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UPPER-TRACT UROTHELIAL CARCINOMA – DIAGNOSTICS AND PROGNOSTIC FACTORS

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UPPER-TRACT UROTHELIAL CARCINOMA – DIAGNOSTICS AND PROGNOSTIC FACTORS
THESIS FOR THE DOCTORAL DEGREE (Ph.D.)

By

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To my patients.

POPULAR SCIENCE SUMMARY OF THE THESIS

Urothelial carcinoma in the upper urinary tract, UTUC, is a rare disease of the cells called transitional or urothelial cells, which line the mucous membrane of the renal calices, renal pelvis and the ureters, as opposed to its "twin", namely, bladder cancer, which is fairly common. The disease most often presents with macroscopic haematuria, i.e., visible blood colouring the urine. Other early symptoms include pain caused by blood clots obstructing the ureter, as an increase in pressure in the renal collecting system elicits pain. Sometimes suspicion of UTUC is caused by a radiological sign that is found when computed tomography (CT) has been performed for other indications. The patients are generally older than 70 years at the onset of the disease and are often frail, with a history of smoking and cardiovascular and renal disease. This complicates both the work-up and the tolerability of surgical and oncological treatments. The patient is investigated with CT using intravenous contrast medium, which is thought to risk deterioration of renal function, and consequently, only patients with fairly good renal function can undergo optimal radiological investigation. As there is a risk for concomitant bladder cancer and its presence would change the order of treatments, a visual inspection of the bladder via endoscopic cystoscopy is performed. Additionally, microscopic investigation of cells shed in voided urine (cytology) helps determine the presence and gravity (low or high grade) of the tumour. The importance of evaluating the tumour meticulously before deciding on the treatment method has increased with the emergence of different surgical techniques and oncological regimes.

The gold standard treatment of nonmetastatic UTUC has been radical nephroureterectomy (RNU), which involves removal of the entire kidney, ureter and a part of the urinary bladder surrounding the ureteral orifice on the affected side, with everything removed in one piece without opening the collecting system. This is a large procedure and leaves the patient with just one kidney. UTUC cannot recur on the same side, but the patient risks being left with insufficient renal function, and UTUC can in rare cases arise in the remaining contralateral urinary collecting system (renal calices, renal pelvis and ureter). The other surgical option is kidney-sparing surgery (KSS) removing the UTUC with laser and endoscopic instruments or by segmental ureterectomy, excising the affected segment of the ureter and anastomosing the remaining parts. Although KSS leaves the patient with unchanged renal function, strict follow-up is mandatory as UTUC tends to recur. Only low-risk UTUC, i.e., noninvasive and low-grade UTUC, is suitable for KSS. Thus, it is crucial that we have methods of evaluating UTUC correctly before removing the entire kidney and ureter, as is done with RNU.

In **Paper I**, we investigated the diagnostic specimens taken at ureterorenoscopy (URS)[1] by comparing the evaluation of grade and ploidy (see below) with the RNU specimen obtained from the same patients when they underwent RNU as final treatment. URS was performed before RNU to obtain a detailed assessment of the number of tumours, tumour size, and tumour location and to secure cancer cells in saline washings (barbotage) from the bladder, renal pelvis and ureter. Minimal biopsies were obtained with small forceps introduced through the working channel of the ureteroscope. The ploidy, i.e., the number of chromosomes sets, which is equal to the DNA content in the nuclei of the tumour cells, was analysed with spectrophotometry and compared in the URS and RNU specimens.

In **Paper II,** we investigated which tumour characteristics were associated with tumour stage and with survival. This is important, as stage determines what surgical techniques could be successful in treating and curing the patient. Patients with noninvasive UTUC can usually be treated with KSS, whereas patients with invasive UTUC (≥pT2) should undergo RNU. Both understaging and overstaging would have negative repercussions for the patient. We found that tumour grade, DNA ploidy and S-phase fraction (SPF), a measure or marker of cell proliferation (how many of the cells that are in the process of dividing), were associated with tumour stage. However, tumour size, location of the tumour and multifocality were not associated with stage. As expected, we found that patients with superficial, low-grade and diploid (normal number of chromosomes) UTUCs lived longer and to a lesser degree died from urothelial carcinoma than patients with invasive, high-grade and aneuploid UTUCs.

We investigated a larger number of patients with UTUC in **Paper III** to explore a finding from Paper II, namely, that the SPF was higher in invasive UTUC than in superficial UTUC and that SPF was associated with survival. Comparing the relative importance of the tumour characteristics that predicted death from UTUC, we found that SPF was the strongest predictive factor. Stage was the other tumour characteristic that predicted death from UTUC. We confirmed that the risk of dying from UTUC increased with increasing SPF. Survival differed depending on stage, grade and ploidy.

In summary, we have shown that the evaluation of ureterorenoscopic samples gives an accurate representation of UTUC when samples are taken in a systematic fashion, with in situ barbotage and even in very small endoscopic biopsies. We have shown that the stage can be indirectly assessed by determining the grade, ploidy and SPF. SPF is also a good predictor of survival.

[1] Visual inspection with an endoscopic instrument specialised for use in the urinary system, all the way up into the kidney. There are “semirigid” and flexible ureteroscopes, and through their working channel additional instruments can be inserted for biopsy, saline washing and treatment of lesions (e.g., tumours) with a laser fibre.

ABSTRACT

Upper-tract urothelial carcinoma (UTUC) is rare, constituting 5-10% of all urothelial cancers. Urothelial carcinoma of the bladder is a common disease and is more studied than UTUC. The incidence of UTUC is rising, with people living longer and, especially, surviving bladder cancer to a greater extent than before; therefore, these individuals are at risk of developing UTUC, as the two diseases are closely linked. As the majority of patients presenting with UTUC are ≥65 years old and have risk factors for renal impairment, as well as for complications from general anaesthesia, large surgical procedures and chemotherapy, it is crucial to make as good a risk assessment as possible prior to deciding with the patient what treatment to undertake. Survival is poor for invasive UTUC, despite radical treatment, but if low-risk UTUC can be identified, that minority of patients can have equal survival if offered kidney-sparing treatment (KSS), generally URS laser ablation.

In **Paper I,** forty-five patients who underwent URS prior to radical nephroureterectomy (RNU) were included, and 43 were included in the final analysis. Samples were analysed, comparing the agreement of grade and ploidy in endoscopic biopsies and in barbotage samples from the renal pelvis and from the ureter (fluid collected from the bladder after instrumentation of the upper tract) with those in RNU specimens. Almost half of the tumours (20/43) were grade 3 (high-grade) cancer. Thirteen of the low-grade cancers were classified as grade 2, and 10 were classified as grade 1. The overall agreement of grade was 94%, with cytology being equal to the histology of biopsies in identifying cancer. Ureteral-barbotage specimens were more accurate than the other samples in 4/16 cases of UTUC located in the ureter.

The same patient cohort that was analysed in Paper I was included in **Paper II**, where the tumour characteristics associated with tumour stage were found to be tumour grade, DNA ploidy and cell proliferation (S-phase fraction, SPF). Five years had passed since inclusion and URS of the last patient; thus, the follow-up time was long in the calculation of survival. Additionally, the risk of death from UTUC in relation to SPF was calculated. An increased risk of death from UTUC with increasing SPF was found. Ploidy was useful for strengthening the assessment of grade 1 (diploid) and grade 3 (aneuploid) but not grade 2 UTUC.

Paper III was a larger study of cancer-specific survival (CSS) and the prognostic role of the different tumour characteristics studied in Paper II associated with survival and with invasive tumour stage. The cohort was extended to include 99 in the final analysis. SPF and stage were confirmed in multiple Cox analysis to be independent prognostic markers. The area under the ROC curve indicated that SPF was a good predictor for both the invasive stage and death from UTUC. CSS stratified by stage, grade and ploidy confirmed the large difference in survival between superficial and invasive stages, between different grades (when using the WHO 1999 classification) and between diploid and aneuploid UTUC. The risk of death from UTUC increased by 17% for every percent increase in SPF.

In summary, with a thorough and systematic work-up, the individual patient´s risk can be estimated after URS so that those with high-risk UTUC can be treated with RNU, whereas patients with low-risk UTUC can consider KSS, i.e., URS laser ablation or segmental ureterectomy. Samples taken at URS are reliable for grading and the determination of ploidy and SPF. Tumour stage and SPF were found to be independent prognostic markers. The tumour characteristics that proved useful for “indirect staging” of UTUC were grade, ploidy and SPF, as these were independently associated with tumour stage. SPF was shown to strengthen risk stratification and can be analysed in barbotage taken at URS. We also showed that the WHO 1999 classification system was more informative than the two-tiered WHO 2004 classification and better predicted CSS.

LIST OF SCIENTIFIC PAPERS

I. Malm C, Grahn A, Jaremko G, Tribukait B, Brehmer M. Diagnostic accuracy of upper tract urothelial carcinoma: how samples are collected matters. Scand J Urol. 2017;51:137-145.
II. Malm C, Grahn A, Jaremko G, Tribukait B, Brehmer M. Predicting invasiveness and disease-specific survival in upper tract urothelial carcinoma: identifying relevant clinical tumour characteristics. World J Urol. 2019 Nov;37(11):2335-2342.
III. Malm C, Jaremko G, Brehmer M. S-phase – an Independent Prognostic Marker in Upper Tract Urothelial Carcinoma (submitted)

CONTENTS

LIST OF ABBREVIATIONS

AUC	area under the curve
CI	confidence interval
CSM	cancer-specific mortality
CSS	cancer-specific survival
CT	computed tomography
DFS	disease-free survival
DSS	disease-specific survival
EMAST	elevated microsatellite alterations at select tetranucleotides
EAU	European Association of Urology
FCM	flow cytometry
FGFR3	fibroblast growth factor receptor 3
GFR	glomerular filtration rate
LG	low-grade urothelial carcinoma
HG	high-grade urothelial carcinoma
HR	hazard ratio
IHC	immunohistochemistry
IVR	intravesical recurrence of urothelial carcinoma
Ki-67	a nuclear antigen present only in the nuclei of cycling cells
KSS	kidney-sparing surgery
LOH	loss of heterozygosity
LVI	lymphovascular invasion
MCTU	multidetector computed tomographic urography
MeSH	Medical Subject Headings
MFS	metastasis-free survival
MMR	DNA mismatch repair genes
MSI	microsatellite instability
mTOR	mammalian target of rapamycin
NGS	next-generation sequencing
OCM	other-cause mortality

OS	overall survival
TP53	tumour suppressor gene coding for phosphoprotein p53
PCR	polymerase chain reaction
PFS	progression-free survival
pre-RNU URS	diagnostic ureterorenoscopy carried out before radical nephroureterectomy
PUNLMP	papillary urothelial neoplasm of low malignant potential
RCC	renal cell carcinoma
RCT	randomised controlled trial
RFS	recurrence-free survival
RNU	radical nephroureterectomy
ROC	receiver operating characteristic
SEER	National Cancer Institute Surveillance, Epidemiology and End Results (SEER) program database
SPF	S-phase fraction
UC	urothelial carcinoma
UCB	urothelial carcinoma of the bladder
URS	ureterorenoscopy
UTUC	upper tract urothelial carcinoma

1 INTRODUCTION/BACKGROUND

1.1 DEFINITION

Upper tract urothelial carcinoma (UTUC) can arise from the urothelium in the renal calices, renal pelvis and ureter. The majority of tumours occurring from the renal calices and renal pelvis through the ureters, urinary bladder and urethra arise from the urothelium (transitional epithelial cells) lining the mucous membranes. Urothelial carcinoma, or transitional cell carcinoma, is defined in the MeSH database as "a malignant neoplasm derived from transitional epithelial cells, made up of epithelial cells tending to infiltrate the surrounding tissues and give rise to metastases. Neoplasm is a new abnormal growth of tissue. Malignant neoplasms show a greater degree of anaplasia and have the properties of invasion and metastasis, compared to benign neoplasms."

1.2 AETIOLOGY AND RISK FACTORS

Inflammation plays a part in the malignant transformation of cells, and many of the known risk factors cause and sustain inflammation. The most important risk factors include some that cannot be modified (older age and male sex) and others that can be modified (exposure to tobacco, aromatic amines, aristolochic acid, arsenic and the analgesic phenacetin). Older age increases the risk of UTUC and affects the prognosis and tolerability of the different treatments. The impact of some risk factors (age, sex, smoking) on prognosis is further discussed below (Section 1.6).

The most common risk factors for developing urothelial carcinoma (UC) are exposure to tobacco and aromatic amines. Tobacco smoking increases the relative risk of UTUC by 2.5-7 times [1]. Aromatic amines can be present in dyes, textiles, rubbers and chemicals. Exposure to aromatic amines (benzidine and β-naphthalene) increases the risk but they are now prohibited in many countries. In some regions where the *Aristolochia fangchi* and *Aristolochia clematis* plants containing aristolochic acid are endemic, notably in the Balkans, parts of mainland China and Taiwan, this is an important risk factor. Aristolochic acid can be found in health food preparations and in bread, where the seeds of these plants are sometimes used. It has been shown to have a causal correlation to UTUC, as metabolites of aristolochic acid cause mutation of the *TP53* gene by inducing DNA breaks, leading to nephropathy[2], end-stage renal disease and increased risk of UTUC [2]. The incidence of UTUC is also relatively high in parts of Taiwan. This is in part due to the presence of Aristolochia plants and in part due to arsenic exposure by water pollution, which is also a risk factor for developing UTUC. Phenacetin analgesics (banned in 1980) and the drugs cyclophosphamide and ifosfamide also increase the

[2] Chinese herbs nephropathy, Balkan nephritis or Balkan endemic nephropathy, which are all caused by exposure to aristolochic acid, are now considered the same disease.

risk. Radiation is a risk factor for the development of UC with a latency period of at least 15 years. Two studies have reported an increased incidence of UTUC in patients who received pelvic radiation 14-25 years earlier [3, 4].

Lynch syndrome is a group of autosomal-dominant inherited diseases associated with germline mutations in mismatch repair (MMR) genes (*MLH1, MSH2* and its modifier *EPCAM, MSH6* or *PMS2*). *MSH2* mutation is the most common mutation found in Lynch-associated UTUC [5–7]. Mutation carriers have a 22-fold increased risk of developing UTUC compared to that in the general population [8]. Testing is performed with immunohistochemistry (IHC) for all four MMR proteins, *MSH2, MSH6, MLH*1 and *PMS2*, or by analysis of microsatellite instability (MSI) in tumour tissue [9]. Presently, Lynch syndrome is the only known hereditary tumour syndrome with an increased risk of UC. Presumably, approximately 2.5-12% of UTUCs are associated with Lynch syndrome, and so are 5% of urothelial carcinoma of the bladder (UCB) [10, 11]. The prevalence will rise, as patients with Lynch syndrome now survive the two more common Lynch-associated malignancies, namely, colorectal and endometrial cancer, to a greater extent than before.

Lynch syndrome should be screened for in the patient's history by considering age of onset of UTUC (<60 years), patient's own and family history of Lynch-associated malignancies, which include ovarian, stomach, hepatobiliary, small bowel, sebaceous gland, central nervous system neoplasms and prostate cancer [12]. The diagnosis of Lynch syndrome is confirmed by germline genetic testing i.e. analysis of DNA isolated in an oral rinse or peripheral blood. Currently, *MLH1, MSH2, MSH6, PMS2,* and *EPCAM* are included in the testing [5, 13]. The advantages of detecting Lynch syndrome are that the patient can be screened for other Lynch-associated malignancies, with the hope for a better prognosis with early detection of colorectal and endometrial cancer, and that family members carrying mutations can be identified and counselled. If diagnosed with advanced UTUC, a patient with defective MMR can often benefit from immunotherapy [14]. However, high MSI seems to have a better prognosis than other UTUCs [15].

1.3 EPIDEMIOLOGY

UCB is a common disease, with an incidence (number of new cases per 100 000) in Sweden in 2019 of 43.2 in males and 14.8 in females. In contrast, UTUC is much less common. Approximately 5-10% of all UCs are UTUCs. The incidence was 4.47 in males and 2.68 in females (i.e., in total 231 new cases in males and 137 in females) in Sweden in 2019. UTUC caused 92 deaths in Sweden in 2019, while 715 patients died of UCB in Sweden the same year. The incidence of UTUC increases sharply after the age of 65 [16]. UTUC is more common among men, with a male-to-female ratio of 2-3:1. Advanced-stage tumours were found to be equally common in males and females in a population-based study of all 930 patients diagnosed with UTUC in Sweden from 1971–1998, including also those not treated with surgery [3]. Patients developing UTUC are generally older than those developing UCB [16].

The incidence of UTUC increased after an initial decrease was seen after banning phenacetin-containing drugs in 1980 [17]. The rising incidence is pronounced in the age group older than 80 years, resulting in an increasing mean age at diagnosis [18, 19]. Additionally, it seems that the higher (invasive) stages have increased the most [20, 21], as opposed to many other cancers that are now often detected at an earlier stage than before. In contrast, stage migration towards earlier stage UTUC was observed in a study of 13 800 SEER-registered UTUCs diagnosed between 1973 and 2005 [19]. The invasive stage at diagnosis is four times more common in UTUC (60%) than in UCB (15%) [3, 22]. The rising incidence of invasive UTUC might also be an artefact, as studies that include only patients who have undergone surgery will have missed advanced UTUC in older patients who did not undergo any treatment. More recently, the SEER database was used in a study which reported an increasing incidence of more advanced stages but a decreasing age-standardised incidence [20]. The *increasing* age-standardised incidence found by some could in part be explained by increasing survival in UCB [19] but also in part by increased use of endoscopy and radiology [17].

A total of 3.1% of the patients in a Swedish population were found to develop contralateral UTUC at a median follow-up time of 46 months [23]. Patients with UTUC but without previous or synchronous UCB have a 38-50% risk of developing UCB within five years [24, 25]. On the other hand, patients with UCB have a 2-3% risk of developing UTUC [26, 27]. The multifocal, synchronous or metachronous development of UC is explained by two hypotheses: clonal development and the field change hypothesis [22]. These are elaborated below in Section 1.4.6.1 Clonogenic theory, field change theory and similarities and differences between UCB and UTUC.

1.4 DIAGNOSTIC WORK-UP

Macroscopic haematuria (56-98%) and flank pain (25-30%) are the most common presenting symptoms [18]. Haematuria can be present in early disease, but the disease can also be silent until it has reached an advanced stage. Late symptoms, indicating advanced disease, include anorexia, weight loss, anaemia, bone pain, and fatigue. Currently, many tumours are incidental findings, as computed tomography (CT) is the dominating radiological method and is used on wide indications, and as survival of UCB has increased. Approximately 15% of patients are asymptomatic at the time of diagnosis [28, 29].

UTUC is characterised according to the TNM classification [30] and by cytological and histopathological grading. In this section, staging, radiological investigations, grading and diagnostic ureterorenoscopy (URS) are discussed.

1.4.1 TNM

The TNM classification is used for staging UTUC [30–32]. Staging of the primary tumour depends on the depth of invasion. Stage pT3 is defined differently for tumours located in the renal pelvis, calyceal system and ureter. The stages are defined as follows:

Primary Tumour (T)

TX Primary tumour cannot be assessed

T0 No evidence of a primary tumour

Ta Papillary noninvasive carcinoma

Tis Carcinoma in situ

T1 Tumour invades the subepithelial connective tissue

T2 Tumour invades the muscularis

T3 Tumour invades beyond the muscularis into the peripelvic fat or the renal parenchyma (applies to the renal pelvis only)

T3 Tumour invades beyond the muscularis into the periureteric fat (applies to the ureter only)

T4 Tumour invades adjacent organs or through the kidney into the perinephric fat

Regional Lymph Nodes (N)[3]

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 but not more than 5 cm in greatest dimension; or multiple lymph nodes, none more than 5 cm in greatest dimension

N3 Metastasis in a lymph node, more than 5 cm in greatest dimension

Distant Metastasis (M)

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

An important difference between UCB and UTUC is the anatomy. The muscular layers of the ureter and renal pelvis are thinner than those of the bladder. The distal third part of the ureter has three muscular layers, whereas the two proximal thirds have only two. This allows the UC to reach a higher stage faster in the proximal ureter than in the distal ureter and the bladder. This may be a reason for the higher proportion of invasive renal pelvis and ureteral tumours compared to UCB at the time of diagnosis [28]. Non-invasive UC accounts for only 40% of newly diagnosed UTUCs compared with 80% of newly diagnosed UCB [33, 34]. Stage is the most important factor determining outcome for the patient. The pathology report should comment on the manner in which the tumour invades the stroma, since tumours with tentacular growth are more aggressive than those that exhibit broad-front or pushing margins [35]. However, which tumours will progress and develop into an advanced stage is not entirely understood. Factors that determine invasiveness and prognosis and how to correctly identify them preoperatively are still needed. Any amount of lymph node metastases is a poor prognostic

[3] According to Sobin and Wittekind, “the regional lymph nodes are the hilar, abdominal para aortic, and paracaval nodes and, for ureter, intrapelvic nodes. Laterality does not affect the N classification.” [32]

factor. Renal parenchymal invasion and high grade are negative prognostic factors [31]. UTUC spreads via the lymphatic system and bloodstream and by direct extension into adjacent organs.

1.4.2 Radiology

Until the 2010s, radiological methods in the work-up of UTUC included excretory urography and retrograde pyelography, and URS was rarely performed. Technical development has greatly improved, and since 2011, multidetector computed tomography urography (MCTU) has been recommended as the first choice in the European Association of Urology (EAU) guidelines [36]. MCTU provides more information than intravenous urography, where mainly contrast defects and obstruction could be detected. There is no single recommended MCTU protocol. During the native phase, calcifications are assessed. During the tissue enhancement phase, which can be either corticomedullar/arterial or venous, contrast enhancement of lesions is detected. UTUCs are well discriminated in this phase, where they, unlike most benign lesions, do show enhancement. During the excretory phase, UTUC can be seen as contrast defects, and obstruction can be assessed [37, 38]. Unfortunately, patients with severe renal impairment cannot undergo MCTU due to the risk of further renal deterioration from the use of intravenous contrast medium, and the sensitivity and specificity for the detection of pathology and for staging are still not sufficient. Possible false positive findings include benign filling defects (prominent renal papillae, sloughed renal papillae, urinary tract calculi, blood clots, suburothelial haemorrhage, polyurethritis cystica, tuberculosis, mycetomata), benign wall thickenings (inflammation, endometriosis), fibroepithelial polyps, crossing blood vessels, and kinks of the ureter. Renal cell carcinoma (RCC), collecting duct carcinoma and renal lymphoma can mimic UTUC and, although malignant, should not be treated with radical nephroureterectomy (RNU) [38, 39].

The performance of MCTU can be assessed on its ability to correctly detect the presence of UC and its ability to stage UTUC. The performance of MCTU in the detection of UTUC (sensitivity) is generally very good, but the reported rates are influenced by the inclusion criteria of the studies. Retrospective studies including only patients who were eventually diagnosed with UTUC will report higher sensitivity than studies including patients based on symptoms that could be due to UTUC. Comparison of measures of performance across studies is difficult, as definitions, classification and calculations of ambiguous findings vary. In a meta-analysis, the pooled sensitivity of six studies was 92% (95% confidence interval CI: 85-96%), ranging from 66 to 100%, and the specificity was 95% (95% CI: 88-98), ranging from 88 to 99% [40]. Another study of 174 renal units, including patients in Papers I and II of this thesis, reported lower specificity: 51%. The discrepancy could be due to different classifications of findings that could not be dismissed [37]. Although the sensitivity and specificity of MCTU for the detection of UTUC are high, the ability to determine the precise stage is not sufficient.

Most studies on radiological staging draw the line between T2 and T3 [41, 42]. However, a clinically crucial line is the discrimination of Ta/T1 from T2, as this can change the choice of

treatment modality. A study of change in enhancement in the tumour using a split bolus protocol did not find a difference in enhancement across stages [43]. Importantly, radiology does not provide a histopathological diagnosis.

1.4.3 Histology and cytology - Grading classifications

1.4.3.1 Collection of specimens

Specimens for definitive diagnosis of UTUC can be assessed by cytological or histopathological methods. Cytology is assessed in specimens collected by voided urine, drip urine, or in situ barbotages (saline washings). Histopathology is assessed in biopsies (obtained via retrograde URS or the percutaneous route) and in RNU specimens. Visual assessment of a lesion seen at URS is not reliable for diagnosis: in one study, 30% of the malignant lesions were visually assessed as benign [44]. In a prospective study, it was reported that few cases of carcinoma in situ (CIS) could be identified solely by visual assessment by URS [37]. The performance of the tests and a discussion of the classification of UC are described in the following section.

1.4.3.2 Urine cytology

As cytology specimens are fixed and dyed cells obtained from fluid (urine or barbotage), they cannot be evaluated on the same criteria as those for a histopathology specimen, in which the cells maintain their orientation and location. In cytology specimens, the cells are evaluated as single entities as well as how they organise in clusters. Also, the context with other cells, including white blood cells, is important. Therefore, the morphological features of the urothelial cell are critical. The degree of nuclear anaplasia is central. Cytological assessment of cells in urine is done either by analysing voided urine or by selective urine sampling where urine is collected from the upper urinary system on a selected side by placing a ureteral catheter to collect drops of urine. However, urine cytology has poor performance for detecting UTUC. Voided and selective urine cytology have a low sensitivity, 17-70%, and negative predictive value, 68%, but a high specificity and positive predictive value [45–49]. Cells with more malignant transformation are less dehiscent and more easily sloughed off the urothelium, both spontaneously and when a saline washing is instilled. Even so, cytological analysis is useful when the urologist understands the limitations of the analysis. The main aim of analysing urine cytology is to detect or negate high-grade UC. The terminology has been clarified in recent publications [50, 51] which also aim to standardise the reporting of the findings. This Paris System for Reporting Urinary Cytology states that the urologist should clearly state what/how and where the specimen was obtained: voided urine or in situ barbotage from the renal pelvis, ureter or bladder, in addition to the patient's history. This influences the evaluation. The cytology report should comment on the following aspects: adequacy of the specimen: (whether the quality is satisfactory for evaluation, general categorisation: presence/absence of epithelial cell abnormality), a descriptive diagnosis, other (any molecular findings). The descriptive diagnosis includes statements on negativity for epithelial cell abnormality, signs of infection (bacterial, fungal, viral); chronic, acute or nonspecific inflammation; cellular changes associated

with chemotherapy or radiation; and epithelial cell abnormalities (atypical urothelial cells, low-grade UC, high-grade UC, squamous cell carcinoma, adenocarcinoma, other specified malignant neoplasms). In a cytological specimen, low-grade carcinoma is scarcely identified and can end up in the category "atypical urothelial cells". Further clarification should be made when possible into "atypical urothelial cells of undetermined significance" or "atypical urothelial cells, cannot rule out high-grade carcinoma" or "atypical urothelial cells, favour neoplasm" [50]. This changes how the patient is further investigated and followed-up and is a potential source of misunderstanding between urologists and pathologists.

1.4.3.3 Barbotage

Cytology can also be performed on barbotage taken in situ in the renal pelvis or bladder. The sample from the bladder is collected after washing out local anaesthesia and any residual urine. The sample from the renal pelvis is collected by slowly instilling saline through the working channel of the ureteroscope and then collected via the same route before contrast medium is instilled. Supposedly, this yields a greater number of urothelial cells in the sample than does voided urine. Barbotage could provide a higher diagnostic yield than voided urine cytology [52, 53]. Urothelial cells collected in this manner are also thought to be more easily assessed cytologically than those exposed to urine for a prolonged period. Both urine cytology and barbotage are stained with the Papanicolaou technique [54]. Theoretically, barbotage cytology could be more representative of the entire tumour than a biopsy, which is important in heterogeneous tumours.

In a study where voided urine and selective cytology were obtained in 116 and 126 patients, respectively, 10 extra malignancies were identified by selective ureteroscopic washings. Selective cytology predicted UTUC with a sensitivity and specificity of 76 and 73%, respectively. Voided urine cytology predicted G2 and G3 in RNU specimens at 43 and 80% sensitivities, respectively, whereas corresponding sensitivities for selective cytology were 65 and 96% [55].

1.4.3.4 Biopsy

Diagnosis based on histopathology of endoscopic biopsies is difficult [56]. The size of the working channel of most flexible ureteroscopes is usually 3.6 Ch [57], which limits both the size of biopsy devices and the technique of securing biopsies. Direct staging of UTUC is not possible as biopsies are small and cannot be taken at depth, as that would cause perforation and risk of tumour seeding [48, 58–60]. Subepithelial connective tissue was found to be present in 59% of biopsies [61]. Thus, determination of invasion of the lamina propria in biopsies is not reliable, and biopsies can mostly be used to assess grade [61–63].

Smaller samples (<2 mm) are less likely to be correctly assessed [63, 64], but even small volume biopsy specimens can, if handled correctly, provide a histopathologic diagnosis. In most cases, biopsies can diagnose and correctly grade UTUC. The results are dependent on the

technique in the operating theatre and correct handling of the specimen in transportation to the pathology laboratory and knowledge of the small volume and delicate nature of UC among staff in the pathology department [57, 61]. Studies [57, 65] comparing different biopsy devices have conflicting results (with a grade concordance of 63-100% between biopsy and RNU specimens), and although the specimen size and quality of specimens in terms of crush artefacts differed in a study [57], the concordance of grade with grade in RNU did not differ.

Higher diagnostic yields can be achieved when combining barbotage cytology and biopsy histology, e.g., by using the liquid that the biopsy was transported in for cytology [47, 66, 67]. Ureteral washings and biopsies both had a sensitivity of 70-80% and seemed to be complementary, with a combined sensitivity of 100% in a study of 39 patients. Biopsy was especially useful in low-grade and papillary urothelial neoplasm of low malignant potential (PUNLMP) cases where cytology is difficult [47]. Although both biopsy and barbotage have high diagnostic yields, there is concern about both under- and overgrading as well as under- and overstaging [68]. A recent large nationwide Dutch study confirmed that the diagnostic yield for grading is high, but staging is more difficult and less reliable [69]. Some researchers have reported high numbers of incorrect grading of endoscopically taken samples [70, 71], possibly because of tumour heterogeneity. Higher yields have been reported in newer studies, possibly reflecting the use of better instruments and more experience with the method [72–74].

As biopsies provide scant material, grading is prioritised. However, if sufficient material is obtained, further analyses are feasible. Bagrodia et al. tested the feasibility of using URS biopsies for genomic characterisation of UTUC, performing next-generation sequencing (NGS) on URS biopsies, and showed a high level of concordance regarding the presence and prevalence of genomic alterations between biopsy and the subsequent RNU specimen, irrespective of where in the (sometimes heterogeneous) tumour the biopsy was taken [75]. Koyama et al. have shown that immunohistochemical investigation of Ki-67 expression can be performed on URS biopsies. They found a statistically significant correlation between Ki-67 expression in ureteroscopic biopsies and high tumour grade, concomitant CIS, and stromal invasion in RNU specimens [76].

1.4.3.5 Classifications

The classification of UC used for UTUC is the same as that for UC located in the bladder and urethra. Almost all studies on the classification of UC have been performed on UCB. Assessment of tumour invasion is more complex in the urothelium than is tumour staging in other organs, as the basement membrane is less distinct and benign conditions, e.g., von Brunn´s nests can mimic invasion. Invasion is important for the definition of cancer in other organs, but a large proportion of UCs are papillary and not invasive; thus, anaplasia plays a relatively greater role for classification. Compared to cancer in other organs, anaplasia or the appearance of the cells of the urothelium is relatively more important for the diagnostics than the relation of the tumour to the lamina propria or basement membrane, as the lamina propria is

often not included in biopsies and the basement membrane is not distinct in the urinary collecting system. This was stressed in the first widespread classification system of UC [77]. Notably, regenerative and inflammatory reactions can give rise to cellular changes resembling malignancy. The first international, systematic, widespread classification and terminology of transitional cell carcinoma was defined in 1973 through an international collaboration of pathologists and urologists supported by the WHO [77]. The term transitional cell carcinoma was later changed to urothelial cell carcinoma in 1998 (ISUP meeting). The grades are described as follows: "grade 1 applies to the tumours that have the least degree of anaplasia compatible with a diagnosis of malignancy; grade 3 applies to tumours with the most severe degrees of cellular anaplasia; and grade 2 lies in between." Seemingly simple, the limitations of the WHO 1973 classification are the lack of unambiguous criteria for cut-offs between these categories. One result of this ambiguity is that category grade 2 has seemingly been used too extensively, particularly in cases of uncertainty.

This classification was updated in 1999, with reappropriation of grade 1, grade 2 and grade 3 but with different criteria compared to 1973. [35]. As most tumours in the WHO 1973 grade 1 group had been observed not to progress, there was a need to sort out those tumours, as they do not merit the label carcinoma and should not be treated as such. The new term for these was PUNLMP. In 1998, the International Society of Urological Pathology, ISUP, made changes to the terminology and refined and modified criteria. Transitional cell carcinoma was from that time on referred to as urothelial carcinoma. Flat intraepithelial lesions were now categorised as either dysplasia or CIS. Papillary tumours are now categorised into four categories: papilloma, PUNLMP, low-grade and high-grade [51]. In 2004, the WHO formally adopted the 1998 ISUP system ("WHO 2004/ISUP system") [78]. One aim was to eliminate the ambiguity and diffuse cut-offs between WHO 1973 grades 1-2 and WHO 1973 grades 2-3. Additionally, introduction of the category "high-grade UC" was meant to clearly identify patients at increased risk of invasive stage or in need of adjuvant instillations (papillary high grade and CIS). In a 2012 update the 2nd International Consultation on Bladder Cancer [51], clarified that neoplasms with grade heterogeneity should be assigned the highest-grade present in the lesion. Additionally, the consensus meeting stated that "generally, invasive UC should be graded as high-grade, irrespective of depth of invasion". Another update was published in 2016, which introduced "urothelial dysplasia" [79]. Urothelial dysplasia is difficult to define, has large interobserver variability, and the risk of progression to CIS is unclear.

The classification of cancer aims to confer information on its prognosis, both in terms of risk of recurrence and risk of death. In UTUC, it would be useful if grading could suffice or act as an indirect marker of stage, and therefore, the correlation of stage and grade is important to elucidate. Grades should be clearly defined and reproducible. Despite decades of attempts to refine the classification of UC, some problems remain. The greatest problem is still the large interobserver variability [79–81]. The WHO 1999 update was introduced in a third of European pathology departments before the lack of reproducibility led to an upswing for the WHO

2004/ISUP system. However, the WHO 1973 and thus presumably also the WHO 1999 classification has been shown to better predict prognosis than does the WHO 2004/ISUP system [82]. The classification systems are a work in progress, and development is ongoing.

Grading is to an extent subjective, much more so than flow cytometry or genetic analysis. Recently, the EAU Non–muscle-invasive Bladder Cancer Guidelines Panel Multicentre Study recommended combining the WHO 1999 and 2004 classifications in a four-tiered system, dividing grade 2 UC into low-grade G2 and high-grade G2 as this better correlates with survival [83].

1.4.4 Ureterorenoscopy

1.4.4.1 Indications

To minimise the time to definitive surgery, patients who are obviously not suitable for kidney-sparing treatment and undoubtedly have UTUC should not be examined with URS. On the other hand, all patients in whom the findings of URS could impact treatment should be offered this investigation. URS enables further characterisation of the tumour. Visual inspection of the tumour determining location, uni- or multifocality, tumour architecture, surface diameter, and importantly, sampling of the tumour for cytological, histological and genetic analysis can all be achieved. The aims are to confirm or reject suspected UTUC, to further characterise the lesion to decide on the treatment modality (kidney-sparing surgery [KSS] or RNU) for that specific patient and to treat the carcinoma in the same session if that is the chosen option [84, 85]. URS is generally performed under general anaesthesia but is feasible under regional anaesthesia. It has been done for decades, but its technical development means that the instruments used have improved much and are still improving [86]. Thin, flexible, durable endoscopes are available. Even so, sometimes the upper urinary tract is not accessible due to stricture or poor vision, often due to bleeding. Untreated urinary infection is a contraindication.

An advantage of performing URS is that cells for cytological assessment can be collected by in situ barbotage, collecting the sample via the working channel of the ureteroscope. Combining radiology, diagnostic URS and cytology gives a more accurate diagnosis and is needed if accidental extirpation of benign kidneys is to be entirely avoided. This has been shown by Favaretto et al. in a study of 324 consecutive patients [87]. Additionally, a study of 58 patients who underwent RNU due to UTUC with different preoperative imaging (36/58 CT) found that 12.8% (5/39) of patients without preoperative histopathology did not have UTUC in the final RNU specimen [88]. Another study of a consecutive series of 113 patients with UTUC, diagnosed by different imaging methods (mainly CT and cytology) and treated between 1996 and 2011, found that 6/113 (5.6%) did not have cancer in the final RNU histopathology. The CT sensitivity was 83.1%. URS was performed in 56 patients, with a sensitivity of 83.9%. Urine cytology had a sensitivity of 60.9% [89].

The EAU guidelines [6] recommend URS with biopsy in cases where the additional information could impact treatment. URS before RNU may improve the preoperative assessment of tumour stage, which should be considered when choosing a surgical approach, with the open technique being recommended for RNU of >pT3. The prediction of advanced stages (>pT2) has been shown to improve from 66% for URS alone to 71% when combining URS and radiology (CT or MRI) [87]. Moreover, URS with biopsy can rule out UTUC in rare cases of metastases to the upper urinary tract or in cases of a collecting duct carcinoma that can be difficult to assess with CT. Centres that routinely perform URS with biopsies when assessing UTUC patients have shown that URS results in significant changes in treatment decisions [90, 91], a reduced rate of misdiagnosis and rate of RNU [84]. The number needed to investigate with URS to avoid one misdiagnosis or one RNU was 5 but is probably not comparable over time, as radiology techniques have evolved greatly in the last two decades that have passed since the investigation of the first patients in that study. A study [49] of patients undergoing URS after urine cytology and radiology (CT or IVU) concluded that URS was important when the voided urine cytology was negative. (See more under Section 1.5.2 RNU).

1.4.4.2 Adverse effects of diagnostic URS: Infection, seeding and risk of intravesical recurrence (IVR)

Adverse effects are likely underreported in the literature but are reported to occur in 3.5-25% of endoscopic upper tract *stone* operations [92]. The most commonly described adverse effects after URS for stone disease are fever (2-28%) and sepsis (3-5%) [93–95]. Other severe complications include ureteral avulsion, strictures, kidney damage, fistulas and severe bleeding requiring transfusion. Mortality is very rare but has been reported [55, 96]. High intrarenal pressure during URS and prolonged URS are well-established risk factors for adverse effects, including bleeding, perforation, infectious complications, stricture, renal impairment and possibly tumour seeding [97–99]. Intrarenal pressure is normally 0-15 mmHg and rises to 60-300 mmHg during URS. This is much higher than the 20-50 mmHg threshold for pyelotubular and pyeloveneous backflow [100, 101].

The risk of tumour seeding due to increased intrarenal pressure during URS is a concern, but little confirmation of this hypothesis exists in the literature. A couple of case reports have been published [102–104]. Ureteral access sheaths have been demonstrated to aid in keeping the pressure down during URS [97, 105] and reducing postoperative complications [106], but in addition to risking hiding small ureteral UTUCs, they can injure the urothelium [107] and thus possibly increase the risk of tumour implantation. Although the sheath hinders contact of cancer cells with the ureter during laser ablation of UTUC located in the renal pelvis, an injured epithelium could be more susceptible to direct tumour implantation of any residual UTUC after URS.

Several studies have reported an increased risk of intravesical recurrence (IVR) after diagnostic URS carried out before RNU. After RNU, IVR is expected in 27-41% of patients [108–110]. In

most studies comparing IVR and pre-RNU, URS patients were not stratified by the method of RNU, i.e., open and laparoscopic operations were mixed or not even specified. In a meta-analysis by Guo et al [111], the risk of IVR was increased in patients undergoing diagnostic URS before RNU (HR 1.51, 95% CI: 1.29–1.77; P < 0.001). However, when comparing other oncologic outcomes, overall survival (OS), cancer-specific survival (CSS), metastasis-free survival (MFS), and recurrence-free survival (RFS) in patients receiving pre-RNU URS with those that did not in the included studies, no difference in OS [112, 113], MFS [112–115], or RFS [115, 116] was found. Interestingly, CSS was higher in patients undergoing URS before RNU [113–115, 117], and the groups differed regarding tumour stage but not grade. A more recent meta-analysis confirmed that MFS, CSS and OS were not affected by conducting a pre-RNU URS [118]. This indicates that URS is indeed important for pre-RNU diagnostic accuracy, enabling the selection of patients for KSS or RNU and the identification of low-risk patients. Further studies [108, 119, 120] confirmed that IVR did not affect oncological outcomes and revealed that IVR was not increased after pre-RNU URS.

Time between URS and RNU affected the IVR in one study [121], showing greater risk when URS was performed in a separate session within one week prior to RNU compared to the performance of URS on the same day as RNU or no performance of URS at all. Two recent systematic reviews and meta-analyses [118, 122] found an increased risk of IVR only in patients who had an endoscopic biopsy taken. The rate of recurrence does not in itself seem to affect survival [123, 124]. Causal inference is difficult as patients are never randomised to KSS or RNU. Adjusting for all differences affecting recurrence risk in the group that is thoroughly examined with URS with in situ barbotage and endoscopic biopsies and the group that goes straight to RNU after MCTU is difficult.

Other methods of refining diagnostics include narrow-band imaging [125] as well as optical coherence tomography [126] and confocal laser endomicroscopy [127, 128], which are under investigation. However, an international multi-institutional evaluation of the oncological impact of different image enhancement technologies, including fibre-optic and digital ureteroscopy, narrow-band imaging (NBI) and Image1-S, did not find any advantage of one technology over the other [129].

1.4.5 Genetics

UC is among the cancers that exhibit the most mutations, but the presence of mutations does not in itself mean that all these mutational changes are necessary for the development of cancer. The genetic changes that seem to be of importance are associated with cell proliferation and cell death. Methods of detecting mutations are rapidly improving and becoming more available in the clinical setting. New oncological treatments are under development or have recently become available, and their effectiveness depends on the type of mutation present.

Two main molecular pathways have been known to be linked to low-risk and high-risk UC. They are to a large extent mutually exclusive and correlate with the WHO 2004 division of UC into low and high grades [50]. The *FGFR3* pathway has been described as the hyperplasia pathway. These genetically stable tumours recur but are not prone to progression and metastasis and have a good prognosis. However, more recent studies on UTUC have shown that *FGFR3* mutations are also present in high-grade UTUC [14], even to the same extent in UCB and UTUC [130]. Invasive UC exhibiting *FGFR3* mutations yields better survival than invasive UC with wild-type *FGFR3* [130]. *FGFR3*-targeted immunotherapy is available and developing; one example is infigratinib, which has yielded a higher response in metastatic UTUC than in UCB patients [131]. The *TP53*-linked pathway is the dysplasia pathway. Approximately 30-60% of UTUCs exhibit altered *TP53*, a cell cycle marker. These genetically unstable tumours often progress from dysplasia to invasive papillary high-grade or to CIS, with shorter survival. A third pathway of importance for UTUC is germline mutations in MMR. MSI-high tumours associated with Lynch syndrome are thought to benefit from immunotherapy in the form of immune checkpoint inhibitors [14]. Additionally, the KSS might be most appropriate in patients with UTUC displaying MSI, as MSI-high is correlated with higher survival [22].

1.4.5.1 Clonogenic theory, field change theory and similarities and differences between UCB and UTUC

As UCB is much more common than UTUC, most studies on UC regarding grading, prognosis and genetics have been performed using UCB specimens and patients. It is important to elucidate whether the results from studies of UCB can be extrapolated to UTUC, as it has implications on the usefulness of prognostic information and effectiveness of immunotherapy and chemotherapy. Differences in the molecular profile of UCB and UTUC can to some extent be explained by differences in the incidence of stages and grades [14]. The differences in the prevalence of mutations between UCB and UTUC with a higher frequency of *FGFR3* and a lower frequency of *TP53* mutations in UTUC were also significant after adjusting for stage and grade in one of the largest studies comparing the genetic profiles of UCB and UTUC [132].

Whether UTUC and UCB are the same disease in different locations or are more diverse and whether UC spreads and recurs from the same clone (clonogenic theory with intraluminal seeding and implantation) or arises from several primary tumours in a genetically unstable mucous membrane (field change theory) are debated [22, 58, 133, 134]. The clonogenic theory explains multifocality and recurrences with migration and grafting of one tumour clone by intraluminal seeding and implantation or by intraepithelial migration of cancer cells [22].

Some studies indicate clonal relatedness between UTUC and UCB: 29 patients with UTUC who subsequently developed UCB were analysed with NGS, and the tumours in the upper tract and bladder of these patients were found to always be clonally related [132]. However, in a larger sample, the researchers did find significant differences in the prevalence of common genomic alterations between UTUC and UCB, with a higher mutational burden and higher frequency of

microsatellite instability in UTUC [132]. In a study comparing specimens with invasive UCB, specimens from patients with benign bladder conditions, normal kidney specimens and UTUC in the renal pelvis, Zhang et al. found “extremely” similar gene expression in UC from the upper and lower urinary tract [135]. In a recent study of IVR after RNU for UTUC [136] found evidence of clonal relation in 73% of UCB arising after RNU for UTUC using targeted DNA sequencing of a panel of 41 genes.

The field change theory explains multifocality and recurrences in distinctly different locations in the urinary tract as caused by development of several tumour clones, owing to the entire urothelium coming into contact with mutagenic agents in the urine. In support of this theory, multifocal UC has been shown to have a unique clonal origin in many cases, as demonstrated by loss of heterozygosity, LOH and X-chromosome inactivation assays arising from independently transformed progenitor cells [137]. Different types of genetic instability (prevalence of specific oncogenic driver molecular alterations) have been found to dominate UTUC and UCB, indicating that UTUC and UCB are distinct entities, e.g., MSI (due to deficient MMR) and DNA promoter hypermethylation are more common in UTUC than in UCB, and elevated microsatellite alterations at select tetranucleotides (EMAST) are more frequent in UCB [138–141]. MSI is much more common in UTUC (15% of sporadic cases) than UCB (3%) [15].

The matter of clonal relatedness, field cancerization and the role of intraluminal seeding for IVR is still unsettled. In a systematic review [142] of 118 tumours (55 UTUCs and 63 IVRs) from primary UTUC and metachronous or synchronous IVR, 94% of IVRs were considered to be clonally related. Although the authors consider seeding to be the most important mechanism for IVR (i.e., clonal relationship between synchronous and metachronous UC in the upper tract and bladder), they state that field cancerization can also contribute to the development of separate tumours in both the upper and lower urinary tract. The two studies using NGS indicate clonal relatedness [132, 143], whereas the one using whole genome sequencing did not [142]. The methods used have evolved, and the older studies (microsatellite technology) are not as reliable as the newer ones, which use NGS and whole genome sequencing. The evolving knowledge about tumour heterogeneity and tumour evolution make comparisons of studies difficult [142].

1.4.6 Proliferation: S-phase and Ki-67 IHC

Proliferation is increased in cancer, and methods of detecting and quantifying proliferation are useful for assessing prognosis in some cancer types, e.g., ovarian and breast cancer [149]. Ploidy and SPF are routinely used for some malignancies, but the number of studies on UC is small, and the method has not been widely implemented. Proliferation can be investigated using DNA histograms, which are a static picture of the proportion of cells in the different parts of the cell cycle. Flow cytometry (FCM) enables the investigation of a high number of cells in a very short time: 30000 cells are investigated in one minute in a standardised and objective manner; thus, FCM could be a complement to histopathology, which is still struggling with intra- and

interobserver variability. However, cells of all types in the sample are included in the output; therefore, this method can only be used as an adjunct to the diagnostic morphologic histopathology assessment [144]. By measuring the proportion of cells in S-phase (S-phase fraction, SPF) in addition to ploidy, a functional property of the tumour is investigated, one which is closely linked to malignancy. In UCB, ploidy has been shown to be linked to tumour grade and the SPF to stage [144–147]. FCM of paraffin-embedded tumours was described in 1983 by Hedley [148]. Validation of DNA histograms analysed in paraffin-embedded specimens compared to fresh samples of the same tumour has shown that the method also works well for the determination of ploidy in archival paraffin-embedded specimens. Measurement of SPF was, however, not successful in some attempts in the 1980s [146, 149]. Improvement of the method has made both the assessment of small amounts of tumour material and the evaluation of SPF possible [150]. Some, but not all, studies of ploidy and SPF in UC using FCM indicate that ploidy and/or SPF is an independent prognostic factor for progression, treatment response and survival [151–157].

A study on paraffin-embedded UCB specimens found no difference in survival between diploid and aneuploid UCB in a total of 77 patients in the era before neoadjuvant chemotherapy. Tumour grade and DNA ploidy were clearly associated, and diploid tumours were grade I in 2/3 of cases, whereas aneuploid tumours were grade II or III in 2/3 of cases. The SPF ranged from 1-35%, with a median of 6.6%. No significant association was found between grade and SPF divided into > the median value or < the median value. The correlation between SPF and grade was not significant [146]. Another study confirmed that paraffin-embedded specimens stored for up to 15 years provided good quality for the measurement of ploidy but found no further prognostic information from ploidy that was not obtained from histology grades. SPF was not studied [147]. In a study of 66 UCB patients who underwent radical cystectomy without any additional treatment, ploidy was an independent prognostic factor. SPF was not analysed [158]. Ploidy and SPF were studied in 24 pT3-4 N0 M0 UCB patients treated with neoadjuvant chemotherapy and radical cystectomy. Ploidy did not correlate with response to chemotherapy or to OS or CSS, but high SPF correlated with both response to chemotherapy and increased OS and CSS [159].

FCM has to a very limited extent been studied in UTUC. Blute et al. analysed 109 UTUC specimens in archival specimens collected from 1960–1975 and found that ploidy added prognostic information in low-stage, low-grade UTUC. SPF was not analysed [160]. Oldbring et al. performed FCM on biopsies from primary tumours in the renal pelvis and ureter and on biopsies from macroscopically normal mucosa operated with RNU in 11 patients. In line with studies of UCB, aneuploidy was detected in all grade 3 and half of grade 2 UTUCs. SPF was also analysed, and high SPF (mean 17.3%) was found in aneuploid grade 3 UTUCs, but low values (4.6%) were found in aneuploid grade 2 UTUCs [161].

Another method of measuring cell proliferation that has been studied in UTUC is the detection of the nuclear protein Ki-67 by immunohistochemistry. This protein is especially present in

malignant tumours, but its prognostic value regarding survival outcomes is conflicting. Overexpression of Ki-67 was significantly associated with worse CSS and OS but not with IVR-free survival in a meta-analysis [162]. However, it is still not settled whether Ki-67 is an independent prognostic marker, and the included studies used different cut-offs. Assessment of Ki-67 IHC is difficult, and despite having incorporated the analysis in routine analysis of UTUC for some years in our pathology department, the value remains unclear.

Analysis of FISH (UroVysion, designed to detect aneuploidy for chromosomes 3, 7, 17 and loss of the 9p21 locus) in focal barbotage does not improve diagnostic accuracy. A study comparing cytology and cytology with the addition of FISH found that cytology alone had low sensitivity (38%) but high specificity (89%) for the detection of UTUC. FISH increased the sensitivity but decreased the specificity [163], but similar to cytology, the method depends on the presence of UTUC cells in the barbotage.

1.5 TREATMENT

Organ-confined, nonmetastatic UTUC can be treated with KSS or RNU. RNU has curative intent in this setting, and KSS can have curative intent in an elective setting or be performed for imperative or palliative indications. KSS includes antegrade and retrograde endoscopic techniques and open or laparoscopic segmental ureterectomy.

1.5.1 Kidney-sparing treatment of UTUC, KSS

The indication for KSS has changed from being deemed palliative or curative in imperative cases to being recommended to be considered a curative treatment and discussed with all patients with low-risk UTUC since the 2017 update of the EAU guidelines [164] Previously, KSS was recommended only in highly selected cases in the 2011 update [36] and in low-risk and in imperative cases from 2015 [165]. The cut-off in size for KSS versus RNU was changed from 1 cm to 2 cm in the 2017 update [164]. In elective cases, KSS has curative intent, and the patients have small, low-grade, low-stage, preferably solitary tumours, with few risk factors for progression. Data from Holmäng et al. indicate that only 5-10% of all UTUCs fulfil these criteria [3]. Imperative indications are either curative, often due to severe renal impairment or UTUC in a solitary kidney, or palliative, in patients with high-grade, multifocal UTUC in patients with severe comorbidities that preclude RNU. These comorbidities include severe pulmonary or cardiovascular disease and old age. RNU can sometimes be delayed, i.e., patients can start off with KSS and proceed to RNU if there is a progression in the grade or estimated stage, or if too many recurrences or other difficulties occur during follow-up, including insufficient compliance. By postponing the loss of renal function, the increased risk for cardiovascular disease will also be postponed [166]. It is important not to miss the window of opportunity to switch to RNU if progression occurs before the cancer becomes locally advanced or metastatic. This time frame is difficult to determine from studies, as it is inconsistently reported. Single-centre studies have reported a “risk of grade migration of 4-19% and [a] risk of stage migration of 8-14%” [33]. Renal unit survival is a relevant measure in the context of

delaying RNU to save renal and cardiovascular function. Recurrence rates and renal unit survival are further discussed in Section 1.6.3 Survival.

KSS may be performed by a retrograde or antegrade approach, depending on the tumour location and size. Retrograde ureteroscopic tumour ablation is less invasive, with a smaller risk of tumour seeding. If the tumour is large or hard to reach, e.g., located in a lower pole calyx with a narrow infundibulum or the patient has a urinary diversion, an antegrade percutaneous approach may be favourable. The incidence of tumour seeding within the nephrostomy tract is extremely rare [102], 0.75% (1/133) at an experienced centre [167]. Segmental ureterectomy can also be performed. There are no randomised controlled trials (RCTs) comparing retrograde URS or percutaneous KSS of UTUC with RNU. A systematic review of retrospective nonrandomised studies indicates that patients with low-grade, non-invasive UTUC fare equally well after KSS as after RNU. The indication for segmental ureterectomy seems to be safe to extend somewhat to include selected patients with high-grade, invasive UTUC [168]. KSS has become more common in recent decades as ureteroscopes have improved with smaller calibre instruments and better optics [33, 169, 170]. The laser technique has also improved.

Different energy sources have been used for endoscopic surgery: electrocautery and different types of lasers, primarily holmium: yttrium-aluminium-garnet (Ho:YAG), neodymium: yttrium-aluminium-garnet (Nd:YAG), thulium: yttrium-aluminium-garnet (Tm:YAG) and combinations thereof [171–175]. Electrocautery has a higher risk of stricture formation, and its use in the ureter is dangerous owing to the variable depth of penetration. With all energy sources, caution is needed, especially when used in the ureter.

1.5.2 Radical nephroureterectomy, RNU

The gold standard treatment of high-risk organ-confined UTUC is RNU. This procedure can be performed by open or laparoscopic surgery. Laparoscopic surgery can be performed with conventional techniques or robot assistance. RNU implicates removal of the entire kidney, ureter and ureteral orifice en bloc along with a part of the urinary bladder, the “bladder cuff”. When performed correctly, RNU has reasonably good oncological results. In other respects, it is a large procedure. There is a risk of significant blood loss, tumour seeding if the urinary collecting system is accidentally opened, nerve palsy, the risks associated with general anaesthesia in this elderly patient group, and general postoperative complications (pain, hernia, ileus, thrombosis, pulmonary embolism, fascial dehiscence, haematoma, acute renal failure, and mortality) [176, 177]. An advantage is that lymphadenectomy can be performed; thus, a more detailed staging is obtained. The curative effect of lymphadenectomy is debated, and a more detailed discussion of the role of lymphadenectomy is beyond the scope of this thesis.

In recent decades, conventional laparoscopic and robot-assisted laparoscopic operations have become more readily available, with many advantages for both patients and surgeons. It is less invasive in the sense that the incisions into the skin, muscles and fascia are smaller, blood loss is

generally smaller, and postoperative recovery is faster with less pain. Moreover, ergonomics for the surgeon is better [178, 179]. However, there is a concern that compared with open surgery, laparoscopic surgery for UTUC could increase the risk of IVR compared with open surgery and lead to metastases in the trocar sites due to tumour seeding promoted by the high intraabdominal pressure necessary to maintain vision and manoeuvrability during the procedure. The scarcity of RCTs in this patient population makes it difficult to assess differences between interventions and techniques. Studies comparing robot-assisted and conventional laparoscopy for UTUC are missing. Studies comparing oncological outcomes for open and laparoscopic RNU suggest that more advanced UTUC (>pT3) should be operated on with open techniques [6, 179, 180]. However, in a patient population with advanced age, it is often a dilemma to choose between the more minimally invasive technique and a technique that is perhaps slightly superior in oncological terms. Furthermore, preoperative discrimination between pT2 and pT3 is not an easy task.

Disadvantages of RNU obviously include loss of nephrons, which is important both because of the risk of developing contralateral UTUC (3%) and because the patient population at risk for UTUC is also at risk for renal impairment due to advanced age, high prevalence of cardiovascular disease and smoking. The median decrease in renal function after RNU was 13 ml/min/1.73 m2 in a study [181]. Renal impairment independently increases the risk of cardiovascular disease and death [182–184]. A retrospective study [185] that described a worse prognosis in patients with lower preoperative renal function found that the prognosis was worse when the estimated glomerular filtration rate (eGFR) did not decline much postoperatively. Renal function after RNU, which entirely reflects the contralateral kidney, was not associated with survival rate, although many patients with locally advanced disease (pT3–4 and/or pN1–2) had reduced renal function at diagnosis and even more so after RNU. Patients with locally advanced disease had a significantly smaller decline in eGFR than those with organ-confined disease, ≤pT2: the median decline in eGFR was 5.7 ml/min/1.73 m^2 (IQR 0–16.7) compared to median 17.9 ml/min/1.73 m^2 (IQR 5.8–26.5, $P < 0.0001$). Annual mortality is high in the population with renal insufficiency: seven times that of the general population [33]. A study of patients with small (T1a–T1b N0 M0) RCC and normal preoperative kidney function demonstrated that KSS resulted in approximately half the risk of developing cardiovascular events relative to radical surgery after adjustment for comorbidities and preoperative cardiovascular risk [166]. The risk of end-stage renal disease could be even greater after RNU for UTUC than after nephrectomy for RCC. After adjustment for age, preoperative renal function, diabetes and cardiovascular disease, the risk of doubling of creatinine or end-stage renal disease two years after surgery was higher (HR 2.90) after RNU than after nephrectomy [186].

The risk of IVR after RNU, occurring in 22-47% of patients [187], may be affected by the method of surgery, although the results are diverging. Additionally, no matter what surgical technique is performed, adherence to oncological principles, including meticulous bladder cuff

excision, is important [188]. The risk of IVR has been reported to be reduced by postoperative intravesical instillation of mitomycin C [189]. The meta-analysis [190] underlying the recommendations of the EAU guidelines [6] found that the laparoscopic approach was an independent predictor for IVR. Other meta-analyses have come to other conclusions regarding IVR and surgical techniques [178, 191, 192]. See also Section 1.4.4.2 above, where the impact of pre-RNU URS on IVR is discussed.

1.5.3 Time delay to definitive surgery

Time delay to definitive treatment most likely affects survival in most malignancies, including UTUC, which can be relatively fast growing. There are a few retrospective analyses of the impact of time to RNU, but the effect of time is not great and may even be contradictory. It is likely that there is a selection in clinical practice of more advanced patients who have quick access to RNU, thereby obscuring the effect of time delay. A study reported worse OS when the time from diagnosis to RNU exceeded 120 days (HR: 1.61), with no difference at shorter time points [193]. Three studies [115, 194, 195] found no significant difference in oncological outcomes (RFS, MFS, 3- and 5-year cancer-specific mortality [CSM], and 5-year CSS); delay was observed in one of these studies due to administration of neoadjuvant chemotherapy in 50% of cases [195] and initial KSS [195] and URS prior to RNU [115]. A theoretical optimal cut-off was calculated to be 30.5 days in a study [196], where CSS and local/distant RFS were not significantly different between the groups receiving RNU within or later than 30.5 days. However, RFS was higher in patients with renal pelvic UTUC undergoing RNU at the later time point, reasonably indicating a selection of more advanced tumours for early surgery. Even though the time to definitive treatment should be kept short and the exact safe interval cannot be determined, the data suggest that it is safe to perform a thorough work-up, including URS, preoperatively. The timing of chemotherapy (neoadjuvant or adjuvant) does affect the schedule. The EAU guidelines recommend that RNU be carried out within 12 weeks “once a decision regarding RNU has been made”. This is further discussed in Section 1.6.3 Survival.

1.5.4 Instillations, systemic chemotherapy and immunotherapy

A meta-analysis of nonrandomised observational case series, including a total of 438 patients with CIS or laser-ablated pTa/T1 in the upper tract who received endocavitary BCG or mitomycin instillation, analysed the rates of cytology response, recurrence, progression, CSS, and OS. No differences between the regimens or instillation approaches (antegrade vs. retrograde vs. combined approach) were found [197]. The indication for BCG as primary treatment in CIS requiring KSS has some support, but instillations after laser ablation of papillary UTUC have not been proven to improve survival or decrease recurrence rates [198].

Cisplatin-based chemotherapy has been used in advanced UTUC for some decades, but the first RCT assessing the efficacy of systemic platinum-based chemotherapy was published in 2020. Chemotherapy was administered post-RNU, i.e., in an adjuvant setting, within three months of RNU. In patients with locally advanced UTUC, gemcitabine–platinum combination

chemotherapy significantly improved disease-free survival (DFS) [199]. UC is a strongly immunogenic disease, as demonstrated by the high efficacy of BCG in CIS of the bladder. UC expresses the immune checkpoint programmed death-ligand 1 (PD-L1), which interacts with the patient's immune cell programmed death 1 (PD-1) receptor, thus evading elimination by the immune system. Data on immunotherapy are scant for UTUC, and data extrapolated from UC are the basis for the treatment of UTUC with monoclonal antibodies targeting PD-1 (pembrolizumab and nivolumab) and its ligand PD-L1 (atezolizumab, avelumab, and durvalumab) either as second-line treatment after platinum chemotherapy or as first-line treatment for cisplatin ineligible patients with PD-L1-positive UTUC [200].

Both the patient and urologist need to be motivated to adhere to a strict follow-up schedule if organ-sparing treatment is chosen. The optimal follow-up schedule is not yet known. EAU guidelines recommend a follow-up schedule based on the risk group of the tumour. Cystoscopy, MCTU, urinary cytology and ipsilateral URS are recommended depending on whether KSS or RNU has been performed, with a weak level of evidence for the recommendations made [6]. A second-look procedure is often appropriate, as cancer was found in half of cases when a second-look was performed after 6 weeks [201].

1.6 PROGNOSIS: PROGNOSTIC FACTORS AND SURVIVAL

When counselling patients about the choice of treatment modality, prognostic factors need to be assessable before performing RNU. This is still a rather weak point in the work-up of UTUC and demands a systematic and thoughtful, multidisciplinary approach. The EAU guidelines have evolved substantially over the last decade, after the first publication in 2005 [6, 36, 164, 165, 202]. Risk stratification into low-risk and high-risk groups based on the assessment of several preoperative factors was introduced in 2015 [165]. Patients are categorised as low risk when all the following criteria are met: unifocal tumour, small size (<2 cm), low-grade cancer on cytology and biopsy, and MCTU indicating superficial, unifocal nonmetastatic disease without signs of pathologically enlarged lymph nodes. There are some predictive models and nomograms that can be used to calculate the risk of invasive UTUC and facilitate treatment decisions [40, 203] or the calculation of survival after RNU [204]. Below, prognostic factors are discussed, first those that can be assessed before RNU and, last, those that are only possible or best to assess after RNU.

1.6.1 Prognostic factors assessable before RNU

1.6.1.1 Age

Higher age is associated with more aggressive tumours and advanced stage at diagnosis. Elderly patients risk not being fit to undergo complete RNU and lymphadenectomy and, unfortunately, do not tolerate chemotherapy to the same extent as younger patients. Age was associated with worse progression-free survival (PFS), CSS and OS but with a very small effect overall in a meta-analysis [205]. Lower CSS and OS with increasing age have also been shown by other

researchers [20, 206, 207]. However, chronological age has been shown to not be an independent prognostic factor for CSS when adjusting for performance status [208], and age in itself should not be used as a factor excluding curative treatment.

1.6.1.2 Smoking

In addition to smoking being one of the most important risk factors for developing UTUC, smokers have an increased risk of recurrence, including IVR and death from UTUC. Smoking is a relatively more important risk factor in females than in males [209, 210]. Heavy, long-term cigarette smoking increases the risk of more advanced disease (high-grade and high-stage tumours, lymph node metastasis, lymphovascular invasion [LVI]) as well as recurrence and progression. Smoking cessation for more than a decade when diagnosed with UTUC seems to result in a better prognosis, and patients should be encouraged to quit smoking [211, 212].

1.6.1.3 Sex

In the literature, sex often does not have an impact on the prognosis of UTUC (CSS) in multivariable models, although women in some studies present with more advanced disease than males [212–214]. The evidence is conflicting, with a study [215] that found that sex was not a prognostic factor in UTUC but described less difference between the sexes in terms of incidence, prognosis and stage at diagnosis for UTUC than for UCB. Another recent large SEER database study including 9208 nonmetastatic UTUC patients treated with RNU found that sex was an independent predictor of CSM, with a 5-year CSM of 30.6% for females and 25.5% for males [20].

1.6.1.4 Renal function

Renal impairment is a risk factor for the development of UTUC, and severe renal impairment precludes the use of adjuvant cisplatin-based chemotherapy. The importance of renal impairment for the prognosis of UTUC has not been extensively studied. Renal impairment is common in these patients, 19-48% [216]. A study [217] pointed to higher stage UTUC in patients with renal impairment, and two other studies [29, 218] have shown that patients with renal insufficiency before RNU have worse survival. The presence and implication of renal impairment, postoperative decline in renal function and the potential difference in survival depending on renal function are important for chemotherapy regimens, possibly affecting the timing of treatment (neoadjuvant or adjuvant) and assessment of the risk-benefit ratio for an individual patient.

1.6.1.5 Location

The prognostic impact of the initial location of the UTUC tumour is debated. Reasonably, UTUC located in the renal pelvis may be detected later than UTUC located in the ureter, i.e., when the stage is more advanced [219], as ureteral tumours could give rise to obstruction with flank pain earlier. Macroscopic haematuria is, however, the dominant symptom in UTUC, and

there should be no temporal difference in that sign between caliceal, renal pelvic and ureteral tumours. There is a hypothesis that ureteral UTUC would spread more easily due to the more extensive periureteral lymphovasculature and the fact that the physical barrier consisting of the renal parenchyma and perirenal fat could delay tumour progression [219, 220]. After adjustment for other patient and tumour characteristics, most importantly stage and grade, tumour location was not an independent prognostic factor in several studies on different patient populations [29, 221, 222]. Three large studies based on the SEER database [223–225] found no difference in CSM based on tumour location. One of the studies [224] found more advanced T and N stages of renal pelvic tumours than ureteral tumours, but tumour location did not independently predict CSM. In contrast, a few smaller studies have shown worse prognosis for ureteral tumours [219, 220, 226, 227].

1.6.1.6 Multifocality

Several studies have found that tumour multifocality predicts worse oncological outcomes [29, 219, 220, 228]. However, multifocality is strongly associated with tumour stage, grade, and lymph node metastasis, and the remaining prognostic value of multifocality after adjustment for these factors is rather small [222]. Multifocality has been described as a manifestation of biologically aggressive disease in patients with organ-confined UTUC and an association between multifocality and both CSM and progression has been found only when restricting the analysis to organ-confined UTUC [229]. The results in a Chinese study deviated from those of most “Western” cohorts, with multifocality predicting a better pathological outcome, i.e., papillary, low-stage UTUC [230]; it was hypothesised that aristolochic acid in the widely consumed traditional Chinese herbal medicines could explain this discrepancy.

1.6.1.7 Size

Although the EAU guidelines changed its recommendation from a 1 cm to 2 cm cut-off for KSS or RNU in 2017 [164], support for this was weak. However, recently, a study of 932 RNUs for nonmetastatic UTUC performed from 2000–2016 showed that the 2 cm cut-off is indeed appropriate, better than a 1 cm or a 3 cm cut-off, for achieving a balance between avoiding unnecessary RNUs and finding invasive UTUCs [231]. Another study used the SEER database and found that greater size predicted a higher rate of the invasive stage almost linearly in a cohort of 4657 renal pelvic UTUCs treated with RNU from 2004–2016 and characterised as predominantly large tumours (median tumour size 3.7 cm, IQR 2.5-5.0 cm) [232]. Tumour size was measured in RNU specimens in these studies, and the tumour size is generally somewhat smaller when measured after preparation in the pathology laboratory than in vivo measured during URS.

1.6.1.8 Grade

Several studies have demonstrated a prognostic role of tumour grade [18, 20, 62, 212, 233–236], although it is not as strong or consistent as that of stage. In a study of all 168 consecutive

UTUCs treated with KSS and RNU at one centre in Canada from 1978–2001, the most important predictor of cancer mortality was tumour grade. Tumour stage was highly correlated with grade (Spearman's rho = 0.59; P < 0.001) [18]. The WHO 2004 classification of 247 reassessed RNU or ureterectomy specimens revealed an 82% association between high-grade UTUC and stage ≥pT2 [195].

1.6.1.9 Other risk factors: pyuria and tumour architecture

Preoperative clinical markers that have been shown to predict outcome include markers of inflammation: C-reactive protein, anaemia, plasma fibrinogen level and pyuria. Inflammation is part of the causal chain of carcinogenesis and progression. Studies [237, 238] have demonstrated an independent association between preoperative pyuria and CSS and OS in patients with locally advanced (pT3/4) UTUC. The sessile tumour architecture has been shown to correlate with worse survival than the papillary tumour architecture [239, 240].

1.6.2 Prognostic factors assessable after RNU

1.6.2.1 Stage

Tumour stage is the most established prognostic factor in UTUC [6, 18, 212, 233–235]. In a SEER database (2004–2016) study of 9208 nonmetastatic UTUCs, Ruvolo et al. confirmed that both stage and grade were important prognostic factors and that CSM increased significantly in patients with high-grade UTUC and in stages T3N0M0, T4N0M0, and T1–4N1–2M0 [20].

1.6.2.2 Other factors

Among other prognostic factors, concomitant CIS has been shown to be an independent predictor of both RFS and CSS in patients treated with RNU for UTUC [241]. The prognostic value of tumour necrosis is controversial. Although several studies have reported an association between extensive tumour necrosis and advanced stage and lower RFS and CSS rates, this was not confirmed after adjustment for other tumour characteristics, including stage, in other studies [212, 242]. A study found that only extensive (>10% of the tumour area) tumour necrosis was associated with recurrence and survival after adjustment for stage, grade, LVI, and lymph node status. The additional prognostic value of extensive tumour necrosis was marginal for recurrence and survival [243].

The prognostic role of lymph node dissection has not been established. It seems that patients with locally advanced UTUC have a survival benefit of lymph node dissection with a cut-off at eight extirpated nodes [212]. The prognostic value of LVI in RNU specimens was demonstrated for metastasis but not CSM in one study [220]. LVI was a strong predictor of poor outcome in other studies, which recommended evaluating LVI status for finding patients with a higher risk of metastases when lymph nodes are not available [244–246].

The prognostic value of alterations in the mammalian target of rapamycin (mTOR) pathway was investigated with immunohistochemistry in 620 UTUC patients who underwent RNU, and PI3K and cyclin D were found to be significant predictors of inferior oncologic (CSM) and clinical (high-grade UTUC, LVI, non-organ-confined disease) outcomes [247]. Further assessment of the predictive role of cell proliferation by immunohistochemical staining for Ki-67 was performed in a multi-institutional study of 475 RNU specimens from UTUC patients [248]. A cut-off of 20% was used for overexpression of Ki-67. It was found to be an independent predictor of RFS and CSS. The authors suggest that the largest effect of Ki-67 could be to predict patients with a lower risk of recurrence or CSM, perhaps sparing them chemotherapy.

Microsatellite instability (MSI) is defined as "the presence of ubiquitous mutations in microsatellite DNA sequences" [212]. It has been demonstrated in several cancer types. MSI is rare in UCB (3%) but common in both sporadic and Lynch syndrome-associated UTUC (15-20%) and is not associated with age. Analysis of MSI can be useful in invasive UTUC and can be expected to be positive in 16% of high-risk UTUCs [15]. Rouprêt et al. retrospectively investigated the prognostic role of high MSI in 80 patients by performing PCR on microdissected archival formalin-fixed paraffin-embedded primary UTUCs from 80 patients operated on between 1990–2002. Significantly higher OS was seen in patients with high MSI [249]. Lynch syndrome should be suspected when high MSI levels are found [15]. MSI-high tumours may respond well to immune checkpoint inhibitors [14].

1.6.3 Survival

As previously described, (in Section 1.6.2.1) survival is highly dependent on stage. Changes in the distribution of stage over time and in different study populations must be considered when comparing survival after different kinds of surgery, such as RNU or KSS, and over time. Compared with the UTUC population receiving KSS, RNU patients usually have a higher stage and grade of UTUC, thereby having a worse prognosis and at the same time being healthy enough to be considered able to survive the operation. When KSS was first introduced, it was only offered for imperative and palliative indications, including a solitary kidney and severe renal, cardiovascular or lung comorbidities. This patient group probably also has a higher risk of other-cause mortality, OCM. Gradually, KSS has been offered electively, also including healthier, younger patients with a small tumour burden. As patients with UTUC have not been randomised to different treatment methods, it is difficult to discern the true effect of the different treatment methods from the selection of stages, grades and other comorbidities.

Survival after RNU (5-year CSS) for patients with all stages of UTUC was well above 70% (72%-86%) in several reports [233, 250–252]. In stark contrast, a study of UTUC in the population of the Australian state of Victoria found a poor overall 5-year survival rate of 32% [253]. For low-grade tumours, CSS seems to be similar after KSS to that after RNU. For high-grade UTUC, the outcome is better after RNU in some studies, and KSS is generally only

recommended for high-grade UTUC in a solitary kidney or in cases of severe comorbidity [33, 169], i.e., for imperative indications. The five-year OS was 75% and 100%, respectively, after KSS and RNU for low-grade UTUC and 25% and 48%, respectively, for high-grade UTUC, but there was no statistically significant difference between the two techniques in one study with only eight patients in the KSS group [254]. There is a general consensus that patients with high-grade or G3 UTUC should only be offered KSS for imperative or palliative indications. However, in an experienced centre, where patients with G3 UTUC were treated with KSS imperatively or palliatively, long-term follow-up revealed that these patients, although they had more recurrences than the lower-grade patients, only exhibited a nonsignificant trend towards decreased OS and RFS [124]. Likewise, in a relatively large study with 160 patients, half of whom were treated with KSS, the survival rates were 54% 2-year OS for endoscopically treated high-grade UTUC, 74% 5-year OS and 87% CSS for low-grade UTUC. The CSS rates in the RNU group for all grades were 58% 5-year OS and 64% CSS, but for low-grade UTUC, the 5-year OS was 88% and the 5-year CSS was 93% [255]. Additionally, no difference in CSS between endoscopically treated KSS and RNU patients in either the low- or high-grade carcinoma subgroups was reported in earlier studies [33, 168, 236, 256–258]. The absence of a significant difference in survival between KSS- and RNU-treated UTUC does not necessarily mean that it is safe to treat high-grade UTUC endoscopically, as there were very few high-grade carcinomas in the KSS groups.

Recurrence rates while under surveillance after KSS have been reported to be 48% to 60% in a systematic review [33]. A wider range of local recurrence rates of 6-71% after KSS was found in a meta-analysis [123]. The corresponding rate was 1-18% after RNU. However, the definition of recurrence varies highly among studies; some include IVR and others, only ipsilateral upper tract recurrences and distant metastasis. A large study from Philadelphia, USA [124] with perhaps the longest follow-up time of consecutive patients treated with KSS reported an RFS of 30% at five years after the patients´ first surgery. Grasso et al. reported recurrence in 77% of KSS-treated patients, and the majority of these lesions were small and described as easily treated. The mean time to recurrence was 12 months [255]. The five-year PFS rates were 85% and 75% in the series published by Grasso et al. and Scotland et al., respectively. [124, 255]. Progression was defined as the development of higher-grade UTUC. Importantly, no association was found between recurrence and OS or CSS [124]. Similarly, recurrence and progression after KSS were not directly related in a predominantly low-risk Italian study population [171].

The rates of progression to RNU have been reported to range from 15% [169], 17% [255], 24% [33], and 28.6% [124] to 80% [259]. The outcome was grade dependent, with 96% survival for G1 and 20% for G3 in a study of endoscopic KSS of UTUC, with 5-year renal unit survival estimated to be 85% [169]. In a small study of 34 KSS patients, it was estimated that delayed RNU, in the sense that patients started with KSS and proceeded to RNU at a later point in time, did not appear to affect survival outcomes [254].

Survival from UTUC is still poor and has not convincingly changed, despite generally improved surgical techniques and anaesthesia over the decades, permitting treatment of older and frailer patients and increased use of adjuvant therapies. The mortality rate in UTUC has mimicked the incidence rate fairly closely in Sweden over the past two decades. This implies that the case fatality has remained constant. Refined diagnostic methods have improved the diagnostic sensitivity and specificity over time, but it is difficult to predict whether this would increase survival rates through stage migration [260]. A study using a cohort of UTUC patients obtained from the Swedish Cancer Registry including all patients diagnosed with UTUC, including those not treated and those diagnosed at autopsy, reported a higher stage and older age at diagnosis compared to those in other reports. This could be explained by their limited selection bias compared to that in the other studies and is not necessarily a true stage migration [3].

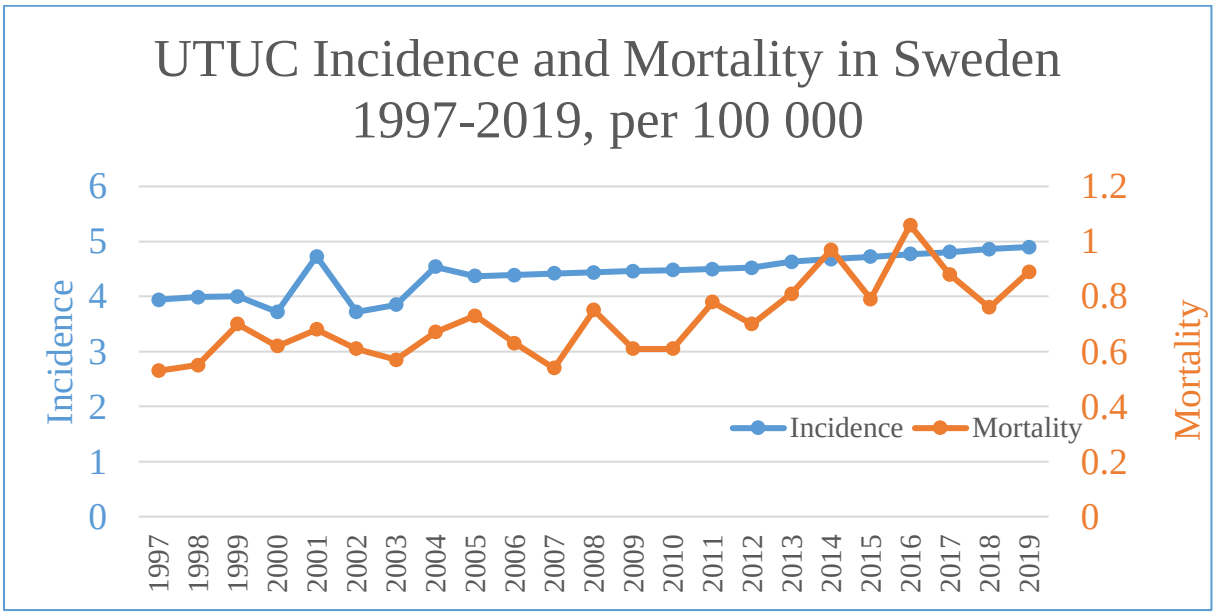


Fig. 1 Incidence and mortality in UTUC in Sweden from 1997–2019, number of new cases per 100 000 people, Data from Statistical areas, Cancer and Cause of death [internet]. Stockholm: Socialstyrelsen/National Board of Health and Welfare, available at: https://www.socialstyrelsen.se/statistik-och-data/statistik/statistikdatabasen

The trend in survival over time varies in different studies. A Norwegian population-based study found that CSS improved from 57% (1999–2008) to 65% (2009–2018) [261]. Accordingly, a US study [21] comparing the periods 1973–1984 and 1985–1996 found no difference in 5-year CSS between the early and late study periods, despite a slightly increased stage-stratified incidence. The incidence rate of in situ UTUC (which includes papillary noninvasive, pTa in the SEER database) increased from 7.2% to 23.1%. [21]. In contrast, a decrease in the 5-year OS rate from 60 to 48% for diagnoses made in 2003–2005 compared with 1985–1987 was found in a slightly older sample from the UK. CSS could not be estimated, as data on causes of death were not available [262]. The unchanged 5-year relative survival (an approximation of CSS) of 57% did not change over time in the Netherlands, despite a 50% increase in incidence over the

25-year period leading up to 2017 [17]. CSS was also unchanged over time in a US study comparing survival during three periods between 1984 and 2004 [235]. The effect of increased use of chemotherapy may be more evident in the next few years. Adjuvant chemotherapy has shown promising effects on cancer-free survival, albeit not yet on decreased CSM [20, 199]. A study has shown that DFS and OS are highly correlated, regardless of adjuvant chemotherapy and tumour stage, indicating that DFS potentially is an appropriate endpoint at shorter follow-up times in trials of immunotherapy or chemotherapy [263].

2 RESEARCH AIMS

The aims of this thesis were to evaluate and improve the diagnostic work-up, risk stratification and treatment outcomes of UTUC by evaluating preoperative prognostic markers to enable personalised treatment options.

The specific aims of each paper are as follows:

Paper I: To determine the accuracy and reliability of ureterorenoscopic work-ups: how accurate are ureterorenoscopic barbotage cytology, biopsy histopathology and ploidy? Additionally, to learn whether barbotage cytology suffices as an alternative to biopsies.

Paper II: To identify tumour characteristics associated with invasive tumour stage in UTUC and to determine tumour characteristics associated with CSS.

Paper III: To further evaluate the usefulness of the proportion of cells in S-phase (SPF) as a predictor of invasiveness and of CSS in UTUC. Additionally, to compare the prognostic values of the WHO 1999 and 2004 classification systems.

3 MATERIALS AND METHODS

3.1 PATIENT COHORTS AND ANALYSES

The study design of **Paper I** was an investigation of diagnostic accuracy. **Papers II and III** were in addition prediction studies, analysing factors predicting invasive stage and survival. The STARD protocol was followed [264]. Between 2005–2012, 148 consecutive patients with suspicious or diagnosed UTUC at Karolinska University Hospital, Stockholm, Sweden, were included in a prospective study of diagnostic procedures [37, 265] and long-term follow-up. Of these, 94 had UTUC as the final diagnosis, and 45 of the 55 patients who were treated with RNU had a diagnostic URS prior to RNU because either the diagnosis was not unambiguous on MCTU or more information was desired. These 45 patients were included in **Papers I and II.** Two patients were excluded from the final analysis due to no remaining UTUC in the RNU specimen and missing an RNU specimen at reassessment. Thus, 43 patients were included in the final analysis. After MCTU or the best alternative imaging depending on renal function, the patients underwent cystoscopy and URS confirmation of UTUC before RNU. KSS was initially only offered in imperative cases, and elective KSS was gradually introduced for small, superficial papillary UTUCs during this time period. The URS protocol is described below and in greater detail in **Paper I.** RNU was performed within 4 weeks of URS. The index tests in paper I were grade and ploidy of the URS samples. RNU specimens were stained with haematoxylin-eosin and assessed with histopathology and FCM. This was the reference standard. Data were collected from patients' electronic charts throughout the study period. Cause of death was checked in the death certificates or in the patients´ electronic charts

In **Paper III,** the previous cohort (n=43) was extended to include a cohort of all consecutive UTUC patients (n=72) operated on with RNU at Stockholm South General Hospital during the same time period (2005–2013). Patients in the Stockholm South General Hospital cohort who had undergone RNU but did not have UTUC as a final analysis were excluded. Both cohorts included open, conventional and robot-assisted laparoscopic RNUs performed with different techniques of bladder cuff removal due to organ-confined UTUC. The index test was SPF analysed with FCM and the reference test was histopathology of RNU specimens.

Statistical analysis was performed using SPSS (IBM, version 22.0) and Microsoft Excel for Mac 2011 (v. 14.3.9) in **Papers I and II** and SPSS (IBM, version 27) in **Paper III**. Descriptive statistics of clinicopathological data were calculated. Normally distributed categorical data were tested for significance using Pearson's chi-squared test or, if the sample size was small, Fisher's exact test. Nonnormally distributed data were tested for significance with the Mann–Whitney *U* test or Kruskal–Wallis (KW) test. Continuous data were tested for significance using Student's t test or analysis of variance (ANOVA), as appropriate. Ninety-five percent confidence intervals were calculated, and statistical significance was set to a level of 0.05.

In **Paper I**, overall agreements were estimated as the proportions of observations with equivalent results from the two diagnostic methods. Associations were tested with Fisher's exact test. Indices of diagnostic validity were calculated.

In **Papers II and III,** Kaplan–Meier curves were constructed for survival, and differences in survival were compared with the log-rank test. Cox regression was used to calculate the associations between death from UTUC and stage, grade, size, multifocality, location, ploidy and rate of proliferation. In **Paper III**, OS and 5- and 10-year CSSs were calculated. OS is the percentage of patients who are still alive. CSS represents the proportion of patients who did not die from UTUC within five and ten years of diagnosis, respectively. Differences in the distribution of SPF were calculated between stages (superficial pTa-1, CIS and invasive ≥pT2 tumours), both WHO 1999 (grades 1, 2 and 3) and 2004 (low-grade, high-grade) classification grades, diploid/aneuploid UTUC, and between patients who died of UTUC and those who did not. The predictive accuracy of SPF in relation to tumour stage and in relation to death from UTUC was measured as the area under the receiver operating characteristic (ROC) curve, with 95% confidence intervals. Multiple Cox regression was performed to evaluate independent predictors of survival. The patients were observed from the date of diagnosis until death or censoring.

3.2 PAPER I

The URS investigation protocol included urethrocystoscopy and bladder barbotage prior to instrumentation of the upper tract. The bladder was first emptied and then slowly filled with saline during inspection looking for concomitant UCB. Approximately 250 ml of barbotage fluid was collected. Then, a thin Foley catheter was placed and closed during URS. Semirigid nontouch ureteroscopy [266] was performed: a semirigid ureteroscope was introduced as high up in the ureter as feasible, usually to the crossing of the iliac vessels. A floppy-tip nitinol guidewire was inserted to the level of the tip of the semirigid ureteroscope. Maintenance of the guidewire at this exact position in the ureter, when changed to a flexible ureteroscope that was backloaded onto this guidewire, was ensured by fluoroscopy. Once the flexible ureteroscope was introduced into the ureter, the guidewire was removed, and the flexible ureteroscope was advanced up to the renal pelvis. The entire collecting system, including the calices and renal pelvis, was inspected prior to collection of the renal pelvic barbotage sample, which was taken through the flexible ureteroscope, using passive irrigation and aspiration with a 10 ml syringe, collecting 48 ml for cytology and 24 ml for FCM. Endoscopic biopsy was conducted with a safety guidewire in place using Piranha^{TM} 3 Ch ureteroscopic biopsy forceps (Boston Scientific Nordic AB, Helsingborg, Sweden). The biopsy specimen was not entered into the working channel of the ureteroscope, as it would risk getting stuck. Instead, the ureteroscope was removed entirely. The biopsy specimen was placed in formalin in a test tube and kept in the vertical position during transportation. The fluid that dripped down via the ureter when the ureterorenoscope was in place in the ureter was collected through the uretheral catheter placed in the urinary bladder; this was the ureteral barbotage. Barbotage samples were sent fresh to the

pathology laboratory. Both bladder (ureteral) and renal pelvis barbotages were treated in the same manner and analysed for cytology with Papanicolaou staining or FCM. The contents of the biopsy test tube were poured through a lens paper sieve, through which it was stained with haematoxylin-eosin. After securing the barbotage samples, contrast medium was instilled through the working channel to ensure that the entire urothelial surface had been inspected, without risking artefacts in cytology that could arise from contact with the contrast medium.

All specimens from URS and RNU were initially assessed by specialised urocytologists/uropathologists and then reassessed by one experienced uropathologist. He was not blinded to the aim and design of the study. Tumours were graded according to the three-tiered WHO 1999 classification system, categorising tumours into grades 1, 2 and 3. Determination of WHO 2004 grading (low- or high-grade) was later added, and according to the experience of the uropathologist, grade 1 and 2 UTUCs in this specific cohort were all low-grade, and grade 3 UTUCs were all high-grade. Cell anaplasia was assessed based on "irregular cell size, variation in nuclear shape and membrane thickness, chromatin texture, hyperchromasia, mitotic figures and change in nucleocytoplasmatic ratio". Apoptosis, cellular debris and the degree of inflammation were noted. The characteristics of cell aggregates were studied carefully: to a variable degree, tumorous cell proliferation appeared as epithelial clusters in addition to exfoliated single cells in almost all cytological specimens. The histopathology and FCM of the RNU specimen were the reference standards. Comparisons were made of the histology grade in endoscopic biopsies and the cytology grade in barbotage specimens, cytology specimens collected from the renal pelvis and ureter, and grade in histology of RNU specimens. DNA ploidy in barbotage specimens was correlated with ploidy and grade in RNU specimens.

3.3 PAPER II

The same prospective consecutive cohort of patients as that in Paper I was included, and they were diagnosed with UTUC verified by imaging and URS before undergoing RNU. The same samples as those described in Paper I were used. In Paper II, tumour characteristics were analysed for the prediction of invasive stage and death from UTUC. CSS rates were calculated in 2018, meaning that the follow-up period was long for all participants. Stages were categorised as superficial (pTis and pTa-1) or invasive (≥ pT2). Tumour size was measured during URS comparing it with the guidewire, laser fibre or tip of ureteroscope or by placing the tip of the ureteroscope next to one end of the tumour. The urologist placed the tip of her index finger on the shaft of the ureteroscope at the urethral meatus, keeping it on the shaft as the instrument was removed thus far as the tip of the instrument reached the opposite end of the tumour, measuring the length between meatus and index fingertip. However, the size used for calculating agreements was tumour size in the RNU specimen, as measured by the pathologist, because these data were eventually more complete. The tumour shrank to some extent when dried and fixed.

3.4 PAPER III

This cohort of 115 patients who underwent RNU with UTUC as the final diagnosis was analysed retrospectively by reassessment of the histology in RNU specimens and by FCM to determine the ploidy and SPF in RNU specimens. The index test was SPF, and the reference test was the RNU specimen. The primary endpoint was the prognostic role of SPF.

3.5 ETHICAL APPROVALS AND CONSIDERATIONS

The studies were approved by the Regional Board of Ethics and were performed in accordance with the Declaration of Helsinki [267]. Informed consent was obtained from all patients.

Patients included in these studies on UTUC diagnostics received the same work-up and treatment as would be offered to them if they chose not to participate with their data. The risk of undergoing RNU of a nonmalignant kidney or small low-grade UTUC was smaller for patients participating in this study than for patients receiving routine healthcare elsewhere. The standardised work-up as outlined in the study protocol described in Paper I was not actually a reality outside of studies in many hospitals during the study period but would have been the care offered to the patient cohort of Papers I and II even if they chose not to participate in the study. The chance of privacy violation was small, as the same clinicians treating the patients also conducted the study. Additionally, data were deidentified when entered in the database and processed statistically. In the centres involved, data collected from patients included in the study were the same as those entered into the hospital charts of the patients not participating in the study. The risk for the individual patients concerning data handling was small.

4 RESULTS

4.1 PAPER I

Forty-five patients were included. Two were excluded from the final analysis because in the reference test, the RNU specimen was not available for reassessment and showed no remaining tumour after laser ablation during URS. The median age at diagnosis was 68 years (range: 34-89). The female:male ratio was 11:32. Almost half of the tumours (20/43) were grade 3 (high-grade) cancer: out of the 23 low-grade cancers, 13 were grade 2, and 10 were grade 1.

Almost all UTUCs were identified by both cytology and histopathology, and the overall agreement in grade was 94% in endoscopic biopsies and 91% in barbotage cytology. Regarding grade, the agreement between URS biopsies and subsequent RNU specimens was statistically significant for both the 1999 and 2004 WHO classifications ($P = 0.014$ and $P = 0.017$, respectively). Endoscopic biopsy was obtained in 36/43 patients; it identified all cancers as pathological, but the grading was not correct in all cases. In two cases, the biopsies were classified as “atypia”, and in one case, they were classified as “urothelial carcinoma”. Barbotage cytology correctly graded 39/43 of all UTUCs, i.e., a 91% agreement, ($P = 0.007$ and $P = 0.014$, respectively, for WHO 1999 and 2004 classifications) and was sensitive even for the detection of low-grade tumours. The four specimens that were not correctly graded were described as inflammation and atypia. Barbotage cytology was as reliable as histopathology of endoscopic biopsy. In cytology as well as in histopathology, agreements in grade between barbotage and RNU specimens were statistically significant for both 1999 and 2004 WHO classifications. Barbotage specimens were also useful for the determination of cell proliferation and ploidy using FCM. This enhanced the diagnostic accuracy for grade 1 and grade 3 UTUCs.

Ureteral barbotage provided additional information. In 4/16 patients with ureteral UTUC, this sample enabled more accurate grading than was achieved with renal pelvis barbotage or biopsy. In another two patients with UTUC located in the ureter, the ureter barbotage sample was the only URS specimen that revealed cancer. One patient with pTaG1 in the RNU specimen had G2 cells in the ureteral barbotage, and the other patient had pTaG2 in the RNU specimen but G1 in the ureteral barbotage. Neither of these two patients underwent endoscopic biopsy, and their renal pelvic barbotages were benign.

Agreement of tumours identified by barbotage cytology and biopsy histopathology

	Cytology of barbotage specimens positive for malignancy		Histology of biopsy specimens positive for malignancy	
Grade in nephroureterectomy specimen (*n*)	*n* (total)	%	*n* (total)	%
Low				
G1 (10)	7 (10)	70	8 (8)	100
G2 (13)	13 (13)	100	11 (12)	92
High				
G3 (20)	19 (20)	95	15 (16)	94
(43)	39 (43)	91	34 (36)	94

Table 1. Agreement of Tumours Identified by Barbotage Cytology and Biopsy Histopathology, [268]. *Reproduced with permission from Taylor & Francis Group, Informa UK Limited* http://www.tandfonline.com/.

Accuracy of grading in ureterorenoscopic biopsies compared with nephroureterectomy specimens, according to WHO 1999 and 2004 classification

Nephroureterectomy / URS biopsy		Low		High
		Grade 1	Grade 2	Grade 3
Low grade	Grade 1	6 [a]	6 [b]	0
	Grade 2	2 [c]	3 [a]	5 [b]
High grade	Grade 3	0	2 [c]	9 [a]
UTUC nonspecific grade		0	0	1 [b]
Atypia		0	1	1 [b]
Correct grade WHO 1999		6/8 (75%)	3/12 (25%)	9/16 (56%)
Correct grade WHO 2004		17/20 (85%)		9/16 (56%)
Total number		8	12	16

Table 2. Accuracy of grading in URS biopsies compared with nephroureterectomy specimens, according to WHO 1999 and 2004 classification. Considering all tumours, 18/36 (50%) were graded correctly according to the WHO 1999 classification, whereas 26/36 (72%) were graded correctly according to the WHO 2004 classification. [a]Graded correctly in histological analysis of endoscopic biopsy; [b]biopsy undergraded the tumour; [c]biopsy overgraded the tumour. Modified from Paper I [268], *with correction of superscript letter assigned to the number 2 in the Grade 2 column that should be the letter "c", designating that biopsy overgraded the tumour. Reproduced with permission from Taylor & Francis Group, Informa UK Limited,* http://www.tandfonline.com/.

4.2 PAPER II

The patient cohort was identical to that in Paper I. Inclusion of patients ended in 2012, and patients were observed from the date of diagnosis until death or censoring at the end of the study, April 2018. After a median follow-up of 95 months (range 4–144 months) or 7.9 years (range 0.33-12 years), 16/43 patients had died: 10 from UC and six from other causes. The short follow-up time was due to patient death soon after inclusion, as no patient was lost to follow-up. Patient and tumour characteristics are presented in table 3.

Tumour stage was associated with tumour grade ($P < 0.001$), SPF ($P = 0.004$) and ploidy ($P = 0.045$). Size, location and multifocality were not associated with stage. The median SPF was 5.7 (IQR 2.6-12). The SPF was significantly higher in invasive UTUCs than in superficial UTUC ($P = 0.011$) but did not differ between papillary and nonpapillary (CIS) superficial UTUC ($P = 0.482$). Grade was significantly associated with both ploidy ($P < 0.001$) and SPF, except for CIS, compared with G1 or G2. The distributions of SPF across stages and grades are shown in Figures 2 and 3.

The tumour characteristics that were significantly associated with CSS were grade, stage and SPF ($P = 0.044$, 0.023 and 0.006, respectively). Location, multifocality, ploidy, tumour size, and history of UCB were not associated with CSS. Overall CSS at the end of the study period was 77% (95% CI: 70-92%). The 5-year CSS was 78% (95% CI: 67-92%); stage-stratified 5-year CSS rates were 88%, 50% and 100% for superficial, invasive and CIS tumours, respectively (log-rank, $P = 0.012$). The five-year grade-stratified CSSs were 100% for G1, 85% for G2, 53% for papillary G3, and 100% for CIS only (log-rank, $P = 0.022$).

The hazard ratio (HR) for death from UC increased by 25% for every 1% increase in the SPF for superficial tumours ($P = 0.027$). However, for the invasive tumours, no association was seen between increasing SPF and risk of death ($P=1.00$).

	All Patients	pTis-1	≥ pT2	Diploid	Aneuploid
Number of patients	43	31	12	22	21
Age at diagnosis		P = 0.63		P = 0.49	
Mean (min, max)	68.8 (34-89)	68.3 (34-87)	70.1 (48-89)	67 (34-83)	70.7 (50-89)
Median (IQR)	68 (63-77)	68 (63-77)	69 (63.3-79.3)	68 (59.8-77)	70 (63.5-80.5)
Female:male ratio		P = 0.42		P = 0.80	
	11:32	9:22	2:10	6:16	5:16
Smoking status, n					
Current smoker	13	10	3	9	4
Ex-smoker	15	10	5	8	7
Never-smoker	15	11	4	5	10
Bladder cancer		P = 0.33		P = 0.87	
Prior to UTUC	15	13	3	9	7
Syn- or metachronous	7	5	2	3	4
No bladder cancer	21	13	7	10	10
UTUC stage, n				P = 0.033	
Superficial (pTa-pT1)	26	26	-	16	10
Invasive (>pT2)	12	-	12	3	9
CIS only	5	5	-	3	2
UTUC grade, n		P << 0.001		P << 0.001	
G1	10	10	-	10	-
G2	13	12	1	7	6
G3	20	9	11	5	15
UTUC size, n		P = 0.15		P = 0.85	
<15 mm	8	6	2	3	5
>15 mm	27	18	9	15	12
CIS only	5	5	-	3	2
Unknown	3	2	1	1	2
Number of tumours, n		P = 0.66		P = 0.87	
No visible tumour	3	3	-	1	2
Unifocal	22	15	7	12	10
Multifocal	18	13	5	9	9
Ploidy, n		P = 0.03			
Diploidy	22	19	3	22	-
Aneuploidy	21	12	9	-	21
Cause of death		P = 0.02		P = 0.29	
UC	10	4	6	3	7
Other than UC	6	4	2	4	2
Unknown	1	0	-	-	-
Patient still alive	26	23	4	15	12

Table 3. Patient and tumour characteristics of the patient cohort in Papers I and II.

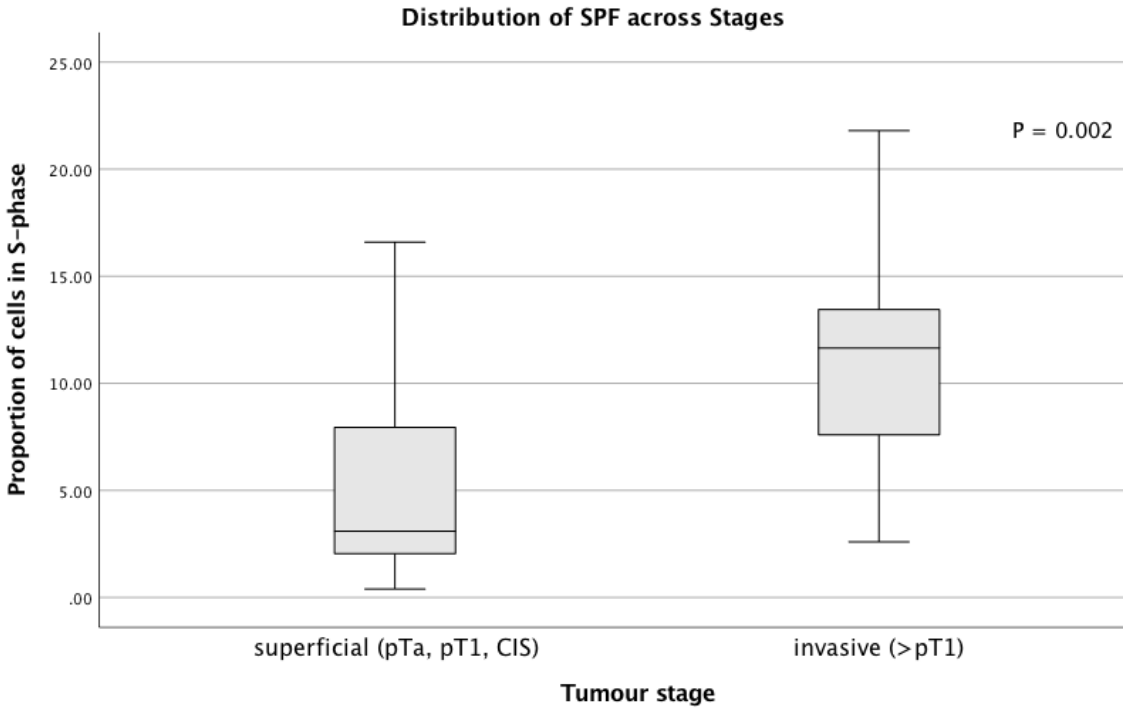


Fig 2. Distribution of SPF across stages. The median SPF (%, IQR) for superficial UTUC was 3.1 (2.0-9.2) and for invasive UTUC was 11.7 (7.2-13.7).

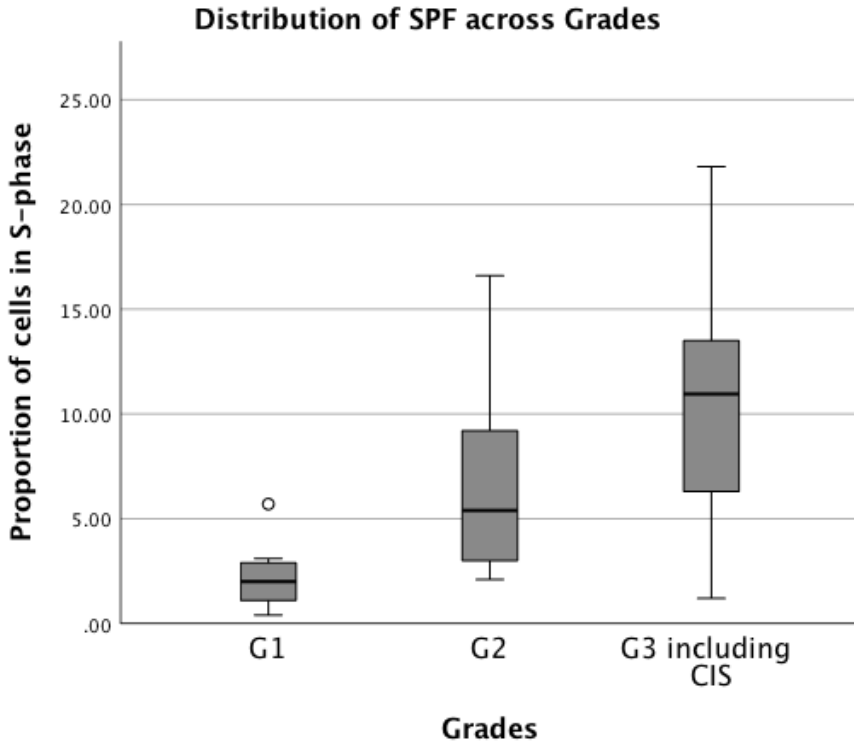


Fig 3. Distribution of SPF across grades. The median SPF (%, IQR) for G1 was 2 (1-5.4), for G2 was 5.4 (2.95-10.2), and for G3 was 10.1 (6.1-13.7). KW-test, P = 0.048 (G1-G2), P << 0.001 (G1-G3), P = 0.391 (G2-G3).

4.3 PAPER III

A total of 99 patients remained in the final analysis, after exclusion due to missing tumours, missing RNU specimens or other final diagnoses than UTUC. The median age was 70 years (IQR 63-78). Patients were followed for a median of 7.6 years (IQR 3.1-11.9). The median SPF in the whole cohort was 7% (IQR 2.7-11.3%), and the mean SPF was 5.2% (95% CI: 5.9-8.1%, range 0.4-26%). The distribution of SPF was significantly different across stages (superficial pTis-Ta-T1 vs. invasive ≥pT2, $P < 0.001$), grades ($P << 0.001$), ploidy ($P << 0.001$) and cause of death ($P << 0.001$). The SPF was not different between CIS and papillary superficial tumours or between CIS and G1/low-grade UTUC. The difference between the distribution of SPF was not significant between grade 1 and CIS (t test, $P = 0.293$) or between low-grade tumours and CIS ($P = 1.00$).

As none of the patients eligible for final analysis (those whose RNU specimen was available for reassessment) were lost to follow-up, all patients were observed from the date of diagnosis until death or censoring on November 20, 2020. During the follow-up of up to 14.4 years (median: 7.6 years, IQR: 3.1-11.9), a total of 61 patients died, and of these, 32 died of UTUC. The 5- and 10-year CSS rates for all patients were 69% (95% CI: 60-79%) and 67% (95% CI: 60-79%), respectively. The 5-and 10-year OS rates for all patients were 70.7% and 68.7%, respectively. Kaplan–Meier curves showed statistically significant differences in CSS among tumour stages (log-rank $P << 0.001$), ploidy (log-rank $P = 0.007$) and WHO 1999 grades (log-rank $P << 0.001$). However, the CSS was not significantly different between the WHO 2004 grades (log-rank, $P = 0.065$). Factors that were significant using Cox regression were analysed together in a multiple Cox regression: tumour stage, tumour grade, ploidy and SPF. In the multiple Cox regression, SPF (HR 1.13, $P = 0.012$) and stage (HR 2.65, $P = 0.043$) were found to be independent prognostic markers of CSM. The strongest prognostic factor was SPF. Although the invasive tumours were statistically significantly larger than the superficial tumours, tumour size did not predict death from UTUC. This larger study confirmed the finding in Paper II that the risk of dying from UTUC increased with increasing SPF, and the HR was 1.17 (95% CI: 1.10-1.25, $P<0.001$), i.e., the risk of dying from UTUC was 17% greater with every one percent increase in SPF.

The areas under the ROC curves of SPF in relation to invasive stage and death from UTUC were 0.8 (95% CI: 0.705-0.894) and 0.77 (95% CI: 0.67-0.87), respectively.

Patients who died from UTUC differed from those who did not regarding stage ($P<0.001$), ploidy ($P=0.012$), presence of UCB at any time point ($P<0.05$), and WHO 1999 grades ($P<0.001$), but not WHO 2004 grades. Additional differences between these two classifications were also seen in that the estimated survival differed in low-grade UTUC assessed as G1 tumours compared with low-grade G2 tumours. The estimated survival also differed between high-grade G2 and high-grade G3 UTUC. Low-grade G1 and low-grade G2 had different SPFs, as did high-grade G2 and high-grade G3 UTUC.

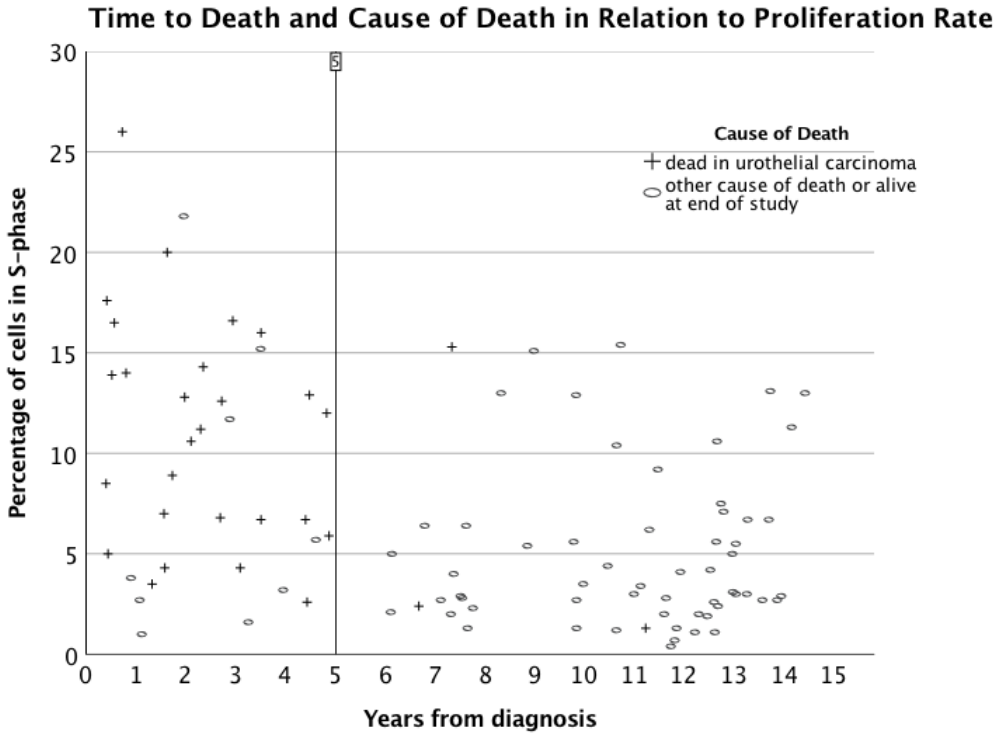


Fig. 4. The majority of deaths from UTUC that occurred during the 14.4-year follow-up period occurred within the first five years; thus, the 5- and 10-year CSSs did not differ. The SPF was higher in patients who died from UTUC than in those who died of other causes or were still alive at the end of the study. By five years from RNU, 29/32 (91%) of the deaths from UTUC that were recorded during the total follow-up of up to 14.4 years had already occurred. Patients without results for the SPF were excluded; thus, the number of deaths from UC according to this figure was 30, and the total number of patients was 96.

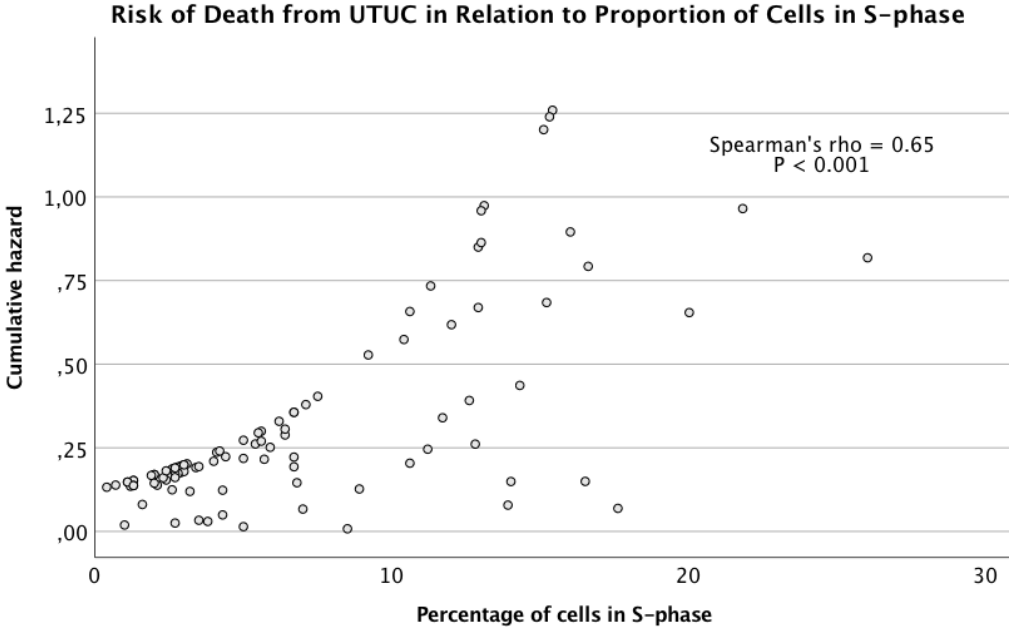
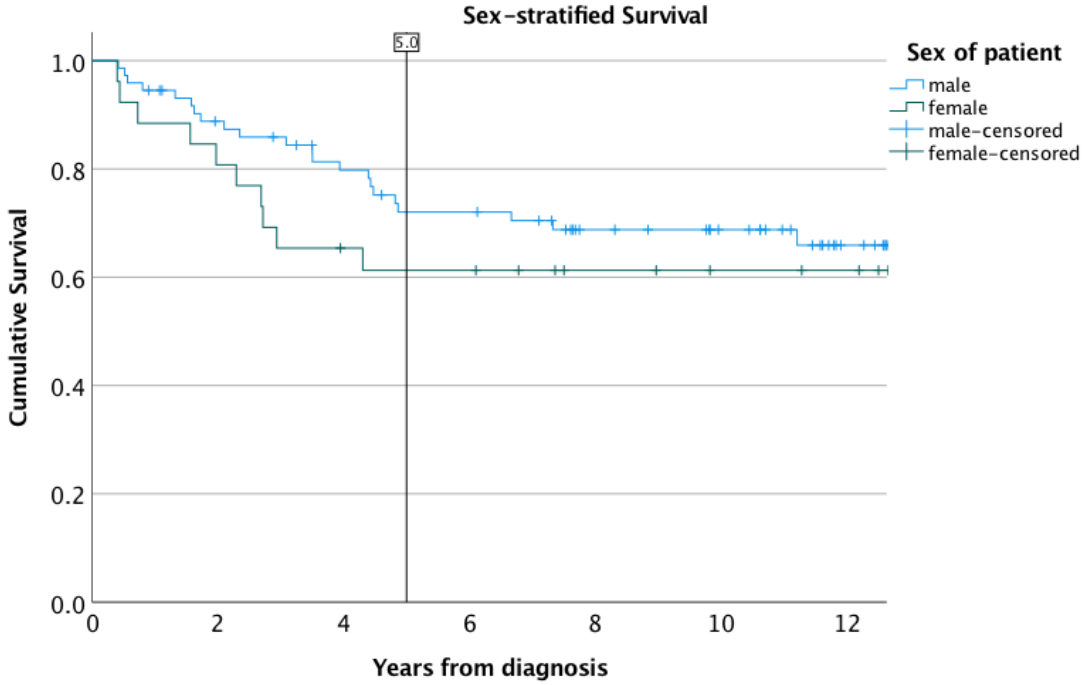


Fig. 5. Spearman's rho $\rho = 0.65$ indicated that there was a strong correlation between the SPF and the risk of dying from UTUC. This correlation was statistically significant and clinically relevant.

Among our patients treated with RNU, there was no statistically significant difference in survival between males and females (log-rank test, P = 0.394, Fig. 3) or in tumour stage at diagnosis (Fisher's exact test, P = 0.355, Fig 4), which was one of the two independent predictors of survival. However, there was a statistically significant difference between the presence of G3 tumours in males and females, with more invasive G3 tumours in males (Fisher's exact test, P = 0.046) (Table 3, Fig. 4). The strongest predictive factor of CSS in our study was the distribution of SPF, which did not differ between the sexes.



Number at risk							
Year	0	2	4	6	8	10	12
Male	73	61	52	46	36	30	16
Female	26	21	16	15	11	9	8

Fig. 6. Kaplan–Meier curve depicting survival stratified by sex. Log-rank test P = 0.394, not significant, i.e., survival did not differ between men and women in our patient cohort.

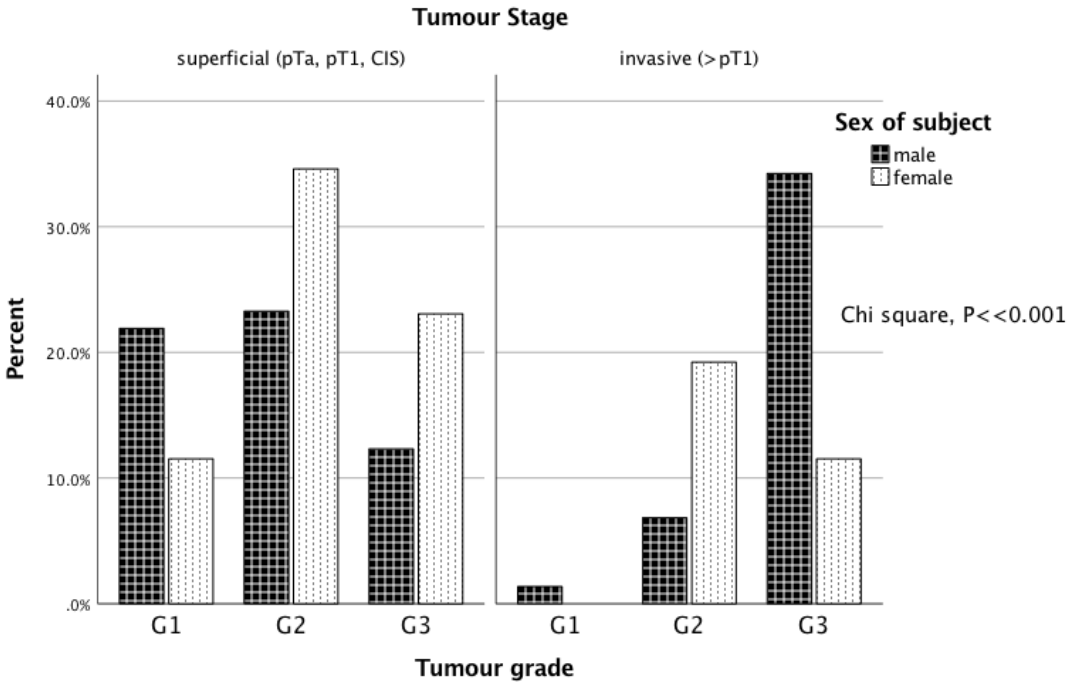


Fig. 7. Distribution of tumour stages and grades among male and female patients. NB y-axis represents proportion of patients.

5 DISCUSSION AND POINTS OF PERSPECTIVE

The work-up of UTUC must involve a careful consideration of different aspects, including the physical and mental status of the patient, comorbidities and anaesthesiologic risks, radiologic findings, presence or history of UCB, and in cases that are not obvious candidates for RNU, the findings of URS. During URS, the number, location and size of tumours, as well as ploidy, SPF and grade, should be assessed. The aim is to achieve as good a prediction of stage as possible before RNU or KSS. The present study demonstrates that samples collected at URS are useful and reliable for assessing UTUC prior to making decisions on the final surgical method. The study strengthens the role of cytology of barbotages, which are easy to secure, in showing that low-grade UTUC can also be detected and graded in in situ barbotage specimens, which is in line with contemporary findings [53]. Diagnostic yield of UTUC and especially of low-grade UTUC was higher using in situ barbotage, compared with studies on voided urine cytology [45]. Ploidy and SPF analysed with FCM can be analysed in barbotages and are a useful complement for risk stratification. Barbotages are presumably better at yielding a representative diagnosis of the entire tumour when it is heterogenous, so even though barbotage and biopsy were equal in our study, we recommend taking both kinds of samples. We did, however not demonstrate this, and it might be more important in larger tumours. Additionally, CIS, which is often not visible with white light endoscopy, is underdetected using just inspection and biopsy [269, 270]. MCTU is even in cases when the patients have good enough renal function to undergo the optimal investigation not in itself sufficient work-up, but was seen to aid in the detection of CIS, which was not detected with URS visual inspection in 15/16 cases of CIS only, in a study including the patient cohort from papers I and II [37].

We confirmed that stage was associated with grade, ploidy and SPF. Although grade correlated with stage, it does not entirely suffice as a proxy for stage. Ploidy was strongly associated with stage but could not be applied to distinguish between superficial and invasive stage in G2 UTUC in our study. The combination of grade, ploidy and SPF performed on URS samples are all important pieces of information to be considered together. A robust correlation between grade and stage would imply that grade could be trusted as a surrogate for stage [271]. Such a correlation has been reported with the WHO 1999 classification. Holmäng and Johansson examined the prognostic role of stage and grade in 555 primary UTUCs (without previous UCB) operated on with RNU and found that tumour grade only had a small additional prognostic value compared with tumour stage in pT3 UTUC, in that the WHO 1999 classification revealed a significantly lower 5-year CSS in G3 (25%) than G2 (49%) pT3 tumours ($P < 0.0037$). Grade and stage had an excellent correlation in PUNLMP, grade 1, and low-grade: 144/146 (98.6%) of them were stage Ta. Similarly, in grade 3, 204/214 (95.3%) were invasive. However, using the WHO/ISUP 2004 classification, grade and stage were not correlated: 114/409 (27.9%) high-grade UTUCs were noninvasive, and most of them were classified as grade 2 with the WHO 1999 classification system [272]. The advantage of WHO 1999 classification of better predicting stage and prognosis, that we found in paper III and the

usefulness of considering both the WHO 1999 and 2004 classifications have been stressed in recent publications [82, 83]. Consequently, grade 2 UTUC should, in addition, be labelled as low or high grade, to provide better prognostic information. Ploidy was associated with stage, but could not in itself distinguish between superficial and invasive stage in our study.

Independent predictors of prognosis in our study were stage, grade and SPF. In univariable analysis, ploidy also predicted CSS. Long-term CSS as studied in the present studies was useful for evaluation of SPF, but survival can differ in studies with different inclusion criteria and survival studies are preferably designed as registry studies. The CSS rates in the present study were comparable to several studies [233, 250, 251] but lower rates have been reported [17, 261].

The strong prognostic value of SPF reported in Paper III could be a watershed. Lipponen et al. [154] found that aneuploidy and high SPF predicted both metastases (lymph node and distant) as well as lower CSS in studies on UCB. The mean SPF in our patient cohort (7%, range 0.4-26) was in level with that reported in UCB patients by Lipponen et al. [146]: 6.6% (range 1-35) but lower than the mean 10.3% (range 2.7-20.3) in the eleven UTUC patients investigated by Oldbring [161]. Although a very small study, Oldbring also observed that aneuploid grade 2 differed from aneuploid grade 3 UTUC regarding the level of SPF. This difference was confirmed in our study, although not as extreme. We found a mean SPF of 8.1% and 12.9% in aneuploid grade 2 and grade 3, respectively, whereas Oldbring reported means of 4.6% and 17.3%. In line with a study on UCB [158], we also found that ploidy was a predictor of death from UC in UTUC patients in univariable analysis, but in multiple Cox, it was not found to be an independent predictor.

The causal relationship of different factors was not investigated with the present study design, but the identification of predictors is even so clinically useful. Paper I was referred to in the EAU guidelines [6] and in the Swedish guidelines on UC [273]. Causal relations can be studied using RCT or observational/aetiological study designs. At the centre of both routine health care and clinical research is the balance between risk and benefit for the individual patient. Research is not ethical if not well conducted, including its leading to robust conclusions through good methodology. The level of evidence of scientific studies depends on the design and quality of conduct. The method with the highest scientific value is the RCT. However, the treatments that are compared in an RCT must be hypothesised to be theoretically equally effective (the equipoise requirement). If not, it would not be ethical to randomise patients to the alternative treatment arms. With the present knowledge of UTUC, it would be possible to randomise low-risk patients to RNU or KSS, but in reality, few patients would accept randomisation, usually having a strong preference for a particular treatment modality. Therefore, interpretation of survival data will be more complex.

RCT design facilitates minimising possible biases that can lead to results and interpretations that are not (internally) valid. Tests of diagnostic tests can be designed as RCTs but often are

not, as in our studies. The patients would then be randomised to the test of interest or the gold standard test and then prospectively followed up, and their prognosis be evaluated. More commonly, the diagnostic accuracy of a test is studied with a cross-sectional design. When the validity of a test is measured in this manner, it is important that the subjects are a consecutive series of patients, representative of the diagnosis, that the time elapsed between the index test and the reference standard is kept short, that the person performing and evaluating the test is blinded to the other test and that the result of the index test is not considered for the reference, gold standard test result. The present studies have both cross-sectional and prediction study designs.

Due to the rarity of UTUC, the number of patients in the present studies is small. Although not smaller than that in many studies of UTUC, this limited number leads to uncertainty of the results, as reflected in the wide confidence intervals in Papers I and II. The small sample size also entails a risk of missing correlations or finding false correlations. In the present study, which was performed with real-world data in a clinical setting, consecutive patients were included, and a good representation of stages and grades was achieved, except for the most advanced metastatic stage, as these patients were generally not treated with cytoreductive surgery or biopsied. This also results in good external validity or generalisability of the results to other UTUC populations. However, diagnostic accuracy depends on the prevalence of UTUC in the studied population, and this was high in our cohort. The results should be interpreted as a recommendation of a diagnostic method, i.e., use of a spectrum of tests under a stringent protocol, rather than focusing on the numbers presented for measures of performance. Including patients from more hospitals than the two in our cohort would increase the sample size and statistical power and is often done when studying rare diseases. The advantage of performing a single-centre study is that it could be easier to ascertain the quality of the data, and thereby the internal validity. The internal validity reflects whether the tests or measures show what we mean for them to show, i.e., the “quality” or “correctness” of the studies. It depends on quality of observations or measurements. The internal validity in our studies would be quite high, as the time elapsed between samples (URS and RNU) was short, did not vary much and was never longer than a month. Additionally, all URS procedures were performed or supervised by a single person. This also applies to the FCM and reassessment of histopathology and cytology, respectively. Urothelial grading is afflicted by considerable interobserver variability, and thus, the internal validity is higher with one observer, but this compromises the external validity. Having no dropouts from the studies and using the same instruments both increase the internal validity. The chosen reference test, histopathology and FCM of the RNU specimen is a hard endpoint, as is the endpoint death from UTUC. However, we could in some cases not be sure if the patients died from UTUC or UCB. The data quality was otherwise good, as we had access to patients´ charts and could assess plausible cause of death if the death certificate in the chart was not convincing.

Study of survival is well examined with observational study design using the National Quality Registry of Urinary Bladder Cancer [274], where (almost) all cases diagnosed in the country are registered. However, survival was not the primary endpoint in Papers II and III. The primary aim in Paper III was to validate SPF as a predictor of invasive stage and then as a prognostic factor. This variable is not available in the National Quality Registry of Urinary Bladder Cancer. A multicentre study would have improved the sample size, statistical precision and generalisability (external validation). Unfortunately, the National Quality Registry of Urinary Bladder Cancer was not useful for studies of UTUC despite including some data on UTUC. No information on cytology, ploidy or SPF was included in the registry. Registry data on endoscopic biopsy include compulsory information on stage, although this is often not assessible. Preoperative stage and grade are compulsory when entering data, but many patients undergoing RNU do not have reliable data for these variables. Furthermore, conversion from KSS to RNU is not possible to discern in the registry. Consequently, the reliability of UTUC data is poor.

Survival is usually presented as overall, relative or cancer-specific survival. OS and relative survival are more robust endpoints, whereas CSS can be flawed by biases such as errors in the cause of death reported in death certificates. The kind of estimate used depends upon the purpose of the study and on the properties of the patients and disease investigated. Both cancer fatality and age influence the difference between the different measures [275]. Relative and CSS rates are used when the effect of only the studied cancer on survival is desired, as the effect of death from other causes is supposed to be eliminated. In relative survival analysis, the patient population with the diagnosis of interest is compared with an external group, usually the general population. The difference in survival is then assumed to be due to the studied disease [276]. The difference between the relative survival rate, or CSS, and OS is the proportion of patients who die from other causes [275]. Usually, CSS is highest, followed by relative survival and OS. When proportions of cancer-related death are high, differences in survival rates will be small. Whereas CSS is prone to misclassification regarding the cause of death, relative survival may be biased when the cancer studied has risk factors in common with diseases with high mortality, which is the case with smoking in UTUC. Relative survival is generally regarded as preferable to cause-specific survival, but in those cases, the general population will also have a different risk of death from these other causes, introducing bias to relative survival. On the other hand, all-cause mortality or OS are hard endpoints that are free from bias [277]. Patients with UTUC are generally old and have a high degree of comorbidities; thus, OS and CSS are not expected to differ much but still caution to what rate is reported is urged when comparing and interpreting survival in different studies.

Despite large improvements in radiologic and URS methods and the advent of genomic analyses complementing cytologic grading and the different methods of measuring proliferation, there is still a need for better prediction of prognosis in individual patients. Radiology is still not sufficient for the determination of stage. There is still no marker in clinical

use for predicting which low-risk UTUC patients will progress and die from UTUC despite radical surgery, possibly benefitting from additional oncologic treatment. The future probably lies in further development of genetic or other markers that hopefully can be secured in a relatively minimally invasive manner. Biomarkers in fluid biopsies, voided urine or barbotage samples would be helpful to further strengthen pre-RNU risk stratification. To increase survival, further development of oncological treatment is important, as CSS is meagre despite radical surgical treatment.

6 CONCLUSIONS

Paper I found that in situ barbotage cytology identified 91% of all UTUCs, including low-grade UTUCs. This is a high rate compared to that for techniques used in other studies. Barbotage cytology and endoscopic biopsy histology were equally efficient in detecting cancer. Even so, both in situ barbotage and biopsy should be performed in addition to complete URS. In clinical practice, there is a risk that the biopsy material will perish during handling in the operating theatre or in the laboratory or that the material is indeed insufficient. Hence, it would be unwise to omit barbotage. If there is no visible lesion, cytology is a reliable method of finding CIS. Barbotage is also useful for FCM of ploidy and SPF, which in this study strengthened the diagnostic accuracy of grade 1 and grade 3 tumours. Barbotage could also capture cancer cells that are more representative of the whole tumour, which is relevant when the tumour is heterogeneous.

As stage is often not determined in URS biopsies and never in cytology, in **Paper II,** we examined the feasibility of indirect staging of UTUC by analysing the associations of stage with other tumour characteristics. The only tumour characteristics associated with stage were grade, DNA ploidy and SPF. Factors prognostic for CSM were tumour grade, stage and SPF, but not DNA ploidy. Correct tumour grading is essential in the diagnosis of UTUC. Ploidy strengthens the assessment. The role of SPF was further explored in the next paper.

In **Paper III,** we evaluated the proportion of cells in S-phase (SPF) as a predictor of invasiveness and CSS in upper tract urothelial carcinoma (UTUC) and found that the SPF was a good test for predicting both invasiveness and CSS, as indicated by the area under the curve (AUC) of the ROC analysis. Therefore, the SPF can improve the risk stratification of UTUC if used as an add-on test and can be performed with in situ barbotage, endoscopic biopsy or RNU specimens. Furthermore, considering both the WHO 1999 and 2004 classifications together will better predict CSS than using one system alone, and we recommend that pathologists report tumour grades using both classifications. Interobserver variability may be lower with fewer categories, but more clinically relevant information is conveyed using both systems in parallel.

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