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Immunohistochemical studies on the biological and clinical significance of Interleukin-1-Beta / Interleukin- 1 Receptor-Antagonist axis in invasive bladder cancer

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1 Introduction

1.1 Epidemiology

Bladder cancer (BCa) is one of the most commonly diagnosed cancers in the male population and this continues to be relevant even when both genders are considered. In the European Union, the age-standardized incidence rate is 20 in men and 46 in women (1). The lifetime risk of developing BCa is 1.1% in men and 0.27% in women (2). Men are diagnosed with BCa three to four times more frequently than women, traditionally attributed to exposures and lifestyle. Prostatic enlargement with urinary stasis and retaining urine-containing carcinogens might be additional factor (3, 4). Two different entities of BCa are to be considered: low-grade (LG) and high-grade (HG) tumors. The latter group also includes carcinoma in situ (CIS). Nevertheless, tumors confined to the mucosa (Ta) are superficial and LG, while those invading subepithelial tissue (T1) or muscle (T2) are usually HG tumors. This is the main reason for different treatment and follow-up approaches between these two entities, according to numerous epidemiologic studies (5). Almost 75% of BCa patients present with Ta or T1 disease. Among this population, LG tumors have high incidence of tumor recurrence, but low probability of tumor progression. On the other hand, HG tumors have high propensity for disease progression, especially if categorized as muscle invasive disease (MIBC) (1, 6).

1.2 Aetiology

1.2.1 Main risk factors

Smoking has been identified as the most significant risk factor for BCa. The aromatic amines and hydrocarbons within the tobacco smoke, which undergo renal excretion, are linked to the development of BCa. The risk of BCa increases with lifetime smoking duration and intensity (1, 7). Although smoking cessation has shown to improve the survival rate of lung cancer patients, there is no strong evidence in reduction of mortality rate for patients with BCa. These observations suggest potential genetic variations, epigenetic alterations, or immune responses as distinguishing features comparatively to other cancers (8, 9). A recent study identified genetic polymorphisms related to aggressive BCa (10, 11). Moreover, early onset of disease implies a hereditary pattern of BCa genesis (12). This is important to emphasize since BCa is one of the most expensive cancers to treat, associated with significant worldwide economic burden (13, 14). The overall high-mutation rates of MIBC are quite similar to those found in melanoma or non-small cell lung cancer. Most BCa mutations are clonal, suggesting that mutagenic activity occurs early in cancer development (10). However, genetic screening for this type of cancer has not yet been suggested as a form of preventative care. Occupational exposure to aromatic amines and polycyclic hydrocarbons is the second most significant risk factor, accounting for about 10% of all cases (1).

1.2.2 Environmental factors

The association between metabolic factors and drinking habits remain uncertain. However, exposure to pelvic ionizing radiations, especially in form of external beam radiotherapy (e.g. localized prostate cancer therapy) might increase the risk of developing a secondary cancer - primary BCa (1, 15).

1.3 Pathophysiology and molecular biology

Urothelial carcinoma includes tumors of the bladder, upper urinary tract, and proximal urethra (2). Histologically, 75% of cancers represent pure urothelial carcinoma, while 25% show variant histology. Genetic analysis of BCa revealed instability of MIBC, unlike non-muscle invasive bladder cancers (NMIBC). In this regard, two different carcinogenic pathways have been described – papillary and non-papillary (12, 16). The first one is characteristic mainly for NMIBC, with fibroblast growth factor 3 (FGFR3) – activating mutations as the landmark of majority Ta tumors. Nevertheless, a mutation of this type of gene is very rare in MIBC, with a frequency of less than 10%. Researchers speculate that the papillary carcinogenic pathway has a better prognosis and lower mortality rate (12). Molecular categorization, mainly based on mRNA expression profiles, defines several molecular subtypes (luminal, basal/squamous, neuronal) that dictate clinical behavior, such as response to chemotherapy or immunotherapy (2, 10) (Figure 1). The genomic profile of LG NMIBC is defined by well-known mutation patterns, with predominantly a non-papillary pathway. On the other hand, the most common suppressor gene mutations in MIBC are the transformation-related protein 53 (TP53), retinoblastoma 1 (RB1), human epidermal growth factor receptor 2 (ERBB2) and phosphatase and tensin homolog (PTEN) (17-

19). Moreover, PTEN mutation is associated with a poor disease profile and an early progression to metastatic disease, especially if a dual mutation with TP53 is present (20, 21)

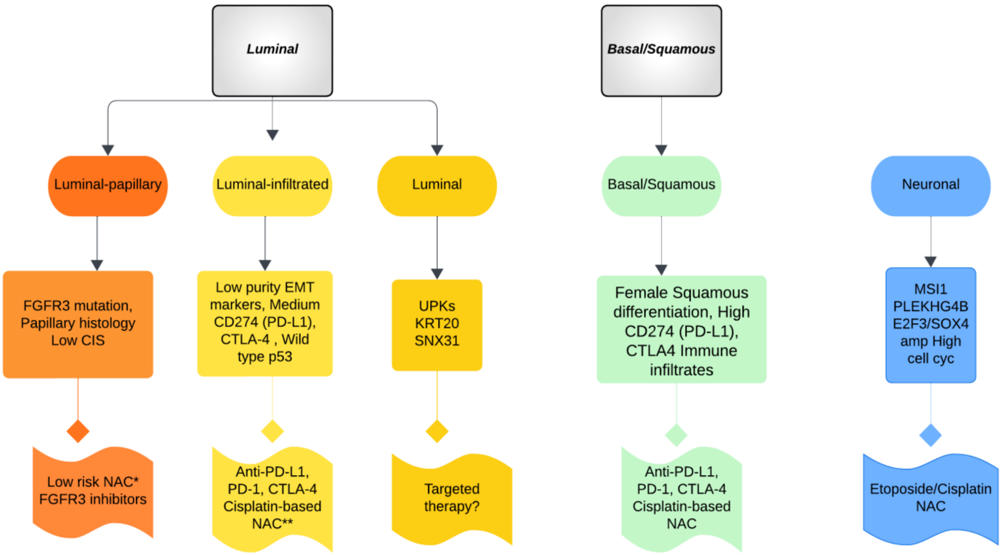


Figure 1. Proposed schema of molecular categorization of bladder cancer. Adapted from Robertson AG, et al. 2017 (10).

1.4 Tumor staging and grading

1.4.1 Staging and classification system

To be able to fully create a risk assessment for a bladder cancer, it is essential to be able to determine whether or not the disease is organ confined, metastatic, or if concurrent upper-tract tumors are present. As such, all new cancers require a degree of radiological staging. The **T**umor, **N**ode, **M**etastases (TNM) classification is the standard staging system, used to address the extent of BCa involvement and degree of progression. Tumors confined to the mucosa or invading lamina propria are classified as Ta and T1, respectively, while intra-epithelial, HG tumors confined to the mucosa are classified as CIS (1). Moreover, tumors invading musclurais propria (T2) or beyond (T3-T4) have been classified as MIBC. In addition, metastatic disease is classified according to involvement of the (non)-regional lymph nodes or remote sites.

Ideally, in order to stage patients adequately, the histopathologic and radiographic TNM classifications are used conjunctively. Preoperative staging is based on the imaging assessment and histopathology from the transurethral resection of bladder tumor (TURBT) (22). The latest TNM classification is presented in Table 1.

T- primary tumor
Tx- Primary tumor cannot be assessed
T0- Primary tumor not detected
Ta - Non-invasive papillary tumor
T1- Tumor invades sub-epithelial connective tissue
T2 - Tumor invades muscles
T3 - Tumor invades perivesical tissue
T3a – Microscopically
T3b - Macroscopically (extravesical mass)
T4 - Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, or abdominal wall
T4a - Tumor invades prostate stroma, seminal vesicles, uterus, or vagina
T4b - Tumor 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 (hypogastric, obturator, external iliac, or presacral)
N2 - Metastasis in a multiple regional lymph node in the true pelvis (hypogastric, obturator, external iliac, or presacral)
N3 - Metastasis in common iliac lymph node(s)
M - distant metastasis
M0 - No distant metastasis
M1a - Non-regional lymph nodes
M1b - Other distant metastases

Table 1. 2017 TNM classification of BCa. Adapted from Witjes et al., 2021 (1).

1.4.2 Lymphovascular invasion

The presence of vascular invasion in cystectomy specimens is an independent prognostic factor for cancer free survival, while lymphatic invasion alone has no significant influence, in multivariate analysis (23, 24). However, in histological specimens obtained after transurethral resection (TURB), there is an increased risk of pathologic upstaging, followed by worse disease outcome (25, 26).

1.4.3 Histological grading

There are currently two available grading systems for classification of NMIBC. The World Health Organisation (WHO) published a histological grading system for urothelial cancer in 1973, which distinguished between grade 1 (G1), grade 2 (G2), and grade 3 (G3) categories (27). The updated version, initially published twenty years ago, included papillary urothelial neoplasm of low-malignant potential (PUNLMP), LG, and HG tumors. The system has been updated further in both 2016 and again in 2022, with a significant shift of patients between the two – increase in the number of HG patients (WHO, 2004) due to inclusion of a subset of G2 patients with a more favorable prognosis in comparison to the G3 category (WHO, 1973) (1, 28, 29). Conversely, MIBC are always considered HG urothelial tumors, which explains why no further prognostic information can be provided by grading of these tumors. However, identification of morphological subtypes is a significant prognostic tool in MIBC and may substantially influence the treatment protocol (30, 31).

The following subtypes of BCa are currently used (1):

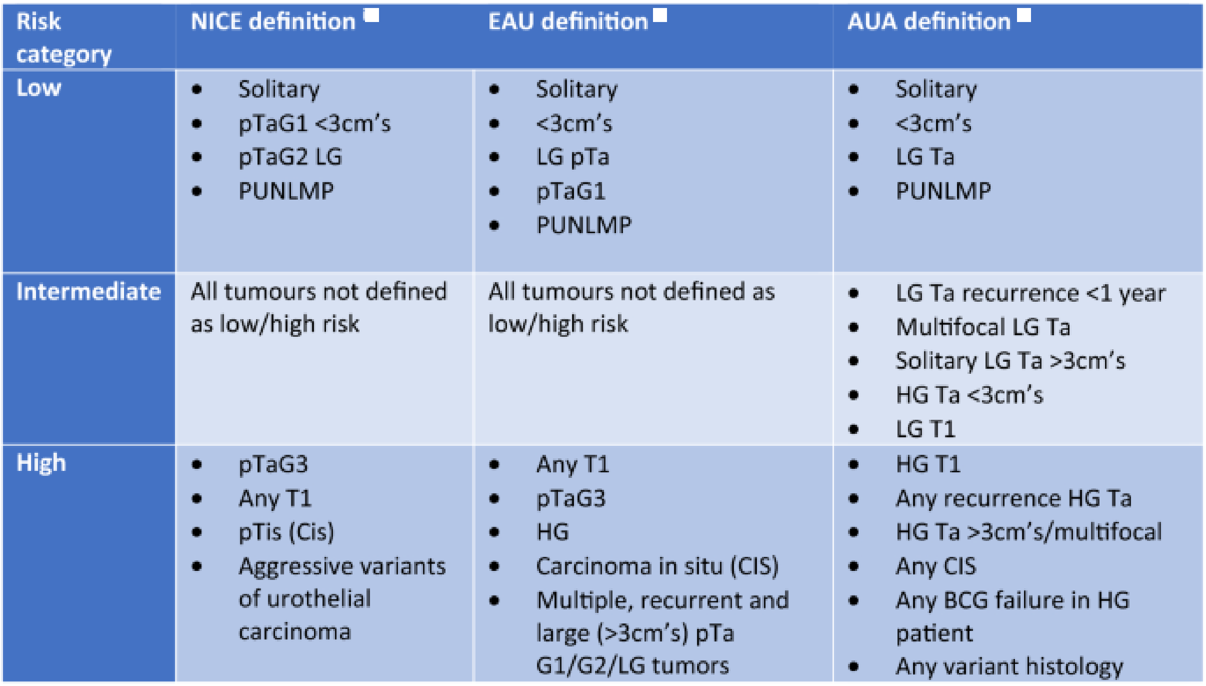
- Urothelial carcinoma (≥ 90% of cases);
- Urothelial carcinomas with partial squamous and/or glandular or divergent differentiation;
- Micropapillary urothelial carcinoma;
- Nested/microcystic variant;
- Large nested variant;
- Microtubular urothelial carcinoma;
- Plasmacytoid, signet ring;
- Lymphoepithelioma-like;
- Sarcomatous urothelial cancer;

- Urothelial carcinomas with neuroendocrine differentiation.

  According to new WHO 2022 recommendations, all BCa subtypes are considered HG (32). The percentage of subtype in the specimen must be reported since, as mentioned above, it has been shown to be of prognostic value (33).

1.4.4 Risk groups of NMIBC

Non-muscle invasive BCa represents a very heterogeneous disease with vastly different levels of risk at each end of the spectrum. It is important to note that NMIBC encompass, clinically and biologically, incredibly distinct tumors. Therefore, the most accepted method is to divide the disease into low-, intermediate-, and high-risk. This definition defines the diagnostic and treatment algorithm, and improve disease outcome (12). The EAU, AUA, and NICE guidelines broadly agree to place NMIBC patients into three groups, based upon their risk of recurrence and progression. Among these, the majority (60-70%) are staged as pTa, followed by pT1 (20-30%), and CIS lesions (up to 10%) (12, 34).



Risk category	NICE definition	EAU definition	AUA definition
Low	• Solitary • pTaG1 <3cm's • pTaG2 LG • PUNLMP	• Solitary • <3cm's • LG pTa • pTaG1 • PUNLMP	• Solitary • <3cm's • LG Ta • PUNLMP
Intermediate	All tumours not defined as low/high risk	All tumours not defined as low/high risk	• LG Ta recurrence <1 year • Multifocal LG Ta • Solitary LG Ta >3cm's • HG Ta <3cm's • LG T1
High	• pTaG3 • Any T1 • pTis (Cis) • Aggressive variants of urothelial carcinoma	• Any T1 • pTaG3 • HG • Carcinoma in situ (CIS) • Multiple, recurrent and large (>3cm's) pTa G1/G2/LG tumors	• HG T1 • Any recurrence HG Ta • HG Ta >3cm's/multifocal • Any CIS • Any BCG failure in HG patient • Any variant histology

Table 2. Risk stratification of NMIBC - Comparison of EAU, AUA, and NICE guidelines. Courtesy of Jaffer A. et. al., 2022 (35): iv- NICE-National Institute of Cancer Examination; EAU- European Association of Urology; AUA- American Urological Association.

1.4.5 Muscle-invasive bladder cancer (MIBC)

Approximately 20-30% of patients with BCa present with muscle invasive disease (T2a-T4b) (36). The diagnosis of MIBC is confirmed after TURB and the pathological report. Complete staging is achieved with the addition of radiological imaging in the form of computer tomography (CT) imaging of the chest, abdomen, and pelvis (1). All patients with MIBC are considered high-risk with the hazard of occult nodal disease ranging between 18% and 45% (22). Despite providing excellent local control, surgery alone only provides a five year survival of around 50% when combining all stages of disease, except in case of metastatic disease (37, 38). Since only a minority of patients with metastatic BCa show long-term response to palliative treatments, neoadjuvant cisplatin-based chemotherapy (NAC) has become the standard of care for patients in this setting with adequate performance status and renal function; Level 1 evidence demonstrated a five percent survival improvement over five years (39, 40). However, the small number of patients showing a positive treatment response (30-40%) has led to several trials investigating the effectiveness of immunotherapy (ICI) in neoadjuvant setting. Unfortunately, there is currently no strong proven evidence of ICI advantage. Selecting the patients in regard to the likelihood of ICI or chemotherapy response might help improve patient outcomes through avoiding overtreatment in non-responders (12). Prognostic biomarkers should be able to improve our understanding of complex tumor biology and to enhance early diagnosis of aggressive BCa subtypes, thus preventing early progression and cancer-specific death.

1.5 Diagnostic evaluation

1.5.1 Presentation and diagnosis

The evaluation of patients with haematuria should involve a medical history, physical examination, imaging, and cystoscopy. However, use of molecular marker tests are rising in popularity, driven by the low sensitivity of urinary cytology (41). The most common presentation of BCa is visible (gross) haematuria, but sometimes isolated microscopic haematuria or irritative voiding symptoms, with a tumor incidentally discovered on imaging, may be an initial presentation pathway (2). The patient history should identify the timing of gross haematuria, number of episodes, and any antibiotics/cultures obtained. Frequent haematuria treated with antibiotics and a negative culture are concerning for cancer (22). Evaluation of macrohematuria involves cystoscopy and imaging of the upper urinary tract, with CT urography. A guideline-recommended work-up is presented in Table 1 (1, 2).

Definition	**Work-up**
Asymptomatic microscopic hematuria	
≥3 RBCs per HPF on properly collected urinalysis In the absence of benign cause: infection, menstruation, vigorous exercise, medical renal disease, trauma, recent urologic procedure.	• Low risk: shared decision-making between repeat urinalysis in 6 mo. or cystoscopy and renal ultrasound • Initially low risk with haematuria on repeat urinalysis: reclassify as intermediate and pursue workup accordingly • Intermediate risk: cystoscopy and renal ultrasound • High risk: cystoscopy and axial upper tract imaging: CT urogram preferred, MRI urogram if contraindication to CT • If negative work-up, repeat urinalysis within 12 months, if negative – discontinue further evaluation if positive, initiate shared decision making regarding additional evaluation
Gross hematuria	
Visible blood in the urine Rule out similar benign causes	• Cystoscopy for bladder evaluation • Axial upper tract imaging: CT urogram • CT urogram is preferred; alternative - MRI urogram or renal ultrasound if contraindication to both CT and MRI

Table 3. Diagnostic evaluation for bladder cancer. Adapted from Lenis AT, et al., 2020 (2).

1.5.2 Cystoscopy and enhanced diagnostics

The diagnosis of BCa primarily depends on cystoscopic bladder examination and histological evaluation of sampled tissue (1). Cystoscopy is an office-based procedure performed with flexible camera, approximately five millimeters in diameter, inserted through the urethra using topical intraurethral anaesthetic lubricant. Enhanced cystoscopy with narrow band imaging or blue light cystoscopy (BLC) improves the sensitivity and specificity for identifying tumors (2). Photodynamic diagnosis (PDD) of bladder cancer is dependent upon fluorescent signal from neoplastic cells after intravesical instillation of fluorophore, detected by a blue light-emitting cystoscope. The fluorophore in current use for PDD is a substrate incorporated in the heme biosynthesis pathway (42). Many studies have suggested that the addition of fluorescence-assisted blue light cystoscopy to standard, white light cystoscopy leads to better visualization of bladder tumors at the time of TURBT (42-45). Both, the American and European Urological Association support the use of BLC in order to increase detection rate and to decrease disease recurrence (46, 47).

Narrow-band imaging (Olympus®) is another enhanced diagnostic tool, which relies on filtering out red light from white light, resulting in green and blue bands with different tissue penetration depths, resulting in enhancement of mucosal and submucosal vasculature (42, 48). Similar to PDD, a number of studies have determined superiority of narrow-band imaging over white light cystoscopy, in terms of detection of cancerous lesions, with greatest utility in detection of CIS. Finally, enhanced endoscopic imaging platform has been developed by Karl Storz (Professional Image Enhancement System - IMAGE 1 S®), which does not require special equipment or intravesical administration of fluorophore. This endoscopic system utilizes conventional white light endoscopy and creates digitally contrasted images with four unique software-based visualisation modes (Spectra A, B, Clara, Clara Chroma modality).

1.5.3 Urinary cytology

Urine cytology is an additional diagnostic tool for detection of HG urothelial carcinoma. It is based on examination of voided urine or bladder-washing specimens for exfoliated cancer cells. Although technological improvements have been introduced to the process, urinary cytology has so far been limited to a mean sensitivity of 50% (49). The sensitivity of voided urine cytology for detection of urothelial neoplasm is higher for HG and G3 tumors (84%), but low (16%) in LG and G1 tumors (50). The sensitivity in CIS detection is 28-100% (51).

Cytology is a regular part of the diagnostic algorithm for patients with suspected HG urothelial cancer, particularly as an adjunct to cystoscopy. However, it is not a proper tool for detection of LG tumors, unless complemented by cystoscopy (52). Although the detection of LG tumor requires ancillary studies, the chance of clinical progression of such a lesion is low. For HG carcinomas and CIS, which is difficult to detect through cystoscopy, cytology provides a high degree of diagnostic accuracy (52).

Cytological interpretation is user-dependent and evaluation can be influenced by urinary tract infections, stones, or intravesical instillations (53, 54).

Figure 2: Urinary cytology for high-grade urothelial cancer.

1.5.4 Imaging modalities

Ultrasound (US) is usually performed as adjunct to physical examination and represents first step in imaging evaluation. It has moderate sensitivity to wide range of abnormalities in both, upper and lower urinary tract. Although it detects intraluminal masses within the bladder, as well as kidney masses and hydronephrosis, it is not sensitive enough to determine the true source of haematuria (55, 56). Moreover, since US cannot reliably exclude the presence of upper tract urothelial cancer, CT remains an irreplaceable tool in BCa diagnostics and staging. Filling defect and hydronephrosis are main radiological signs found on CT urography, but the necessity of performing CT once the bladder tumor has been detected is questionable (47). The incidence of upper tract urothelial cancers is low (1.8%), but increases significantly in tumors located in the trigone; Therefore, CT urography seems to be reasonable in HG tumors and in tumors located in the trigone (47, 57). The role of multiparametric magnetic resonance imaging is still questionable in BCa diagnosis and staging, although, recent publication reports strong predictive staging potential of MRI-VIRADS (Vesical Imaging-Reporting and Data System) (58). However, it is important to emphasize that CIS cannot be diagnosed with any of the available imaging methods alone (47). Pre-treatment staging of MIBC is well established, with progressive imaging techniques gaining popularity. Although positron emission tomography scan (PET/ CT) is currently not recommended as routine staging, it has proven prognostic value in MIBC. In fact, many medical centers recommend 18F-fuorodeoxyglucose - FDG PET/CT as preferable imaging tool in detecting more malignant diseases. The reason behind this lies in the 20-40% higher detection rate when compared to conventional imaging (CT/MRI) (22).

1.5.5 Molecular biomarkers

Biomarkers have utility throughout the spectrum of disease in urothelial cancer, from aiding diagnosis to guiding initial therapy selection or definitive surgical management, radiotherapy, or other systemic therapy.

1.5.5.1 Prognostic urinary biomarkers

Numerous urinary markers have been developed in order to overcome limitations of cytology and imaging diagnostic. UroVysion (FISH), Nuclear Matrix Protein (NMP) 22R, Fibroblast Growth Factor Receptor (FGFR) 3/Telomerase Reverse Transcriptase

(TERT) and microsatellite analysis might help in detection in those patients with NMIBC who also present with negative cystoscopy and urinary cytology, in that they are more likely to develop disease recurrence and possibly progression (59-61). However, the prognostic value of urinary markers in MIBC is still debatable and currently no urine-based biomarkers exist that reliably predict therapeutic response in this patient group. The most promising of the abovementioned diagnostic tools is FGFR3, whose high mutation burden might identify patients with favorable disease at the time of cystectomy. This is supported by the fact that the luminal BCa subtype usually carries FGFR3 mutations and expresses papillary morphology, which altogether signifies less aggressive cancer behavior. This could be another valuable tool in guiding adjuvant treatment and follow-up strategies after RC, besides tumor stage (pT), nodal status (pN), or lymphovascular invasion (LVI) (62). Additionally, previous results from UroSEEK gene panel revealed alterations in 11 mutated genes, which may be possible treatment targets (63). Eich, et al. (63) discovered that more than 90% of bladder tumors were found positive for at least one genetic alteration within the panel, with FGFR3 mutations occurring more often in LG, non-invasive BCa, while the opposite was true for p53 (64). This data implies the importance of continuing the quest for appropriate biomarkers in urine, tissue, and blood in order to improve early detection or guide adjuvant treatment of this deadly disease.

1.5.5.2 Prognostic tissue biomarkers

There is growing evidence showing that BCa comprises a heterogeneous group of diseases beyond conventional histopathology. Elaborative immunohistochemistry (IHC) investigations have aimed to determine the expression of specific cancer-associated molecules, thus gauging prognostic significance of selected histopathologic characteristics (65). The most widely used prognostic, tissue-based molecular markers for NMIBC are p53 and Ki-67, both potent cell cycle regulators (66, 67). Ki-67 is a DNA-binding nuclear protein that is expressed throughout the cell cycle in proliferating cells (68). This biomarker is a powerful predictor of tumor development and several studies have proven its predictive potential in terms of both tumor recurrence and progression, as well as response on intravesical therapy (69-71). Additionally, Ki-67 has been recognized as a positive predictor of cancer specific mortality (CSS) in patients with pT1 at the time of RC (72). Its expression is measured on immunohistochemistry (IHC), through measuring IHC intensity and obtaining final H-scores, similar to several biomarkers, including those from IL-1 family. The advantage of IHC is accessibility, low cost, and promising results, even when subtyping BCa in routine clinical practice. On the contrary, some reports claim that Ki-67, as well as p53, are only associated with adverse prognostic features without benefit in terms of guiding adjuvant treatment and follow-up strategies after RC (62). This controversy, as well as many others, require additional research. On the same token, other molecular biomarkers, which have demonstrated to be predictive of intravesical therapy response, have recently been introduced to the literature, including cell cycle regulators, apoptosis inhibitors, cell adhesion molecules, and proliferative markers (73).

In MIBC, the main role of tissue biomarkers is to predict cancer behavior and response to perioperative chemotherapy. Over-expression of several molecular markers such as changes in vascular endothelial growth factor (VEGF), human epidermal growth factor receptor (EGFR), or RNA expression affecting the PI3K/Akt/mTOR pathway are common in invasive BCa, which have led to a thorough investigation of these markers as diagnostic tools (22, 74, 75). According to recent data, four molecular subtypes of BC have been identified, with most prominently basal and luminal forms (1). The basal group shows strong expression of EGFR3 and chemosensitivity to cisplatin-based chemotherapy. Conversely, the luminal type seems to show over-expression of other growth factor receptors (ERBB2 or ERBB3) and is resistant to chemotherapy (31, 76). Moreover, several well-designed studies determined significant mutation burden in genes for ERCC2 among patients with good response to chemotherapy (77-79). The positive predictive value of ERRC2 in T2N0 BCa has been reported to be as high as 89% (79). Among patients with luminal type MIBC, p53 gene expression leads to chemoresistance (80). The role of biomarkers as predictors for response to neoadjuvant immunotherapy have also been assessed and again, basal group tumors showed better response. Significance of positive program-cell death ligand (PD-L1) expression in improving prediction rate is inconsistent among studies (81, 82), but still, this marker is the most commonly used when deciding upon immunotherapy in advanced BCa. Moreover, it seems that autophagy, a dynamic process that prevents the transformation of normal cells into tumors, promotes tumorigenesis and gemcitabine resistance in BCa by activating Protein-kinase B (AKT) (83). The hyperactivation of this marker has already been linked to BCa metastases, the major cause of death in BCa patients (84). Since no single agent has been identified as the ideal predictor of cancer behavior and response to immuno- or chemotherapy, the complexity of the tumor-immune interaction and utility of predictive biomarker panels compared with single markers alone should be highlighted.

Several new markers have been investigated within the last years, with interleukins suggested to be the most promising. Interleukin 1-alfa (IL-1α) and interleukin 1-beta (IL-1β) bind to the type 1 IL-1 receptor and have similar biological activity (85). However, these are encoded by two different genes, with a low degree of sequence homology. Several trials have been investigating the possible clinical implications of various markers; Human competitive IL-1 inhibitor, Anakinra, decreased proliferative rate of tumors refractory to standard therapies (86, 87). With the addition of the chemotherapy-associated role of IL-1β (88), the importance of the IL-1β/IL-1RA axis in future research of anti-cancer strategies might be significant. In contrast, Bevacizumab (a monoclonal antibody targeting VEGF-A) in combination with neoadjuvant chemotherapy, in patients undergoing radical cystectomy, was unable to improve OS in examined patients (22, 89). A phase II trial (TUXEDO) of cetuximab (monoclonal antibody against EGFR) in combination with concurrent chemoradiation therapy with either mitomycin C and 5-FU or cisplatin in MIBC is currently underway and the first results thereof are expected soon. Mammalian target of rapamycin (mTOR), cell proliferation regulator, has to date not found clinical trial implementation. In perspective, results of these studies should allow better understanding of the immunological concept behind BCa. The goal is to identify which tumors benefit from immunological support, or rather, which tumors progress under immunostimulation.

1.6 Treatment

1.6.1 Transurethral resection of bladder tumor (TURBT)

The goals of TURBT in NMIBC is to establish accurate pathological diagnosis/staging and completely remove all visible lesions. It is a crucial procedure in the management of BCa, both diagnostically and therapeutically, whose quality has been shown to have a significant impact on the outcome of patients with BC (90). The most common indication for performing a TURBT is the presence of a suspicious lesion or papillary tumor during cystoscopy. Positive cytology in the absence of any suspicious finding in cystoscopy is another indication. In these circumstances, quadrant biopsies by cold-cup biopsies or loop resections should be performed (90).

After TURBT, as significant risk exists for the presence of a residual tumor, especially in patients with T1 tumors or TaG3 tumors (91). Approximately 30-35% of patients with T1 tumors in initial resection will eventually be found to have muscle-invasive disease. Therefore, a second resection is recommended in each patient with pT1 tumor or HG tumors after first resection or patients with incomplete initial resection who are not planned for immediate cystectomy (92). The significance of second resection seems to be particularly emphasized in patients without muscle tissue in the initial resection (93). The second resection should be performed within two to six weeks after the initial resection (1, 90). The most common complications of TURBT include post-operative bleeding, bladder perforation, urinary tract infection, and hydronephrosis.

1.6.1.1 Special tumor locations during TURBT

Difficulties during TURBT may be encountered depending on tumor location. For example, if the tumor is located near the orifice, the surgeon must avoid coagulation current in order to prevent hydronephrosis. Patients developing symptoms due to ureteral obstruction can be treated by subsequent stent insertion. Alternatively, if the tumor is located at the bladder dome, the tumor-resectoscope distance can be reduced by emptying the bladder in order to facilitate resection. The close proximity to the peritoneum and bowel should garner particular attention not to perforate the bladder during resection (90). If the tumor is located in bladder diverticula, it is difficult to resect. In contrast to a physiological bladder wall, diverticula usually do not contain a muscularis propria layer. Therefore, the risk of perforation during TURBT is increased and the pathologic evaluation of tumors resected from bladder diverticula might be challenging as well. Since pathologic staging might be inaccurate in these tumors, imaging using cross-sectional techniques is important. In patients with infiltrating tumors, a complete diverticulectomy or even radical cystectomy may provide better oncologic outcome compared to a TURBT (90, 94).

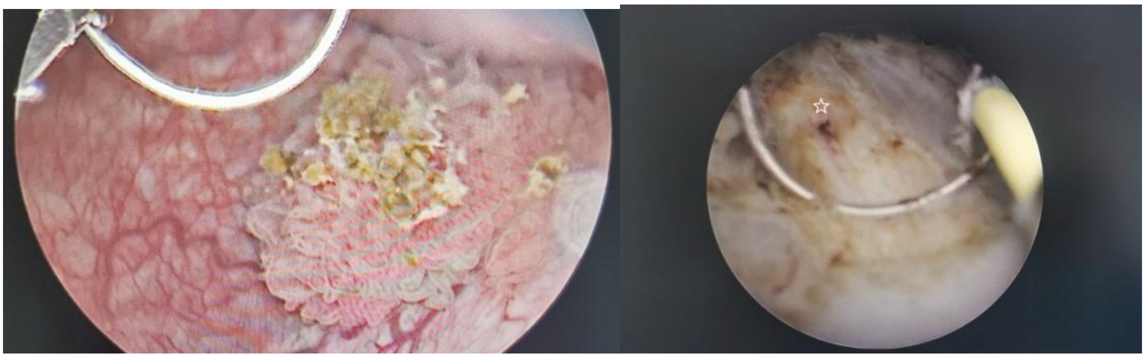


Figure 3. Tumor near the orifice - asterisk depicts right orifice after tumor resection. Courtesy of Dr. Kavaric P., Urology Clinic, Clinical Center of Montenegro.

1.6.2 Perioperative intravesical therapy

Immediate, single intravesical instillation of chemotherapy, within 24 hours after TURBT has been the standard of care in low- and select intermediate-risk BCa patients (47). Mitomycin C (MMC) or epirubicin are the most common chemotherapy drugs used for this purpose, but pirarubicin as well as gemcitabine, have also shown to lower the intravesical recurrence rate (95, 96). Given these assumptions, timely application of chemotherapy following TURBT and sufficient indwell-time of an adequate dose need to be achieved (97) (Table 4). Also, limited impact of single, immediate instillation has to be suspected in case of greater load of tumor cells or inherent propensity to develop novel recurrence, i.e. in multiple, large, and poorly differentiated tumors. The need for further adjuvant intravesical therapy depends on prognosis. In low-risk groups, single instillation is a definite treatment and only regular follow-up is indicated. While in intermediate groups, repeat instillations (with or without previous adjuvant instillation) improve patients' survival (47).

A Practical Guide on Single Immediate Instillation of Chemotherapy
Consider single immediate chemotherapy in patients with a tumor number of a maximum of 7, a tumor size with maximum of 3 cm, and a number of prior tumor recurrences per year with a maximum of 1.
Assure lack of perforation and lack of propensity of haemorrhage following TURBT, such as insufficient coagulation or persistent haemorrhage despite sufficient coagulation.
Use any chemotherapy agent you are familiar with for intravesical instillation; mitomycin C and epirubicin have been reported most commonly.
A common schedule is 40 mg of mitomycin C.
Apply single immediate instillation of chemotherapy within the first 2 hours following TURBT as a single dose via the indwelling catheter; close the catheter by a respective clamp.

Table 4. Guide on instillation chemotherapy. Adapted from Burger M, et al., 2021 (97) : Kamat AM, Black PC, editors. Bladder Cancer: A Practical Guide.

Bacille Calmette-Guerin (BCG), live, attenuated form of mycobacterium bovis, is currently recommended by international guidelines for intermediate- and high-risk NMIBC in order to decrease the risk of tumor recurrence and progression (98). Its instillation decreases recurrences at three years by 70% and progression by almost 30%, compared to TURBT alone (99). BCG is introduced into the bladder for 90 to 120 minutes, once weekly for six weeks, during an induction phase. If the patient tolerates the treatment and responds well, assessed by control cystoscopy, a maintenance regimen should be continued for one to three years (2, 47). In conclusion, BCG has been proven superior to TURBT alone or TURBT followed by intravesical therapies such as MMC, epirubicin, or interferon to decrease recurrences. It is currently recommended by international guidelines as the standard of care for intermediate- and high-risk NMIBC (46, 47, 100).

1.6.3 Radical cystectomy

Standard treatment for patients with MIBC consists of RC, pelvic lymph node dissection, and urinary diversion, with or without neoadjuvant chemotherapy (NAC). Radical cystectomy is recommended in patients with T2–T4a, N0M0 disease, very high-risk NMIBC, BCG refractory, or BCG-unresponsive NMIBC (2, 47). Cystectomy should not be delayed more than three months after diagnosis, since negative survival effect is to be expected. In men, standard RC includes removal of the bladder, prostate, seminal vesicles, distal ureters, and regional lymph nodes, while standard RC in women includes removal of the bladder, the entire urethra and adjacent vagina, uterus, distal ureters, and lymph nodes (1, 101). The extent of node dissection during cystectomy remains controversial, as the first prospective randomised study found no survival benefit of extended dissection over limited dissection in patients undergoing cystectomy (102). Results from a similar study, the SWOG S1011, were recently published to show that besides an increased node yield and a higher pathologic N stage, no other benefits of extended lymphadenectomy have been observed (103). Urinary diversion at time of cystectomy may take several forms: incontinent or continent (2).

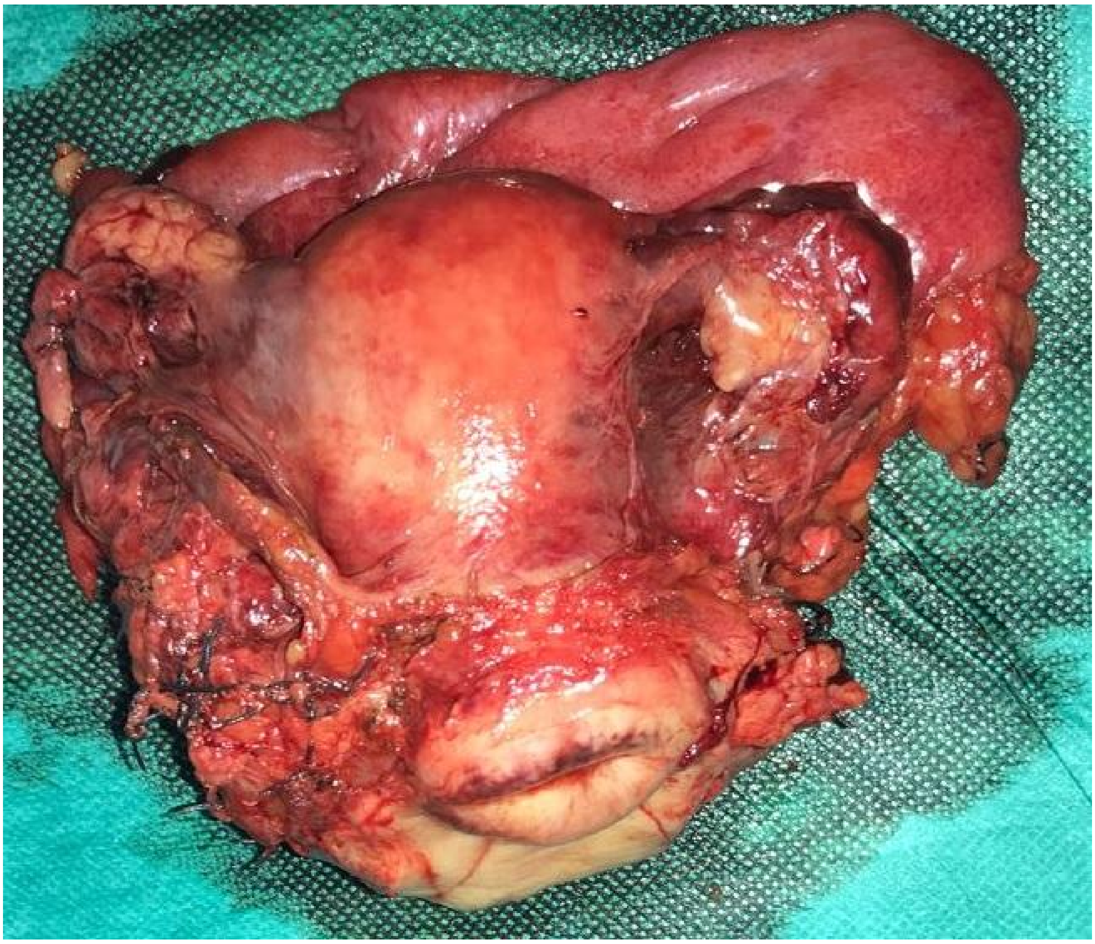


Figure 4. Radical cystectomy specimen in female patient. Courtesy of Dr. Kavaric Petar, Urology Clinic, Clinical Center of Montenegro.

The following forms of urinary diversion are currently available (1, 2):

A) Urine is drained outwards into an external reservoir (plastic bag): The drainage to the outside can be done via alloplastic material (percutaneous nephrostomy). This form is mainly used for temporary urinary diversion. Other options include implanting the ureters directly into the skin or inserting a piece of intestine between the ureters and the skin (conduit).

B) Urine diversion into the colon that is not switched off through the implanting the ureters into the sigmoid colon (ureterosigmoidostomy). The ureter colon implantation is the oldest form of urinary diversion and currently only used in rare cases.

C) Draining the urine into a reservoir formed from intestines, whose emptying function is guaranteed through self-catheterization with a continent external stoma or through the external one urethral sphincter by connecting the reservoir the urethral stump after cystectomy.

D) The reservoirs with continent stoma are called “continent supravesical urinary diversion,” while the orthotopic created reservoirs are referred to as “intestinal replacement bladders” or “neobladders”. What both techniques have in common is that they should imitate the reservoir function of the urinary bladder with different emptying mechanisms.

Selection of the urinary diversion type should stem from a well-informed discussion between the patient, the family, and the surgeon about the risks, benefits, and postoperative expectations as well as incorporate patient- and tumor-specific factors.

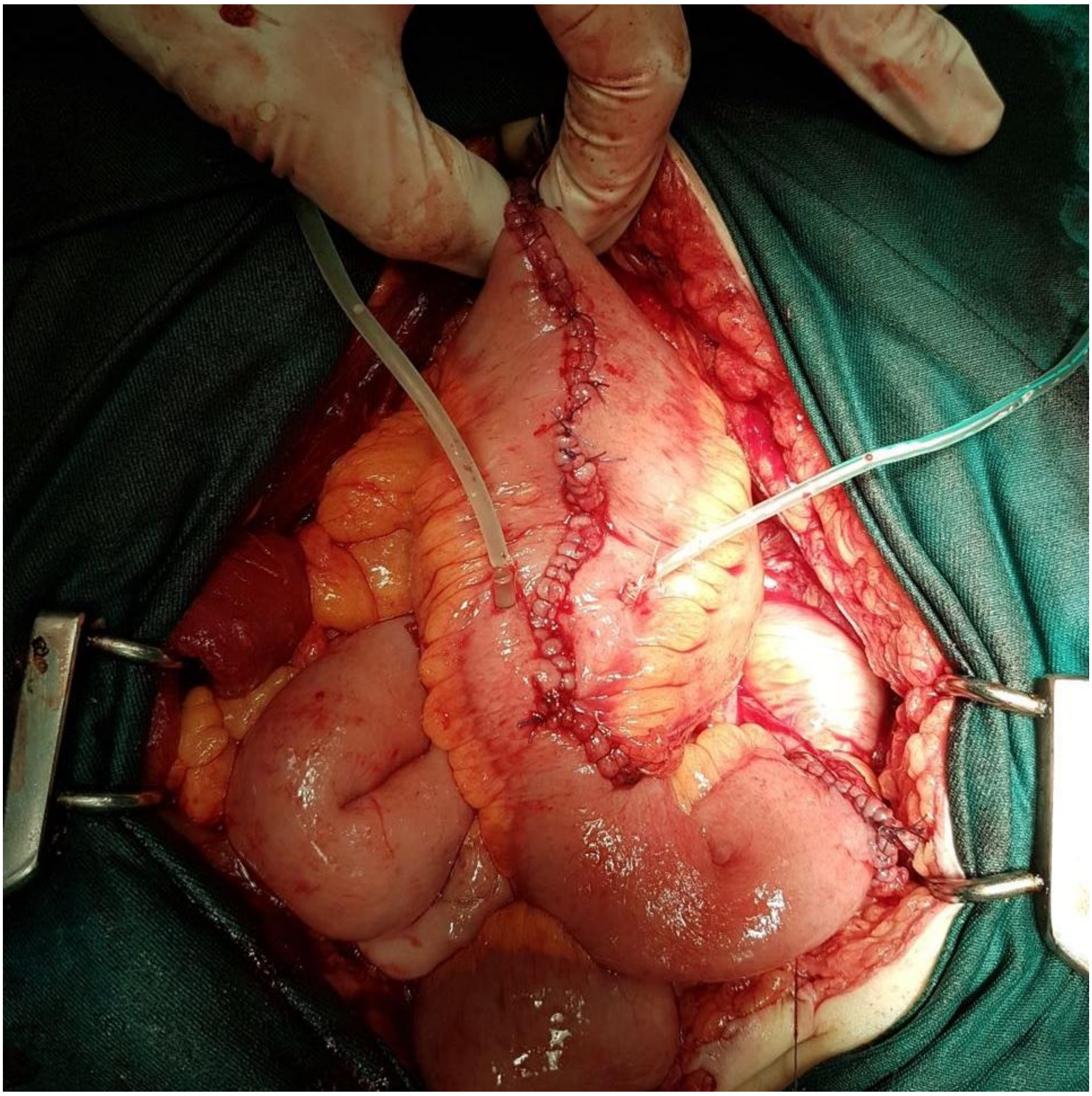


Figure 5. Orthotopic neobladder, modified Hautmann technique; Djordjevic & Vukovic, 2021 (104). Courtesy of Dr. Djordjevic Dejan, Euromedic Hospital, Belgrade, Serbia.

Figure 6. Stoma nipple within urostomy bag after ileal conduit urinary diversion. Courtesy of Dr. Djordjevic Dejan, Euromedic Hospital, Belgrade, Serbia.

1.6.4 Neoadjuvant vs. adjuvant therapy

Neoadjuvant chemotherapy (NAC) prior to RC in T1-T4/N0M0 cancer is recommended in both, the AUA and EAU guidelines (1, 2, 46). The rationale for NAC includes early treatment of micro metastatic disease, better compliance, and successful administration comparing to the adjuvant setting. Moreover, the opportunity to study in vivo response with better understanding of tumor biology is also important. However, treatment related delays or disease progression during NAC are emphasized as major drawbacks of this therapy and may impact definitive treatment (105). The available data from Phase III randomised trials supports the use of neoadjuvant, cisplatin-containing chemotherapy in patients with MIBC with a moderate, but statistically significant improvement in overall survival (106-109). The main differences in trial designs were the type of chemotherapy and the number of cycles provided. Before embarking on treatment schedule, cisplatin eligibility of the patient must be confirmed, since up to up to 50% of patients will be cisplatin-ineligible for one or more of the following reasons: ECOG performance status two or greater, creatinine clearance less than 60 ml/min, hearing loss grade two or greater, and hearth failure Class III or more (2). The difference in cisplatin regime (gemcitabine/cisplatin (GC) vs. methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC)) has not been confirmed in prospective studies. If cisplatin-based NAC is not an option, RC should be performed without further delay.

It is unclear, however, if patients with non-urothelial histology will also benefit from NAC (1). According to a recent update cited 2021, NAC was associated with pathological downstaging for all MIBC histological subtypes, with improved OS for patients with pure urothelial cancer, sarcomatoid variant and neuroendocrine carcinoma (110).

Future clinical trials are being planned where neoadjuvant treatments are being tailored based upon a tumor’s genomic classification. Valid predictive biomarkers guiding the future neoadjuvant treatment are still being waited on. What is already known is that specific mutations, particularly in ERBB2, ERCC2, and DNA repair genes, may predict the response to neoadjuvant chemotherapy (22). Moreover, it seems that the basal subgroup of MIBC shows good response to cisplatin-based chemotherapy, unlike the luminal subgroup (111).

1.6.5 Trimodal therapy

The morbidity associated with cystectomy led to a growing interest in bladder-sparing treatments, such as trimodal therapy (TMT) and partial cystectomy with neoadjuvant chemotherapy (112). TMT of MIBC comprises transurethral resection of the bladder tumor (TURBT) followed by radical radiotherapy (RT) with a concurrent radiosensitizing agent (113). Chemotherapy used in TMT is often combination of cisplatin/fluorouracil or palicitaxel, cisplatin alone, or combination of fluorouracil with mitomycin C and these function as radiosensitizing agents as well as systemic treatment for micrometastatic disease (2). A recent retrospective study provided evidence that similar oncological outcomes between RC and TMT are to be expected, except for select patients with MIBC (114). These results support TMT, however, only in the setting of multidisciplinary shared decision making and among suitable candidates.

1.6.6 Targeted therapies and antibody-drug conjugates

Tumor angiogenesis plays a crucial role in tumor migration, growth, and metastasis, making it an attractive therapeutic target. Fibroblast growth factor receptor (FGFR) signalling is altered in many malignancies promoting oncogenesis and its intracellular pathway mainly through phosphatidylinositol 3-kinase (PI3K)/Akt pathway (115). Since urothelial cancers harbour high frequency of somatic alterations in FGFR3, therapy targeting this receptor was recently introduced in treatment algorithm of metastatic bladder cancer. Erdafitinib is now FDA approved as second line treatment for tumors harbouring FGFR3 mutations (116). Moreover, antibody-drug conjugate Enfortumab vedotin, acting as an anti-necin-4 antibody, showed promising results in treating refractory metastatic disease, in selected group of patients (117). These and several other agents (PARP or HER2 inhibitors) are being evaluated in metastatic urothelial cancer and cross-sectional imaging is utilized on a regular basis to assess for response to treatment.

1.7 Objectives of the study

From aiding in diagnosis to guiding therapy selection, predictive biomarkers are important tools in managing BCa. They might even prompt timely abandonment of ineffective treatments, leading to more successful oncological care. Our understanding of specific mechanisms of tumor behavior and molecular structures are key to designing predictive markers that offer insight into innate tumor biology and therapeutic susceptibility. IL-1 axis has not been widely investigated in invasive BCa, while autophagy and proinflammatory markers in addition to their role in cancer genesis is well defined and described in literature. However, prognostic value of combining IL-1 axis, autophagy, and inflammatory markers has not been investigated so far.

Therefore, our study aimed to:

1. Determine the expression patterns of IL-1ß/IL-1RA axis in invasive BC;
2. Assess the prognostic role of IL-1ß in terms of survival outcome;
3. Determine the correlation of IL-1β and IL-1RA with biomarkers Ki-67 and AKT, thus gaining further insight in their molecular relevance in BC.
4. Look at correlation for the IL-1 axis with the autophagy pathway.

2 Materials and methods

2.1 Study design

Our study was a retrospective institutional-based clinical study, with oncological and clinical data derived from database of Urology Clinic, Eberhard-Karls University of Tuebingen.

2.2 Target population

Patients of both genders with invasive urothelial carcinoma of the bladder who underwent a radical cystectomy and who attended the Department of Urology, Tuebingen University Hospital from February 1996 through December 2010. The sample size included 194 patients, divided into discovery and confirmatory groups, where expression of four biomarkers (IL-1ß, IL-1RA, AKT and Ki-67) were stained in cystectomy specimens for tissue microarray (TMA) and immunohistochemistry (IHC). Corresponding histopathological benign urothelium tissue samples from surrounding areas were also processed for TMA and IHC and stained for the same biomarkers.

2.3 Patients database

In Excel©-Table of Urology Clinic in Tuebingen, following patients' data were extrapolated: Name, surname, sex, age, date of surgery, type of urinary diversion, previous transurethral resections, and perioperative instillation therapy. Results of biomarker tissue expression (AKT, Ki-67, IL-1ß, and IL-1RA) were documented through H-score histochemical scoring system for interleukins and AKT, while Ki-67 score was expressed as the percentage of the number of immune-stained nuclei among the total number of nuclei of tumor cells (118). Additionally, several other data were addressed:

- Application of neoadjuvant or adjuvant chemotherapy (MVAC or GC protocol);

- Disease recurrence, local or distant;
- Survival outcomes (overall, cancer specific, and recurrence free survival).

2.4 Inclusion criteria

a. Patients with invasive urothelial BCa who underwent radical cystectomy and various urinary diversions;
b. Stage T1-4a cancer, with or without CIS;
c. Any N, M0, or M1 tumor stage;
d. Any tumor grade

2.5 Exclusion criteria

a. < T1 bladder tumors;
b. Variant histology;
c. Previous radiotherapy;
d. History of non-urothelial malignancy.

2.6 Perioperative evaluation

Standard radiological imaging included abdominal MRI or computed tomography. At least two experienced pathologist assessed tumor grade and stage, using the WHO 2016 TNM classification system. Patients were examined postoperatively every three months for the first year, then semi-annually for the second, third, and fourth year and annually thereafter. Time to recurrence (RFS), cancer specific survival (CSS), and overall survival (OS) were assessed (119).

2.6 Tissue microarray, immunohistochemistry and scoring

To obtain representative scores for TMA construction, parallel hematoxylin and eosin stained sections were used to identify a representative core position within the specimens (119). IHC staining was carried out according to the antibody manufacturers' instructions. Tissue slides were incubated overnight at 4 °C with a human IL-1 beta/ IL-1F2 (AF-201, R&D systems Inc., USA) and IL-1 RA/ IL-1F3 (AF-280, R&D systems, USA) polyclonal goat immunoglobulin, respectively, in dilutions 1:10 and 1:300, in real antibody diluent (DAKO, Glostrup, Denmark) (120). After three more washing steps, visualization was performed with Dako Liquid DAB-Substrat Chromogen System K3467 (DAKO, Glostrup, Denmark) and counterstaining with haematoxylin, as indicated by the manufacturer (121). Two or more cores of every invasive BC and corresponding normal bladder tissue were then integrated.

TMAs were evaluated in a blindly by two independent reviewers, and divergent results were reevaluated. For the results, IL-1β and IL-RA cytoplasmatic cellular staining was scored using a four-point scale (0, no staining; 1+, light staining at high magnification; 2+, intermediate staining; 3+, dark staining of linear membrane at low magnification.) Expressions were then quantified by the histochemical scoring system / H-score 0-300 and a quotient IL-1ß/IL-1RA was built for further calculations. Microscopic analysis was performed at x100 and x400 magnifications (119).

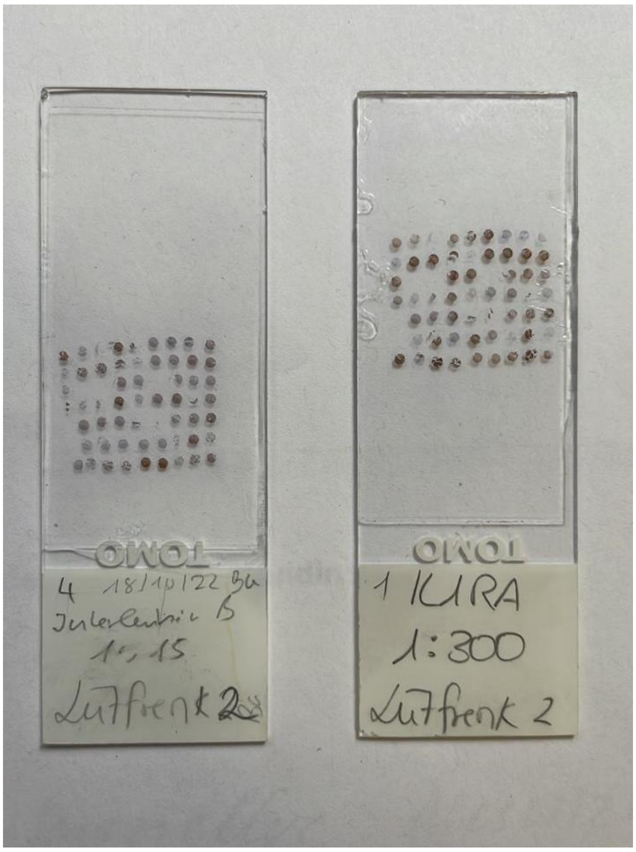


Figure 7. Tissue microarray of IL-β and IL-RA in dilution 1:15 and 1:300

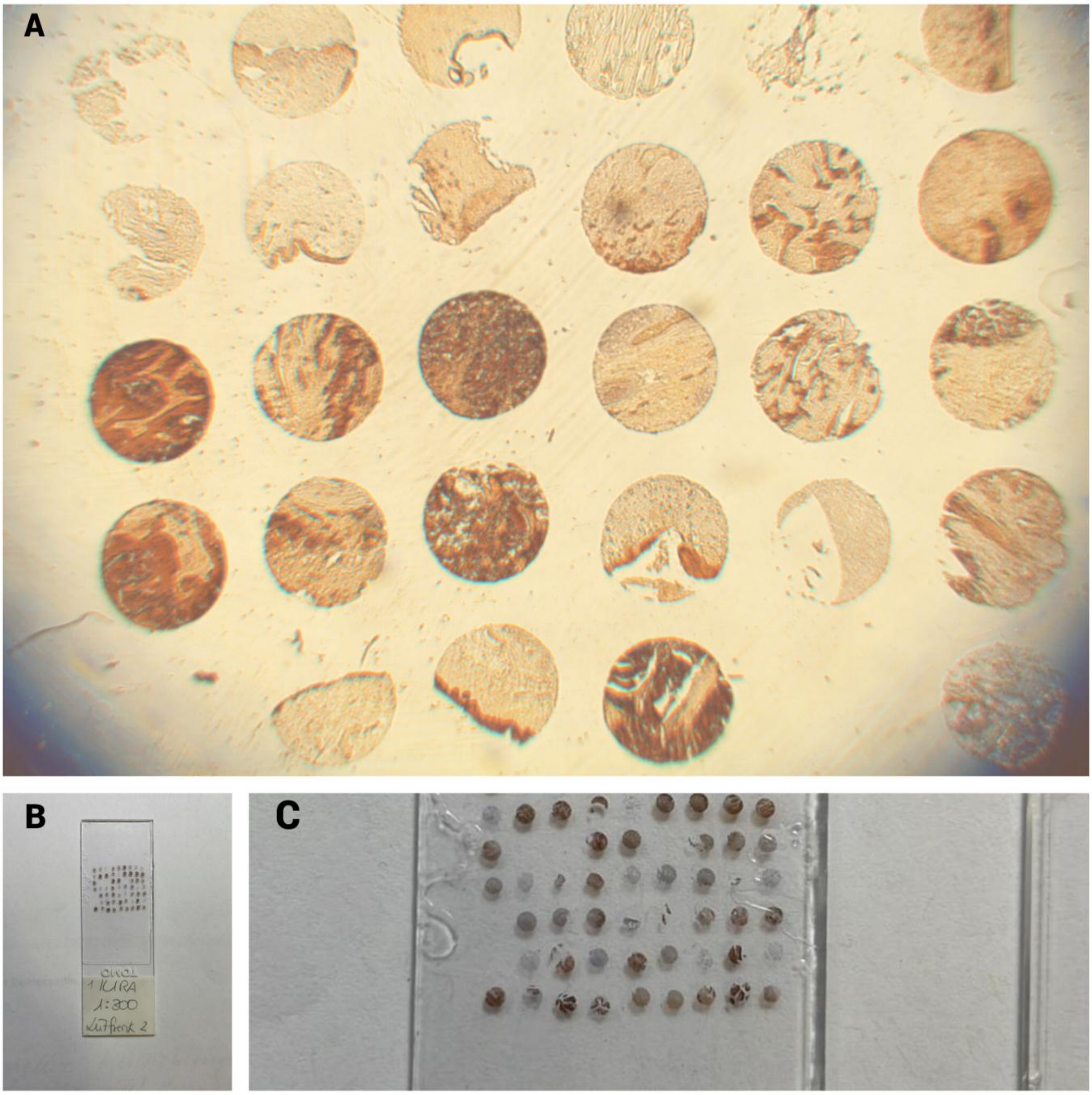


Figure 8. Tissue microarray and cytoplasmatic staining score for IL-RA: a) x20 magnification; b and c) macroscopic appearance.

For AKT, epithelial zones were scored according to the intensity of staining of the cytoplasm, nucleus, or membrane and the same scoring system was used. Individual results were demonstrated in the cellular staining classes and compared to clinical and histopathological data of the second cohort, whereas clinical disease course was evaluated in the first group. The Ki-67 score was expressed as a percentage of the number of immune-stained nuclei among the total number of nuclei of tumor cells, regardless of the immunostaining intensity. Counting was performed in three representative selected fields of the BC tissue section at x400 magnification. Ki-67 score ranged from 0-100% and its cut-off level was 15%, where “low immunoreactivity” was defined by nuclear staining of < 15% and “high” for staining ≥ 15% (119).

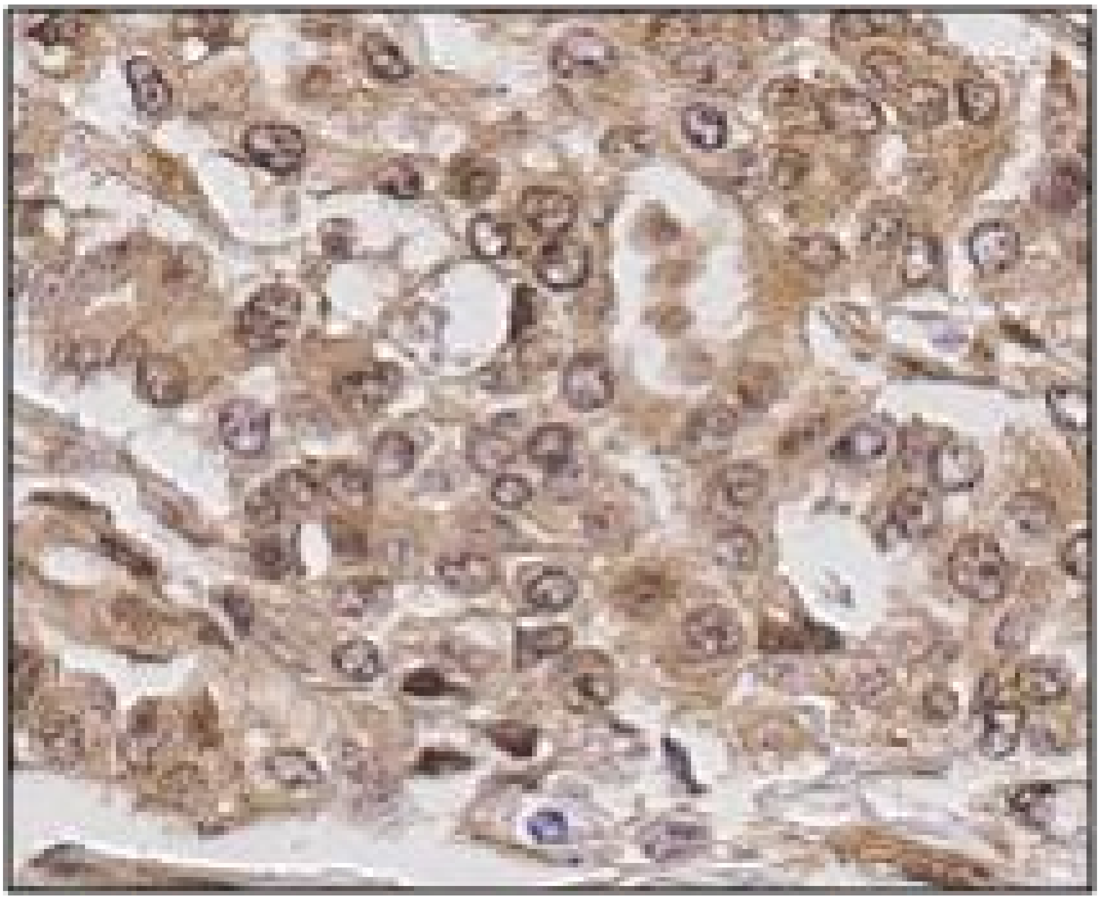


Figure 9. High cytoplasmatic and nuclear expression of AKT, magnification x400

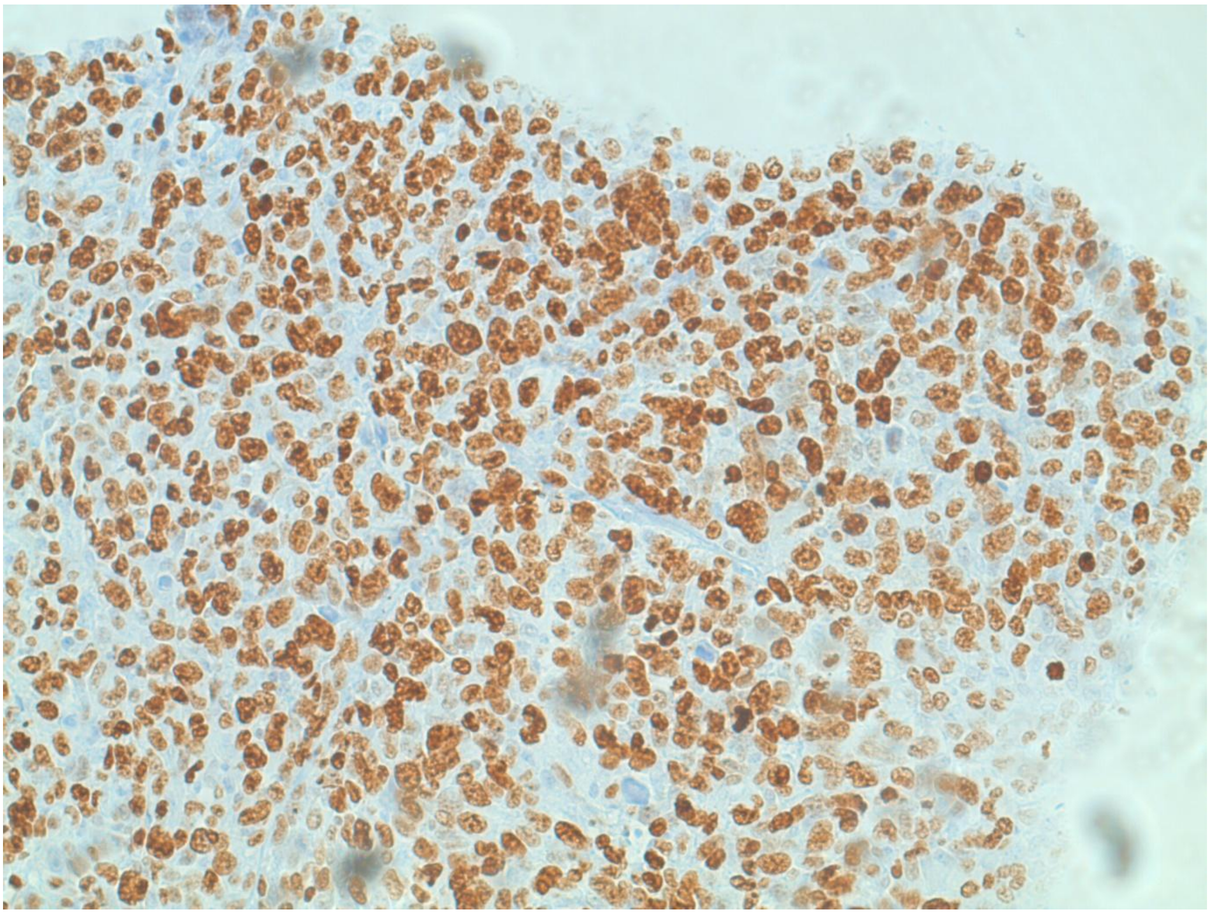


Figure 10. High nuclear staining (>15%) of Ki-67, magnification x200.

2.7 Statistical analysis

The expression pattern of IL-1ß, IL-1RA, AKT, and Ki-67 was determined in cystectomy specimens and dependent on the variable's category, results correlated with clinicopathologic parameters by Wilcoxon Kruskal-Wallis tests, Chi-square tests, or linear regression analyses. For univariate analysis, the Fisher's exact/Pearson chi-square test was used for nominal data and the Student's t-test for scaled data. All P-values were two-sided with $P < 0.05$ considered to indicate statistical significance. Values were given as mean, median, and standard deviation for all continuous variables or as median (range) for nonparametric variables. Kaplan–Meier analyses were used to estimate recurrence-free (RFS), cancer-specific (CSS), and overall survival (OS) by the log-rank test. Uni- and multivariate analyses were performed to assess the impact of IL-1 axis on relevant clinical and pathologic variables. P-values <0.05 were considered significant. JMP, version 16.2 (SAS Institute, Cary, USA) was used for statistics (119).

2.8 Ethical considerations

2.8.1 Confidentiality

The confidentiality of all participants admitted to this study is protected to the fullest extent possible. The study participants are not identifiable by name in any report or publication resulting from data collected in this study.

2.8.2 Ethical committee approval

The study was approved by the institutional review board of the University of Tuebingen No. 279/2013BO2 on March 1st , 2023.

3 Results

3.1 Preoperative data

3.1.1 Number of patients

The study included 194 patients who were treated by radical cystectomy for invasive BCa, between February 1996 and December 2010. Patients were retrospectively enrolled and selected in two groups: 102 in a discovery and 92 patients in confirmatory group. Correlation of IL-1 axis with AKT was examined within first cohort, while correlation with Ki-67 was determined in second group (119).

3.1.2 Sex

78 males and 24 females were recruited for the first, while 67 males and 25 females were selected for the second group, respectively (Figure 11).

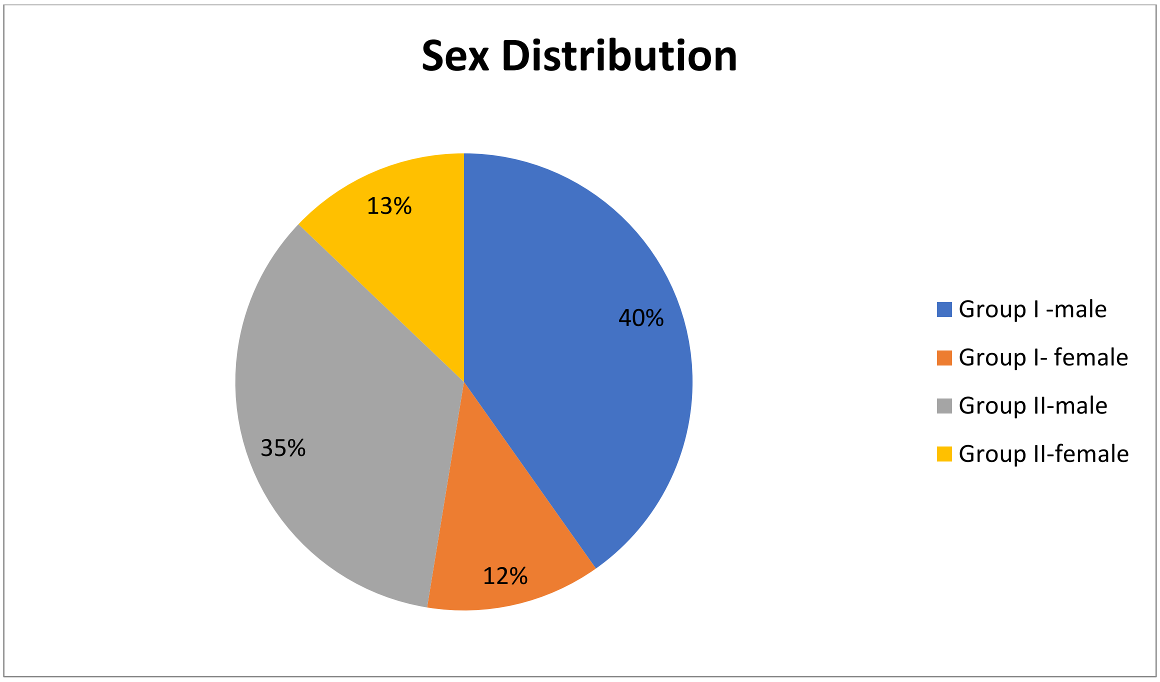


Figure 11. Sex distribution within groups (percentage).

3.1.3 Age

Median age at the time of cystectomy was 69 (59-74) years within the discovery and 65 (55-72) years within the confirmatory group, respectively.

3.2 Pathology

3.2.1 Tumor stage (T)

Predominant pathological tumor stage in both groups was pT3 (n=47 vs.42, p=0.55). Within second group nine patients had pT1 (9.78%), unlike the first group, in which only muscle-invasive cancer pathologies was included in the study. Moreover, pathological samples with CIS were present in only two patients within second group, whereas the first group only had concomitant CIS occurrences which was present in five patients (Figure 12).

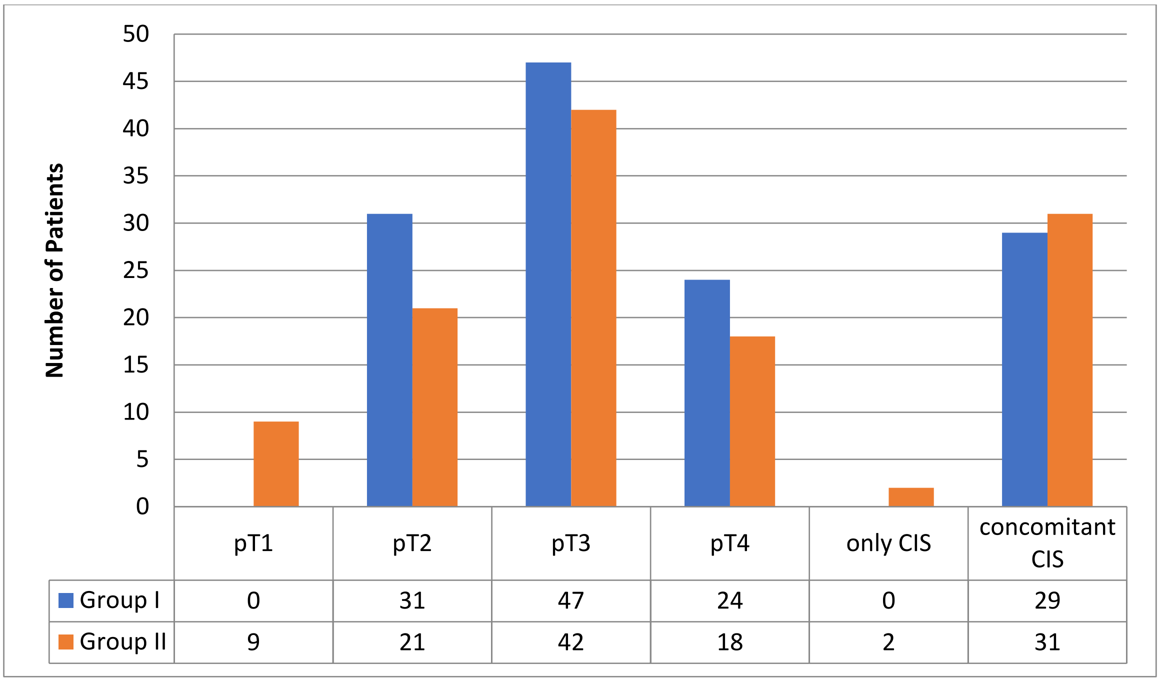


Figure 12. Tumor stage distribution across the groups

3.2.2 Tumor grade (G)

The most common tumor grade among groups was G3 (n=78 vs. 71, p=0.94), with G1 present only in one sample from the second group (Figure 13).

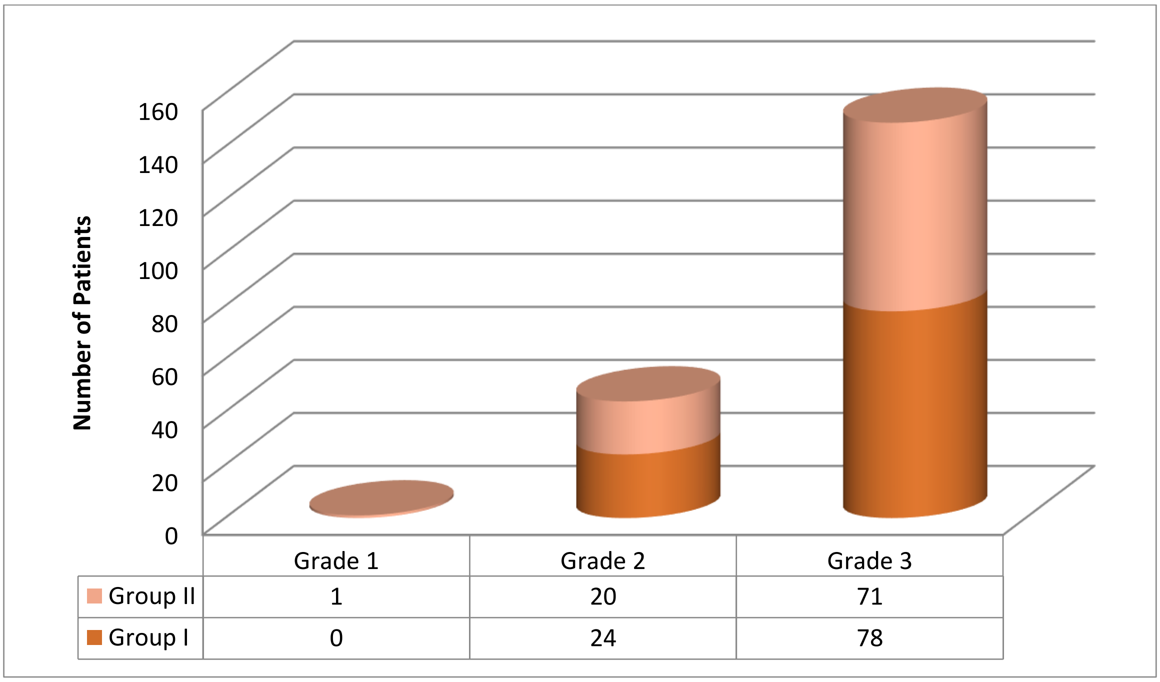
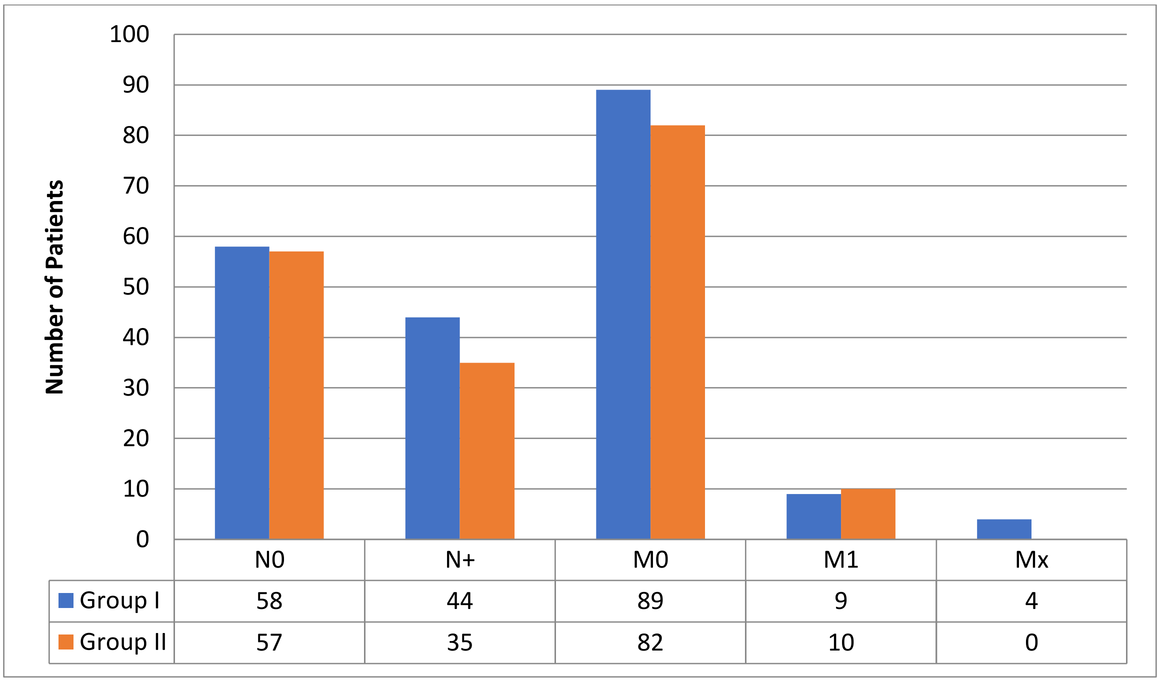


Figure 13. Tumor grade distribution among groups.

3.2.3 Nodal (N) and metastatic status (M)

Majority of patients in both groups developed neither lymph node nor distant metastatic disease (n=58 vs. 57 for N0 and n= 89 vs. 82 for M0 status, p=0.5 for both, respectively) (Figure 14).

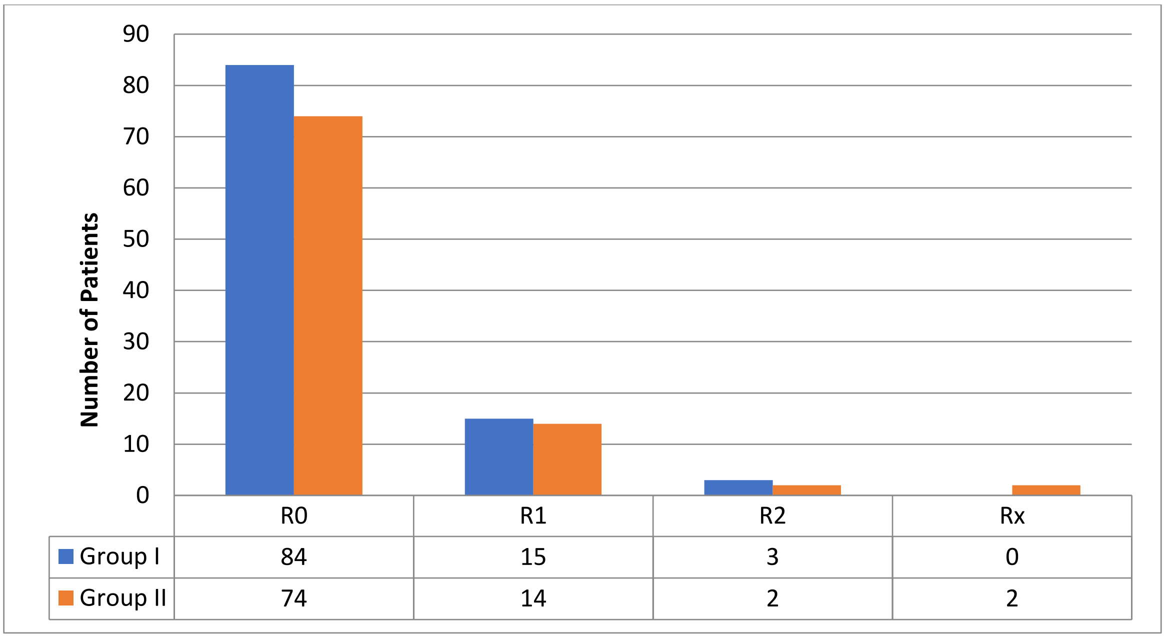


	N0	N+	M0	M1	Mx
🟦 Group I	58	44	89	9	4
🟧 Group II	57	35	82	10	0

Figure 14. Nodal status and occurrence of metastatic disease at the time of cystectomy.

3.2.4 Resection margin status

Negative surgical margins were detected in more than 80% of patients within groups (n=84 vs. 74, p=0.03), while R2 was present in less than 3% of patients (Figure 15).



	R0	R1	R2	Rx
■ Group I	84	15	3	0
■ Group II	74	14	2	2

Figure 15. Resection margin status at the time of cystectomy.

3.3 Expression characteristics of IL-1β

Mean cytoplasmic expression of IL-1β in BCa and surrounding benign urothelium tissue was 185 vs. 120 (p=0.004) (Figure 16). High expression levels of IL-1β were detected only in small number of benign urothelial tissues (H-score: 240, Figure 17). Therefore, Il-1ß was significantly overexpressed in invasive cancer tissue compared to benign urothelium (p<0.001) (119).

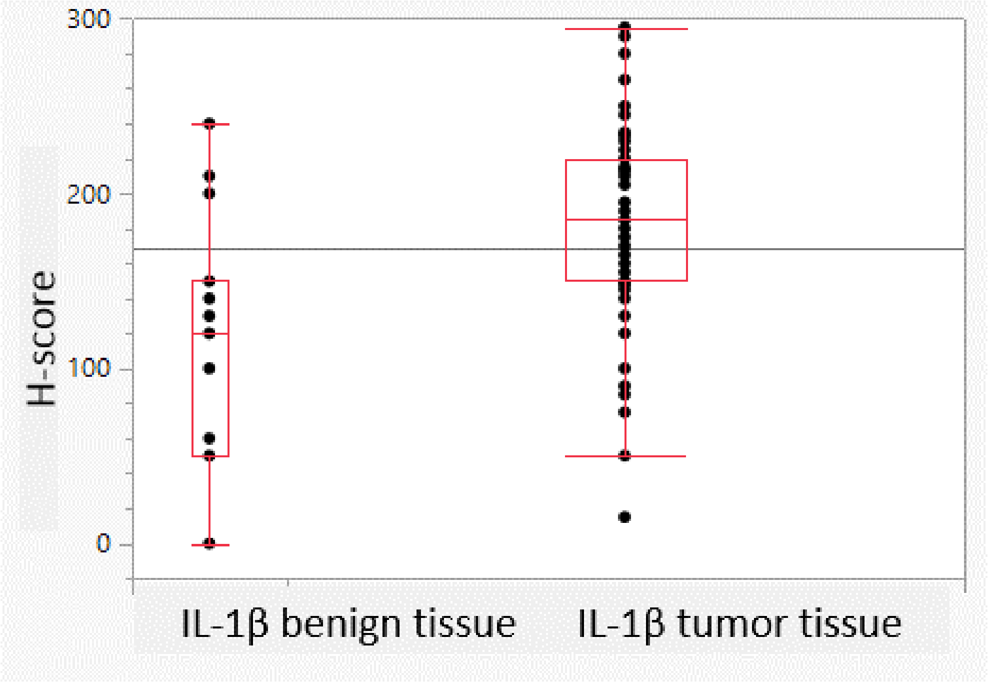


Figure 16. Expression levels of IL-1β in benign and malignant tissue.

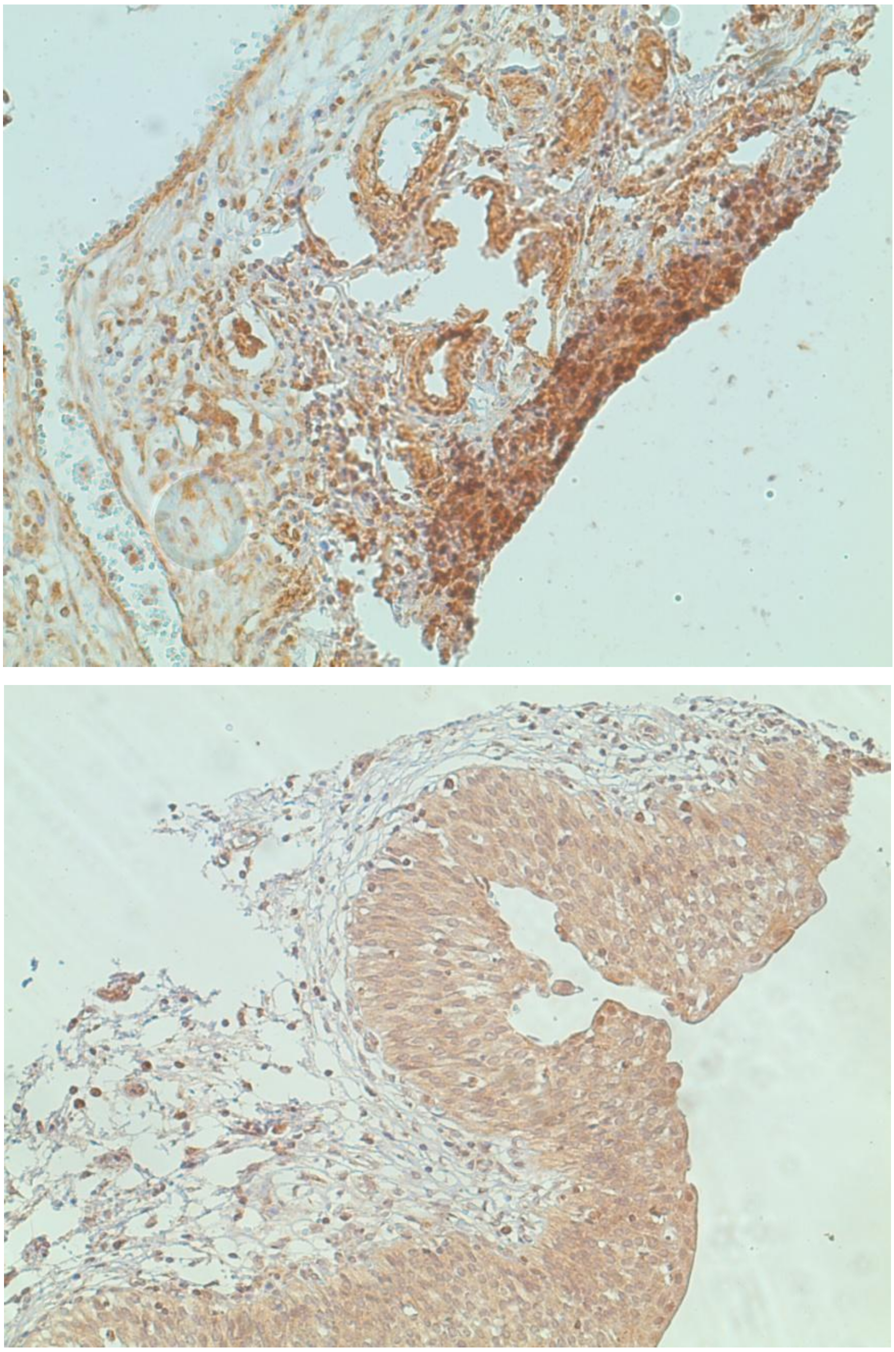


Figure 17. High expression level of Il-1β (malignant tissue, upper image) and low expression level (benign tissue, lower image); p=0.004, Wilcoxon Kruskal-Wallis tests.

3.4 Expression characteristics of IL-1RA

Mean cytoplasmic expression of IL-1RA in BCa and surrounding bladder tissue was 190 vs. 110, p=0.004 (figure 18). As for IL-1β, high expression of IL-RA was detected in few benign tissue samples (H-score: 280) (Figure 19).

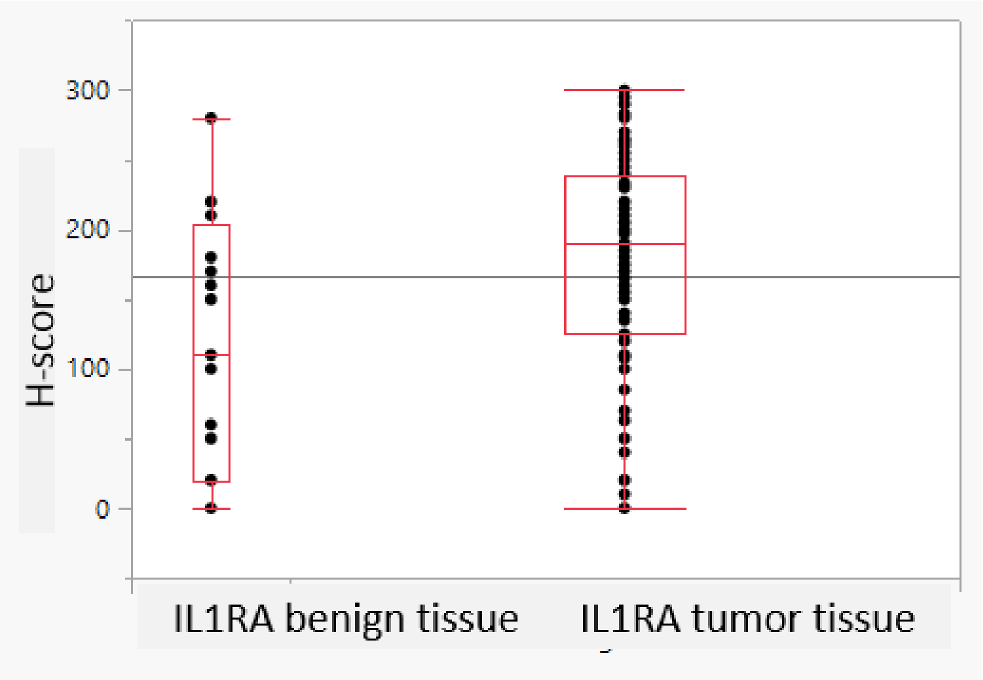


Figure 18. IL-1RA cytoplasmatic expression in malignant and benign tissue; p =0.004, Wilcoxon Kruskal-Wallis tests.

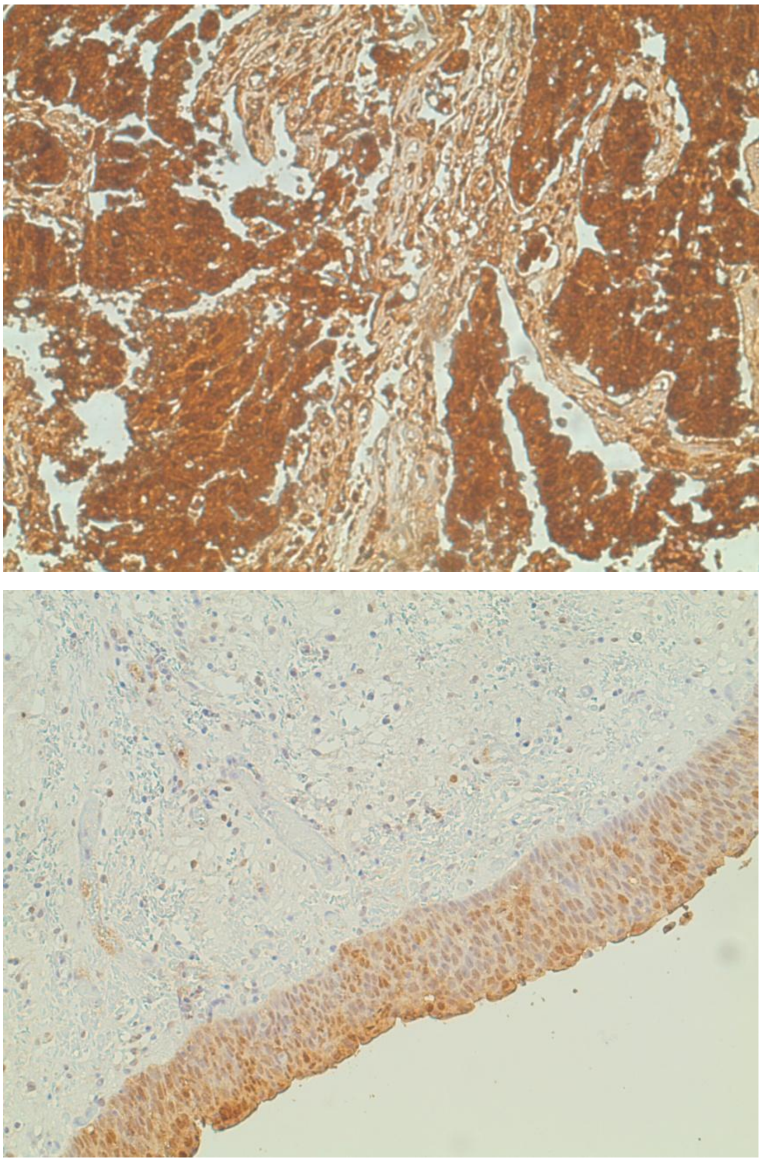


Figure 19. High expression (cancer tissue) vs. low expression (benign tissue) of IL-1RA (H score: 300 vs. 90)

3.5 Correlation of IL-1β with histopathologic parameters

Figure 20. depicts the correlation of IL-1β expression with histopathologic variables in univariate analysis. High expression level was observed in patients with vascular invasion (210 vs. 183, p<0.02) and lymphatic invasion (210 vs. 180, <0.05), as well as in G3 BCa (193 vs. 188, <0.04) (119).

Histopathology variables	IL-1β (H-score)
G2	188
G3	**193***
T2	205
T3	190
T4	190
lymphatic invasion	**210***
vascular invasion	**210***
N0	185
N1	185
N2	210
N3	198
M0	185
M1	210

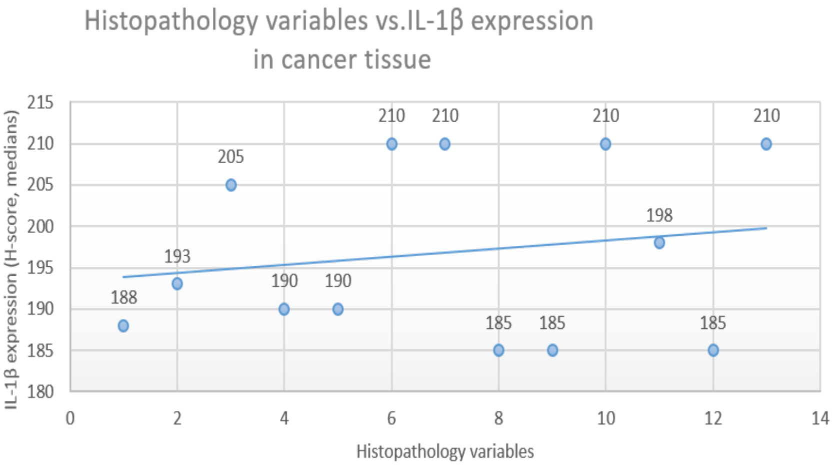


Figure 20. Univariate analysis of correlation between IL-1β expression and histopathologic variables.

3.6 Correlation of IL-1RA with histopathologic parameters

There was no significant correlation between adverse tumor features (tumor stage, lymphovascular invasion, N+ or M+ status) and level of IL-RA expression. Nevertheless, low expression of IL-1RA was associated with HG (G3) cancer (p=0.002), although G3 BCa showed lower median IL1-RA expression when compared to G2 (185 vs. 250, <0.003) (Figure 21).

Histopathology variables	IL-1RA (H-score)
G2	**250****
G3	185
T2	178
T3	200
T4	200
lymphatic invasion	200
vascular invasion	203
N0	194
N1	200
N2	205
N3	175
M0	194
M1	200

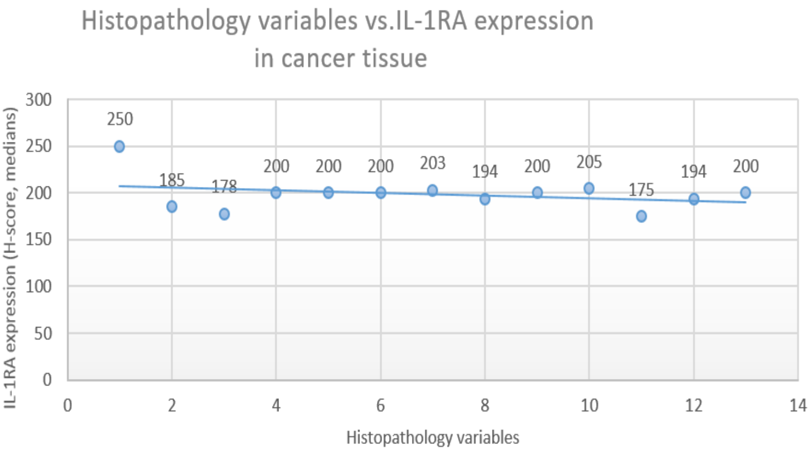


Figure 21. Univariate analysis of correlation between IL-1RA expression and histopathologic variables.

3.7 IL-1 ratio and histopathologic parameters

Positive correlation between IL-1 ratio and tumor grade (p=0.002) was observed with higher IL-1 ratio in G3 cancer (1.10 vs. 0.66, p=0.001) (Figure 22).

Histopathology variables	IL-1β/IL-1RA ratio (cut-off 0.7)
G2	0,7
G3	**1,1****
T2	1
T3	1
T4	0,9
lymphatic invasion	1
vascular invasion	1
N0	1
N1	0,9
N2	1,1
N3	1
M0	1
M1	1

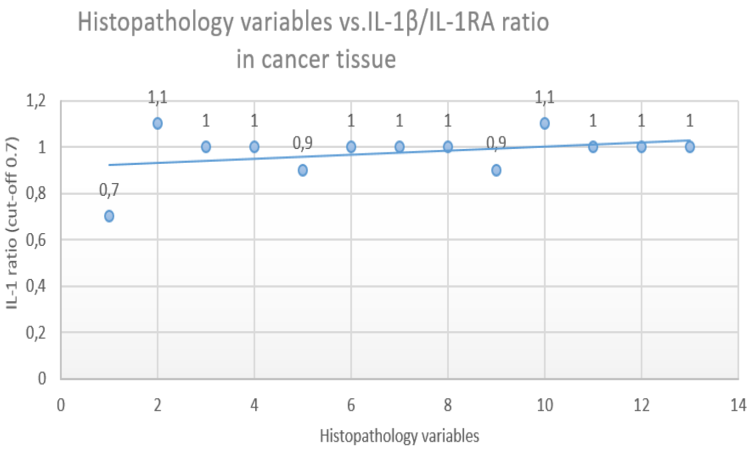


Figure 22. Univariate analysis of correlation between IL-1 ratio and histopathologic variables.

3.8 Combined scores and clinical data

3.8.1 Positive corelation of histological parameters with combined biomarker scores

There was a significant correlation with vascular invasion when Ki-67+ *and* IL-1ß+ combined, while higher tumor grade and combined scores of Ki-67+ *or* IL-1ß+ demonstrated statistical significance as well (Figure 23 and 24).

Histopathology variables	Ki-67+ **and** IL-1β+
>T2 vs. T2	0,61
N+ vs. N0	0,97
M+ vs. M0	0,28
≥G2 vs. <G2	1
L+ vs. L0	0,93
V+ vs V0	0,02

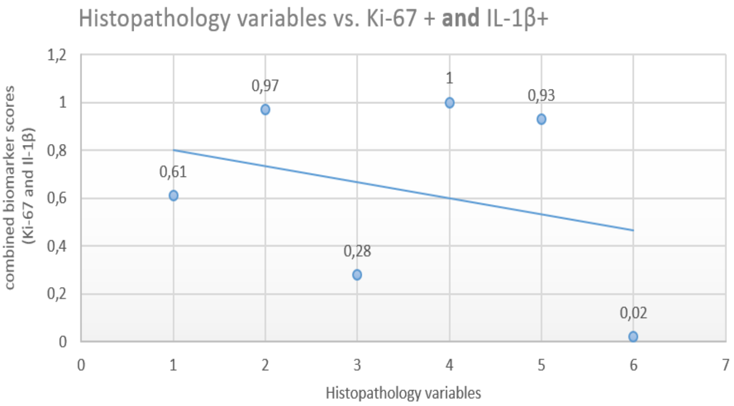


Figure 23. Correlation between combined Ki-67+ and IL-1ß+and histopathologic variables.

Histopathology variables	Ki-67+ **or** IL-1β+
>T2 vs. T2	0,67
N+ vs. N0	0,25
M+ vs. M0	0,4
≥G2 vs. <G2	0,02
L+ vs. L0	0,51
V+ vs V0	0,92

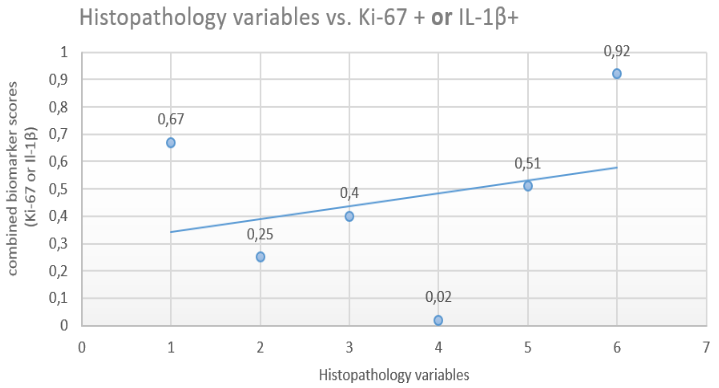


Figure 24. Correlation between combined Ki-67+ or IL-1ß+and histopathologic variables.

3.8.2 Correlation of histological parameters with IL-1RA associated combined biomarker scores

No statistical significance was demonstrated between Ki-67+ *and/or* IL-1RA and histological parameters (Figure 25 and 26).

Histopathology variables	Ki-67+ **and** IL-1RA
>T2 vs. T2	0,81
N+ vs. N0	0,39
M+ vs. M0	0,46
≥G2 vs. <G2	0,52
L+ vs. L0	0,3
V+ vs V0	0,33

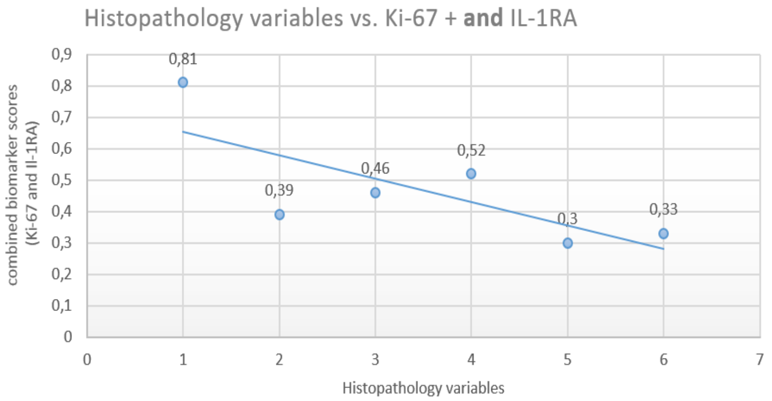


Figure 25. Correlation between combined Ki-67+ or IL-1RA and histopathologic variables.

Histopathology variables	Ki-67+ **or** IL-1RA
>T2 vs. T2	0,06
N+ vs. N0	0,54
M+ vs. M0	0,56
≥G2 vs. <G2	0,57
L+ vs. L0	0,7
V+ vs V0	0,9

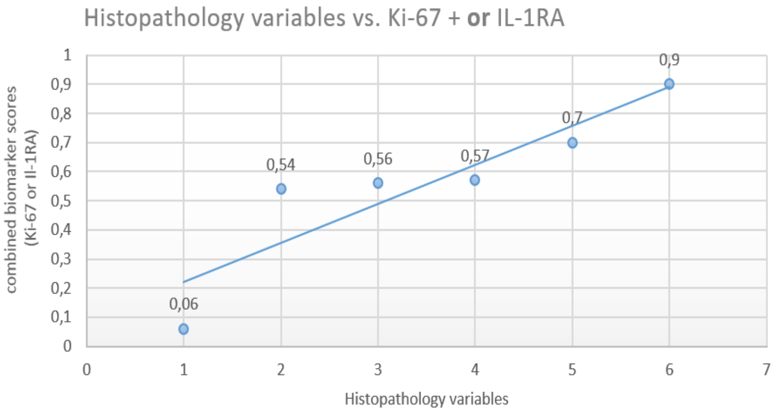


Figure 26. Correlation between combined Ki-67+ or IL-1RA and histopathologic variables.

3.9 Correlation of IL-1 axis with Ki-67 and AKT expression

3.9.1 Correlation of 1 IL-β with Ki-67 and AKT expression

Regarding potential associations to proliferation or autophagy, IL-1β was associated with higher AKT expression in the BCa cells (p=0.04, Figure 27). Additionally, high expression scores of Ki-67 (>15%) also correlated with the intensity of IL-1β expression (p=0.01, Figure 27) (119).

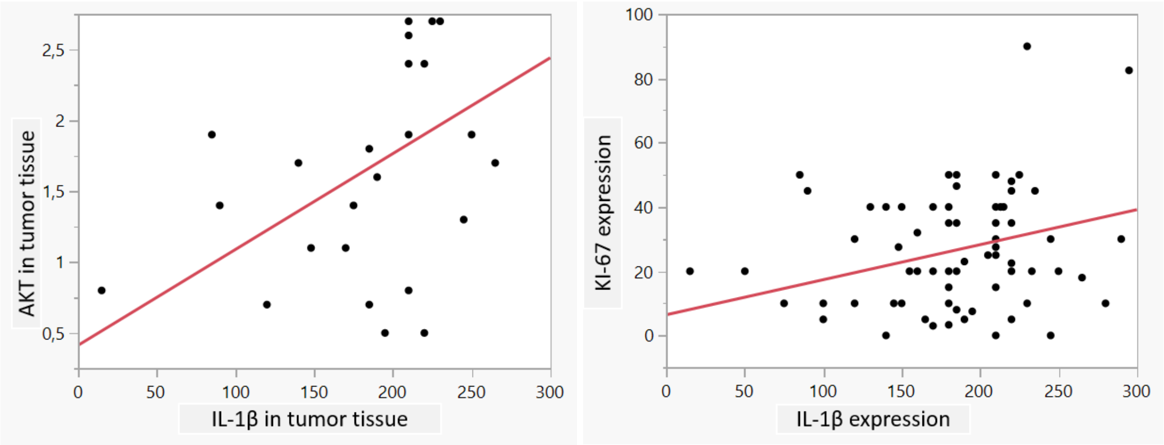


Figure 27. Correlation of IL-1β with Ki-67 and AKT in tumor tissue.

3.9.2 Correlation of IL-1 ratio with Ki-67 and AKT

IL-1 beta/IL-1RA ratio, with specific cut-off value (0.7), was associated with higher AKT expression in cancer cells (p=0.009). Nevertheless, high Ki-67 scores did not correlate significantly with IL-1 beta/IL-1RA ratio, although positive trend towards correlation was observed (p=0.08) (Figure 28).

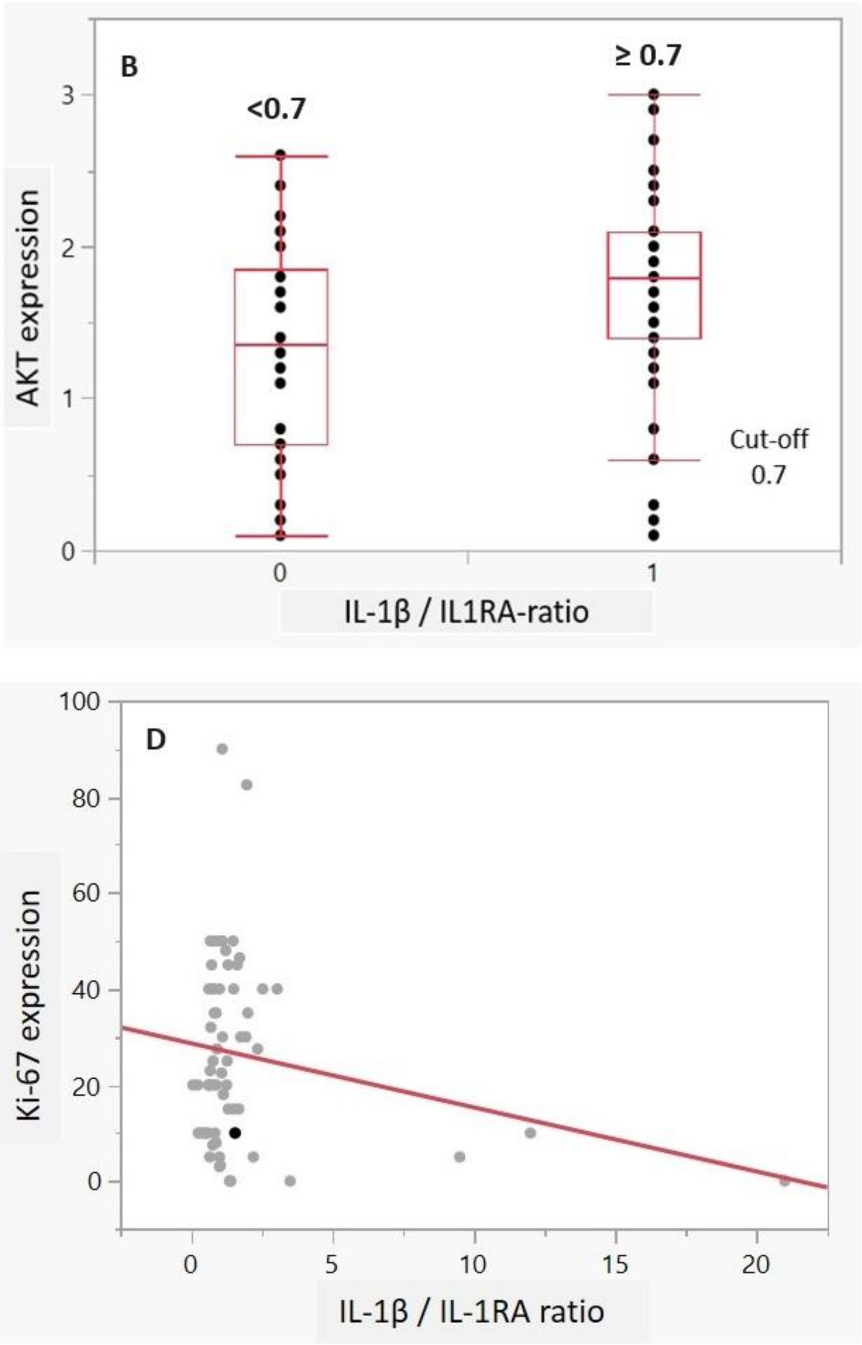


Figure 28. Correlation of IL-1β/IL-1RA ratio with Ki-67 and AKT expression in tumor tissue.

4 Survival analysis

4.1 IL-1β and survival analysis

Survival analysis revealed improved RFS, CSS as well as OS in case of IL-1ß high expression (p<0.02, <0.03 and <0.006, respectively, Figures 29-31).

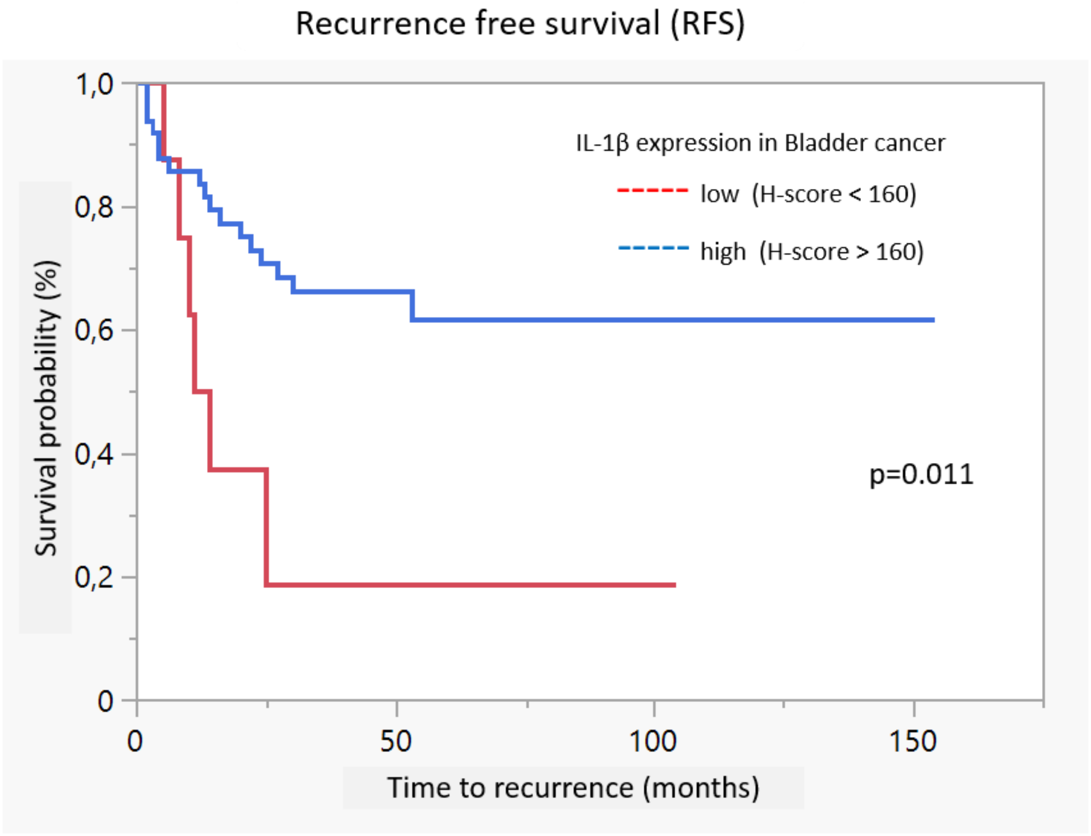


Figure 29. Kaplan-Meier analysis for recurrence free survival in according to cytoplasmatic cellular staining of IL-1β within tumor cells. Adapted from Vukovic M et al., 2024 (119).

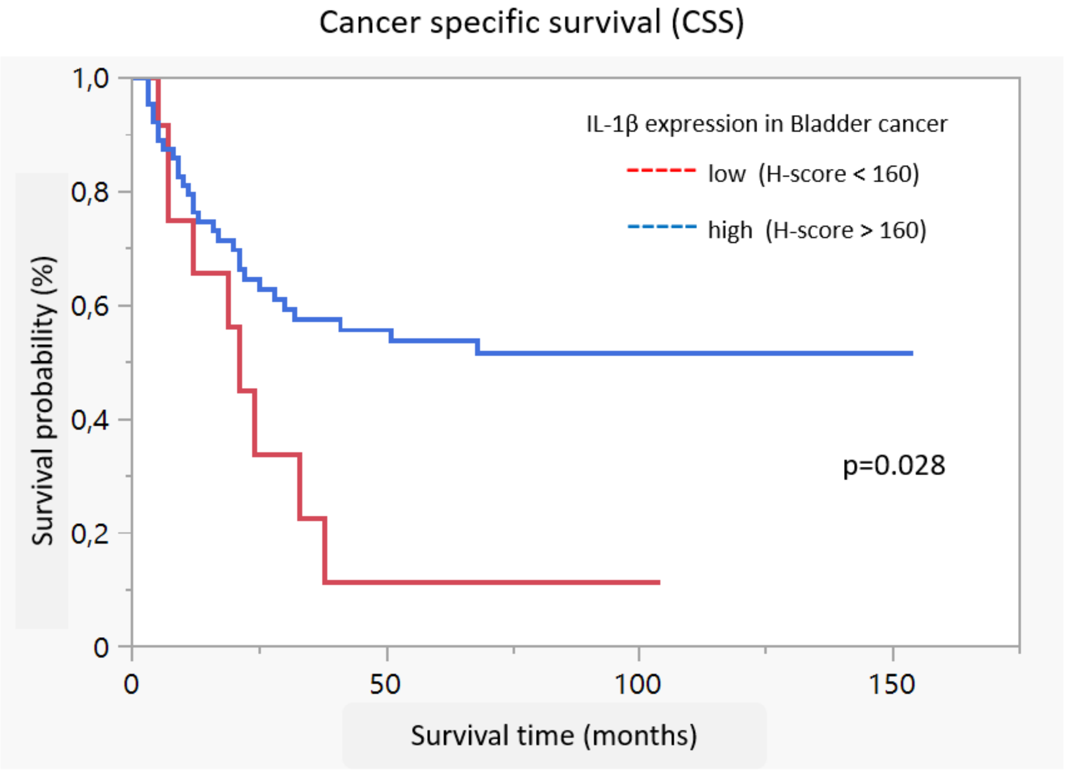


Figure 30. Kaplan-Meier estimates for cancer specific survival in dependence of cytoplasmatic cellular staining of IL-1β within tumor cells. Adapted from Vukovic M et al., 2024 (119).

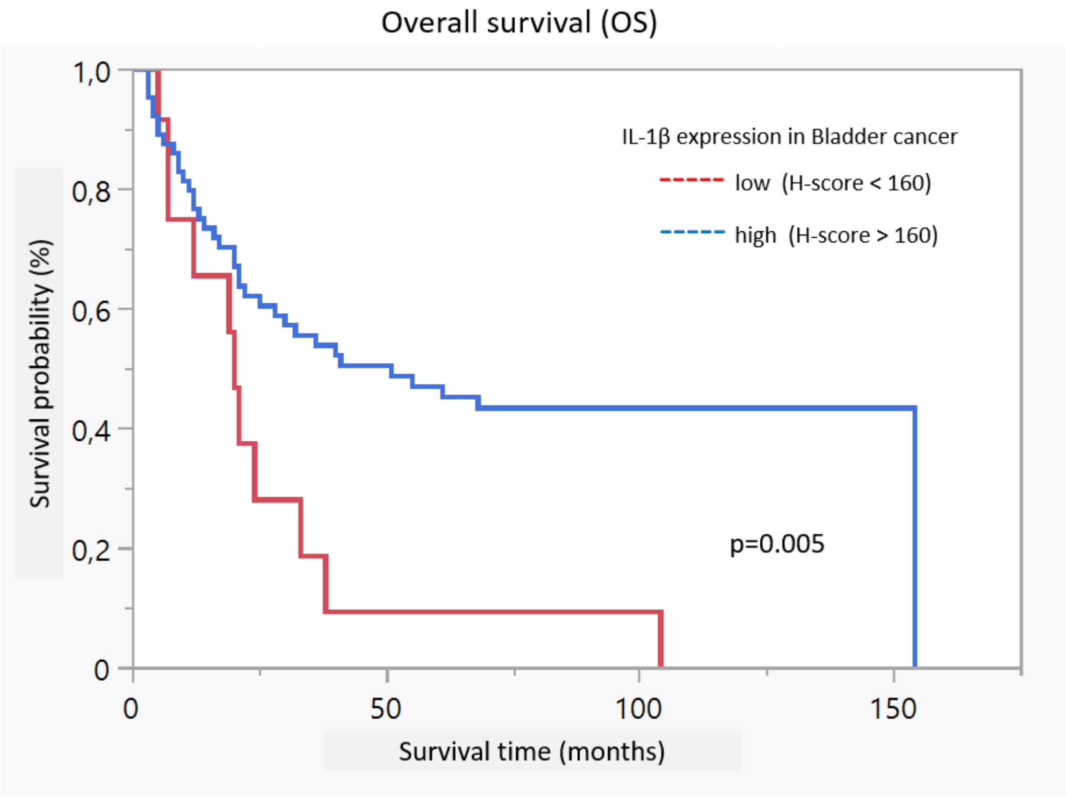


Figure 31. Kaplan-Meier estimates for overall survival in dependence of expression levels of IL-1β within tumor cells. Adapted from Vukovic M et al., 2024 (119).

4.2 IL-1RA and survival analysis

Significant correlation could not be found regarding IL-1RA expression or IL-1ß/IL1RA-ratio and survival outcomes (p=0.17, 0.19 and 0.6 for IL-RA, respectively, Figures 32-34).

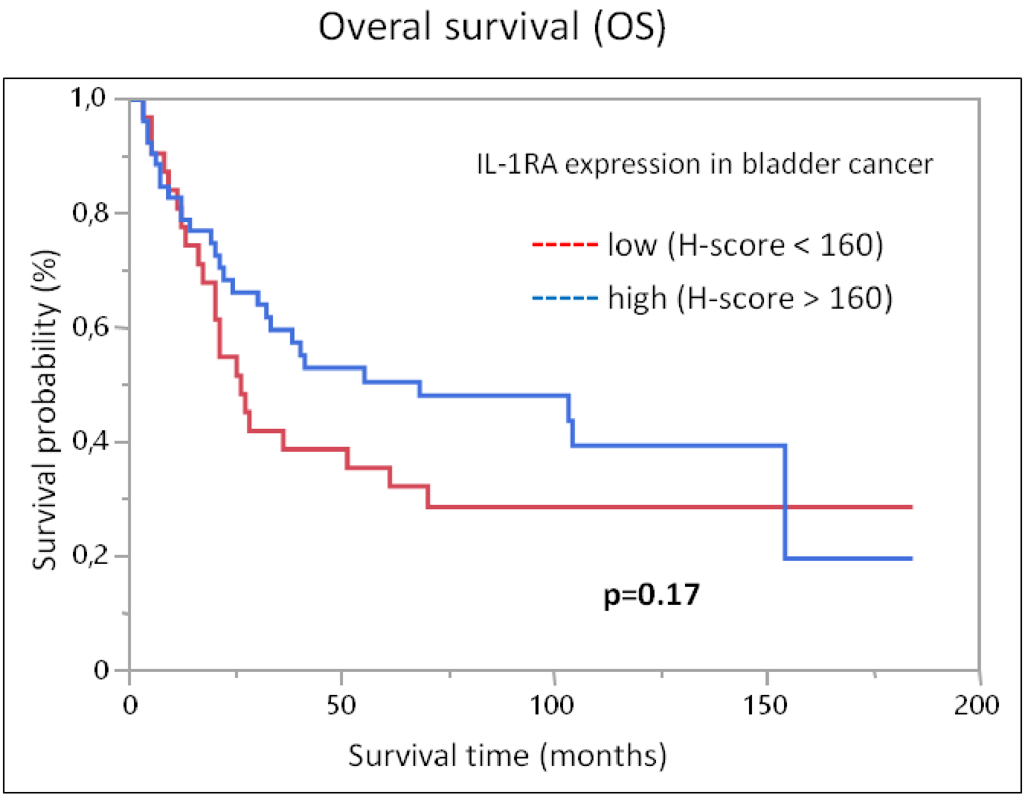


Figure 32. Kaplan-Meier estimates for overall survival in dependence of expression levels of IL-1RA within tumor cells. Adapted from Vukovic M et al., 2024 (119).

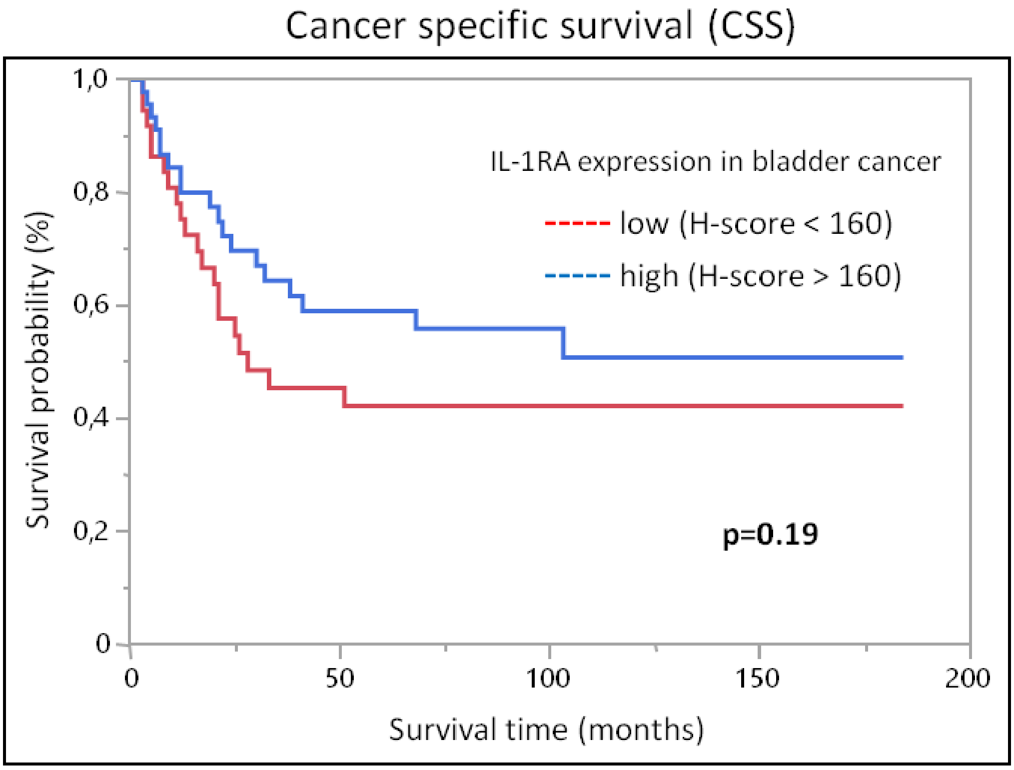


Figure 33. Kaplan-Meier estimates for cancer specific survival in dependence of cytoplasmatic cellular staining of IL-1RA within tumor cells. Adapted from Vukovic M et al., 2024 (119).

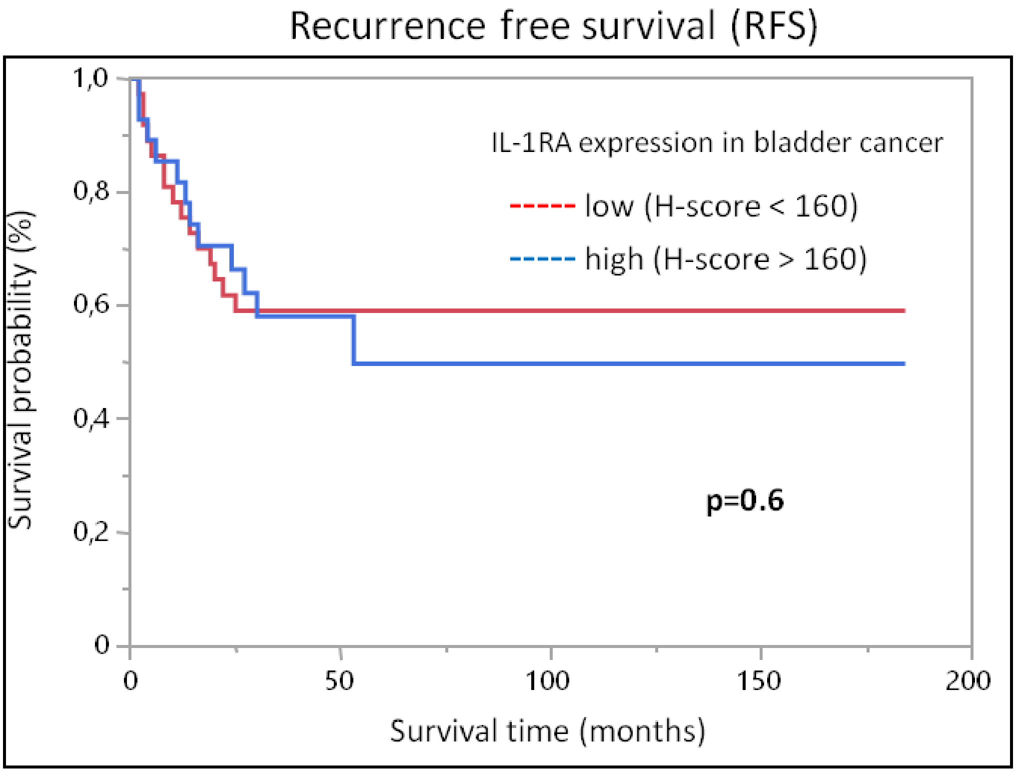


Figure 34. Kaplan-Meier analysis for recurrence free survival in according to cytoplasmatic cellular staining of IL-1RA within tumor cells. Adapted from Vukovic M et al., 2024 (119).

5 Univariate and multivariate analysis

All investigated parameters were associated with CSS and OS in univariate analysis, with IL-1β as a single variable associated with OS, as well. In multivariate analysis, only IL-1ß was confirmed as an independent predictor of RFS, CSS, as well as OS (Table 5).

Variable	Univariate analysis			Multivariate analysis		
Recurrence (RFS)	P	HR	95% CI	P	HR	95% CI
Pathological stage >T2 vs. T2	**0.0099**	3.25	1.33-7.95	0.1339	2.14	0.79-5.80
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	-		
IL-1β Low vs. High tumor expression (cut-off 160)	**0.0172**	3.16	1.23-8.16	**0.0265**	2.97	1.14-7.77
Age, year	0.2397	1.02	0.99-1.06	-		
Variable	Univariate analysis			Multivariate analysis		
CSS	P	HR	95% CI	P	HR	95% CI
Pathological stage >T2 vs. T2	**0.0107**	2.53	1.24-5.17	0.3407	1.51	0.65-3.49
Nodal status N+ vs. N0	**0.0309**	1.96	1.06-3.62	0.3170	1.48	0.69-3.17
Metastasis status M+ vs. M0	**0.0015**	3.56	1.63-7.79	0.0504	2.58	1.00-6.65
Grade ≥G3 vs. < G3	**0.0069**	3.62	1.42-9.22	0.1247	2.38	0.79-7.18
IL-1β Low vs. High tumor expression (cut-off 160)	**0.0343**	2.27	1.06-4.84	**0.0460**	2.35	1.02-5.42
Age, year	**0.0047**	1.05	1.01-1.08	0.3421	1.02	0.98-1.06
Variable	Univariate analysis			Multivariate analysis		
OS	P	HR	95% CI	P	HR	95% CI
Pathological stage >T2 vs. T2	**0.0020**	2.79	1.45-5.33	0.1240	1.85	0.85-4.03
Nodal status N+ vs. N0	**0.0258**	1.85	1.08-3.17	0.1909	1.60	0.79-3.24
Metastasis status M+ vs. M0	**0.0036**	3.13	1.45-6.73	0.1148	2.12	0.83-5.37
Grade ≥G3 vs. < G3	**0.0173**	2.31	1.16-4.60	0.1611	1.94	0.77-4.88
IL-1β Low vs. High tumor expression (cut-off 160)	**0.0082**	2.54	1.27-5.06	**0.0249**	2.40	1.12-5.18
Age, year	**0.0031**	1.05	1.02-1.08	0.1274	1.03	0.99-1.07

Table 5. Univariate and multivariate Cox regression analyses for recurrence-free survival (RFS), cancer-specific survival (CSS) and overall survival (OS) in the first cohort. Adapted from Vukovic M et al., 2024 (119).

6 Discussion

BCa is the most common cancer of the urinary tract and ranks fifth among cancers in men in western countries (47). Early diagnosis of bladder cancer is mainly confirmed by cystoscopy, after gross haematuria. Based on urine or urinary cells, only a few molecular markers have been approved so far, by the Federal Food and Drug Administration (FDA). These include BTA stat, BTA TRAK, NMP22 BladderChek Test, NMP22 ELISA, UroVysion, and uroCyt (122). Urine soluble markers should be able to ensure a primary diagnosis, follow-up control, and screening of high-risk populations. New biomarkers have been described in serum and urine, including nucleic acid or protein-based tissue biomarkers. Moreover, tissue-based biomarkers have also been emerging over the past few decades, aiming to predict tumor response and/or progressive potential of the disease (123). However, there are currently no FDA-approved predictive tissue-based biomarkers for therapy in BC.

6.1 Molecular subtypes and associated gene mutations

Similar to other cancers, BCa also exhibits tumor heterogeneity, resulting in differences in morphology, gene expression, proliferation, metabolism, aggressiveness, and drug resistance. Moreover, it seems that MIBC exhibit similar mutation rate to those of melanoma and non-small cell lung cancers (10). Tumor heterogeneity may cause the failure of cancer therapy, including chemotherapy and targeted immunotherapy. Therefore, it is highly important to elucidate the molecular mechanisms behind this event in urologic cancers. Two main molecular subtypes of MIBC have been described, luminal and basal/squamous, and this differentiation might be of therapeutical significance, leading to a more specified treatment response (10). This means that basal tumors show better response to NAC with consecutive prolonged survival, unlike tumors expressing p53 associated gene expression (p53 like-subtype), where bone metastases and resistance to chemotherapy are more common (12). In addition, luminal subtype show better OS across the studies, regardless of treatment choice.

Moreover, based upon different clinical behavior of non-invasive and invasive BCa, two distinct carcinogenic pathways have been suggested: the papillary and non-papillary pathway. Furthermore, the papillary pathway gives rise to LG tumors, unlike non-papillary, which leads to dysplasia/CIS or invasive, HG tumors (12). Papillary pathway means genomic stable LG disease with FGFR3 mutations in almost all LG Ta tumors. On the other hand, this mutation is rare in G3-T1 NMIBC (30%) and in MIBC (10%) (76, 124, 125). Therefore, cancers harboring this mutation are associated with low recurrence rate and favorable prognosis. This leads to conclusion that this gene might be excellent prognostic biomarker. However, we still need tissue markers that could change the treatment algorithm and prognosis for more aggressive, locally advanced, or metastatic disease. Understanding the molecular landscape of invasive BCa and its association with complex cellular mechanisms including autophagy, proliferation, or interleukin expression might bring new perspective to the poor prognosis of advanced BCa.

6.2 True predictive biomarker might improve treatment success

Molecular biomarkers are already used to estimate the treatment efficacy and to improve the survival of patients with various cancers. Despite this, prognostic tissue biomarkers in invasive BCa are still scarce, although emerging literature evidence has risen over the course of the last decade. Reasons behind this are likely multifactorial, including difficulties in profiling intratumoral heterogeneity, the lack of standardization in research methodology or complex dynamics of cellular processes (123). An ideal predictive biomarker should be measurable before the start of treatment and able to provide information on the likelihood of response to a specific therapy. The growing body of literature deals with neoadjuvant therapy related tissue biomarkers, evaluating the efficacy of neoadjuvant chemo or immunotherapy in MIBC. In this regard, different pathways have been identified, with DDR, ERCC2, ATM and FANCC as the most common predictors of cellular response to platinum-based chemotherapy. On the other hand, patients who had tumors with immune cell infiltration and high PD-1 protein expression exhibited favorable response to immunotherapy (77, 123, 126). This study investigated the interrelation of several tissue biomarkers and prognostic impact of IL-1 axis in invasive BCa, based upon cellular IHC expression within tumor tissue on cystectomy specimen.

6.3 IL-1 family in bladder cancer

IL-1β is a member of the IL-1 family of cytokines and may act as mediator of inflammatory response in cancer, involved in various cellular activities, including cell proliferation, differentiation and apoptosis (119, 127). Although many studies have determined the significant role of IL-1β in the occurrence and development of malignant tumors, none of these have been performed on urothelial tissue (120). However, due to complex repercussions on the course of cancer, both tumor promoting and inhibitory functions of IL-1 beta have been described though its specific role in carcinogenesis of urothelial cancer is yet to be determined (88). On the other hand, it seems that IL-1RA might play significant role in BCa carcinogenesis (128). This potential biomarker, encoded by the *IL1RN* gene, is a potent competitive antagonist to interleukin-1 (IL-1) and thereby is mainly involved in the regulation of inflammation. A downregulation of IL-1RA expression on IHC staining of MIBC tissue samples has been already described, with significantly reduced expression in invasive comparing to non-invasive disease (129). This confirms that IL-1RA might be recognized as important factor in bladder carcinogenesis and high expression might be related to tumor aggressiveness (128).

Our study investigated the expression patterns of IL-1β and IL-1RA in invasive BCa and a significant overexpression was observed when compared with normal bladder tissue. Moreover, IL-1ß expression was stronger in patients with adverse prognostic features (HG tumors (G3) and/or lymph-vascular invasion), unlike the IL-1RA, where lower expression correlates with a high tumor grade, resembling results of a German research group (128). Additionally when considering our study, IL-1RA was also significantly overexpressed in invasive BCa, as compared to benign urothelium, while significant co-expression between IL-1β and IL-1RA was observed only in benign tissue. We found a positive correlation between IL-1β expression and each survival pattern (CSS, OS, RFS). Similar results have been described in colorectal cancer (120). Alternatively, additional member of IL-1 family (IL-1α) showed higher IHC expression in tumors, rather than within stromal cells, with low expression levels associated with an increased risk for cancer-specific death (130). This might lead to conclusion that IL-1 family is important for BCa cell biology, and that measurements of these cytokines may be useful in pre-treatment characterization of urothelial cancer.

6.4 Autophagy in bladder cancer

Autophagy, an intracellular recycling system, has a significant impact on tumorigenesis with both tumor-promoting and tumor-supressive roles. The significance of this process is depicted through interrelations with interleukins, cytokines, and other immuno-modulating cells, which leads to modification of inflammatory factors and cellular homeostasis. One of the most questioned roles of autophagy is the cytoprotective role, which has been postulated as one of the causes of chemo-resistance of targeted tumor cells (131). Whether or not targeting the specific autophagy pathways might be a potential therapeutic strategy for preventing cancer progression or therapy resistance, should be answered in the near future. Autophagy initiation is mediated by several signal-sensing kinases. It has been addressed that bladder cancer heterogeneity and high mutational burden might be induced by frequent mutations in several signalling pathways, including cell-cycle genes, receptors tyrosine kinase, PI3K/AKT/mTOR, and chromatin regulatory gene mutations (132). Interactions between IL-1 axis and autophagy markers in invasive BCa is still largely unknown. It is noteworthy that recent evidence suggests the IL-1β/1RA axis regulates cell proliferation, migration, clone formation, and apoptosis *in vitro* via autophagy mechanisms (120). Additionally, it seems that autophagy controls production of IL-1β and pro-inflammatory cytokines through at least two separate mechanisms, predominantly with sequestration into autophagosomes. This effect might be both, stimulatory and inhibitory (133-136). According to results of our study, both IL-1β as well as IL-1β/IL-1RA ratio correlated positively to AKT expression in BCa. The true prognostic potential of this relation, is still unknown despite strong evidence of prognostic potential of IL-1β/IL-1RA axis in invasive BC modulated by AKT pathway (119) (Figure 35).

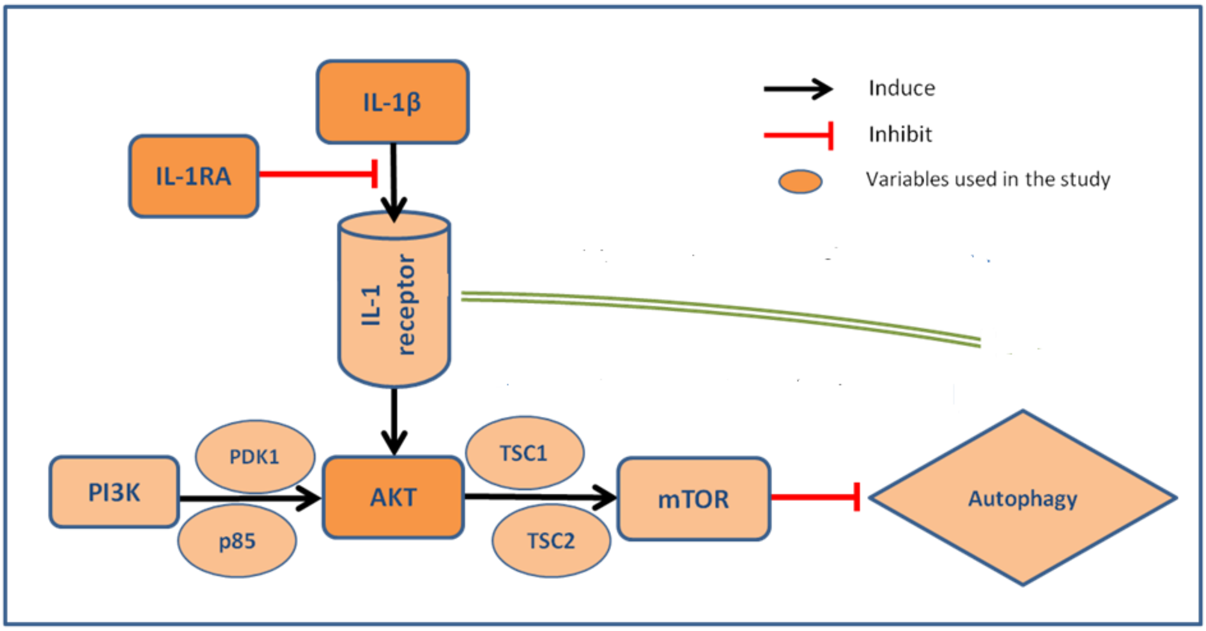


Figure 35. Possible relationship between IL-1β/IL-1RA axis and autophagy signaling pathway in invasive bladder cancer. With permission from Vukovic M et al., 2024 (119).

6.5 Ki-67 in bladder cancer

Ki-67 is a nuclear protein, associated with cellular proliferation. High expression of this proliferative marker within tumor tissue usually indicates poor survival and higher tumor recurrence in several cancers (137). Moreover, Ki-67 overexpression has been associated with poor outcomes for non-invasive urothelial BCa as well (72). Nevertheless, prognostic impact of proliferative markers in invasive BCa is yet to be determined, despite recent reports demonstrating worse survival if high Ki-67 expression is determined in a cystectomy specimen (138). Additionally, this biomarker may be a positive predictor of PD-L1 expression, thus aiding in the selection of candidates for post-chemotherapy MIBC (72). This study aimed to investigate if IL-1β/1RA axis might express positive correlation with Ki-67, suggesting better diagnostic and prognostic role of combined biomarkers, instead of each one separately. Indeed, results have shown that high expression of IL-1β within cancer tissue correlates positively with high staining of AKT and Ki-67 in tumor cells, thus improving prognostic potential of tissue markers, when combined. Finally contingency analysis confirmed significant association of combined expressions of Ki-67 *and* IL-1β, as well as Ki-67/ IL-1 ratio, with high tumor grade and vascular invasion within tissue specimen (119)

6.5 Pathways and interrelations of analysed biomarkers

The observed interrelations of IL-1β expression with proliferation and markers of autophagy suggests an approach for multivariate immunohistochemical profiles integrating improved prognostic and presumably also predictive accuracy by combining several markers. Indeed, IL-1β high expression showed positive correlation with both, AKT and Ki-67 high staining in tumor cells, unlike IL-1 RA (119). This supports previous studies reporting a high Ki-67 proliferation index in MIBC and more aggressive disease in general (139, 140). Although Ki-67 could serve as an independent prognostic factor in patients with invasive BCa, this is not the case with IL-1 axis or AKT. Autophagy has shown contrasting effects in the treatment of BCa. Therefore, a better understanding of its role in cancer treatment is crucial for the selection of effective drugs to target the autophagic pathway (141). However, data concerning IL-1β expression in BCa is currently scarce and results of our research might open new horizons in this regard. To the best of our knowledge, the present work is the first to evaluate the prognostic utility of IL-1β in invasive BCa (119). Therefore, the combined analysis of interleukins with autophagy and proliferation markers could bring new insight in a more detailed analysis of BCa behavior and its malignancy potential. Our results demonstrated significant correlation of combined Ki-67 and IL-1β score with vascular invasion, as well as with higher tumor grade. Although our analysis requires large-scale validation, it suggests a potential mechanism-based selection strategy, in which patients with higher expression of IL-1β in BCa tissue may have better immune response and potentially, better response to cancer treatment. The same conclusion has been reached by Rébé and Ghiringhelli, although it is emphasized that IL-1β may behave as a both, a cancer promoter and an anti-tumor interleukin, depending on tumor stage and origin. In fact, chemotherapy or radiation can trigger the production of IL-1β by either cancer cells or tumor infiltrating cells and influence the behavior this interleukin. Therefore, IL-1β may alternatively favor or inhibit therapy-mediated anti-tumor immune response (88). Based on these facts, many antibodies have already been developed to block IL-1β. The IL-1RA anakinra is one of the most widely used in pre-clinical studies, acting as a competitive inhibitor of IL-1α and IL-1β receptors (142, 143). Results showed that this IL-antagonist decreased proliferative rate of certain tumors, refractory to standard therapies. However, specific role in BCa has not been investigated thus far. Our study will potentially influence future research in this regard, considering that the IL-1β/IL-1RA axis has shown promising prognostic results, especially when combined with other biomarkers such as Ki-67 and AKT.

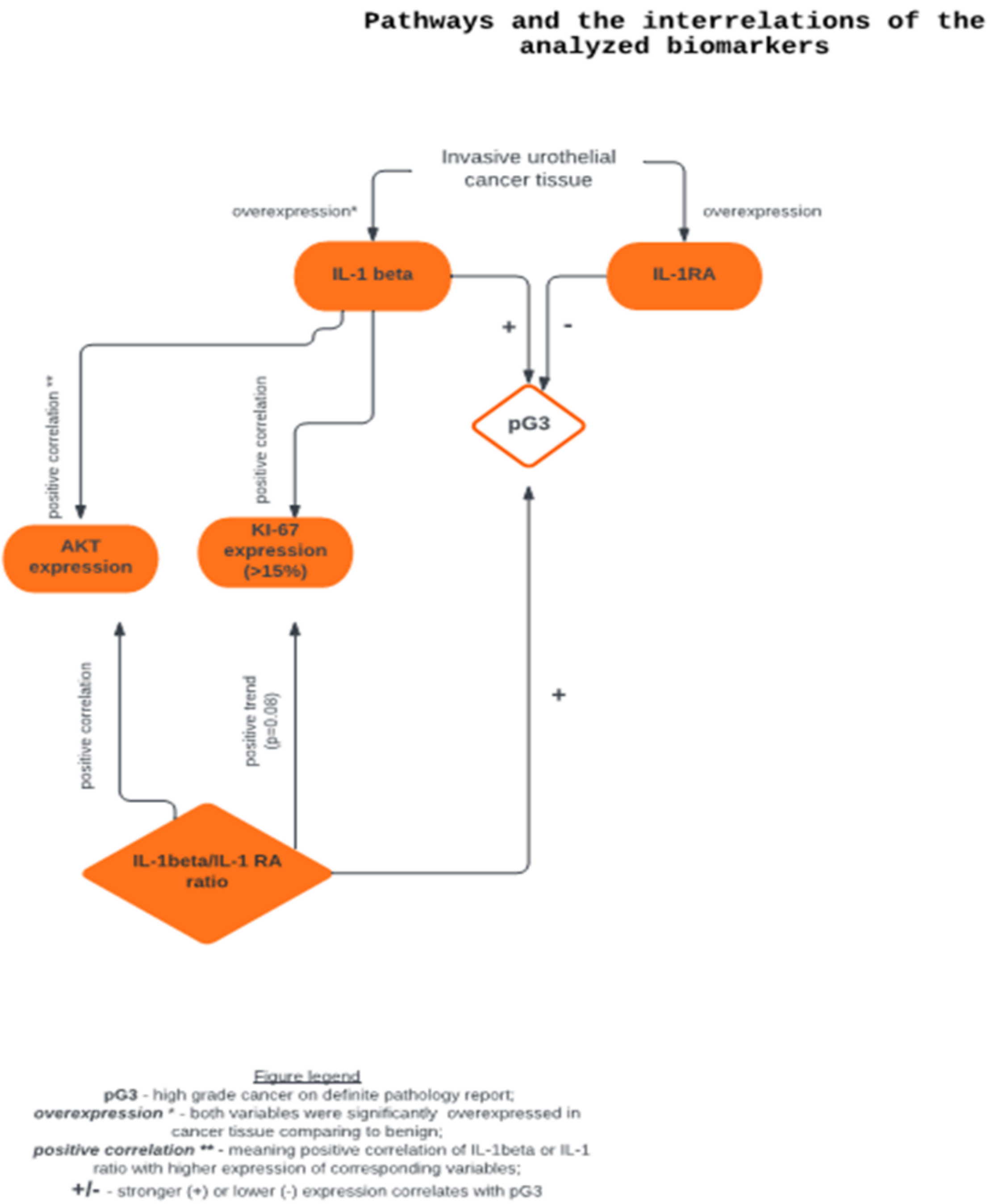


Figure legend
pG3 - high grade cancer on definite pathology report;
***overexpression** ** - both variables were significantly overexpressed in cancer tissue comparing to benign;
***positive correlation** *** - meaning positive correlation of IL-1beta or IL-1 ratio with higher expression of corresponding variables;
+/- - stronger (+) or lower (-) expression correlates with pG3

Figure 36. Possible interrelations of the analysed biomarkers in invasive bladder cancer.

6.6 Future directions of IL-1β/IL-1RA axis and its potential as prognostic biomarker

The prognostic role of IL-1β and its axis in BCa is still unknown. Another member of the IL-1 family (IL-α) has high expression in urothelial cancer tissue and this expression significantly correlates to worse OS and PFS (21). Interestingly, our results revealed positive correlation of survival pattern and high expression of IL-1β in cancer tissue (119). In addition, it seems that specific threshold values for IL-1β/IL-1RA-ratio (cut-off 0.7) might be given to determine frequency of aggressive tumors. This supports the previous, decade-long efforts dedicated to quantification of predictive biomarkers of immunotherapy in BCa. Nevertheless, future directions in this regard should be based on genetic analysis with determination of most common gene alterations, with the final goal to determine real-time prognostic and predictive biomarker for monitoring patients with urothelial cancer (144). Recently introduced circulating-tumor DNA (ctDNA) has emerged as a powerful biomarker in oncology because of its utility in monitoring disease relapse and treatment response in multiple cancer types, including BCa. Additionally, gene encoding IL-1RA (IL1RN) and ILRN mRNA levels seem to regulate migration and tissue invasion capacities of BCa cell lines (128). Since analysis of frequently mutated genes in invasive BCa suggests potential targets for personalized treatment and predictors of treatment response, future work on IL-1 genetic profile might bring new light in this regard. According our results, IL-1β is a promising clinical tool in future treatment and prognosis of invasive BCa.

7 Conclusion

Based on the results of this first analysis, IL-1β may be reliable prognostic biomarker of BCa malignancy potential. Moreover, high expression of this cytokine predicts better survival among these patients. To determine whether or not the observed association is causal, an additional mechanistic and genetic work-up is needed. Nevertheless, to the best of our knowledge, the present work is the first to evaluate the prognostic utility of the IL-1β and IL-1 axis for invasive BCa patients after RC. Using IHC tumor tissue expression of IL-1β and IL-1RA, we determined higher expression of these cytokines in more aggressive tumors with positive correlation between proliferation and autophagy markers. The additional predictive value of IL-1β was presented through better survival patterns with higher IL tissue expression. The exact explanations for these opposing effects are still unknown. The micro-environment, grade, and molecular subtype of the cancer may all participate in this divergence. In any case, the potential use IL-1β blockers should be carefully considered in a clinical context. More research is needed to further evaluate the role of IL-1β/1RA axis in urothelial cancer, especially the potential of IL-1β in the context of newly emerging biomarkers in invasive BCa.

8 Abstract

Interleukin beta (IL-1ß), a member of the IL-1 cytokine family, was shown to have prognostic impact in oesophageal and colorectal cancer. IL1 receptor antagonist (IL-1RA) binds to the IL-1 receptor as competitive antagonist and is mainly involved in the regulation of cell proliferation and inflammation. Previous data indicate a role of IL-1 and IL-1RA imbalance in urothelial carcinoma (UC) where pharmacologic inhibition of IL-1 signalling might be considered as therapeutic option. The aim of our study was to assess expression patterns and investigate the prognostic role of IL-1ß and IL-1RA in invasive UC. Moreover, we evaluated their interaction with markers of autophagy (AKT) and proliferation (Ki-67) in urothelial bladder cancer.

The study included 194 patients selected into two independent groups, 102 in a discovery and 92 patients in confirmatory group, who underwent radical cystectomy for UC between February 1996 - December 2006 and June 2003 - December 2010, respectively. Specimen from cancer tissue and benign surrounding urothelium ($n=22$ and $n=39$) were processed to a tissue microarray and immunohistochemically stained for IL-1ß, IL-1RA, AKT and Ki-67. Expression was quantified by the histochemical scoring system (H-score 0-300) and the ratio of IL-1ß/IL-1RA was assessed. Scores in UC and benign tissue were compared to clinical data in both cystectomy cohorts and correlated to Ki-67 and AKT expression. Relationships with outcome were analyzed using Wilcoxon Kruskal-Wallis tests, Chi-square tests or linear regression analyses, dependent on the variable's category. Kaplan–Meier analyses were used to estimate recurrence-free (RFS), cancer-specific (CSS) and overall survival (OS) by the log-rank test.

Both, Il-1ß and IL-1RA were significantly overexpressed in invasive UC compared to benign urothelium in both cohorts ($p<0.005$). In benign urothelium, Il-1ß and IL-1RA were co-expressed ($p<0.0004$, confirmed <0.05). IL-1ß was overexpressed in patients with vascular invasion (210 vs. 183, $p<0.02$), lymphatic invasion (210 vs. 180, <0.05) and with G3 UC (192 vs. 188, <0.04). Furthermore, G3 UC showed lower IL1-RA (<0.003) and IL-1ß/IL-1RA ratios scored significantly higher (1.10 vs. 0.66, <0.003). Survival analysis revealed favourable recurrence free (RFS)-, cancer specific (CSS)-, and overall survival OS) in case of high IL-1ß expression (H-score$\geq$160 each, $p<0.02$,

<0.03 and <0.006, respectively). Multivariate analyses revealed an independent prognostic impact of (low) IL1ß expression on RFS, CCS and OS. IL-1ß as well as IL-1ß/IL-1RA ratio was positively correlated to AKT expression (p<0.05 and <0.01, respectively). Additionally, high staining of Ki-67 (>15%) correlated with higher expression pattern of IL-1β (p=0.01).

Overexpression of IL-1ß and IL-1RA is frequently found in UC. Interestingly, co-expression is found only in benign urothelium. Strong correlation to dedifferentiation of UC was observed between the members of the IL-signalling axis, especially IL-1ß. Moreover, a prognostic significance was observed for IL-1ß protein expression in UC. The observed link between the IL-1ß / IL-1RA axis and AKT signalling may indicate possible autophagy activation processes beside the known oncologic effects of AKT activation.

9 Zusammenfassung

Interleukin-Beta (IL-1ß), ein Mitglied der IL-1-Zytokinfamilie, wurde als prognostischer Faktor bei Speiseröhren- und Darmkrebs identifiziert. Der IL-1-Rezeptor-Antagonist (IL-1RA) bindet als kompetitiver Antagonist an den IL-1-Rezeptor und ist hauptsächlich an der Regulierung der Zellproliferation und Entzündung beteiligt. Frühere Daten deuten auf eine Rolle des Ungleichgewichts von IL-1 und IL-1RA beim Urothelkarzinom (UC) hin, wobei eine pharmakologische Hemmung der IL-1-Signalübertragung als therapeutische Option in Betracht gezogen werden könnte. Ziel unserer Studie war es, die Expressionsmuster von IL-1ß und IL-1RA im invasiven UC zu bewerten und deren prognostische Rolle zu untersuchen. Darüber hinaus haben wir ihre Wechselwirkungen mit Markern der Autophagie (AKT) und der Proliferation (Ki-67) im Urothelkarzinom der Blase untersucht.

Die Studie umfasste 194 Patienten, die in zwei unabhängige Gruppen unterteilt wurden: 102 Patienten in der Entdeckungsgruppe und 92 in der Bestätigungsgruppe, die sich zwischen Februar 1996 - Dezember 2006 bzw. Juni 2003 – Dezember 2010 einer radikalen Zystektomie aufgrund eines UC unterzogen hatten. Proben aus

Tumorgewebe und umgebendem gutartigem Urothel (n=22 bzw. n=39) wurden zu einem Gewebemikroarray verarbeitet und immunhistochemisch für IL-1ß, IL-1RA, AKT und Ki-67 gefärbt. Die Expression wurde durch das histochemische Bewertungssystem (H-Score 0-300) quantifiziert, und das Verhältnis von IL-1ß/IL-1RA wurde ermittelt. Die Werte im UC- und im gutartigen Gewebe wurden mit klinischen Daten in beiden Zystektomie-Kohorten verglichen und mit der Ki-67- und AKT-Expression korreliert. Die Beziehungen zum klinischen Ausgang wurden unter Verwendung von Wilcoxon-Kruskal-Wallis-Tests, Chi-Quadrat-Tests oder linearen Regressionsanalysen, abhängig von der Variablenkategorie, analysiert. Kaplan-Meier-Analysen wurden verwendet, um das rezidivfreie Überleben (RFS), das krebsspezifische Überleben (CSS) und das Gesamtüberleben (OS) mithilfe des Log-Rank-Tests zu schätzen.

Sowohl IL-1ß als auch IL-1RA waren in invasiven UC im Vergleich zu gutartigem Urothel in beiden Kohorten signifikant überexprimiert (p<0,005). Im gutartigen Urothel wurden IL-1ß und IL-1RA gemeinsam exprimiert (p<0,0004, bestätigt <0,05). IL-1ß war bei Patienten mit Gefäßinvasion (210 vs. 183, p<0,02), lymphatischer Invasion (210 vs. 180, <0,05) und G3-UC (192 vs. 188, <0,04) überexprimiert. Außerdem wiesen G3-UC niedrigere IL1-RA-Werte auf (<0,003), und das IL-1ß/IL-1RA-Verhältnis war signifikant höher (1,10 vs. 0,66, <0,003). Die Überlebensanalyse zeigte ein günstiges rezidivfreies (RFS), krebsspezifisches (CSS) und Gesamtüberleben (OS) bei hoher IL-1ß-Expression (H-Score ≥160 jeweils, p<0,02, <0,03 und <0,006). Multivariate Analysen zeigten einen unabhängigen prognostischen Einfluss von niedriger IL-1ß-Expression auf RFS, CSS und OS. IL-1ß und das IL-1ß/IL-1RA-Verhältnis korrelierten positiv mit der AKT-Expression (p<0,05 bzw. <0,01). Darüber hinaus korrelierte eine hohe Ki-67-Färbung (>15 %) mit einem höheren IL-1ß-Expressionsmuster (p=0,01).

Eine Überexpression von IL-1ß und IL-1RA wird häufig beim UC gefunden. Interessanterweise tritt die gemeinsame Expression nur im gutartigen Urothel auf. Es wurde eine starke Korrelation zwischen der Dedifferenzierung von UC und den Mitgliedern der IL-Signalkaskade, insbesondere IL-1ß, beobachtet. Darüber hinaus wurde eine prognostische Bedeutung der IL-1ß-Proteinexpression bei UC festgestellt. Der beobachtete Zusammenhang zwischen der IL-1ß/IL-1RA-Achse und der AKT-

Signalgebung könnte auf mögliche Autophagie-Aktivierungsprozesse neben den bekannten onkologischen Effekten der AKT-Aktivierung hinweisen.

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11 List of abbreviations

AKT - protein kinase B

AUA - American Urological Association

BCa - bladder cancer

BCG - Bacille Calmette-Guerin

BLC - blue light cytoscopy

CIS - carcinoma in situ

CSM - cancer-specific mortality

CSS - cancer specific survival

CT - computer tomography

EAU - European Association of Urology

ECOG - Eastern Cooperative Oncology Group

EGFR - human epidermal growth factor receptor

ERBB2 - human epidermal growth factor receptor 2

ERRC2/3 - growth factor receptors

FDA - Food and Drug Administration

FGFR3 - fibroblast growth factor 3 receptor

G1,G2,G3 - tumor grade (I, II, III)

GC - gemcitabine and cysplatin

HER2 inhibitors - human epidermal growth factor receptor 2

HG – high-grade tumor

LG – low-grade tumor

ICI - immunotherapy

IHC - immunohistochemistry

IL-1β - interleukin 1-beta

IL-1RA - interleukin receptor antagonist

Ki-67 - nuclear protein that is associated with cellular proliferation

LVI - lymphovascular invasion

MIBC - muscle-invasive BCa

MRI - magnetic resonance imaging

MVAC - methotrexate, vinblastine, doxorubicine and cisplatin

NAC - neoadjuvant cisplatin-based chemotherapy

NICE - National Institute for Health and Care Excellence

NMIBC - non muscle-invasive BCa

OS - overall survival

PARP inhibitors - poly ADP ribose polymerase inhibitor

PDD - photodynamic diagnosis

PD-L1 - program-cell death ligand

PET/ CT - positron emission tomography scan

PTEN - phosphatase and tensin homolog deleted on chromosome 10

PUNLMP - papillary urothelial neoplasm of low-malignant potential

RB1 - retinoblastoma susceptibility gene

RC - radical cystectomy

RFS - recurrence free survival

TERT - telomerase reverse transcriptase

TMA - tissue microarray

TMT - trimodal therapy

TNM - staging system that includes the extent of the tumor (T), extent of spread to the lymph nodes (N), and presence of metastasis (M)

TP53 - tumor suppressor gene for protein p53

TURBT - transurethral resection of bladder tumor

VEGF - vascular endothelial growth factor

WHO - World Health Organisation

12 Erklärungen zum Eigenanteil

Im Rahmen dieser Dissertation habe ich eigenständig die folgenden Schritte durchgeführt: Zunächst habe ich die Literaturrecherche zur Identifikation relevanter Studien und Publikationen durchgeführt. Anschließend habe ich die Probanden für die Studie rekrutiert und die erhobenen Daten gemäß den ethischen Richtlinien verarbeitet. Die statistische Analyse der Daten wurde mit Hilfe von SPSS durchgeführt, wobei ich eigenständig die deskriptiven Statistiken und Regressionsanalysen erstellte. Die Interpretation der Ergebnisse und deren Diskussion basieren auf meinen eigenen Überlegungen und einer kritischen Auseinandersetzung mit der bestehenden Literatur. Ich habe außerdem die Grafiken und Tabellen erstellt und die Manuskripte für die Publikation vorbereitet. Die abschließende Erstellung und Strukturierung der Dissertation habe ich ebenfalls selbstständig vorgenommen.

Die Idee zur Konzeption dieser Studie wurde im Bereich Uro-Onkologie der Universitätsklinik für Urologie Tübingen entwickelt. Daran beteiligt waren Prof. Dr. med. Arnulf Stenzl, Prof. Dr. med. Igor Tsaur, Prof. Dr. med. Steffen Rausch, Dr.med. Tilman Todenhöfer, Dr.med. Sebastian Jersinovic, Dr.med. Thomas Lüftrenk, Dr.med. Jorge M.Chamlati und Dipl.-Biol. Jörg Hennenlotter. Herr Prof.dr.med.Steffen Rausch und Jörg Hennenlotter haben diese Arbeit aktiv betreut, gaben Hilfestellung bei der statistischen Auswertung sowie Inspirationen und Korrekturvorschläge bei der schriftlichen Ausarbeitung der Dissertationsschrift.

13 Publications

Parts of this dissertation have already been published in the following publication:

- Vukovic M, Chamlati JM, Hennenlotter J, Todenhöfer T, Lütfrenk T, Jersinovic S, Tsaur I, Stenzl A, Rausch S. Interleukin-1β/Interleukin (IL)-1-Receptor-Antagonist (IL1-RA) Axis in Invasive Bladder Cancer-An Exploratory Analysis of Clinical and Tumor Biological Significance. *Int J Mol Sci*. 2024 Feb 19; 25(4):2447.

14 Acknowledgement

The results of this dissertation were primarily based on our work published in the *International Journal of Molecular Sciences*, entitled "*Interleukin-1β/Interleukin (IL)-1receptor antagonist (IL1-RA) axis in invasive bladder cancer – an exploratory analysis of clinical and tumor biological significance*" (*ijms-2839711; doi: 10.3390/ijms25042447*), for which we received written approval from the editors for citation. The whole research project was supported by EUSP scholarship grant of the European Association of Urology (S-2021-0013).

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