Prognostic and Predictive Factors in Bladder Cancer

TAMMER HEMDAN
Abstract


Bladder cancer is a potentially curable malignancy; however in regards to the state of current therapy regimens, a plateau has been reached in both the non-muscle and muscle invasive types. To obtain effective treatment, and consequently a decreased mortality, it has become imperative to test and understand aspects affecting therapy response. The aim of this thesis is to illustrate a better understanding of clinical factors affecting therapy response using new drug combinations and new tumor markers alongside established risk criteria. In Paper I we reported the 5 year follow up from a multicenter, prospectively randomized study and we evaluated the 5-year outcomes of BCG alone compared to a combination of epirubicin and interferon-a2b in the treatment of patients with T1 bladder cancer. Treatment, tumor size and tumor status at second resection were independent variables associated with recurrence. Concomitant Cis was not predictive of failure of BCG therapy. Independent factor for treatment failure was remaining T1 stage at second resection. In Paper II &III we investigated the validity of emmprin, survivin and CCTα proteins as biomarkers for response and survival before neoadjuvant cisplatin chemotherapy. Bladder tumor specimens were obtained before therapy from a total of 250 patients with T1-T4 bladder cancer enrolled in 2 randomized trials comparing neoadjuvant chemotherapy before cystectomy with a surgery only arm. Protein expression was determined by immunohistochemistry (IHC). Patients in the chemotherapy cohort with negative emmprin and CCTα expression had significantly better overall survival (OS) than those with positive expression. In Paper IV primary end point was examining STMN1 as prognostic factor in bladder cancer. Analysis was performed on three bladder cancer patient cohorts using IHC, western blot and a bladder cancer cell line. High levels of STMN1, expression correlated to shorter disease-specific survival and the growth and migration of the cells were significantly reduced when transfecting the cells with STMN1 siRNA. Conclusion Risk assessment and predictors of outcomes could help in individualized treatment and follow up. Biomarkers will become more important for treatment choices in bladder cancer management.

Keywords: bladder cancer, BCG, cisplatin, STMN1, emmprin, survivin, CCTα, biomarker, immunohistochemistry, IHC, tissue microarray, TMA

Tammer Hemdan, Department of Surgical Sciences, Urology, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

© Tammer Hemdan 2016

ISSN 1651-6206
urn:nbn:se:uu:diva-282607 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-282607)
To my family
Main Supervisor: **Per-Uno Malmström, M.D.,PhD, Professor**  
Department of Surgical Sciences Uppsala University

Co-supervisor: **Ulrika Segersten, PhD,**  
Associate Professor  
Department of Surgical Sciences Uppsala University

Faculty opponent: **Peter J.Boström, M.D., PhD,**  
Associate Professor,  
Department of Urology,  
Turku University Hospital, Finland

Examination committee: **Gunilla Enblad, M.D.,PhD, Professor**  
Department of Immunology, Genetics and Pathology, Experimental and Clinical Oncology

**Lars Egevad, M.D., PhD, Professor**  
Department of Oncology-Pathology, Radiumhemmet,  
Karolinska Universitetssjukhuset Solna, Stockholm, Sweden

**Michael Häggman, M.D.,PhD,**  
Associate Professor  
Department of Surgical Sciences Uppsala University
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


III  Hemdan T, Malmström PU Segersten U; Choline Phosphate Cytidylyltransferase-α (CCT-α) a novel predictor of response to cisplatin neoadjuvant chemotherapy in urothelial cancer of the bladder. *Submitted*


Reprints were made with permission from the respective publishers.
Contents

1. Introduction.................................................................................................................. 13
   1.1 Epidemiology ........................................................................................................ 13
   1.2 Histopathology and staging .............................................................................. 13
   1.3 Carcinoma in situ (CIS).................................................................................... 14
   1.4 Sub-staging of T1 non-muscle-invasive bladder cancer ......................... 16
   1.5 Natural history of bladder cancer ................................................................. 16
   1.6 Patient selection & screening: criteria, rationale & limitations.............. 17
   1.7 Role of biomarkers; potential benefits and definition ......................... 18
   1.8 Clinical uses of cancer biomarkers ............................................................. 19
   1.9 Prognostic markers ......................................................................................... 20
   1.10 Predictive markers ......................................................................................... 20
   1.11 Methodological aspects of marker evaluation ...................................... 21
   1.12 Prognostication and risk assessment ...................................................... 22
   1.13 Host & environmental related prognostic factors .................................. 23
   1.14 Conventional prognostic factors ............................................................... 24
   1.15 Contemporary prognostic factors .............................................................. 27
   1.16 Current Predictive Tools ........................................................................... 30
      MIBC ............................................................................................................. 31

2. Diseases Management ............................................................................................ 33
   2.1 Non-Muscle invasive Bladder Cancer .................................................. 33
   2.2 Intravesical therapy ......................................................................................... 34
      2.3 Intravesical Immunotherapy ........................................................................ 34
         2.3.1 BCG ...................................................................................................... 34
         2.3.2 BCG failure ........................................................................................ 35
   2.4 Intravesical chemotherapy ........................................................................... 36
      2.4.1 Mitomycin C .......................................................................................... 36
      2.4.2 Interferon α .......................................................................................... 36
      2.4.3 Epirubicin .............................................................................................. 37
   2.5 Muscle Invasive Bladder Cancer (MIBC) .............................................. 37
   2.6 Absolute effect of Chemotherapy ............................................................... 39
   2.7 The anti-cancer agent cisplatin – cytotoxicity and resistance .............. 39
      2.7.1 Mode of action ...................................................................................... 39
      2.7.2 Toxicity & resistance ............................................................................ 40
Abbreviations

ACT  adjuvant chemotherapy
ANN  artificial neural networks
AUA  American Urological Association
AUC  area under the curve
BC   bladder cancer
BCG  Bacillus Calmette-Guérin
CCS  cancer specific survival
CCTα choline-phosphate cytidylyltransferase alpha
CDDP cisplatin
CIS  carcinoma in situ
CRP  C-reactive protein
CTC  circulating tumor cells
CUETO Club Urológico Español de Tratamiento Oncológico
DFS  diseases free survival
DSS  disease-specific survival
EAU  European Association of Urology
EORTC European Organization for Research and Treatment of Cancer
ERCC1 excision repair cross complementing 1
GU   genitourinary
HGTCC high-grade papillary urothelial carcinoma
HPF  high power field
IBCG international bladder group
ICUD International Consultation on Urologic Diseases
IHC  immunohistochemistry
IPOP immediate postoperative instillation of chemotherapy
ISUP The International Society of Urological Pathology
ISUP International Society of Urological Pathology
IVP  intravenous pyelogram
LGTC low-grade papillary urothelial carcinoma
LMP  low-grade cancer
LN lymph nodes
LVI lymphovascular invasion
MIBC muscle-invasive bladder cancer
MMC mitomycin C
MMP metalloproteinases
MVD microvessel density
NACT neoadjuvant chemotherapy
NCCN National Comprehensive Cancer Network
NER nucleotide excision repair
NMIBC non-muscle-invasive bladder cancer
NNT number needed to treat
OS overall survival
PFS progression-free survival
RC radical cystectomy
ROC receiver operating characteristic
STMN1 stathmin 1
SWOG Southwest Oncology Group
TCC transitional cell carcinoma
TNM tumor, node, metastasis
TUNEL Terminal deoxynucleotidyl transferase
TUR transurethral resection
TURB transurethral bladder resection
UC urothelial carcinoma
UCB urothelial carcinoma of the bladder
UCC urothelial cell carcinoma
WHO World Health Organization
1. Introduction

1.1 Epidemiology
Bladder cancer (BC) is a disease of the environment and age. The lifetime probability (from birth to death) of developing bladder cancer is 3.81% in males and 1.15% in females [Siegel R et al, 2013]. It is the second most frequent genitourinary malignancy and the most expensive cancer to care for from diagnosis to death. As life expectancy increases, the number of patients with bladder cancer also expands. The incidence and mortality rates associated with bladder cancer vary by country, ethnicity, gender, and age. It ranks fourth in incidence in men older than 70 years (after prostate, lung, and colorectal cancers), and its incidence steadily increases with age [Ferlay J et al, 2008]. It is estimated that approximately 100,000 new cases each year in Europe are diagnosed. Nonetheless interpretation of incidence rates is difficult, since the rates recorded in various cancer registries may partly reflect different diagnostic criteria [Hankey BF et al, 1991; Lynch CF et al, 1991]. The most frequently diagnosed in the western world is transitional cell carcinoma, now known as urothelial carcinoma (UC) [Kumar et al, 2005]. Interestingly this disease has a biphasic presentation, where approximately 70% presents with non-muscle-invasive tumors and the remainder present with muscle-invasive disease.

1.2 Histopathology and staging
The bladder consists of the urothelium and the underlying layers of lamina propria, muscularis propria (detrusor muscle) and perivesical fat, surrounded in parts by the adventitia. Bladder cancer staging follows these layers with increasing depth of invasion associated with increasing pathologic stage [Amin MB et al, 2013; Babjuk M et al, 2013; Edge SB et al, 2010].

The initial grading system of 1973 had a high inter-observer variation due to the lack of clear definitions for the three pathologic grades with increasingly high percentage of tumors classified as grade 2 [Amin MB et al, 2013; Pan CC et al, 2010; Burger M et al, 2008; MacLennan GT et al, 2007]. In 2004 World Health Organization and the International Society of Urological Pathology (ISUP) introduced a new grading system detailed histologic criteria
to decrease the inter-observer variability with the intermediate grade (grade 2), which was the subject of controversies now disregarded [Amin MB et al., 2013; Pan CC et al., 2010; Burger M et al., 2008; MacLennan GT et al., 2007]. It evolved the three-stage system with grades 1–3 to a two-stage system differentiating between low-grade and high-grade tumors; low-grade papillary urothelial carcinoma (LGTCC), and high-grade papillary urothelial carcinoma (HGTCC) [Eble JN et al., 2004]. LGTCC is defined as urothelium of any thickness that retains overall architecture and polarity but with scattered hyperchromatic nuclei and occasional mitotic figures that are normal and restricted to the lower layers of the urothelium. HGTCC is defined by moderate to marked architectural disturbance that includes frequent branching and fusion of papillary fronds. Loss of polarity, nuclear pleomorphism, irregular chromatin distribution, and prominent nucleoli are frequently present. In addition, denudation may be marked, resulting in naked fibrovascular cores without any adherent urothelium [Eble JN et al., 2004; Amin MB et al., 2013]. The risk of recurrence and progression increase proportionately, with grade using this outline. The 2004 WHO classification [Eble et al., 2004] also separated between non-invasive (NMIBC) and invasive bladder cancer (MIBC), and the term “superficial bladder cancer” has been omitted. This grouping is based on molecular analyses, which found identical molecular alterations in muscle-invasive tumors and “superficially invasive” (pT1) bladder cancers [Sauter and Mihatsch 1998]. pT1 disease is defined by invasion into the lamina propria, which consists of loose connective tissue containing blood vessels, lymphatics and thin, wispy muscle called muscularis mucosae. pT2 disease involves cancer invasion into the large smooth muscle bundles of the muscularis propria (detrusor muscle). Identification of pT2 disease is critical in most patients for the decision to perform cystectomy versus conservative therapy. Staging beyond pT2 can only be performed on cystectomy specimens, as fat may be present in all layers of the bladder wall; thus documentation of pT3 disease is only possible when full thickness sections of the bladder wall are available for review.

To date, though the prognostic value of both grading systems has been validated, the published comparisons of these two grading systems, however, have not clearly confirmed that the new one is superior in terms of reproducibility [Amin MB et al., 2013; May M et al., 2010; van Rhijn BW et al., 2010]. The EAU guidelines recommend using both grading systems as long as the prognostic role of the WHO 2004 is not validated in prospective trials [Babjuk M et al., 2008].

1.3 Carcinoma in situ (CIS)

In both non-invasive and invasive urothelial carcinoma patients, concomitant CIS is associated with high-risk of tumor progression and even metastasis.
From a clinical perspective, extent of disease (focal/multifocal), coexistent invasive carcinoma and recurrence were the principal determinants of clinical outcome in recent studies on CIS [Witjes, 2004]. The WHO 2004 classification simplifies the classification of urothelial dysplasia and separates reactive changes from urothelial dysplasia and carcinoma in situ (CIS). This led to an increase in the diagnostic accuracy of the histopathological diagnosis of flat urothelial lesions. CIS is an obligate precursor of invasive urothelial carcinoma. Primary CIS accounts for less than 1%–3% of urothelial carcinomas. Concurrent CIS can be found in 45%–65% of muscle-invasive and in 5%–19% of non-invasive urothelial tumors [van der Meijden et al, 2005]. Histologically CIS shares histologic features with HGTCC, with disrupted polarity, nuclear pleomorphism and enlargement, prominent nucleoli and frequent mitotic figures high in the urothelium are often present it is characterized by flat urothelium with high cytological atypia with large hyperchromatic nuclei and frequent mitosis. In the diagnostic criteria of CIS, WHO 2004 classification were refined to emphasize that cytological changes including nucleomegaly, hyperchromasia, pleomorphism, and mitotic activity in the mid and upper urothelium were key markers for the diagnosis (Figure 1). However, the infiltrations of the entire thickness of the urothelium by these cells are no longer required for the diagnosis of CIS.

Figure 1 Histopathology (provided by Bladder Cancer Diagnosis, Therapeutics, and Management, 2010©).
1.4 Sub-staging of T1 non-muscle-invasive bladder cancer

The varied prognosis of T1 NMIBC underscores the heterogeneous nature of this disease as the clinical outcomes vary from patient to patient. A critical challenge involves our ability to accurately stage pT1 tumors. Numerous studies have demonstrated that sub-staging of T1 NMIBC (pT1a/pT1b/pT1c) is an important predictor of progression [Holmang S et al., 1997; Smits G et al., 1998; Hasui Y et al., 1994; Angulo J et al., 1995; Orsola A et al., 2005]. Two studies specifically addressed T1 sub-staging in patients treated with BCG therapy. Kondylis et al. [Kondylis F et al., 2000] found no difference in progression for sub-stage, and Orsola et al. [Orsola A et al., 2005] found a significant difference in progression-free survival (PFS) for T1a compared to T1b + T1c. One of the limitations, with pT1 sub-staging is that the muscularis mucosae and vascular plexus is not able to be identified in up to 35% of cases [Holmang S et al., 1997; Smits G et al., 1998; Hasui Y et al., 1994; Angulo J et al., 1995; Orsola A et al., 2005]. Cheng et al. [Cheng L et al. 1999] explored alternative approaches to sub-staging, and measured depth of invasion by micrometer. Additionally, Van der Aa et al. [van der Aa M et al., 2005] defined microinvasive pT1 as a single spot of invasion seen only within one high-power field (under the microscope) and classified pT1 cases not meeting these two criteria as extensive invasive pT1. While these data support pathologic sub-staging, the optimal system has yet to be determined and substaging is not routinely applied in routine pathologic assessment. While a variety of molecular markers have been investigated to provide a better assessment of NMIBC prognosis [Stein J et al., 1998; Karam J et al., 2006; van Rhijn B et al., 2003; Spruck III C et al., 1994], to date the value of such markers over conventional clinicopathologic variables is not clear.

1.5 Natural history of bladder cancer

It should be recognized that the true natural history of untreated bladder cancer in the modern era remains largely unknown. Bladder cancer is rarely found incidentally at autopsy and since almost all bladder cancers cause signs and symptoms and become diagnosed, and virtually all bladder cancers are treated at least by transurethral resection, we really only know the “treated history” [LeeR et al., 2000]. Urothelial carcinoma of the bladder, which is by far the most common histologic type of bladder cancer in most parts of the world, is a very heterogeneous disease. It is considered to develop along two separate pathways. On the one side of the spectrum are the small solitary low-grade cancers (or LMPs) confined to the urothelium which rarely progress and typically do not pose a threat to patient's life, while on the other side are the high-grade muscle-invasive lesions that produce early metastases.
and cause death despite aggressive treatment. Several prognostic factors can be correlated to this behavior including histologic grade, the depth of penetration into the bladder wall (stage), the appearance of vascular/lymphatic invasion, and the presence of carcinoma in situ (CIS). Although clinically useful, histologic risk assessment is not a sufficiently sensitive discriminant in determining the specific biologic potential of a particular cancer. Therefore, an assessment of the genetics and molecular biology of a tumor and of the entire process of carcinogenesis is warranted [LeeR et al, 2000].

1.6 Patient selection & screening: criteria, rationale & limitations

Despite the adoption of increasingly sophisticated multidisciplinary treatment protocols bladder cancer mortality has not been significantly reduced for the past 30 years. This dramatically contrasts with the significant reduction in mortality obtained on cardiovascular and infectious diseases [Watson J et al, 2013]. Depending on the stage of cancer at time of diagnosis the survival rates vary. Of patients submitted to radical cystectomy because of muscle-invasive disease, 57% has muscle invasion at presentation, while 32% has been initially diagnosed with non-muscle-invasive disease that progressed despite the treatment [Vaidya et al, 2001]. This data suggest there are a percentage of patients with superficial disease who are about to die because of progression, and the identification of this group of patients prior to establishment of invasive disease, as well, might contribute to increase the survival of patients affected by BC.

The way to reduce mortality of BC include the diagnosis of the illness at early stages, the improvement of treatment of superficial BC to avoid progression, and the improvement of treatment of muscle-invasive disease to avoid metastatic spreading. This could be achieved by a screening program; however, the role of BC screening remains controversial since no prospective, randomized studies have been conducted to demonstrate that screening reduces mortality in BC. The concept underlying bladder cancer screening is to diagnose diseases in an asymptomatic population before symptoms of the disease develop and to improve outcomes compared to the natural history of the disease. The National Cancer Institute published a statement in which three requirements were determined to be fulfilled to have efficacy of screening [NCI cancer screening overview, 2007.]: 1) Screening leads to earlier disease detection than if the cancer would have been detected because of symptoms. 2) Earlier detection and therefore earlier treatment can improve the overall outcome of the disease. 3) Prospectively collected screen-
ing results show a decrease in disease-specific mortality and an improvement in overall survival.

There are potential issues with screening that need to be considered in bladder cancer. Demonstration of a survival benefit or at a minimum downstaging from muscle invasion to non-muscle-invasive disease is necessary before bladder cancer screening can be recommended. To demonstrate this would require a large prospective study targeting a population at sufficient risk and would ideally incorporate a cost-effective endpoint. Demographic facts have to be taken into account when discussing screening for bladder cancer. There is a risk for over diagnosis of indolent disease which may lead to overtreatment. The accuracy of the screening test is also important since false positive results can lead to patient anxiety and unnecessary testing with attendant complications and cost. Furthermore, false-negative findings in a cohort at risk may lead to a false sense of security. Finally, benefits of population-based screening should outweigh the risks and show cost-efficacy before widespread utilization. A screening test is most effective when the targeted disease either displays a high incidence or a high mortality [Wilson J et al, 1968]. Bladder cancer has a lower incidence and mortality than breast and colon cancer, but higher incidence rates than cervical cancer [Siegel R et al, 2013]. However, the incidence rates include all grades (low and high grade) and stages (invasive as well as non-invasive). Since the low-grade cancers are in most cases not life-threatening, their early detection will have minimal impact on survival and the impact of screening on the detection of high-grade or invasive cancers is most critical [Larre S et al, 2013].

1.7 Role of biomarkers; potential benefits and definition

To reduce bladder cancer morbidity and mortality thereby alleviating both the economic and social costs caused by bladder cancer, there is an urgent need to develop novel tumor biomarker tests which are sensitive and specific enough for early diagnosis, for staging and monitoring disease progression and for predicting and monitoring therapeutic response, paving the way to a “personalized” cancer treatment [Makawita S et al, 2010]. Intriguingly, the state of art on cancer biomarkers research shows interesting and promising results together to clamorous failures.

The Biomarkers Definitions Working Group of the National Institutes of Health defines a biomarker as a cellular, biochemical, and/or molecular (including genetic and epigenetic) characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [Biomarkers Definitions Working Group, 2001]. A cancer biomarker, in particular, is a “biological molecule produced either by the tumor cell or by human tissues in response to cancer that is objectively measured and evaluated as an
indicator of cancerous processes within the body” [Fuzery AK et al, 2013]. Alternatively, a tumor marker may be defined as a “molecule that indicates the presence of cancer or provide information about the likely future behavior of a cancer (i.e., likelihood of progression or response to therapy”) [Duffy MJ et al, 2013].

In fact, an ideal tumor marker should be: (i) easily, quickly measured, not too expensive; (ii) it must be drawn from readily available sources, such as blood or urine; (iii) it should have a high sensitivity and an high specificity; (iv) its levels should vary rapidly in response to treatment; (v) its basal level should permit a risk stratification and prognostic evaluation; (vi) most importantly, its monitoring should be linked to pathophysiology of cancer, above all in term of evolution. Regrettably, none of the present markers meets all the illustrated characteristics.

At present, two main experimental approaches are used to find out new valid tumor markers with acceptable level of specificity and sensitivity, i.e. genomic and proteomic. These approaches could also theoretically overcome a major problem in the discover of valid cancer biomarkers, that is the very low concentrations of markers obtained from tissues with small, early-stage cancer lesions. Until now, as cancer associated markers, research is mainly searching for any cell products including proteins (enzymes, serum proteins, metabolites, receptors, carcinoembryonic proteins, and oncoproteins) and more recently DNA, RNA (also microRNA) and whole cells (circulating tumor cells – CTCs), encoded/contained by suppressor/promoter genes which could have a significant pathogenetic role in transformation/dedifferentiation, proliferation, and metastasis of tumor cells and that can be observed in tissues and/or biological fluids. There is also a current need to stress the right integration of the biomarker level with the clinical picture and the other diagnostic and prognostic parameters.

1.8 Clinical uses of cancer biomarkers

In recent years, research on cancer pharmacology has focused on three perspectives. First, mapping of tumor cell resistance against chemotherapy has aimed at development of more efficient drugs. Second, treatment adjustment after characterization of the tumor has provided an individualized treatment regimen. Third, development of targeted drugs that hit a defined molecular pathway in the tumor cell has evolved as a result of increased knowledge in molecular biology.

The different types of cancer biomarkers that can be used in multiple clinical settings depend on the disease stage (and hence on patient status). Biomarkers, indeed, can be accounted for before cancer diagnosis (in risk as-
assessment and screening for premalignant lesions or early invasive disease), at diagnosis (in staging, grading, and selection of initial therapy) and after diagnosis (in monitoring therapy, selecting additional therapy and detecting recurrence). “Consequently, the spectrum of cancer patient status can range from unaffected individuals who are concerned about whether they should adopt preventive or screening strategies, to patients with early-stage disease for whom considerations of appropriate primary (surgery and radiation) and adjuvant systemic therapies (chemotherapy, hormone therapy, biological therapy or various combinations of these therapies) are critical, to those who are free of disease but are concerned about recurrence, and finally to patients with established metastatic disease” [Paoletti C et al, 2014]. Remarkably, some biomarkers are only used in a specific setting, whereas other ones can serve in more than one mode [Henry NL et al, 2012]. In this regard, tumor biomarkers might be useful for: (1) risk assessment, (2) screening for early cancer detection, (3) diagnosis, (4) prognosis, (5) selection and monitoring of anticancer therapy [Henry NL et al, 2012; Paoletti C et al, 2014; Prensner JR et al, 2012].

1.9 Prognostic markers

Practically, prognostic markers predict “the natural course of an individual cancer, distinguishing good outcome tumors from poor outcome tumors, and they guide the decision of whom to treat and/or how aggressively treated” [Sawyers CL et al, 2008]. Prognostic markers are therefore particularly important at the time of initial diagnosis of malignancy and in cancers that vary widely in patients’ outcome (e.g. prostate and breast cancer) [Duffy MJ et al, 2008; Duffy MJ et al, 2014]. However, as emphasized by Duffy and Crown [Duffy MJ et al, 2014], “no prognostic marker can accurately predict outcome for an individual patient; it provides a probability estimate of outcome for a heterogeneous population of patients”. Importantly, prognostic markers may be crucial to reduce overtreatment of patients with indolent malignancy and so minimizing the side effects of adjuvant systemic therapies, and to avoid under-treatment of patients with aggressive and life-threatening malignancy for which would be recommended to receive the most appropriate local and systemic therapy [Duffy MJ et al, 2014]. In the last years, hundreds of prognostic biomarkers have been proposed, but few have progressed to clinical use.

1.10 Predictive markers

Predictive markers are molecules that “provide upfront information as to whether or not a patient is likely to benefit from a specific therapy” [Duffy
MJ et al, 2011]. Predictive biomarkers assess the likelihood that the tumor will respond to the drug, and thereby allow a level of personalization to be introduced into the treatment regimen [La Thangue NB et al, 2011]. There are a small number of predictive biomarkers that have found clinical utility [August J, 2010], and others are gaining clinical acceptance as objective measurements that inform on the clinical response to the drug (i.e., only patients expressing the marker will respond to the specific treatment or will respond to a greater degree than those without the marker) [La Thangue NB et al, 2011; Sargent DJ et al, 2005]. Predictive markers, by prospectively differentiating populations of “responder” from “non-responder” patients, can guide the choice of anticancer therapy [Sargent DJ et al, 2005] thereby saving patients from unnecessary side effects [Duffy MJ et al, 2014]. At the same time, predictive markers might result in considerable cost savings (especially for the new biological therapies), as anticancer drugs would be used only in patients likely to derive benefit.

1.11 Methodological aspects of marker evaluation

The efficacy of a bladder cancer marker is determined by its performance characteristics.

Prevention of false positive tests results is critical for bladder cancer screening because false positive tests yield unnecessary costs, potential complications associated with confirmatory testing (i.e., cystoscopy and upper tract evaluation), and undue patient anxiety. False negative tests, on the other hand, potentially render false reassurances which can result in adverse outcomes should the patient delay seeking evaluation. Marker accuracy is often characterized by sensitivity, specificity, and positive predictive value. These performance characteristics are not completely determined by the intrinsic properties of the marker; rather they are also dependent on the cohort undergoing marker testing [Nielsen et al, 2006].

To be clinically useful, a cut-point needs to be established which dichotomizes the result into a positive or negative test. A useful method for evaluating the performance characteristics of a marker is the receiver operating characteristic (ROC) curve. The perfect marker with 100% sensitivity and 100% specificity would have an area under the curve (AUC) = 1. On the other hand, an AUC = 0.5 indicates that the marker has a 50–50 chance of correctly detecting the presence of cancer. Various methods to designate the “optimal” cut-point have been proposed (Zweig and Campbell 1993; Nielsen et al, 2006), but the clinical application of the marker greatly influences the appropriate cut-point. In a screened population, the false positive rate should be very low to avoid unnecessary diagnostic work-ups, especially if these work-ups are expensive or invasive, as in bladder cancer [Nielsen et al, 2006]. In a surveillance protocol, however, the consequences of failing to
detect cancer outweigh the consequences of false-positive tests in patients with a known history of bladder cancer. As a result, specificity becomes more important for markers used to screen an asymptomatic population whereas sensitivity is more important for cancer detection during surveillance [Nielsen et al, 2006].

Development of ROC curves and subsequent marker cut-points are subject to bias from unrealistic control populations and over fitting [Baker, 2003]. As pointed out by Nielsen et al. [Nielsen et al, 2006], the characteristics of the control arm in a study population is a key factor in determining the performance features of biological markers for bladder cancer detection.

Overfit bias occurs because so many different marker cut-points can be generated for the available, sometimes limited data. By chance alone, one of these cut-points is bound to provide excellent accuracy. In order to avoid over fitting bias, internal validation is often performed. Internal validation can be performed by randomly splitting the data into a training set and a validation set. A few of the marker cut-points with the best performance obtained from the training set can then be selected to be tested in the validation set. In addition, further confirmation of appropriate marker cut-point can be evaluated in a prospective analysis from a separate population [Baker, 2003].

1.12 Prognostication and risk assessment

Bladder cancer is a heterogeneous disease. The clinical goal in bladder cancer management is to predict the fate of an individual tumor and to select the optimal individual treatment. This has led the way for the risk stratification of histopathologic features including tumor grade, depth of invasion, multiplicity, size, and morphology, the presence of vascular or lymphatic invasion, and the presence or absence of CIS. With high risk of recurrence and progression and the need for prognosis/predictive response to chemotherapy, further biomarker testing is warranted. Prognostication and risk assessment are essential for treatment decision-making, patient counseling, and determination of eligibility for clinical trials. The extent of this malignancy is highly correlated with its prognosis and with the options to treat it. Clinicians can only decide the appropriate therapy if they are informed as completely as possible how bladder cancer has affected the parent organ and its surroundings, the lymph nodes and the distant organs by possible metastases. These staging systems provide useful estimates of survival outcome; however, the inherent heterogeneity of tumor biology, patient characteristics, and variability in the thoroughness of surgical staging lead to significant variation in outcomes within each stage category. Furthermore,
current staging systems for urothelial carcinoma of the bladder (UCB) do not incorporate important clinical, pathological, and molecular markers of disease outcome. That said, many patients with UCB are elderly and have significant comorbidities, thus competing risks are important in evaluating outcomes and choosing personalized therapies. Therefore an adequate staging system is necessary. Such a staging system should be adopted by a majority of clinicians, radiologists and pathologists. A number of modalities are performed in combinations in order to achieve an accurate prognosis and prediction of outcome: transurethral resection (TUR), bimanual palpation before and after TUR, radiological imaging techniques and pathohistological examination. Despite these combinations and the improvement of all methods available, the inaccuracy of staging in bladder cancer is 30–50% [Richie JP et al 1998]. This is especially true in those patients for whom bladder preservation is preempted. Once the cystectomy specimen and the removed lymph nodes after radical cystectomy are available, the staging and grading is much more complete and reliable [Hall RR et al, 1999] In patients with non-muscle invasive bladder cancer (NMIBC), predictors of outcomes could help in the decision making regarding follow-up scheduling, administration of intra-vesical instillation therapies (immediate postoperative instillation of chemotherapy (IPOP) and/or adjuvant) [Sylvester RJ et al, 2004], and/or early radical cystectomy (RC). In patients with muscle invasive bladder cancer (MIBC) who underwent RC, an accurate prediction of the presence of lymph node metastasis and the probability of disease recurrence is essential for selecting patients who might benefit from neoadjuvant and/or adjuvant systemic chemotherapy [Meeks JJ et al, 2012].

1.13 Host & environmental related prognostic factors

Incidence of bladder cancer rises monotonically with age: the disease is rare prior to age 35 and two-thirds of the cases occur in people aged 65 or older [American Cancer Society et al, 1998; Liu L et al, 1998]. Age at diagnosis has been shown to be associated with disease recurrence, disease progression [Fernandez-Gomez J et al, 2009; Kluth LA et al, 2013], cancer-specific mortality [Kluth LA et al, 2013], and response to Bacille Calmette-Guérin (BCG) [Saint F et al, 2003; Joudi FN et al, 2006; Herr HW, 2007; Shariat SF et al, 2010; Rosevear HM et al, 2011]. Worse outcomes in elderly patients could be attributed to changes in the biologic potential of the tumor and host as well as to differences in quality of care (i.e. less aggressive treatment in the elderly) and comorbidities [Shariat SF et al, 2010; Gray PJ et al, 2013]. Nielsen et al. have shown in a study of 888 patients who underwent RC for UCB that higher age at RC is associated with extra vesical disease, pathologic upstaging, and higher cancer-specific mortality. These findings have been subsequently validated in several studies [Nielsen et al, 2007;
Thrasher JB et al, 1994; Clark PE et al, 2005]. The influence of gender on the incidence, staging, prognosis, and survival in UCB has been poorly investigated and understood [Fajkovic H et al, 2011]. Despite UCB being more common in men, women with bladder cancer have worse survival than men in both non-muscle invasive [Fernandez-Gomez J et al, 2009; Kluth LA et al, 2013(b)] and muscle-invasive bladder cancer [May M et al, 2013; Otto W et al, 2012]. The impact of prior recurrences on outcomes of patients with NMIBC has also been assessed by several studies [Sylvester RJ et al, 2006]. In a study comparing primary and recurrent tumors, Alkhateeb et al. have shown that recurrent tumors were at higher risk of disease progression [Alkhateeb SS et al, 2010] Likewise results of large epidemiological studies also suggest the existence of familial bladder cancer, and first-degree relatives appear to have an increased risk for the disease [American Cancer Society 1998; Kiemeney LALM et al, 1996]. Smoking exposure has long been the best-established causative agent for UCB but race and obesity has likewise been reported as factors [Freedman ND et al, 2011; Kluth LA et al, 2013; Yee DS et al, 2011]. Finally lab values like C-reactive protein (CRP) evaluated at the time of RC was independently associated with a higher risk of cancer specific mortality [Gakis G et al, 2011]. Also preoperative albumin is associated with a higher risk of overall survival in UCB patients after RC [Gregg JR et al, 2011]. Anemia has been found to be an adverse prognostic factor in patients treated with radiation therapy for muscle-invasive bladder cancer [Gospodarowicz MK et al, 1989; Greven KM et al, 1990]. The opposite has been reported in surgically treated bladder cancer [Bassi P et al 1999].

1.14 Conventional prognostic factors

The anatomical extent of bladder tumor, or the depth of wall invasion, is universally accepted as the most important prognostic factors from large patient series [Amin MB et al, 2013]. T1 tumors have higher rates of disease recurrence, disease progression, and cancer-specific mortality compared to Ta tumors [Fernandez-Gomez J et al, 2009; Sylvester RJ et al, 2006; Fernandez-Gomez J et al, 2008]. Additionally, several studies have reported on the prognostic interest of sub-staging according to invasion in T1 tumors above (T1a), in (T1b), or beyond the muscularis mucosae (T1c) [Chang WC et al, 2012; Orsola A et al, 2005]. Prognosis is substantially related to the presence of organ confined or extravesical disease respectively. The five-year survival ranges from 70% for T2 tumors to 10% for T4 tumors. [Frazier HA et al 1993; Bassi P et al, 1999; Lerner SP et al, 1992; Pagano F et al, 1991; Pollack A et al, 1995]. The clinical TNM staging system combines a pathologic evaluation of the TUR specimen with findings from examination under anesthesia and preoperative radiograph-
ic imaging. Unfortunately, inaccuracies in clinical staging are highlighted in that up to 40% of patients are up-staged and about 25% are down-staged after pathologic assessment of the RC specimen, thus limiting the prognostic accuracy of clinical staging system [Shariat SF et al, 2007]. The problem of understaging has significant implications such as in counseling patients for neoadjuvant chemotherapy [Meeks JJ et al, 2012].

Tumor differentiation, namely grade, is designed to reflect the degree of tumor cell anaplasia. The WHO/ISUP system classifies bladder tumors into papillary urothelial neoplasms of low malignant potential, or papillary carcinomas of low or high grade [Epstein JI et al, 1998] It has been shown that patients with grade 3 and high-grade tumors are at highest risk of disease recurrence and progression; that said, grade 3 seems to have worse outcomes than high-grade tumors due to the heterogeneity in the subgroup of high-grade tumors [Amin MB et al, 2013; MacLennan GT et al, 2007; Lopez-Beltran A et al, 2004]. While tumor grade is one of the most important predictors of disease recurrence and progression after TUR and/or intravesical immunotherapy, the predictive power after RC is limited [Kirkali Z et al, 2005]. One of the reasons may be because most patients undergoing RC have high-grade disease [Stein JP et al, 2001; Shariat SF et al, 2006]. In NMIBC tumor size has been shown to be associated with disease recurrence and progression, with the most commonly used cut-off being 3 cm [Sylvester RJ et al, 2006; Denzinger S et al, 2007; Fernandez-Gomez J et al, 2008]. Multifocality represents a more controversial prognostic factor, which correlates with disease recurrence rather than disease progression [Fernandez-Gomez J et al, 2008]. Concomitant CIS is a validated prognostic factor for both disease recurrence and disease progression in NMIBC [Fernandez-Gomez J et al, 2008; Kakiashvili DM et al, 2011].

In 2000, the SWOG has shown that CIS generally responds favorably to BCG [Lamm DL et al, 2000]. Palou et al. have recently evaluated the incidence of CIS in the prostatic urethra (routinely evaluated by biopsy) in 146 patients with primary T1G3 NMIBC treated with BCG [Palou J et al, 2012]. The authors reported an incidence of 10% in the prostatic urethra, which was associated with both disease recurrence and progression. These findings suggest that prostatic urethra involvement should be evaluated routinely in all patients suspected of having high-grade tumor or in case of presence of CIS in the bladder. The strongest predictor of risk for later urethral disease is transitional cell carcinoma (TCC) involvement of the prostate [Montie JE et al, 1994].

Numerous studies have shown that the presence of lymphovascular invasion (LVI) defined as the presence of carcinoma in the endothelial lining or in the vascular wall, predicts disease progression and cancer-specific mortality [Kunju LP et al, 2008; Cho KS et al, 2009; Streeper NM et al, 2009;

Furthermore, the prognosis of invasive bladder cancer is directly related to the extent of the nodal involvement, namely the number and size of the positive nodes. Patients with N1 disease seem to benefit from pelvic node dissection and radical cystectomy, as evidenced by similar outcome in those with node negative disease and similar P-stage of the primary tumor. Nonetheless, the observed benefit quickly disappears when more than one node is involved. In node-positive patients, one of the largest single-center reports of patients treated with RC, the recurrence-free survival at 10 years for patients with eight or fewer positive lymph nodes was significantly higher than in those with more than eight positive lymph nodes (40 % vs. 10 %, respectively) [Stein JP et al, 2001]. In pathologic node-negative patients, increasing the number of LNs has also been suggested to result in better survival [May M et al, 2011; Herr HW et al, 2002].

The presence of lymph node metastasis is the most important pathologic prognostic factor after RC, with associated 15–30 % 5-year survival rates [Bochner BH et al, 2006; Stein JP et al, 2007; Herr HW et al, 2004]. Multi-institutional series of patients treated with RC have shown that approximately 70–80 % of patients with pathologic node-positive disease experience disease recurrence compared to 30–40 % of patients with extravesical disease and pathologically negative lymph nodes within 5 years of surgery [Bochner BH et al, 2006; Karakiewicz PI et al, 2006; Shariat SF et al, 2006]. Tarin et al. have recently investigated impact of lymph node involvement by location on disease outcomes using the 2010 TNM staging system [Tarin TV et al, 2012].

Of 591 patients treated with radical cystectomy with mapping pelvic lymph node dissection, 114 patients (19 %) had lymph node involvement and 42 patients (7 %) had pN3 disease. The authors could demonstrate that lymph node location was not associated with outcomes; however, the number of positive lymph nodes was associated with worse oncologic outcomes. Interestingly, a similar disease recurrence-free survival for patients with pN3 disease compared to pN1 or pN2 tumors has been shown.
Positive surgical margins have been shown in the Frazier series to be associated with a poor outcome and to be an independent predictor of survival [Frazier HA et al, 1993] Transitional cell carcinoma encompasses more than 90% of all bladder cancers. The remaining proportion of patients have pure squamous cell carcinomas or adenocarcinoma, rarely mixed histotypes [Mostofi FK et al, 1973] Even though a direct relationship between tumor extent and survival has been demonstrated for such histotypes, no reliable information is available regarding the comparative impact of histological type on outcome [Raghavan D et al, 1997; Ghoneim MA et al, 1997].

Also, Kamat et al. reported intravesical BCG therapy to be ineffective against micropapillary UC, thus suggesting RC as the optimal treatment strategy for non-muscle invasive micropapillary UC before disease progression [Kamat AM et al, 2006]. Additional sections to ponder would be prior response to therapy: progression is exceedingly high in those that recur/do not respond to BCG. Equally data on the pathology of the re-staging TURB and outcome is of interest. Particular in T1 patients those without invasive changes on the second TUR are more likely to respond to BCG whereas residual T1 on the restaging predicts strongly for progression despite BCG. Postponement of radical cystectomy recently, in several studies the time from diagnosis to treatment has been evaluated and shown that patients who were treated with RC later than 3 months after initial diagnosis were of increased risk of extravesical stage, node-positive disease and worse outcomes compared to those treated within 3 months [Chang SS et al, 2003; Lee CT et al, 2006].

1.15 Contemporary prognostic factors

Next to tumor properties and the former history of an individual patient, more sophisticated procedures may be used to evaluate the likelihood of treatment response, recurrence, progression and survival. According to the target molecules and methodology, prognostic markers are classified into the following groups: (1) chromosomal alterations and DNA/nucleotide-based markers, (2) cDNA microarray mRNA expression analysis and gene expression signatures, (3) proto-oncogenes/oncogenes, (4) tumor-suppressor genes, (5) cell cycle regulators, (6) angiogenesis-related factors, and (7) extracellular matrix adhesion molecules. This forms the basis for future strategies to manage bladder cancer. Thanks to the achievements of molecular biology both carcinogenesis and tumor progression can be related to cytogenetical and molecular alterations and are increasingly better understood. The following steps are mainly discriminated: • Immortalization • Proliferation • Apoptosis • Angiogenesis • Decreased Cell Adhesion • Invasion • Metastasis.
In the 1970s, Folkman et al. showed that new capillary blood vessels are necessary for a cancer to expand beyond a diameter of 2mm [Gimbrone MA Jr et al, 1974]. Therefore, neovascularization is a prerequisite for both the progression of the primary tumor and for the growth metastatic lesions. This process is designated as tumor angiogenesis. Bladder cancer cells can stimulate more angiogenesis than normal urothelium. Traditionally, angiogenesis has been quantified by microvessel density (MVD: >100 microvessels per hpf) [Barbieri CE et al, 2010]. Thrombospondin-1 is an extracellular matrix glycoprotein which has been shown to be a potent angiogenesis inhibitor. The correlation between tumor recurrence and thrombospondin-1 expression has been studied by Grossfeld et al. in 163 patients with invasive bladder cancer [Grossfeld GD et al, 1997]. Robert et al shows that micro vessel count correlates significantly with the presence of occult lymph-node metastases [Roberts JT et al, 2005]. As cisplatin is still regarded the main active drug in urothelial bladder cancer treatment, it is biologically plausible that the expression of an established modifier of the cellular platin response correlates with treatment efficacy [Hoffmann AC et al, 2010]. Several gene products have been described to modify the cellular response to chemotherapeutic agents in vitro and to correlate with clinical outcome in vivo. For example, excision repair cross complementing 1 (ERCC1) is a component of nucleotide excision repair (NER) pathway, a major repair mechanism of DNA damage induced by platin compounds reacting with DNA and forming inter- and intra-strand cross links. The balance of DNA damage to DNA repair dictates tumor cell death or survival after cisplatin therapy [Metzger R et al, 1998]. ERCC1 expression as detected by immunohistochemistry as well as gene expression has been linked to response and survival in other studies with platin-based therapies [Hoffmann AC et al, 2010; Bellmunt J et al, 2007; Kim KH et al, 2010]. Another important example is the multidrug resistance gene 1 (MDR1). It encodes an integral membrane protein named P-glycoprotein (Pgp) or an ATP-binding cassette subfamily B which acts as an energy-dependent cellular efflux pump [Simon G et al, 2007]. Anticancer drugs were found to induce MDR1 gene [Pastan I et al, 1991].

Physiologically, cell division and cell death are subject to a multiplicity of regulatory pathways, yielding a delicate balance. In cancer cells this balance is disrupted. Proliferatory pathways are activated, resulting in a net growth of the respective tissue. Among others, Shiina et al. and Clasen et al. have investigated bladder cancer proliferation by examining the immunohistochemical positivity for Ki67, a marker of cell proliferation.[Shiina H et al, 1999; Clasen S et al, 1997]. Ki67 was clearly increased in bladder tumor specimens, as compared with normal mucosa, and correlated with tumor stage and tumor grade, demonstrating a faster cell-cycle transition. Cell-cycle control is the key step underlying cell division and proliferation. Several genes and gene products associated with regulation of the
cell-cycle have been the subject of recent intensive investigation. Two of these, the p53 and Rb tumor suppressor genes have been shown to be important prognostic factors for patients with bladder cancer. P21 is a negative cell-cycle regulator transcriptionally regulated by p53 that also prevents the inactivation of Rb. Abnormal immunohistochemical staining of p21 is reported to be a risk factor for progression of TCC in patients with wild type p53 expression [Stein JP et al, 1996].

Aneuploidy has been associated with decreased survival in both low- and high-stage transitional cell carcinomas, although the correlations are better and more consistent for low-stage tumors [Lipponen PK et al, 1992]. Aneuploidy correlates well with histological grade and with lymph node metastasis [Lipponen PK et al, 1992].

An altered regulation of the programmed cell death (apoptosis) is another way to overcome the physiological balance between cell proliferation and cell death. Several previous investigations have shown altered apoptotic rates in bladder cancer tissue [Lara PC et al, 1999; Lipponen PK et al, 1994; King ED et al, 1996]. Key genes regulating apoptosis are the pro-apoptotic bax gene and the anti-apoptotic genes bcl-2 and bcl-X. Interestingly, the bcl-2/bax ratio correlated with immunohistochemical p53 accumulation, suggesting a crosstalk among bcl-2, bax and p53, potentially affecting drug-induced apoptosis and regulating resistance to chemotherapy [Ye D et al, 1998].

Nuclear morphometry, an image analysis technique for evaluation of nuclear shape, correlates with metastasis [Borland RN et al, 1993]. Loss of expression of blood groups A, B and H antigens in bladder cancer have long been recognized as prognostic indicators for patients with bladder cancer [Aprikian AG et al, 1993; Goliganin D et al, 1995; Summers JL et al, 1983]. However, in carefully performed studies, the loss of ABO antigens was found to be relatively common and provides no prognostic information [Aprikian AG et al, 1993; Goliganin D et al 1995; Summers JL et al, 1983].

Decreased cell adhesion and motility of the tumor cell are other fundamental requirements for the acquisition of invasive properties. Selectins, integrins and cadherins are those substances responsible for cell/ cell contact and interaction. Thus, a subsequent loss of adhesion molecules will be associated with an increased capability of a given cell to leave the cellular formation and invade the surrounding tissue. Bringuer et al. showed that in 49 patients with bladder cancer, decreased E-cadherin expression is significantly correlated with tumor stage, tumor grade and survival in this series.

Immortalization remains a fundamental step in carcinogenesis. Physiologically, every cell that has reached terminal differentiation is
prone to senescence and will subsequently undergo cell death. One of the key reasons for this is the abundance of telomerase expression in terminally differentiated cells. In bladder cancer, even high-differentiated tumors have been found to be positive for telomerase activity as determined by the TUNEL assay. As a consequence this observation has been translated into a diagnostic use [Kitsukawa SI et al 1999; Müller M et al 1998; Ohyashiki K et al 1998; Rahat MA et al 1999; Ramakumar S et al 1999; Yokota K et al 1998].

Tumor invasion requires destruction of the extracellular matrix in general, and particularly the degradation of the basal membrane. Laminin, a component of the basement membrane, is known to be sensitive to several proteases, including plasmin. Metalloproteinases (MMP) represent another group of proteases used by the tumor cell to invade the surrounding tissue. Emmprin is a modulator of MMPs and is upregulated in bladder carcinoma compared to benign urothelium [Muraoka et al, 1993]. Excess expression has also been correlated with tumor progression and development of metastases in several types of cancer.

Many factors are involved in tumor metastasis, with a decreased cell–cell adherence, increased cell motility and the potential of tissue invasion being prerequisite for the metastatic process. Shirahama et al. investigated the expression of binding sites of fucose binding proteins in patients with invasive bladder cancer [Shirahama T et al 1993]. Overall, actuarial and cancer-specific survival was significantly worse in patients with strong expression of fucose-binding protein-binding sites.

1.16 Current Predictive Tools

The current EAU-guidelines acknowledge the need to ad prognostic factors into risk tables and nomograms for clinical use. The combination of clinical and pathologic variables marks more accurate clinical predictions of outcome than the use of a single variable alone.

**NMIBC**

In 2006 (EORTC) Genitourinary (GU) group developed a scoring system and risk tables [Sylvester RJ et al, 2006], based on data from 2,596 patients diagnosed with Ta/T1 tumors, who were randomized in seven previous EORTC-GU group trials. The scoring system was built on the six most relevant clinical and pathologic predictors of outcomes such as tumor stage and grade, number of tumors, tumor size, concomitant CIS, and prior recurrence rate. However, the study was limited by the low number of patients treated with BCG, the high rate of IPOP, and the fact that no re-TUR was performed. Therefore, the Club Urológico Español de Tratamiento Oncológico
(CUETO) developed a scoring model, which predicts the short- and long-term probability of disease recurrence and progression in 1,062 patients with NMIBC from four CUETO trials that compared the efficacy of different intravesical BCG treatments [Fernandez-Gomez J et al, 2009]. These patients received 12 instillations during 5–6 months; however, neither immediate postoperative instillation nor re-TUR was performed. The scoring system was based on seven factors including age, gender, prior recurrence status, number of tumors, tumor stage, tumor grade, and the presence of concomitant CIS. Though many clinicians are using these scoring systems in daily practice, to date, only few studies have externally validated both these models [Fernandez-Gomez J et al, 2011; Hernandez V et al, 2011; Xylinas E et al, 2013]. Furthermore, these validation studies have reported an overestimation of both the risks of disease recurrence and progression, especially in the high-risk group of patients [Fernandez-Gomez J et al, 2011; Hernandez V et al, 2011; Xylinas E et al, 2013].

MIBC

The reported discordance between staging on transurethral bladder resection and on RC pathology in the literature ranges from 20 to 80 %. Correct staging in BC has direct implications for its management.

Pre-cystectomy nomograms provide only a modest increase in accuracy and reasons for this may include differences in TUR technique, non-standardized use of restaging biopsies, inaccuracy and variable use of preoperative imaging, and variability in the pathologic evaluation. Specifically, Mitra et al. developed a pre-cystectomy decision model to predict pathologic upstaging and oncologic outcomes in clinical stage T2 BC. The study describes a cross-validated decision tree generated using pre-cystectomy variables aiming to stratify patients with cT2 tumors based on the risk of pathologic upstaging and adverse oncologic outcomes. This model can be potentially employed as a tool for making clinical decisions with respect to neoadjuvant chemotherapy (NACT) in these patients [Mitra AP et al, 2012].

The integration of other pathologic prognostic markers, for example LVI in addition to molecular markers of disease, possibly will enhance predictive accuracy of pre-cystectomy nomograms [Shariat SF et al, 2008]. Nevertheless, they demonstrate that the combined use of clinical and pathologic variables, which cannot always be integrated within look-up tables, results in more accurate predictions than the use of a single variable.

Only few studies have demonstrated a significant improvement in predictive accuracy, when biomarkers were added to established predictors in the predictive tool setting [Karam JA et al, 2007; Shariat SF et al, 2008; Catto JW et al, 2003; Qureshi KN et al, 2000]. One of these studies, for example, demonstrated in 191 pTa-3N0M0 patients following RC that
the addition of a panel of five well-established cell cycle regulatory bi-
biomarkers (p53, pRB, p21, p27, and cyclin E1) improved the predictive
accuracy of competing-risk nomograms and survival in these patients. In
comparison, two smaller studies have added biomarkers to standard
clinicopathologic features using pre-cystectomy prediction tools (ANN and
neuro-fuzzy modeling) [Catto JW et al, 2003; Qureshi KN et al, 2000].
2. Diseases Management

2.1 Non-Muscle invasive Bladder Cancer

The significance of the initial surgical management of NMIBC by TURBT cannot be overstated.

It serves both diagnostic and therapeutic purposes. The EAU-guidelines emphasizes a competently performed initial surgery which will set the foundation for the management of the patient and optimize patient outcome. Similarly, follow-up must be systematic and vigilant in order to minimize the risk and consequences of tumor recurrence or progression. An increasing body of evidence also now links the quality of the initial TURBT to prognosis. The aims of the TURBT are to obtain a specimen of sufficient size and quality to enable accurate histological characterization, to determine the depth of invasion and the presence or absence of both lymphovascular involvement and abnormal urothelium adjacent to the primary lesion.

Repeat TURBT is now a standard of care in selected cases of high-grade non-muscle-invasive urothelial carcinoma and forms part of the key recommendations of treatment guidelines published by the EAU, American Urologic Association (AUA) and International Consultation on Urologic Diseases (ICUD) [Burger M et al 2013; Hall MC et al 2007]. Re-resection serves three main purposes: (1) to ensure complete clearance of the primary tumor, (2) to minimize the risk of under staging, and (3) to provide prognostic information to aid decision-making. In addition, there is some evidence that re-resection may improve response to intravesical treatment.

Persistent tumor after initial resection has been observed in 33-53% of T1 patients and in 41.4% of TaG3 tumor [Brauers A et al, 2001]. Therefore a second resection has a substantial risk reduction [Schips L et al, 2002] and increase recurrence-free survival [Grimm M-O et al, 2003; Divrik RT et al, 2006]. The possibility that a T1 tumor has been under staged and muscle-invasive disease detected by second resection ranges from 4-25%, precise staging is therefore important. While TURB by itself can remove a Ta, T1 tumor wholly, these tumors commonly recur and can progress to MIBC.
2.2 Intravesical therapy

Existing EAU guidelines recommend administration of some form of intravesical therapy for low-, intermediate-, and high-risk NMIBC. Immediate postoperative instillation of intravesical chemotherapy has been shown to reduce tumor recurrence with a variety of agents, and is now routinely recommended by the EAU for all cases of NMIBC [Babjuk M et al, 2012; Hall MC et al, 2007]. The proposed mechanism is that the chemotherapeutic agent destroys any residual tumor or circulating tumor cells released after TURBT, thereby preventing tumor cell implantation and reducing tumor recurrence. However, this theoretical mechanism of action has not been proven in any definitive matter. Guidelines offers no further reference for adjuvant therapy for those with low-risk disease, but backs intravesical bacillus Calmette-Guerin (BCG) or chemotherapy for those with intermediate-risk disease and those at high risk for progression recommending induction BCG with maintenance therapy [Pawinski A et al, 1996]. Chemotherapy and immunotherapy are the two primary forms of intravesical therapy.

2.3 Intravesical Immunotherapy

2.3.1 BCG

Since its institution by Morales et al. in 1976, intravesical Mycobacterium bovis bacillus Calmette-Guérin (BCG) has served as a fundamental, adjuvant therapy in the treatment of non-muscle invasive bladder cancer [Morales A. et al, 1976]. According to guidelines, urologists prefer the use of BCG to intravesical chemotherapy to a ratio of 2:1. BCG is the first-line treatment for carcinoma in situ because of its proven track record. Despite overwhelming success, BCG is fraught with potential adverse reactions and its use must be balanced with possible harm to eventual patients. Following the instillation of BCG, both soluble and cellular immune mediators appear to mediate an antineoplastic response, stimulating both the innate and adaptive immune system. BCG first binds fibronectin, a glycoprotein which mediates cell adhesion. Subsequent internalization of BCG into benign and malignant urothelial cells occurs, initiating a cascade of inflammatory responses. This activation promotes cell-mediated antitumor activity, conferred by CD8+ T cells, natural killer (NK) cells, and macrophages [Askeland EJ et al, 2012]. In a meta-analysis, BCG decreased tumor recurrence by 43% on average compared to 16-21% using intra-vesical chemotherapy, depending on the agent [Amling C. L., 2001]. In several studies,
BCG has been shown to decrease the rate of relapse and increase the relapse-free interval making it one of the most successful immunotherapies used in the clinic to date [Raghavan D. et al, 1990]. Still, there remains a necessity for new types of therapies due to an overall 65% recurrence rate, a 30% progression rate, BCG resistance in up to one-third of patients and side effects in >90% treated [Alexandroff A. B et al, 1999; Amling C. L., 2001; Schenk-Braat E. A. et al, 2004]. Meta-analyses have demonstrated that BCG therapy may reduce the risk of tumor progression [Jahnson S et al, 2005]. BCG maintenance therapy seems to be suggestively superior in preventing recurrences than regimens with mitomycin C (MMC) or epirubicin [Lazica DA et al, 2013; Brausi M et al, 2002]. Nevertheless, BCG causes considerably more side effects [Sylvester RJ et al, 2004]. While BCG is a very effective treatment, there is an agreement that not all patients with NMIBC should be treated with BCG due to the risk of toxicity [Abern MR et al, 2013; Perlis N et al, 2013].

As a result of these trials, several evidence-based guidelines established by AUA, EAU, and the National Comprehensive Cancer Network (NCCN) have been drafted to recommend indications where BCG therapy is warranted. The three major indications include: (1) adjuvant induction cycle for intermediate risk tumors (low-grade Ta), (2) adjuvant induction cycle plus maintenance therapy for high-risk tumors (high-grade Ta, T1), and (3) Induction cycle plus maintenance therapy for primary treatment of carcinoma in situ (CIS).

2.3.2 BCG failure

The international bladder group (IBCG) tried to emphasize the importance of distinguishing recurrence from treatment failure. Recurrence refers to reappearance of disease, any grade, T category, or CIS after completion of therapy. Failure is any recurrence or progression that occurs during intravesical therapy [Lamm D et al, 2008]. There are four subgroup categories of BCG failure [Nieder AM et al, 2005; O’Donnell M et al, 2009]: 1. BCG intolerance: recurrent disease in an intolerant BCG patient and/or side effects. 2. BCG resistance: recurrence or persistence of lesser stage or grade after initial course which resolves with further BCG instillations. 3. BCG relapse: recurrence after initial resolution. 4. BCG refractory: non-improving or worsening disease despite BCG.
2.4 Intravesical chemotherapy

2.4.1 Mitomycin C

It is an alkylating agent which causes DNA cross-linking, strand breakage, and ultimately inhibition of synthesis. It is the most broadly studied and commonly used intravesical chemotherapy, with roles in the perioperative, adjuvant, and BCG failure settings. The guidelines support the use of a single instillation of MMC in the immediate postoperative period to reduce the risk of recurrence in patients with NMIBC. However, it has also been used as a weekly instillation in various maintenance schedules. In patients at intermediate or high risk of recurrence, one immediate instillation of chemotherapy can be followed by further instillations of chemotherapy or a minimum of 1 year of BCG [Babjuk et al, 2008; Hall et al, 2007]. Data from the EORTC metanalysis of 23 studies have confirmed that the average net benefit for single perioperative MMC is about 14% at 1–3 years and 7% at 7 years [Pawinski et al, 1996]. Lamm et al. performed a meta-analysis of five controlled trials and reported that the recurrence rate was reduced by 15% [Lamm, 1992]. The advantage of MMC was 15% (52% recurrences in the control groups versus 37% in the MMC group) [Lamm, 1992]. A long-term effect on recurrence and disease progression was not demonstrated [Lamm, 1992]. The response rates of MMC seem higher than other chemotherapeutic drugs. Superiority of BCG over MMC for intermediate risk is not well established. MMC maintenance therapy is controversial. Malmström et al. found that maintenance BCG was superior in preventing recurrence compared to maintenance MMC, although no difference was found for progression and survival [Malmström et al, 1999]. The side effects are attributed to local toxicity and typically occur after several instillations [de Groot and Cone-mans, 1991]. The most common side effects are frequency, chemical cystitis, and allergic skin reactions due to contact dermatitis [Smith et al, 1999].

2.4.2 Interferon α

Interferon-α (INF-α) is a cytokine produced by the immune system in response to insults such as tumor cell growth. They are natural glycoproteins that mediate host immune responses such as the stimulation of phagocytes, inhibition of nucleotide synthesis, up regulation of tumor antigens, cytokine release, enhanced natural killer cell activity, and activation of T and B lymphocyte [Naito et al, 1991]. Among the subtypes, interferon-α has been the most extensively studied either with chemotherapy or BCG [Bercovich et al, 1995; Stricker et al, 1996] It’s efficacy is dose dependent [Beldegrun et al, 1998; Torti et al, 1988]. Interferon as a solitary agent is more expensive and less effective than BCG or intravesical chemotherapy in eradicating residual disease, preventing recurrence of papillary disease, and treating CIS (20–
43% complete response). As a prophylactic agent, interferon alone demonstrated recurrence rates that were generally inferior to those of BCG alone [Glashan, 1990; Kalble et al, 1994], although it can occasionally be effective in patients who have failed BCG with 15–20% complete response. However, there are no data to demonstrate superior efficacy of BCG with interferon compared with BCG alone as initial treatment, and BCG remains standard therapy for frontline management of high-risk disease. It has been utilized in conjunction with BCG in patients with high-risk NMIBC who failed BCG therapy. A US multicenter phase II study, involving 1,007 patients compared the effects of INF-α plus reduced dose BCG in BCG failure patients to a cohort of BCG naïve patients who received the same INF-α dose but with a standard dose BCG protocol. The response rate in the group of patients who failed BCG was 45% compared to 59% in the BCG naïve group [Joudi F et al, 2006]. In conclusion, INF-α/BCG can be a reasonable option for patients who failed previous BCG therapy even if further studies have to confirm the results.

2.4.3 Epirubicin

Anthracyclines are a class of chemotherapeutic agents commonly used throughout oncology. They serve as intercalating agents, thereby blocking DNA and RNA synthesis, and as inhibitors of topoisomerase II, preventing transcription and DNA repair mechanisms. Most studies have shown that perioperative epirubicin reduces the recurrence rate by 13–27% [Liu et al, 2002; Rajala et al, 2002; Okamura et al, 1994]. When compared to BCG, epirubicin display higher rates of recurrence in most trials [Shelley MD et al, 2010]. Long-term follow-up of EORTC 30911, comparing epirubicin, BCG, and BCG plus isoniazid for intermediate- and high-risk patients, showed that any BCG therapy decreased recurrence, distant metastases, and cancer-specific death rates, though not progression [Sylvester RJ et al, 2010]. As a result, anthracyclines are not frequently used in the adjuvant setting. Maintenance therapy has shown benefit in some studies, however most of them showed no significant benefit [de Groot and Conemans 1991; Smith et al, 1999; Eto et al, 1994; Kondo et al, 1999; Torelli et al, 2001; Okamura et al, 1998]. Evidence is strongest for immediate postoperative instillation of anthracyclines over TURBT alone [Gudjonsson S et al, 2009].

2.5 Muscle Invasive Bladder Cancer (MIBC)

According to existing guidelines radical cystectomy is broadly accepted as the gold standard of treatment for patients with muscle-invading tumors. Despite improvements in surgical techniques, peri-operative care, and early diagnosis following cystectomy up to 50% of patients may develop
metastases within two years [Sternberg CN et al, 1995]. Neoadjuvant chemotherapy (NCT) offers the earliest opportunity to target the distant micro metastases that are responsible for most failures. Therefore EAU guidelines recommend both definitive treatment within 3 months of diagnosis, and neoadjuvant cisplatin-based combination chemotherapy for non-disseminated cT2-T4a bladder cancer. [Griffiths G et al, 2011; Svatěk RS et al., 2011; Malmström PU et al, 1996; Sherif A et al, 2002; Sherif et al, 2004]. The recommendation specifies cisplatin-containing combination chemotherapy without specifying a particular regimen. Due to toxicity, patients should be in the optimum state of health to tolerate chemotherapy in the pre-surgical setting [Calabro F et al, 2009]. Learning from studies in the metastatic setting, histopathology evaluation of the cystectomy specimen offers in vivo prognostic information about chemotherapy sensitivity thus suggesting pathologic complete remission (pCR) (downstaging) as a surrogate prognostic marker for improved survival [Sonpavde G et al, 2009; Rosenblatt R et al, 2012; Winquist E et al, 2004; Schultz PK et al, 1994; Petrelli F et al, 2013]. In fact the most significant prognostic factor for survival appears to be attainment of pT0 status [Schultz PK et al, 1994]. Response to chemotherapy is a prognostic factor of extreme importance, and should be considered when making clinical decisions. Nevertheless, with neoadjuvant therapy concerns remain of delaying definitive curative cystectomy and the possibility of disease progression in a small subset of patients while on chemotherapy and fraction of patients, e.g., pT2a (superficial muscle-invasive disease), is overtreated with NCT.

Alternatively, adjuvant chemotherapy (ACT) strategy permits complete pathological assessment and risk stratification before offering chemotherapy to high-risk patients (with extravesical or node-positive disease). Since the cystectomy is performed immediately, there is no delay in definitive treatment. The chief disadvantage is delay in systemic therapy for occult metastases while treatment for the primary tumor is emphasized and response in vivo cannot be assessed. Randomized adjuvant chemotherapy trials currently in the literature are not definitive, due to inadequate numbers, premature closure, deviations from protocol entry, patient selection, subset analysis, and failure to treat at relapse. The most quoted is the Skinner study [Skinner DG et al, 1991]. This was the first randomized prospective trial that showed a significant increase in time to progression and survival in patients who were randomized to receive chemotherapy. The dominant prognostic factor was extent of regional lymph node involvement at the time of cystectomy. Sternberg CN et al recently published results from adjuvant trial accessing immediate vs. deferred cisplatin-based combination chemotherapy after radical cystectomy. The author found no significant improvement in overall survival with immediate treatment although improved progression-free survival was noted [Sternberg CN et al, 2015].
2.6 Absolute effect of Chemotherapy

Absolute risk reduction is the change in the risk of an outcome of a given treatment or activity in relation to a comparison treatment or activity [Laupacis A et al, 1988]. The inverse of the absolute risk reduction, number needed to treat, is an important measure in pharmacoeconomics. The number need to treat (NNT) represents the number of patients, on average, that must be treated to result in one additional outcome. The ideal NNT is 1, where everyone improves with treatment and no one improves with control. The higher the NNT, the less effective is the treatment [Hutton JL, 2010]. If a clinical endpoint is devastating enough (e.g. death), drugs with a low absolute risk reduction may still be indicated in particular situations. We have to acknowledge that neoadjuvant treatment (chemotherapy, radiation) does not benefit every patient and delays curative treatment for a significant percentage of patients with MIBC (40%). Patients may receive chemotherapy unnecessarily because a more-complete operation may have indicated a greater likelihood of surgical cure. It is also unlikely that NACT will compensate for a poor operation in which significant tumor volume has been left behind in the pelvis. An improved risk stratification of clinical stage T2 (cT2) patients can potentially identify candidates who may derive maximal benefit from this approach. Clinical stage T2 patients, who are pathologically upstaged at cystectomy have significantly worse prognosis than their counterparts who are not upstaged. The identification of such candidates who may be subsequently upstaged represents a strategy for selecting those patients who may benefit the most from NACT, whereas other patients can undergo early RC [Mitra AP et al, 2012(b)]. Despite evidence supporting perioperative chemotherapy, few randomized studies compare neoadjuvant and adjuvant chemotherapy for bladder cancer [Wosnitzer MS et al, 2012]. Consequently, the standard of care regarding the timing of chemotherapy for locally advanced BC remains controversial.

2.7 The anti-cancer agent cisplatin – cytotoxicity and resistance

2.7.1 Mode of action

Cis-diamminedichloroplatinum (II) (CDDP) is the first of a group of platinum coordination complexes with antineoplastic activity to be studied in humans and has been a clinical success during three decades. [DeConti RC et al, 1973]. Cisplatin binds primarily to purine bases in DNA and forms DNA-protein and DNA-DNA adducts where intra-strand adducts are believed to be the most toxic. DNA distortion by the adducts results in binding of damage recognition proteins which hinder both replication and transcrip-
tion [Siddik ZH, 2003], inhibit nucleotide excision repair [Chaney SG et al, 2005] and promote apoptosis [Siddik ZH, 2003]. Three platinum antitumor drugs are available in the clinic; cisplatin, carboplatin and oxaliplatin. They form the same type of adducts at the same sites on the DNA [Chaney SG et al, 2005]. Carboplatin and cisplatin are cross-resistant and share the same range of clinical activity [Wang D et al, 2005].

2.7.2 Toxicity & resistance
The main clinical obstacle with platinum based chemotherapy is resistance and toxicity. Gastrointestinal toxicity is common for all platinum drugs whereas treatment with cisplatin can result in both ototoxicity and peripheral neuropathy. Furthermore, nephrotoxicity is also common and limits the dose interval for cisplatin even if it partially can be treated medically with pre-hydration, mannitol and diuretics [Yao X et al, 2007]. Although cisplatin is a very potent inducer of apoptosis, some tumors are intrinsically resistant to cisplatin and some tumors that are originally sensitive to platinum can eventually acquire resistance, resulting in treatment failure. Cisplatin resistance is multifactorial and understanding of resistance mechanisms is required to successfully identify relevant biomarkers. Cisplatin resistance can be due to inactivation of the drug, increased DNA-repair, decreased apoptosis signaling or decreased drug accumulation. The resistance can be acquired through chronic drug exposure or it may be an intrinsic phenomenon of the tumor cell [Siddik ZH, 2003]. Resistance can be a consequence of intracellular changes that either prevent cisplatin from interacting with DNA, interfere with DNA damage signals from activating apoptosis, or both. Reduced DNA damage may be caused by decreased drug accumulation, increases in the amounts of intracellular thiol capable of inactivating the drug, and/or an enhanced rate of DNA adduct repair. In general, several mechanisms are encountered simultaneously and a high resistance is a net effect of several unrelated mechanisms. Evidence indicates that reduced drug accumulation, due to inhibited drug uptake or increased efflux, is a significant mechanism of cisplatin resistance [Siddik ZH, 2003]. The mechanisms behind cisplatin resistance have mainly been studied using highly cisplatin-resistant cell lines, generated by repeated exposures of a sensitive parental cell line to increasing concentrations of the drug. These models are argued to clinically correspond to resistance developed by chronic drug exposure. In contrast, mechanisms behind variations in intrinsic sensitivity are less well-studied.

Hence the success in isolating a predictive biomarker is understanding tumor biology and elucidation mechanism of action of the tested drug. We know that target cells lose their sensitivity to cisplatin as a result of a wide panel of genetic or epigenetic defects but the exact molecular mechanisms of cisplatin resistance is not fully understood. Galluzzi L et al. clarifies these altera-
tions (i) affect processes that precede the actual binding of cisplatin to its targets (pre-target resistance); (ii) potentiate the ability of cells to repair the molecular damage caused by cisplatin (on-target resistance); (iii) impair the transmission of signals that normally relay such a cisplatin-induced damage to cell senescence or apoptosis (post-target resistance) or (iv) stimulate the delivery of pro-survival signals that antagonize cisplatin cytotoxicity although they are normally not elicited by this drug (off-target resistance) [Galluzzi L et al, 2014]. Thus, CDDP resistance is generally multifactorial, that is, it relies on the activation of several, non-overlapping mechanisms that concur to limit the cytostatic/cytotoxic effects of CDDP at multiple levels.
3. Background to methodology and materials

3.1 Tissue microarray & Reference pathology

In 1986 Battifora described a novel method where tissue samples are embedded in paraffin and subsequently cut in sections that are laid on glass slides to be stained, most often with haematoxylin-eosin. In our work representative tumor tissue where marked using reference pathologist and cylinders subsequently transferred to a recipient paraffin block with a consistent cylinder depth using automated tissue microarray [Kononen J et al, 1998]. After this process the tissue samples could be visualized and evaluated using a light microscope [Battifora H, 1986]. Some of the obvious advantages of using TMA include: i) large number of cases can be assessed simultaneously ii) reduced amount of archival tissues, cost and time iii) control tissues can be placed directly on the actual study slides iv) reproducibility of the staining reaction, better reliability of the interpretation v) consecutive slides can be stained with hematoxylin and eosin (H&E) for morphology or with other antibodies against the same or other molecular targets. This permits comparison of multiple targets in virtually identical, histologically highly controlled regions of the tissues. A potential drawback of testing a marker in a TMA is that a small core may not be representative of the whole tumor, given the likelihood of heterogeneity. Camp et al. examined the correlation between cores and whole sections and came to the conclusion that sampling of two cores of 0.6mm is sufficient [Camp RL et al, 2000].

3.2 Proteomics/Immunohistochemistry

Protein research can be divided into separation-based or probe-based procedures. The second group depends on antibodies binding to specific antigens and includes immunohistochemistry (IHC). Proteome is a word used for all proteins expressed at a certain time and under defined circumstances by a genome. The proteome can refer to all proteins expressed in a particular cell, tissue or organism. The word is a blend of “protein” and “genome”. Antibody-based proteomics is a term used when protein-specific antibodies are used to explore the proteome [Uhlen M et al, 2005]. IHC is widely used in routine histopathology as a complementary tool in the diagnosis of different lesions [Warford A et al, 2004]. This technique relies on an antibody recog-
nizing a specific antigen and through the process of IHC the presence of this particular protein can be visualized on a glass slide containing the tissue sample. There are several confounders related to this method that should be mentioned. The IHC staining results are dependent on various factors, like the specificity and sensitivity of the antibody, fixation of the tissue and on what antigen retrieval method and detection system is employed [Paavilainen L et al, 2010; Leong AS et al, 1989]. Therefore it is important to standardize the staining procedure as much as possible. In addition, the evaluation is subjective and therefore lacks high reproducibility, (both intra- and inter-observer) which can cause problems (Figure 2).

Figure 2 Tissue microarray.

3.3 Antibodies

Antibodies are a type of proteins called immunoglobulins, and they are produced by specialized B lymphocytes in the body as a response to invading pathogens; antigens. The antibody binding site on the antigen is called an epitope. There are different ways of producing antibodies. Monoclonal antibodies are antibodies that are made by identical immune cells that are all clones of a unique parent cell, in contrast to polyclonal antibodies which are made from several different B-cells. Monoclonal antibodies have monovalent affinity, in that they bind to the same epitope. They are highly specific and renewable, some epitopes having modified conformation may not be recognized by a monoclonal antibody and the production is both time-consuming and expensive. On the other hand polyclonal antibodies recognizes several different epitopes on a protein which makes them highly sensitive regardless of protein modifications and folding. Polyclonal antibodies may cross-react with other proteins, and are not reproducible since re-immunization with the same antigen in the same animal species will yield a different pool of antibodies [Lipman, N.S. et al, 2005; Dabbs D, et al, 2006].
3.4 Western blot & cell culture

The **western blot** or protein **immunoblot** is a probe based method to detect specific protein in a protein mixture extracted from a cell or a tissue sample [Burnette, 1981]. It uses gel electrophoresis to separate native protein depending on their weight. Thereafter they are transferred onto a membrane usually nitroceulullose or polyvinylidene difluoride (PVDF) where they are stained with antibodies specific to the target protein [Towbin H *et al*, 1979; Renart J, 1979]. In that way the particular protein and its predicted size can be visualized. The gel electrophoresis step is included in western blot analysis to resolve the issue of the cross-reactivity of antibodies. **Cell culture** is the process by which cells are grown under controlled conditions, generally outside of their natural environment. We have used the cell line T24, a transitional-cell carcinoma cell line that originates from a muscle invasive bladder tumor from an 81 year-old female. The main advantage of using cell line for research is its immortality; the cells can be grown indefinitely in culture. This simplifies analysis of the biology of cells which may otherwise have a limited lifetime.

3.5 Proteins

3.5.1 Emmprin

Extracellular matrix metalloproteinase inducer (emmprin), also known as basigin or CD147, is a glycoprotein that is enriched on the surface of tumor cells and stimulates production of several matrix metalloproteinases by adjacent stromal cells. It stimulates the production of interstitial collagenase (MMP-1) but also forms a complex with MMP-1 at the tumor cell surface [Guo H *et al*, 2000]. It is a member of the immunoglobulin superfamily, which plays fundamental roles in intercellular recognition involved in various immunologic phenomena, differentiation, and development [Miyauchi *et al*., 1991; Kanekura *et al*., 1991]. Inhibition of emmprin with a monoclonal antibody induced significant inhibition of cell growth [Min Jueng Kang *et al*, 2013].

3.5.2 Survivin

Survivin, which is a member of the inhibitor of apoptosis protein (IAP) family, regulates two essential mechanisms in the cell; it blocks apoptosis by inhibition of caspase activation and it is a regulator of mitosis [Tamm *et al*, 1998]. The up-regulation of survivin expression in cancer cells seems to be independent of the cell cycle. The subcellular localization of survivin in tumors (cytoplasmic and nuclear) may indicate survivin activity and serve as a
predictive marker [Engels et al, 2007]. Down-regulation of survivin has been shown to sensitize tumor cells to apoptosis [Chawla-Sarkar et al, 2004] and halt tumor progression by blocking angiogenesis [Altieri, 2003].

3.5.3 Choline-phosphate cytidylyltransferase alpha (CCTα)

The CDP-choline pathway, first identified by Eugene Kennedy in 1956, is the predominant mechanism by which mammalian cells synthesize phosphatidylcholine (PC) for incorporation into membranes or lipid-derived signaling molecules [Gibellini F et al, 2010; Kennedy EP et al, 1956]. This is aided by the rate-limiting enzyme CCTα, an amphitropic enzyme [Cornell R. B. et al, 2006]. In rapidly dividing cells like tumor cells, there is increased expression and activity as a result of increased demand for PC synthesis.

3.5.4 STMN1 (stathmin 1)

An oncogene is a gene that has the potential to cause cancer [Wilbur et al, 2009] In tumor cells, they are often mutated or expressed at high levels [Croce CM, 2008] STMN1’s or oncoprotein 18 plays a central role in regulation of the cell cycle. STMN1 can cause uncontrolled cell proliferation when mutated and not functioning properly. Most normal cells will undergo a programmed form of rapid cell death apoptosis when critical functions are altered. Activated oncogenes can cause those cells designated for apoptosis to survive and proliferate instead [Croce CM, 2008]. Most oncogenes require an additional step, such as mutations in another gene, to cause cancer.

3.5.5 Ki-67

The Ki-67 protein (also known as MKI67) is a commonly used marker of cell proliferation and has been studied extensively in almost all cancers. Because Ki67 is expressed only by cells actively engaged in the cell cycle, positive immunostaining is considered a good correlate of biological ‘aggressiveness’. Numerous studies have identified Ki67 as an independent prognostic marker of disease recurrence, progression and disease-specific survival [Liukkonen T et al, 1999; Pfister C et al, 1999; Wu TT et al, 2000]. The fraction of Ki-67-positive tumor cells (the Ki-67 labeling index) is often correlated with the clinical course of cancer. While variation in study design and assay interpretation has resulted in a number of contradictory reports, a large multicenter study has confirmed that Ki67 is independently associated with disease recurrence and overall survival and could be used to predict which patients might benefit from preoperative systemic chemotherapy [Margulis V et al, 2009].
3.5.6 p53

The International Cancer Genome Consortium has established that the p53 gene is the most frequently mutated gene (>50%) in human cancer, indicating that the p53 gene plays a crucial role in preventing cancer formation [Surget S et al, 2013]. P53 gene encodes proteins that bind to DNA and regulate gene expression to prevent mutations of the genome [Arnold J. Levine et al, 2010] thus, functions as a tumor suppressor [Surget S et al, 2013] and has been described as "the guardian of the genome" [Read, A. P. et al, 1999]. P53 acts as a break of the cell cycle and apoptosis enabling DNA repair before cell division [Bond et al, 2004; Bond et al, 2005]. If the DNA is not repaired effectively, apoptosis is instead initiated. Stress signals dramatically increase the half-life of the p53 protein [Appella et al, 2001].

3.6 Hospital records

The data assembled from the hospital records of the individual patients served as a basis for the entire project, and they were collected from the archives of the treating hospitals. The archives were carefully screened, and information on aspects such as clinical tumor characteristics (size and multiplicity), details of treatment given and any histologically confirmed recurrence and/or progression, and eventual death from UCB were documented.
4. Aim and rationale of the thesis

To study the predictive power of clinical characteristics and biomarkers in the current management of urinary bladder cancer.

4.1 Specific aim paper I
In a multicenter, prospectively randomized study we evaluated the 5-year outcomes of Bacillus Calmette-Guérin alone compared to a combination of epirubicin and interferon-α2b in the treatment of patients with T1 bladder cancer. Histological grade and concomitant carcinoma in situ was assessed as potential predictive factors.

4.2 Specific aim paper II
To validate whether expression of emmprin and survivin in tumor samples taken before therapy could be used as a predictive marker of chemotherapy response.

4.3 Specific aim paper III
In a bladder tumor setting, test the novel CCTα protein as biomarker for chemotherapy response.

4.4 Specific aim paper IV
To explore STMN1 prognostic significance in disseminated urinary bladder cancer and its possibility as a therapeutic target.
5. Material and methods

Paper I

5.1 Study design and participants

The Nordic Urothelial Cancer Group conducted between 1999-2006 a prospective, randomized, multicenter study involving 256 patients registered at 20 urologic units in Sweden (n = 206), Norway (n = 26), and Finland (n = 24). The study protocol was designed to meet the standards of the Helsinki Declaration, including written informed consent signed by the patients. Ethics approval was granted by the Medical Faculty Ethical Committee of Umeå University (Dnr 98-145). The inclusion criteria were patients with recently detected T1 G2–G3 urinary bladder cancer. Good performance status (PS) was one of the conditions, as the protocol suggested cystectomy if T1 disease persisted or recurred and if progression was detected at the 6-month follow-up. Exclusion criteria were (1) recurrent bladder tumor of any stage; (2) muscle-invasive bladder tumor at a second-look resection; (3) involvement of the urethra, prostate (ducts or stroma), or upper urinary tract; (4) hydronephrosis; (5) anticoagulation with warfarin; (6) a history of radiotherapy or systemic chemotherapy; (7) prior endovesical treatment with the investigational drugs other than a single instillation of chemotherapy, including epirubicin after TURB; (8) history of tuberculosis; and (9) immune deficiency and other malignancy (except basal cell carcinoma of the skin).

Pretreatment examination studies comprised physical examination, blood analysis, cytology, bladder volume, urine culture (if needed), and chest x-ray. An intravenous pyelogram (IVP) within the last 6 weeks was also required. All patients underwent an initial TURB of all visible tumors followed by a second-look resection, including bladder mapping and biopsy of the prostatic urethra within 4–8 weeks. Randomization was done by computer through a telephone service at the Oncology Centre at Umeå University after the second look.

Patients received treatment with either one ampoule (1-8 x 10^8 CFU, 2 ml in 100 ml saline) of BCG (OncoTICE, Organon Teknika, Boxtel, the Netherlands) or the combination of 50 mg of the dry substance epirubicin (Farmorubicin, Pharmacia GmbH, Erlangen, Germany) and 10 million units (dissolved in 100 ml of saline) of IFN-α2b (Intron A, Schering-Plough, Kenilworth, NJ, USA). Both regimens were given as induction treatment for 6
weeks followed by 3 week maintenance therapy for 2 years (treatment schedule in Figure 3).

The trial was early stopped due to slow recruitment. Six of the randomized patients were excluded because of violation of inclusion criteria. Of the lasting eligible 250 patients, 198 were men and 52 women. One hundred twenty-six patients were randomly assigned to the BCG arm and 124 patients were allocated to the combined, experimental arm. Stratification was based on histologic grade and associated carcinoma in situ (CIS). The other tumor characteristics size and multiplicity of the two groups were also well balanced.

**Figure 3** Treatment and assessment schedule. TURB = Transurethral resection of the bladder*; R = randomization; Pex = multiple biopsies; BCG = bacillus Calmette-Guérin; IFN = interferon-α2b; Epi = epirubicin.* If no tumor, TURB at primary location; second look refers to TURB at the primary tumor location plus mapping.

---

**Paper II-III**

**5.2 Study design and participants**

A Nordic collaborative group assessed the effectiveness of chemotherapy prior to cystectomy compared to cystectomy only in two consecutive trials. The studies included 620 patients who were diagnosed with T1G3, T2-T4aNXM0 urothelial bladder cancer from 1985 until 1997. The intended chemotherapy for the patients included in the first trial was two cycles of
cisplatin 70 mg/m² i.v. and doxorubicin 30 mg/m² i.v. It was planned that all patients in the experimental arm as well as in the control arm would receive preoperative irradiation. The irradiation consisted of 4 Gy daily for five consecutive days. In the second trial three cycles of cisplatin 100 mg/m² i.v. and methotrexate 250 mg/m² i.v. were planned. Cystectomy included a lymph node dissection of the obturator fossa. In both trials randomization was stratified by country. Adjuvant chemotherapy has seldom been used and never been recommended in the national guidelines.

For these studies tissue specimens from transurethral resection were obtained from the Swedish patients after ethics committee approval (reference number 2008:136). The selection process is shown in Figure 4. The survival analyses were carried out on an intention to treat basis.

**Figure 4** Flowchart showing inclusion of patients from the Nordic randomized combined trials.
Paper IV

5.3 Study design and participants

Patients from Uppsala University Hospital included in cohort I was diagnosed with bladder cancer between 1984 and 2005. Use of these patient samples for protein profiling was approved by the regional ethical review board of Uppsala (reference number 2005:339). The tumor material compromises a wide-range tissue microarray (TMA) of prospectively collected primary tumors ($n=115$ Ta, $n=115$ T1 and $n=112$ T2–T4). Clinicohistopathological data are presented in Table 1.

Table 1 Clinico-histopathological characteristics for the patients in cohort I.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no of cases</td>
<td>342</td>
</tr>
<tr>
<td>Follow-up time (mo.)</td>
<td>54.2</td>
</tr>
<tr>
<td>Range (mo.)</td>
<td>1 – 223</td>
</tr>
<tr>
<td>Average age (years)</td>
<td></td>
</tr>
<tr>
<td>$\leq$ 72</td>
<td>167 (49)</td>
</tr>
<tr>
<td>$&gt;$ 72</td>
<td>175 (51)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>259 (76)</td>
</tr>
<tr>
<td>Female</td>
<td>83 (24)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>Ta</td>
<td>115 (34)</td>
</tr>
<tr>
<td>T1</td>
<td>115 (34)</td>
</tr>
<tr>
<td>T2–4</td>
<td>112 (33)</td>
</tr>
<tr>
<td>WHO Grade (2004)</td>
<td></td>
</tr>
<tr>
<td>LG</td>
<td>82 (24)</td>
</tr>
<tr>
<td>HG</td>
<td>260 (76)</td>
</tr>
<tr>
<td>Recurrence (Ta/T1)</td>
<td></td>
</tr>
<tr>
<td>Frequent</td>
<td>61 (27)</td>
</tr>
<tr>
<td>Few</td>
<td>85 (37)</td>
</tr>
<tr>
<td>None</td>
<td>39 (17)</td>
</tr>
<tr>
<td>N/A*</td>
<td>45 (20)</td>
</tr>
<tr>
<td>Progression within 5 yrs (Ta/T1)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>31 (13)</td>
</tr>
<tr>
<td>No</td>
<td>103 (45)</td>
</tr>
</tbody>
</table>

Abbreviations: mo. = months, N/A = not available * Insufficient follow-up data.

At follow-up of the prospective material, the non-muscle invasive patients were categorized as having none, few or frequent recurrences. The group ‘few recurrences’ was defined as less than three recurrent tumors within 18 months, whereas the group ‘frequent recurrences’ was defined as three or
more recurrences within the same time period. Progression was defined as shift of the tumor into a higher stage. Months to progression within 5 years were on average 23.3 months (s.d., 14.4 range 2.0–55.0 months). Follow-up times for non-recurrent and non-progressing cases were ≥4 and ≥5 years, respectively. In addition to progression-free survival (PFS), the follow-up also included overall survival (OS) and disease-specific survival (DSS). The end points of OS, PFS and DSS were calculated from the date of surgery to the date of event or last follow-up. Cohort I has been published previously [Fristrup et al, 2012; Lindén et al, 2013; Boman et al, 2013a, b].

Validation cohort II. Patients in cohort II were 239 Swedish T1–T4 patients included in two randomised controlled trials [Sherif et al, 2004]. Clinical and histopathological data of the cohort are presented in Table 2. Primary end points were OS, DSS and cisplatin response. OS and DSS were calculated from the date of study inclusion to event or last follow-up date. Cisplatin response was defined as downstaging to no or non-invasive tumor in the cystectomy specimen. Ethical permit to use these samples for protein expression evaluation was obtained from regional ethical review board of Uppsala (reference number 2008:136).

Table 2 Clinico-histopathological characteristics for the (T1-T4)-patients in validation cohort II.

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no of cases</td>
<td>239</td>
</tr>
<tr>
<td>Follow-up time (mo.)</td>
<td>48.5</td>
</tr>
<tr>
<td>Range (mo.)</td>
<td>1 – 126</td>
</tr>
<tr>
<td>Average age (years)</td>
<td></td>
</tr>
<tr>
<td>≤ 63</td>
<td>98 (41)</td>
</tr>
<tr>
<td>&gt; 63</td>
<td>141 (59)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>196 (82)</td>
</tr>
<tr>
<td>Female</td>
<td>43 (18)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>3 (1)</td>
</tr>
<tr>
<td>T2</td>
<td>100 (42)</td>
</tr>
<tr>
<td>T3</td>
<td>108 (45)</td>
</tr>
<tr>
<td>T4</td>
<td>28 (12)</td>
</tr>
<tr>
<td>Number of patients</td>
<td></td>
</tr>
<tr>
<td>Clinical trial 1</td>
<td>59 (25)</td>
</tr>
<tr>
<td>Clinical trial 2</td>
<td>180 (75)</td>
</tr>
<tr>
<td>Number of patients</td>
<td></td>
</tr>
<tr>
<td>Cisplatin-based arm</td>
<td>117 (49)</td>
</tr>
<tr>
<td>Control arm</td>
<td>122 (51)</td>
</tr>
</tbody>
</table>
Cohort III represents a retrospective primary tumor /matched metastases whole-section material from 90 patients treated at Uppsala University Hospital between 1976 and 2003. The 90 patients were divided into two main groups: 70 primary tumors having one corresponding metastasis and 20 primary tumors matched with multiple metastases (two or three metastases). 10 of the 46 regional lymph glands were sentinel nodes from eight patients (two patients with two sentinel lymph glands each). Primary tumor tissues from 24 of the 90 patients are present in cohort I and use of this material has the same ethical review board number as for cohort I (see above). Histopathological and clinical data are presented in Table 3. This cohort has been studied previously [Gardmark et al, 2005].

Normal tissue
In addition, normal/non-malignant urothelium tissue specimens from sixth bladder cancer patients and one patient with benign bladder condition were also analyzed for STMN1 protein expression.

Table 3 Clinico-histopathological characteristics for the patients in the primary tumor/ matched metastases-material (cohort III).

<table>
<thead>
<tr>
<th>Variable</th>
<th>No. Cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total no of cases</strong></td>
<td>90</td>
</tr>
<tr>
<td><strong>Average age(years)</strong></td>
<td></td>
</tr>
<tr>
<td>≤ 66</td>
<td>43 (48)</td>
</tr>
<tr>
<td>&gt; 66</td>
<td>47 (52)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>66 (73)</td>
</tr>
<tr>
<td>Female</td>
<td>24 (27)</td>
</tr>
<tr>
<td><strong>Source of primary tumor</strong></td>
<td></td>
</tr>
<tr>
<td>Transurethral resection</td>
<td>76 (84)</td>
</tr>
<tr>
<td>Cystectomy</td>
<td>14 (16)</td>
</tr>
<tr>
<td><strong>Metastatic location</strong></td>
<td></td>
</tr>
<tr>
<td>Regional lymph gland</td>
<td>46 (40)</td>
</tr>
<tr>
<td>Distant lymph gland</td>
<td>7 (6)</td>
</tr>
<tr>
<td>Liver</td>
<td>8 (7)</td>
</tr>
<tr>
<td>Lung</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Skeleton</td>
<td>4 (3)</td>
</tr>
<tr>
<td>Intestinal</td>
<td>19 (16)</td>
</tr>
<tr>
<td>Prostate</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Vagina</td>
<td>3 (3)</td>
</tr>
<tr>
<td>Other</td>
<td>23 (20)</td>
</tr>
</tbody>
</table>
6. Procedures

6.1 Paper I

For the first 2 years follow-up entailed cystoscopy and cytology every third month if there was no recurrence, every 6 months until 5 year from the start of the treatment. The primary end point was recurrence-free survival at 6 months. Secondary end points were side-effects of the two treatments, time to failure of the treatment, progression, cancer-specific survival and overall survival. All recurrences had to be verified by histopathology, and progression was defined as muscle-infiltrative tumor or metastatic disease. Treatment failure was defined in our study as patients who progressed in their disease, received cystectomy or underwent radiotherapy. Crossover to the other treatment arm was recommended if a patient had remaining CIS or stage Ta recurrence. Cystectomy was recommended for recurrence of T1 tumors and for progression in stage (T2 or higher). Patients deemed unsuitable for cystectomy or patients with widespread disease were treated according to the procedures of the clinic.

6.2 Paper II-IV

Representative tissue areas were identified by a pathologist and two cores (1mm) were obtained from these areas. The tissue microarrays were constructed using an automated instrument, ATA-27 (Beecher Instruments, Sun Prairie, WI, USA). Automated immunohistochemistry (IHC) was performed as previously reported using an Autostainer 480 instrument® (Lab Vision, Fremont, CA, USA) [Segersten MU et al, 2009]. For paper II the tissue slides were incubated for 30 minutes with the monoclonal antibody emmprnin (sc-21746, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1/100 prior to using a dextran polymer visualization system (UltraVision LP HRP polymer®, Lab Vision) for detection of the protein expression. For survivin the polyclonal (ab-469, Abcam) 1/400 was used. For paper III the rabbit polyclonal antibody CCTα, HPA035428 1/75 (Sigma-Aldrich, St. Louis, MO, USA) was used. In paper II&III negative controls were produced by using Universal Negative Control Serum (A. Menarini Diagnostics, Berkshire, UK) instead of the primary antibody. For paper IV the primary mouse monoclonal STMN1 antibody (sc-48362, Santa Cruz Biotechnology, Santa
Cruz, CA, USA) diluted 1/250 was used. For p53 the primary mouse monoclonal p53-DO7 (DAKO, Glostrup, Denmark) and Ki67-MIB1 (Dako) diluted 1/500 and 1/600, respectively was used. Negative controls were produced by using PBS instead of primary antibody.

6.2.1 Annotation of IHC paper II-IV

Paper II was a validation study so methods were generally replicated from earlier studies [Als AB et al., 2007; Margulis V et al., 2009]. The staining was evaluated by two independent observers who were blinded to clinical and outcome data. Discordant scores were reviewed by a third scorer who made the final decision. Membrane staining was scored for emmprin and nuclear staining for survivin. The extent of staining in the tumor was scored as the number of cells stained: negative ≤ 10 % and positive >10 % as in the study by Als [Als AB et al., 2007].

For paper III the biomarker testing had no previous literature guidelines for manual scoring in the bladder, so we used our own lab H-score from HPA. The staining was evaluated by pathologist blinded to clinical and outcome data. The extent of staining in the tumor was scored as the number of cells stained: 0 - < 20 % (score 1), 20 - 50 % (score 2), >50 - 75, (score 3), > 75% (score 4). Intensity was scored as negative (score 0), weak (score 1), medium (score 2) or high (score 3). The product of the intensity- and extent scores were calculated to obtain a semi quantitative H-score. Analysis for the different CCTα score groups showed that categories 1-12 had similar outcomes when assessing all patients, thus a dichotomized distribution called the final score (0) negative and (1-12) as positive was used for analyzing the treatment arms separately.

In paper IV, evaluating the STMN1 expression, a previously published scoring system of IHC was used [Segersten et al., 2009]. Intensity was scored as weak (score 1), medium (score 2) or high (score 3). The extent of staining was scored as the number of cells stained :< 25 % (score 1), 25–75% (score 2), >75% (score 3). The final H-score was calculated as the product of the intensity- and extent scores. Score category 0: negative (score 0); score category 1: weak expression (score 1–3); score category 2: moderate/strong expression (score 4–9). Moreover, IHC analyses of the p53- and Ki67-protein levels were performed according to the protocol described above. Scoring and cutoff value of >20% cells stained as positive, for both p53 and Ki67, were according to previous publications [Saint et al., 2004; Margulis et al., 2006]. The scoring was performed, for each protein, by two independent observers.
6.3 Additional procedure for paper IV

6.3.1 Cell culture and transfection

A human muscle-invasive urinary bladder cell line, T24, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) was cultured in RPMI-1640 (Life Technologies, Carlsbad, CA, USA) with 5% FBS (Life Technologies) and 1% penicillin/streptomycin (Life Technologies). The cells were transfected in six-well plates with a 5’ siRNA (AM16708, Life Technologies) and a 3’ siRNA, (s8093, Life Technologies), directed against the STMN1 mRNA using Lipofectamine (Life Technologies) as a transfecting agent according to the manufacturer’s protocol. Two controls were used, one with only the transfection agent lipofectamine and one adding scrambled siRNA (4390843, Life Technologies) without a specific mRNA target. The 3’ siRNA was used in the following proliferation assay.

6.3.2 Proliferation assay

Transfected T24 cells (as described above) were seeded in 96-well plates and incubated for 24 h allowing the cells to attach and grow. MTS (3-(4, 5-dimethylthiazol-2-yl) -5- (3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium) assay from Promega (Fitchburg, WI, USA) was used to measure cell viability after 24, 32, 48, 56, 84, 104 and 128 h of seeding.

6.3.3 Invasion assay

The effect of STMN1 on the invasive ability of T24 cells was evaluated using a 96-well extracellular matrix (ECM) chamber assay (Cellbiolabs Inc, San Diego, CA, USA). Briefly, the lower chambers were filled with RPMI-1640-medium (Life Technologies), using 10% FBS as an attractant component. T24 cells transfected with STMN1 3’ siRNA and scrambled siRNA as described above were seeded in the upper chambers in serum-free medium. After incubation for 20 h at 37°C, the cells migrating through the ECM membrane were lysed and quantified using CyQuant GR Dye (Cellbiolabs Inc). The fluorescence was read at 480 nm/520 nm.

6.3.4 Western blot

The Western blot analysis was performed according to previously published protocol [Lindén et al, 2012] with the differences that the total cell protein lysates were of T24 cell line origin and the following antibodies were used: (i) primary antibodies; the monoclonal mouse anti-STMN1 antibody (sc-48362, Santa Cruz Biotechnology) also used for IHC (see above) and the polyclonal rabbit anti-β actin antibody (RB-9421, Waltham, MA, USA), (ii)
secondary antibodies; sheep anti-mouse (NA931V, GE Healthcare, Little Chalfont, UK) for STMN1 and goat anti-rabbit (sc-2004, Santa Cruz Biotechnology) for β-actin.
7. Statistical analysis

In paper I, intention-to-treat was basis of all our statistics. The event-free period was calculated according to the Kaplan-Meier model. Comparison between the groups was performed using the log-rank test. Multivariate analyses were performed with the Cox proportional hazards regression model. All tests were two-tailed, and \( p < 0.05 \) was considered statistically significant.

In paper II-IV, statistical analyses were performed using the SPSS 20.0® / SPSS 22.0® (IBM SPSS Statistics, USA) software. For measuring the interrater agreement of scores Cohen’s kappa coefficient was calculated. Overall and cancer specific survival curves were constructed according to Kaplan-Meier, and differences between groups were studied using the Log rank test. Univariate and multivariate Cox’s proportional hazards regression analyses were performed, adjusting for patient inclusion in one of the two trials, treatment allocation to cystectomy with or without neoadjuvant chemotherapy, age, gender and T categorization. Number needed to treat was calculated with Prism (version 6, GraphPad Software, Inc.).

In paper IV correlation analyses of protein expression with the clinical variables age, gender, stage, grade and recurrence were assessed using the Spearman’s or the Pearson’s \( \chi^2 \) (two-sided tests) when appropriate. For recurrence, the statistical analyses were performed in three groups (none, few or frequent recurrences) and in two groups (recurrence: yes or no). Survival analyses for OS, DSS and PFS were performed using log-rank test and survival curves were estimated by the Kaplan–Meier method. Univariate and multivariate Cox’s proportional hazards regression analyses were performed, adjusting for age, gender, T-stage and grade. In the statistical analysis, comparisons between following score categories were performed: (0 vs. 1 vs. 2), (0 vs. 1, 2) or (0, 1 vs. 2). A p-value <0.05 was considered significant.

The in vitro experiments were evaluated using Student’s t-test (two-sided) and the analyses are based on at least three independent experiments. For cell proliferation and invasion assays, number of replicas in each experiment were six at least 20 wells, for treated and untreated cells, to determine significant difference (p-value < 0.05).
8. Results

8.1 Results paper I

The total elapsed time for the study is now 6.9 years; all statistical calculations were however done at the end of the five-year period. During the follow-up 33 patients had cystectomy and 15 were given radiotherapy (Table 4). The Consort diagram (Figure 5) portrays the different finales. In a multivariable analysis the type of intravesical therapy, size and tumor status at second resection were independent variables associated with recurrence. When this study was performed per treatment arm tumor size was the only independent factor in the BCG group while this was the case for tumor status at second resection, age and concomitant cancer in situ in the other group. Independent factor for both progression and treatment failure was T1 stage at second resection. Regarding cancer specific survival none of the variables were independent, however, T1 stage at second resection was of borderline significance ($p=0.076$) (Table 5). The recurrence free rate at the five years follow-up 38% were in the combination arm as opposed to 59% in the BCG arm ($p=0.001$). The recurrence free survival is depicted in Figure 6. This was also studied according to the stratification criteria. Patients with associated carcinoma in situ in the combination arm had a significantly worse outcome ($p<0.001$) compared to the rest of the subgroups, including those with carcinoma in situ in the BCG arm (Figure 7). The result for the other endpoints for combination and BCG treated were: free of progression 78 and 77%, free of treatment failure 75 and 75%, cancer specific survival 90 and 92%, respectively. None of these differences were substantial. Crossover after relapse was instituted in 10 and 30 of the BCG and the combination treated patients respectively. Second line BCG resulted in a recurrence-free rate at two years of 63% (9/33) while 30% (3/10) obtained this with the combination therapy (Figure 8).
Table 4 Secondary treatments up to 60 months.

<table>
<thead>
<tr>
<th>Treatment type</th>
<th>BCG</th>
<th>Epirubicin/Interferon –α2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crossover</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>Cystectomy</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Radiation</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 5 Flow of patients during follow-up.
Figure 6 Recurrence-free survival of patients according to randomization arm (p = 0.001).

Figure 7 Recurrence-free survival in subgroups according to CIS status. BCG, no CIS vs. Epi, no CIS: p=0.38, BCG, no CIS vs. BCG, CIS: p=0.41, BCG, no CIS vs. Epi, CIS: p<0.001, EPI, no CIS vs. Epi, CIS: p=0.13, EPI, no CIS vs. Epi, CIS: p<0.001.
Figure 8  Recurrence-free survival after crossover. (Stage at crossover Ta-28, T1-2, Tis -10) p=0.007.
Table 5  Multivariate analysis for risk of recurrence, treatment failure and cancer specific death at 60 months for all patients and according to treatment arm.

<table>
<thead>
<tr>
<th>Variables</th>
<th>All patients</th>
<th>BCG</th>
<th>Epirubicin+Interferon 2α</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>HR</td>
<td>95.0% CI for Exp(B)</td>
</tr>
<tr>
<td>Treatment (BCG,Epi)</td>
<td>0.002</td>
<td>1.883</td>
<td>1.26</td>
</tr>
<tr>
<td>Sec look, T1</td>
<td>0.001</td>
<td>2.267</td>
<td>1.453</td>
</tr>
<tr>
<td>Size (&lt;3, &gt;3)</td>
<td>0.008</td>
<td>1.689</td>
<td>1.145</td>
</tr>
<tr>
<td>Multiplicity (1, &gt;1)</td>
<td>0.115</td>
<td>1.365</td>
<td>0.927</td>
</tr>
<tr>
<td>Sex (Male, Female)</td>
<td>0.703</td>
<td>0.912</td>
<td>0.569</td>
</tr>
<tr>
<td>Age (&lt;67, ≥67)</td>
<td>0.559</td>
<td>0.894</td>
<td>0.615</td>
</tr>
<tr>
<td>TIS (No, Yes)</td>
<td>0.238</td>
<td>1.278</td>
<td>0.851</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment (BCG,Epi)</td>
<td>0.936</td>
<td>0.977</td>
<td>0.557</td>
</tr>
<tr>
<td>Sec look, T1</td>
<td>0.435</td>
<td>1.329</td>
<td>0.651</td>
</tr>
<tr>
<td>Size (&lt;3, &gt;3)</td>
<td>0.087</td>
<td>1.641</td>
<td>0.951</td>
</tr>
<tr>
<td>Multiplicity (1, &gt;1)</td>
<td>0.408</td>
<td>1.229</td>
<td>0.703</td>
</tr>
<tr>
<td>Sex (Male, Female)</td>
<td>0.286</td>
<td>1.061</td>
<td>0.319</td>
</tr>
<tr>
<td>Age (&lt;67, ≥67)</td>
<td>0.663</td>
<td>1.129</td>
<td>0.654</td>
</tr>
<tr>
<td>TIS (No, Yes)</td>
<td>0.279</td>
<td>1.374</td>
<td>0.772</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment (BCG,Epi)</td>
<td>0.892</td>
<td>1.071</td>
<td>0.4</td>
</tr>
<tr>
<td>Sec look, T1</td>
<td>0.168</td>
<td>2.329</td>
<td>0.701</td>
</tr>
<tr>
<td>Size (&lt;3, &gt;3)</td>
<td>0.554</td>
<td>0.68</td>
<td>0.19</td>
</tr>
<tr>
<td>Multiplicity (1, &gt;1)</td>
<td>0.554</td>
<td>0.68</td>
<td>0.19</td>
</tr>
</tbody>
</table>
8.2 Results paper II

The status of immunoreactivity could be assessed for emmprin in 241 (96%) patients and for survivin in 236 (94%) patients of 250 included patients. Baseline characteristics are shown in table 2. Biomarker distribution of the patients according to treatment arm are depicted in Table 6. Illustrations of the different staining patterns are depicted in Figure 9. There was no significant difference in biomarker distribution between stages (T1-2/ T3-4). The inter-observer agreement of survivin scores (negative/positive) was 0.62, and for emmprin it was 0.61.

Table 6 Biomarker distribution according to treatment arm.

<table>
<thead>
<tr>
<th></th>
<th>Neoadjuvant arm</th>
<th>Cystectomy arm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative/positive</td>
<td>60/59</td>
<td>59/58</td>
</tr>
<tr>
<td>Emmprin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative/positive</td>
<td>87/34</td>
<td>83/37</td>
</tr>
</tbody>
</table>

Figure 9 Sample immunohistochemical images of emmprin (a, b) and survivin staining (c, d) negative and positive expression (x20 magnification).

Figure 10 depicts the overall survival in months from randomization in the whole cohort (n=250) with results comparing both arms. When analyzing the
OS in the chemotherapy cohort, patients with negative emmprin expression had a significantly higher overall survival compared to their counterparts in the positive emmprin group, 71% versus 38%, \( p<0.001 \). This was also the case for CSS where the results for patients with negative emmprin expression was 76% and for patients with positive emmprin expression it was 56%, \( p=0.027 \) (data not shown). Nevertheless, in the cystectomy cohort, emmprin expression was not associated with either OS or CSS (46 % and 35%, \( p=0.23 \), and 55% and 51%, \( p=0.64 \)), Figure 11.

![Figure 10](image-url)  

**Figure 10** Overall survival in months from randomization in the whole cohort (n=250) showing results comparing both arms.
**Figure 11 a-b** Overall survival in months from randomization in emmprin positive and negative patients (n=121) (a) receiving chemotherapy, and (b) patients (n=120) receiving only surgery (cystectomy arm).
Figure 12 a-b Overall survival in months from randomization according to treatment in emmprin negative patients (n=170) (a) and in emmprin positive patients (n=71) (b).
Table 7 Multivariate analysis of overall survival and cancer specific survival for all patients.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Overall survival</th>
<th>Cancer specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p</td>
<td>HR</td>
</tr>
<tr>
<td></td>
<td>Lower</td>
<td>Upper</td>
</tr>
<tr>
<td>Trial</td>
<td>0.301</td>
<td>1.282</td>
</tr>
<tr>
<td>Gender</td>
<td>0.352</td>
<td>1.25</td>
</tr>
<tr>
<td>Average Age</td>
<td>0.755</td>
<td>1.065</td>
</tr>
<tr>
<td>Emmmprin negative/positive</td>
<td>0.017</td>
<td>1.64</td>
</tr>
<tr>
<td>Neoadjuvant/Cystectomy arm</td>
<td>0.004</td>
<td>1.729</td>
</tr>
<tr>
<td>T1-2/T3-4</td>
<td>0.026</td>
<td>1.556</td>
</tr>
</tbody>
</table>
When analyzing score categories and outcome the emmprin negative patients had an absolute risk reduction of 25% in OS (CI 11-40) and NNT of 4 (CI 2.5-9.3)(Figure 12). In multivariable analyses including emmprin status, trials, treatment arm, stage, age and gender only emmprin status, stage and treatment arm were associated with OS, while only arm correlated to CSS (Table 7). Downstaging (to p-T0, Ta, Tis) among those with negative emmprin expression occurred in 44% after chemotherapy and in 21% of the controls, (p=0.004). Regarding survivin, a trend for improved outcome for those with negative survivin expression in the chemotherapy cohort was found (p=0.146), but survivin expression was not significantly associated with outcome (data not shown).

8.3 Results paper III

Clinicopathological data of the TMA cohorts are given in table 8. The number of tumor samples was 93% (241) of totally 250 included patients. Illustrations of the nuclear staining patterns are depicted in Figure 13. There was no significant difference in biomarker distribution between stages (T1-2/ T3-4), p=0.908. Biomarker distribution of the patients according to treatment arm are depicted in Table 8.

### Table 8 Biomarker distribution of CCTα according to treatment arm.

<table>
<thead>
<tr>
<th>Biomarker Distribution</th>
<th>Neoadjuvant arm</th>
<th>Cystectomy arm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCTα negative/positive</td>
<td>38/83</td>
<td>32/88</td>
</tr>
</tbody>
</table>

**Figure 13** Sample of immunohistochemical images of CCTα (a) negative and (b) positive expression (x20 magnification).

Patients with lack of expression (negative) enrolled in the cystectomy arm showed a worse 5 year overall survival than positive expressed patients, 12% versus 50 %, p<0.001 respectively (Figure 14a). Whereas negatively expressed patients in the treatment arm mimic similar outcome as those positively stained, 54 % and 67% p=0.025 (Figure 14b). Equivalent result where
found in CSS (data not shown). Allocating the negatively and positively expressed tumors over respective treatment arm, the consequence of neoadjuvant chemotherapy over these patients both in overall survival (Figure 15 a&b) and cancer specific survival becomes more apparent (data not shown).

Figure 14 (a-b) Overall survival (OS) in months from randomization in CCTα positive and negative patients (a) not receiving neoadjuvant therapy (cystectomy arm) (n=120), and (b) patients receiving neoadjuvant therapy (n=121).
Figure 15 (a-b) Overall survival (OS) in months from randomization according to treatment in (a) CCTα negative patients (n=70) (b) and in CCTα positive patients (n=171).
When evaluating score categories and outcome, the negatively stained tumors by the CCTα antibody had an absolute risk reduction of 33% (CI 12-55) with NNT of 3. The corresponding result for positive tumors was an absolute risk reduction of 11% (CI -3-25) with NNT of 10. In multivariate models that also included trial, gender, age, treatment arm and pathological stage, CCTα expression was related as follows to OS (p=0.001; HR= 0.464, 95% CI 0.296-0.725) and CSS (p=0.002, HR= 0.449, 95% CI 0.268-0.754) (Table 9 a-b). No correlation to downstaging (to p-T0, Ta, Tis) among those with negative CCTα expression was noted (neoadjuvant arm p=0.402; cystectomy arm p=0.177).

Table 9 a&b Multivariate analysis of outcome for all patients and according to OS and CSS.

a) Overall survival

<table>
<thead>
<tr>
<th>Variables</th>
<th>p</th>
<th>HR</th>
<th>95.0% CI for HR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>Trial</td>
<td>0.140</td>
<td>1.403</td>
<td>0.895</td>
</tr>
<tr>
<td>Gender</td>
<td>0.243</td>
<td>1.325</td>
<td>0.827</td>
</tr>
<tr>
<td>Average Age</td>
<td>0.242</td>
<td>1.259</td>
<td>0.856</td>
</tr>
<tr>
<td>CCTα negative/positive</td>
<td>0.001</td>
<td>0.464</td>
<td>0.296</td>
</tr>
<tr>
<td>Neoadjuvant/Cystectomy arm</td>
<td>0.001</td>
<td>2.063</td>
<td>1.385</td>
</tr>
<tr>
<td>T1-2/T3-4</td>
<td>0.041</td>
<td>1.481</td>
<td>1.016</td>
</tr>
</tbody>
</table>

b) Cancer specific survival (CSS)

<table>
<thead>
<tr>
<th>Variables</th>
<th>p</th>
<th>HR</th>
<th>95.0% CI for HR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lower</td>
</tr>
<tr>
<td>Trial</td>
<td>0.364</td>
<td>1.266</td>
<td>0.760</td>
</tr>
<tr>
<td>Gender</td>
<td>0.484</td>
<td>1.224</td>
<td>0.695</td>
</tr>
<tr>
<td>Average Age</td>
<td>0.967</td>
<td>1.009</td>
<td>0.649</td>
</tr>
<tr>
<td>CCTα negative/positive</td>
<td>0.002</td>
<td>0.449</td>
<td>0.268</td>
</tr>
<tr>
<td>Neoadjuvant/Cystectomy arm</td>
<td>0.001</td>
<td>2.202</td>
<td>1.380</td>
</tr>
<tr>
<td>T1-2/T3-4</td>
<td>0.176</td>
<td>1.350</td>
<td>0.874</td>
</tr>
</tbody>
</table>
8.4 Results paper IV

A clear, distinct cytoplasmic STMN1 staining was observed in the urinary bladder cancer tissue, both non-muscle- and muscle invasive tumors, as well as in bladder cancer metastases (Figure 16). The majorities (five out of seven) of the investigated non-malignant bladder tissue specimens were STMN1-negative (Figure 16A) and the rest (two out of seven) were classified as having category 1 intensity. The pattern of the IHC stainings was in concordance with a previous bladder cancer publication of STMN1 stainings (Wosnitzer et al., 2011) and with the human protein atlas validation of the antibody in normal and bladder cancer tissue. The following inter observer $k$ values were obtained for manual IHC scoring of: STMN1: 0.73 (cohort I), 0.71 (cohort II), 0.76 (cohort III); p53: 0.70 (cohort I); Ki67: 0.75 (cohort I).

Figure 16. Cytoplasmic STMN1 expression in different urinary bladder cancer tumor stages. (A) Normal (x20) with negative expression, (B) low-grade Ta tumor (x20) with weak expression, (C) high-grade T1 tumor (x20) with moderate/strong expression and (D) high-grade T2 tumor (x20) with moderate/strong expression. Moderate/strong expression of STMN1 in metastatic urinary bladder cancer exemplified in E. A high-grade T3 primary tumor (x20) and (F) matching sentinel lymph node metastasis (x20).
Cohort I

Correlation of STMN1 expression to clinical data

In the statistical analysis examining the correlation between STMN1 expression, score categories: (0 vs. 1 vs. 2), (0 vs. 1, 2) or (0, 1 vs. 2), and basic and survival variables, following results were significant. Statistical calculations of the cytoplasmic STMN1 expression, in the 342 TMA tumors, revealed that STMN1 strong staining (tumors belonging to score category 2) significantly correlated to higher stage (p<0.01), high grade (p<0.001) and shorter OS and DSS (Figure 17).
Figure 17. Kaplan–Meier plots describing the correlation of STMN1 to survival. In advanced bladder cancer patients (cohort I), STMN1 overexpression was correlated to shorter (A) OS in T2–T4 patients and to shorter (B) DSS in T1–T4 patients. In cohort I (C) as well as in cohort II (D) T2–T4 patients with moderate/strong STMN1-expressing tumors had a higher risk of cancer-specific death within 2 years. Log-rank test results are displayed for each Kaplan–Meier plot. The p-values are the adjusted values from the multivariate analyses, correcting for stage, age and gender. (Negative exp. =dotted line, neg./weak or weak exp. =dashed line and mod./strong exp. =solid line).
Staging expression, overall survival, disease specific survival & cox analysis

Adjusting for stage, age and gender using multivariate Cox’s proportional hazards regression, analyses resulted in following hazard ratios (HRs), confidence interval (CI) and P-values: in T1–4 patients: DSS (0, 1 vs. 2: HR=1.83, 95% CI 1.09–3.08; p=0.02); in subgroup T2–T4 patients: OS (0 vs. 2: HR=1.77, 95% CI 1.02–3.07; p=0.04) and DSS (0, 1 vs. 2: HR=2.04, 95% CI 1.13–3.68; p=0.02). In addition, in muscle invasive bladder cancer, log-rank analysis of DSS (STMN1 score: 0 vs. 2) demonstrated to be of borderline significant (p=0.053, data not shown). However, when correcting for stage, age and gender, there was significantly shorter DSS for patients with tumors staining strongly for STMN1 (HR=2.1, 95% CI 1.04–4.25; p=0.04) compared with those with negative tumors. There was no correlation between STMN1 expression and age, gender, recurrence or PFS.

Cohort II, validation

In cohort II, we were able to validate that high tumor expression of STMN1 is correlated to higher risk of death in cancer within 2 years in T2–T4 patients (Figure 17D, Table 10). We were not able to validate in cohort II that shorter 5-year OS was associated to overexpression of STMN1, as seen in cohort I (Figure 17A). STMN1 expression was not related to age, gender, stage (T1–2 vs. T3–4) or cisplatin response. As cohort II only consists of three T1 tumors and the rest T2–T4 tumors (Table 2), we could not perform survival analysis on T1–T4 group or Spearman’s or Pearson’s χ²(analysis on all tumor stages, grade, recurrence or PFS.
Table 10 Univariate and multivariate Cox regression analyses regarding DSS (2 years) in cohort I and II (T2-T4 patients).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cohort I (T2-T4, n=112)</th>
<th></th>
<th>Cohort II (T2-T4, n=236)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
<td>Univariate</td>
<td>Multivariate</td>
</tr>
<tr>
<td></td>
<td>HR (95% CI) p-value</td>
<td>HR (95% CI) p-value</td>
<td>HR (95% CI) p-value</td>
<td>HR (95% CI) p-value</td>
</tr>
<tr>
<td>Age (≤av.age vs &gt;av.age)</td>
<td>2.2 (1.24–3.88) 0.007</td>
<td>2.33 (1.30–4.18) 0.004</td>
<td>1.69 (0.98-2.9) 0.057</td>
<td>1.45 (0.84-2.52) 0.19</td>
</tr>
<tr>
<td>Gender</td>
<td>0.81 (0.46-1.44) 0.48</td>
<td>0.73 (0.40-1.31) 0.29</td>
<td>1.33 (0.72-2.45) 0.36</td>
<td>1.11 (0.59-2.06) 0.75</td>
</tr>
<tr>
<td>Stage (T2 vs. T3,4)</td>
<td>1.59 (0.84-2.99) 0.15</td>
<td>2.0 (1.04–3.83) 0.037</td>
<td>1.62 (0.96-2.74) 0.073</td>
<td>1.55 (0.91-2.62) 0.11</td>
</tr>
<tr>
<td>STMN1 (0,1 vs. 2)</td>
<td>1.84 (1.04–3.26) 0.037</td>
<td>2.04 (1.13–3.68) 0.019</td>
<td>1.93 (1.16–3.22) 0.012</td>
<td>1.76 (1.04–2.99) 0.030</td>
</tr>
</tbody>
</table>
Cohort III, STMN1 expression in primary tumors and matched metastases

Representative images of the immune reactivities of metastatic tissues are supplied in Figure 16E and F, and the STMN1 expression in the patient material containing primary tumor and metastases is presented in Table 3.

In the total tumor material, 83% (75 out of 90) of the primary tumors and 79% (92 out of 116) of the metastases were STMN1-positive (score category 1 or 2). The expression of STMN1 in matched metastases was stronger in 19% (17 out of 90), weaker in 27% (24 out of 90), the same in 44% (40 out of 90) or heterogeneous expression among several metastases/patient in 10% (9 out of 90), compared with primary tumor.

In addition, for 70% (63 out of 90) of the patients, STMN1 was expressed both in primary and matched metastases. In the subgroup with only one matched metastasis (70 patients), 81% (n=57) of the primary tumors and 74% (n=52) of the metastases stained positive for STMN1 (Figure 18A).

Figure 18 Immunoreactivity score of the STMN1 protein in the metastatic cohort III. (A) Distribution of score categories of primary tumors and metastases from patients with single metastasis. White=score category 0, light grey=score category 1 and dark grey=score category 2. (B) Distribution of score categories of primary tumors and metastases from patients with multiple metastases. White=score category 0, light grey=score category 1 and dark grey=score category 2 and striped=the score categories of the matching metastases are heterogeneous. (C) Summary of primary tumor immunoreactivity in relation to their matching metastases. White=all metastases are in a lower score category than the primary tumor, light grey=all metastases are in the same score category as primary tumor, dark grey=all metastases are in a higher score category than the primary tumor and striped=the score categories of the matching metastases are heterogeneous.
For the majority of the patients 69% (n=48), both primary tumor and matched metastasis expressed STMN1 (score category 1 or 2). The highest score category 2 was observed for 40% (n=28) of the primary tumors and 39% (n=27) of the metastases (Figure 18A). In addition, 50% (n=35) of the metastases were in the same score category as its primary tumor (Figure 18C). Interestingly, 20% (n=14) of the metastases showed a higher expression than matched primary tumors (Figure 18C). Notably, 4 of these 14 patients had an entirely negative primary tumor.

In the subgroup of patients with multiple metastases, 95% (19 out of 20) presented at least one metastasis positive for STMN1 (Figure 18B). Subgrouping further, we observed that 35% (n=7) of the patients had all their metastases in the higher score category 2 (Figure 18B) and for three patients all metastases had a higher STMN1 score than corresponding primary tumors (Figure 18C). For the primary tumors, only two were negative and 85% (n=17) of the patients had a positive primary tumor in combination with at least one positive metastasis. For 75% (n=15) of the patients all tumors, both primary tumor and all matched metastases, were positive, belonging to either score category 1 or 2.

In the primary matched metastasis material (90 patients), there were 10 sentinel lymph nodes of 46 regional lymph gland metastases. For all eight patients with sentinel nodes, both primary and matched sentinel nodes were STMN1-positive (score category 1 or 2). Six of the ten sentinel lymph glands had an overexpression of STMN1 (score category 2).

**Additional results**

**Correlation of STMN1 to p53 and Ki67 protein expression**

The protein expression of p53 and the proliferation marker Ki67 were evaluated in bladder cancer tissue represented by cohort I. High STMN1 expression correlated to increased protein expression of p53 (p<0.001) high protein levels of Ki67 (p<0.001).

**siRNA knockdown of STMN1 expression**

The effort to knockdown the expression of STMN1 in the T24 cell line resulted in a partial knockdown with the 5′ siRNA and a complete knockdown with the 3′ siRNA, as can be seen in figure 19. When inspecting the cell cultures with microscopy, before lysing, there was no morphological difference between treated cells and controls.
Figure 19 Western blot experiment for evaluation of the effect of siRNAs on STMN1 expression. STMN1 3’ siRNA completely inhibited STMN1 protein expression in T24 cells, whereas the 5’ siRNA only reduced the expression. The β-actin signal was used as loading control.

Cell proliferation in T24 cells

To evaluate the role of STMN1 in bladder cancer cell proliferation, we performed MTS assays of T24 cells incubated with the STMN1 3’ siRNA, which had best effect to decrease STMN1 protein levels in the cells. We observed that the STMN1 3’ siRNA significantly reduced the cell proliferation at 48 (p=0.0065), 56 (p=0.0005), 84 (p=0.0037), 104 (p=0.0001) and 128 hrs (p=0.0011) after seeding the siRNA-treated cells (Figure 20).
Figure 20 The effect of STMN1 on T24 cell proliferation. The viability of T24 cells transfected with STMN1 siRNA (○) or scrambled siRNA (▼) was significantly different (p<0.01) from 48 h after seeding until the experiment ended after 128 h. Values are the means of three independent experiments with six replicates each, for both treated cells and controls (±SD).

Invasion assay

To determine the role of STMN1 in bladder cancer cell migration, STMN1 3’ siRNA-transfected T24 cells were analysed in an ECM chamber assay. The STMN1 3’ siRNA-treated cells demonstrated a significantly reduced invasion rate (p<0.0001) compared with control cells (Figure 21).
Figure 21 Cell migration ability of T24 cells transfected with STMN1 siRNA scrambled siRNA. Downregulation of STMN1 significantly (p<0.0001) reduced the invasiveness of the T24 cells. Values (Reference Fluorescence Unit, RFU) are the means of four independent experiments with at least 20 replicates each, for both treated cells and controls (±SD). Each of the four experiments was significant even separately (p≤0.01).
9. Discussion

9.1 Paper I

Conferring to current guidelines concomitant CIS, histological grade and to some extent subcategorizing T1, are chief elements that should be considered when approaching the management of NMIBC. They are collectively regarded as a display of poor prognosis. That was the reason for stratifying for these factors. It is common presently to include any T1, irrespective of grade as belonging to the high-risk non-muscle invasive bladder cancer category. In our 24 months analysis grade (2 or 3) was an independent factor for recurrence but this was not seen at the longer follow-up [Duchek M et al, 2010]. Nor was it an important factor for the other endpoints. This is in line with ICUD-EAU references that any invasive tumor should be called high grade [Amin MB et al, 2012]. The presence of concomitant CIS with T1 tumors was shown to be a risk factor in several studies [Bianco FJ Jr et al, 2004; Solsona E et al, 2004; Masood S et al, 2004; Orsola A et al, 2005], and increased the risk for progression six fold, according to the EORTC risk tables [Sylvester RJ et al, 2006]. Curiously we now show that this was not evident with the recommended modern management as opposed to non-BCG therapy. This is more in agreement with results from the CUETO group that found only a modest increased risk [Fernandez-Gomez J et al, 2008]. Adjuvant chemotherapy with BCG has been used for more than thirty years. At present we know much about the strengths and limitations of this therapy. The later has stimulated efforts to find new drugs or combinations with better efficacy and less toxicity. One example is the Nordic CIS trial which tested a combination of mitomycin-C and BCG [Kaasinen E et al, 2003]. Regrettably the results with alternative therapies in this, and other trials, have been disappointing. The difference in their studies was that no second resection was performed and BCG maintenance was shorter. Additional change in risk grouping should be incorporation of the tumor status at second resection. The prognostic significance of stage at second resection was found in an analysis of a large case series several years ago [Herr HW et al, 2007]. We found that those with remaining T1 tumor at second resection had a more than twofold increased risk for recurrence, compared to those with less tumor burden; questioning if bladder sparing is a viable alternative for these patients. Therefore, it seems now evident that risk tables have to be updated with information based on presently recommended management of
high-risk tumors. Irrespective of the contemporary treatment and relatively large size our trial has some limitations. One is that the study never employed the planned number of patients making subgroup analysis more uncertain. Other confines are that the number of patients receiving single instillation of chemotherapy after TURB, and those excluded because stage T2 or higher at second resection were not registered. No central pathologic review has been performed on the total material but review of the samples from the Swedish patients (number 177) showed a concordance of more than 90% with local pathology. Lastly patients unfit for major surgery was not included as secondary cystectomy was a recommendation. We have earlier reported our results after crossover in a trial comparing mitomycin-C and BCG [Malmström PU et al, 1999]. Crossover treatment was in that study successful in 39% of patients with second line BCG compared to 63% in the present trial. The former trial had different inclusion criteria making comparisons difficult. The benefit with the cross-over possibility is that non-responding patients sooner will have an alternate therapy and that this therapy can be registered as opposed to outside protocol treatment. Disadvantage is that this could be a confounder when measuring endpoints other than primary recurrence. The five-year cancer specific survival in this trial is more than 90%. This is better than the 83% reported with immediate cystectomy [Denzinger S et al, 2008]. Nonetheless, randomized comparisons are needed to evaluate this endpoint.

9.2 Paper II & III

The important finding in these studies are that two of the three candidate biomarkers, emmprin and CCTα, were predictive of response to neoadjuvant chemotherapy prior to cystectomy for locally advanced bladder cancer. Survivin had no predictive value regarding response to this treatment. In a previous study by Als et al. performed on a retrospective series of locally advanced and/or metastatic bladder cancer following cisplatin-based chemotherapy to identify molecular markers [Als AB et al, 2007]. Using protein expression assessed by IHC a strong correlated to response to chemotherapy was shown. In their multivariate analysis emmprin and survivin expression were both independent prognostic markers for poor outcome, together with the presence of visceral metastases. In another innovative study Vaezi et al. presented CCTα as a new alternative protein biomarker for prognosis in a case series of head and neck tumors [Vaezi AE et al, 2014]. In our series we validate the emmprin findings in the neoadjuvant setting. Our survivin results showed a trend toward better survival for survivin-negative tumors but no significant difference. The available control group lends the opportunity to compare with neoadjuvant therapy showing that both emmprin and CCTα negative patients clearly benefit from neoadjuvant chemotherapy in compar-
ison to the positive patients. Presently few biomarkers have shown potential, a major drawback has been methodological and thus applying the same evidence-based medicine standards as other types of medical interventions have been proposed [McShane LM et al, 2012]. In this type of study, specimens that are collected, processed, and archived during the course of a prospective trial are analyzed retrospectively to test the clinical utility of a tumor marker. Our bio-bank has that design and is thus suitable for validation of candidate biomarkers, both the prognostic value in the surgery only arm and the predictive value in the neoadjuvant arm. Additional benefit compared to other studies is that the tumor specimens were obtained from the diagnostic transurethral resection. Limitations of this investigation ought to be noted. Concerning the material, the trials had different chemotherapy combinations that are not considered standard today. Nonetheless, both trials were included in the meta-analysis that is the basis for recommendations in the current guidelines, and none of the newer combinations has been proven to be more effective than those used earlier. Furthermore, in the first trial preoperative radiotherapy was given to all patients as was routine at that time. In our multivariate analysis the trial is included to control for these discrepancies. Additional limitation is the limited lymph node dissection stipulated at the time and exclusion of the non-Swedish specimens due to ethical policy issues, but as the trials were stratified by country the risk of confounding in this respect is minimal. The survival benefit was greater in the biobank material than in the original combined trials. When comparing stages (T1-2/3-4) between the original material and the biobank subset we found more advanced tumors in the later. It is probable that with smaller tumors it was often the case that no histological material was left for IHC analyses. Concerning methodological limitations variables include pre-analytic, analytic, and post-analytic factors. Pre-analytically, different tissue handling, storage time preparation and fixation, diverse reagents, different staining methods between hospitals could all be factors. We did not find apparent differences when comparing expression between larger contributors. Analytically to reduce the number of methodological variables we have chosen to use tissue microarrays and an automated autostainer, but tumor heterogeneity is well known for protein markers and generalization of results obtained with this technique has been questioned [Gudjonsson S et al, 2011]. Consequently no universally accepted standardization guidelines have been developed. This approach removes differential antigen retrieval and staining conditions as possible confounders [Bussolati G et al, 2008]. The manual scoring method is subjective, and thus open to human variability. Separating negative versus all degrees of positive staining facilitates the scoring. Kappa values between 0.61-0.80 are considered good, but improvements in reproducibility are clearly necessary in order to introduce this method in clinical routine [Altman DG et al, 1991]. Post-analytically we concentrated on survival in the outcome analysis, which was the primary endpoint in both trials. Survival analysis was carried out accord-
ing to intention to treat, and preferably analyses should also be performed according to treatment given. In our database we cannot identify the exact chemotherapy that was given, but as reported earlier 80% of the patients allocated to chemotherapy received all treatment that was planned [Sherif A et al, 2004]. Our results demonstrate that emmprin and CCT protein expression could be useful for selecting patients for neoadjuvant chemotherapy, while survivin was not useful. Clearly it is too early to introduce this biomarker in the clinic with additional validation must be performed first.

9.3 Paper IV

In this paper we have been able to demonstrate the significance of the protein STMN1 both as a prognostic marker and therapeutic target in bladder cancer. Raised STMN1 levels are associated with shorter OS and DSS. In the in vitro experiment STMN1 was related to cell invasion, but link to STMN1 protein expression and tumor recurrence in the NMIBC was not observed. In muscle-invasive patients, we could validate the association between STMN1 and DSS in an independent cohort, the statistical analysis showed that STMN1 is not a strong prognostic factor. Conversely, we could not validate the association between low STMN1 levels and increased cisplatin sensitivity in muscle-invasive bladder cancer, which is in concordance with other studies [Jiang et al, 2013]. Our access to a distinctive metastatic bladder cancer cohort, including both the primary tumors and matching metastases made it possible to determine the expression levels of a therapeutic target protein in applicable tissue. When STMN1 protein levels were reduced by using siRNA-targeting, we detected a significant reduction in vitro of tumor cell proliferation and cell invasiveness. This is in agreement with other studies [Baldassarre et al, 2005; Hsieh et al, 2010; Jeon et al, 2010; Zheng et al, 2010; Lei et al, 2011; Phadke et al, 2011; Tan et al, 2012; Byrne et al, 2013; Chen et al, 2013]. Our results indicate that STMN1 is of significance for tumor cell proliferation and migration also in bladder cancer. Conjoining these cell model results with knowledge that STMN1 is expressed in primary tumors prone to metastasize, and also in bladder cancer metastases, strongly suggests that STMN1 is a relevant treatment target in advanced muscle-invasive bladder cancer. In non-muscle-invasive cancer, STMN1 might function as a therapeutic target, even though in these tumors STMN1 is expressed to a lesser extent and not correlated to progression as mentioned above. We found in bladder cancer a correlation between protein levels of STMN1 and p53 respectively Ki67 like formerly published in hepatocellular cancer [Singer et al, 2007]. We are well mindful of the fact that the increase in p53 protein in cancer does not totally correspond to all variants of mutated p53 [Knowles, 2006; Oren and Rotter, 2010]. Still, it is an indication that there is an affiliation between STMN1 and p53 in bladder cancer, as has been reported in other cancers [Yuan et al, 2006; Singer et al,
The significant correlation in bladder cancer tissue between high levels of the proliferation marker Ki67 and increased STMN1-protein expression are in concordance with our in vitro results, where STMN1 is recognized to be involved in cell proliferation.
10. Conclusion

In this thesis diverse bladder cancer cohorts, covering all stages, of the diseases were analyzed for clinicopathological prognostic and predictive factors using new drug combinations and molecular markers.

Paper

I) Five-year follow-up data from patients with T1G2-3 tumors of the bladder established the superiority of BCG for recurrence prevention compared to combination of epirubicin + interferon-α2b. Contrary to current risk tables concomitant CIS could not be identified as a predictive factor for poor outcome after BCG therapy.

II) The protein emmprin could be verified as an independent predictive and prognostic factor for response and survival after cisplatin-containing chemotherapy in patients with advanced bladder cancer.

III) Introducing protein CCTα as a novel predictive biomarker in advanced bladder cancer patients.

IV) STMN1 is a promising prognostic biomarker in bladder cancer with the potential to act as therapeutic target.
The search for more sensitive and specific biomarkers of bladder cancer continues. The current oncology personalized medicine paradigm is closely linked to biomarkers and based on matching patients to therapies based on the use of pre-treatment markers. Yet, tumors are genetically unstable, and this is the most efficient way for them to evolve [Beckman RA, 2009; Beckman RA, 2010; Beckman RA et al, 2006]. As a consequence, heterogeneity not only exists between patients, but within patients, such that no two tumor cells are alike within a single patient. With this in mind present prognostic factors derived from clinical, histopathologic and imaging techniques are apparently not able to distinguish sufficiently the ‘responders’ from the ‘non-responders’. Using biomarkers to make vital therapeutic decisions early on can reduce timelines and increase the likelihood of clinical success. Key principles of which are validation of selected biomarker against clinical benefit endpoints. Molecular markers, such as p53, bcl-2, Ki-67, RB and p21 have been added to the diagnostic tools [Fradet Y, 1990]. It is believed that they represent the true identity and behavior of bladder cancer. Despite numerous published studies, the number and quality of biomarkers available for use in bladder cancer remains disappointingly low. While remarkable progress in elucidating the complexities of signaling pathways driven by genetic, epigenetic, and endogenous/exogenous environmental factors, only now is the scientific community extensively utilizing biomarkers to comprehend individual risk assessment in terms of the evolving neoplasm. The current problems with the molecular markers are twofold. Using different thresholds and determination methods, results are not comparable and more standardization is needed. Although proven to be successful in the hands of some investigators, these markers have not been tested using large cohorts of patients in randomized prospective clinical trials. Once validated, it is expected that molecular markers will have to be added to the TNM system. Subsequently patients will benefit from a more refined TNM system in the future. It is expected that staging will improve in the future. Further progress might be achieved by combining existing biomarkers into larger, more powerful panels, or through the discovery of new biomarkers that robustly outperform current diagnostic tests and clinical indicators. Proteomics, in particular, is a rapidly developing field of research which offers the opportunity to obtain a real-time assessment of global protein expression and has been used to identify tumor-specific proteins. By comparing pro-
tein expression between patients with bladder cancer and healthy controls, or proteins expressed by invasive and non-invasive tumors, it is hoped that tumor-specific protein profiles can be identified which have diagnostic and prognostic value. But, like genomics, proteomics is a complex and expensive technique and currently lacks the reproducibility required for routine diagnostic and prognostic testing [Bell AW et al, 2009]. Regardless of the approach taken to identify biomarkers, it is essential that future studies are subjected to rigorous testing which must include prospective multicenter trials. The multimodality management of genitourinary neoplasms serves as a reflective model for the management of other malignancies. Future research will focus toward furthering our understanding of the molecular basis for bladder carcinogenesis, along with development of risk prediction models that integrates genetic and environmental factors to predict individualized probability for bladder cancer development, prognosis, and guide treatment. Both individual patients and healthcare providers are set to benefit from a new era of ‘personalized medicine’ in which biomarkers will direct the use of increasingly targeted and potentially more cost effective therapeutic agents.

Målet med denna avhandling är att öka bringa en bättre förståelse för hur kliniska faktorer kan påverka terapisvar, genom att utvärdera nya läkemedelskombinationer och tumörmarkörer tillsammans med etablerade kända riskkriterier.


Framtidens onkologiska behandling bygger på att matcha patient och behandling, för att därigenom uppnå en individuellt riktad terapi. Detta kan ske genom att använda sig av biomarkörer: särskilda indikatorer (i detta fall proteiner) som kan indikera svar på behandling. Blåscancer uppvisar en kraftig olikhet, inte bara patienter emellan, utan även inom den enskilde patienten. Inga två tumörceller är likadana. Med detta i åtanke är nuvarande prognostiska faktorer som härör från kliniska, histopatologiska behandlingar och avbildningstekniker (röntgen), inte tillräckliga att skilja patienter som kan tänkas svara på behandling (så kallade ”responders”) från de som inte kom-
mer att göra det ("icke-responders"). Användningen av biomarkörer för att tidigt kunna fatta viktiga terapeutiska beslut, kan minska tidsåtgång och öka sannolikheten för klinisk framgång och överlevnad.

I delarbete II & III analyserade vi proteinuttrycket i tumörvävnaden från totalt 250 patienter med aggressiv blåscancer. Patienterna delades upp i två randomiserade grupper. I dessa jämförde vi effekten mellan grupperna; de som fick preoperativ kemoterapi, mot den andra gruppens cystektomi (borttagning av urinblåsan) och fann att två proteiner (emmprin och CCTα) var av värde som prediktiva faktorer för patienter som fått denna kombinationsbehandling.

I delarbete IV analyserade vi proteinet STMN1 som länge varit en känd prognosindikation i andra cancerformer. I vårt material av matchande primärtumörer och metastaser var överrepresentationen av STMN1 kopplad till sämre prognos och överlevnad. Vi undersökte även möjligheten att använda detta som terapeutisk mål, genom att se hur blåscancertumörceller reagerade när man stängde av produktionen av det specifika proteinet.

Framtida forskning kommer att styras in mot att främja vår förståelse av den molekylära grunden för blåscancer - tillsammans med utvecklingen av riskprognosmodeller som integrerar med genetiska och miljömässiga faktorer - för att förutsäga individuell sannolikhet för cancer i urinblåsan.
13. Acknowledgement

It is a pleasure to thank the many people who made this thesis possible.

I would like to distinguish my supervisor and mentor Professor Per-Uno Malmström, for giving me this opportunity and sharing his wisdoms, intelligence and visions. For highlight the most critical point in every project, and for inviting me into your home. You constantly made sure I had adequate understanding and knowledge in every aspect of our work.

My co-supervisor and friend, Associate Professor Ulrika Segersten, for her immaculate inputs and encouragement, and for sharing her wisdom, knowledge and life experience. It feels that this is just the beginning to hopefully greater things to come.

I am grateful to all patients, who have contributed with the samples I have used in this study. Without their contribution, this work would not have been possible.

To the pathologists, Christer Busch, who have classified and chosen representative areas of all the tissues in the materials used in this thesis. And who sat with me a whole year teaching me the basics of the microscope.

To pathologist Maysaa Abdulla, who has helped us with our biomarker scoring.

Special thanks to Staffan Jahnson, for his impeccable inputs and all of his help.

I also would like to acknowledge Kenneth Wester and Evelina Sjöstedt for their assistance.

To my fellow group members, Mårten Lindén and Henrik Ugge.

To Lisa Wernroth, who always delivered on our questions on statistics.

To Amir Sherif, Milos Duchek and Trul Gårdmark, for paving the way with their previous work.
Thanks to all co-workers at the lab, for the best conversations during morning fika and lunch.

To Eva Johansson and Anna-Bill Axelsson, for always giving me kind words of encouragement and support.

To Göran Sahlén and Sam Ladjevardi, for continuously asking how it’s going with my research. To Frej Filén for being a true colleague. To all my colleagues at The Department of Urology, for their thoughtful support and understanding.

To Johanna Hagstrand and Sigfrid Linnérs memorial fund and Lions fund for providing the financial support making it all possible.

To my family, for believing in me.
14. Referencer


Dabbs, D. Diagnostic immunohistochemistry, (Churchill Livingstone, China, 2006).

de Groot AC, Conemans JM (1991) Systemic allergic contact dermatitis from intravesical instillation of the antitumor antibiotic mitomycin C. Contact Dermat 24:201


Herr HW, Donat SM and Dalbagni G: Can restaging transurethral resection of T1 bladder cancer select patients for immediate cystectomy? J Urol 2007; 177: 75.


Kanekura T, Miyauchi T, Tashiro M, Muramatsu T. Basigin, a new member of the immunoglobulin superfamily: genes in different mammalian species, glycosylation changes in the molecule from adult organs and possible variation in the N-terminal sequences. Cell Struct Funct. 1991 Feb;16(1):23-30..


Mitra AP, Skinner EC, Miranda G, Daneshmand S. A pre-cystectomy decision model to predict pathological upstaging and oncological outcomes in clinical stage T2 bladder cancer. BJU Int. 2012(b);111:240–8.


Sauter G, Mihaetsch MJ (1998) Pussycats and baby tigers: non-invasive (pTa) and minimally invasive (pT1) bladder carcinomas are not the same! J Pathol 185:339–341


Sylvester RJ, van der Meijden AP, Oosterlinck W, Witjes JA, Bouffioux C, Denis L, Newling DW, Kurth K. Predicting recurrence and progression in individual patients with stage Ta T1 bladder cancer using EORTC risk tables: a combined analysis of 2596 patients from seven EORTC trials. Eur Urol. 2006;49(3):466–75.


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)