALANCING PREVENTION AND IATROGENICITY'

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With the completion of the REBACARE trial – a unique study in the niche of the relatively rare disease that UTUC is – it is time to reflect, evaluate, and gather insights for optimizing future studies. While this trial has yielded valuable outcomes and may contribute to guideline adaptions and discussions, there are certainly areas for improvement in potential follow-up studies.

The study design was strongly influenced by the period in which it was initiated. Ideally, the REBACARE trial would have had a two-arm (preoperative vs. postoperative instillation) or even a three-arm design (including a control group without instillation). However, by 2016, evidence had already shown that postoperative instillation reduced the risk of intravesical recurrence (IVR), leading to the EAU guideline recommendation in 2017 for a single postoperative instillation with chemotherapy. As a result, including a group without perioperative instillation was deemed unethical. Additionally, a second arm with only postoperative instillation was not feasible because, at that time, a substantial proportion of patients ultimately did not receive the postoperative instillation – partly because the treatment had only recently been introduced and partly due to urologists' concerns about potential extravasation. These factors led to the 'suboptimal' single-arm design of the REBACARE trial.

To still enable a comparison, a retrospective cohort was established, comprising patients who did not receive perioperative chemotherapy instillation. However, strict exclusion criteria (such as ' no bladder carcinoma in history', 'surgery after 2000, and ' no intravesical or systemic chemotherapy within two years post-surgery') made it significantly more challenging than expected to assemble a large cohort. Furthermore, due to the rarity of UTUC, it took three years to prospectively include 190 patients – an important feasibility consideration for future trials. For comparison, the ODMIT-C trial (postoperative instillation after RNU) took ten years to complete. Additionally, guideline adjustments during a trial's duration can significantly impact outcomes. Although the REBACARE trial ultimately included 190 prospectively enrolled patients and 247 in the retrospective cohort, the sample size was insufficient for the proposed propensity score matching.

Another issue is the possible effect of continuous saline irrigation following the preoperative instillation considering IVR risk. It remains unclear whether the reduced IVR risk was due to the chemotherapy or the irrigation, a debate that also exists regarding transurethral bladder tumor resection. However, this additional step was crucial for protecting both the patient and the surgical team from chemotherapy exposure and for obtaining ethical approval for the study. While the exact contribution of chemotherapy versus saline irrigation may never be fully determined, we believe this approach outweighs the potential complications of postoperative MMC leakage. Moreover, implementing this measure in contemporary clinical practice is feasible.

But what do we propose as the next step? The most pressing question remains: is preoperative instillation superior to postoperative instillation, or vice versa? This critical question warrants a new study and we believe that this future trial should meet the following criteria:

- A prospective, two-arm, randomized controlled design, comparing preoperative and postoperative instillation with chemotherapy (MMC/epirubicin);
- A third, retrospective arm of patients who did not receive any form of intravesical instillation for additional comparison;
- A realistic prespecified risk reduction target of 20-30%;
- Exclusion of patients who have received systemic chemotherapy or other types of intravesical instillation;
- Histologically confirmed IVR to prevent detection bias;
- Exclusion of patients with a history of bladder cancer to rule out pan-urothelial disease, which carries a higher IVR risk;
- Sufficiently large cohort of >250 patients per arm to enable propensity score matching;
- Completion within a reasonable timeframe to minimize potential confounders introduced by prolonged study periods;
- An international, multi-institutional collaboration among specialized UTUC centers to ensure consistency in surgical techniques and follow-up;

- A minimum follow-up of two years.
- Urine samples, as well as tumor tissue, must be collected to molecular characterize all UTUC and IVR and to assess the feasibility of using urine markers in diagnosis and follow-up.

By pooling resources and expertise across institutions and borders, we can overcome the limitations of small sample sizes and generate more robust, statistically significant findings. This approach represents the best path forward for producing high-quality, practice-changing research in UTUC – research that patients need and deserve!

PART V

APPENDICES

SUMMARY

Chapter 1 general introduction, which proving a background on urothelial cancer of the upper urinary tract (UTUC) an UTUC treatment. UTUC is relatively rare compared to bladder urothelial carcinoma (UBC), leading to limited knowledge and literature. Originating in the renal pelvis or ureter, UTUC arise typically from the urothelium. Diagnosis often involves CT-urography, cystoscopy, and biopsy, though detection and risk-stratification remain challenging. Standard treatment for localized, non-metastatic UTUC is a radical nephroureterectomy (RN), with kidney-sparing surgery (laser coagulation, distal ureterectomy) as an option in selected low-risk cases. Although radical surgery, UTUC are known to be at high risk of developing intravesical recurrences during the years following surgical treatment. Up to 47% of the patients will eventually be diagnosed with such a recurrence and in daily clinical practice we are yet not able to prevent patients for this risk fully. This thesis aims to clarify the biology behind de development of UTUC and IVR, assess whether a preoperative instillation with chemotherapy before radical surgery lowers the risk of IVR, and evaluate quality of life outcomes for patients in the Netherlands.

In **Chapter 2** we examined the trends in incidence and survival rates for UTUC in the Netherlands over a 25-year period (1993-2017). We found that the incidence of UTUC has increased. In absolute numbers; in 1993 400 patients were diagnosed with UTUC were this number has risen up to 800 diagnoses yearly in 2017. The survival rates, however, did not improve over this time period. Despite advancements in diagnostic and therapeutic approaches, the lack of improvement in survival suggests potential limitations in current treatment strategies or delayed diagnoses. These results underscore the need for enhanced awareness, earlier detection, and more effective therapies to improve UTUC outcomes.

When two tumors within the same patient share the same molecular mutations, so a common origin, this is known as clonality. For quite a time the debate is ongoing if IVR are ‘seeds’ of the primary UTUC. In **Chapter 3**, we systematically

reviewed whether synchronous (simultaneous) and metachronous (sequential) intravesical recurrences and UTUC are clonally related. The outcomes suggest that seeding of tumor cells seems to be the most important mechanism of developing IVR as >80% of paired IVR and UTUC were related in these cohorts. In **Chapter 4**, we have assessed this theory of seeding and the outcome of chapter 3 in a retrospective cohort of 15 UTUC patients having IVR during follow up. To investigate the clonal relationship between both entities, targeted DNA sequencing was performed (41 genes) on both tumors within the same patient. In 73% of the patients a clonal relation of both tumors was indeed detected, so, seeding of tumor cells seems the most important mechanism in developing IVR. This knowledge could support the development of more predictive and personalized treatment approaches for patients with UTUC, with a focus on preventing intravesical recurrences

Known from urothelial carcinoma of the bladder, some molecular subtypes are associated with better or worst outcomes and different responses to different targeted therapies. However, if and how these findings extend to UTUC remains unclear. In **Chapter 5**, a deep-learning workflow was designed to predict certain molecular subtypes for UTUC from routine histopathological H&E slides, widely used by pathologist for the diagnosis of UTUC. The workflow showed to be effective in predicting certain UTUC subtypes, which were linked to specific clinical outcomes and treatment responses. By identifying these subtypes directly from H&E slides, this workflow could support more personalized treatment decisions and indicate which patients would gain the most from further molecular analysis.

Patients diagnosed and treated for UTUC are known to be at high risk of developing intravesical recurrence (IVR) in the years following surgery. The postoperative chemotherapy instillation following radical surgery, as currently recommended in the European Association of Urology guidelines for UTUC, has been shown to effectively lower the risk of IVR. However, a significant proportion of patients do not receive this instillation, as clinicians fear extravesical leakage of the chemotherapy. **Chapter 6** introduces the REBACARE trial, in

which patients receive a single intravesical instillation of chemotherapy *before* radical surgery for UTUC, while the urinary tract anatomy remains intact. This approach is hypothesized to enable more patients to receive this treatment and to be as effective as a postoperative instillation in reducing the risk of IVR. In **Chapter 7**, the REBACARE trial results are presented, with 170 UTUC patients enrolled from 18 Dutch hospitals. The final analysis showed no overall benefit of preoperative chemotherapy instillation in reducing the risk of IVR. However, other important findings emerged: (1) diagnostic ureterorenoscopy (URS) during UTUC work-up is a significant risk factor for IVR, likely due to tumor cell seeding; (2) in patients who underwent diagnostic URS, the chemotherapy instillation was not beneficial, likely because disseminated tumor cells had already implanted; but (3) preoperative instillation did show added value for patients who did *not* undergo diagnostic URS.

In **Chapter 8**, the short-term changes in health-related quality of life (HRQoL) among patients undergoing radical surgery for upper urinary tract urothelial carcinoma (UTUC) were assessed as part of the REBACARE trial. Patients experienced a decline in HRQoL immediately following surgery, particularly in physical and social functioning. Even an improvement in emotional function was observed. Postoperative complications were negatively associated with social functioning. However, most patients will eventually return to baseline levels three months following surgery. These outcomes can be used to counsel UTUC patients before and after surgery and contextualize recovery following treatment.

In **Chapter 9**, the main findings are summarized and future perspectives are discussed. Based on the findings of this thesis we underscore the fact that most intravesical recurrences are based on seeding, a diagnostic URS should be performed judiciously and when performed a postoperative instillation should be considered and when no diagnostic URS is performed a preoperative instillation with chemotherapy before radical surgery for UTUC is recommended.

NEDERLANDSE SAMENVATTING

Hoofdstuk 1 biedt een algemene inleiding over het urotheelcarcinoom van de hogere urinewegen, in het Engels 'UTUC' genoemd, en de behandeling ervan. UTUC is relatief zeldzaam in vergelijking met blaaskanker, wat leidt tot beperkte kennis en literatuur. UTUC ontstaat meestal in het nierbekken of de ureter en ontwikkelt zich doorgaans vanuit het urotheel; de binnenbekleding van de urinewegen. De diagnose wordt gesteld middels CT-urografie, een cystoscopie en een biopt (via ureterorenoscopie), hoewel detectie en risicostratificatie uitdagend blijven. De standaardbehandeling voor gelokaliseerde, niet-metastatische UTUC is een radicale nefro-ureterectomie (RNU), waarbij niersparende chirurgie (lasercoagulatie, distale ureterectomie) een optie is in geselecteerde laagrisicopatiënten. Ondanks radicale chirurgie zijn UTUC-patiënten bekend met een hoog risico op het ontwikkelen van een intravesicaal recidief (IVR) in de jaren na de chirurgische behandeling. Tot 47% van de patiënten wordt uiteindelijk gediagnosticeerd met een dergelijk recidief en in de dagelijkse klinische praktijk zijn we nog niet goed in staat om dit risico te verlagen. Dit manuscript heeft al doel de biologie verantwoordelijk voor de ontwikkeling van UTUC en IVR te onderzoeken, te beoordelen of een preoperatieve instillatie met chemotherapie vóór radicale chirurgie het risico op IVR verlaagt, en de uitkomsten van de kwaliteit van leven voor patiënten in Nederland te evalueren.

In **hoofdstuk 2** hebben we de trends in incidentie en overlevingspercentages voor UTUC in Nederland over een periode van 25 jaar (1993-2017) onderzocht. We constateerden dat de incidentie van UTUC is toegenomen. In absolute cijfers; in 1993 werden 400 patiënten gediagnosticeerd met UTUC, terwijl dit aantal in 2017 is gestegen tot 800 diagnoses per jaar. De overlevingspercentages zijn echter in deze periode niet verbeterd. Ondanks vooruitgang in diagnostische en therapeutische benaderingen suggereert het gebrek aan verbetering in overleving mogelijke beperkingen in de huidige behandelingsstrategieën of vertraagde diagnoses. Deze resultaten benadrukken de noodzaak van verhoogde bewustwording, vroegtijdige detectie en effectievere therapieën om de uitkomsten van UTUC te verbeteren.

Wanneer twee tumoren binnen dezelfde patiënt dezelfde moleculaire mutaties delen, wat wijst op een gemeenschappelijke oorsprong, staat dit bekend als klonaliteit. Al geruime tijd is er een debat gaande over de vraag of IVR 'zaadjes' zijn van de primaire UTUC. In **hoofdstuk 3** hebben we systematisch beoordeeld of synchrone (gelijktijdig) en metachrone (opeenvolgend) intravesicale recidieven en UTUC klonale verwantschap vertonen. De uitkomsten suggereren dat de intraluminale verspreiding van tumorcellen het belangrijkste mechanisme is voor het ontwikkelen van IVR, aangezien in >80% van de patiënten het IVR en UTUC klonaal met elkaar gerelateerd waren. In **hoofdstuk 4** hebben we deze theorie en de uitkomst van **hoofdstuk 3** beoordeeld in een retrospectieve cohortstudie van 15 UTUC-patiënten die IVR ontwikkelden tijdens de follow-up. Om de klonale relatie tussen beide entiteiten te onderzoeken, werd DNA-sequencing uitgevoerd (41 genen) op beide tumoren binnen dezelfde patiënt. Bij 73% van de patiënten werd inderdaad een klonale relatie tussen beide tumoren gedetecteerd, wat aantoont dat het verspreiden van tumorcellen het belangrijkste mechanisme lijkt te zijn bij de ontwikkeling van IVR.

Van urotheelcarcinoom van de blaas is bekend dat bepaalde moleculaire subtypen zijn geassocieerd met betere of slechtere uitkomsten en verschillende reacties op verschillende gerichte therapieën. Het is echter onduidelijk of, en hoe deze bevindingen zich relateren tot UTUC. In **hoofdstuk 5** werd een deep-learning workflow ontworpen om bepaalde moleculaire subtypen voor UTUC te voorspellen op basis van routinematige histopathologische H&E-preparaten, die veelvuldig door pathologen worden gebruikt voor de diagnose van UTUC. De workflow bleek effectief in het voorspellen van bepaalde UTUC-subtypen, die gerelateerd waren aan specifieke klinische uitkomsten en behandelingsresponsen. Door deze subtypen direct uit H&E-preparaten te identificeren, kan deze workflow meer gepersonaliseerde behandelingen adviseren en aangeven welke patiënten het meest profiteren van verdere moleculaire analyse.

Patiënten die gediagnosticeerd en behandeld zijn voor UTUC lopen een hoog risico op het ontwikkelen van intravesicale recidieven (IVR) in de jaren na de operatie. De postoperatieve chemotherapie-instillatie na radicale chirurgie,

zoals momenteel aanbevolen in de richtlijnen van de European Association of Urology voor UTUC, heeft aangetoond effectief te zijn in het verlagen van het risico op IVR. Echter, een aanzienlijk percentage patiënten ontvangt deze instillatie niet, omdat clinici vrezen voor extravesicale lekkage van de chemotherapie. **Hoofdstuk 6** introduceert de REBACARE-trial, waarin patiënten een enkele intravesicale instillatie van chemotherapie ontvangen vóór radicale chirurgie voor UTUC, terwijl de anatomie van de blaas intact blijft. Deze benadering is gebaseerd op de hypothese dat meer patiënten deze behandeling kunnen ontvangen en dat het uiteindelijk even effectief zal zijn als een postoperatieve instillatie in het verlagen van het risico op IVR. In **hoofdstuk 7** worden de resultaten van de REBACARE-trial gepresenteerd, met 170 UTUC-patienten die hebben geparticipeerd en zijn geïncludeerd in 18 Nederlandse ziekenhuizen. De uiteindelijke analyse toonde geen algemeen voordeel van de preoperatieve chemotherapie-instillatie in het verlagen van het risico op IVR. Andere belangrijke bevindingen kwamen echter naar voren: (1) Een diagnostische ureterorenoscopie (URS) tijdens de diagnostiek voor UTUC is een significante risicofactor voor IVR, waarschijnlijk door de intraluminale verspreiding van tumorcellen door deze operatietechniek; (2) bij patiënten die een diagnostische URS ondergingen, was de preoperatieve instillatie met chemotherapie niet bijdragend in het verlagen van het risico of IVR, waarschijnlijk omdat de gedissemineerde tumorcellen al waren geïmplanteerd; maar (3) een preoperatieve instillatie toonde echter wel toegevoegde waarde voor patiënten die geen diagnostische URS hadden ondergaan.

In **hoofdstuk 8** werden de verandering op de korte termijn in de kwaliteit van leven van patiënten die radicale chirurgie ondergingen voor UTUC onderzocht, als onderdeel van de REBACARE-trial. Patiënten ervaarden een daling in de kwaliteit van leven, onmiddellijk na de operatie, met name in de fysieke en sociale functionaliteit. Er werd echter wel een verbetering in de emotionele functionaliteit waargenomen. Postoperatieve complicaties waren negatief geassocieerd met de sociale functionaliteit. De meeste patiënten zullen echter uiteindelijk binnen drie maanden na de operatie terugkeren naar hun basisniveau. Deze uitkomsten kunnen worden gebruikt om UTUC-patiënten voor en na de operatie voor te lichten en het herstel na de behandeling te contextualiseren.

In **hoofdstuk 9** worden de belangrijkste bevindingen samengevat en toekomstige perspectieven besproken. Op basis van de bevindingen van deze thesis benadrukken we dat de meeste intravesicale recidieven het gevolg zijn van intraluminale verspreiding. Over het uitvoeren van een diagnostische URS moet zorgvuldig worden besloten en wanneer deze wordt uitgevoerd, moet een postoperatieve instillatie in overweging worden genomen. Wanneer er geen diagnostische URS is uitgevoerd, wordt een preoperatieve instillatie met chemotherapie vóór radicale chirurgie voor UTUC aanbevolen.

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ABOUT THE AUTHOR

Thomas van Doeveren was born in the grayest city of the Netherlands, Spijkenisse, on the 21st of August in 1991. In 2009, he completed secondary school at the Blaise Pascal in Spijkenisse. In 2009 he studied Technical Management at the Technical University of Delft. From 2010 until 2018 he studied medicine at the University of Rotterdam including one year as fulltime board member of the Medical Faculty Association Rotterdam (MFVR). After graduation, he started working on his PhD at the urology department of the Erasmus University Medical Center in Rotterdam under supervision of prof. dr. J.L. Boormans, dr. K.K.H. Aben and dr. P.J. van Leeuwen resulting in this thesis. After 2 years of research, he started in 2018 working as a resident not in training at the Franciscus & Vlietland Hospital in Rotterdam. In 2022 he started his traineeship in Urology. From 2022 until 2024 he was working in the IJsselland Hospital in Cappelle a/d Ijssel at the General Surgery department as part of this traineeship. Currently he is working at the department of Urology in the Erasmus Medical Center in Rotterdam and will continue in the Amphia Hospital in Breda.

ERASMUS MC PHD PORTOFOLIO

Name PhD student	Thomas van Doeveren
Erasmus MC Department	Urology
PhD period	Januari 2018 – Januari 2025
Promotor	Prof. Dr. J.L. Boormans
Supervisors	Dr. K.K.H. Aben & Dr. P.L. van Leeuwen

PhD training	Year	ECTS
Courses		
Endnote	2017	0.2
Basis introduction on SPSS	2017	1.0
BROK (Basic course rules and organisation for clinical researchers	2018	1.5
Basic course on 'R'	2018	2.0
Scientific Integrity	2018	0.3
Biomedical English writing	2019	2.0
Photoshop and illustrator CC	2019	0.3
NGS in DNA diagnostics Course	2019	1.0
Course on coaching bachelor students	2019	0.2
Biostatistical methods: basic principels	2020	5.7
(inter)national conferences		
Sophia research day, The Netherlands	2018	0.3
NVU voorjaarsvergadering, The Netherlands	2018, 2019	0.6
NVU najaarsvergadering, The Netherlands	2018, 2019	0.6
SEOHS symposium, The Netherlands	2018	0.3
MOLMED Medicine day, The Netherlands	2019	0.3
34th Annual EAU congress, Spain	2019	1.0
IBCN congress, Denmark	2019	1.0
35th Annual EAU congress, Amsterdam	2020	1.0
Refeeravond Erasmus MC, The Netherlands	2022	1.0

Presentations		
Oral presentation DUOS day, the Netherlands	2018	0.7
Poster presentation at the EAU, Spain	2019	0.7
Guided postertour presentation at the EAU, Spain	2019	0.7
Oral presentation at the NVU, The Netherlands	2019	0.7
Oral presenation refeeravond Erasmus MC, The Netherlands	2019	0.7
Oral presentation IBCN, Denmark	2019	0.7
Oral presentation DUOS day, The Netherlands	2019	0.7
Poster presentation at the EAU, The Netherlands	2020	0.7
Oral presentation refereeravond Erasmus MC, The Netherlands	2020	0.7

Teaching	**Year**	**ECTS**
Physical examination of men	2018, 2019	1.0
Urogenital trauma	2018, 2019	1.0
Supervision interns	2018	1.0
Intervision on Coaching	2019	0.2
Coaching bachelor students	2020	2.0
Journal club	2022	3.0
Onderwijs Urologie	2022	1.0
Total		**35.8**

ABBREVIATIONS

AI	Artificial Intelligence
CES	Clinical Effect Size
CIS	*Carcinoma in situ*
CT	Computed Tomography
CTC	Common Toxicity Criteria
CTCAE	Common Terminology Criteria for Adverse Events
DNA	DesoxyriboNucleic Acid
d-URS	diagnostic UreteroRenoScopy
EAPC	Estimated Annual Percentage of Change
EAU	European Association of Urology
EORTC-QLQ	European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire
ESR	European age-Standardized incidence Rates
FFPE	Formalin-Fixed Paraffin-Embedded
FGFR	Fibroblast Growth Factor Receptor
HRQoL	Health Related Quality of Life
IVP	Intravenous Pyelography
IVR	Intravesical Recurrences
KSS	Kidney Sparing Surgery
LND	Lymph Node Dissection
MMC	MitoMyCin
MSI	Microsatellite Instability
NAC	Neo-Adjuvant Chemotherapy
NCR	Netherlands Cancer Registry
NGS	Next-Generation Sequencing
RNA	Ribonucleic Acid
RNU	Radical NephroUreterectomy
TMA	Tissue MicroArray
UBC	Urothelial Cell Carcinoma of the Bladder

UTUC	Upper Tract Urothelial (cell) Carcinoma
UUT	Upper Urinary Tract
WES	Whole Exome Sequencing
WGS	Whole Genome Sequencing
WSI	Whole Slide Image

DANKWOORD

Het. Is. Klaar. ON-GEL-OFE-LIJK.

Datgene wat jaren op de achtergrond sluimerde, dan weer op de voorgrond stond, onderwerp was tijdens ‘rustige’ diensten en waar toch altijd tijdens de vakantie ergens aan gewerkt moest worden… de promotie. Het is leerzaam geweest, een pure uitdaging voor de planning, waarin ook tijd gemaakt moest worden voor liefde, de ‘twee mooiste kinderen ooit’ die tijdens deze periode in ons rijke leven zijn bijgevoegd, de wens om veel te kunnen sporten, m’n vrienden en familie, en de opleiding! Het was een tijd waarin ik heb geleerd om op het juiste moment te pieken, mezelf niet te overwerken en keuzes te maken op allerlei pijlers in het leven. Maar wat ben ik trots dat dit boekje er is gekomen. Een overwinning voor mezelf, maar ik denk ook echt voor de huidige kennis op het gebied van deze, toch nog, relatief zeldzame tumor.

Hoewel je je echt wel eens ‘alleen’ hebt gevoeld gedurende dit traject, is dit een prestatie waaraan heel, heel veel mensen hebben bijgedragen. Mijn naam prijkt weliswaar op de voorkant, maar het is echt een team-effort. Dat maakt het ook een geweldig proces; naast wetenschappelijke ervaring opdoen, is het ook echt een kans om te leren omgaan en te werken met allerlei karakters, persoonlijke belangen en verschillende expertises en interesses.

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Joost! Op een onverwachts moment stond je achter me tijdens mijn onderzoeksstage die ik bij jou deed: “Luister jij TECHNO!!!??? En ken jij MACEO PLEX!!” Nou, toen wist ik dat het wel goed zat! Ik was op zoek naar een plek voor mijn onderzoeksstage bij de urologie, en jij had net de Grant binnengesleept voor het opzetten van de REBACARE-trial. Vanaf het begin was het plan dat de onderzoeksstage een moment was om te peilen of we een eventueel promotietraject rondom deze trial zagen zitten. Dit was voor mij achteraf echt

de perfecte springplank. Vanaf het eerste moment wist ik dat jouw werkwijze bij mij past: scherp, kritisch, maar met heel veel ruimte voor eigen tijdsindeling. Als je maar levert… Daarnaast heb je me altijd gesteund in mijn 'onrust' om toch ook snel de kliniek weer in te duiken; om m'n grote 'masterplan' uit te voeren! Voor mij ben je echt een voorbeeld. Je straalt rust uit, hebt mega veel kennis van zaken, bent sociaal sterk en deelt mijn mening dat 'als je Avicii wil horen, je maar lekker 538 opzet'. Ik hoop nog heel veel met je te maken te hebben in de toekomst. Het enige wat nog op de palmares moet komen, is dat we dan toch echt een keer als echte fanboys Maceo samen gaan zien!

Pim. Wat betreft Maceo hebben we jou ook gewoon nodig: de drie musketiers op de techno-toer. Tijdens mijn onderzoekstijd hebben we elkaar eigenlijk echt te weinig in het echt gezien. Maar wat kon jij altijd door mijn ingeleverde stukken heen prikken. Mail van Pim: Tatsssss… 'ja, daar had ik inderdaad nog niet aan gedacht'. Dank hiervoor.

Katja. Door jou ben ik een tijd minimaal één dag in de week ondergedompeld geweest in het IKNL'se. Een wereld op zich!! Een totaal andere manier van kijken naar de geneeskunde, wat voor mij echt heeft bijgedragen aan een bredere blik op deze wereld. Van micro naar macro!! Ook jij wist mij altijd op scherp te zetten! Ik zal niet ontkennen: ik heb af en toe een momentje van frustratie gevoeld, maar ik ben echt van mening dat door jouw aanpassingen en adviezen onze stukken naar een hoger niveau zijn getild!! Dus heel, heel erg dank daarvoor. Ook dank dat je altijd aanwezig was bij mijn presentaties op de EAU, altijd in gezelschap van mijn IKNL-vriendin **Anke**! Bij deze wil ik ook **alle medewerkers van IKNL** bedanken die betrokken zijn geweest bij de uitvoering van de REBACARE-trial. Zonder jullie inzet en begeleiding had ik deze studie nooit zo vlot en vlekkeloos tot een einde kunnen brengen. Niet alleen wij, maar ook de patiënten die hebben deelgenomen aan deze studie, hebben deze resultaten aan jullie te danken! In het bijzonder wil ik **Marjan, Jessica, Margriet, Joline en Rogier** bedanken.

Graag wil ik **de kleine en grote commissie** bedanken voor hun bereidwilligheid om mij te begeleiden tijdens dit laatste deel van mijn promotietraject. Ik ben vereerd tegenover jullie te mogen staan.

De onderzoekstuin! Door mijn langdurige promotietraject (7 jaar!!!!!!) heb ik heeeuuuul veel collega's mogen meemaken. Sorry voor iedereen die ik vergeet te noemen, maar… **Ilse, Toscane, Daniel, Henk, Ivo, Sebastiaan, Michelle, Rosa, Tess, Sophie, Olga, Felice, Dennis, Christiaan, Mathijs, Arnout, Jan, Frank-Jan, Maaike, etc**.: wat een klapper van een tijd is het geweest. Ik heb uiteindelijk maar 2,5 jaar fulltime in de tuin mogen bivakkeren, maar het was altijd gieren. Er werd zooooooo veeeeeel geouwehoerd. Bij elkaar komen in Sebas' Coffeecorner, vliegende skippyballen, broodje Dynamite bij Dennis, Nijmegen volledig op z'n kop gezet… In het Erasmus duren de promotietrajecten relatief lang, maar deze werktuin heeft daar zeker aan bijgedragen. Als de laatste was aangekomen om te beginnen met werken, pakte de eerste alweer de spullen in om te gaan; een continue stroom van verplaatsingen van personen. We hebben heel wat congressen meegemaakt, waarbij Barcelona toch wel echt het hoogtepunt was; wat een zieke hut had Ilse toch geregeld. En wat hebben we gelachen. Ik had dit nooit willen missen.

Één grande investigador wil ik apart noemen: mijn uro-matador, Joep 'de knalraket' de Jong. Wat ben jij toch een unieke vent, een speciaaltje. Ik denk ook echt dat de wereld nog één van jou niet had aangekund. Geen energie? Dat ken jij niet. Grenzen? Bestaan niet. Kunnen we er iets moois van maken? Dan maken we er iets geweldigs van. Met jou op congres kreeg alles een gouden randje. Ieder congres werden we wel op een ochtend wakker, lepeltje-lepeltje in kostuum, en was er een story for life gecreëerd. Eén blik was genoeg en we lieten iedereen achter en stapten in een taxi naar een party. Eén blik was genoeg en we stonden op één of andere Nederlandse verjaardag in Barcelona, waar we binnenkwamen omdat jij opeens Spaans sprak, wat toch Italiaans bleek te zijn. Er is heel wat gebeurd bij je, en dat je wat gas hebt teruggenomen, is meer dan logisch. Heel volwassen ook, maar dat ik kan zeggen dat ik met de pre-Joep heb mogen onderzoeken, dat maakt me blij.

Hannah, miss blue eyes. Het is alweer 11 jaar geleden dat we tegen elkaar aanliepen op een feestje tijdens ADE. De chemie was er direct!! Ik heb jou nooit meer losgelaten en jij mij nooit meer. Je maakt me compleet, je 'leest me' zonder dat ik een woord hoef te zeggen, je bent er altijd op het moment dat ik zelf nog niet besef dat ik je even extra nodig heb… 'now I've got a woman at home, she treats me well', zoals Ben Howard volgens mij gewoon over jou heeft geschreven. Je

woonde eerst in Amsterdam, ik nog in Rotterdam… wat mij betreft één van de meest fijne periodes in mijn/ons leven. Het was een grote ontdekkingstocht, we waren altijd op pad en hebben beide steden samen uitgespeeld. Uiteindelijk ben je afgedaald naar Rotterdam en zijn we begonnen om een heel, heel fijn leven samen op te bouwen. Nu ook met twee fantastische kinderen: Lot en Flip. Het gezinnetje is wat ons betreft compleet en naast fantastische vriendin blijk je ook nog een extreem fijne moeder te zijn. Ik heb het vaker gezegd, maar dit promotietraject, ten tijde van mijn opleiding tot uroloog en de tropenjaren, had nooit en te nimmer afgekomen als jij mij daar niet die onvoorwaardelijke steun in had gegeven. Het was echt niet makkelijk, maar je hebt altijd achter me gestaan, begrepen dat dit traject ook tijd opsnoepte… Daarnaast was je ook degene die juist af en toe op de rem trapte: nu ff niet, ga sporten, spreek af met vrienden, ben heel even met mij. Ik ben je daar zo intens dankbaar voor en het is een bijzonder besef dat jij zo'n groot aandeel hebt in zoveel facetten van mijn leven. We hebben nog mooie plannen in het verschiet en ik kijk nu al uit naar de toekomst die voor ons ligt… I love you.

Lot. De wijze eerste. En helaas ga jij me heeeeeel wat geld kosten als je ouder wordt. Can't say no ;) Je bent zo'n intense schat. En een sociaal wonder. Waar je ook bent, jij hebt alweer een nieuw vriendinnetje gemaakt. En je betrekt je broertje hier altijd bij. Jullie hebben een fijne symbiose: hij trekt je de achtbaan in waar je nooit in had gedurfd, maar jij zorgt er wel voor dat er tenminste een riempje vast wordt gedaan. Fantastisch ook hoe je soms verbanden legt die niemand anders ziet; sterker nog, je hebt het geschopt tot de stellingen!! Blijf zo geïnteresseerd in alles… 'maar, waarom dan…?' Dan wordt de wereld waarschijnlijk een stuk mooier door alles wat je gaat bereiken! Ik hou zo kneiterveel van je.

Flip. The rocketman! Het motto in jouw korte leven tot nu toe: 'Hoezo zou ik dit nog niet kunnen?' Ondanks dat ik al meerdere hartverzakkingen heb mogen meemaken, tot bungelend aan één voet boven een afgrond, mag ik hopen dat je deze eigenschap blijft houden. Ik kijk nu al uit naar de momenten dat je me gaat uitdagen op de wielrenfiets of mountainbike, me in de muur beukt met karten (zoals ik bij m'n eigen pa), etc. Je gaat me scherp houden en hopelijk vooral jong!! Jij gaat er komen; no boundaries for you!! … en dat je ALSJEBLIEFT ook de knuffelkont blijft die je bent. Ook van jou hou ik zielsveel.

Pa en Ma, Martin en Nicolien, Paps en Mams, St. Martinus en Vinolien. Tja, wat kan je anders wensen als kind. Door jullie stabiliteit hebben wij dat ook gekregen. Door jullie humor en het altijd openstaan voor gekkigheid lachen jullie kinderen en kleinkinderen nu ook aldoor. Een idee of carrièrepad was nooit te gek, als er maar over gecommuniceerd werd, met wel het motto: 'prima, maar je weet je einddoel'. Door jullie enthousiasme over de zorg heb ik dat ook gekregen. De keuze om dokter te worden was daarom geen verrassing (al hebben jullie me alsnog gesteund in de rebelse keuze voor de TU Delft). Een promotietraject was voor jullie geen onbekend terrein. Ik geloof ook zeker dat door jullie gecreëerde basis dit traject nu tot het eind is gekomen. Jullie gaan nu een heel andere fase van het leven in: het pensioen. Ga ervan genieten, van jullie kinderen en kleinkinderen, maar ook zeker weer van de tijd voor jullie zelf. Dat wij daar een groot deel van bij mogen zijn!!

Zussies, Sophie en Lisanne. M'n tweeling-bro's. Veel broers ervaren een strijd met hun zus/zussen. Zeker als het er dan ook nog twee zijn. Ik heb dat nooit zo ervaren. Onze jeugd kende alleen maar hoogtepunten in mijn herinneringen. Op vakantie hadden we niet veel vrienden nodig, we hadden elkaar. Jullie hebben een bizarre, soms onbegrijpelijke taal die alleen tweelingen kunnen hebben. Nog geen half woord is genoeg. Toch denk ik dat ik bij jullie ook niet meer dan één woord nodig heb om jullie te begrijpen, en jullie mij. Ook jullie zijn in de zorg beland; Sophie, jij eerst voor het implementeren van Hix als consultant en nu als beleidsmedewerker, Lisanne nu als applicatiebeheerder Hix. Wat hilarisch is, want ik zeik natuurlijk als arts altijd over hoe *** Hix werkt en jullie zeggen dan unaniem dat ik het ten eerste waarschijnlijk weer niet goed gebruik en ten tweede omdat artsen altijd iets te zeiken hebben! Het fijne is wel dat jullie dus totaal op de hoogte zijn van de perikelen in het ziekenhuis en ik dan ook altijd ff lekker m'n hart kan luchten; zowel over het klinische aspect als over het promotietraject. Ook mijn zussies worden groot. Sophie en Ruud, jullie hebben Lena aan de wereld geschonken; de nieuwe wereldkampioen surfen, want je ziet het al, die geniet wel van dat chille 'surfersleventje' ;) En Lisanne en Maurice, jullie gaan hopelijk trotse ouders worden van een prachtig kind! Ik ben blij jullie broer te zijn; dat we de broer-zussen-etentjes maar weer eens flink gaan oppakken!!

Ankie en Camiel, jullie zijn en waren altijd op de hoogte van alle stapjes binnen dit traject. Altijd geïnteresseerd. En ik kan wel zeggen, Ankie, dat door jouw oppasdagen de promotie niet nog langer heeft geduurd!

Riekert en Ans, ook jullie hadden tomeloze interesse in mijn PhD. Altijd vragen hoe het ging en hoe het ervoor stond. Riekert, door jouw achtergrond in de farmaceutische industrie had je altijd scherpe meningen en las je mijn stukken steevast, wat ik altijd heel erg waardeerde.

Oma Trees, 92 alweer. En jij leeft van mijlpaal naar mijlpaal. Eerst Lot, toen Flip, toen Lena, nu de promotie; het aankomende andere draakje staat nog op de planning! Je doet het toch maar. Als ik je spreek, is het altijd eerst: 'hoe is het met je studie?' Daarna: 'hoe is het met de kinderen?' Je bent trots en haalt energie uit alles wat iedereen bereikt. Helaas heb je laatst de stap moeten zetten richting het verzorgingstehuis in het decadente Heemstede, maar daar lijk je snel je draai gevonden te hebben.

Robin. M'n maat!! Wat begon op het moment dat we onze ene voet nog maar net voor de andere voet konden zetten op de atletiekbaan, is uitgegroeid tot een levenslange vriendschap. De hele dag met lego spelen in de tuin, naar de McDonald's met het team en Paul als we het clubrecord estafette weer hadden aangescherpt, onze eerste dronkenschap op het AB-toernooi van HSC, jouw 'salsa-pasjes' eerst in de Soos in Zuidland en daarna 't Fust op Stadhuisplein, de gestoorde BOB-busritjes terug naar Spijkenisse, de kipnuggets bij de Mac om onze verontwaardiging te bespreken waarom ik nou uit de VOF was gezet terwijl ik alleen een fles drank had gejat en 'heeee, ik ben gewoon geneeskundestudent, ik doe geen vlieg kwaad', onze interrail-reis door Europa waar we het budget tijdens de eerste dagen in Berlijn al hadden uitgegeven en waar jij m'n net stukgelopen lange relatie binnen no time deed vergeten door de soepelste wingman-acties die ik gezien heb (iets met PvdA)… tot ook de afgelopen jaren waar ik altijd wel wat te zeiken had op de promotie en het traject. Jouw nuchtere blik en het feit dat je zelden meeging in die emotie, hebben mij vaak weer vooruit gekregen. We zijn elkaar maar een paar keer een beetje uit het oog verloren: tijdens onze studententijd en tijdens een berucht Pleinvreesfeestje in de Factory 010 ;). Ik heb je echt mega hoog zitten. Je tomeloze inzet voor een eigen bedrijf, je maatschappelijke visie op de wereld en je loyaliteit naar alles en iedereen. Ooit gaat het je allemaal uitbetalen op de manier hoe jij je dat al jaren voor je ziet, en gaan jij en lieve Tess daar heerlijk van genieten. Dat we samen oud mogen worden, klagend over de wereld die we hopelijk allebei wel een stukje beter hebben gemaakt op ons eigen vlak.

Omar. M'n andere maat. Jij bent wat mij betreft de belichaming van mijn innerlijke onrust dat er meer was te ontdekken in de wereld. Door jou heb ik dat mogen ervaren! Het begon allemaal op een ADE-avond waar we spontaan met z'n tweeën heen gingen en sindsdien is het AAN! Wat hebben wij een hoop meegemaakt en ik durf wel te zeggen dat we Rotterdam redelijk uitgespeeld hebben en binnenstebuiten hebben gekeerd. Oneindige avondjes op je balkon, ouwehoeren over van alles, dansen op talloze feesten, en ook nog eens mijn mooiste verjaardagscadeau ooit georganiseerd… Naast mijn nachtburgemeester ben je in de daguren ook een hele speciale maat voor me. Je innerlijke rust, je andere kijk op veel zaken en je luisterend oor zijn heel vaak van veel waarde voor me geweest. Op het moment van schrijven heb je een zeer fijne relatie met Daan; dit is je/jullie zo gegund, jullie hebben elkaar gevonden en god mag hopen dat het zo blijft. Wat ons betreft: je gaat hopelijk nog de rest van je leven last van me hebben, en laten we hopen dat ons plan tot uitwerking komt; met z'n tweeën in een verzorgingstehuis terechtkomen waar we alleen maar platen gaan draaien!!

Volmarijnstraat 9. Daniel, Jeffrey, David, Yvonne en Lieneke. Mijn roomies, mijn Volmarijntjes… WAT EEN TIJD was dat. Zes fantastische jaren in dat paleis aan de Volmarijnstraat; a dream came true. Dit vond natuurlijk allemaal plaats vóór mijn promotie, maar als een periode me gevormd heeft en invloed heeft gehad op mijn jaren daarna, is dit er wel één! **Daniel:** My literary boy!!! Jij verslindt de boeken waar je bij staat. Daarvan heb ik er ook een hoop in mijn schoot geworpen gekregen van je. Jij was het rustige baken in huis (nou ja, op gala's toch wel vaak mijn running mate; NAAN!!!!), je hebt een zeer brede interesse, je ouwehoert hier graag over en ik geniet echt altijd van onze discussies over zaken breder dan geneeskunde. Zet een platenspeler en een fles bourbon neer en wij vermaken ons wel op de dinsdagavond! Even op de koffie gebeurt de laatste tijd ook steeds vaker en daar geniet ik van. Ik heb het gevoel dat Mette en Flip elkaar weleens kunnen gaan vinden als dikke maten, en dan mag de wereld zich wel zorgen gaan maken!! **Jeffrey:** mijn verdiepingsgenoot: de eerste blik die ik van jou kreeg staat me nog altijd bij; jij had blijkbaar die nacht nachtdienst gehad en ik ging een plankje ophangen. Ik had het gevoel dat ik vanuit een diep dal omhoog moest klimmen, maar jezus, wat hebben wij het goed gehad op die eerste verdieping. Je heerlijke gezeik over alles en iedereen, je mega aanstekelijke lach, de 'horse-whisperer', je frikandellenworp naar een

stel veganisten… maar ook gewoon je fijne adviezen, altijd degene die even opbelt, en een vriend voor het leven. Bij mij geldt 'uit het oog, uit het hart', heb je ooit zo soepel duidelijk gemaakt, maar jij zorgt er persoonlijk voor dat dit wel goedkomt. **David**: al vroeg hadden we bedacht dat het toch het allermooist zou zijn als we samen in één huis terecht zouden komen. Nou, zo geschiedde. En wat een verdomd lekkere tijd was het. Jij hebt me kennis laten maken met de totaal gestoorde kant van het leven; de Dordrecht–Strijen style!! Door jou ben ik tegen techno aangelopen… nog bedankt daarvoor. Maar je hebt me vooral geleerd af en toe dikke schijt te hebben aan wat dan ook. Een megafijne eigenschap die jij tot in de puntjes onder de knie hebt: "Mail tijdens mijn vakantie? Dat wis ik gewoon. Als het belangrijk was, mailen ze nog wel een keer." Ik hoop dat we vaker gaan mountainbiken en dat ik hopelijk ooit de bochten zo aan kan snijden als jij nu kan. **Lieneke:** Het stamhoofd van huize Volmarijn. De eerste, de beste. Degene met het torenkamertje!! Je bent een schat, maar hebt ook zeker je eigen mening klaar. En ook een fijne andere kijk op de wereld soms; heerlijk was dat aan de eettafel. Fijn ook hoe jij jezelf altijd opsloot in de week voor een toets en jezelf dan trakteerde op een hoop blikjes energiedrank, goede thee en lekker beleg!! Ik kan het weten, want jouw thee was mijn thee ;) Sorry dat ik zo vaak je kastje heb leeggeroofd, woeps!! **Yvonne:** Last but not least, de veroveraar en baas van David. Ook jij bezat een torenkamer, maar dan die met het dakterras… of was het toch een balkon!? Jouw komst betekende een upgrade in het eten. Van 'AH Basic' pasta-roomkaas naar langzaam gestoofde Aziatische… dingen! De kunst was zo lang mogelijk wachten met het appje 'wie eet er mee vanavond', in de hoop dat jij het eerder stuurde! Volmarinoooos, jullie hebben mijn studententijd gemaakt tot wat-ie is geworden; dat we elkaar altijd mogen blijven zien!!

It moaie Fryslân, mon chérie! Fijne vrienden, reisgenoten naar Donlevade en Fryslân. Daan, Simone, Daniel, Isabelle, Sjoerd en Elise. We hebben allemaal onze verhalen en carrières, fantastische kinderen en de tropenjaren die we samen meemaken. We kunnen bij elkaar terecht en het echt over alles hebben. Ik teken voor nog een hoop voorjaarsreisjes samen!!