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Immunhistochemische Untersuchungen zur Biologie und klinischen Bedeutung von Nektin-4 bei muskelinvasivem Blasenkrebs

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For Alissia,

When you walk,
always go all the way.
In spring, to the flower,
In summer, to ripe wheat,
in autumn, to a full shelf,
in winter to the snow queen,
in a book, to the last line,
in life, to the real truth,
in myself, to the blush across one cheek and the other.
But if you don't come the first or the second time
to the deck and the real metal
try: again and again and again.

(Tone Pavček)

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1.0 INTRODUCTION

1.1 Bladder cancer

1.1.1 Epidemiology

Bladder cancer (BC) is the eleventh most commonly diagnosed cancer worldwide. The worldwide age-standardized incidence rate (per 100,000 persons /year) is 9.5 for men and 2.4 for woman. In Europe, the highest age-standardized incidence rate has been reported in Belgium (31 in men and 6.2 in women) and the lowest in Finland (18.1 in men and 4.3 in women) (International Agency for Research on Cancer, 2021). The incidence and mortality of BC has decreased in some registries, possibly reflecting the decreased impact of causative agents (Teoh *et al.*, 2020). Unfortunately, the incidence rate is rising in underdeveloped countries, where the industrialization has led to carcinogenetic exposure (Wein *et al.*, 2016).

1.1.2 Etiology

Urothelial cancer is a cancer of the environment and age. There is a strong association between environmental toxins and urothelial cancer formation (Wein *et al.*, 2016).

1.1.2.1 Smoking

Previous studies indicate that the population attributable risk of bladder cancer for tobacco smoking is 50% to 65% in men and 20% to 30% in women, and that current cigarette smoking triples bladder cancer risk relative to never smoking (Freedman *et al.*, 2011). The incidence of bladder cancer is directly related to the duration of smoking and the number of cigarettes smoked per day (Brennan *et al.*, 2000). Starting to smoke at a younger age increases the risk of death from bladder cancer (Al Hussein Al Awamlh *et al.*, 2019). Tobacco smoke contains carcinogens such as beta-naphthylamine and polycyclic aromatic hydrocarbons. These particles promote inflammation in the bladder cells and culminates in deoxyribonucleic acid (DNA) -adduct formation and permanent genetic mutation. Such mutations can promote carcinogenesis (National Center for Biotechnology Information, 2010).

Electronic cigarettes (E – cig.) are marketed as a safe alternative to tobacco to deliver nicotine. A study by Tang MS *et al.* recently showed that mice exposed to short term E-cig smoke sustained extensive DNA damage in lungs and induces bladder urothelial hyperplasia. That implicate E-cig smoke as a lung and potential bladder carcinogen in mice (Tang MS *et al.,* 2019).

1.1.2.2 External exposure to chemicals

Occupational exposure to chemical carcinogens such as polycyclic aromatic hydrocarbons, nitrosamines, aromatic amines and arsenic are the second risk factors for urothelial tumors (Zeegers *et al.,* 2001). These compounds are commonly found in the industrial production of dyes, paint, metal, rubber or petroleum products. The primary culprits are the aromatic amines that bind to DNA. They can enter the system and cause bladder cancer from inhalation or through skin absorption (Burger *et al.,* 2013).

1.1.2.3 Radiotherapy

In the Nieder *et al.* study (population-based cohort study) has been concluded that in male patients that have been treated with radiotherapy for localized prostate cancer there is an increased risk of bladder cancer compared to patients undergoing surgical radical prostatectomy (Nieder et al., 2005).

1.1.2.4 Chronic urinary tract infection and bladder irritation

Schistosoma haematobium infection is a clear contributor to the formation of squamous cell carcinoma (SCC) of the bladder (Abol-Enein, 2008). Infection with human papillomavirus (HPV) has no major role in the pathogenesis of SCC of the urinary bladder (Westenend *et al.,* 2001). Several studies suggested that chronic bacterial infection may play a role in bladder cancer formation. Chronic bladder stones, catheter use and infections are tightly contracted with bladder carcinoma. The mechanism of neoplasms formation remain unknown (Abol-Enein, 2008).

1.1.2.5 Gender

Men are more likely to develop bladder cancer than woman. Woman, however, usually present with locally advanced disease and have a worse survival outcome. In a meta-analysis of 27,912 patients from Liu S. *et al.* it has been discovered that women survival outcome after radical cystectomy (RC) compared to the male gender is worse (Liu S. *et al.,* 2015).

1.1.2.6 Genetics

In one study, Figueroa *et al*. identified four susceptibility loci associated with bladder cancer risk. Two of them achieved genome-wide statistical significance: rs10936599 on 3q26.2 ($P = 4.53 \times 10(-9)$) and rs907611 on 11p15.5 ($P = 4.11 \times 10(-8)$). Two notable loci that approached genome-wide statistical significance were also further identified: rs6104690 on 20p12.2 ($P = 7.13 \times 10(-7)$) and rs4510656 on 6p22.3 ($P = 6.98 \times 10(-7)$). To understand bladder carcinogenesis connected with these four loci, fine-mapping and laboratory further investigation is required (Figueroa *et al.,* 2014).

1.1.3 Staging and classification systems

The TNM classification is a system to describe the spread of cancer cells in the patient's body. T stands for size of the tumor and any spread into a nearby tissue; N describes spread of cancer cells to nearby lymph nodes and M stands for spread of cancer cells to other parts of the body – metastasis. For bladder cancer staging, TNM classification 2017 eighth edition is recommended (Brierley, TNM Classification of Malignant Tumors, 2016).

1.1.3.1 TNM classification of urinary bladder cancer (2017, 8th edition)

T-Primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Ta	Non-invasive papillary carcinoma
Tis	Carcinoma in situ
T1	Tumour invades subepithelial connective tissue layer
T2	Tumour invades muscle
	T2a Tumour invades superficial muscle
	T2b Tumour invades deep muscle
T3	T3 tumours -invade perivesical tissue
	T3a -microscopically
	T3b-macroscopically (extravesical mass)
T4	Tumour invades any of the following: prostatic stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
	T4a Tumour invades prostatic stroma, seminal vesicles, uterus or vagina
	T4b Tumour invades pelvic wall or abdominal wall
N-Regional lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node in the true pelvis
N2	Metastasis in multiple regional lymph nodes in true pelvis
N3	Metastasis in common iliac lymph node(s)
M-Distant metastasis	
M0	No distant metastasis
	M1a Non-regional lymph nodes
	M1b Other distant metastasis

Table 1: TNM Classification of urinary bladder cancer (2017, 8th edition). From EAU Guidelines 2022.

When staging after neoadjuvant chemotherapy and cystectomy is done, it has to be written as ypTNM. For example ypT0N0 is associated with positive chemotherapy response and prognosis (Martini *et al.,* 2019).

1.1.4 Pathology

Histologically, 90% of bladder cancers are urothelial carcinomas and 5% are squamous cell carcinomas. In less than 2% of the patients, adenocarcinoma or another histology variant can be seen. In about 80% of the patients with urothelial carcinoma the tumor is non-muscle invasive. It can be a flat lesion or carcinomas in situ (CIS) and high-grade (HG) or low-grade (LG) papillary tumors. In muscle invasive bladder cancer, the urothelial carcinoma infiltrates into the muscular layer of the bladder. These are always high-grade tumors (Wein *et al.,* 2016).

1.1.4.1 Histology of urothelial cancer

Non-muscle invasive bladder cancer (NMIBC) is either a papillary urothelial neoplasm of low malignant potential (PUNLMP), high-grade (HG) or low-grade (LG) urothelial cancer and/or carcinoma in situ (CIS).

1.1.4.1.1 Urothelial carcinoma in situ (CIS)

Urothelial carcinoma in situ is a high-grade, flat, non-invasive tumor and is a precursor lesion for invasive cancer. It has a high tendency to spread in a different part of the urinary tract (Akhtar *et al*., 2019). CIS is positive for cytokeratin 20. NMP 22 is also present in tumor cells (Mati *et al*., 2020). Macroscopically, CIS is a red flat lesion in the mucosa and can be falsely interpreted as inflammatory changes.

1.1.4.1.2 Papillary urothelial neoplasm of low malignant potential (PUNLMP)

PUNLMP is usually small papillary lesion located on trigonum. It consists of small number of atypical cells (Jaworski *et al*., 2019). PUNLMP is not associated with progression. It recurs in 35% of patients. They are exceptionally rare in children. In a case of high risk relapsed PUNLMP in pediatric patients, intravesical chemo-instillation (Mitomycin-C or MMC) may allow complete remission (Maurizi *et al.,* 2019).

1.1.4.1.3 Low-grade (LG) urothelial carcinoma

Low-grade urothelial carcinoma is typically papillary. Occasionally mitotic figures can be seen. Low-grade tumors include deletion of 9q and alterations in FGFR-3, HRAS and PI3K. (Cordon- Cardo, 2008). LG tumors are rare, associated with infiltration in the bladder wall but often with recurrences.

1.1.4.1.4 High-grade (HG) urothelial carcinoma
High-grade urothelial carcinoma has, in more than 80%, a tendency to grow into the bladder wall if left untreated. Deletions of 2q, 5 q, 10q and 18q as well as alterations of TP21 and TP27 along with TP53 can be identified in genetic studies. (Cordon- Cardo, 2008).

1.1.4.2 Histologic spectrum of urothelial carcinoma
In order to include distinct growth patterns of urothelial carcinoma, a wider spectrum of histological variants of urothelial cancer has been identified. Currently 10 variations of urothelial carcinoma have been described. In the following, we will focus only on the most common bladder cancer variants.

1.1.4.2.1 Plasmacytoid variant
This variant is characterized by sessile and non-papillary tumor growth pattern. The plasmacytoid cells usually invade through the bladder wall and into perivesical structures at the time of diagnosis (Kaimakliotis, *et al*., 2014).

1.1.4.2.2 Glandular tumor differentiation (adenocarcinoma)
Glandular tumor differentiation represents approximately 6% of urothelial cancer cases. It is the presence of two glandular spaces within the tumor. It is important that this glandular differentiation, for example on the anterior wall of the bladder, is not confused with urachal adenocarcinoma. There have been a few cases of cystitis glandularis transforming into adenocarcinoma of the bladder. A precise endoscopic evaluation is therefore recommended (Zhou *et al*., 2021).

1.1.4.2.3 Micropapillary differentiation
It usually occurs with advanced stage and metastases at the diagnosis. The five-year overall survival rate is 51%, suggesting that most patients died from their cancer. Certain patients with micropapillary bladder cancer may respond to intravesical bacillus Calmette-Guérin (BCG). Survival rate can be improved with early radical cystectomy (Willis *et al.,* 2015).

1.1.4.2.4 Nested variant
It is a very aggressive form. It can be confused with Brunn nests in cystitis cystica or inverted papilloma (Wein *et al.,* 2016). In the study from Beltran *et al*., the median overall survival was 21 months (Beltran *et al*., 2014).

1.1.4.2.5 Clear cell variant
70% of bladder carcinomas will have regions with clear cell differentiation. These regions are not necessarily associated with poor prognosis. That can be confused with metastases of renal clear cell carcinoma (Wein *et al.,* 2016).

Neoadjuvant chemotherapy may be beneficial for patients with micropapillary, plasmacytoid, sarcomatous, and mixed variants, and especially for patients with neuroendocrine differentiation (Veskimäe *et al.,* 2019).

Lymphovascular invasion (LVI) at the time of TURB is associated with a worse prognosis (Kim *et al.* 2014).

1.1.5. Diagnosis

1.1.5.1 Signs and symptoms
The most common symptom of bladder cancer is in 85% of patients painless visible hematuria which is associated with advanced disease. Lower urinary tract symptoms such as irritation can be associated with CIS. Microhematuria is associated with bladder tumor in less than 4% (Ramirez *et al.,* 2015).

1.1.5.2 Diagnostic evaluation

1.1.5.2.1 Urinary cytology and novel urinary biomarkers
Urinary cytology with cystoscopy is the most commonly used test for detection and follow-up for patients with bladder cancer. With its average sensitivity of 48% (16% for LG tumors and 84% for HG tumors) and its specificity of 86% it still represents a gold standard for detecting tumor cells in urine (Yafi *et al.,* 2015). However, voided urinary cytology has a low sensitivity (especially in patients with low grade tumors),

a long learning curve and a high interobserver variability, thus requiring experts on cytology. To improve these problems various kinds of urine-based tests for diagnosis and monitoring of bladder cancer (BC) have been evaluated. FDA has approved UroVysion, uCyt+, BTA, and NMP22 as urine markers for BC. Promising novel urinary tumor biomarkers, such as URO17, ADX-BladderTM, UBC Rapid and Xpert Bladder® are showing high sensitivities in prospective multicenter trials, and may lead to clinical use and FDA approval (Kavalieris *et al.*, 2017).

1.1.5.2.1.1 NMP22 kit and NMP22 BladderCheck (Alere, Waltham, MA, USA)
Both are ELISA tests that target the NMP22 protein, which is expressed to a higher degree in BC cells than in normal urothelium. It can be found in urine during apoptosis of urothelial and bladder cancer cells (Lee and Kim, 2020). The overall sensitivity and specificity of the NMP22 kit (which is used for surveillance) are 52–59% and 87–89%, respectively. The sensitivity and specificity of NMP22 BladderChek (which is used for diagnostic purposes) are 62–75% and 70–83%, respectively. The limitation of these tests is the high false-positive-rate caused by detection of benign conditions (Chou *et al.*, 2015).

1.1.5.2.1.2 ImmunoCyt/uCyt+ assay (Scimedx Inc., Denville, NJ, USA)
The ImmunoCyt/uCyt+ assay is the only test available for surveillance of the patients and combines urine cytology and immunohistochemical staining using fluorescent-labeled monoclonal antibodies. The assay detects exfoliated BC cells using the carcinoembryonic antigen and two BC-associated mucins (LDQ10 and M344). An overall specificity from 62% to 84% and a sensitivity from 67% to 100% have been observed (Lee and Kim, 2020).

1.1.5.2.1.3 UroVysion (Vysis, Abott Molecular Inc., Chicago, IL, USA)
The UroVysion uses a multi-target fluorescence in situ hybridization assay to detect aneuploidy in chromosome copy numbers 3, 7, 17, and 9p21 and identifies loss of the P16 tumor-suppressor gene (Mowatt *et al.*, 2010). The test has a sensitivity of between 57.1– 84% and specificity of between 78–92%. UroVysion has shown higher accuracy compared to voided urinary cytology, especially for low-grade BC. However, UroVysion lacks cost-effectiveness and simplicity (Savic *et al.*, 2009).

1.1.5.2.1.4 UBC® Rapid Test (Polymedco Inc., Cortlandt, NY, USA)
The BTA stat and BTA TRAK tests detect the human complement factor H and complement factor H-related proteins that are involved in cancer cell growth in previously diagnosed BC patients for the purpose of surveillance. The sensitivity and specificity of BTA stat are 57–83% and 60–92%, respectively, while those of BTA TRAK are 73–77% and 45–81%, respectively (Tabayoyong and Kamat, 2018).

1.1.5.3 Imaging
Imaging of the bladder and upper urinary tract allows clinical staging and assessment of lymph nodes involvement or organ metastases.

1.1.5.3.1 Ultrasound
Ultrasonography plays an important role in the evaluation of the urinary tract. It can detect hydronephrosis, renal masses and tumors in the bladder but it cannot replace CT urography (Moslemi *et al.,* 2011).

1.1.5.3.2 Computed abdominal tomography
A contrast-enhanced CT scan with CT urography, is routinely performed to exclude involvement of upper urinary tract and spread of disease into other organs. In the case of muscle invasion, exclusion of pulmonary metastases with CT scan of the lung is necessary (Witjes *et al.,* 2021).

1.1.5.3.3 Multi-parametric magnetic resonance imaging
MRI is mainly used in patients with severe renal impairment, allergy to iodine-containing contrast agents or young patients to avoid radiation. The local extent of bladder cancer is important for prognosis, planning and assessment of response to neoadjuvant treatment (Compérat *et al.*, 2022).

The Vesical Imaging-Reporting and Data System (VI-RADS) has been introduced to provide preoperative bladder cancer staging and assessing the presence of muscle invasion in the pre-TURBT (trans-urethral resection of bladder tumor) setting. VI-RADS scale consists of a five point score which predicts the likelihood of muscle-invasive disease (MIBC). VI-RADS scores 1 and 2 indicate low probability of MIBC; VI-RADS 3 indicates equivocal likelihood; VI-RADS 4 indicates MIBC is likely to be present and 5 indicates MIBC or invasion out of bladder wall.

VI-RADS act as a predictive tool indicating patients with non-muscle invasive bladder cancer (NMIBC) for secondary resection (re-TURBT) or to monitor radiological response of MIBC patients after neoadjuvant systemic therapy (Del Giudice *et al.*, 2020).

1.1.5.3.4 Cystoscopy

White light cystoscopy remains the gold standard for detection of bladder tumors and surveillance of patients after NMIBC. The main limitations of cystoscopy are its limited sensitivity for flat lesions (CIS) detection, and lower compliance and discomfort for the patients. CIS can be better seen under blue light cystoscopy. It has been established that addition of hexaminolevulinate (Hexvix) increases identification of cancerous lesions, including those of high grade (including CIS) and reduces cancer recurrence rates (Compérat *et al.*, 2022, Stenzl *et al.*, 2022).

1.1.5.3.5 Transurethral resection of bladder tumor

Transurethral resection of bladder tumor (TURBT) is a gold standard and a fundamental step in the treatment of NMIBC (Compérat *et al.*, 2022). Initial complete resection of all visible tumors with a precise operating technique has a major impact on recurrence rates. Hexaminolevulinate (Hexvix) in addition to white light cystoscopy (WLC) is recommended by the European Association of Urology guidelines to improve the visibility of bladder tumors and to exclude carcinoma in situ (CIS). More than 20 well-designed trials in over 4000 patients showed a reduction in recurrence rate of up to 34% with the addition of concomitant blue light cystoscopy (Stenzl *et al.*, 2022).

1.1.6 Non muscle invasive bladder cancer (NMIBC)

1.1.6.1 EAU NMIBC 2021 scoring model

EAU Guidelines panel have created a new prognostic factor risk groups using both the WHO 1973 and WHO 2004/2016 classification systems, grade, concomitant CIS, number of tumors, tumor size, age and tumor stage to predict risk of progression into muscle invasive disease (Sylvester *et al.*, 2021). Predicting recurrence, other models such as EORTC (European Organisation for Research and Treatment of Cancer) scoring model can be used (Sylvester *et al.*, 2005).

Clinical composition of the EAU NMIBC prognostic factor risk groups

Risk group	
Low Risk	• A primary, single, Ta/T1 LG/G1 tumour < 3 cm in diameter without CIS in a patient < 70 years
	• A primary Ta LG/G1 tumour without CIS with at most ONE of the additional clinical risk factors (see above*)
Intermediate Risk	Patients without CIS who are not included in either the low, high or very high-risk groups
High Risk	• All T1 HG/G3 without CIS, EXCEPT those included in the very high-risk group • All CIS patients, EXCEPT those included in the very high-risk group
	Stage, grade with additional clinical risk factors: • Ta LG/G2 or T1 G1, no CIS with all 3 risk factors • Ta HG/G3 or T1 LG, no CIS with at least 2 risk factors • T1 G2 no CIS with at least 1 risk factor
Very High Risk	**Stage, grade with additional clinical risk factors:** • Ta HG/G3 and CIS with all 3 risk factors • T1 G2 and CIS with at least 2 risk factors • T1 HG/G3 and CIS with at least 1 risk factor • T1 HG/G3 no CIS with all 3 risk factors

- Additional clinical risk factors are*:
- Age > 70
- Multiple papillary tumors
- Tumor diameter > 3 cm

Table 2: Clinical composition of the new EAU NMIBC prognostic factor risk groups (EAU Guidelines., 2022)

1.1.6.2 Disease management

1.1.6.2.1 Single shot, post TURB intravesical instillation of chemotherapy

In four large meta-analyses it has been shown that single shot chemotherapy destroys circulating tumor cells and prevents them from seeding. It reduces the five-year recurrence rate by 14% compared to TURB only (Sylvester RJ *et al.*, 2004). In one study, continuous saline irrigation prevented early recurrences as well (Mahran *et al.*, 2018).

Any perioperative adjuvant treatment should be done in the first 24 hours after TURB, otherwise tumor cells are covered with extracellular matrix (Soloway, Masters, 1980). There were no significant differences in efficacy between the different chemotherapy agents (mitomycin C – MMC, adriamycin, epirubicin, pirarubicin) (Sylvester RJ *et al.*, 2004).

1.1.6.2.2 Intravesical bacillus Calmette-Guérin (BCG) immunotherapy

In a meta-analysis from Malmström *et al.* demonstrated that BCG after TURB is superior to TURB alone, or TURB plus chemotherapy for preventing recurrence of NMIBC. In comparison to intravesical chemotherapy is associated with more side effects such as symptoms of cystitis, hematuria, epididymitis and fever (Malmström et al., 2009). According to the EAU guidelines BCG should be administered to patients in the intermediate risk group as six weekly induction doses followed by three weekly maintenance doses at 3, 6 and 12 months. BCG should be administered to patients in the high and very high risk groups as six induction doses followed by three maintenance doses at 3, 6, 12, 18, 24, 30 and 36 months. When BCG maintenance is applied over three years it reduces the recurrence rate compared to a one year maintenance scheme in high risk patients, however, without any benefit in progression or overall survival (OS) (Oddens *et al.,* 2013).

1.1.6.2.3 Early radical cystectomy (RC) for patients with NMIBC

Patients should be informed about the benefits and complications of early cystectomy and presented with possible alternative treatments. It is reasonable to propose immediate RC in those patients with NMIBC who are at very high risk of disease progression and with BCG-unresponsive tumors (Raj *et al.,* 2007).

1.1.7 Muscle invasive bladder cancer

1.1.7.1 Disease management

Standard treatment for muscle invasive bladder cancer with the intent to cure is still radical cystectomy (RC) with bilateral iliac lymphadenectomy, with or without neoadjuvant or adjuvant systemic chemo- or immunotherapy depending on tumor stage and patient's general condition. The five-year survival rate depends on the extent of the disease and may be as low as remain low as 48% in non-organ confined (pT3+) disease (Ghoneim *et al.,* 1997).

1.1.7.2 Neoadjuvant chemotherapy (NAC)

Cisplatin based NAC has been introduced to improve survival rate in the patients with cN0M0 disease. Patients after NAC with a positive response are determined as a < ypT1, ypN0 and negative surgical margins. In a comparative study based on data from the National Cancer Database from patients treated with NAC and RC vs. RC alone showed that disease determined as < ypT2 after NAC was associated with decreased risk of death (HR: 0.85, 95% CI: 0.79–0.91) compared to RC alone, whereas > pT2 was associated with increased risk of death (HR: 1.46, 95% CI: 1.34–1.60). Delayed surgery due to NAC does not have a negative impact on survival. Neoadjuvant cisplatin combination chemotherapy improves OS in five years for 8% (Pfail *et al.,* 2020).

1.1.7.3 Radical treatment with radical cystectomy (RC) and urinary diversion

RC with lymphadenectomy can be given as a treatment option to patients with a longer life expectancy. Performance status influence the choice of type of urinary diversion. In patients with bladder tumors, radical cystectomy should be performed within a three month time frame, including neoadjuvant therapy. As described in a systematic review and meta-analysis of delay in radical cystectomy and the effect on survival, delay of > 3 months has a negative effect on OS (HR: 134, 95% CI: 1.18–1.53) (Russell *et al*., 2020).

1.1.7.3.1 Indications for radical cystectomy (EAU Guidelines 2022)

- Bladder cancer T2–T4a, N0-Nx, M0 disease;
- BCG unresponsive NMIBC;
- Extensive papillary disease that cannot be sufficiently controlled with TURB and intravesical therapy.

1.1.7.4 Radical cystectomy in men

Radical cystectomy in men include removal of the bladder, prostate, seminal vesicles and pelvic lymph nodes (below the bifurcation of the aorta). It can be performed by open surgery or minimally invasive. Apart from the primary urothelial cancer of the bladder histological analysis of the removed specimen may reveal incidental prostate cancer in approximately 21–50% of cases, which is usually of no prognostic significance (Damiano *et al.,* 2007). Sexual preservation strategies for younger men

are still controversial, and four different methods have been described: prostate sparing, prostatic capsule sparing, seminal vesicles preserving and autonomous nerve sparing cystectomy. In a multivariate analysis, from Kessler *et al.* where 381 men after RC and ileal orthotopic bladder substitution were included, the rate of daytime continence was significantly higher in patients with attempted nerve preservation (hazards ratio [HR] 1.4, 95% confidence interval [CI] 1.05–1.87). Nighttime continence was significantly better in patients younger than 65 years (HR 1.39, 95% CI 1.07–1.8). In a multivariate analysis erectile function recovered significantly more often in patients with nerve sparing (HR 2.59, 95% CI 1.24–5.39) and in those younger than 65 years (HR 2.98, 95% CI 1.83–4.85) (Kessler *et al.*, 2004).

1.1.7.5 Radical cystectomy in women

Radical cystectomy in women historically included total anterior pelvic exenteration including bladder, urethra, anterior vagina, uterus, cervix and regional lymph nodes (below the bifurcation of the aorta). As in men it can be performed by open surgery or minimally invasive. Women generally present with locally advanced tumors, with higher stages than in men. Pelvic floor disorders and sexual dysfunction should be discussed prior to RC.

Sexual-function preserving RC with orthotopic neobladder in well-selected patients, may be comparable to normal RC when we talk about oncological outcome and it may lead to an improved postoperative sexual and urinary function (Veskimäe et al., 2017).

1.1.7.6 Robotic-assisted laparoscopic cystectomy (RARC)

Robotic-assisted laparoscopic cystectomy has grown in popularity. It has been introduced to reduce the morbidity in comparison to traditional open surgery. From the Pasadena Consensus Panel from 2015 it can be concluded, that most operative, oncologic, functional, and complication outcomes are similar in comparison to open RC. RARC is associated with less blood loss and longer operating time, particularly with intracorporeal neobladder reconstruction (Wilson *et al.,* 2014).

1.1.8 Metastatic muscle-invasive bladder cancer

1.1.8.1 Introduction

Cisplatin based systemic combination chemotherapy is a standard regimen for a patients with metastatic progress.

1.1.8.2 First-line systemic therapy

According to EAU Guidelines from 2022, patients with metastatic BC can be divided into three categories (Table 3): fit for cisplatin- based chemotherapy, fit for carboplatin- based chemotherapy and unfit for any platinum based chemotherapy (Cathomas *et al.,* 2022).

Platinum-eligible Cisplatin-eligible	Carboplatin-eligible	Platinum-ineligible
ECOG PS 0–1 and GFR > 50–60 mL/min and Audiometric hearing loss grade < 2 and Peripheral neuropathy grade < 2 and Cardiac insufficiency NYHA class < III	ECOG PS 2 or GFR 30–60 mL/min or not fulfilling other cisplatin-eligibility criteria	GFR < 30mL/min ECOG PS > 2 ECOG PS 2 and GFR < 60 mL/min Comorbidites > Grade 2

ECOG = Eastern Cooperative Oncology Group; GFR = glomerular filtration rate; NYHA = New York Heart Association; PS = performance status.

Table 3: Definitions of platinum-eligibility for first-line treatment of metastatic urothelial carcinoma. (EAU Guidelines 2022)

First line systemic regimen in for cisplatin fit patients include gemcitabine/ cisplatin (GC) and MVAC (methotrexate, vinblastine, adriamycin plus cisplatin) with overall survival of 13.8 and 14.8 months, respectively. Because of lower toxicity of GC, it became a standard chemotherapy regimen (von der Maase *et al.,* 2005).
Carboplatin can be used for patients unfit for cisplatin, with the benefit of improved tolerability but with the cost of decreased efficacy (Galsky *et al.*, 2011).

1.1.8.3 Switch maintenance with immunotherapy after platinum-based chemotherapy for patients who achieved stable disease

As described in a JAVELIN Bladder 100 Clinical Trial, where 700 patients without progression/stable disease after 4–6 cycles of cisplatin based chemotherapy (gemcitabine + cisplatin/ carboplatin) were analyzed, addition of maintenance avelumab (PD-L1 inhibitor) vs. best supportive care (BSC) significantly prolonged overall survival (median overall survival, 21.4 months vs. 14.3 months). Avelumab became a standard of care for all patients that achieve disease stabilization after 4–6 cycles of first line cisplatin based therapy (Powles *et al.,* 2020).

1.1.8.4 Treatment of patients unfit for any platinum-based chemotherapy

Optimal treatment in this population of patients is still not established. Pembrolizumab (programmed cell death ligand PD – 1 inhibitor) has been approved from FDA regardless of PD-L1 status (cut off point 10%) as a first line treatment in these patients based on multicenter, single-arm, phase 2 study (KEYNOTE-052) (Balar *et al.,* 2017).

1.1.8.5 Second-line immunotherapy for patients with progress after cisplatin based therapy

Pembrolizumab has showed median overall survival of 10.3 months as compared with 7.4 months in a chemotherapy group (paclitaxel, docetaxel, or vinflunine) in a phase III trial of 542 patients who had a tumor PD-1 ligand (PD-L1) combined positive score (the percentage of PD-L1-expressing tumor and infiltrating immune cells relative to the total number of tumor cells) of 10% or more (Bellmunt *et al.,* 2017).

The phase III RCT (IMvigor211) included 931 patients comparing atezolizumab with second-line chemotherapy (paclitaxel, docetaxel or vinflunine) did not meet its primary endpoint of improved OS for patients with high PD-L1 expression with 11.1 months (atezolizumab) vs. 10.6 (chemotherapy) months. Atezolizumab was the first checkpoint inhibitor approved by the FDA for metastatic UC as a second line immunotherapy (Powles *et al.,* 2018).

The nivolumab (PD-1 inhibitor) was approved by the FDA based on the results of a single-arm phase II trial (CheckMate 275), enrolling 270 pretreated patients with platinum based therapy. The primary endpoint of ORR was 19.6%, and OS was 8.74 months for the entire group (Sharma *et al.,* 2017).

1.2 Nectin cell adhesion molecule

1.2.1 Cell- cell interactions

Mammalian organs are composed of cells that can interact homotypically or heterotypically. Unlike a homotypic bond, where cells of the same type connect to each other, a heterotypic bond connects cells of different types to each other. Roughly, three different groups of intercellular interactions can be distinguished: asymmetric homotypic, symmetric homotypic, and heterotypic (Figure 1). Symmetric homotypic junctions between cells of the same type can be found for example in intestinal epithelium or between vascular endothelial cells. Asymmetric homotypic intercellular interactions are observed between axons and dendrites. Heterotypic interaction between cells can be seen for example between Sertoli and germ cells (Rikitake *et al.* 2012).

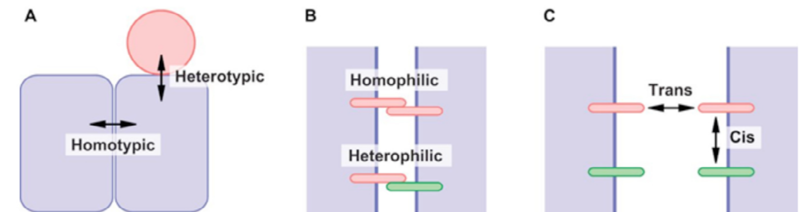


Figure 1: Types of cell- cell interactions: **(A)** Cells adhesion can be homotypical or heterotypical. **(B)** Homophilic means interactions between molecules of the same type, heterophilic means forming interactions between two different molecules. **(C)** Cis interaction: cells interact on the surface of one cell. Trans interaction: cell adhesion molecules on the surface of one cell interact with adhesion molecules on the surface of an opposing cell (Rikitake *et al.,* 2012).

1.2.2 Molecular structure of Nectin and interactions between surface of the cells

Nectins are Ca2+ independent transmembrane immunoglobulin like molecules. The family comprised of Nectin 1–4 which are encoded by the PVRL1–4 genes. They are involved in homophilic and heterophilic cell-cell interactions. Their heterophilic trans interaction is much stronger than homophilic. (Sakisaka *et al.*, 2004). Nectins consists of three immunoglobulin-like loops (V-type and two C2-type loops) in their extracellular part with a transmembrane (TM) segment and a cytoplasmic tail (Fig. 2A). Through a cytoplasmatic tail Nectin binds Afadin, which establishes intracellular communication. The V, C, C part are involved in cell growth, proliferation and apoptosis, when they bond with growth factors (Reymond *et al.,* 2000). As we can see on the Fig. 2B, Nectins are forming V shaped cis dimers, and lateral cluster formation with trans interaction (Satoh-Horikawa *et al.*, 2000).

Nectins can interact with other transmembrane proteins, such as Nectin-like molecules (Necls, also known as CADMs) and T cell immunoreceptor on the surface of opposing cells (Ikeda *et al.,* 2003). Nectin-1 can also interact with Nectin-3, Nectin-4 and Necl-1 (CADM3) (Stanietsky *et al.*, 2009) (Fig. 2C). Cells expressing different Nectins tend to aggregate in a mosaic pattern (Fig. 2D).

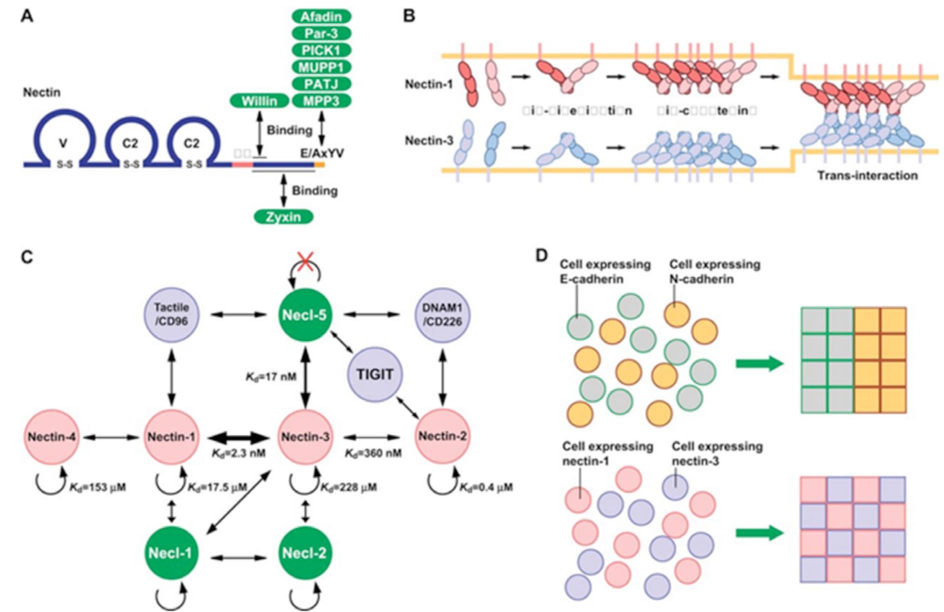


Figure 2: Molecular structure of Nectin and interactions between surface of the cells. **(A)** Nectins consists of three immunoglobulin-like loops (V-type and two C2-type loops) in their extracellular part with a transmembrane (TM) segment and a cytoplasmic tail. **(B)** Nectins are forming V shaped cis dimers, clusters and trans interactions **(C)** Nectin interact with other transmembrane proteins, such as Necls and T cells. **(D)** Nectins tend to aggregate in a mosaic pattern.

1.2.3 Function of Nectins in cellular environment

In a normal cellular environment, Nectin-1 and Nectin-2 can be predominantly found in organs of the immune system (B cells and monocytes) (Shimizu and Takai, 2003), Nectin-3 in testes and placenta and Nectin-4 predominately in embryonic and placental tissue including trachea and esophagus (Sakisaka and Takai, 2004).
Nectin can be receptors for virus entry. Nectin 1–2 for herpes simplex virus and Nectin-4 for measles virus (Mühlebach *et al.,* 2011).

1.2.4 Nectins in diseases

Mutations in human PVRL4 (which encodes nectin-4) cause an ectodermal dysplasia-syndactyly syndrome that is characterized by the combination of hair and tooth abnormalities, alopecia and cutaneous syndactyly. (Ahmad *et al.* 2018, *Brancati et al.* 2010) Mutation in PVRL 1 (which encodes nectin-1) cause palate – ectodermal dysplasia syndrome with cleft lip (Sözen *et al.*, 2001).

1.2.4.1 Role of Nectin-4 in carcinogenesis

Nectin-4 has been found to be overexpressed in numerous malignancies, including bladder cancer (BC), pancreas cancer (PC), ovarian cancer (OC), colorectal cancer (CRC) and lung cancer (LC)(Liu *et al.*, 2021). Exact mechanism in tumorgenesis has been not established, but in literature can be found, that Nectin-4 is involved in tumor angiogenesis and cell proliferation (Carmeliet *et al.*, 2000). It has also been established, that overexpression of Nectin-4 in different tumor tissue, correlates with poor prognosis (Deng *et al.*, 2019).

1.2.4.2 Nectin-4 in tumor angiogenesis

Angiogenesis is a crucial step for tumor spread, invasion and expansion. In literature can be observed that Nectin-4 promotes tumor angiogenesis via the activated PI3K/AKT signaling pathway. Nectin-4 expression correlate with a considerable amount of vascular endothelial growth factor (VEGF).

Its ectodomain play a soluble form of Nectine. Under hypoxic conditions, soluble form interact with Integrin ß4 to enhance angiogenesis. It can be detected in serum and is associated with disease progression. Ectodomain of Nectin-4 is associated with hypoxia induced lymphatic tumor cell spreading (Fig. 3).

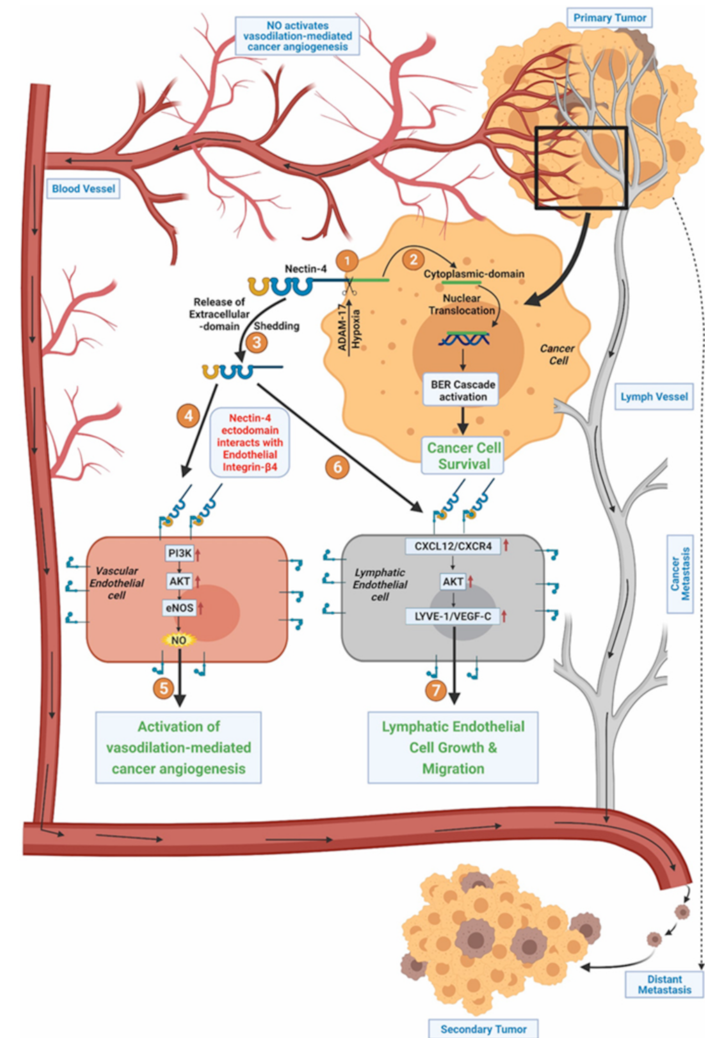


Figure 3: Nectin-4 mediated cancer progression. **(1)** Hypoxic condition **(2)** Endodomain of Nectin-4 goes into the nucleus and induces DNA repair, leads to cancer cell survival. **(3, 4)** Ectodomain of Nectin-4 binds with vascular endothelial Integrin β4 and activates AKT/PI3 path way. **(5)** Elevated levels of NO lead to the vasodilation and cancer angiogenesis. **(6)** Binding ectodomain of Nectin-4 to lymphatic endothelial cells triggers the lymphatic tumor cell spreading. **(7)** (Chatterjee *et al.*, 2021)

1.2.4.3 Nectin-4 expression in different tumor tissues

Several studies have shown overexpression of Nectin-4 in different cancer tissues, such as, bladder cancer (BC), ovarian cancer (OC), breast cancer (BrC) and pancreatic cancer (PC). In literature can be observed, that Nectin-4 expression is related to the poor prognosis of affected patients (Pavlova NN *et al.*, 2013, Derycke MS *et al.*, 2010, Akano A *et al.*, 2009). In a research from Fabre-Lafay S, *et al.* reported that Nectin-4 is present on the cell surface (bound form) as well as in the serum (free form). Both are overexpressed in tumor tissue and play a role as tumor marker (Fabre-Lafay *et al.*, 2007).

1.2.4.4 Nectin-4 expression in different subtypes of urothelial carcinoma

Hoffman-Censits *et al.* have investigated different subtypes of bladder cancer. Patients have been scored according to H-score (intensity of staining) into negative (H 0–14), weak (H 15–99), moderate (H 100–199) and strong (H 200–300) groups. In general, 80% of muscle invasive bladder cancer showed strong staining. 10 cases of MIBC showed squamous differentiation with a strong staining in 57%. In the plasmacytoid variant, 25% of tumors were seen in a strong H-staining group, with a complete lack of Nectin-4 expression in sarcomatoid and small cell carcinomas (Hoffman-Censits *et al.*, 2021).

Similar results were found in the study by Tomiyama *et al.* where 66% of upper tract urothelial carcinoma (UTUC) samples were highly positive for Nectin-4. Tumor progression and risk of poor progression-free survival (PFS) were in a correlation with upregulation of Nectin-4 (Tomiyama *et al.*, 2020).

The reported response rate of EV in bladder cancer patients is around 40% (Rosenberg *et al.*, 2020), which is much lower than the rate of positivity for Nectin-4 described in studies above. To initiate microtubule disruption, EV has to bind with Nectin-4 on the surface of tumor cells. Therefore, without or with low expression of Nectin-4, there cannot be any expected response. In the following, we will look in detail at the action of Enfortumab Vedotin (EV), a new drug target for the treatment of advanced bladder cancer.

1.3 Antibody drug conjugates (ADC)

Antibody-Drug Conjugates (ADCs) are novel anti-cancer drugs, where monoclonal antibodies (specific to a tumor-specific antigen) are coupled with a strong biological drug (Chatterjee *et al.*, 2021). FDA approved, enfortumab vedotin (EV), for the treatment of metastatic urothelial cancer (Tomiyama *et al.*, 2020).

The monoclonal antibody of EV is coupled with a microtubule-disrupting agent called monomethyl auristatin E (MMAE). Binding of EV to the V-C-C domain of the Nectin-4 antigen leads to a complex internalization and lysosome mediated cleavage of the valine-citrulline linker. Consequently, MMAE in the target cells combine with the microtubules and cause a microtubule disassembly. This ultimately leads to death of cancer cells (Rosenberg *et al.*, 2019) (Fig.4).

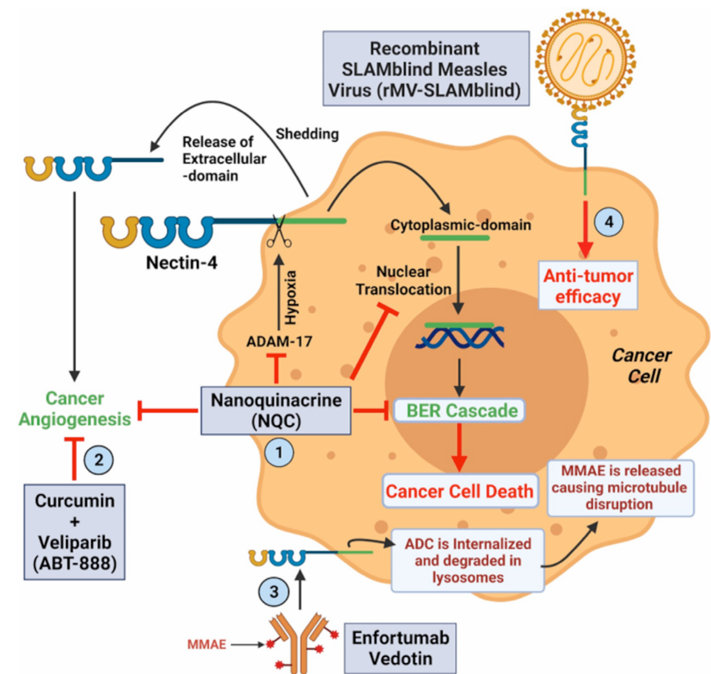


Figure 4: Targeting Nectin-4 with EV. **(3)** Enfortumab Vedotin, coupled with monomethyl auristatin E (MMAE) binds to Nectin-4 protein and initiates microtubule disruption. This ultimately leads to cell death of cancer cells (Chatterjee S et al, 2021).

The effectiveness of EV have been so far published in the following studies:
Recently in 2020 at the American Society of Medical Oncology (ASCO), Rosenberg presented break through results from EV-103 trial. Patients received a combination treatment with EV plus PD-1 inhibitor pembrolizumab or chemotherapy as a first line treatment. The ORR, CR and PR rates were 73.3%, 15.6% and 58% with progression free survival (PFS) time of 12.3 months (Hoimes et al., 2020).

In a EV-201, phase II, single-arm study, EV was administered to 125 patients with metastatic urothelial carcinoma, who were previously treated with platinum based combination chemotherapy and anti–PD-1/PD-L1 therapy. Overall response rate (ORR) was achieved in 44% of patients with a complete response rate (CR) of 12% and partial response rate (PR) of 32% (Rosenberg *et al.*, 2019).

In a EV- 301, a randomized phase III study, the efficacy of enfortumab vedontin versus chemotherapy (standard docetaxel, paclitaxel, or vinflunine) in previously treated patients with metastatic UC has been assessed. EV significantly prolonged survival in comparison to chemotherapy (median OS 12.88 vs. 8.97 months, HR 0.70, CI 95%, p = 0.001) (Powles *et al.*, 2021).

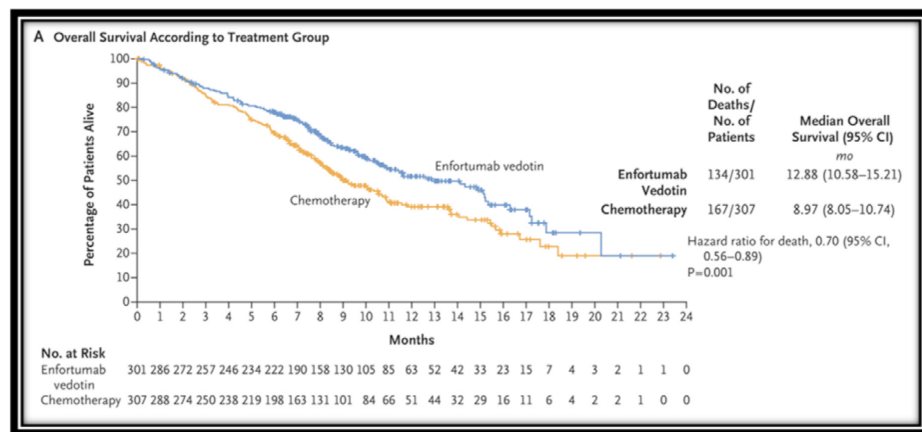


Figure 5: EV- 301, a randomized phase III study from Powles T et al., J Clin Oncology 39, 2021 (supp 6; abstr 393) – presented at ASCO GU. Abbreviations: CI, confidence interval; HR, hazard ratio; OS, overall survival.

1.4 Scope of study

Formerly, the Enfortumab Vedontin therapy has been FDA approved according to the two general accepted studies, EV 101 and EV 301 study. In the EV 101 study almost all tumors (96.7%) displayed strong Nectin-4 expression according to the H-score. In the EV 301 study a prolonged patients' overall survival (OS) in comparison to chemotherapy (standard docetaxel, paclitaxel, or vinflunine (median OS 12.88 vs. 8.97 months, HR 0.70, CI 95%, p = 0.001) was observed (Rosenberg *et al.*, 2019). However, the extent and percentage of Nectin-4 expression could not be specified. From a urooncologist's point of view, bladder tumors should highly express Nectin-4 in order to achieve positive treatment results with EV. The immunohistochemical staining method allows us to quantify the level of receptor expression (Kononen *et al.*, 1998). Most of the studies suggest that all UCs highly express Nectin-4 sufficient for a therapy response with EV and thus no IHC tissue analysis is necessary prior to treatment.

In a study from Klümper *et al.* Nectin-4 expression significantly decreased during metastatic spread ($p < 0.001$; median H-score = 40) of disease with 39.4% of metastasis lacking Nectin-4 expression. Absence or weak membranous Nectin-4 expression (34.0% of the cohort) was associated with a significantly shortened PFS on EV (log rank $P < 0.001$) (Klümper *et al.*, 2022). In light of the recent discussion on the analysis of Nectin-4 expression as a predictive biomarker in mUC and the prospect of EV involvement in earlier stages of UC, we performed an IHC analysis of two independent groups of patients with predominantly MIBC.

Therefore, main goal of the present work was to investigate Nectin-4 expression in tissue microarrays of two independent cohorts of patients with predominantly muscle invasive bladder cancer. Our goal was to confirm or decline whether nearly all bladder cancers overexpress Nectin-4. The expression, including the associated staining protocol, has been compared with clinical and demographic patient data to evaluate, if a need for confirming biomarker prediction before treatment with EV could be initiated.

The second aim of this study was to compare clinical data and course of disease according to Nectin-4 expression. We compared the expression of Nectin-4 in benign tissue to gain insight into the biology and pathology of Nectin-4.

2.0 MATERIALS AND METHODS

2.1 Type of study and approval of the local ethics Committee

This study was designed as a retrospective clinical study. The study included two independent cohorts of patients who underwent radical cystectomy for UC.

The Ethics Committee of the Medical Faculty of the Eberhard-Karls-University Tübingen granted its approval after regular application and examination the study and the preparation of the present medical doctoral thesis. Project number: 279/2013BO2 from March 1st, 2023.

2.2 Patients

The study included two independent cohorts of patients who underwent radical cystectomy in the period from 1996 to 2006 at the Department of Urology of the University Hospital of Tübingen. At the time of the cystectomy, each of the included patients had urothelial bladder carcinoma ≥ pT1.

The first database of 299 patients who underwent radical cystectomy between December 1984 and December 2006 was established at the University Clinic of Urology in Tübingen. The database was supplemented with other parameters with the aim of an optimal oncological characterization of the patient population at the time of cystectomy. Finally, the upgraded database included first name, surname, date of birth, sex, age at the time of surgery, date of surgery, surgical method, TNM stages, R status, grading and CIS. In addition, the database includes information about previous instillation therapy, neoadjuvant chemotherapy and adjuvant chemotherapy. Furthermore, it includes information about follow-up such as presence of recurrence, date of recurrence, calculated time to recurrence, tumor-related death, date of tumor-related death, calculated time to tumor-related death, calculated overall survival or last overall survival with last patient observation, respectively. The basis for

the creation of the database were pathology reports, physicians' letters, radiological consults, consultation with urologists and data from the Comprehensive Cancer Center.

2.2.1 Database

The formal results analysis database contains the following categories and columns: Name, First Name, Gender, Date of Birth, Date of Surgery, Pathology Number, Block Number Carcinoma, Block Number Benign Tissue, TMA Number, Array Position Tumor, Array Position Benign Tissue, T, N, M, G, Cis, Therapy, Recurrence, Tumor Dependent Death, Overall Survival and Evaluation of Nectin-4 expression.

2.2.1.1 Exclusion criteria

- Bladder tumor entities other than urothelial tumors (such as adenocarcinomas, squamous cell carcinomas etc.);
- Lack of availability of paraffin blocks;
- Incomplete pathology reports;
- Patients with cystectomy specimens without muscle-infiltrating bladder carcinoma and without urothelial carcinoma components.

2.2.3 Tissue microarray

The method was first described in 1998 by Kononen. The tissue microarray (TMA) method is used to examine a large number of tissues simultaneously. For this purpose, tissue punches (donor) are taken from different tissues using punch cylinders and these are placed in an empty paraffin block (recipient). The tissue cylinders should be between 0.6 mm and 3 mm in diameter and 3 to 4 mm in length. With this method we are able to embed up to 1000 punches from different tissue samples in a paraffin block and examine them simultaneously. It is also possible to take several samples from one block. Due to the small diameter of the punches, the original block (donor) will not be damaged. Even very small amounts of tissue can be preserved. The advantage is that the same conditions, such as room temperature and incubation time, exist for all tissue samples during staining. This ensures effective comparability between the punches. In addition to the TMA, numerous other

characterizations of tumor samples such as in situ hybridization of DNA or RNA and detection of RNA or DNA by PCR can be done (Kononen *et al.*, 1998). The efficiency, economy, and accuracy of this technology have been repeatedly confirmed in recent years (Brunswick, Chung, & Hewitt, 2004).

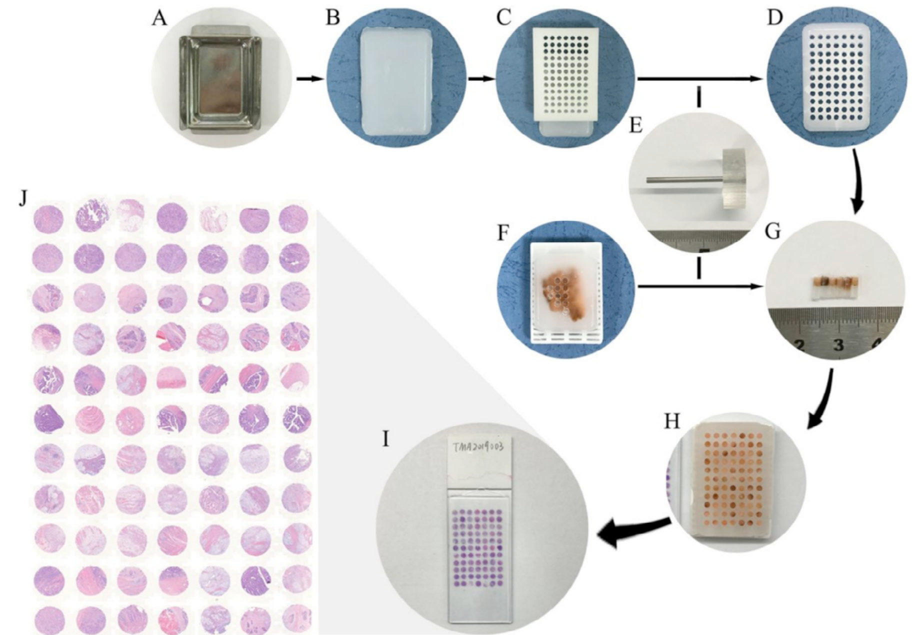


Figure 6: Technique for tissue microarray preparation (Chen YJ *et al.*, 2020)

2.2.4 Immunohistochemistry

Immunohistochemistry (IHC) is a method that allows us to visualize antigens (Ag) on a cell surface using labeled antibodies (Ab). The first description of the method dates back to the year 1941, when Coons detected pneumococcal antigens with labeled antibodies. Thus describing for the first time the combination of histology and biochemistry on a single slide (Coons AH, Creech HJ & Jones RN, 1941). In the following years, this method was developed further and further.

Roughly speaking, there are direct and indirect immunohistochemical methods. In the direct staining method, antibodies are used to detect the protein (Ag) structures. In the first step, the antibodies are added to the tissue. Through the antigen-antibody reaction, the antibody binds specifically to the antigen. After the addition of a substrate, for example 3,3'-Diaminobenzidin (DAB), the target proteins are made visible to the human eye. In this case, it is referred to as immunofluorescence. Another possibility is to link the primary antibody to an enzyme. Here, too, the primary antibody forms a fixed antigen-antibody bond. If a specific substrate is added to the enzyme, the enzyme-substrate reaction results in the formation of a dye, which then becomes visible.

Indirect staining involves the use of a secondary antibody. This secondary Ab is directed against the Fc fragment of the primary antibody. In this case, the enzyme is linked to the secondary antibody. Also in this case, the enzyme-substrate reaction described above takes place with the formation of a dye, which becomes visible and can be seen as an indicator for a specific epitope. Through the use of immunohistochemistry, it is possible to determine not only the presence, but also the localization of a protein. In the case of cytoplasmic proteins, the cytoplasm is stained. If proteins are localized on the cell membrane or cell nucleus, they are stained accordingly. A further feature for the characterization of tumor tissue is the quantification of the antigen quantity. For this purpose, staining intensity can be used (Matkowskyi KA *et al.*, 2003).

In summary, immunohistochemistry can be used to evaluate tumor tissues with regard to the occurrence, distribution and quantity of their specific antigens.

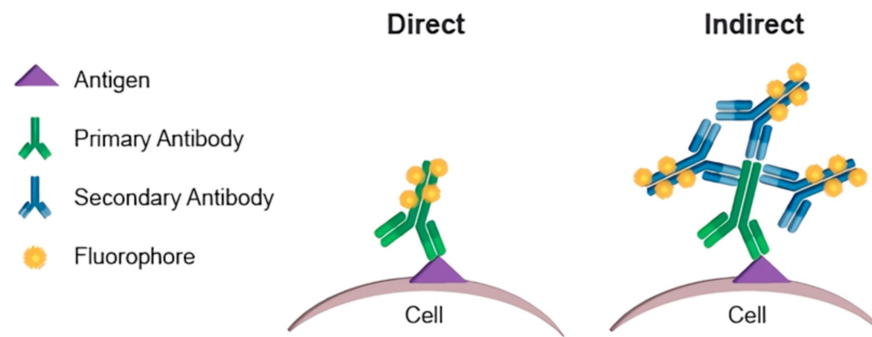


Figure 7: The principle of direct and indirect immunofluorescence. (Flow cytometry – creative Biolabs. Antibody-creativebiolabs.com. Accessed January 22, 2023. https://www.antibody-creativebiolabs.com/flow-cytometry.htm)

2.2.4.1 Course of immunohistochemistry

In the present study one immunohistochemical stain against Nectin-4 protein is used on the tissue. The indirect staining method was performed in the clinical laboratory of the Department of Urology at the University Hospital Tübingen. Prior to immunostaining, all reagents were brought to room temperature.

2.2.4.2 Staining protocol against Nectin-4

Before starting the staining, the sections were deparaffinized. For this purpose, the sections were incubated at 52°C and then treated two times for 10 minutes with the solvent, xylene.

Subsequently, they were rehydrated in a descending alcohol row. The sections were immersed twice for five minutes in 100% ethanol, twice for five minutes in 96% ethanol, and once for five minutes in 70% ethanol.

In the third step, the endogenous peroxidase was reduced. This is to reduce the background coloration caused by the endogenous peroxidase. For this purpose, the preparations were incubated in three percent hydrogen peroxide solution (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 minutes. The sections were rinsed once for five minutes with *Aqua destilata* (*A. dest.*).

To unmask and intensify the staining result, the sections were boiled in citrate buffer at pH 6.0 for 25 minutes in the microwave (Moulinex Mikro-Chef MO 500, Moulinex, Alençon, France) and left in the buffer for 30 minutes to cool at room temperature.
The sections were subsequently rinsed twice for five minutes with TBST (TRIS buffered Saline with Tween 20). To block non-specific staining, the sections were incubated in the block solution with 1,5% horse serum in Antibody Diluent (DAKO S0809, DAKO; Glostrup, Denmark) for 30 minutes.

Subsequently, the block solution was tipped off and the primary polyclonal rabbit antibody (Thermo Fisher, Waltham, MA, USA) in a dilution 1:2000 was added. The primary antibody was incubated overnight for 16 hours at 4°C.

The sections were then washed again with TBST three times for five minutes each.
The slides were next incubated with secondary antibody HRP One-Step Polymer (Zytomed Systems Berlin, ready-to-use, 150 µL per slide) for 40 minutes at room temperature. An additional three times rinse was performed for five minutes each with TBST. The staining reaction was subsequently initiated with a staining substrate consisting of DAKO Liquid DAB, Substrate (Agilent Santa Clara, CA, USA) K3467 and buffer from the ImmPACT ™DAB kit (Vector Laboratories, Burlingame, USA). That was incubated for two minutes at room temperature. This was followed by two additional rinses with TBST for five minutes each.

For counterstaining, the sections were incubated for 30 seconds with Mayer's hematoxylin (150 µL per slide, Agilent Santa Clara, CA, USA). To raise the pH, these were subsequently rinsed with tap water for seven minutes, resulting in increased blue coloration of cell morphology and nuclei.

Finally, for dehydration, the sections were treated with 96% ethanol twice each for three minutes, then with 100% ethanol for two times for five minutes, and finally with xylene two times for five minutes.

To fix the staining, the sections were further covered with Vecta-Mount (Vector Laboratories, Burlingame, USA) mounting medium and a coverslip.

2.2.4.2.1 Used solvents

- 1x TBST = 100 ml 10 × TBS + 900 ml *aqua dest.* + 2 ml Tween 20
- 3% H_2O_2 = 7,5 ml 30% H_2O_2 + 242,5 ml Aqua dest
- Citrate buffer = 9 ml standard solution A + 41 ml standard solution B + 450 ml *aqua dest*
- Standard solution A= 5,3 g citric acid monohydrate in 250 ml *aqua dest.*
- Standard solution B = 14,7 g Natriumcitrat (tri-natriumcitrat-dihydrate) in 500 ml *aqua dest*.
- Block solution (for one slide) = 2.25 µL horse serum + 150 µL DAKO Antibody Diluent, S0809
- Primary antibody 1:2000 (for one slide) = 0,1 µL Nectin-4 antibody + 150 µL DAKO Antibody REAL Diluent

Pre-dilution (for one slide):

- 1 µL Antibody + 99 µL Diluent (1:100)
- 7.5 µL + 142,5 µL Diluent (1:20) ≥ 1:2000

2.2.5 Assessment of the expression behavior

To evaluate the TMAs, the slides were observed using microscope Carl Zeiss Axioskope SIP 45814 with medium brightness constantly over the evaluation. A first impression could be gained at 10 × and 20 × magnification. For a more detailed observation and assessment of the cellular distribution, 40 × magnification was used. The observation of the anonymized punches was performed without the knowledge of the pathological features of the tissue. Only the K-number of the pathology and the number of the donor paraffin block were assigned to each dot. Therefore, unambiguous assignment was not possible. The results were recorded on a documentation sheet created individually for each of the eight TMAs using Excel. The arrangement of the dots on the TMA blocks corresponded to the arrangement of the fields on the documentation sheet used (see Fig.7 and 8).

The software for images capturing was ProgRes Capture Pro 2.10.0.1 – Jenoptic (Fremont, CA, USA).

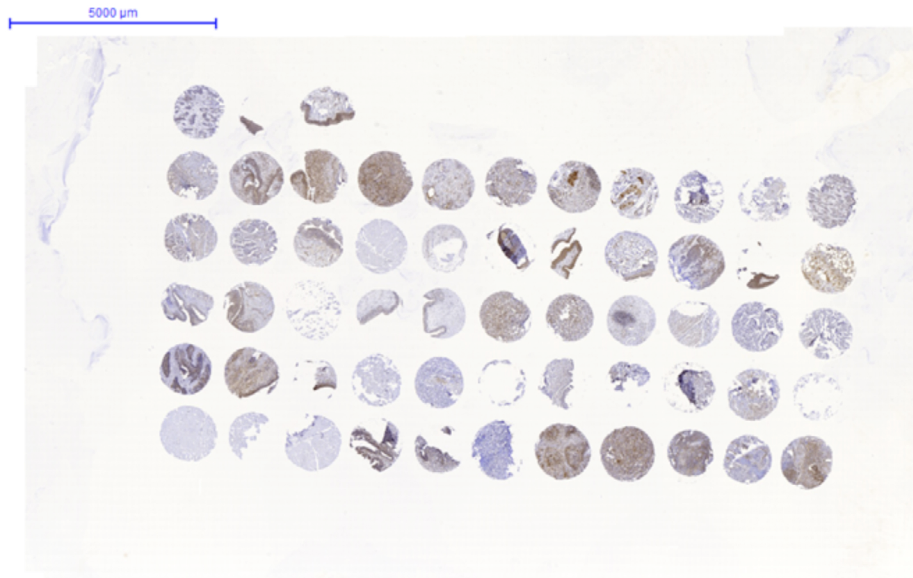


Figure 8: Immunohistochemically stained TMA slide. Own recording.

	A	B	C	D	E	F	G	H	I	J	K
1	K 21667/2006 Block 29 Tumor	K 21667/2006 Block 29 benigne	K 21667/2006 Block 29 benigne								
2	K 1010/2006 Block 30 Tumor	K 10636/2006 Block 15 benigne	K 10636/2006 Block 18 Tumor	K 10636/2006 Block 19 Tumor	K 19054/2006 Block 20 Tumor	K 19054/2006 Block 19 Tumor	K 19054/2006 Block 19 benigne	K 19054/2006 Block 19 benigne	K 20795/2006 Block 15 Tumor	K 20795/2006 Block 19 Tumor	K 21667/2006 Block 29 Tumor
3	K 15208/2006 Block 17 Tumor	K 15208/2006 Block 19 Tumor	K 15208/2006 Block 17 benigne	Schweinemuskel	K 15208/2006 Block 19 benigne	K 17320/2006 Block 37 Nerv??	K 17320/2006 Block 37 benigne	K 17320/2006 Block 40 benigne	K 16674/2006 Block 4 Tumor	K 16674/2006 Block 1 benigne	K 1010/2006 Block 30 Tumor
4	K 13756/2006 Block 9 benigne	K 13756/2006 Block 44 Tumor	K 13756/2006 Block 40 Tumor	K 13756/2006 Block 31 benigne	K 13756/2006 Block 31 benigne	K 14273/2006 Block 32 Tumor	K 14273/2006 Block 32 Tumor	K 14273/2006 Block 32 benigne	K 14273/2006 Block 32 benigne	K 14949/2006 Block 26 Tumor	K 14949/2006 Block 26 Tumor
5	K 8296/2006 Block 14 Tumor	K 8296/2006 Block 6 benigne	K 8296/2006 Block 14 benigne	Schweinemuskel	K 9818/2006 Block 29 Tumor	K 9818/2006 Block 32 Tumor	K 9818/2006 Block 29 benigne	K 9818/2006 Block 26 benigne	K 13750/2006 Block 9 Tumor	K 13750/2006 Block 13 Tumor	K 13750/2006 Block 11 benigne
6	Schweinemuskel	Schweinemuskel	Schweinemuskel	K 5719/2006 Block 19 Tumor	K 5719/2006 Block 19 Tumor	K 4089/2006 Block 1 Tumor	K 5794/2006 Block 2 Tumor	K 5794/2006 Block 2 Tumor	K 6266/2006 Block 11 Tumor	K 6266/2006 Block 5 Tumor	K 8296/2006 Block 6 Tumor

Figure 9: Display of the corresponding evaluation table. Yellow: Pig muscle/liver as orientation guide. Own recording.

The slices were evaluated in one run. The cytoplasmatic Nectin-4 expression was evaluated according to H-score and subsequently entered into the existing database with clinical-pathological and other collected data of the patients.

2.2.6 H-score

In the present study the H-score (P (percentage of positive cells) × I (intensity)) was used for both tumor and normal tissue. For the determination of the H-score, the sum of all urothelial tumor cells including benign urothelium in one dot is set as 100%. For each dot, the percentage number of cells corresponding to the respective intensity from 0 (no staining), 1 (low staining), 2 (medium staining) to 3 (strong staining) has been determined (Maygarden *et al.,* 1994).

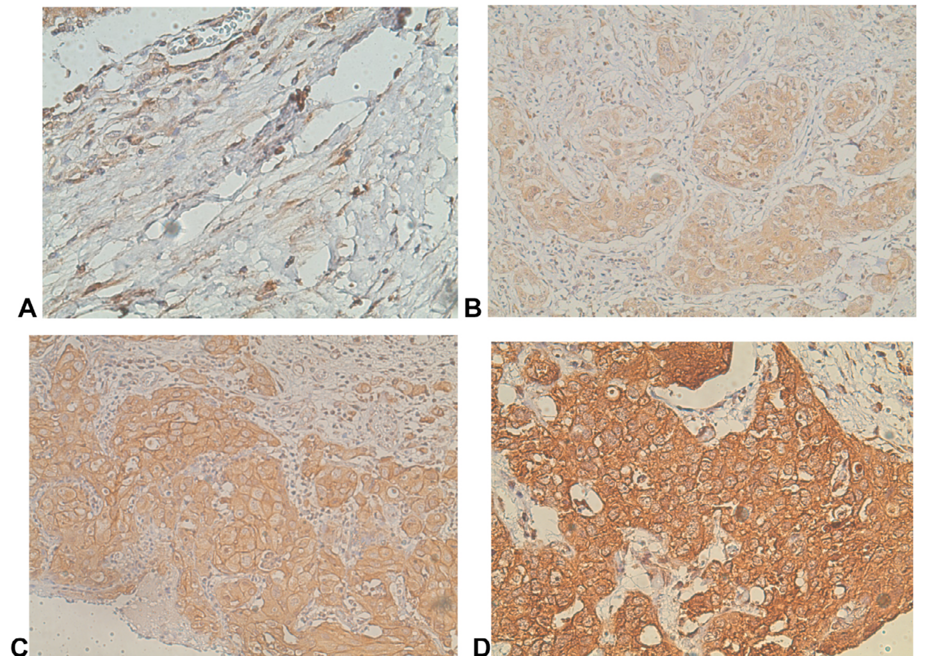


Figure 10: Samples of malignant TMA´s (200 x magnification) with **(A)** (0 – no staining) **B** (1 – low staining), **C** (2 – medium staining) and **D** (3 – strong staining).

2.2.7 Statistics

The statistical analysis of the collected data was performed with the program JMP (version 16, SAS Institute Inc., Cary, NC, USA). The collected data have been entered from the Excel spreadsheet into the statistical program for evaluation. The following tests were performed: Test for normal distribution using quantile-quantile plots (Q-Q plots), Spearman Rho correlation, Wilcoxon Kruskal-Wallis test, Kaplan-Meier plots, univariate and multivariate logistic regression analyses. Significance was assumed when the probability of error was $p \leq 0.05$. Quantile-quantile plots (Q-Q plots) were used to test the collected data for distribution. It was found that there was no normal distribution. Therefore, only non-parametric tests were used.

For the demographic and other variables of the patient population such as age at surgery, sex and TNM stage, median standard deviation SD and frequency distributions with quartiles were collected. Also for the data of the follow-up, tumor-specific survival and overall survival, statistical parameters and frequency distributions were collected.

For the outcome measures, staining intensity the statistical parameters and frequency distributions were also collected. This was followed by a comparison of the tumor and normal tissues using the Wilcoxon/Kruskal-Wallis test and the following chi-square approximation (Kruskal, 1952). For better assessment, dichotomization was performed as follows: The Age at the time of surgery in $0 < \text{median}$ and $1 \geq \text{median}$ (median: 69 years), T stage $0 < pT3$ and $1 \geq pT3$, N stage in $0 = N0$ and $1 \geq N1$. The comparison of the outcome variables Nectin-4 expression in tumor tissue with the TNM stages was performed in univariate analyses using Wilcoxon/Kruskal-Wallis test and Chi-square approximation (Kruskal WH, 1952).

The association between patient characteristics such as age and sex with the outcome variables was performed in single-factor analysis using the Wilcoxon/Kruskal Wallis test and associated chi-square approximation.

For the outcomes of occurrence of recurrence, tumor-dependent death, and overall survival, statistical parameters (median, SD, mean) and frequency distributions with quartiles were collected. Kaplan–Meier

analyses were used to determine recurrence-free (RFS), cancer-specific (CSS) and overall survival (OS) by the log-rank test (Kaplan, 1958; Mantel, 1966) with dichotomized Nectin-4 expression into $0 < \text{median}$ and $1 \geq \text{Median}$.

Uni- and multivariate Cox proportional hazard analyses were performed to assess the impact of Nectin-4 together with other assumed influencing variables on patients' outcome (Cox D, 1972).

2.2.8 Data protection

The handling of patient data was carried out in compliance with the medical confidentiality and in compliance with data protection requirements. Only persons who are subject to the duty of confidentiality were involved in the examinations and collection of clinical history data.

After completion of the clinical data collection phase, the patient collective was completely anonymized. Name and date of birth were removed and patients were assigned sequential numbers instead. Traceability, for example by birth dates or initials, is thus ruled out.

2.2.9 Used software

- Statistical Analysis: JMP 10.0® (Version 16, SAS Institute Inc., Cary, NC, USA)
- Literature management: BibGuru (Paperpile LLC,245 First Street, 18th Floor
- Cambridge, MA 02142,USA).
- Database creation: Microsoft Excel 2011® (Microsoft Germany, Germany)
- Dissertation writing: Microsoft Office Word 2020® (Microsoft Germany, Germany)
- Viewing and analysis of scans: ProgRes Capture Pro 2.10.0.1 – Jenoptic (Fremont, CA, USA).

3.0 RESULTS

3.1 Patients characteristics

The study included two independent cohorts consisting of discovery group (n = 103) and confirmation group (n = 97) patients who underwent radical cystectomy for invasive BC in the University Hospital Tuebingen. The patients had at least tumor stage pT1 and larger. The radical cystectomy took place between 1996 and 2006.

3.1.1 Characteristics of the first collective – discovery group (n = 103)

3.1.1.1 Gender and age distribution at the time of operation

The discovery group contained 103 patients at the time of evaluation with a smaller, female collective of 24 patients (23.3%) and a male collective, with 79 patients (76.7%) (Figure 11a and b).The range of age at time of surgery was from 32 to 84 years old, with a median age at time of surgery of 69 years old. The mean was 65.9 years old and the standard deviation was 10.8 years.

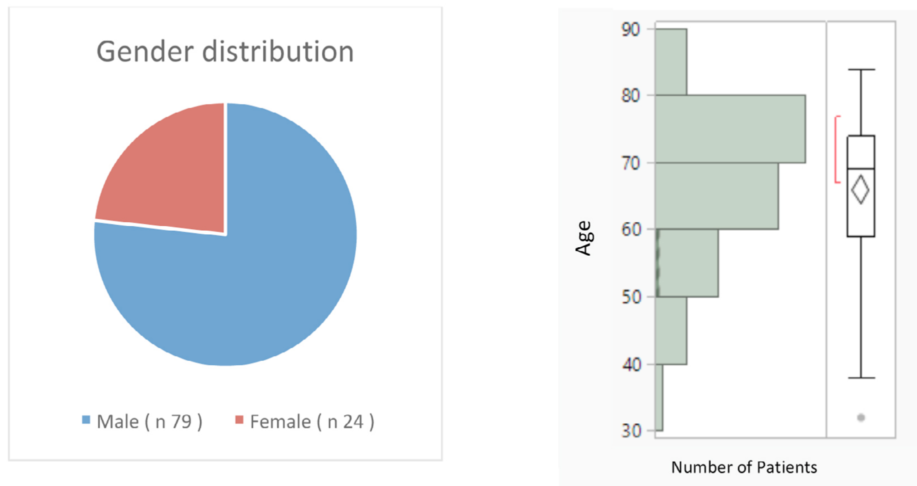


Figure 11: 11a (left) and 11 b (right): Gender and age distribution at the time of operation

3.1.1.2 TNM classification, grading and R status

TNM values after radical cystectomy were available for each patient. The distribution of T stage presented as follows: T1 – T2b was present in 31 patients (30%) with T1 presentation in one patient (0,7%), T2a was present in 16 patients (15,6%), and T2b in 14 patients (13,7%). Furthermore, stage T3 was present in a total of 48 (47.2%) of the patients. T3a category contained 24 patients (23.6%) as well as T3b with 24 patients (23.6%). Finally, 24 patients presented with stage T4 with T4a presentation in 18 patients (17.6%) and T4b in six patients (5.9%).

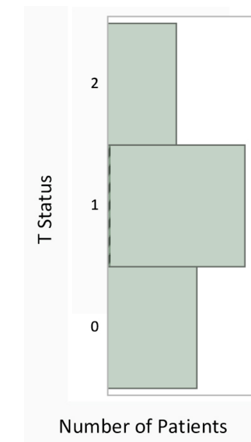


Figure 12: T Distribution: 0 (T1- T2b) in 31 patients, 1 (T3a-T3b) in 48 patients and 2 (T4a-T4b) in 24 patients

Lymph node status revealed the following results: Lymph node status was not determined in four of the 103 patients. It was determined that N0 was staged in 58 patients (58.2%), N1 in 24 patients (24.5%), N2 in 15 patients (15.3%) and finally N3 in two patients (2%).

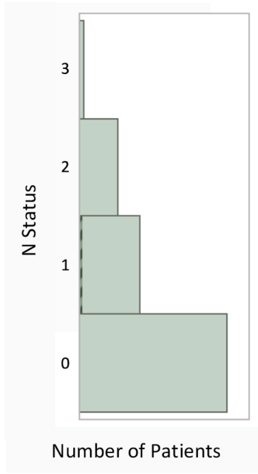


Figure 13: Node Distribution: 0 (N0 – 58 patients), 1 (N1 – 24 patients), 2 (N2 –15 patients), 3 (N3 – 2 patients).

When determining metastasis status, no values were obtained in four patients, while metastasis was present in nine patients (9.9%), and a further 90 patients (90.1%) were without metastasis.

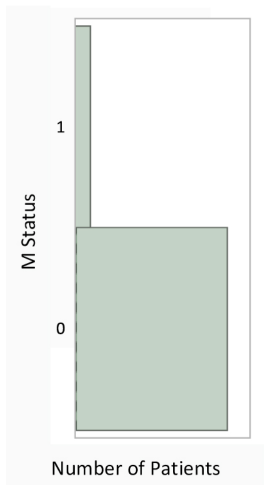
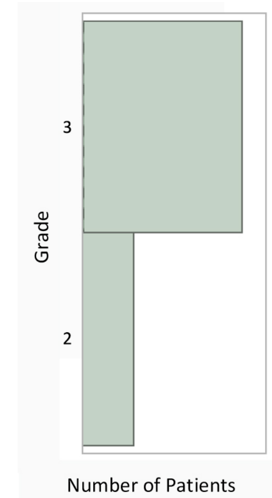


Figure 14: M Status: 1 (M1 in 9 Patients), 0 (M0 in 90 patients)

To complete the characteristic data summary, grading was performed in all 103 patients and was distributed as follows: G2 was obtained in 25 patients (23.5%) and G3 in 78 patients (76.5%). The resection status obtained revealed the following: R0 in 84 patients (81.4%), R1 in 16 patients (15.7%), and R2 in 3 patients (2.9%).



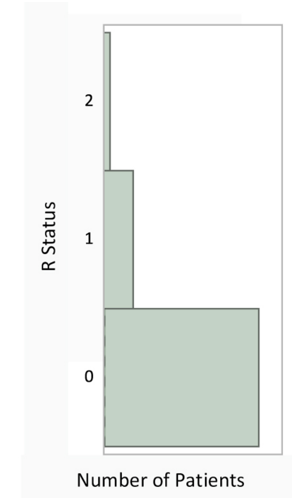


Figure 15: 15 a (left) G2 in 25 patients and G3 in 78 patients. **15 b (right)** R Status: R0 in 84 patients, R1 in 16 patients and R2 in 3 patients.

Characteristics of the first collective	median	n	%
Age distribution (n = 103)	65.9		
Gender (n = 103) Male Female		 79 24	 77.7 23.3
T-Status (n = 103) T1 T2a T2b T3a T3b T4a T4b	T3a	 1 16 14 24 24 18 6	 0.7 15.6 13.7 23.6 23.6 17.6 5.9
N-Status (n = 99) N0 N1 N2 N3	0	 58 24 15 2	 58.2 24.5 15.3 2
M-Status (n = 99) M0 M1	0	 90 9	 90.1 9.9
Grading (n = 103) G1 G2 G3	3	 0 25 78	 23.5 76.5

Table 4: Tabular representation of the characteristics of the first collective.

3.1.2 Characteristics of the second collective – conformation group (n = 97)

3.1.2.1 Gender and age distribution at the time of operation

The conformation group contained 97 patients at the time of evaluation. The female collective contained 27 patients (27.8%) and male collective contained 70 patients (72.2%) in (Figure 16a). The range of age at the time of surgery was 40 to 83 years old, with a median age at time of surgery of 65.5 years old. The mean was 64.4 years old, and the standard deviation was 10.5 years.

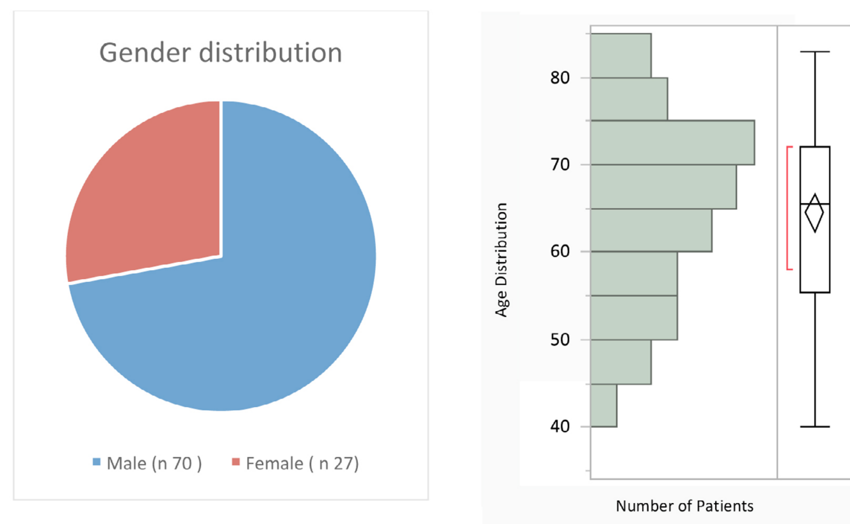


Figure 16: 16a (left) and 16b (right): Gender and age distribution at the time of operation

3.1.2.2 TNM classification, grading and R status

TNM values after radical cystectomy were available for each patient. The distribution of T stage presented as follows: T1 – T2b was present in 31 patients (32%) with T1 presentation in 9 patients (9%), T2a was present in 9 patients (9%), and T2b in 13 patients (13,4%). Stage T3 was present in a total of 43 (45%) of the patients. T3a contained 22 patients (22.6%) and T3b with 21 patients (21.6%). Stage T4 was presented in 23 (23%) patients with T4a presentation in 16 patients (17.4%) and T4b in 7 patients (7%).

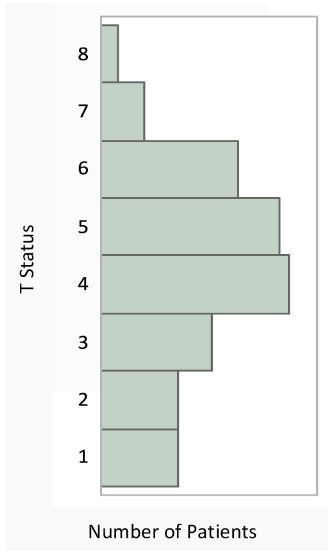


Figure 17: T Distribution 1,2,3 (T1- T2b) in 31 patients, 4,5 (T3a-T3b) in 43 patients and 6,7,8 (T4a-T4b) in 23 patients

Lymph node status was observed and revealed the following results: Lymph node status was not determined in 3 of the 97 patients. Furthermore, N0 was determined in 61 patients (64.8%) and N+ in 33 patients (35.2%)

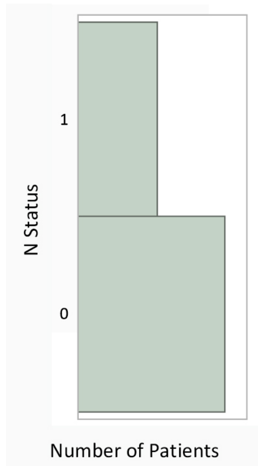


Figure 18: Nodes Distribution: 0 (N0 61 patients), 1 (N+ 33 patients).

In further compliance with the TMN classification, metastasis was also determined in conformation group, in which 11 patients presented with metastasis (11.4%) and 86 patients (88.6%) were without metastasis.

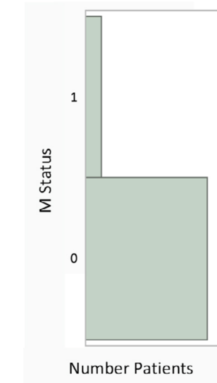
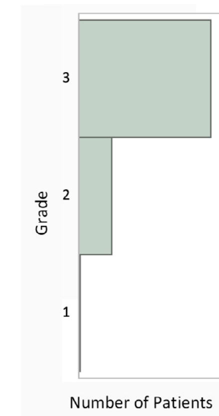


Figure 19: M Status: 1 (M+ in 11 Patients), 0 (M0 in 86 patients)

Grading was performed in 96 patients and was distributed as follows: G1 was determined in 1 patient (1%), G2 was obtained in 19 patients (19.7%), and G3 in 76 patients (79.3%). The resection status obtained revealed the following: R0 for 77 patients (81,2%), R1 for 16 patients (16,8%), and R2 for 2 patients (2%).



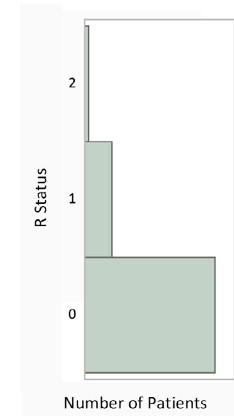


Figure 20: 20 a (left) G1 in 1 patient, G2 in 19 patients and G3 in 76 patients. **20 b (right)** R stage: R0 in 77 patients, R1 in 16 patients and R2 in 2 patients.

Characteristics of the second collective	median	n	%
Age distribution (n = 97)	64.4		
Gender (n = 97)			
Male		70	72.2
Female		27	27.8
T-Status (n = 97)	T3a		
T1		9	9
T2a		16	15.6
T2b		14	13.7
T3a		24	23.6
T3b		24	23.6
T4a		18	17.6
T4b		6	5.9
N-Status (n = 94)			
N0		61	64.8
N+		33	35.2
M-Status (n = 99)	0		
M0		86	88.6
M1		11	11.4
Grading (n = 96)	3		
G1		1	1
G2		19	19.7
G3		76	79.3

Table 5: Tabular representation of the characteristics of the second collective.

3.2 Description of staining behavior in normal and tumor tissue

The Nectin-4 expression could be detected by immunohistochemistry and viewed under a light microscope. The expression of the Nectin-4 was shown in both, normal and tumor tissue (see Fig. 21 and 22). On the TMA, the well assessable punctures were evaluated. The staining ranged from light to dark brown. The evaluation was performed as described in chapter 2.2.5. This evaluation referred to cytoplasmic expression in tumor and normal tissues. In general, epithelial cells were stained, with a particular affinity for the urothelium. The staining extended equally from the basal cell layer to the apically located luminal cell layer. The tumor cells were either diffusely distributed or visible as massive clusters.

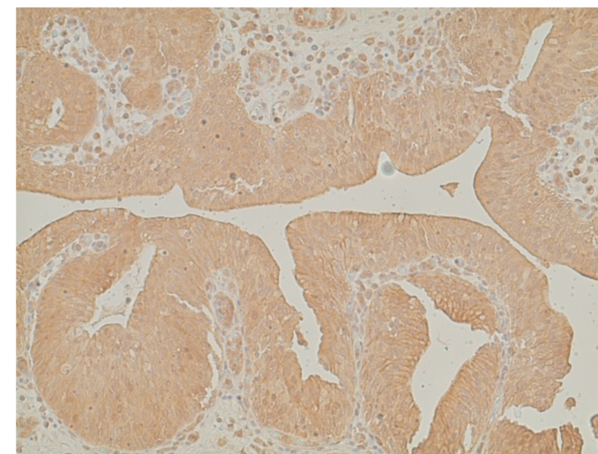


Figure 21: Benign urothelium (200 × magnification) light microscopy. Own recording.

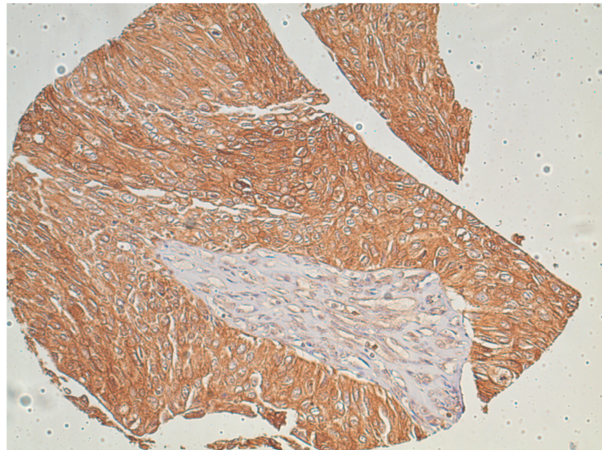


Figure 22: Clusters of urothelial carcinoma (200 x magnification) light microscopy. Own recording.

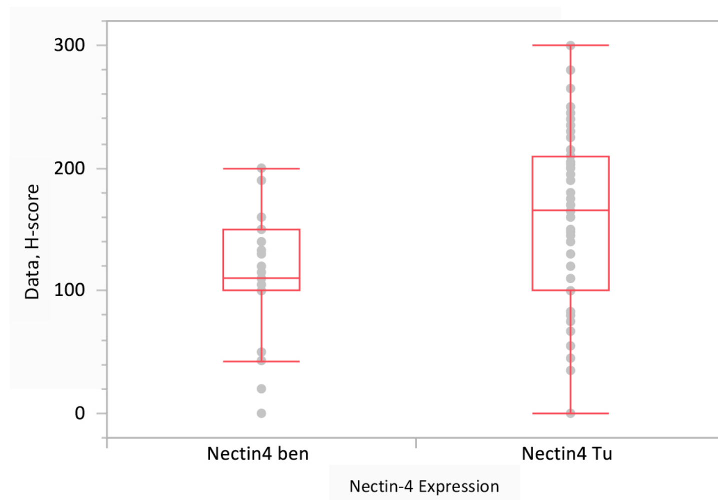


Figure 23: Single factor analysis of Nectin-4 expression in tumor tissue according to Nectin-4 expression in benign tissue (Wilcoxon/Kruskal-Wallis-Tests p = 0.0016). Median H-score benign tissue 110 vs. median H Score malignant tissue 165.

Benign tissue on average stains with less intensity, but still more than expected. Benign tissue was removed from the tumor-free part of the urothelium. Given the consistently medium to high staining intensity of benign tissue for Nectin-4, we hypothesize that the whole urothelium is genetically altered in the presence of tumor and that the microscopic appearance of benign tissue already shows the immunohistochemical features of malignant tissue with higher expression of Nectin-4.

3.3 Correlations with demographic data (age, gender)

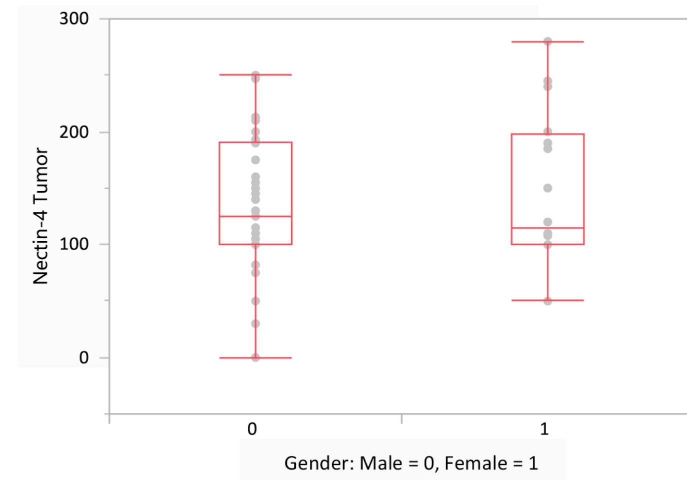


Figure 24: Single factor analysis of Nectin-4 expression in tumor tissue male vs. female (Wilcoxon/Kruskal-Wallis-Tests p = 0,4861). Median H-score male 125 vs. median H-score female 115.

Comparison showed no significant result of p = 0.4861 in single factorial analysis using Wilcoxon/Kruskal-Wallis test. In the male population was median H-score higher than in the female population.

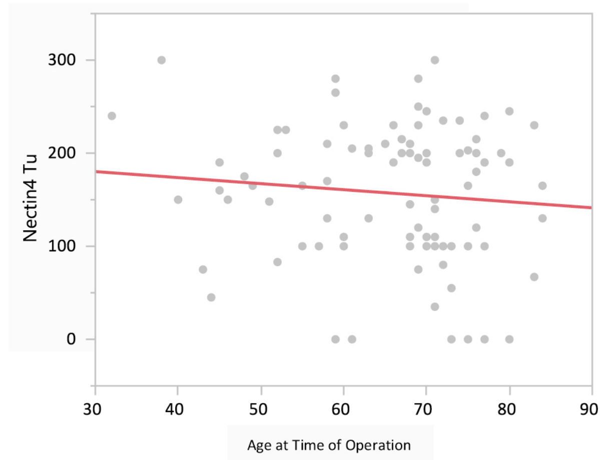


Figure 25: Bivariate Analysis of Nectin-4 expression in tumor cells in comparison to age distribution (p = 0,3497)

There was no statistical significance observable in the collected data.

3.4 Correlation to clinical characteristics – discovery group

3.4.1 Correlation with TNM classification and grading

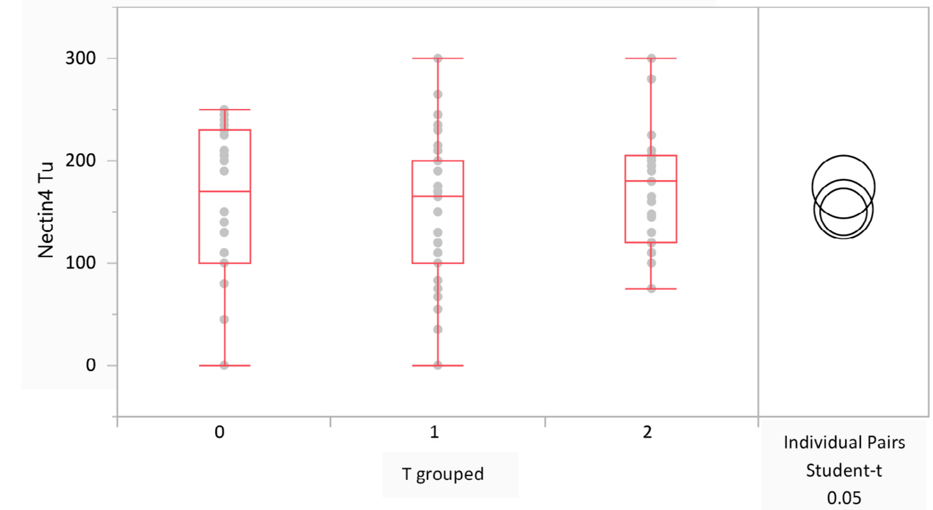


Figure 26: Single factorial analysis of Nectin-4 expression in tumor tissue grouped by T-stage where 0 = T1–2b, 1 = 3a+3b, 2 = 4+CIS (Wilcoxon/Kruskal-Wallis-Tests p = 0,6367).

Comparison of different T stage and Nectin-4 expression in tumor tissues showed no statistical correlation (p = 0.6367). Moreover, statistical analysis was unable to show that depth of infiltration leads into higher expression of Nectin-4.

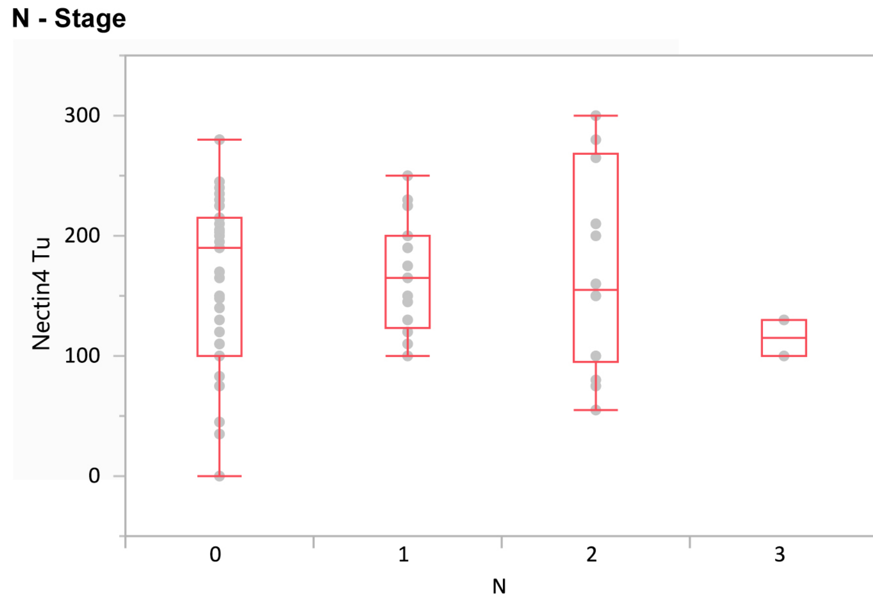


Figure 27: Single factorial analysis of Nectin-4 expression in tumor tissue grouped by N-Stage where 0 = N0, 1 = N1, 2 = N2, 3 = N3 (Wilcoxon/Kruskal-Wallis-Tests p = 0,7689)

Comparison of different N stages and Nectin-4 expression in tumor tissues showed no statistical correlation (p = 0.7689). In addition, statistical analysis revealed a decrease in the mean staining-value as the number of positive lymph nodes increased.

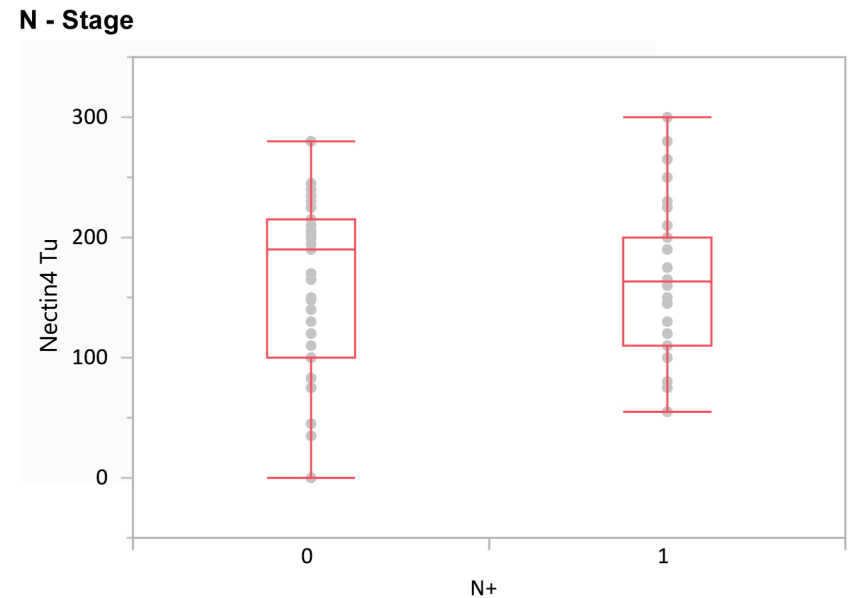


Figure 28: Single factorial analysis of Nectin-4 expression in tumor tissue grouped by N-Stage 0 = N0, 1 = N+ (Wilcoxon/Kruskal-Wallis-Tests p = 0,9965).

Also when comparing N+ only, no statistical correlation is revealed (p = 0.9965). Statistical analysis has again revealed a decrease in the mean staining value in N + disease. Hypothetically, we are inclined to believe that poorly differentiated tumors, which indirectly affect N+ disease, show poorer expression of Nectin-4.

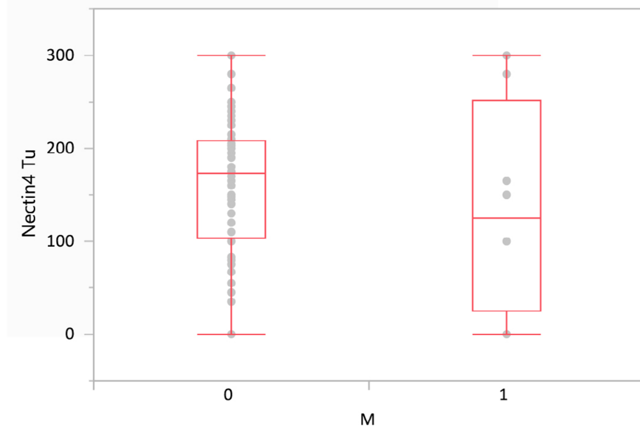


Figure 29: Single factorial analysis of Nectin-4 expression in tumor tissue grouped by M-stage (Wilcoxon/Kruskal-Wallis-Tests p = 0,4323).

Comparison of M-stage and Nectin-4 expression in tumor tissues according to H-score showed no statistical correlation (p = 0.4323). Statistical analysis has again revealed a decrease in the mean staining-value in M + disease.

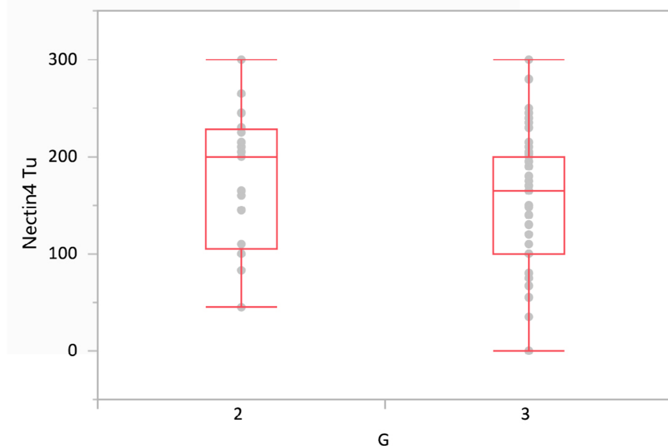


Figure 30: Single factorial analysis of Nectin4 expression in tumor tissue grouped by G-stage (Wilcoxon/Kruskal-Wallis-Tests p = 0,1181).

Comparison of G-stages and Nectin-4 expression in tumor tissues according to H-score showed no statistical correlation (p = 0.1181). Statistical analysis has revealed a decrease in the mean staining value in G3 disease. We can see trends of lower expression along progression of MIBC.

3.5 Correlation to clinical characteristics – confirmation group

3.5.1 Correlation with TNM- classification and grading

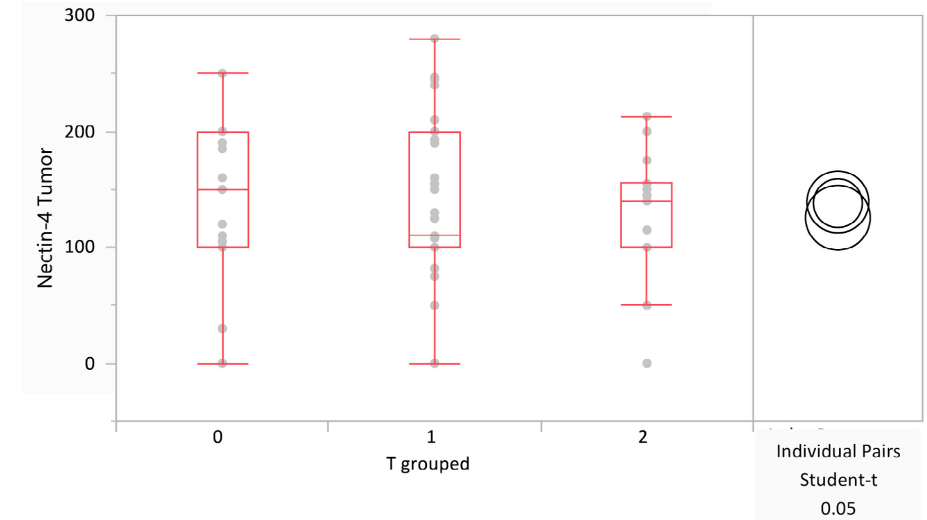


Figure 31: Single factorial analysis of Nectin4 expression in tumor tissue grouped by T-stage where 0 = T1–2b, 1 = 3a+3b, 2 = 4+CIS (Wilcoxon/Kruskal-Wallis-Tests p = 0,6810).

Comparison of different T-stages and Nectin-4 expression in tumor tissues showed no statistical correlation (p = 0.6810). Statistical analysis was unable to show that depth of infiltration leads into higher expression of Nectin-4.

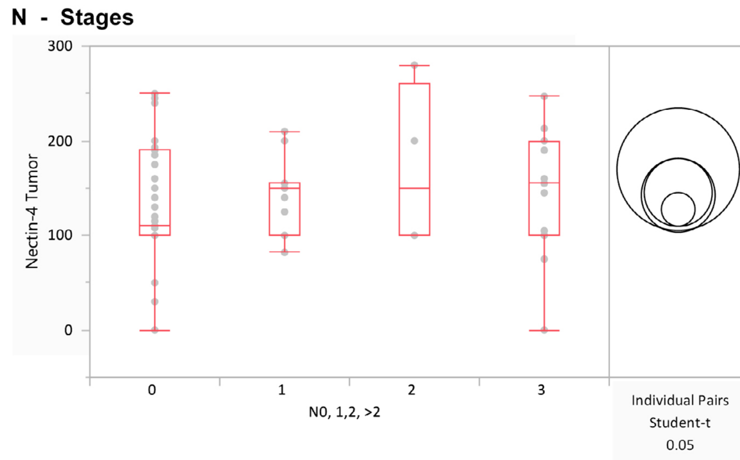


Figure 32: Single factorial analysis of Nectin-4 expression in tumor tissue grouped by N-stage where 0 = N0, 1 = N1, 2 = N2, 3 = N3 (Wilcoxon/Kruskal-Wallis-Tests p = 0,6541).

Comparison of different N-stage and Nectin-4 expression in tumor tissues showed no statistical correlation (p = 0.6541). Statistical analysis did not show a significant difference in staining values when we talk about a higher number of positive lymph nodes. This confirms the hypothesis from discovery group.

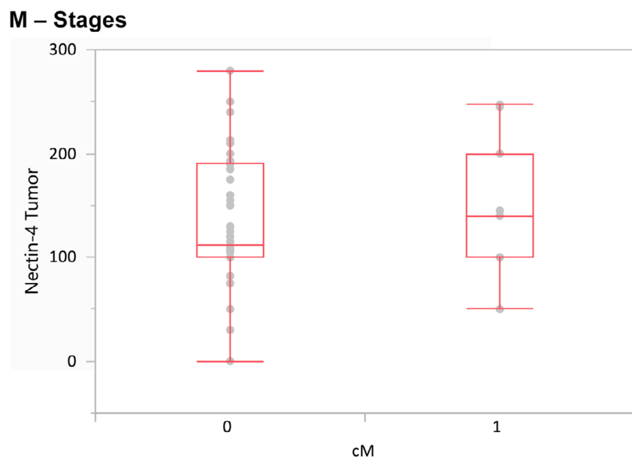


Figure 33: Single factorial analysis of Nectin-4 expression in tumor tissue grouped by M-stage (Wilcoxon/Kruskal-Wallis-Tests p = 0,4323).

Comparison of M Stadiums and Nectin-4 expression in tumor tissues according to H-score showed no statistical correlation (p = 0.4323).

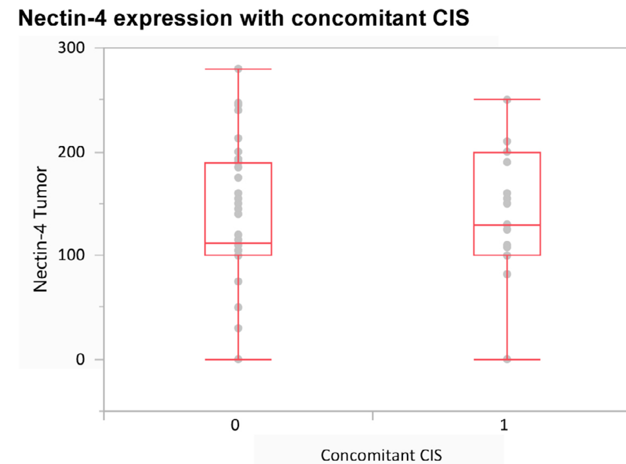


Figure 34: Single factorial analysis of Nectin-4 expression in tumor tissue with concomitant CIS (Wilcoxon/Kruskal-Wallis-Tests p = 0,3407)

Comparison of CIS and Nectin-4 expression in tumor tissues according to H-score showed no statistical correlation (p = 0.3407).

3.6 Correlation to course of the disease

3.6.1 Time to event (TTE) at H-score 220

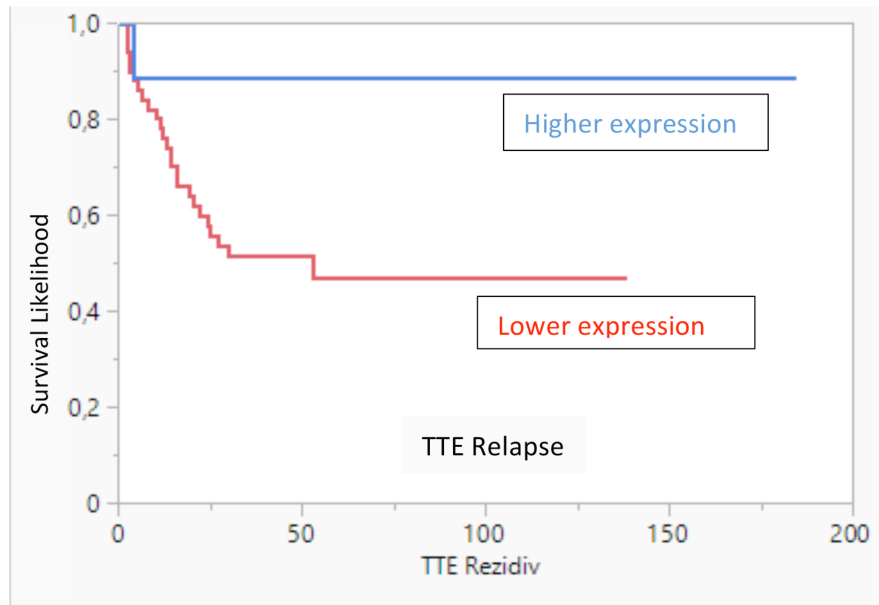


Figure 35: Estimated probability of relapse by H-score 220.

For 27 of the 103 patients (=26.2%), data on time to event (TTE) could be collected.
Patients developed relapse of disease at H values below 220 at 4 months, while patients with H values above 220 remained relapse-free for as long as 33 months.
There was almost statistical significance (Wilcoxon Test $p = 0.0779$), but given the low number of patients, below 220, the statistical analysis is not reliable.

3.6.2 Cancer specific survival (CSS) – cut off point of 220 (H-score)

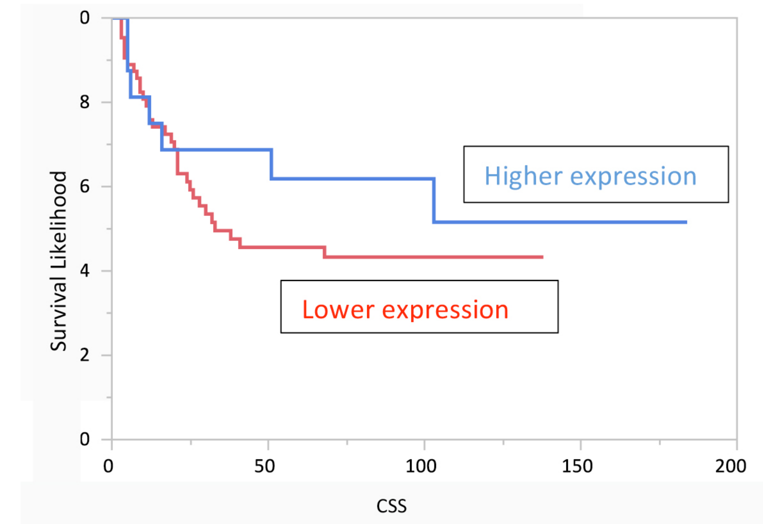


Figure 36: Kaplan-Meier-Curves for cancer specific survival from patients with high (H-score ≥ 220, blue) and low expression of Nectin-4 (H-score < 220, red).

For 39 patients data on cancer-specific survival (CSS) could be collected. The median time to death was 40 months for patient with H-score < 220 and 69 months for the patients with H-score > 220. There was no statistical significance (Log rank Test $p = 0.4285$).

3.6.3 Overall survival (OS) with cutoff point of 220 (H-score)

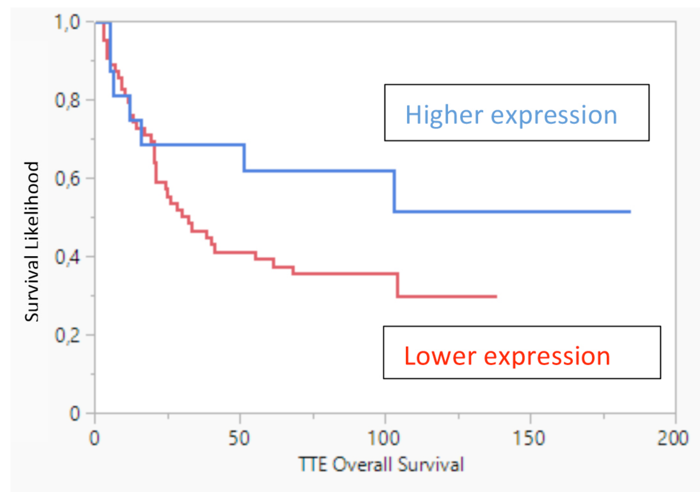


Figure 37: Kaplan- Meier- Curves for overall survival (OS) from patients with high (H-score ≥ 220, blue) and low expression of Nectin-4 (H Score < 220, red).

For 46 patients data on overall survival (OS) could be collected. The patients died tumor-independently between 51 and 69 months after surgery. The median probability of tumor-independent death for patients with an H-score < 220 was 51 months and for patients with an H-score > 220 it was 69 months. There was no statistical significance (Wilcoxon Test p = 0.3117).

3.6.4 Uni- and multivariate analysis

Known demographic and clinical parameters were assessed in univariate Cox proportional hazard analyses for prognostic significance (Table 6, mid columns). Then, all significant parameters were assessed in multivariate analyses, together with Nectin-4, to prove for potential independent impacts (Table 6, left columns).

A Recurrence	Univariate analysis			Multivariate analysis		
	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI
Pathological stage T>2 vs. T2	**0.0099**	3.25	1.33–7.95	0.16	1.92	0.77–4.81
Nodal status N+ vs. N0	0.0879	1.88	0.91–3.88	–		
Metastasis status M+ vs. M0	–			–		
Grade ≥G3 vs. <G3	0.0531	1.30	0.60–2.83	–		
Nectin 4 low vs. high tumor expression (cut-off 220)	0.09	5.66	0.77–41.79	0.13	4.61	0.61–34.57
	Effect Likelihood **0.0236**					
Age, year	0.2397	1.02	0.99–1.06	–		

B CSS	Univariate analysis			Multivariate analysis		
	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI
Pathological stage T>2 vs. T2	**0.0107**	2.53	1.24–5.17	0.25	1.59	0.72–3.51
Nodal status N+ vs. N0	**0.0309**	1.96	1.06–3.62	0.21	1.60	0.76–3.33
Metastasis status M+ vs. M0	**0.0015**	3.56	1.63–7.79	0.11	2.16	0.85–5.50
Grade G≥3 vs. G<3	**0.0069**	3.62	1.42–9.22	0.26	1.78	0.65–4.88
Nectin 4 low vs. high tumor expression (cut-off 220)	0.43	1.39	0.61–3.17	0.91	1.05	0.44–2.51
Age, year	**0.0047**	1.05	1.01–1.08	**0.0423** Effect Likelihood	1.04	1.00–1.07

C OS	Univariate analysis			Multivariate analysis		
	P	Hazard ratio	95% CI	P	Hazard ratio	95% CI
Pathological stage T>2 vs. T2	**0.0020**	2.79	1.45–5.33	0.15	1.74	0.83–3.65
Nodal status N+ vs. N0	**0.0258**	1.85	1.08–3.17	0.0673	1.92	0.95–3.84
Metastasis status M+ vs. M0	**0.0036**	3.13	1.45–6.73	0.21	1.81	0.71–4.58
Grade G≥3 vs. <G3	**0.0173**	2.31	1.16–4.60	0.41	1.45	0.60–3.46
Nectin 4 low vs. high tumor expression (cut-off 220)	0.19	1.73	0.77–3.89	0.47	1.37	0.58–3.27
Age, year	**0.0031**	1.05	1.02–1.08	**0.0191** Effect Likelihood	1.04	1.01–1.08

Table 6: Univariate and multivariate Cox regression analyses for recurrence-free survival (RFS), cancer-specific survival (CSS) and overall survival (OS) in the discovery cohort.

Univariate analysis shows that T-stage and Nectin-4 expression influence relapse, but both lose their independent predictive value in multivariate analysis (see Table 6 above). All parameters examined, including age, T, N+, M and G, were significantly associated with CSS and OS. Multivariate analyses showed that patient age still had an independent predictive effect (see Table 6 above, right columns).

3.7 Correlations to some other biomarkers

3.7.1 Nectin-4 expression in correlation with somatostatin-II receptor, insulin receptor substrate 1 (IRS1) and IL 1 b receptor expression

As a point of interest, we present below the results of other biomarker studies that have been performed on the same group of patients. Somatostatin receptors (SSTR) have been shown to be important for tumor biology and prognosis in various types of cancer. In a study from Mass M., *et al.* expressions of SSTR1–4 were shown to be significantly decreased in BC as compared to benign urothelium (Mass *et al.*, 2020), where SSTR3 expression played independent marker of improved CSS and OS. IRS-1 is able to integrate different signaling pathways, which indicates its possible role in cancer progression (Tartare-Deckert S *et al.*, 1955). The present data show that low IL1RA (interleukin 1 receptor antagonist) levels in bladder cancer cell lines correlate with their increased invasive potential. Secreted IL1RA (sIL1RA) antagonizes the effects of IL1a or IL1b through inhibition of IL1 binding to surface receptors (Schneider L, Liu J, Zhang C, *et al.*, 2021).

3.7.1.1 Comparison of cytoplasmatic somatostatin II receptor expression and Nectin-4 expression in tumor cells

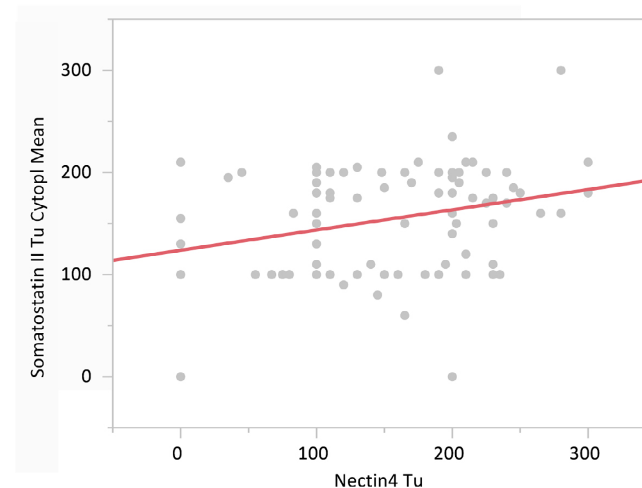


Figure 38: Bivariate analysis of cytoplasmatic somatostatin II receptor according to Nectin-4 expression in tumor cells.

Comparison of cytoplasmic expression of SSTR2 and Nectin-4 in tumor tissues showed a significant correlation. This means that higher cellular Nectin-4 expression of BC cells has positive correlation with stronger SSTR2 expression in tumor tissue, which might have prognostic value ($p = 0.0115$) (Mayer L., 2021).

3.7.1.2 Comparison of insulin receptor substrate 1(IRS1) expression and Nectin-4 expression in tumor cells

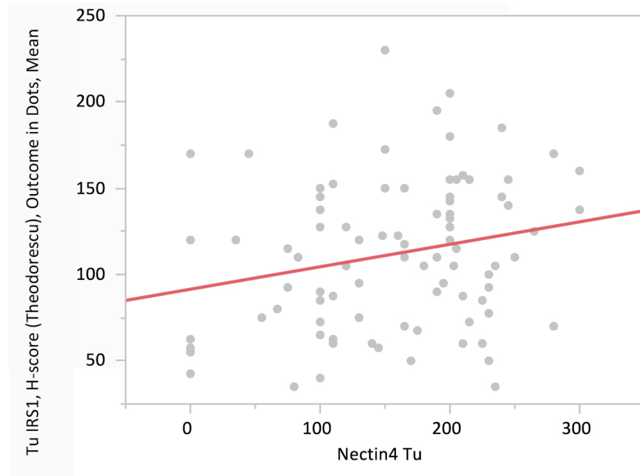


Figure 39: Bivariate analysis of insulin receptor substrate 1(IRS1) according to Nectin-4 expression in tumor cells

Comparison of IRS1 and Nectin-4 in tumor tissues showed a positive correlation ($p = 0.03$). This means that higher cellular Nectin-4 expression of BC cells has positive correlation with stronger IRS1 expression in tumor tissue, which might have prognostic and therapeutic value.

3.7.1.3 Comparison of Nectin-4 expression according to IL1β in tumor cells

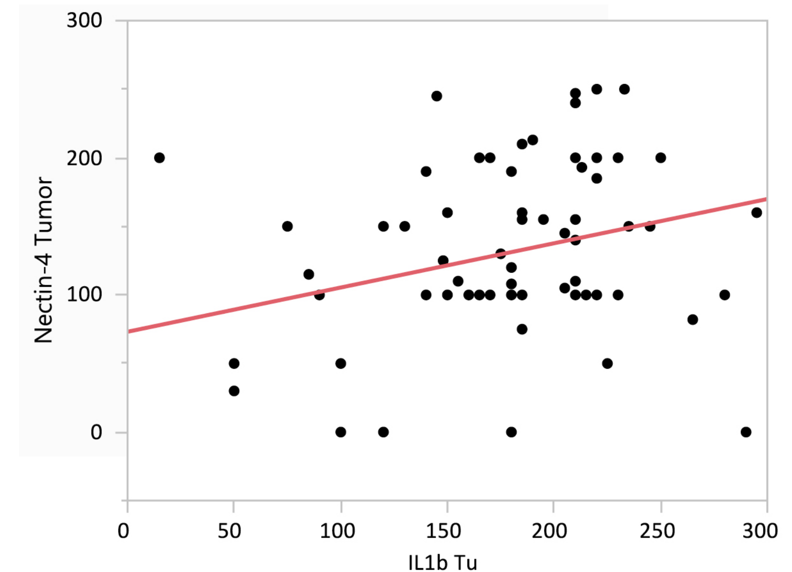


Figure 40: Bivariate analysis of Nectin-4 according to IL1 β in tumor cells

In Figure 40 it can be seen that higher expression of IL-1β correlate with higher Nectin-4 expression in BC cells ($p = 0.015$). Survival analysis revealed favorable RFS, CSS, and OS in case of high IL-1ß expression ($p < 0.02$, < 0.03 and < 0.006, respectively) (Vukovic M *et al.*, 2023).

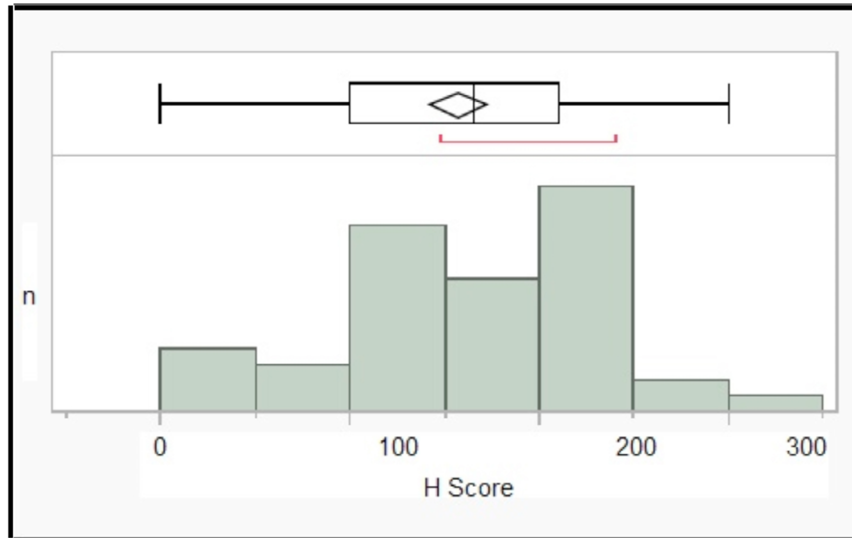
3.8 Staining characteristics and biomarker statements

Tissues from histologically proven BC and from benign urothelium were processed to a tissue microarray and immunohistochemically stained by the reported method and quantified by the histochemical scoring system (H-score 0–300). Results were then transferred into four classes. Each cohort was divided into four 25%-classes of successive increasing staining and distribution was demonstrated for each the two BC cohorts.

The expressions within each patient in discovery cohort are lined up as follows:

- 25% low intensity (0–99)
- 25% mild intensity (100–165)
- 25% moderate intensity (166–209)
- 25% strong intensity (210–300)

3.8.1 Expression of Nectin-4 in tumor tissue (n = 90) (discovery group)



100.0%	Maximum	300
99.5%		300
97.5%		294,5
90.0%		240
75.0%	Quartil	210
50.0%	Median	165
25.0%	Quartil	100
10.0%		56,2
2.5%		0
0.5%		0
0.0%	Minimum	0

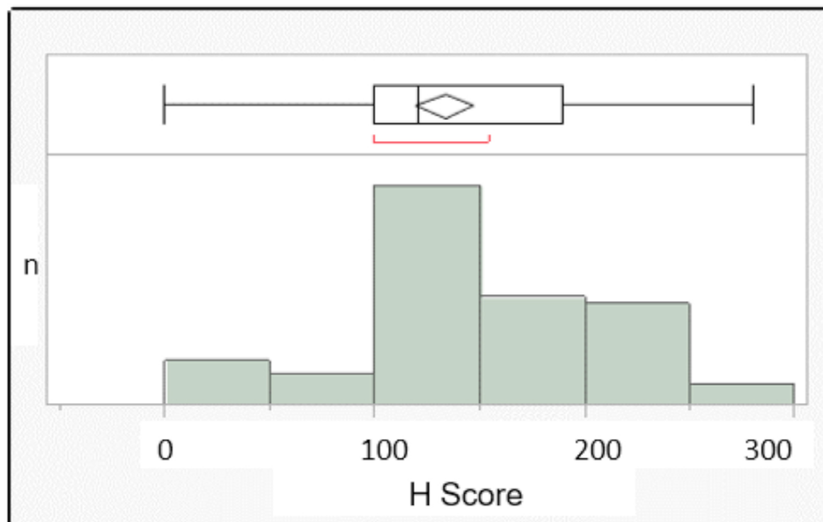
Figure 41: Distribution of Nectin-4 expression in muscle invasive bladder cancer

Each group (25% low, 25% mild, 25% moderate, 25% high) contained 22.5 patients.
No expression could be seen in 2.25 patients (2.5%) with high expression in only 22.5 patients. Maximum expression was 300 (H-score) in 0.5%. Mean expression score in BC tissue was 157 ($p < 0.002$).

3.8.2 Expression of Nectin-4 in tumor tissue (n = 83) (confirmation group)

The expressions within each patient in conformation group are lined up as follows:

- 25% low intensity (0–99)
- 25% mild intensity (100–119)
- 25% moderate intensity (120–189)
- 25% strong intensity (190–300)



100.0%	Maximum	280
99.5%		280
97.5%		250
90.0%		206
75.0%	Quartil	190
50.0%	Median	120
25.0%	Quartil	100
10.0%		50
2.5%		0
0.5%		0
0.0%	Minimum	0

Figure 42: Distribution of Nectin-4 expression in patients with muscle invasive bladder cancer (confirmation group).

Each group (25% low, 25% mild, 25% moderate, 25% high expression) contained 20.75 patients. No expression could be seen in 2 patients (2.5%) with high expression in only 20.75 patients. Maximum expression was 280 (H-score) in 0.5%. Mean expression score in tumor tissue were 133 ($p < 0.005$).

With regard to Nectin-4 expression, the two groups were comparable with mean staining score 133/157.

4.0 DISCUSSION

Immunotherapy and target therapeutics have been a breakthrough in the treatment of metastatic bladder cancer. We are facing challenges by treatment in patients with bladder tumors because of comorbidities and side effects, that can compromise functional status. Despite this breakthrough in treatment, this disease remains generally incurable.

Cisplatin based systemic combination chemotherapy has become a standard regimen and a first-line-therapy for patients with metastatic progress. For the patients with progression who were previously treated with platinum based combination chemotherapy and anti–PD-1/ PD-L1 therapy, an antibody-drug conjugate enfortumab vedontin has been approved by FDA (Alt *et al.*, 2020).

There are indications in the literature that almost all muscle-invasive bladder tumors display strong expression of Nectin-4 (Jonathan Rosenberg *et.al.*, 2020).

However, no prior immunohistochemical analysis of staining for Nectin-4 is nowadays required to initiate treatment. The reported response rate of EV in bladder cancer patients is around 40% (Rosenberg *et al.*, 2020), which is much lower than the rate of positivity for Nectin-4 described in literature (Hoffman-Censits *et al.*, 2021, Tomiyama E *et al.*, 2020). Treatment with enfortumab vedintin is challenging, mainly because of possible severe side effects. In EV- 301 study treatment-related adverse events at any grade has been seen in 93.9% of patients (Powles T *et al.*, 2021). From a onco-urologist's point of view, EV has to bind with Nectin-4 on the surface of tumor cells. Therefore, without or with low expression of Nectin-4, there cannot be any expected response. Bladder tumors should express Nectin-4 well if we want to achieve beneficial treatment results with EV without additional risk to the patient.

In the prospect of EV involvement in earlier stages of UC (neoadjuvant setting), an IHC analysis of two independent groups of patients with predominantly MIBC from cystectomy samples was performed.

In two independent groups of patients (n = 90) and (n = 83) the cytoplasmatic Nectin-4 expression was evaluated according to H-score (0–300).

In the first cohort (*discovery group)* with 90 patients, no expression could be seen in 2.25 patients (2.5%) with high expression in only 22.5 patients. Maximum expression was 300 (H-score) in 0.5% of patients. Mean expression score in BC tissue were 157 ($p < 0.002$).

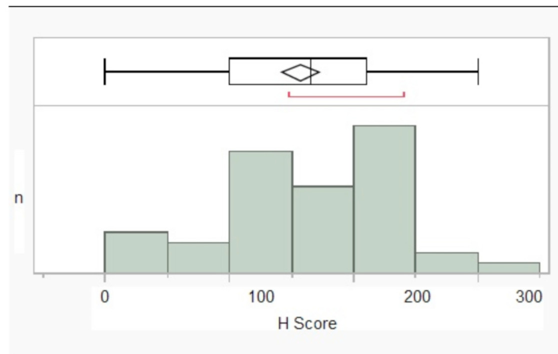


Figure 43: Distribution of Nectin-4 expression in muscle invasive bladder cancer (discovery group).

In the second cohort, no expression could be seen in 2 patients (2.5%) with high expression in only 20.75 patients. Maximum expression was 280 (H Score) in 0.5%. Mean expression score in tumor tissue were 133 ($p < 0.005$).

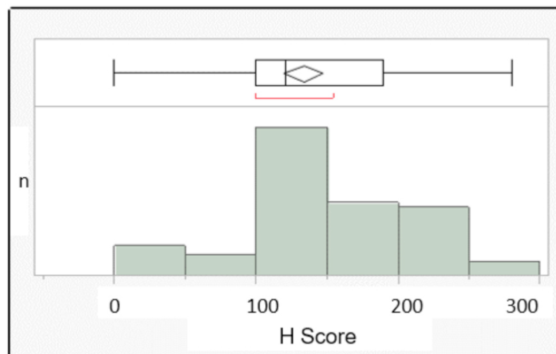


Figure 44: Distribution of Nectin-4 expression in patients with muscle invasive bladder cancer (confirmation group).

With regard to Nectin-4 expression, the two groups were comparable with mean staining score 133/157 ($p < 0.002$ / $p < 0.005$).

Furthermore, we saw trends of lower expression along progression of MIBC in the confirmation group. Comparison of different T-stages and Nectin-4 expression in tumor tissues showed assumption that depth of infiltration leads into lower expression of Nectin-4 ($p = 0.6367$).
Statistical analysis has also revealed a decrease in the mean staining value in N + disease. Hypothetically, we are inclined to believe that poorly differentiated tumors, which indirectly affect N+ disease, show poorer expression of Nectin-4. ($p = 0.9965$)
Comparison of M-Stages and Nectin-4 expression in tumor tissues according to H-score indicates decrease in the mean staining value in M + disease. (Wilcoxon/Kruskal-Wallis-Tests $p = 0,4323$).

Comparison of G-Stages and Nectin-4 expression in tumor tissues revealed a decrease in the mean staining value in G3 disease (Wilcoxon/ Kruskal-Wallis-Tests $p = 0.1181$). This was also statistically confirmed by metastasis analysis in the study by Klümper *et al.* where membranous Nectin-4 expression significantly decreased during metastatic spread (Wilcoxon matched pairs $P < 0.001$; median H-score 40; interquartile range, 0–140) with 39.4% of metastasis lacking membranous Nectin-4 expression. Absence or weak membranous Nectin-4 expression (34.0% of the cohort) was associated with a significantly shortened PFS on EV (log-rank $P < 0.001$) (Klümper *et al.*, 2023).

In our study, cancer-specific survival (CSS) revealed that patients died tumor-specifically between 40 and 69 months after surgery. The median probability of death for the patient with H-score < 220 was 40 months and for the patients with H-score > 220 was 69 months. There was no statistical significance (Wilcoxon Test $p = 0.5031$).
That can be the statistical basis for the poor efficacy of EV in metastatic cancers.

Overexpression of IL-1ß and IL-1RA (IL- 1 receptor antagonist) is frequently found in BC, with a prognostic significance observed for IL-1ß protein expression. The observed link between the IL-1ß / Nectin-4 expression may indicate possible autophagy activation processes beside

the known oncologic effects of AKT activation (a key molecular regulator) and EV effect after binding with Nectin-4 on the surface of tumor cells.

Despite Nectin-4 expression and clinical effect seen in patient after EV therapy, most patients do not achieve durable response. We are witnessing biological gap between in literature described high rate of Nectin-4 overexpression and results from our study.
The data from our study support the recommendation of EV treatment in early stages of bladder cancer, such as BCG-unresponsive NMIBC or as neoadjuvant treatment for MIBC. Immunohistochemical staining for Nectin-4 is recommended prior to the introduction of EV in metastatic bladder cancer, as this would increase the efficacy of the treatment and reduce side effects in patients with low Nectin-4 expression.

5.0 ABSTRACT

Introduction & objectives: Nectin-4, a membrane protein involved in cell adhesion, has been recently introduced as a target of the novel antibody drug conjugate Enfortumab-Vedotin (EV). However, uniform Nectin-4 overexpression in BC was reported and no predictive capacity from Nectin-4 staining was reported. In the present trial we aimed to determine expression in two independent BC cohorts and further evaluate an alternative IHC interpretation approach for Nectin-4 in advanced BC.

Materials & methods: The study included two independent cohorts consisting of n = 97 and 103 patients (70 × male, median age 65.5 years, 31 × T ≤ 2 – 43 × T3 – 23 × T4, 76 × G>2, 11 × M1 and 79 × male, 69 years, 31 × T ≤ 2 – 48 × T3 – 24 × T4, 78 × G > 2, 9 × M1, median follow-up 44.5 months, respectively) who underwent radical cystectomy for invasive BC. Tissues from histologically proven BC and from benign urothelium (n = 22 and 39) were processed to a tissue microarray and immunohistochemically stained by reported methods (polyclonal rabbit antibody, dilution 1 : 2000, incubation 16 h at 4°, quantified by the histochemical scoring system / H-score 0–300). Results were transferred into four classes: Each cohort was divided into four 25%-classes of successive increasing staining and distribution was demonstrated for each the two BC cohorts. Results were compared to clinical and pathological features.

Results: Mean expression scores in BC and parallel benign urothelium tissue were 133 and 81 / 157 and 114 ($p < 0.005$ / 0.002) with benign urothelium expression correlated with that of corresponding BC (< 0.02). The expressions within each patient cohort are lined up as follows: Expression in 25% of the cohort scored low (0–99), of 25% 100–119, of 25% 120–189 and of 25% strong (190–300) in the first cohort, the second distributed low (0–99) of 25%, 25% 100–165, 25% 166–209 and 25% strong 210–300. Furthermore, we saw trends of lower expression along progression of MIBC

Conclusion: The assumption that nectin-4 is ubiquitously overexpressed in all urothelial carcinomas, as described in the literature, cannot be confirmed in our results. It could therefore be postulated that the current use of EV without prior determination of the nectin-4 receptor status should be critically reviewed. Furthermore, the question arises to what extent nectin-4 expression could be a predictive biomarker in metastatic bladder cancer.

ZUSAMMENFASSUNG

Einleitung und Ziele: Nectin-4, ein Membranprotein, das an der Zelladhäsion beteiligt ist, wurde kürzlich als Ziel des neuen Antikörper-Wirkstoff-Konjugats Enfortumab-Vedotin (EV) vorgestellt. Es wurde über eine einheitliche Überexpression von Nectin-4 bei Blasenkarzinom (BCa) aber keine Vorhersagekraft einer Nectin-4-Färbung berichtet. In der vorliegenden Studie wollten wir die Expression in zwei unabhängigen BCa Kohorten bestimmen und einen alternativen IHC-Interpretationsansatz für Nectin-4 bei fortgeschrittenem BK weiter evaluieren.

Materialien und Methoden: Die Studie umfasste zwei unabhängige Kohorten mit n = 97 bzw. 103 Patienten (70 Männer, medianes Alter 65,5 Jahre, 31 Pat. T ≤ 2 – 43 Pat. T3, 23 Pat. T4, 76 × G > 2, 11 Pat. M1 und 79 Männer, 69 Jahre: 31 Pat. T ≤ 2 – 48 Pat. T3 – 24 Pat. T4, 78 Pat. G > 2, 9 × M1, medianer Beobachtungszeitraum 44,5 Monate), die sich einer radikalen Zystektomie bei invasivem BCa unterzogen. Gewebe von histologisch nachgewiesenem BCa und von gutartigem Urothel (n = 22 und 39) wurden zu einem Gewebe-Microarray verarbeitet und nach den bekannten Methoden immunhistochemisch angefärbt (polyklonaler Kaninchen-Antikörper, Verdünnung 1 : 2000, Inkubation 16h bei 4°, Quantifizierung nach dem histochemischen Scoring-System / H-Score 0–300). Die Ergebnisse wurden in vier Klassen eingeteilt: Jede Kohorte wurde in vier 25%-Klassen mit sukzessive-zunehmender Färbung unterteilt, und die Verteilung wurde für jede der beiden BCa-Kohorten bestimmt. Die Ergebnisse wurden mit klinischen und pathologischen Merkmalen verglichen.

Ergebnisse: Die mittleren Expressionswerte in BCa Proben und parallelem gutartigem Urothelgewebe betrugen 133 und 81 bzw. 157 und 114 (p < 0,005 / 0,002), wobei die Expression des gutartigen Urothels mit der des entsprechenden BK korrelierte (< 0,02). Die Ausprägungen innerhalb jeder Patientenkohorte sind wie folgt angeordnet: In der ersten Kohorte war die Expression bei 25% der Patienten niedrig (0–99), bei 25% 100–119, bei 25% 120–189 und bei 25% stark (190–300), in der zweiten bei 25% niedrig (0–99), bei 25% 100–165, bei 25% 166–209 und bei 25% stark 210–300. Außerdem sahen wir einen Trend zu einer geringeren Expression im Verlauf des muskelinvasiven BCa.

Schlussfolgerung: Die Annahme, dass Nectin-4 bei allen Urothelkarzinomen wie in der Literatur beschrieben ubiquitär überexprimiert ist, lässt sich in unseren Ergebnissen nicht bestätigen. Man könnte daher postulieren, dass die derzeitige Verwendung von EV ohne vorherige Bestimmung des Nectin-4-Rezeptorstatus kritisch überprüft werden sollte. Weiters erhebt sich die Frage inwieweit die Nectin-4-Expression ein prädiktiver Biomarker bei metastasiertem Blasenkrebs sein könnte.

6.0 ABBREVIATIONS

Ab	Antibody
ADC	Antibody drug conjugates
Ag	Antigen
ASCO	American Society of Medical Oncology
BC	Bladder cancer
BrC	Breast cancer
BCG	Bacillus Calmette-Guérin
BK	Blasenkrebs
BSC	Best supportive care
BTA	Bladder tumor antigen
CI	Confidence interval
CIS	Carcinoma in situ
CR	Complete response rate
CSS	Cancer specific survival
CT	Computed tomography
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
EAU	European Association of Urology
E-cig.	Electronic cigarettes
EV	Enfortumab Vedontin
FDA	Federal drug agency
FGFR-3	Fibroblast growth factor receptor 3
GC	Gemcitabine/ cisplatin
Hexvix	Hexaminolevulinate
HG	High grade
HPV	Human papillomavirus
HR	Hazard ratio
HRAS	Harvey Rat sarcoma virus
IHC	Immunohistochemical analysis
IL 1 β receptor	Interleukin 1 b receptor
IL1RA	Interleukin 1 receptor antagonist
IRS1	Insulin receptor substrate 1
LG	Low grade
LVI	Lymphovascular invasion
MIBC	Muscle invasive bladder cancer

MMAE	Monomethyl auristatin E
MMC	Mitomycin -C
MRI	Multi-parametric magnetic resonance imaging
mUC	Metastatic urothelial cancer
MVAC	Methotrexate, vinblastine, Adriamycin, cisplatin
NAC	Neoadjuvant chemotherapy
NMIBC	Non-muscle invasive bladder cancer
NMP 22	Nuclear matrix protein 22
OC	Ovarian cancer
OOR	Overall response rate
OOS	Overall survival
PC	Pancreatic cancer
PCR	Polymerase chain reaction
PD -1	Programmed cell death protein 1
PD-L1 inhibitor	Programmed cell death ligand 1 inhibitor
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PR	Partial response rate
PUNLMP	Papillary urothelial neoplasm of low malignant potential
PVRL	Poliovirus receptor-related protein
Q-Q plot	Quantile-quantile plot
R status	Resection status
RARC	Robotic-assisted laparoscopic cystectomy
RC	Radical cystectomy
RNA	Ribonucleic acid
SCC	Squamous cell carcinoma
SD	Standard deviation
SSTR	Somatostatin receptors
TM	Transmembrane
TMA	Tissue microarray
TNM	Tumor, node, metastasis
TP 21	Tumor suppressor protein 21
TP 27	Tumor suppressor protein 27
TP 53	Tumor suppressor protein 53
TTE	Time to event
TURBT	Transurethral Resection of Bladder Tumor
UC	Urothelial cancer

UK	Urothelial Karzinom
UTUC	Upper tract urothelial carcinomas
VEGF	Vascular endothelial growth factor
VI-RADS	Vesical Imaging-Reporting and Data System

7.0 DECLARATION OF OWN CONTRIBUTION

I, Sebastian Jeršinovič, hereby declare that I have written this thesis independently. I have not used any other resources than those indicated and have marked all passages taken over verbatim or in terms of content as such.

The idea for the conception of this study originated in the Department of Urology of the Urological Clinic of the University of Tübingen in collaboration with Prof. Dr. med. Dr. h.c. Arnulf Stenzl, the former Medical Director of the Urological Clinic of the University of Tübingen, Prof. Dr. Steffen Rausch, my supervising doctoral advisor from the University Clinic of Urology Tübingen and Dipl.-Biol. Jörg Hennenlotter, the scientific supervisor of this work.

This work made use of an existing TMA block and database. The creation of the collective and the basic database, as well as the preparation of the TMA were done by Mrs. Katharina Teepe and Dr. med. Thomas Lüttfrenk.

All other data were collected by me personally. The statistical evaluation and interpretation of all data was done by me after guidance by Dipl.-Biol. Jörg Hennenlotter.

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