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CURRENT AND POSSIBLE FUTURE DIAGNOSTIC METHODS FOR UPPER TRACT UROTHELIAL CARCINOMA

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Cover illustration: Possible future histopathologist assessing a current diagnostic method for the upper urinary tract. ©Alexandra Grahn

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my patients, who inspire me to do research;

To Janne and Hasse, who are no longer with us but were truly inspirational in their knowledge, humbleness, and curiosity; and

To Danuta Golonka. You made this possible. I am forever grateful.

POPULAR SCIENCE SUMMARY OF THE THESIS

Upper tract urothelial carcinoma (UTUC) is a type of cancer affecting the inner lining of the upper urinary tract. It is rare and mainly occurs in patients over 60 years of age. Patients with low-risk disease have a good prognosis, whereas those with high-risk disease have a poor prognosis. Traditionally, the standard treatment has been surgical removal of the kidney and adjacent ureter. However, this is a major surgical procedure that carries significant risks. Local treatment using endoscopic laser ablation has been proven equally effective in the low-risk group, thus reducing the risks related to large surgery and decreased kidney function. Hence, it is very important to separate patients who have high-risk UTUC and require prompt radical surgery from those who have low-risk disease and can safely benefit from local treatment. This thesis includes four studies on the current and possible future diagnostic methods for UTUC to aid treatment choices for a more personalized management of this disease.

Study I compared a type of CT (Multiphase CT urography, MCTU), which was new at the time of the study, to other imaging techniques used at that time and visual inspection during endoscopy. The results showed that MCTU was superior to the other imaging modalities used at that time and that MCTU and endoscopy had different advantages. However, it should be noted that none of the methods were 100% accurate. *Study II* evaluated the accuracy of samples collected during endoscopy of the upper urinary tract. Analysis of cytology and biopsies correctly identified almost all cancers but were not always correct in grading the tumour. Additionally, the results revealed that counting the number of chromosomes in the cancer cells could help differentiate between aggressive and nonaggressive UTUC.

Studies III and *IV* were conducted to evaluate the potential of new diagnostic methods to try to compensate for the shortcomings of the current diagnostic methods. *Study III* was a very small study that suggested that 3D imaging could differentiate aggressive from nonaggressive UTUC. In *study IV*, we looked at patterns of gene mutations in the tumours and found that they were associated with tumour aggressiveness and long-term prognosis.

In conclusion, the current diagnostic methods have different strengths and weaknesses, as well as room for improvement. Using a combination of the current methods and new techniques will likely improve the diagnostic work-up for UTUC, which is necessary for selecting the right treatment strategy for each individual patient.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Urotelial cancer i de övre urinvägarna är cancer i slemhinnan i urinledare och njurens samlingssystem. Cancerformen är ovanlig, dödlig och drabbar främst äldre. Målet för avhandlingens fyra delarbeten var att utvärdera befintliga och nya diagnosmetoder för urotelial cancer i de övre urinvägarna, för att bidra till att förbättra diagnostiken och därmed möjligheterna till individanpassad behandling.

Urotelial cancer i de övre urinvägarna delas in i hög- och lågrisktyp. Den aggressiva högriskformen leder till döden hos cirka hälften av patienterna inom fem år från diagnos, medan den icke-aggressiva lågriskvarianten har betydligt bättre prognos. Under senare år har det visat sig att lokalbehandling, där njuren bevaras, hos patienter med lågriskcancer är lika säker som större kirurgi där hela njuren och urinledaren tas bort. Patienter med högriskcancer behöver radikal behandling, medan patienter med lågriskcancer kan besparas de risker och biverkningar som kommer med större kirurgi och minskad njurfunktion, och ändå få samma behandlingsresultat. Det är viktigt att man vid diagnos korrekt kan fastställa om där finns tumör eller ej och tumörens allvarlighetsgrad, så att man kan välja rätt behandling.

I *studie I-II* utvärderade vi de diagnosmetoder vi använder idag, röntgen och endoskopisk undersökning av de övre urinvägarna; ureteroskopi. I *studie I* jämförde vi en typ av CT-undersökning (MCTU) som var ny vid tidpunkten för studien, med andra röntgenundersökningar som var vanliga då, samt med visuell bedömning av ureteroskopi. Studien visade att MCTU var bättre än de andra röntgenundersökningarna. MCTU var bättre än ureteroskopi på att hitta cancermisstänkta förändringar, till priset av att alla förändringar som hittades inte var cancer. Ureteroskopi var bättre på att fastställa om de förändringar som upptäcktes verkligen var cancer eller ej. Ingen av undersökningstyperna kunde dock hitta alla tumörer. Därför är det viktigt att kombinera diagnosmetoderna, samt att kombinera ureteroskopi med provtagning av sköljvätska för cellprov och vävnadsprover från tumörmisstänkta förändringar.

I studie II studerade vi hur träffsäkra proverna från de övre urinvägarna var. Vi tittade både på cellprover och små vävnadsbitar som tagits vid ureteroskopi. Både cellproverna och vävnadsbitarna var väldigt bra för att hitta cancern, men kunde inte alltid fastställa rätt allvarlighetsgrad. Att räkna kromosomer i cancercellernas cellkärnor kunde hjälpa till att skilja på de minst respektive mest aggressiva tumörerna: de minst allvarliga tumörerna hade normalt antal kromosomer och de aggressiva hade avvikande antal kromosomer.

I studie III-IV utvärderade vi två nya diagnostiska metoder. Studie III undersökte om 3D-mikroskopi, ett sätt att titta på tumören i tre dimensioner, kunde skilja på aggressiva och icke-aggressiva tumörer. Vi fann att det var skillnader i blodkärlens struktur i aggressiva och icke-aggressiva tumörer. 3D-mikroskopi kan möjligen användas som en prognostisk markör i framtiden och studeras vidare. I studie IV studerade vi genmutationer i tumörvävnad och fann

att aggressiva och icke-aggressiva tumörer hade olika mutationsmönster, och att dessa dessutom kunde knytas till tumörens långtidsprognos, det vill säga huruvida patienten fick metastaser och/eller dog av sin cancer.

Sammanfattningsvis undersökte vi befintliga och nya möjliga diagnosmetoder för urotelial cancer i övre urinvägarna. Våra resultat visade att MCTU och att ta prover i samband med ureteroskopi förbättrar diagnostiken och det används idag som standard. Våra studier visade också att det finns styrkor och svagheter hos dessa diagnosmetoder; det är viktigt att känna till dem och kombinera de olika metoderna för att få en så korrekt diagnos som möjligt, så man kan välja individanpassad behandlingsmetod. Nya diagnosmetoder, som 3D-mikroskopi och genetisk analys kan troligtvis tillföra information och förbättra diagnostiken och riskklassificeringen vid urotelial cancer i de övre urinvägarna, men behöver studeras mer i kliniska sammanhang.

Patienter är olika och behöver olika typ av behandling beroende på tumörens allvarlighetsgrad och patientens övriga hälsotillstånd. Korrekt riskklassificering av tumören krävs för säker individanpassad behandling. © Alexandra Grahn

ABSTRACT

Background

Urothelial carcinoma is a type of cancer originating from the mucus membrane of the urinary tract. It most commonly occurs in the bladder but may also occur in the upper urinary tract, then called UTUC. UTUC is mainly detected in sexagenarians and older individuals. The gold standard of treatment has been nephroureterectomy (RNU), but this is a major surgery that carries the risk of significant peri- and postoperative morbidity. In addition, the associated decrease in kidney function affects whether these patients can receive adjuvant chemotherapy. Kidney saving surgery (KSS), such as focal laser ablation via ureterorenoscopy (URS), is increasingly recommended for selected patients, as several studies have reported similar disease-specific survival (DSS) outcomes in patients with low-risk UTUC, irrespective of the surgical method used (KSS vs. RNU). KSS has a significantly lower perioperative morbidity rate but a higher recurrence rate, so these patients require vigilant monitoring and follow-up. For more personalized treatment, it is crucial to distinguish patients with aggressive UTUC who require radical surgery and adjuvant treatment from those with nonaggressive disease who can safely benefit from KSS. This thesis comprises 4 studies on the current and possible future diagnostic methods for UTUC.

Aim

The overall aim of these studies was to improve the diagnostic work-up, to aid treatment choices and, thus, to improve the survival of patients with UTUC. *Study I* aimed to assess the diagnostic accuracy of radiographic and endoscopic methods; *study II* aimed to evaluate the samples acquired during URS; *study III* aimed to investigate whether 3D imaging could be used in the diagnostic work-up; and *study IV* aimed to determine whether gene mutations in the tumour could be correlated to tumour stage, grade and long-term prognosis.

Patients and methods

The studies are based on a prospective cohort of patients referred for diagnostic work-up or treatment of UTUC during the period 2005-2012. *Study I* also included patients without UTUC who were subject to investigation. Histopathological and cytological assessments were used as reference standards. *Studies II-IV* included only patients with UTUC, and RNU specimens were used as a reference standard. The statistical methods used were binary classification tests in *studies I-II*, descriptive statistics in *study III*, and principal component analysis, hierarchical clustering and analysis of variance in *study IV*.

Results

Study I showed that multiphase CT urography (MCTU) had superior diagnostic accuracy compared to other imaging modalities and that MCTU and URS had different strengths. None of the methods were 100% accurate, emphasizing the importance of sample collection during URS.

Study II found that the cytology results of in situ barbotage and histopathology each separately identified almost all cancers but were not always correct in grading the tumour. In addition, there was a significant correlation between tumour grade and ploidy in G1 and G3 tumours, aiding in the interpretation of ambiguous samples.

In *study III*, 3D imaging could differentiate between superficial low-grade and invasive high-grade UTUC among 4 samples.

Study IV showed that the mutational patterns in the tumour correlated with tumour stage, grade and long-term prognosis.

Conclusion

None of the current diagnostic methods are 100% accurate; they all have different strengths and weaknesses. Our results showed that MCTU should be regarded as the preferred imaging modality (unlike at the time of the study) and that the diagnostic accuracy of cytology could be greatly improved if analysed in focal barbotage. The diagnostic accuracy can most likely be improved by using a combination of these modalities. New diagnostic methods, such as analysis of tumour gene mutations and 3D imaging, may add important information to the diagnostic process.

LIST OF SCIENTIFIC PAPERS

I. **Diagnostic accuracy of computed tomography urography and visual assessment during ureterorenoscopy in upper tract urothelial carcinoma**

Grahn A, Melle-Hannah M, Malm C, Jaderling F, Radecka E, Beckman M, and Brehmer M.

BJU Int. 2017;119(2):289-97. doi:10.1111/bju.13652

II. **Diagnostic accuracy of upper tract urothelial carcinoma: how samples are collected matters**

Malm C, *Grahn A*, Jaremko G, Tribukait B and Brehmer M.

Scand J Urol. 2017;51(2):137-45. doi:10.1080/21681805.2017.1295102

III. **Volumetric imaging: a potential tool to stage upper tract urothelial carcinoma**

Grahn A, Tanaka N, Uhlén P and Brehmer M.

World J Urol. 2019;37(11):2297-302. doi:10.1007/s00345-019-02682-1

IV. **Genomic profile – a possible diagnostic and prognostic marker in upper tract urothelial carcinoma**

Grahn A, Eisfeldt J, Malm C, Foroughi Asl H, Jaremko G, Tham E and Brehmer M.

BJU Int. 2021. doi:10.1111/bju.15566

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LIST OF ABBREVIATIONS

BCG	Bacillus Calmette-Guerin
CIS	Carcinoma in situ
CT	Computed tomography
DFS	Disease-free survival
DSS	Disease-specific survival
EAU	European Association of Urology
ESKD	End-stage kidney disease
G	Grade
HNPCC	Hereditary nonpolyposis colorectal cancer
IVU	Intravenous urography
KSS	Kidney sparing surgery
LNU	Laparoscopic RNU
LR-	Likelihood ratio for negative test results
LR+	Likelihood ratio for positive test results
MCTU	Multiphase CT urography
MMR	Mismatch repair
MSI	Microsatellite Instability
NGS	Next-generation sequencing
NNT	Numbers needed to treat
NPV	Negative predictive value
ONU	Open RNU
OS	Overall survival
PET-CT	Positron emission tomography-computed tomography scan
PNRT	Percutaneous nephroscopic resection of the tumour
PPV	Positive predictive value
PUNLMP	Papillary urothelial neoplasm of low malignant potential
RCT	Randomized controlled trial
RNU	Radical nephroureterectomy
RR	Relative risk
SNRUBC	Swedish National Quality Registry for Urothelial Carcinoma
SU	Segmental ureterectomy
TMB	Tumour mutational burden
TNM	Tumour, node, metastasis classification
UC	Urothelial carcinoma
UCB	Urothelial carcinoma of the bladder
URS	Ureterorenoscopy
UTUC	Upper urinary tract urothelial carcinoma

1 INTRODUCTION

Upper urinary tract urothelial carcinoma (UTUC) is a lethal cancer that mainly affects patients over 60 years of age. Disease-specific survival (DSS) is closely related to the aggressiveness of the tumour. Patients with high-risk disease have a 5-year DSS of <50%, and for those with low-risk disease, the corresponding figure is 80-90% (1-3). Potentially curative radical surgery carries significant comorbidities and risks for this patient group. Curative intending radical nephroureterectomy (RNU) has an overall complication rate of 15-40% (33% Clavien–Dindo ≥3) and a perioperative mortality rate of 0.7-1.6% (4). Moreover, the loss of a kidney leads to decreased kidney function, which may lead to further morbidity and mortality. In a recent large register study (5) on patients with renal cancer (whose treatment also affects kidney function), Lundstam et al. reported a 17-fold increased risk for end-stage kidney disease (ESKD) in the renal cancer group compared to the control group within the first year after diagnosis. Furthermore, the 5-year DSS rate was 29% among patients with renal cancer combined with ESKD compared to 64% among those with renal cancer alone. UTUC patients are reported to have a significantly higher risk of creatinine doubling and/or ESKD than patients with renal cancer (hazard ratio 3.13) after RNU (6). Hurel et al. (7) reported ESKD before treatment in 19% of a cohort of 476 patients with UTUC.

Kidney sparing surgery (KSS) is increasingly being advocated due to its substantially lower risk of peri- and postoperative morbidity (1, 8-11) at the cost of a higher risk of recurrence and the need for more invasive follow-up procedures (1, 12-14). Choosing the most suitable treatment modality requires robust and reliable preoperative risk stratification. Over the past decade, there have been major changes in the recommended diagnostic work-up and treatment of UTUC due to research advancements and further technical development. Despite extensive research, the currently used diagnostic methods and risk stratification methods are still in need of improvement.

2 LITERATURE REVIEW

2.1 INCIDENCE

Urothelial carcinoma (UC) is a type of cancer originating from the mucous membrane of the urinary tract. It mainly occurs in the bladder (UCB). Urothelial carcinoma can also occur in the upper urinary tract and is then called upper tract urothelial carcinoma (UTUC). Although UTUC is rare, the annual incidence, reported to be approximately 2 cases per 100 000 inhabitants, is increasing in the western world, especially among certain groups and in certain geographical areas (3, 15, 16). The incidence of UTUC in Sweden is reported to be 3-3,5/100 000 (17). Notably, in the Swedish National Quality Registry for Urothelial Carcinoma (SNRUBC), UTUC of the ureter and UTUC of the renal pelvis are reported as separate entities, including patients with multifocal tumours involving both the ureter and renal pelvis; thus, some patients are registered twice in SNRUBC. However, this likely does not fully explain the observed increase in cases, which is also being reported worldwide. In 2020, 103 patients in Sweden died from UTUC (18). The peak incidence is in patients aged 70-90 years old, and the male-to-female ratio is 3:1. Up to 60% of UTUC patients have invasive disease at diagnosis (19), but a stage migration towards localized disease has been reported (4, 20).

2.2 RISK FACTORS

The urothelium is exposed to toxins excreted in the urine, and UTUC has been linked to several environmental factors. The most documented risk factors for UTUC are smoking and exposure to aristolochic acid. The relative risk (RR) of developing UTUC is increased 2.5–7 times by smoking. Interestingly, the cessation of tobacco consumption decreases the risk again by 60-70% after >10 years of being tobacco-free. Balkan endemic nephropathy and nephropathy caused by the consumption of Chinese herbs predispose individuals to UTUC. Aristolochic acid, produced by Aristolochia plants (Figure 1) commonly found in the Balkan area and in certain Chinese herbal remedies, causes a *TP53* mutation (codon 139 (A:T→T:A)) that has mainly been identified in patients with UTUC and either of the abovementioned nephropathies (21).

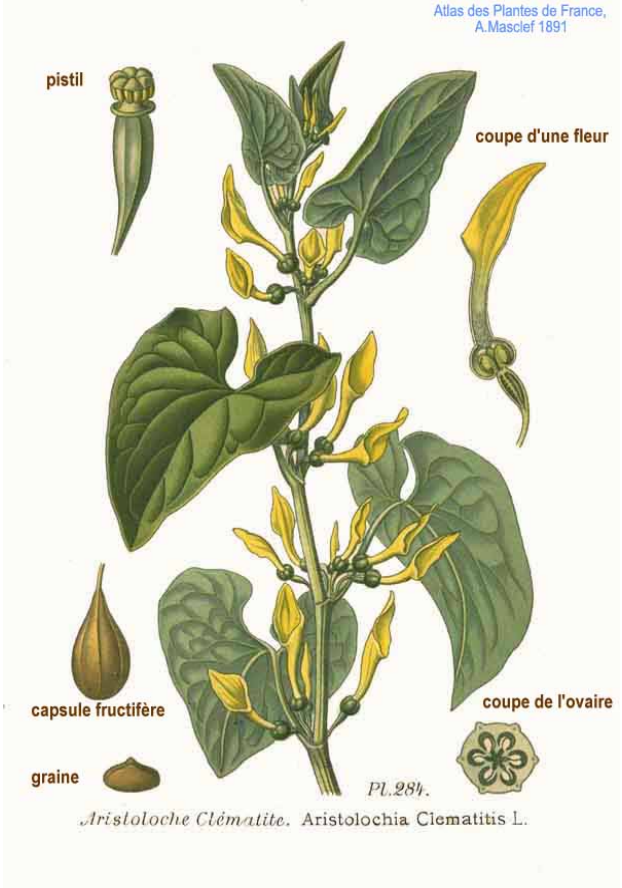

Fig. 1. Aristolochia clematis. Image from public domain. https://commons.wikimedia.org/wiki/File:284_Aristolochia_clematitis_L.jpg

The high incidence of UTUC reported in Taiwan has been linked to arsenic in drinking water (3). Historically, UTUC has also been linked to certain aromatic amines and the analgesic phenacetin. The latency between exposure and cancer development is approximately 20 years, and since these substances have been phased out since the 1960s and 1980s, respectively, these risk factors play a decreasing role.

2.2.1 Lynch syndrome

A total of 5-10% of UTUC cases may be hereditary and linked to hereditary nonpolyposis colorectal cancer (HNPCC)/Lynch syndrome (19, 22). Lynch syndrome is an autosomal dominant condition that predisposes individuals to the development of certain cancers. It is caused by germline mutations in DNA mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6* or *PMS2*). Lynch syndrome associated UTUC is most common in *MSH2* mutation carriers (3). An acquired additional mutation or hypermethylation of the counterpart MMR gene causes microsatellite instability (MSI), a condition that makes the genome more vulnerable to damage and is linked to a broad spectrum of different cancer types (22). UTUC is the third most common malignancy associated with HNPCC/Lynch syndrome after colorectal cancer and endometrial cancer. Patients affected by HNPCC/Lynch have a 22 times higher risk of developing UTUC than the general population (23). HNPCC/Lynch syndrome-associated UTUC should be suspected when onset occurs before 60 years of age or if other HNPCC-related cancer(s) are noted in the patient history or are reported in close relatives (28).

2.3 SYMPTOMS

The most common symptoms of UTUC are haematuria (70-80%) and flank pain (20%). Late symptoms, indicating metastatic disease, are weight loss, fatigue, fever and night sweats (3).

2.4 PROGNOSIS

Patient prognosis depends on the tumour grade and stage: the 5-year disease-specific survival (DSS) is <50% for high-risk disease and 80-90% for low-risk disease (3, 24). Low-risk UTUC patients have similar DSS rates regardless of whether KSS (laser ablation via URS) or RNU is performed. Unfortunately, the prognosis of advanced disease is grim; for metastatic UTUC, the 2-year DSS is 16-29%, and the 5-year DSS is 8-16%, with a median survival time, irrespective of treatment, of 7-12 months (25).

2.5 HISTOLOGY AND CLASSIFICATION

Most cases of UTUC have urothelial origin, but variant histology, such as squamous cell carcinoma, does occur and is associated with a worse prognosis.

2.6 TUMOUR STAGE

The 2016 TNM (tumour, node, metastasis) classification is used for staging (26) (see Table 1). Compared to urothelial carcinoma of the bladder (UCB), the muscle layers of the ureter are substantially thinner (27). This may be why UTUC becomes muscle-invasive at an earlier stage and why a significantly higher proportion of patients have muscle-invasive disease at diagnosis (60% vs. 20% for UCB). In addition, genetic differences between UCB and UTUC may play a role.

Lymph nodes mainly affected by metastasis are the hilar, abdominal paraaortic, paracaval and intrapelvic nodes, depending on location in the upper tract of the tumour. Tumour stage is considered one of the strongest prognostic factors but is difficult to assess before RNU (28-31).

T – Primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Noninvasive papillary carcinoma
Tis/CIS	Carcinoma in situ
T1	Tumour invades subepithelial connective tissue
T2	Tumour invades muscularis
T3	Tumour invades beyond muscularis
T4	Tumour invades adjacent organs or though the kidney into perinephric fat
N - Regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No metastasis to regional lymph nodes
N1	Metastasis in a single lymph node ≤2 cm
N2	Metastasis in multiple lymph nodes or a single lymph node >2 cm
M - Distant metastasis	
M0	No distant metastasis
M1	Distant metastasis

Table 1. Stage classification of UTUC.

2.7 TUMOUR GRADE

The grading system for UCB is also used for UTUC. There are two different grading systems currently in use, WHO 1973/1999 and WHO 2004/2016. The European Association of Urology (EAU) guidelines use both systems since most published studies use the WHO 1973/1999 classification (3).

The WHO 1973 grading system is based on cell anaplasia and includes grades (G) 1, 2 and 3, with G3 being the most aggressive form. A development of WHO 1973 is the WHO 1999 grading system, which is used in Sweden. This system regards papillary urothelial neoplasm of low malignant potential (PUNLMP) as a lower grade than G1 *(32)*. The WHO 2004/2016 grading system is divided into PUNLMP, low- and high-grade. The aim of the WHO 2004 classification was to decrease interobserver variability and create three distinct categories, where PUNLMP is not labelled cancer and the classification of tumours into the other two categories are more tightly linked to prognosis and genetic stability (33). There is no direct translation between the systems. WHO 2004 low-grade is equal to WHO 1973/1999 G1, and high-grade is equal to G3. However, the 1973/1999 G2 guidelines overlap with both low- and high-grade tumours in WHO 2004/2016, see Figure 2. Carcinoma in situ (CIS) is defined as G3 cells growing in a non-exophytic superficial manner.

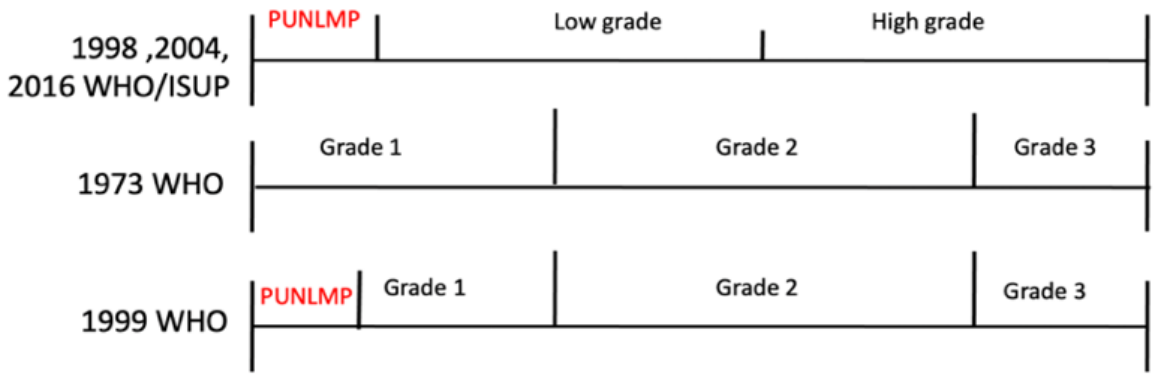

Fig 2. Grading systems. Reproduced with permission by Georg Jaremko.

2.8 CORRELATION OF STAGE, GRADE AND PROGNOSIS

There is a strong correlation between stage and grade. Holmäng et al. (34) showed a correlation of >95% between stage and grade when using the WHO 1999 classification for PUNLPM, G1 and G3 (PUNLMP and G1 being superficial and G3 being invasive). However, when the WHO 2004 classification was used, the correlation between stage and grade was 28% for high-grade tumours. High-grade tumours that were classified as noninvasive were mainly G2. Holmäng et al. also showed that the WHO 1999 classification system offers a more nuanced assessment of DSS. Grade can be assessed from ureteroscopic biopsies and focal cytology and is used for prognostic assessment prior to therapy selection. Using the grading system that offers the best correlation between stage and grade has been an argument for the application of the WHO 1999 classification. Two recent articles have advocated using a combination of the WHO 1973/1999 and WHO 2004/2016 systems (35, 36), resulting in the categories “low-grade G2” and “high-grade G2” with different prognoses.

2.9 CURRENT DIAGNOSTIC WORK-UP

2.9.1 Radiology

The recommended modality for the diagnosis of UTUC has changed over the past decades due to advances in research and technological development, see Table 2 below.

Authors	Year	Results
Gatewood et al. (37)	1982	Case series exploring computed tomography (CT) as modality for investigation of UTUC.
American Urological Association (38)	2001	Guidelines for the investigation of haematuria: Intravenous urography (IVU) is considered…the method of choice, however…CT has increasingly been used.
Gray Sears et al. (39)	2002	CT in favour of IVU.
Oosterlinck et al. *EAU guidelines* (40)	**2004**	**IVU is still the first choice for examination…CT scan may be useful**
Caioli et al. (41)	2005	CT superior to IVU. Several other studies reported similar results (Dillman 2007, Chow 2007, Cowan 2007, Van Der Molen 2008).
Cowan et al. (42)	2007	High rate of false positive findings on CT.
Rouprêt et al. *EAU guidelines* (43)	**2011**	**CT replaced IVU as the recommended modality, discussion about the CT protocol.**
Rouprêt et al. *EAU guidelines* (44)	**2013**	**CT with an excretory contrast phase *should* be performed.**
Helenius et al. (45)	2014	Advantage of an early contrast phase (corticomedullary phase, CMP)
Rouprêt et al. *EAU guidelines* (19)	**2015**	**CT urography *must* be part of the diagnostic work-up.**
Swedish national guidelines (46)	2019	Diagnostic CT should include at least 3 contrast phases.
Janish et al. Review (47)	2020	Sensitivity of 92% and specificity of 95% was reported for multiphase CT urography.

Table 2. Development of the EAU guidelines. Additional references are shown to illustrate the context for the debate in the literature.

One of the problems with evaluating imaging is that the protocols for imaging modalities have changed. With CT, the timing of the contrast phases affects visualization. Modern MCTU comprises 4 phases. In the native phase, without any contrast, calcifications and the outline of the internal organs are visualized. The early contrast phase (corticomedullary phase, CMP) depicts contrast enhancement of the urothelium, and UC is contrast enhanced in this phase. The nephrographic phase shows contrast enhancement in the kidney tissue, and finally, the excretory phase shows contrast in the renal pelvis and the ureter. This phase is similar to IVU, and UTUC can sometimes be visualized as a contrast defect. One possible reason for the initial reluctance to use CT might have been that for a long time, CT scans were displayed in the axial view, which is much less intuitive, see Figure 3. When the coronal view was introduced, it became easier to correlate CT features with patient anatomy. A coronal view of the excretory phase highly resembles that of traditional IVU, which might be why this particular phase has gained popularity among urological clinicians.

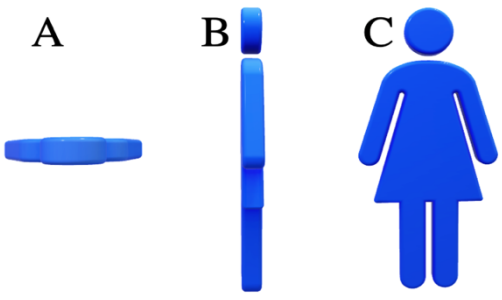

Fig 3. CT projections. © Alexandra Grahn
A. Axial, B. Sagittal, C. Coronal.

The pitfalls of radiologic investigation are associated with the false positive findings that can occur (42). Dillman et al. (48) listed these comprehensively in 2007 as follows: benign filling defects, benign wall thickening and kinks and crossing vessels. Despite technical advancements, these limitations remain, as stated in a review by Martingano et al. in 2020 (49). Crossing vessels can be better assessed by using several contrast phases, but wall thickening and occult filling defects such as prominent papillas (which are common, benign and easily mistaken for tumours on radiographic assessment) require ureteroscopic inspection and sampling. Staging on imaging is decent but lacks the details that are required for clinically relevant treatment choices (50, 51). Last, the contrast agent used in MCTU is nephrotoxic and cannot be given to patients with renal insufficiency, which is a dilemma in these patients.

PET CT was suggested to be beneficial for the primary diagnosis and staging of UTUC by a pilot study by Sassa et al. in 2014 (52), followed by a retrospective study by Asai et al. (53). The trace element for tumour detection in PET CT is excreted in the urine and hence PET CT is not useful the primary tumour detection, but is valuable for metastasis screening. A prospective study of 60 patients by Tanaka et al. in 2016 (54) found that PET CT was superior to conventional CT for detecting metastasis and could add information to guide management.

2.9.2 Endoscopy

Cystoscopy and inspection of the urethra should always be performed to exclude concomitant UCB, which occurs in approximately 17% of patients with UTUC (3). Ureterorenoscopy (URS) is an endoscopic method used to inspect the upper urinary tract. It can be used for visual inspection, for the collection of diagnostic samples (biopsies and barbotage in situ) and to treat UTUC by laser ablation. URS has increasingly been advocated in the diagnostic work-up; see Table 3. Patients with large tumours, obviously not suitable for KSS, should not be subject to URS unless there is diagnostic uncertainty or they are unfit for RNU. Takao et al. (55) reported in a study of 124 patients, that *all* patients with radiographically suspected UTUC in combination with positive voided urine cytology were diagnosed with UTUC. In these cases, URS is not necessary. However, for patients with positive radiographic findings and negative urine cytology, Takao et al. found that only 60% had UTUC. In these patients, URS plays an important role in avoiding RNU in patients without UTUC.

Authors	Year	Results
Keeley et al. (56)	1997	URS can be used in the diagnostic work-up.
Rouprêt et al. *EAU guidelines* (43)	**2011**	**URS is useful when there is diagnostic uncertainty.**
Tsivian et al. (57)	2014	Incorrect diagnoses decreased from 15.5% to 2.1% with routine URS (NNT=7.44).
Golan et al. (58)	2015	RNU was avoided in 42% of patients when URS was added to the diagnostic work-up (in half, URS ruled out UTUC, and the rest could be treated via URS).
Rouprêt et al. *EAU guidelines* (19)	**2015**	**Diagnostic URS and biopsy should be performed.**
Rouprêt et al. *EAU guidelines* (3)	**2020**	**The diagnostic work-up consists of CT urography and URS; the latter should be performed with biopsy if imaging and cytology are not sufficient for diagnosis and risk stratification.**

Table 3. The development of EAU guidelines for diagnostic URS. Some additional references are provided for context.

One of the pitfalls of URS is that it is sometimes technically difficult to visualize the entire upper tract; however, complete inspection is achieved in up to 95% of the attempts (44). Second, flat lesions can be difficult to evaluate. El-Hakim et al. (59) noted that visual assessment alone was inaccurate in 30% of the cases. Yamany et al. (60) found missed lesions in 25% of their patients. Nearly 50% of the missed lesions were CIS. Gillan et al. (61) found a high occurrence of CIS in the upper urinary tract, which was significantly under detected. A strict sample protocol, including mandatory focal barbotage even if the upper tract is macroscopically clear, can aid these matters. There are also technical aids in development, such as narrow-band imaging, confocal laser endomicroscopy and optical coherence tomography, to address the difficulty of assessing flat lesions (3). There was no negative impact on oncological outcomes (DSS, recurrence-free survival and metastasis-free survival) when diagnostic URS was performed before RNU, as reported in a study by Nison et al. (62). Finally, there is an ongoing debate regarding whether URS increases the incidence of recurrence in the bladder, and conflicting evidence has been reported in the literature (3); for more details, please see the section on intravesical recurrence.

2.9.3 Samples

2.9.3.1 Cytology

Voided urine cytology is less sensitive for UTUC than for UCB (63). Cytology has a low false positive rate, but negative cytology needs to be judged carefully due to the high rate of false negative results (38), reported to be up to 50% in low-grade UTUC (63). Furthermore, inflammation can cause cellular changes resembling malignancy. Technical aspects are also important – samples should be taken before the use of contrast agents, as these affect assessment (3). Furthermore, cytology from ureteral urine (acquired by selective ureteral catheterization) and in situ barbotage (acquired during URS) increase the detection rate (63, 64). Theoretically, cytology represents a larger portion of the tumour compared to a small biopsy and thus is less sensitive to tumour heterogeneity.

2.9.3.2 Biopsies

Biopsies can be taken during URS. Since the ureter and, thus, the ureteroscope are very thin, the biopsy forceps and samples are tiny, see Figure 4. This results in up to 11% being insufficient for assessment, as reported by Freund et al. (65). Hence, special care and knowledge are needed, both during preparation and for the histopathological assessment. Biopsy sample collection is a challenge because the whole ureteroscope needs to be removed for each sample to prevent stripping the biopsy. There are larger biopsy forceps that can be front-loaded, but they need to be used though an access sheath. A basket may be used for exophytic tumours.

Fig. 4. Piranha 3 Ch ureteroscopic biopsy forceps. Surgical scissors for size reference. Reproduced with permission by Marianne Brehmer.

The accuracy of URS biopsies has been the subject of several studies over the past few decades. Keeley noted in 1997 that ureteroscopic inspection combined with biopsy and cytology could provide accurate information about both tumour grade and stage (56). EAU guidelines 2004 (40) stated that for tumour grading and staging, URS biopsies have a sensitivity of over 80% but a specificity of approximately 60%. Rojas et al. (66), on the other hand, noted that the accuracy of grading biopsy samples obtained via URS was 92% compared to RNU specimens, and the false negative rate was low. Smith et al. (67) noted with concern that there was a change in stage and/or grade in 1/3 patients who underwent close subsequent biopsies, a figure also reported by others (65).

In a recent review by Subiela et al. (68), the stage and grade of URS-obtained biopsy specimens were compared to RNU specimens. They found a positive predictive value (PPV) of 94% for ≥T1 and a negative predictive value (NPV) of 60% for Ta-CIS. The grade-to-grade matching accuracy was 66% for low-grade tumours and 97% for high-grade tumours. However, to obtain these numbers, Subiela et al. translated the WHO 1973 classification of G1 into low-grade tumours and G2-3 into high-grade tumours, which has been debated (see Figure 2), and this translation affects the assessment of grade-to-grade matching. For the studies that used the WHO 1973 classification, the grade-to-grade matching accuracy was 75%, and it was 66% for those using the WHO 2004 classification. The pooled understaging was 46%. The pooled undergrading was 29% for WHO 1973 and 36% for WHO 2004 classification. One possible reason for this discrepancy might be tumour heterogeneity, as not all cancer cells in the tumour are the same. Since the biopsy samples are small, there is the risk that the cells in the biopsy are not representative of the whole tumour.

In summary, the current biopsy and cytology techniques have certain shortcomings, mainly regarding understaging and false negative rates. These methods can be improved with technical measures and by combining the two approaches (69), but there is still a risk of understaging/undergrading the tumour.

2.10 POSSIBLE FUTURE DIAGNOSTIC METHODS

Considering the diagnostic difficulties encountered with UTUC, new diagnostic methods are needed, which are explored in this thesis.

2.10.1 3D imaging

As further research has allowed for a better understanding of tumour biology, it has become clear that tumours do not solely consist of cancer cells; tumours represent a complex structure comprising tumour cells as well as other cells that are normally present in healthy tissue. These other cells are “under the influence” of the surrounding cancer cells, providing a supportive microenvironment that enables tumour cells to flourish. One of the hallmarks of cancer is angiogenesis, which is needed to supply the tumour with nutrients and oxygen to promote further growth (70, 71). Tumour vessels appear abnormal compared to healthy vessels in normal tissue (70), and an increase in angiogenesis has been correlated with more aggressive and invasive characteristics and a worse clinical outcome (72). 3D imaging is a new technique for the three-dimensional assessment of tumour tissues. It can be used to study the 3D structure

of tumour components other than cancer cells, e.g., the blood and lymphatic vasculature. This method has shown promising results for determining tumour stage in patients with UCB, irrespective of where in the tumour the biopsy was taken (73, 74). However, this technique needs further evaluation.

2.10.2 Molecular diagnostics

Molecular diagnostic approaches have shown value in identifying certain tumour mutations in tumour samples and are used in clinical practice for some malignancies, such as lung and breast cancer (75, 76). The prevalence of certain mutations in the tumour has been linked to both prognosis and the response to certain treatments. To understand how tumour mutations are associated with these features, some basic knowledge of molecular and tumour genetics is necessary.

2.10.2.1 Basic molecular genetics

Genes are the blueprints of all the proteins that are necessary for all cellular functions. Genes are housed in chromosomes, and collectively, the complete set of genes in an organism is referred to as the genome. Biologically important sequences (exons) are embedded in larger portions of DNA; some of these "non-exon sequences" regulate how genes shall be expressed (promoters), others the number of times a cell can multiply (telomeres), yet others we do not know the function of. Mutations, i.e., changes or variants in the genome, can occur during DNA replication (copy during cell division) or due to exposure to toxins or radiation. Our cells have numerous mechanisms of DNA repair, including controlled cell death if all other repair options fail. However, some mutations escape these repair mechanisms. The effect of the mutation depends on both its type (the loss of genetic material versus a substitution that does not alter the function of the protein encoded by the gene) and location (in an exon or a promoter versus in a portion of DNA that is not important for protein function). The effect of the mutation can be an evolutionarily beneficial trait (such as genetic variability in the cells of the immune system), but it can also lead to cancer (which is, strictly speaking, acquired traits that are evolutionarily beneficial to certain cells but not the entire organism) (72, 77).

2.10.2.2 Molecular genetic aspects of cancer development

The development of cancer has been described as a multistep mutational process in which cancer cells gradually acquire different abilities, such as escaping cell death and inducing angiogenesis (70). The number of mutations in cancer cells generally exceeds the number of mutations required for carcinogenesis. A key issue in cancer diagnostics and therapy is to identify the mutations that drive cancer development (driver mutations) as opposed to the ones that occur at random and have no effect (passenger mutations) (72). Acquiring many mutations will increase the risk that a mutation will occur that will drive carcinogenesis but also increase the risk of cell death. Many cells die during this process, but some survive and ultimately drive tumour development.

Tumour development was initially described as a monoclonal expansion of a mutated ancestor cell (72, 78), but later articles argued that polyclonal development *within* the tumour occurs over time (79, 80). In other words, although tumour development starts from one single mutated

cell, over time subpopulations will arise within the tumour that have different genetic mutations and thereby different characteristics. This intratumoural molecular heterogeneity was demonstrated in UCB by Warrick et al. (81).This theory of tumour heterogeneity explains the difficulties experienced with histopathological grade-to-grade matching between URS biopsies and RNU specimens and might explain the development of disease relapse after an initial response to a certain therapy. Intratumoural heterogeneity also poses a problem for molecular diagnostics: a certain mutation may not be present in *all* tumour cells. However, supposing that all the cancer cells originated from a single mutated cell of origin implies that there are certain mutations that are common for all the cancer cells in the tumour.

Yap et al. (82) described the mutational development of different cancer clones as a tree, with the trunk representing collective mutations and the branches representing mutations that are present in some but not all subclones. To have value as a diagnostic molecular marker, the mutation must be located in the trunk of this metaphorical mutational tree, see Figure 5. In addition, the mutation should not be occurring in healthy cells.

Fig 5. The metaphorical mutational tree.
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2.10.2.3 Molecular diagnostic approaches in UTUC

Several studies have explored possible molecular markers for the diagnosis and prognosis of UTUC, as summarized in recent reviews (83-86). UTUC and UCB possess not only similarities (83) but also distinct differences (87, 88). For instance, UTUC seems to have a higher proportion of *FGFR3* mutations than UCB (50 vs. 22%) (85). Interestingly, several studies (20, 89, 90) report that synchronous UCB and UTUC tumours from the same patients share mutational profile, i.e., show clonal relatedness, irrespective of which came first. The degree of clonal relatedness was higher in studies that used chromosomal markers or panel sequencing than in those using whole-exome sequencing. Although the studies are small, this indicates that there are indeed common evolutionary pathways in the tumours. The lower degree of clonal relatedness in studies using whole-exome sequencing might be due to more passenger mutations being detected using this methodology.

In a recent review of genomic alterations of UTUC, Hassler et al. (85) 2020 noted differences in the prevalence of the most frequent genomic alterations in the included studies, especially *TP53* and *FGFR3* mutations. This may be because of the difference in the distribution of high/low-grade and primary/metastatic samples studied. *FGFR3* mutations have been linked to superficial (91) low-grade UTUC (92), whereas *TP53* mutations have been linked to high-grade tumours (92), poor prognosis (91) and metastatic disease (93). However, an overlap has been reported; Audenet et al. (89) reported that 31% of the high-grade tumours in their study had *FGFR3* mutations. Following the theory of multistep carcinogenesis, it might be better to look for combinations of mutations rather than single mutations.

Another approach is to study tumour mutational burden (TMB). TMB is the number of coding single nucleotide variants per mega base of sequenced genome. UC is a toxin-induced cancer. Because the kidneys filter toxins from the blood, which are excreted in urine, the mucosa of the urinary tract is exposed to these toxins as urine passes. This constant exposure to toxins might explain why UC has one of the highest TMBs of all malignancies (94). UCB has a higher TMB than UTUC (95), and high-grade muscle-invasive disease has a higher TMB than low-grade superficial disease (87). Yet another angle to consider is ploidy - the number of chromosomes in a cell. Due to the mutational process described above, cancer cells tend to be aneuploid (having a different number of chromosomes compared to healthy cells).

Genomic profiling has been performed on RNU specimens, URS biopsies and circulating tumour DNA from metastatic UTUC patients. Bagrodia et al. (96) showed a high concordance in gene mutations between URS biopsies and RNU specimens, irrespective of where in the tumour the biopsy was taken. These mutations could also be correlated with tumour stage, grade and DSS of the patients (91). This indicates that there are indeed ubiquitous mutations (from the trunk of the metaphorical mutational tree) that can be both identified and correlated with prognosis. The use of genomic alterations as diagnostic and prognostic markers is rapidly expanding, but neither this method nor any of the markers identified have yet qualified for recommendation in diagnostic guidelines (28).

2.10.3 Urinary biomarker tests

Several urinary biomarker tests have been developed for UCB, and although they are reported to outperform voided urinary cytology, their diagnostic accuracy varies. These tests may offer a less invasive diagnostic and surveillance approach, but the number of studies on their effectiveness is limited, so they are not yet recommended in the EAU diagnostic guidelines (97). Most likely, these tests need to be combined with an analysis of anamnestic risk factors to achieve satisfactory diagnostic accuracy (98). Given that analysis of voided urine has an even lower diagnostic accuracy for UTUC than for UCB (63), these tests are even further from clinical use in UTUC patients. However, a recent study reported promising results for the methylation test EpiCheck in detecting high-grade UTUC in urine obtained by selective ureteral catheterization (99).

2.11 TREATMENT

The treatment for organ confined UTUC is surgery in most cases. There are several different surgical methods available.

2.11.1 Radical surgery

Radical nephroureterectomy (RNU) is the surgical removal of the affected kidney, adjacent ureter and bladder cuff. It can be performed with an open or laparoscopic (conventional, robotic or hand-assisted) technique. Irrespective of the technique, this is major surgery for this elderly patient group, which has significant comorbidities (4). As an example, 51% of the patients in *study I* were ≥ASA3 according to the American Society of Anaesthesiologists' classification system. A higher ASA score is associated with worse DSS after RNU (28). Laparoscopic RNU (LNU) has the same oncological results as open RNU (ONU) (8, 100). A few review studies have sought to evaluate the complication rate of RNU, but many of the studies included did not use the standardized Clavien–Dindo system to report complications, making comparisons difficult. Raman et al. (4) noted a 15-40% overall complication rate depending on the different materials and methods used to report complications. Approximately one-third of the reported complications were Clavien–Dindo 3 or greater. ONU is reported to have a shorter operation time but causes greater blood loss and results in a longer postoperative hospitalization stay than LNU. The perioperative mortality rate has been reported to be 0.7-1.6%. Two studies (101, 102) have reported that there is a median reduction in eGFR of 13.1 ml/min/1.73 m^2 after RNU but with a wide range (-15-77 ml/min/1.73 m^2).

2.11.2 Kidney saving surgery

Several approaches to kidney-saving surgery (KSS) have been developed. In segmental ureterectomy (SU), the diseased distal ureter is removed, and the ureter is reimplanted in the bladder. In percutaneous nephroscopic resection of the tumour (PNRT), percutaneous access to the renal pelvis is established, and the tumour is removed using laser ablation or electrocoagulation. Finally, the upper urinary tract can be accessed through URS, and the tumour can be removed with laser ablation. The advantage of URS compared to PNRT is that the urinary tract is left intact (closed system), which minimizes the risk of seeding metastasis. For URS, irrespective of the indication, the complication rates are reported to be 8-14% in older reports (1, 8). However, the development of the URS technique has been profound during the last decade, which has also affected the complication rate. A large multicentre study by de la Rosette et al. (10) found an overall complication rate of 3.5% after URS for stone disease, with a majority being Clavien–Dindo 1-2, and fever was the most common complication. Ulvik et al. (9) reported stricture in 3% of patients. Sepsis does occur, but it is relatively rare, and perioperative mortality is limited to case reports (103). For PNRT, the overall complication rate is reported to be 27%, with 17% requiring transfusion, 2% experiencing kidney failure and 1% requiring emergency RNU or embolization (1).

2.11.3 Instillation treatment

Bacillus Calmette-Guerin (BCG) or mitomycin can be used for the treatment of CIS in the bladder and to decrease recurrence of exophytic tumours. BCG or mitomycin can also be used for instillation into the upper urinary tract via nephrostomy or ureteric catheters. However, the literature on these approaches is scarce, and published studies are nonrandomized observational

case series. Instillations for exophytic UTUC seem to have no effect. For CIS, the pooled cytology response for BCG instillation was 84%, the pooled recurrence rate was 34%, and the pooled progression rate was 16% (104).

2.11.4 Choice of treatment modality

Nonmetastatic, organ confined UTUC can be treated with curative intent by RNU or elective KSS in low-risk tumours. For high-risk tumours, RNU is the first choice, but KSS can also be used in an imperative (single kidney, kidney failure or comorbidities contraindicating RNU) or palliative setting (decrease of symptoms in patients not fit for radical surgery). KSS was initially used to treat imperative cases; however, with further research and clinical experience, it has evolved into an elective treatment. In the EAU guidelines, RNU was considered the gold standard until 2015 (19). KSS using laser ablation during URS was considered for imperative cases or highly selected elective cases during 2011-2013 (43, 44) and considered for low-risk cases and imperative cases during 2015 (19). Since 2017, the KSS has been recommended for all low-risk UTUC cases (105). Several studies have shown that for low-risk UTUC, URS treatment has the same oncological outcomes as RNU (2, 106-112). In a review, Seisen et al. (24) found similar DSS rates for low-grade noninvasive tumours via URS and PNRT compared to RNU. For SU, Seisen et al. (24) found no significant difference in the oncological outcomes of selected patients based on small nonrandomized series. SU is mainly used if there are contraindications for RNU.

Yamada et al. (102) reported that organ-confined UTUC had a larger decline in GFR (17.9 ml/min/1.73 m^2) after RNU compared to locally advanced disease (5.7 ml/min/1.73 m^2), which in turn had a lower preoperative renal function. This is likely due to the advanced cases having a local extension of tumour invading the renal parenchyma or causing ureteral obstruction. These results indicate that the patients with small tumours have more renal function to lose by RNU than those with advanced tumours and further stress the need for a wise choice between KSS and RNU.

KSS offers preserved renal function, lower complication risks and a shorter hospital stay, but the trade-off is a high risk of recurrence. Recurrence in the ipsilateral upper tract after KSS is reported to be 23-93% for all grades (1, 8, 40, 109, 113). Many recurrences are small and can continue to be treated with KSS (2). However, there is also a risk of progression and an increase in disease-specific mortality (1, 110). Hence, the KSS requires vigilant follow-up, repeated procedures, and flexibility to change the treatment strategy if needed. If progression occurs, it is important not to miss the window of opportunity for cure by promptly planning for RNU. Cutress et al. (1) reported in a review that 20% of patients receiving KSS eventually proceeded to RNU; however, it may take several years. Starting with the KSS may spare the patient several years of deteriorated renal function and the associated comorbidities. Several studies (14, 62, 114-116) have found no negative effect on survival in patients who underwent URS investigation and/or KSS before subsequent RNU. However, for invasive tumours, a delay in performing RNU increases the risk of disease progression (3), which is why correct risk stratification is crucial.

2.11.5 Preoperative risk stratification

In the 2015 EAU guidelines (19), a new comprehensive model for risk stratification was launched to address the need for preoperative risk assessment as the number of treatment choices was growing. A summary of the 2022 update (28) is shown in table 4. Stage and grade are robust prognostic factors (3, 14, 29-31, 117, 118), but stage is difficult to assess before RNU both in samples and on imaging (50, 51). Fortunately, there is a strong correlation between tumour stage and grade (34).

Low-risk UTUC (all factors present)	High-risk UTUC (any factor present)
Unifocal disease	Multifocal disease
Tumour <2cm	Tumour ≥ 2cm
Negative for high-grade cytology	High-grade cytology
Low-grade URS biopsy	High-grade URS biopsy
No invasive aspect on CT	Local invasion on CT
	Hydronephrosis
	Previous cystectomy for high-grade UCB
	Variant histology

Table 4. Preoperative risk stratification according to the EAU Guidelines 2022 update.

Several attempts have been made to develop prognostic nomograms. There are six pretreatment models aimed at predicting muscle-invasive/non-organ confined UTUC. These are based on different combinations of biopsy grade, stage, tumour location, size, architecture, multifocality, local invasion or hydronephrosis on imaging, patient sex, age and haemoglobin level (3, 31, 119). Despite these efforts, the EAU model is probably the most commonly used preoperative risk stratification method (3). The criteria have changed over the years due to the incorporation of new research findings. The influence of multifocality and the cut-off for size have both been debated (24, 117, 120-124). The original cut-off for size was based on a study by Keeley et al. 1997 (125), which stated that size >1.5 cm was a strong risk factor for recurrence. The EAU risk stratification used <1 cm as a cut-off for low risk, which was changed to <2 cm in 2017 (105). The EAU risk stratification model can be used to predict DSS, but despite curative-intent treatment, within five years of diagnosis, 10-20% of the patients in the low-risk group and 50% of the patients in the high-risk group die of UTUC (3). This further underlines the need for additional markers for preoperative risk stratification so that patients who require more aggressive treatment can be better identified.

2.11.6 Postoperative risk stratification

There are five prognostic nomograms that use post-RNU data to predict DSS. These are based on different combinations of age, stage, grade, N-stage (lymph node metastasis), lymphovascular invasion, tumour location, architecture and concomitant CIS. Stage is included in all the postoperative nomograms (3). These nomograms can be used to make decisions about adjuvant treatment.

2.11.7 Intravesical recurrence

UCB and UTUC often occur together (synchronous) or one after the other (metachronous). The reason for this is debated, the main theories being the clonogenic theory (intraluminal seeding from the initial tumour) and field cancerization (several primary tumours develop in a genetically unstable urothelium). Synchronous UCB and UTUC are reported in 17% of all UTUC patients (3). Approximately 3% of patients with primary UCB later develop UTUC (126). Metachronous UCB is common after UTUC and is called intravesical recurrence. In the literature, it is not always stated whether the patients had primary UCB when intravesical recurrence is reported (i.e., whether the diagnosis of UTUC or UCB came first), and hence, whether the new UCB is a recurrence of the previous UCB or a true intravesical recurrence of UTUC is unclear. This makes it difficult to interpret and compare these numbers. Intravesical recurrence is reported to occur in 15-61% of cases after URS treatment of UTUC (1, 12-14), in 24% of cases after PNRT (1), and in 17–47% of cases after RNU (3, 12). There is also conflicting evidence regarding whether diagnostic URS causes an increased risk of bladder recurrence (12, 14, 127). However, two recent review articles showed an increased risk of intravesical recurrence (HR = 1.44) (128) after diagnostic URS but no effect on other oncological outcomes or survival (127, 128). Intravesical chemotherapy (Mitomycin C or similar) administered as a single dose 2-10 days after RNU was shown to reduce the risk of intravesical recurrence in two prospective randomized trials (129, 130) and a meta-analysis (131).

2.11.8 Treatment of metastatic disease

The standard treatment for metastatic UTUC is chemotherapy; the use of RNU for metastatic UTUC is controversial. In a retrospective cohort study, Nazzani et al. (25) found a 3- to 4-month survival advantage in patients who received a combination of RNU and chemotherapy. However, this advantage was only shown in patients who survived more than 12 months, and many patients with metastatic UTUC do not survive this long. Chemotherapy was found to have a larger impact on survival than surgery; patients who were not eligible for chemotherapy had a median survival of 3 months versus 8 months for those who received chemotherapy. The POUT trial (132), a randomized controlled trial, reported better disease-free survival (DFS) in patients with locally advanced UTUC who were treated with adjuvant chemotherapy after RNU. Unfortunately, as many as 76% of UTUC patients with tumours suitable for adjuvant chemotherapy are ineligible after RNU due to the associated decrease in renal function (133). Several recent studies (134-138) have shown the benefits of neoadjuvant chemotherapy, but to date, no randomized controlled trials have been published, so it is not recommended in the EAU guidelines. Immune checkpoint inhibitors can be used for metastatic UC, but many UTUC patients are ineligible due to comorbidities.

2.11.9 Cost–benefit analysis

Although the five-year DSS for low-risk UTUC is 80-90% (2, 109), the five-year and ten-year overall survival (OS) rates are reported to be 57–75% and 40–47%, respectively (1). This reflects the vulnerability of this patient group, who has advanced age and comorbidities. New and technically advanced diagnostic methods, such as NGS, are costly and can be debated in this patient group. However, a correct diagnosis and risk stratification decreases the risk for subjecting the patients to treatments that may be inefficacious, painful and expensive, which is beneficial both to the individual patient and society.

A study of the economic aspects of treatment by Pak et al. (139) reported that KSS is a cost-effective option, as it reduces the costs associated with the treatment of renal failure. From a patient perspective, not all UTUC patients are fit for major surgery, and the loss of a kidney may push the patient into renal insufficiency, which is a risk factor for cardiovascular disease and mortality (5, 140-143). Although there is a higher risk of disease recurrence with KSS, in many cases, it provides sufficient oncological control until the patient dies of other causes (109). Correct risk stratification is also important in metastatic UTUC, where the combination of chemotherapy and surgery gives a small survival benefit for patients who survive more than 12 months (25). Since many patients with metastatic UTUC do not, better diagnostic procedures may help select which patients may in fact benefit from cytoreductive surgery.

3 RESEARCH AIMS

The overall aims of the included studies were to improve the diagnostic work-up of patients with UTUC and, in the long run, to hopefully decrease disease-specific mortality and facilitate more personalized treatment and follow-up protocols. The included studies were developed to evaluate and, if possible, improve upon the diagnostic methods already in use, as well as to evaluate new methods to bridge current knowledge gaps in the literature pertaining to risk stratification and molecular prognostic markers.

The specific aims of each study are as follows, as indicated by roman numerals (see List of Scientific Papers):

I-II. To evaluate and compare the diagnostic accuracy of the current radiographic and endoscopic methods and to evaluate the diagnostic accuracy of samples acquired during URS, including in situ barbotage.

III. To analyse whether the 3D tumour structure can be used to differentiate between invasive high-grade and noninvasive low-grade UTUC in a pilot study.

IV. To investigate whether tumour gene mutations can be used to differentiate between different stages and grades of UTUC and to predict long-term prognosis.

4 MATERIALS AND METHODS

4.1 STUDY DESIGN

Studies I-II are cross-sectional studies based on a consecutive, prospectively collected cohort of patients investigated for UTUC. The cross-sectional study design is commonly used to evaluate diagnostic tests, where the studied index test is performed separately to an independent reference standard. In s*tudy I*, the index tests were imaging and visual assessment of URS, in *study II* samples obtained by URS. The reference standard was available cytological and histopathological results in *study I* and histopathological assessment of RNU specimens in *study II*.

Study III is a small hypothesis-generating pilot study, where the index test was 3D imaging, and the reference standard was histopathological assessment of RNU specimens.

Study I*V* is a diagnostic and prognostic prediction study. A prediction study aims to find factors that can predict the risk of an outcome, irrespective of causality (144). In *study IV,* we evaluated the tumour mutational pattern both in a diagnostic and prognostic setting, as we compared it to both histopathological assessment and the outcomes "cause of death" and "development of metastasis".

Studies I-II followed the STARD guidelines for the reporting of diagnostic accuracy studies, and *study IV* followed the REMARK recommendations for tumour marker prognostic studies. STARD and REMARK are guidelines and nomenclature presented by the EQUATOR network, an umbrella organization of collaborations to increase research quality.

4.2 PATIENTS, DIAGNOSTIC WORK-UP AND TREATMENT

The studies are based on a consecutive prospective cohort of patients referred to our tertiary hospital because of suspected UTUC (flank pain, macroscopic haematuria or malignant cytology in combination with negative findings from the lower urinary tract) or for treatment of UTUC diagnosed during the period from 2005-2012. The diagnostic procedure followed a flow chart, including MCTU and URS for diagnostic samples. *Study I* included both patients with and without UTUC; 148 patients with 174 investigated renal units (renal pelvis and adjacent ureter) were included. *Study II* included 43 patients with UTUC who had had a diagnostic URS prior to RNU. In *Study III*, we selected 4 patients with UTUC from the initial cohort. *Study IV* included (more or less) the same patients as *study II* for details; see Figure 6 and attached *Study IV*, Patients and Methods.

RNU was performed within four weeks after URS. When the projects began, the indications for KSS differed from those used today; initially, most patients underwent RNU, and KSS was reserved for imperative cases. Gradually, more patients were offered KSS as the treatment guidelines shifted. This means that we have both long-term follow-up and all tumour grades and stages represented in these studies. This is unique compared to many current cohorts, which tend to include few patients with low-risk disease and use RNU as reference standard.

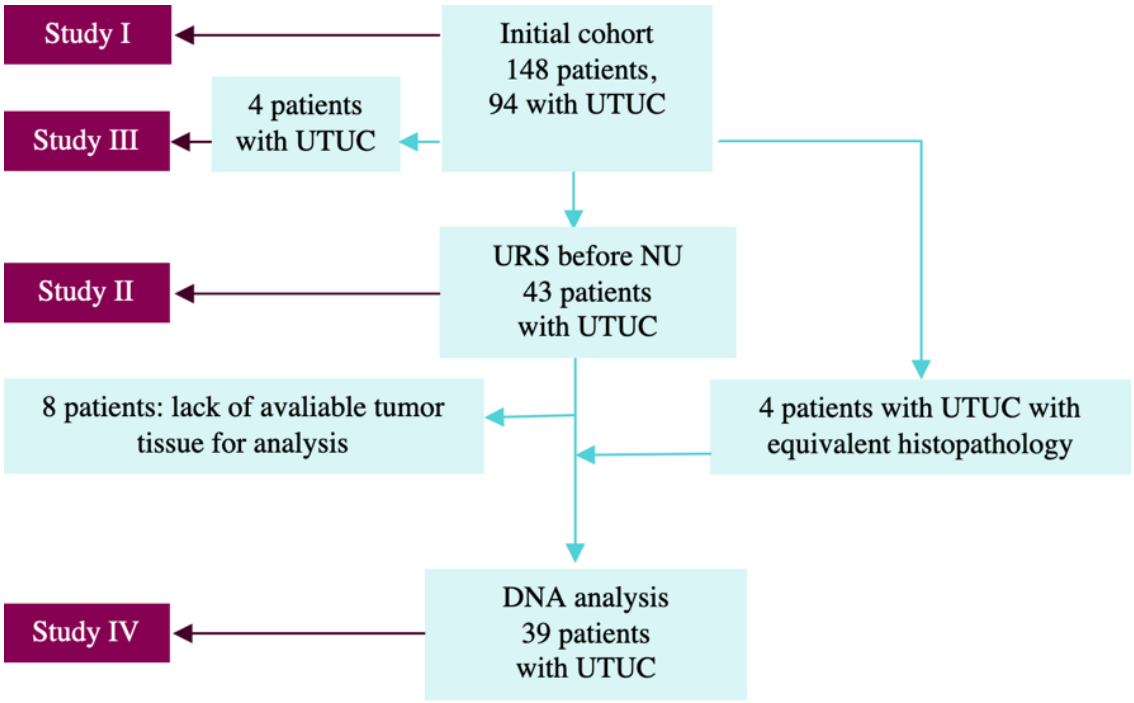


Fig. 6. Flow chart of patients included in the different studies.

4.3 URETERORENOSCOPY

URS was performed in the patients when imaging and cytology assessments were inconclusive for the diagnosis of UTUC. The URS procedure included cystoscopy, the collection of barbotage from the urinary bladder, and then URS with the non-touch technique, first using a semirigid ureteroscope and then a flexible ureteroscope. Barbotage was collected from the renal pelvis, and the fluid that trickled down to the bladder during URS was collected as barbotage from the ureter. Biopsies were taken with a safety guidewire in place using Piranha 3 Ch biopsy forceps (Boston Scientific Nordic AB) in the majority of cases. The entire ureteroscope was removed with the biopsy device to avoid stripping the biopsy, which can occur when it is pulled although the biopsy channel of the ureteroscope. For size assessment of tumours during URS, the tumour was compared to an instrument of known size or measured by aligning the ureteroscope with the proximal margin of the tumour, putting a finger on the ureteroscope at the urethral orifice, and then backing the ureteroscope to the distal margin. The distance that the instrument was backed out is equivalent to the length of the tumour. One urologist performed most of the procedures. The urologist was not blinded to the imaging results.

4.4 RADIOLOGY

At the time of the planning and execution of *study II*, the imaging modality of choice was still being debated. In addition, the optimal CT protocol for the diagnosis of UTUC changed in our hospital during the study period. What is labelled optimal MCTU in the study is a multiphase CT urography entailing *at least* a native, a tissue-enhanced and an excretory phase. The radiologists in *study I* were blinded to the diagnosis of the patients.

4.5 DEFINITION OF DIAGNOSIS AND OUTCOMES

All patients were diagnosed based on histopathological assessment of the samples. When available, the RNU specimen was used for the final diagnosis and reference standard (see the study design for details for the different studies). For size assessment in *study I*, the size of the tumour in the RNU specimen was used as a reference standard. UTUC was regarded as the cause of death if cited as such in the patient records. Metastasis was defined as the presence of recurrent UC in locations other than the bladder.

4.6 DATA MANAGEMENT

All tumour and clinical data were prospectively collected in a database in which personal data was coded. Statistical analyses were performed using IBM SPSS 20, 22 and 23.0, Microsoft Excel for Mac 2011 (14.3.9) and R statistical language. A professional statistician or bioinformatician was consulted for all studies.

4.7 LABORATORY METHODS

4.7.1 Histopathology

Biopsies and RNU specimens were fixed in formalin and stained with haematoxylin-eosin. Cytology was assessed with Papanicolaou staining. Ploidy was analysed using photospectrometry at a rate of 200 cells/s. Samples were initially assessed by specialized uropathologists and later reassessed by a single experienced uropathologist. The reassessment was used in the studies. The pathologist was aware of the aim of the studies but was blinded to the clinical data, except what was stated on the pathology report. In the initial assessment, the WHO 1999 classification was used, and in the reassessment, both the WHO 1999 and 2004 classifications were used. Following the morphological criteria, the G2 tumours in our material should be classified as low-grade according to our collaborating uropathologist. G2 tumours with more advanced abnormalities tended to be classified as G3 in our material. However, the translation between the WHO 1999 and 2004 classifications is debated, and G2 is often translated as high-grade (32, 145). In *study II*, G2 tumours were classified as low-grade, but following discussion and international debate, they were reclassified as high-grade in *study IV*.

4.7.2 3D imaging

3D imaging is a relatively new method to investigate tumour samples. Instead of physically cutting the samples into thin slices, as in conventional histopathology, the sample is left intact, cleared (becoming transparent to visual light), immunolabelled (to highlight interesting targets and/or structures, e.g., vessels) and then imaged with a light-sheet microscope. The recorded data consist of multiple 2D images that, with the help of computer software, can be configured into a 3D image of the tumour, adding a third dimension to the sample, see Figure 7. Depending on the target used, different cell types and intratumoural structures can be segmented out, e.g., tumour vessels.

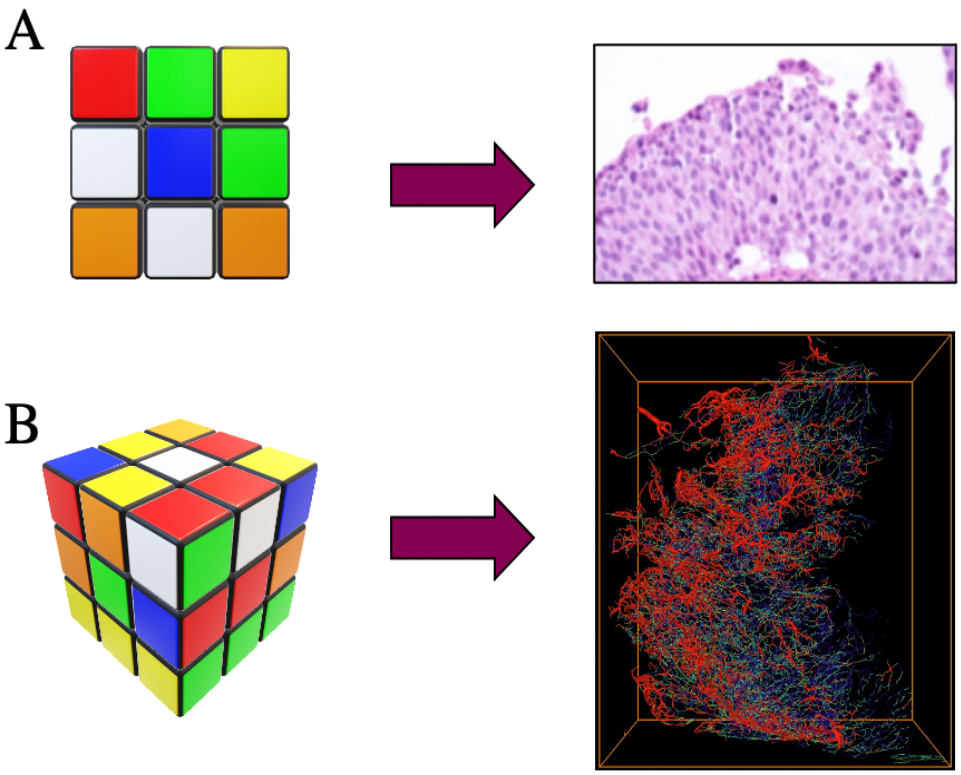


Fig 7.
3D imaging.
A. Conventional histopathology.
B. 3D imaging of vascular network.
© Alexandra Grahn.
Histopathological slide reproduced with permission by Marianne Brehmer, 3D reconstruction with permission from Per Uhlén.

4.7.3 Genetic analysis

Next-generation sequencing (NGS) is a technology used to determine the order of nucleotides in genetic material, i.e., the code in the genome. Very briefly, the process consists of 4 steps:

1. DNA is fragmented, and adapters are ligated to both ends.
2. Adapters bind to the surface of a flow cell, and each fragment is amplified.
3. Fluorescent nucleotides are used to determine the order of nucleotides in the different fragments.
4. Using bioinformatic software, the smaller fragments are aligned and used to configure larger fragments. These are then compared to a reference genome to identify any differences and gene variants.

The identified gene variants can be commonly occurring variants, acquired mutations or artefacts. Depending on the source of the genetic material (fresh or paraffin embedded), the number of artefacts differs. There are several quality parameters that can be used to filter out artefacts (described in detail in supplementary material to *study IV*). Furthermore, whether a gene variant is common in the general population or known to be associated with different diseases or cancer can be evaluated using multiple databases. NGS can be used for the entire genome of an organism, whole-exome sequencing (all exomes) or for selected genes called a panel (146, 147).

For *study IV*, we used a panel including a selection of 388 genes that are commonly mutated in solid cancers. After NGS, we used quality filters to minimize the number of artefacts and excluded known benign and commonly occurring gene variants (unlikely to be causative of a rare cancer) before bioinformatic analysis (Figure 2, *study IV*). Separately, we manually annotated the found mutations, evaluated the effect of the mutation on the protein and selected only known pathogenic mutations for further analysis (Figure 3, *study IV*).

4.8 STATISTICAL ANALYSES

4.8.1 *Studies I-II* – Binary classification tests

For *studies I-II*, we compared a diagnostic test to a reference standard. We used standard methods for evaluating diagnostic tests using 2x2 contingency tables. The different measurements are defined in Figure 8 and below. For *study I*, cytology and histopathology were used as reference standards (i.e., the basis for true diagnosis), and for *study II*, histopathological assessment of RNU specimens was used as the reference standard.

	According to reference standard		
	Have UTUC	Not UTUC	
Positive test	A (true positive)	B (false positive)	PPV A/(A+B)
Negative test	C (false negative)	D (true negative)	NPV D/(D+C)
	Sensitivity A/(A+C)	Specificity D/(B+D)	Accuracy (A+D)/(A+D+B+C)

Fig 8. Overview of binary classification tests.

Definitions:
True positive: patients who test positive for the disease.

True negative: patients who test negative and do not have the disease.

False positive: patients who test positive but do not have the disease.

False negative: patients who test negative but have the disease.

Sensitivity: the proportion of patients who have the disease that is identified by the test (i.e., identification of true positives).

Specificity: proportion of patients who do not have the disease that is identified by the test (i.e., identification of true negatives).

PPV, positive predictive value: proportion of patients who test positive that do in fact have the disease.

NPV, negative predictive value: proportion of patients who test negative that do not have the disease.

Accuracy: proportion of tests that are correct compared to the reference standard.

LR+, likelihood ratio for positive test results: how much more likely it is for a patient with the disease to test positive, compared to a patient without the disease.
LR+ = sensitivity/(1-specificity)

LR-, likelihood ratio for negative test results: how much more likely it is for a patient without the disease to test negative, compared to a patient with the disease.
LR- = (1-sensitivity)/specificity

4.8.1.1 *Aspects of the measurements of diagnostic tests*

If it is necessary to find *all* patients with the disease, then it is suitable to use a test with high sensitivity. However, this comes with a price in terms of more false positive tests, see A, Figure 9. A test with high specificity can rule out disease in healthy patients, which is important if the treatment has strong side effects, see B, Figure 9. PPV and NPV are affected by the prevalence of the disease in the cohort: PPV increases while NPV decreases with the increase in the prevalence of the disease in the cohort. LR+ is good for ruling in a diagnosis. The higher the LR+ is, the better the test. The opposite is true for LR-, which is good for ruling out a diagnosis.

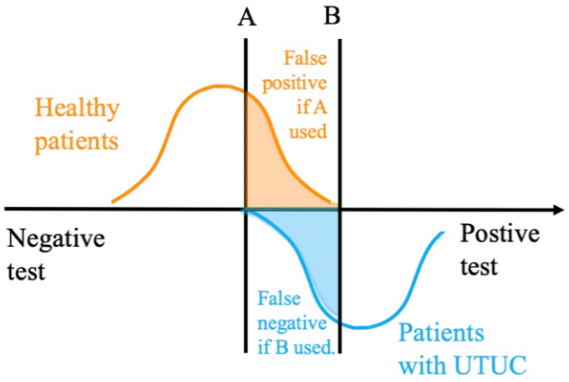


*Fig. 9. Using cut-off A for positive test will find **all** patients with the disease, but also result in many patients without the disease having a positive test, i.e. many false positives. Using cut-off B will result in **only patients with UTUC** having a positive test, but also a larger number of false negatives. In many diseases a cut-off in-between A and B is used. © Alexandra Grahn.*

4.8.1.2 *Fisher's exact test*

For tests of significance, we used Fisher's exact test, which is suitable for smaller sample sizes. The test evaluates the association between two classifications (in this case, the test indicates the presence or absence of UTUC). The test calculates the probability that the set values occurred by chance as opposed to due to an association. The resulting probability, p, is the probability that the null hypothesis is true—in this case, that "no correlation between the index test result and the reference standard". The lower the p value is, the more evidence there is against the null hypothesis. We used 95% confidence intervals and considered $p<0.05$ statistically significant, i.e., low enough to indicate that there was in fact a correlation between the result of the index test and the reference standard. For the statistical calculations performed for *studies I-II,* we used SPSS 22.0, Microsoft Excel for Mac 2011 (v. 14.3.9) and online calculators at http://ktclearinghouse.ca/cebm/practise/ca/calculators/statscalc.

4.8.2 *Study III* – Descriptive statistics

Study III was a small pilot study, so here, we used descriptive statistics only to describe our results. Tests of significance are not applicable in such a small sample size.

The measurements that were described were the density of the vessel marker CD34 in different regions (5-µm z-section) of the samples. The number of regions ranged from 377-506 in the different samples, and these observations formed the dataset for analysing heterogeneity features and constructing 3D models.

Variance: the spread of the observations in the dataset. The square root of the variance is the standard deviation.

Skewness: the symmetry of the distribution of observations. Skew = 0 is a normal distribution, i.e., a symmetrical distribution, whereas skew ≠ 0 indicates an asymmetrical distribution.

Kurtosis: the tails of the distribution. The higher the kurtosis is, the more extreme values the tails entail, see Figure 10.

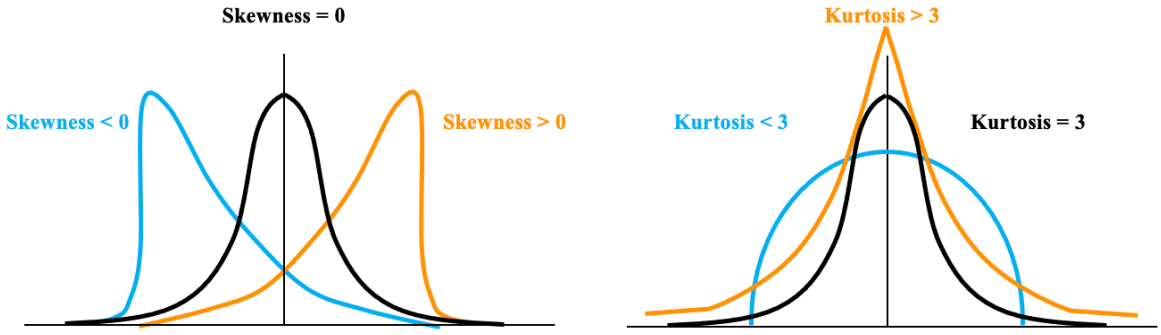


Fig 10. Skewness and kurtosis. © Alexandra Grahn

4.8.3 Study IV – Bioinformatics

The mutational data for *study IV* were a much larger dataset compared to the previous studies, requiring a completely different statistical approach. The results were analyzed in close collaboration with a bioinformatician using R statistical language.

4.8.3.1 Principal component analysis

Principal component analysis (PCA) is not a statistical test but rather a multivariate ordination analysis. It orders the samples in a plane with (normally) 2 axes, PC1 and PC2, with continuous variable values. The parameter with the most variability among the samples is shown on PC1, and that with the second most variability is shown on PC2 (a z-axis can be added for the parameter with the third most variability). Hence, PC1 and PC2 represent a large proportion of the variability among the samples but not 100%. The variability is then graphically illustrated on the plane. If all samples are mixed, then there are no distinct groups within the samples. If the samples are projected in clusters/groups, then there are differences between the groups and similarities within the groups, see Figure 11.

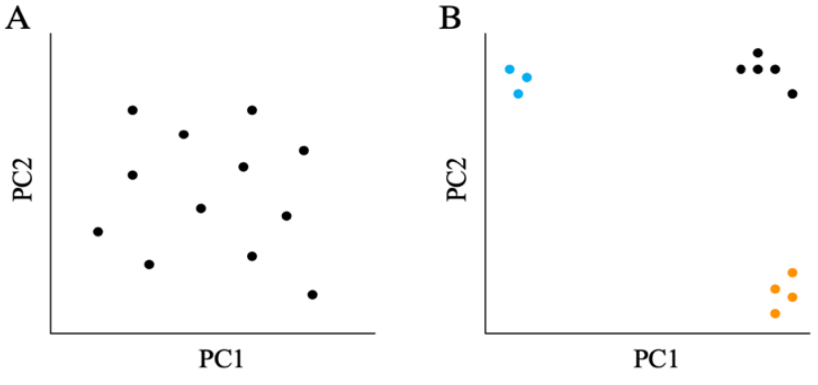
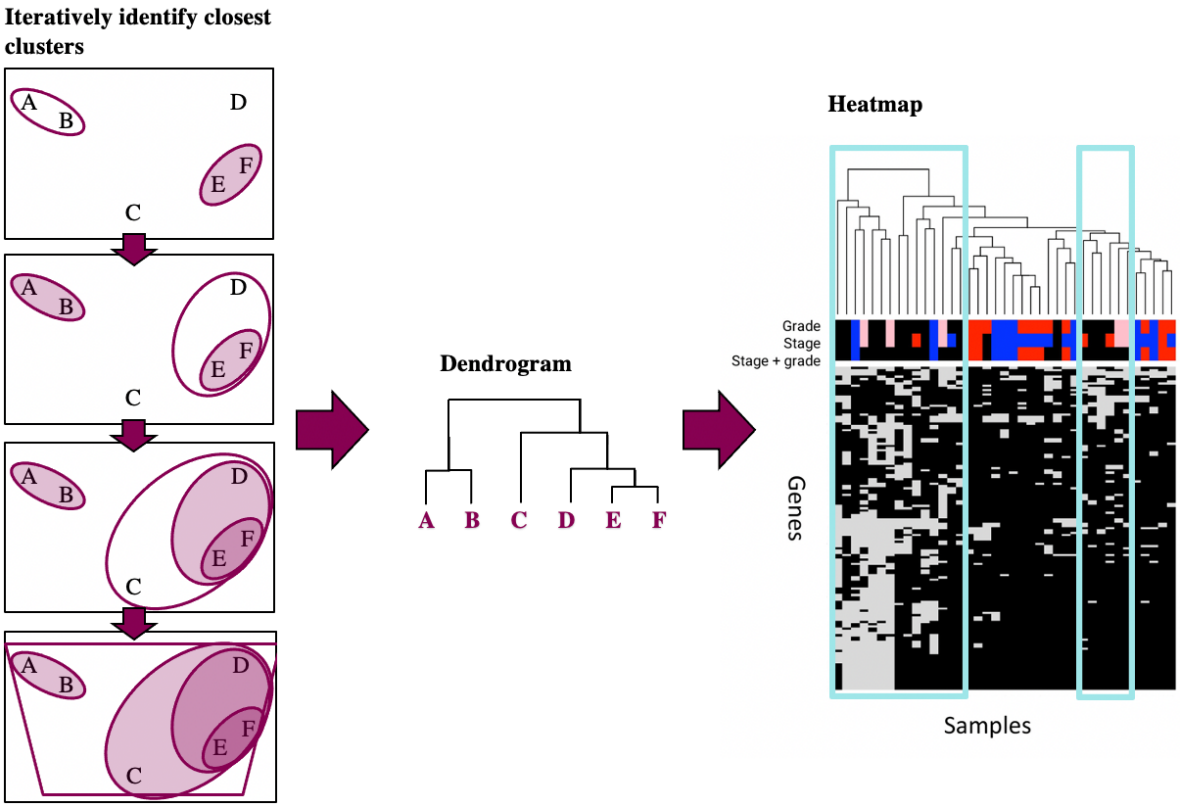


Fig 11. Principal component analysis. A. No distinct groups within the samples. B. Distinct groups with differences, and similarities within the groups. © Alexandra Grahn

4.8.3.2 Hierarchical clustering

Hierarchical clustering is used to illustrate and evaluate the hierarchical relations of different groups of samples or individuals. We used it in *study IV* to evaluate whether samples with the same stage, grade and combined stage and grade had similar tumour mutations. For this method, the computer software counts backwards (agglomerative/bottom-up), pairing the most similar samples in iterative steps. This generates a dendrogram of similarities that can be used to produce a heatmap showing the presence or absence of certain mutations, see Figure 12.

Fig 12. Hierarchical clustering. © Alexandra Grahn



4.8.3.3 Analysis of variance

Analysis of variance (ANOVA) is a collection of methods to, in a structured manner, evaluate whether the variance present in parameters within and between groups is systematic or random. We used repeated ANOVA for comparative analysis in a custom script by our collaborating bioinformatician to compare tumour mutations in the samples based on stage, grade, combination of stage and grade and UTUC as the cause of death. Very briefly, each test group was compared to a control group (e.g., comparing patients who died from UTUC to those who did not). The algorithm searched for mutations present in 4 or more samples in the test group and in less than 50% in the control group. Next, a one-sided binomial test was run to determine whether the test group has a larger proportion of that mutation compared to the control group, generating a p-value for the number of mutation carriers in the test group. In other words, the null hypothesis was "there was no difference in the mutations found", and the alternative hypothesis was that there were differences. Mutations with $p < 0.02$ in the test group were reported as enriched in the test group.

5 ETHICAL CONSIDERATIONS

The studies were approved by the regional ethics committee, and the patients were given oral and written information on the studies before being asked to consent to participate. As stated in the oral and written information to the patients, it was optional to be included in the studies, and they were free to discontinue participation at any point should they wish to. The patients received equal diagnostic work-up and treatment regardless of whether they wished to participate. Patients with deteriorated cognitive ability were not subject to inclusion in our studies. There was no compensation for participation, as the procedures were included in the standard diagnostic work-up. All patient data were pseudonymized and coded.

For all studies, the procedures, potential pain, discomfort, and aspects of integrity were equal to those of standard diagnostic procedures. In some cases, tumours could be ruled out by URS and samples. Although URS is an invasive procedure, it has a low complication rate. Of the 60 first patients subject to inclusion, UTUC could be ruled out in 22%, meaning that RNU could be avoided in 13 patients, 3 of whom would have required subsequent dialysis. RNU is a major surgery with significantly higher perioperative and postoperative morbidity rates, including other issues that arise due to deteriorated renal function and the potential need for dialysis. Furthermore, for low-risk tumours, KSS has the same oncological outcome as RNU. Diagnostic URS involves an extra procedure for the patient if the diagnosis lead to RNU, and there is a higher risk of intravesical recurrence after diagnostic URS, but it has no effect on other oncological aspects or survival (127, 128). However, the advantage of possibly avoiding larger surgery is considered to outweigh the potential risks from this minor procedure. Thus, since many years, diagnostic URS has been included in the standard diagnostic work-up at the included centre. Boorjian et al. (115) showed that URS with biopsy and/or tumour ablation before RNU did not adversely affect the postoperative disease status of patients subject to RNU after URS.

Improved diagnostic work-up is directly beneficial for the included patients in regard to individualized treatment and for those who later relapse, which might be detected earlier and be better characterized due to the results of our ongoing studies. For the genetic study, in theory, integrity is violated to a higher extent than that in the standard diagnostic work-up. However, we only studied a selection of mutations in tumour DNA, not hereditary aspects. This is similar to the evaluation of tumour samples in the standard diagnostic work-up, where certain tumour mutations are investigated with immunohistochemistry; gene sequencing is just a different methodology. MSI can indicate Lynch syndrome, a hereditary syndrome that predisposes patients to develop certain types of cancer at an early age. Our gene panel included an analysis of MSI, but we did not apply for ethical permission to evaluate patients with MSI further in terms of Lynch. Although this would be of academic interest, for the individual patient, the standard diagnostic work-up includes patient history and associated risk factors for Lynch, and should there be any suspicion, the patient is promptly referred for genetic evaluation. In other words, patients with Lynch syndrome are routinely identified, properly investigated and counselled irrespective of our findings.

Performing a randomized controlled trial (RCT) offers a scientifically higher level of evidence compared to the study designs in this thesis. However, since UTUC is a potentially lethal cancer, it is difficult to design an ethically reasonable RTC for the diagnostic work-up.

6 RESULTS

Study I

Compared to non-MCTU imaging, MCTU had significantly higher accuracy, sensitivity and NPV. Compared to MCTU, visual assessment of URS had significantly higher accuracy, specificity and PPV. In summary, MCTU was superior to non-MCTU and had a slightly higher (nonsignificant) detection rate than URS, but the trade-off was a higher false positive rate. URS was better at excluding patients who did not have UTUC and resulted in a higher proportion of patients with UTUC who tested positive. Quite interestingly, in our material, MCTU was better at detecting CIS (seen as contrast enhancement of the urothelial lining) than visual assessment of URS.

Study II

Almost all cancers were identified by both barbotage cytology and histopathology, but grading was not always accurate. However, grade-to-grade matching was statistically significant with both the 1999 and 2004 WHO classifications for both barbotage cytology and URS biopsies. Additionally, low-grade UTUC could be detected using the in situ barbotage method. A statistically significant correlation was found between grade and ploidy in G1 and G3 RNU specimens.

Study III

Using descriptive statistics, considering the density of the vessel marker CD34, differences were found in 2 invasive high-grade tumours relative to 2 superficial low-grade tumours and normal urothelium. The vascular network was more chaotic in the advanced tumours relative to less aggressive tumours and to normal urothelium. There was also a difference observed between superficial low-grade tumours and normal urothelium in this regard.

Study IV

In the blinded assessment, PCA, hierarchical clustering and assessment of the variant count identified 14 tumours with different mutational patterns. Of these, 12 were ≥T2 or G3 tumours of any T. However, there was no enrichment of lethal or metastatic UTUC in this group.

In the unblinded assessment, we found different mutational profiles corresponding to grade, stage, and combined grade + stage. All WHO 1999 grades had different mutational profiles. *FGFR3* mutations were associated mainly with G1 tumours, whereas *TP53* mutations were identified only in G3 tumours.

Patients who died from UTUC or had metastasis had a mutational profile similar to invasive G3 irrespective of the histopathological assessment results.
No patient with TaG1 and a known pathogenic *FGFR3* mutation died from UTUC.
Genes commonly mutated in patients who died of UTUC and/or had ≥T2 and/or G3 tumours were *TP53*, *HRAS*, *ERBB2* and *MGA*.

There was a statistically significant difference in the TMB between superficial and invasive tumours and between G2 and G3 tumours. In other words, the more malignant the tumour, the higher the tumour mutational burden.

7 DISCUSSION

In *study I*, we found a sensitivity of 89% and specificity of 51% for the use of MCTU for UTUC, which is lower than that described in a recent review (47), which reported a pooled sensitivity of 92% and specificity of 95%. CT technology has developed further since our study, which most likely affects these results, but the study methodology also plays a part in this difference. Many studies that have reported on the diagnostic accuracy of CT for UTUC were not primarily designed to evaluate UTUC but rather haematuria or UC (47, 148-150), and many studies have had a very low prevalence of UTUC. As an example, in a review from 2010, all but one of the included studies had fewer than 10 cases of UTUC (151). In other studies, the radiologists were not blinded to the diagnosis of the patient (41, 48), or further investigation, such as URS, was only performed in patients with positive imaging findings (41, 48, 152-154). Undiscovered tumours, i.e., false negatives, could increase the sensitivity in these studies. In the review by Janisch et al. (47), 6 of the included 13 studies were used in the pooled calculations of sensitivity and specificity (the rest included only patients with UTUC and hence could not be included in these calculations). Of these, two studies had <10 patients with UTUC (150, 155), and in two of the remaining 4 studies, URS was only performed for patients with positive findings on imaging (154, 156).

Compared to these studies, *study I* was very stringent in that 97% of the patients had a complete URS, and they all had the same reference standard (histopathological assessment of samples). In our study, the radiologists were blinded to the diagnosis of the patients and the results of URS, although they were aware of the purpose of the study. This might have affected the high prevalence of the assessment "cancer cannot be excluded". After many discussions, we chose to treat this assessment as "cancer" in our analysis, as that is how we regard this assessment in clinical practice. This led to a higher rate of false positive findings in the imaging assessment, which lowered the specificity and PPV for imaging in our study.

Furthermore, we had a high prevalence of UTUC in our cohort. Since we wanted to study the diagnostic accuracy for UTUC, we wanted to include patients who were subjected to this investigation specifically for UTUC, not merely those with haematuria or similar. The high prevalence affected the PPV and NPV, but this finding should be considered in the setting of the investigation of patients with a high risk of having UTUC (which is the setting in which this kind of investigation should be conducted). Wang et al. (157) published a study conducted in 2004-2005 with a similar setup to *study I*; they also had a high prevalence of UTUC and used histopathology as the reference standard. In their secondary assessment, the radiologist was blinded. They found a sensitivity, specificity and accuracy of 85%, 98% and 96%, respectively. Importantly, in their study, only patients with positive radiographic findings had a URS, so the proportion of false negative patients might be underestimated. Furthermore, they used additional alternatives for diagnostic assessment and regarded a "probable presence of UTUC" as "cancer" and an "equivocal presence of UTUC" as "not cancer". This might have contributed to the fewer false positives and, hence, to their comparably higher specificity. In addition, all MCTU examinations were performed according to a 4-phase protocol, which was not the case in our study. The 4-phase protocol was later shown to be slightly superior to 3-phase protocols (158) for the detection of UTUC.

Nonetheless, our study indicated that MCTU had better diagnostic accuracy for detecting UTUC than the other imaging modalities. However, these modalities comprise a heterogeneous group, mostly consisting of CT with other phases. The heterogeneity of this group has been questioned, but we chose not to divide it into the different modalities included to avoid too many small groups and because keeping them together in a single group better mirrored the clinical reality at the time, as different referring physicians used different imaging modalities depending on their choice. At the time of the study, MCTU was still not accepted as a standard imaging modality, as it is today.

The visual assessment of URS had different strengths compared to MCTU but was not 100% accurate, highlighting the fact that it should always be combined with sampling. We found that 84% of the UTUCs were found during visual assessment by URS; 15 of the 16 missed lesions were CIS only. This is similar to but higher than that reported by Yamany et al. (60), who found that URS identified the number and location of tumours in 75% of cases. In their study, they used RNU specimens as the reference standard for all patients. We did not use this approach in this study, which could have led us to miss some false negatives on URS, rendering a higher accuracy. However, in our study, we routinely performed barbotage in situ of the upper tract, which Yamany et al. did not. Gillan et al. (61) performed a study to specifically investigate the detection rate of CIS using URS. They also used RNU specimens as the reference standard in a cohort of 300 patients. They found CIS in 65 patients, 39 of whom had undergone URS prior to RNU; 29/39 cases of CIS were missed by visual assessment of URS. Voided urinary cytology was positive only in 38% of the cases in which it was taken, and barbotage cytology was not routinely performed. Our results combined with those of Yamany, and Gillan emphasize the importance of taking samples during URS, including barbotage cytology, also of the macroscopically clear upper tract.

In *study I,* we did not calculate how the combination of the diagnostic modalities improved diagnostic accuracy, but this has been done in other studies. Favaretto et al. (159) reported an increased accuracy when combining URS and imaging compared to URS alone and Kleinmann et al. (69) reported that cytologic evaluation increased diagnostic accuracy. Tsivian et al. (57) found a significant decrease in incorrect diagnoses with routine URS, and Golan et al. (58) reported that RNU could be avoided in 42% of the patients if URS was added to the diagnostic procedure.

In *study II*, we evaluated the diagnostic accuracy of samples taken during URS using RNU specimens as the reference standard. Histopathological assessment of URS biopsies and cytology of in situ barbotage each identified almost all cancer cases (94% and 91%, respectively); biopsies identified 95% of low-grade and 94% of high-grade UTUC, and cytology identified 87% of low-grade and 95% of high-grade tumours. Messer et al. (63) studied the accuracy of voided urine cytology in a similar but larger cohort of UTUC patients treated with RNU. They found positive urine cytology in only 40% of cases, and 85% of the patients with negative voided urine cytology in their study had high-grade UTUC. For patients with high-grade UTUC, they found positive urine cytology in 54% of cases. Using the in situ barbotage method, we found positive cytology results in 95% of the high-grade UTUCs. Messer further concluded that acquiring selective ureteral cytology and regarding “atypia” as cancer significantly increased the sensitivity to 71% and 74%, respectively. Dev et al. (160)

reported a sensitivity for voided urine cytology and in situ barbotage cytology of 63% and 76%, respectively, also counting atypia as cancer. If we also counted atypia as cancer, 100% of the cancers could be identified in the cytological analysis of in situ barbotage. Our findings in this study have also been confirmed by Zhang et al. (64), who found that high-grade UTUC was correctly identified by cytological analysis in 50% of voided urine samples and in 90% of in situ barbotage specimens.

In our material, the correlation between grade in cytology and biopsies compared to RNU specimens was statistically significant for both WHO 1999 and 2004. In a recent metanalysis of 23 studies, Subiela et al. (68) found a grade-to-grade match between URS biopsy and RNU specimens of 66% for low-grade and 97% for high grade. Our results for biopsies were 85% for low-grade UTUC and 56% for high-grade UTUC. For cytology, we found a grade-to-grade match of 65% for low-grade and 40% for high-grade tumours.

Compared to others in the literature, our grade-to-grade matching rate was lower, especially regarding high-grade tumours. Regarding cytology, we found a significantly higher detection rate compared to other studies. Here, we believe that the barbotage technique can improve the cell yield and thus the diagnostic accuracy. Consistent with Messer et al., we believe that the *method* for acquiring material for cytology matters: the closer you are to the tumour, the higher the diagnostic accuracy. Although we found a very high PPV (94%) and LR+ (19) for high-grade cytology, the sensitivity for high-grade UTUC was only 41%. The strength of barbotage cytology is not in its ability to correctly grade the tumour but in its ability to *identify cancer* if it is present, so it is a very good rule-in test. Our results also underline the importance of judging atypia as a possible sign of pathology in the upper urinary tract. Here, ploidy can aid further decisions: atypia combined with aneuploidy should raise concern and warrant further vigilant follow-up, as aneuploidy is correlated with high-grade tumours (74% of our G3 tumours were aneuploid, whereas all G1 tumours were diploid).

The high accuracy of our study has raised questions about reproducibility and whether the high accuracy is due to the proficiency of our collaborating pathologist. Although he is undoubtedly skilful, we would like to emphasize that this is most likely a result of a successful collaboration among all participants and all procedures in this study - from the method of acquiring barbotage cytology, to the handling of the specimens at the pathology department, to the assessment by the pathologist. The results of Zhang et al. (64) also confirm that such a high accuracy is possible in the right setting and with the right methodology.

Study III was a very small pilot study to investigate whether 3D imaging could differentiate aggressive from nonaggressive UTUC. Naturally, the results are merely a proof of concept and for hypothesis generation. This approach worked for UCB and has a potentially strong advantage for UTUC if it can evaluate tumour invasiveness despite superficial samples. There is a growing understanding that a tumour not only consists of cancer cells but also a large variety of normal supporting cells that are “under the influence” of the cancer cells, which help support cancer growth in different ways (161). 3D imaging is interesting because it offers a 3D view of the tumour and is a unique way of studying the 3D structure of these supporting noncancer cells in tumours. The idea here is that the structure of these supporting cells can contribute valuable information about the characteristics of the tumour.

Different markers and, thus, different tumour structures can be studied with 3D imaging. For our assay, we used the marker CD34. Cells in the umbilical cord and bone marrow normally express CD34, as well as vascular endothelial progenitor cells (162). These are involved in angiogenesis, which is important to tumour development, including growth, progression, ability to metastasize and drug resistance (161). Tumour-derived endothelial cells have an abnormal morphology and phenotype, causing a disorganized vasculature that not only provides the tumour with oxygen and nutrients but also affects immune cell infiltration. Our study showed an increasingly tortuous vascular structure with increasing tumour aggressiveness. Since tumour vessels seemingly affect several essential hallmarks of cancer development (70), studying the tumour vasculature may add prognostic information to the study of tumour cells only.

The next step is to investigate whether 3D imaging can also be used for URS biopsies. These specimens are smaller than the 3 mm punch biopsy specimens collected during the pilot study, which generated datasets of 377-506 measurements of CD34 density. URS biopsies will render significantly smaller datasets, and whether this is sufficient for quantification and differentiation between tumour grades and stages remains to be determined. The assessment of these small URS biopsies has required extensive method development. This work has been carried out by other colleagues in our collaboration, and the results will be published separately. However, the findings seem promising.

Study IV adds long-term follow-up data on the evaluation of gene mutations as prognostic markers. Although smaller than other published studies (91, 163), this study has a significantly longer follow-up time. Our median follow-up time was 10.6 years (range, 0.40-14.4 years), and all patients were followed until death or the end of our study. Patients with a short follow-up time died shortly after inclusion, and the last patient was included in 2012. Regarding survivors only, the median follow-up duration was 11.5 years (range, 9-14 years). The corresponding number for Bagrodia (83 patients) was 3.2 years (range, 0.1-17.3 years), and for Fuji (199 patients), it was 4 years (range, 0.1-19 years) (163; supplementary methods). One could argue that a long follow-up time is less important for high-risk UTUC patients with a 5-year DSS of <50%, but in our work, we had one patient with a G1 tumour who died from UTUC 11 years after diagnosis. These kinds of findings are lost in studies with a shorter follow-up period. In regard to this particular tumour, we found mutations that were otherwise found in tumours with a more advanced histopathological stage and grade. It is highly interesting that an analysis conducted at diagnosis could predict such a long-term prognosis.

We studied how tumour mutations at diagnosis affect long-term prognosis. Most likely, mutations supervene over time, as the phenotype of the tumours tends to change gradually. These mutations probably also affect prognosis, although it is not known to what extent. The plausible effect of later mutations was not investigated in *study IV*. In other malignancies, certain mutations have been linked to targeted therapies, such as MSI for immune checkpoint inhibitors. Most likely, similar mutations can also be found in UTUC. Several studies are investigating molecular markers for UC, which may be included in clinical practice in the future (see Points of perspective).

Several studies have investigated molecular markers in UTUC, as presented in recent reviews by Hassler 2020 and De Lorenzis 2021(85, 86). Although several mutations have been linked to prognosis, the most consistent mutations reported are *FGFR3* and *TP53*. *FGFR3* has been linked to less aggressive UTUC (91, 92), whereas *TP53* has been linked to aggressive disease and a poor prognosis (91-93). However, there is a significant overlap, with up to 31% of high-grade tumours having *FGFR3* mutations (89), and although they have been reported to be mutually exclusive (which also includes *HRAS* mutations), there are tumours that express more than one of these mutations. In our work, we found an initial frequency for *FGFR3* mutations that was similar to that reported in Audenet et al. for high-grade disease, but this was markedly reduced after careful manual annotation, counting only mutations that were known to have a pathogenic effect. This annotation removed much of the overlap, with only two remaining tumours having a combination of *FGFR3* + *TP53* or *FGFR3* + *HRAS* mutations. These tumours were T3G3 and T1G2, respectively, and both patients died of UTUC, which suggests that the presence of *TP53* or *HRAS* mutations overrides the possible advantageous effect of *FGFR3*. Our methodology and results suggest an approach to interpret the coexistence of these mutations. Some studies on tumour mutations in UTUC have investigated certain predefined types of mutations in oncogenes and tumour suppressors (91, 92). We explored all types of mutations in all genes sequenced. Although it was more labour intensive, we chose this method because we found it to be more comprehensive. Our results were similar to others published, which confirms that their method of screening is sufficient.

Finally, our bioinformatic analysis, which is unique among the current literature, showed that in a blinded setup, the tumour mutational profile could be linked to histopathological grade and stage. The comparative bioinformatic analysis showed that certain mutations were enriched in different tumour stages and grades, indicating that the same evolutionary pathways are involved in carcinogenesis in different categories of tumours. To return to the metaphor of tumour mutations as a tree, tumours with the same stage and grade share the same trunk of mutations. Certain mutations were also enriched in the tumours of patients who died of UTUC, indicating that these can be used as prognostic markers and, when detected, warrant more aggressive treatment and follow-up.

7.1 STRENGTHS AND LIMITATIONS

7.1.1 Sample size

One limitation of our studies is the small cohort sizes. Unfortunately, this is the reality when performing single-centre studies on rare diseases, and this is reflected in the sample size of many studies on UTUC. However, this may lead to misinterpreted correlations and wider confidence intervals. Of course, larger multicentre studies are needed. Studies based on national registers are also an alternative to accomplish a larger sample size, but unfortunately, the Swedish National Quality Registry for Urothelial Carcinoma (SNRUBC) does not cover the aspects evaluated in the present studies. An advantage of a smaller cohort is that in-depth analyses are feasible. Our methodology regarding checking all types of detected mutations and whether they had a known pathological effect would be extremely time-consuming in a larger cohort. *Study III* is a small pilot study, and this should be taken into consideration when interpreting the results.

7.1.2 Validity

Validity estimates how well the study can be correlated to real world events. Internal validity is concerned with the study design and the reliability of the observed causality of the studied events. External validity regards generalizability. In the included studies, the small size of the study cohorts gave us good control over the conditions, and the few involved urologists, pathologists and radiologists allowed for a high level of consistency in the assessments, i.e., good internal validity. Reassessment by two agents in each setting would have adjusted for interobserver variability to a greater extent than the current setup and likely would have increased external validity, but this was not done due to resource limitations.

We used a stringent reference standard for all studies. In *study I*, there was possible bias, as biopsies were dependent on visual URS findings. However, barbotage cytology is not, and the reference standard was based on all available histopathological and cytological assessments. Theoretically, a better experimental setup would have been to use RNU specimens for all patients, but that would have resulted in several patients having an RNU despite not having UTUC, which is not ethical. We used blinding to a greater extent than many other published studies on imaging, but the urologists were not blinded to the imaging results, which mirrors clinical practice. In *study II*, the reassessing pathologist was aware of the aim of the study. In *studies III-IV*, the laboratory work and rendering of data, including the bioinformatic analysis in *study IV*, were performed by colleagues blinded to the clinical outcome of the patients.

In *study IV*, we used tumour tissue for genetic analysis. We did not have blood samples for comparison to germline DNA (i.e., constitutional), as this was a historical cohort, and we lacked ethical approval for that. A comparison to germline gene variants would have strengthened our results, guaranteeing that we did not misinterpret any constitutional gene variants as cancer related. However, we used a panel including only known genes that are commonly mutated in solid cancers and took several steps to include only mutations that have a known pathogenic effect. In rare cases, pathogenic variants in some of these genes, such as *TP53*, can occur as constitutional variants. For instance, constitutional pathologic *TP53* variants have a prevalence of 1/5000 - 1/20000 (164) and predispose to Li Fraumeni syndrome, which has an almost 100% lifetime risk of developing cancer at a young age but is rare in patients with UC; thus, we did not expect any of our patients to carry constitutional *TP53* variants. In other words, we feel confident that, due to our stringent data filtration, the mutations we present in the manually annotated material (Figure 3, *study IV*) are highly likely to be both pathogenic and related to UTUC. Naturally, blood samples for comparison to germline DNA should be added to future study designs.

Regarding generalizability, the inclusion of consecutive patients decreased selection bias. All our studies were carried out in cohorts with a very high prevalence of UTUC (all patients in *studies II-IV*) and by professionals who subspecialize in urothelial carcinoma/UTUC and who are proficient in the surgical, laboratory and diagnostic methods used herein. The results are hence most likely not reproducible in a setting outside of a specialized tertiary referral centre and the resources thereof. UTUC is a rare disease, so perhaps the diagnostic work-up and treatment of this condition should be centralized and carried out in specialized centres, as is the

case with other rare malignancies. Regarding validation in other independent cohorts, several studies have demonstrated the superiority of MCTU over other imaging modalities (42, 48, 152, 165, 166), and the findings of Yamany (60), Gillan (61) and Kleinmann (69) underline the importance of collecting both biopsies and in situ barbotage during URS. Regarding *study II*, Zhang et al. (64) found a similarly high detection rate for cytology of in situ barbotage. For *studies III-IV*, our group plans to perform further validation in independent cohorts.

7.1.3 Treatment of uncertain test results

For all diagnostic tests, there are ambiguous test results. In our studies, there were cases in which the index test could not exclude cancer. In *study I*, we regarded an assessment of "cancer cannot be excluded" as "cancer", as in clinical practice. There was a higher proportion of "cancer cannot be excluded" in the imaging assessments than in visual assessments of URS. Whether this reflects the higher diagnostic uncertainty of imaging or the particularities of the involved doctors is unclear. Second assessment by a second clinician would have improved this uncertainty. In *study II*, we chose to regard "atypia" as "not cancer". We chose to regard "atypia" as being further from "cancer" than "cancer cannot be excluded", although to a certain extent, this is lexical semantics. The treatment of these uncertain test results affected our results, as illustrated in Figure 13. In *study I*, this treatment shifted the results towards A, causing an increase in healthy patients with a (false) positive test, lowering specificity and PPV for imaging. In *study II*, counting atypia as not cancer, this shifted the results towards B: lowering the number of false positives and, hence, increasing the specificity and PPV.

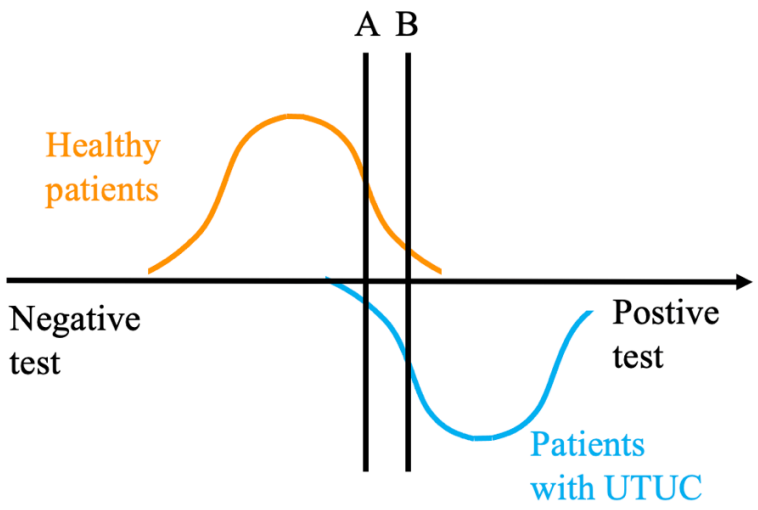


Fig 13. Trade-off in diagnostic tests. © Alexandra Grahn

In study IV, we used several quality parameters to exclude artefacts, and we removed mutations that were common in the general population from the analysis. For the in-depth analysis, we included only mutations with a known pathogenic effect that occurred in three patients or more in our work. This naturally increases the risk of omitting rare and unknown mutations, but our bioinformatic analysis partly compensated for this. However, since our cohort was small, it was not sufficiently powered to detect rare mutations. Furthermore, we aimed to build on current knowledge and look at known pathogenic mutations from a long-term perspective.

8 CONCLUSIONS

UTUC is a potentially lethal cancer (3). However, the loss of a kidney after treatment can also be lethal (5). Hence, a key issue in UTUC is how to optimize the diagnostic work-up to allow for the best possible individualized treatment to be selected. Current diagnostic methods have different strengths and weaknesses, as shown in *studies I-II*. The key is to perform the diagnostic investigations necessary to reliably stratify tumour risk before the treatment modality is chosen and to perform these investigations in the best possible way.

Studies I-II show that there are ways to improve the accuracy of diagnostic methods that were new at the time these studies were conducted. To perform MCTU when not contraindicated, and always take samples during URS, including barbotage in situ for cytology, will improve the diagnostic accuracy. Nonetheless, there are still certain pitfalls, such as the low specificity in MCTU and undergrading in samples, which necessitate the development of new diagnostic and prognostic markers.

In *study III*, 3D imaging identified differences in the vasculature in two invasive and two noninvasive tumours despite the superficiality of the biopsies. In *study IV*, we found that tumour mutations at diagnosis correlated with tumour stage, grade and long-term prognosis. The results of *study IV* agree with the findings in the current literature and illustrate that the correlation between tumour mutations and prognosis continues over a longer timeframe than previously published and suggests a way to interpret the co-occurrence of certain key tumour mutations. Adding new diagnostic modalities will hopefully improve the diagnostic accuracy and allow for more personalized treatment to be provided to UTUC patients.

The diagnostic work-up of UTUC is like building Legos with children. All the pieces are important to gain an understanding of the whole picture, and all participants have a slightly different point of view. If you get along, and everyone does their part, the results can be great.
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9 POINTS OF PERSPECTIVE

9.1 INCLUDED STUDIES IN THE PERSPECTIVE OF CURRENT LITERATURE

The diagnostic work-up and risk stratification of UTUC is a dynamic field of research, and currently, many studies are challenging and changing older paradigms. The inclusion of URS in the diagnostic work-up and the application of KSS were introduced in a wider setting in the EAU guidelines as late as 2015 (19) - the same year their system of risk stratification was launched. A recent study (167) proposed a new 3-tier risk stratification system, where not only low-risk but also some intermediate-risk (multifocal low-grade tumours up to 2 cm) UTUC patients may be considered for KSS. For safe individualized treatment and follow-up, the current diagnostic work-up and risk stratification approaches need further improvement.

When *study I* was planned, there was a debate on which imaging modality to use. When it was carried out, CT seemed superior, but the CT protocols were debated. For a long time, the excretory CT phase was considered the most important phase since it resembles IVU. Within the last decade, CT urography, including at least three phases (preferably four) and including an early contrast phase for enhancement of the urothelium, proved to be superior to other radiographic methods, both in our study and in other works, and is now the gold standard both for diagnostic work-up and follow-up (28, 45, 47, 158, 168).

Study II suggested an improved protocol for sample collection during URS, including in situ barbotage cytology, which had a very high detection rate in our study. Barbotage is easier to perform than biopsy, and the results are theoretically more representative of a heterogenous tumour than a small biopsy sample. This may decrease the known problem of biopsy undergrading, which is probably due to tumour heterogeneity. Adding an analysis of ploidy, especially to cases assessed as atypia, adds further diagnostic information. This study is cited both in national and European guidelines (28, 168).

Study III hints that 3D imaging maybe can offer a possible solution to the problem of small superficial biopsies. Further studies on this method, including its use with URS biopsies, are necessary and underway.

Finally, *study IV* showed that tumour mutational patterns could be used to differentiate different tumour grades, stages and prognostic outcomes in a long-term perspective. In a few cases, the pattern of tumour mutations showed more agreement with the clinical outcome than the histopathological assessment. The methodologies used in *study IV* are well established and are being implemented in clinical practice, which facilitates validation, reproducibility, and possible clinical use in the future. Furthermore, our institution has initiated a molecular tumour board to incorporate molecular characterization into oncological treatment choices, so there is good infrastructure for further studies on tumour mutations.

Several studies have evaluated the molecular subtypes of UCB and their correlation with clinical outcome. Kamoun et al. (169) published a consensus of the molecular classifications of UCB in 2020. They found a strong correlation between overall survival and the consensus classes. Since there are genetic differences between UCB and UTUC, these classes probably

cannot be directly applied to UTUC; however, Fuji et al. published a proposal for the molecular classification of UTUC in 2021 (163). If these results are consistent in further studies, the analysis of tumour mutations may be a valuable complement to histopathological assessment in the future.

9.2 FUTURE RESEARCH AND CLINICAL IMPLICATIONS

9.2.1 More treatment options and genetic analyses

KSS as a treatment for UTUC is increasingly being advocated (3, 19, 43, 167). The POUT trial showed improved disease-free survival in patients with locally advanced UTUC who received adjuvant chemotherapy after RNU (132), and there is a growing body of evidence to support the survival benefit conferred by neoadjuvant chemotherapy to UTUC patients (134-138). Neoadjuvant chemotherapy is particularly interesting in UTUC, as the most effective chemotherapeutic drugs for UC are nephrotoxic, so many patients are not eligible for adjuvant chemotherapy after RNU due to decreased kidney function.

If the molecular classification suggested by Fuji et al. (163) can be validated and linked to long-term prognosis, as for UCB (169), this can complement current risk stratification models (28, 119, 167). UCB molecular subtypes have been correlated with the response to adjuvant therapy (170, 171), which may also be used for UTUC. Furthermore, Kamoun et al. (169) suggested that the molecular similarities between the subtypes of UCB and other malignancies may be used to find new treatment alternatives. This is an expanding field, and many interesting studies have recently been published. Hopefully, ongoing studies, such as the POUT-T trial (https://directory.biobankinguk.org/Profile/Biobank/GBR-1-114), can elucidate molecular markers for diagnosis, prognosis, response to chemotherapy and disease recurrence. In the future, molecular classification may guide not only the choice of surgical strategy (KSS vs. RNU) but also decisions regarding neoadjuvant/adjuvant treatment and the follow-up schedule and -methodology.

9.2.2 Liquid biopsies in diagnostic work-up and follow-up

Tumour mutations in UTUC have been investigated in FFPE tumour tissue (91) and URS biopsies (96) with good concordance. They have also been investigated in circulating tumour DNA (in blood samples) in metastatic UTUC (93) and urinary sediment (163, 172). If we can link certain mutations to certain outcomes, there are very interesting possibilities for liquid biopsies, i.e., sampling and analysis of nonsolid biological tissue, such as blood, urine or in situ barbotage. The use of liquid biopsies could lead to less invasive diagnostic work-up and follow-up if we know that to look for.

9.2.3 The future begins now

An interesting case report illustrating a future scenario was recently published by Blumendeller et al. (173). A patient with high-risk UTUC developed recurrence after initial RNU and palliative chemotherapy. Genetic analysis of the tumour revealed a high TMB, indicating susceptibility to immunotherapy, which was administered and was initially successful, followed by personalized neoepitope-derived multipeptide vaccine and later pembrolizumab. Liquid biopsies were concurrently used to evaluate treatment, and a known tumour-specific variant in the *MLH1* gene was detected in circulating tumour DNA in plasma. The number of mutated molecules per mL of plasma correlated very well with the treatment response as assessed by imaging.

Although this was a case report, the findings illustrate very well how molecular classification and liquid biopsies can guide personalized treatment choices, adjuvant treatment and follow-up. I'm truly looking forward to the development of this field!

Although some hurdles and pitfalls remain in the diagnostic work-up of UTUC, the horizon is expanding in this field of knowledge. The author in the Annapurnas, Nepal 2005.
© Bidhan Kunwar.

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