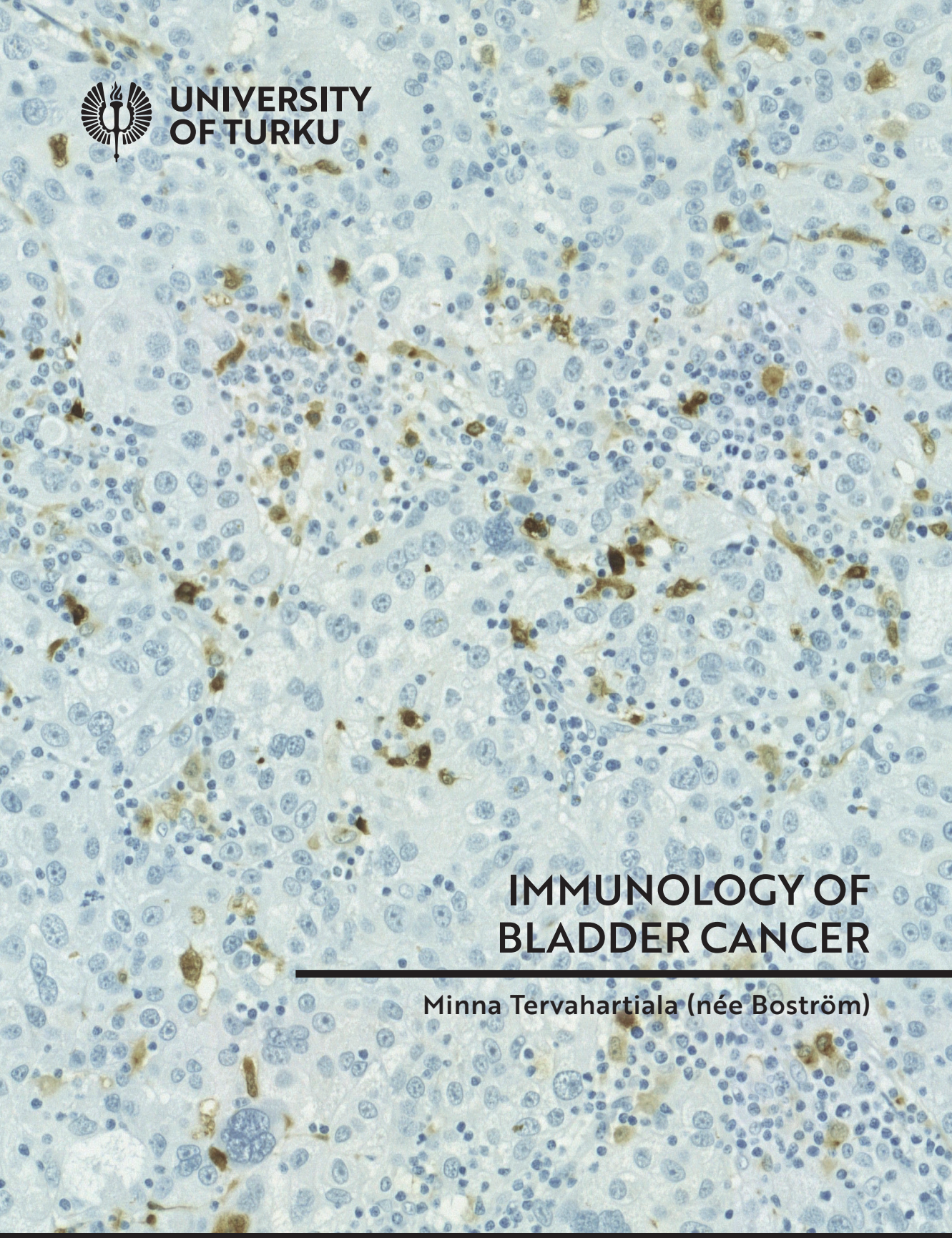

UNIVERSITY OF TURKU

IMMUNOLOGY OF BLADDER CANCER

Minna Tervahartiala (née Boström)

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA - TOM. 1440 | MEDICA - ODONTOLOGICA | TURKU 2019

UNIVERSITY OF TURKU

IMMUNOLOGY OF BLADDER CANCER

Minna Tervahartiala (née Boström)

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. D OSA - TOM. 1440 | MEDICA - ODONTOLOGICA | TURKU 2019

University of Turku

University of Turku
Faculty of Medicine
Department of Medical Microbiology and Immunology
Turku Doctoral Programme of Molecular Medicine (TuDMM)
MediCity Research Laboratory, Turku, Finland

Supervised by

Professor Sirpa Jalkanen, M.D., Ph.D.
MediCity Research Laboratory,
Department of Medical Microbiology
and Immunology,
University of Turku, Turku, Finland

Docent Peter J. Boström, M.D., Ph.D.
Department of Urology,
Turku University Hospital,
Turku, Finland

Reviewed by

Professor Risto Renkonen, M.D., Ph.D.
Faculty of Medicine and HUSLAB,
University of Helsinki and Helsinki
University Hospital, Helsinki, Finland

Docent Amir Sherif, M.D., Ph.D.
Department of Surgical and
Perioperative Sciences,
Urology and Andrology,
Umeå University, Umeå, Sweden

Opponent

Docent Fredrik Liedberg, M.D., Ph.D.
Department of Urology,
Skåne University Hospital,
Lund University, Malmö, Sweden

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

Cover Image: Minna Tervahartiala

ISBN 978-951-29-7742-0 (PRINT)
ISBN 978-951-29-7743-7 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Grano Oy - Turku, Finland 2019

You don't grow when you're comfortable
So fix your ponytail and try again

TURUN YLIOPISTO
Lääketieteellinen tiedekunta
Tutkimuslaboratorio MediCity, Lääketieteellinen Mikrobiologia ja Immunologia, Turku (2019)

MINNA TERVAHARTIALA: Virtsarakon syövän immunologia
Väitöskirja, 170 s.
Molekyylilääketieteen tohtoriohjelma (TuDMM)
05/2019

Tiivistelmä

Virtsarakon syöpä on sairaus, jonka ennuste vaihtelee laajalti taudin vaikeusasteen mukaan. Paikallisilla, rakon lihakseen ulottumattomilla tautimuodoilla on hyvä ennuste, mutta ne myös uusiutuvat herkästi ja saattavat edetä vaikeammaksi taudiksi. Lihakseen ulottuvalla taudilla on huonompi ennuste ja kuolleisuus on korkea. Suuren uusiutumisriskin vuoksi virtsarakon syöpä on yksi kalleimmista syöpäsairauksista hoitaa.

Väitöskirjan tavoitteena oli tutkia immunologisia tekijöitä virtsarakon syövässä. Makrofageja tunnistavien molekyylien, CD68, MAC387 ja CLEVER-1, ennusteellista merkitystä virtsarakon syövässä tutkittiin immunohistologisin keinoin. Molekyylejä tutkittiin virtsarakon syövän taudin kulkua ennustavina sekä neoadjuvantin sytostaattihoidon vastetta ennustavina tekijöinä. Lisäksi CD73-molekyyliä tutkittiin virtsarakon syövän eri solutyypeissä.

Tutkimustulokset osoittivat, että kasvainmakrofagit liittyvät huonoon ennusteeseen virtsarakon syövässä. MAC387- ja CLEVER-1-molekyylit ennustavat neoadjuvantin kemoterapian vastetta leikkauspotilailla. Myös CD73-molekyyli ennustaa kuolleisuutta virtsarakon syöpään, mutta CD73:n esiintyminen ja merkitys eri solutyypeissä virtsarakonsyöpäkasvaimissa vaihtelee kuitenkin suuresti.

Yhteenvetona voidaan todeta, että CD68, MAC387 ja CLEVER-1 toimivat ennusteellisina tekijöinä virtsarakon syövässä. Makrofageja voidaan mahdollisesti käyttää ennustamaan virtsarakon syövän taudinkulkua ja tunnistamaan potilaat, joiden uusiutumisriski on kohonnut. MAC387 ja CLEVER-1 ovat myös mahdollisia neoadjuvantin sytostaattihoidon vasteen ennustetekijöitä. Myös CD73-molekyyli toimii ennusteellisena tekijänä virtsarakon syövässä, mutta tämän molekyylin merkitys vaihtelee eri solutyypeissä. Tutkimus herättää uusia kysymyksiä immunologisista tekijöistä virtsarakon syövässä ja lisää tutkimuksia tarvitaan tulosten vahvistamiseksi.

Avainsanat: Virtsarakon syöpä, makrofagit, ennusteelliset tekijät, immunologia

UNIVERSITY OF TURKU
Faculty of Medicine, MediCity Research Laboratory, Department of Medical Microbiology and Immunology (2019)

MINNA TERVAHARTIALA: Immunology of bladder cancer
Doctoral Dissertation, 170 pp.
Turku Doctoral Programme of Molecular Medicine (TuDMM)
05/2019

Abstract

Bladder cancer (BC) is a highly prevalent disease with wide variety in outcome. Non-muscle invasive tumors have good prognosis but are prone to recur and, furthermore, have a major risk of progression. Muscle-invasive disease has significantly poorer survival with high mortality. Due to the high recurrence rate of the disease, BC is one of the most expensive cancers to treat.

The aim of the present study was to investigate the roles of the immunological biomarkers in BC. Macrophage markers CD68, MAC387, and CLEVER-1 were evaluated as prognostic factors in BC. Muscle-invasive bladder cancer patients receive neoadjuvant chemotherapy before definitive surgery and the predictive role of the biomarkers in neoadjuvant treated patients was also investigated. In addition, the prognostic role of CD73 in different cellular components of bladder tumors was studied.

The results of the study show, that tumor-associated macrophages associate with poor outcome in BC. In contrast, CLEVER-1 positive vessels show protective role in BC. MAC387 and CLEVER-1 are predictive biomarkers in neoadjuvant treated patients. CD73 has also a prognostic role in BC. However, the expression of CD73 varies greatly between different cellular components.

As conclusion, CD68, MAC387, CLEVER-1, and CD73 have prognostic and predictive role in BC. Macrophage markers are potential prognostic biomarkers in BC. MAC387 and CLEVER-1 could separate the patients who would benefit from the neoadjuvant chemotherapy from patients who might suffer from delayed operation and toxicity without the benefit from the treatment. CD73 has a prognostic role in BC, but it is important to investigate the expression in different cells and not to draw conclusions based on the expression status only on one cell type in the tumor. New questions about immunological factors in BC arise from the study and new studies are needed to validate the results.

Keywords: Bladder cancer, macrophages, prognostic biomarker, predictive biomarker, immunology

Table of Contents

List of original publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals (I-III):

I Boström MM, Irjala H, Mirtti T, Taimen P, Kauko T, Ålgars A, Jalkanen S, and Boström PJ. Tumor-Associated Macrophages Provide Significant Prognostic Information in Urothelial Bladder Cancer. PLoS One. 2015;10(7)

II Tervahartiala M, Taimen P, Mirtti T, Koskinen I, Ecke T, Jalkanen S, and Boström PJ. Immunological tumor status may predict response to neoadjuvant chemotherapy and outcome after radical cystectomy in bladder cancer. Scientific Reports. 2017;7(1)

III Koivisto M*, Tervahartiala M*, Kenessey I, Jalkanen S, Boström PJ, and Salmi M. Cell-type-specific CD73 expression is an independent prognostic factor in bladder cancer. Carcinogenesis. 2018;40(1)

Introduction

Cancer is a heterogenous group of diseases with different prognosis and treatment alternatives depending on the type of cancer. Even among same cancer type, survival can vary widely and the aggressiveness of the treatment modalities needs to be chosen individually according to the risks of the disease. However, it is not simple to predict patient's prognosis and the risk for recurrence and progression of the disease. Biological markers are needed to guide the decision-making among the treatment alternatives. It is important to be able to identify accurately patients with worse prognosis and higher risk for recurrence, progression, and death. Prognostic biomarkers are designed to aid in this. In addition, biomarkers are needed to identify which patients will benefit from specific treatments and to which patients the treatment causes possibly only toxic side effects and delay for the more effective treatment. Predictive biomarkers are used to separate responders from non-responders to treatment to limit the side effects and optimize the treatment.

Bladder cancer (BC) is a disease requiring new biomarkers to the clinical work. Biomarkers are needed to identify the patients with more aggressive disease and to guide the clinicians to select effective treatment alternatives to each patient. Local disease is readily treatable and the mortality is low. However, BC is prone to recur and progress to more aggressive and potentially fatal invasive disease. The continuous follow-up causes major social costs and individual inconveniences or decreased quality of life. After metastatic spread of the disease treatment alternatives are limited and the mortality is high.

In addition to the need for prognostic biomarkers, there is a lack of predictive biomarkers to select the right treatment options to each BC patient. Neoadjuvant chemotherapy prior to the surgery is effective to up to half of the patients. The prediction, who will benefit from the treatment (responders) and who will suffer from the toxicity of the chemo agents and delay of the surgery (non-reponders), is challenging. New immunotherapies have brought light to the treatment of advanced BC. However, there are no tools to predict the most suitable patients to these treatment options, either.

Immunological processes are known to have a major effect to development of cancer. Immunological cells are a crucial part of the tumor microenvironment. Immune cells and mediators have been studied widely as prognostic and predictive biomarkers in different cancer types, but more knowledge is needed to specify their possible role as biomarkers in BC.

Review of literature

1 Cancer

Cancer is a heterogeneous group of diseases with abnormal cell growth and potential to invade and metastasize. Over one hundred types of cancer affect humans and the symptoms vary according to the origin of the tumor.[1] In 2015, the incidence of all new cancers was 18.6 million.[2] In Finland, there were 33 000 new cancer patients in 2015 (610 per 100 000) and 270 000 patients were living with diagnosed cancer.[3]

Cancers are caused by several factors acting simultaneously. The risk of cancer increases with age, but the most important lifestyle exposures are tobacco smoking, dietary factors, alcohol consumption, and obesity.[3, 4] Fundamentally, cancer is a disease of tissue growth dysregulation, where the tumor develops from cells escaping the normal cell control and acquiring several capabilities required to form a malignant tumor. "The hallmarks of cancer" by Douglas Hanahan and Robert A. Weinberg constitute an organizing principle for rationalizing the complexities of malignant disease.[5] The development of tumor depends upon changes between the cancer cells and their normal neighboring cells. The hallmarks include (I) self-sufficiency in growth signals; (II) insensitivity to inhibitory signals; (III) resistance of the programmed cell death or apoptosis; (IV) the indefinite capability to replicate; (V) stimulation of angiogenesis to supply nutrients to tumors; and (VI) the capability to invade and spread to distant sites. In 2011, the hallmarks were updated with two new emerging hallmarks, deregulated metabolic pathways and evading the immune system. In addition, genome instability and inflammation were added to the list as enabling characteristics, since their acquisition leads to the development of the cancer hallmarks. Thereby, inflammation has a dual role in cancer development: on one hand, it can be tumor promoting assisting the cancer development by e.g. supplying growth factors and proangiogenic factors and supporting invasion and metastasis, but on the other hand, inflammatory cells also fight against cancer cells and malignant cells need to require a capability to evade the immune destruction to develop an invading and metastasizing tumor.

In 2015, cancer killed 8.8 million people worldwide and 12 200 in Finland..[3, 6] Traditionally, cancer is treated with surgery, chemotherapy, and radiation therapy. However, new therapies and diagnostic tools are studied constantly. Due to the massive cancer research, the knowledge about preventing cancer is increasing, diagnoses are made in early phase of the disease, and the overall cancer mortality is decreasing.

2 Bladder cancer

2.1 Epidemiology and etiology

BC is globally the ninth most common cancer with estimated 430 00 new diagnosed cases worldwide in 2012 (the age-adjusted incidence rate of 5.3/100 000). It is the 13th most common cause of cancer death and the most common malignancy involving the urinary system.[7, 8] Three-quarters of all cases are in men, and majority of the BC cases are occurring in the western countries.[9] The median age at the diagnosis is 69 years in men and 73 in female. Ninety percent of the people with BC are older than 55.[10] In Finland, BC is the fourth most common cancer in male (incidence 34/100 000) and 15th in female (incidence 11/100 000), with 1200 new diagnoses in 2012. The 5-year surveillance of BC in Finland is 74%.[3]

Tobacco smoking is the most important risk factor for BC and explains partly the differences in incidences between geographical regions and gender.[11, 12] Occupational exposure to different chemicals in painting, rubber and aluminium industries is considered as the second most important risk factor for BC.[11] The most common parasitic infection after malaria, Schistosoma haematobium, a parasitic worm in the urinary tract, causes BC in its endemic regions in Northern and sub-Saharan Africa.[13] Although BC is not an inherited disease, the risk for BC is almost two-fold higher in first-degree relatives.[14] Inherited genetic factors, such as N-acetyltransferase 2 (NAT2) variants and gluthathione S-transferase-µ1 (GSTM1)-null genotypes, have been considered to increase the risk for BC via sensitizing to extrinsic carcinogens, such as tobacco smoke.[11]

2.2 Diagnosis of bladder cancer

The most common symptom of BC is gross painless haematuria (in 80-85% of the BC patients). The incidence of urological cancer in patients with macroscopic hematuria varies between 19 and 24%. (20) Unexplained urinary frequency, urgency, and irritative voiding symptoms are also possible signs of a urinary tumor. (21) Fatigue, weight loss, renal failure, respiratory symptoms, and a suprapubic palpable mass are signs of an advanced or metastatic disease.[15]

When a diagnosis of BC is suspected, the initial assessment consists of urine cytology, cystoscopy, and radiological investigation of the upper urinary tracts. Cystoscopy with pathological investigation of the specimens gives most often the diagnosis of the disease. Carcinoma in situ (CIS), often macroscopically invisible tumor, may be diagnosed with combination of cystoscopy and cytology.[16] However, positive urinary cytology may originate from anywhere in the urinary

tract, while negative cytology does not exclude tumor (specificity of 86-94%, sensitivity 35-48%).[17, 18]

Transurethral resection of bladder tumor (TUR-BT) is performed to establish the diagnosis and the stage of the disease. The resection includes muscularis propria to evaluate the stage of the tumor. Photodynamic diagnosis (PDD, fluorescence/blue-light cystoscopy) is a useful tool to target the surgery.[19, 20] During the cystoscopy, the mobility of the bladder and pelvic organs are assessed with a rectal/vaginal bimanual examina-tion. A second TUR-BT 2 to 6 weeks after the initial procedure might be required to reduce the risk of understaging in high-risk NMIBC. Guidelines recommend re-TUR after incomplete initial resection, for high-risk tumors (recurrent, multi-focal, over 3 cm, high grade tumor), and for any T1 tumors especially if the initial resection does not include detrusor muscle (the staging and grading of BC is reviewed in the next Chapter).[21-22] Repeated TUR-BT for high-grade T1 tumors results in upstaging and thus, a change in the management, in 24-49% of patients.[24]

Imaging (computed tomography, CT, or magnetic resonance imaging, MRI) may be used for assessment of local invasion and to detect extravesical T3b or higher staged tumors. In case of invasive disease, a metastatic assessment is performed, including abdominal/pelvic and chest CT, liver function tests, and serum creatinine and electrolytes.[25] Lung and liver metastases are common metastatic sites, but bone and brain metastases are rare at the time of diagnosis of invasive BC and the investigations are indicated only based on the symptoms.[26]

2.3 Classification of bladder cancer

BC is a heterogeneous disease and can be classified in several ways to help clinicians to select right treatment modalities and predict the patient survival. At the time of diagnosis, pathologists assess the histology of the BC from TUR-BT specimens. Pathologists also grade the tumor sample to describe the aggressiveness of the disease. The stage of the disease describes the anatomical extent of the disease with possible invasion and metastases, and thus, guide the use of the treatment alternatives. In addition, studies have recognized distinct molecular subtypes of BCs, which vary according to their survival profiles.

2.3.1 Histology

Urothelium, the epithelial cells lining the bladder wall, is a transitional epithelium consisting of basal cells, intermediate cells and umbrella cells.[27] In normal and healthy urothelium, the basal cells attach to the basement membrane and constitute

the urothelial stem cells. Umbrella cells, which make up the upper layer of the urothelium, form a protective barrier for toxic components in the urine.

Morphologically, BC tumors can be divided into papillary (predominant type), solid, and mixed types.[28] The most of the BCs (90-95%) are pure urothelial carcinomas (transitional cell carcinomas).[29] However, BC is a heterogeneous disease consisting also urothelial carcinoma with divergent differentiation or non-urothelial carcinoma. Urothelial carcinoma may consist partially with squamous and/or glandular differentiated histology. Additional variant histologies include tumors with micropapillary or microcystic histology, small cell carcinoma, sarcomatoid carcinoma, and lymphoepithelioma.[30] Furthermore, BC can have plasmocytoid, giant cell, signet ring, diffuse or undifferentiated histology or they can have a nested variant histology. Squamous cell differentiation is the most commonly found non-urothelial histological type occurring in 20% of the urothelial carcinomas.[31, 32] Different histological types have their own distinct biological behaviors and may have different responses to different treatment modalities. In general, it is considered that variant histology subtype increases the risk of more advanced disease and metastasis and, thus, poorer prognosis, but the results from multivariate analyses are controversial.[32-35]

2.3.2 Cancer stage

BC is classified according to Tumor, Node, Metastasis (TNM) classification system to describe the anatomical extent of the disease (Table 1).[36] Invasion to the muscularis propria is the strongest predictor of tumor recurrence and progression.[37] The division of the tumors to non-muscle invasive (NMIBC) and muscle-invasive (MIBC) is widely used. Pathological staging (pT-category) by TUR-BT is the gold standard, but can be limited by the quality of the specimens and cautery and distortion artifacts, whereas clinical staging (cT-category) involving bimanual examination, cystoscopy, and cross-sectional radiographic assessment is unfortunately often inaccurate (over or understaging may occur as high as 40% of the cases).[38-40]

At the time of diagnosis, approximately 85% of patients have a non-muscle invasive, local disease, while 15% have already a muscle-invasive disease with regional lymph node or distant metastases. NMIBC can be divided into papillary and flat tumors.[41, 42] Papillary tumors include Ta (70% of the NMIBC tumors) and T1 tumors (20% of the NMIBCs). Flat tumors are Carcinoma in situ tumors (Tis), which consist 5-10% of the NMIBCs. 50 to 70% of the non-invasive tumors will recur and 10 to 20% progress to MIBC.[25, 28, 43] However, the progression is mainly limited to high-grade disease and carcinoma in situ. To detect which patients will

progress to invasive disease remains a challenge, and thus, BC patients require repetitive investigations and long follow-up.

Advanced BC may extend locally into the surrounding tissues, such as the prostate, uterus, sacral vertebra, and further to the retroperitoneal soft tissues.[44] BC can metastasize via blood vessels or lymphatics. Distant lymph nodes and lungs are the most common sites of metastases, but liver, bones, skin, and brain metastases occur, too. BC patients with metastatic disease have a poor survival, only three to six months, despite the treatment.[45]

Table 1. TNM classification adapted from UICC 2009.[36]

T - Primary tumor	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
NMIBC (non-muscle invasive bladder cancer)	
Ta	Non-invasive papillary carcinoma, confined to the mucosa
Tis	Carcinoma *in situ* (flat tumor confined to the mucosa).
T1	Tumor invaded to the subepithelial connective tissue/lamina propria
MIBC (muscle invasive bladder cancer)	
T2	Tumor invaded to the muscle
T2a	Tumor invaded to the superficial muscle (inner half)
T2b	Tumor invaded to the deep muscle (outer half)
T3	Tumor invaded to the perivesical tissue
T3a	Tumor invaded to the perivesical tissue microscopically
T3b	Tumor invaded to the perivesical tissue macroscopically
T4	Tumor invades extravesical organs
T4a	Tumor invaded to the prostate, uterus or vagina
T4b	Tumor invaded to the pelvic wall or abdominal wall
N – Lymph nodes	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single lymph node in the true pelvis
N2	Metastasis in multiple lymph nodes in the true pelvis
N3	Metastasis in common iliac lymph node(s)
M – Distant metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

2.3.3 Tumor grade

Tumor grading correlates to the aggressiveness of the tumor, predicts the prognosis of the disease, and helps on the selection of the treatment. BC tumors are graded histologically according to the classification of the World Health Organization

(WHO, 1973) to describe the appearance of tumor cells and cellular anaplasia.[46] In 2004 WHO and the International Society of Urological Pathology (ISUP) published revisions to the 1973 grading system to allow more standardized pathologic description.[47] The grading systems are described in detail in Table 2 and the representative examples of the histological BC samples of different grades are shown in Figure 1.

In the 1973 classification, Grade 1 tumors have a significantly better prognosis than Grade 2 or 3 tumors.[37] They have an orderly arrangement and minimal architectural abnormalities and mitotic figures are rare or absent (Figure 1). Grade 3 tumors, on the other hand, demonstrate extreme nuclear abnormalities, disordered architecture and frequent mitotic activity. Grade 2 is an intermediate group of tumors and it is considered more of a diagnosis of exclusion.

The 2004 classification system is considered superior to the 1973 system, although they are recommended to be used simultaneously.[37, 48] The two grading systems correlate well only at the ends of the grading spectrum; approximately 40% of the Grade 2 tumors are classified as high-grade in the 2004 system.[28] Papillary urothelial neoplasms of low malignant potential (PUNLMP) have a low probability of progression, but they are not pure benign tumors either with recurrence rates up to 60% a and progression rate up to 8%.[49] As majority of the MIBC tumors are high-grade tumors, the 2004 system is particularly important with non-invasive tumors, in which about 50% of the tumors are low-grade.[28, 37]

Table 2. BC grading systems, adapted from EAU guidelines on non–muscle-invasive urothelial carcinoma of the bladder.[48]

1973 WHO grading system	
Urothelial papilloma	
Grade 1	Well differentiated
Grade 2	Moderately differentiated
Grade 3	Poorly differentiated

2004 WHO and ISUP grading system	
Urothelial papilloma	
PUNMLP	Papillary urothelial neoplasm of low malignant potential
Low grade	Low-grade papillary urothelial carcinoma
High grade	High-grade papillary urothelial carcinoma

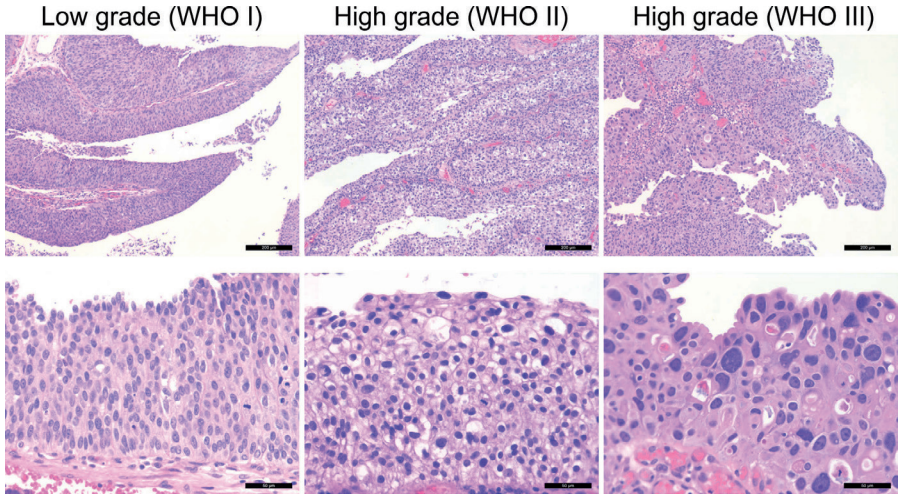

Figure 1. Representative BC tumors with different grading according to the 2004 and 1973 grading systems: Low grade/WHO Grade 1, high grade/WHO Grade 2, and high grade/WHO Grade 3. Scale bar 200μm in the upper row and 50μm in the lower row. Graph. courtesy of Dr. Pekka Taimen, Department of Pathology, Turku University Hospital.

2.3.4 Carcinoma *in situ*

Carcinoma in situ (CIS) is a flat, non-invasive, often multifocal, and by definition, high-grade bladder tumor. It can be easily missed at cystoscopy, although it is nearly always detected with urine cytology.[16, 42] Five to ten percent of all patients with NMIBC have CIS.[42] CIS is classified into three different clinical types: (I) primary (isolated CIS and no previous or concurrent papillary tumors), (II) secondary (detected during follow-up of previous non-CIS tumor), and (III) concomitant (in the presence of any other urothelial tumor). CIS tumors should be excluded from Ta and T1 tumors because these tumors have a different natural behavior pattern than papillary tumors. CIS is highly malignant and has a much higher risk of progression than Ta or T1 tumors: approximately 54% of the CIS tumors progress to MIBC in contrast to 10-20% of other NMIBCs.[37, 42, 50, 51] CIS is also a strong predictor for ureter involvement, and thus, increases the risk for recurrence in the upper urinary tract after RC.[52] However, its aggressiveness varies greatly and it is considered unpredictable tumor type.

2.3.5 Molecular subtypes of bladder cancer

Studies have shown, that different cancers can be grouped into molecular subtypes, which have common gene expression patterns and biological characteristics, such as survival outcomes and therapy responses. Large-scale mRNA expression profiling and DNA sequencing studies have demonstrated, that as many other cancers, BC can be divided into subtypes according to tumors gene expression. In particular, NMIBCs and MIBCs have been shown distinct underlying molecular biology: NMIBCs are characterized by activating FGFR3 (fibroblast growth factor receptor-3) mutations, whereas MIBCs are characterized by frequent p53 mutations.[53-57] For MIBCs, dichotomization to luminal tumors (urothelial differentiation) and basal squamous tumors (basal cell or squamous differentiation) is broadly used. According to different studies, MIBCs can be divided at least into five molecular subgroups: (I) luminal papillary/uroA, (II) luminal infiltrated/p53-like, (III) luminal/genomically unstable, (IV) basal squamous, and (V) neuroendocrine subtype.[58-64] In addition, a recent meta-cohort analysis from 2411 BC samples identified six molecular subtypes of BC (MC1-6), which overlap with subtypes from previous reports: MC1/neural-like, MC2/luminal, MC3/papillary-like, MC4/HER2-like, MC5/squamous-cell carcinoma-like, and MC6/mesenchymal-like subtypes.[65] However, further studies are still needed, and additional subtypes may emerge. There is also evidence, that BC is often molecularly heterogenous and different molecular subtypes can emerge in one tumor.[66] The subtypes display prognostic significance and associate with different benefit from systemic therapies. Basal squamous and neuroendocrine tumors express high levels of epithelial-to-mesenchymal transition (EMT) biomarkers and are aggressive, more prone to invade and metastasize than luminal subtypes.[59, 63, 64] However, basal squamous tumors tend to respond most to neoadjuvant chemotherapy, which improves the prognosis.[67, 68] Luminal papillary/uroA tumors are low-stage tumors with the longest disease-specific survival (DSS) and overall survival (OS).[59, 69] However, the response to neoadjuvant chemotherapy varies and they have the lowest response rate to new checkpoint inhibitors.[63, 68, 70] On the other hand, the luminal/genomically unstable and the luminal infiltrated/p53-like tumors appear to be chemoresistant but particularly sensitive to immune checkpoint blockade therapies.[63, 68, 70, 71] Molecular subtyping could aid clinicians to select right treatment modalities to each patient in the future, but it is still long way to get the subtype classifications in the clinical practice.

2.4 Treatment of bladder cancer

Bladder tumors were mentioned the first time in literature by Lacuna in year 1551. During the 16th and 17th centuries the first surgeries targeting bladder tumors were performed.[72] Primary TUR-BT procedures (bladder tumor electro-resection) were done in 1910 by Edwin Beer, and TUR-BT, as such, was introduced in the 1930s.[73] Since then, TUR-BT has become the primary avenue for diagnosis and removing the tumor tissue. Today there is a variety of BC treatment guidelines published by organizations such as European Association of Urology (EAU), American Urological Association (AUA), National Comprehensive Cancer Network (NCCN), and National Institute for recommended treatments in order to facilitate the decision making of the clinicians.[21] TUR-BT remains as the gold standard in the treatment of NMIBC whereas muscle-invasive tumors are treated with cystectomy with or without systemic therapies. Immunological treatment alternatives have taken a leap recently in patients with advanced disease. The treatment modalities for BC are summarized in Table 3.

Table 3. Summary of bladder cancer treatment.

NMIBC
Low-risk patients (solitary, small, low-grade Ta): TUR-BT + single immediate chemotherapeutic instillation (mitomycin)
High-risk patients (multiple, recurrent, large, T1 and/or G3, high-grade and/or CIS): TUR-BT + adjuvant BCG RC (considered for high-grade, multiple or at difficult sites to resect T1 tumors high grade + CIS)
MIBC
Neoadjuvant chemotherapy + RC (RC + adjuvant chemotherapy) (TUR-BT + radiation therapy, chemotherapy)
METASTATIC BLADDER CANCER
1. line chemotherapy (cisplatin-based: gemcitabine/cisplatin or MVAC[1]) - If ineligible: gemcitabine/carboplatin or CPI (pembrolizumab, atezolizumab) 2. line chemotherapy (non-platinum-based) or CPI (pembrolizumab, nivolumab, atezolizumab, avelumab, duvalumab)

[1] MVAC; methotrexate, vinblastine, adriamycin, cisplatin

2.4.1 NMIBC

NMIBC is the most prevalent type of bladder cancer. A complete trans-urethral resection of bladder tumor (TUR-BT) is recommended for all NMIBC patients for the initial diagnosis and treatment. Management protocols are based on the patients risk of recurrence and progression.[23, 74] NMIBC is divided into low, intermediate, and high-risk groups. The 5-year recurrence rate varies from 50 to 70% and the progression rate varies from 10 to 30% in TUR-BT treated NMIBC tumors. High stage and grade, large tumor size, multifocality, high number of previous recurrences, and the presence of concomitant in situ associate with recurrence and progression. Other negative prognostic factors are the presence of lymphovascular invasion (LVI), histological variants, and greater depth of invasion.[28]

For low risk patients (solitary, small, primary low-grade Ta tumor) a single immediate chemotherapeutic instillation (most commonly mitomycin) is recommended after the resection of the tumor to reduce the recurrence.[75-77] Intravesical chemotherapy destroys tumor cells floating in the irrigation fluid and urine after TUR-BT and it has an ablative effect on residual tumor cells at the site of the resection and on small overlooked tumors.[74, 78] High-risk patients do not benefit from the immediate instillation of chemotherapy.[79] Patients with high-risk disease (multiple, recurrent, large, T1 and/or G3 and/or CIS) are suggested to have further adjuvant therapies with intravesical drugs after complete TUR-BT. Bacillus Calmette-Guérin (BCG) instillations are considered to be the most effective treatment alternative to prevent the risk of tumor recurrence and progression.[23, 28, 80-82] Despite BCG has been used for treatment of BC for over 40 years, the exact mechanism of the antitumor effect of the treatment is still unknown (see Chapter *3.7 Immunity in bladder cancer* for more about the mechanism of the BCG treatment). The optimal schedule of the BCG regimen has been broadly discussed, but it is still not fully known, either. However, at least one year of maintenance BCG is required, and three-year maintenance is considered even more effective to prevent recurrence. The procedure is generally well tolerated, but can be associated with complications including irritative voiding symptoms, such as frequency, urgency, and dysuria. BCG instillations can also potentially cause bladder fibrosis and contracture. Major complications related to BCG treatment can appear after systemic absorption of the drug. Complications include fever, arthritis, granulomatous prostatitis, BCG sepsis, disseminated tuberculosis, and death. Thus, contraindications, such as immunosuppression, should be respected.[25, 48] For patients with recurring tumors after BCG, RC should be considered.[48, 83]

Patients, who experience disease progression from NMIBC to MIBC, have worse prognosis than those with primary muscle-invasive disease.[84] Thus, radical cystectomy (RC) should be considered for high-grade, multiple T1 tumors; T1 tumors located at difficult site to resect; residual T1 tumors; or high-grade tumors

with CIS, especially for patients with longer life expectancy.[23, 85] Despite being superficial tumors, high-grade NMIBCs are aggressive. High-grade T1 BC has 50% progression rate and 30% mortality in 15 year follow-up time.[86] The staging accuracy for T1 tumors by TUR-BT is low, and 27-51% of the patients are upstaged to MIBC at RC.[40]

BC is prone to recur and it is potentially lethal without aggressive treatment in about one third of the patients. The high recurrence rate makes the follow-up an important component of an effective management. Due to the high recurrence rate, a substantial risk of progression, and intensive treatment and follow-up protocols, BC is one of the most expensive cancers to manage.[87] The recommended schedule for follow-up varies among BC guidelines and the follow-up protocol should reflect the patients degree of risk.[23] The first cystoscopy after primary TUR-BT at three months is an important prognostic indicator for recurrence and progression.[88] According to the EAU guidelines, low-risk patients should undergo cystoscopy yearly for 5 years.[89] After this, the risk of recurrence is low and discontinuation of cystoscopies can be considered. High-risk patients should undergo cystoscopy every 3 month for a period of 2 years and every 6 months after that until 5 years, and then annually. In addition, upper urinary tract imaging should be performed every 12-24 month.[25] Because recurrence after 10 years of tumor-free interval is possible for intermediate or high-risk patients, life-long follow-up is recommended.

2.4.2 MIBC

Approximately every fifth BC patient has already at the time of diagnosis an invasive disease with poor prognosis. The standard treatment for localized muscularis propria invasive BC is radical cystectomy. The operation includes en bloc removal of the anterior pelvic organs; the bladder, prostate, seminal vesicle, and distal ureters in men and bladder, uterus, ovaries, anterior vaginal wall, and distal ureters in females.[90] An extended bilateral pelvic lymphadenectomy is recommended.[91] Traditionally, RC is the treatment of choice for tumors with T2-4a, N0-Nx, M0. RC can be considered with patients having a single lymph node metastasis in the true pelvis, but curative results are unlikely in N2-3 cases. Other indications include high-risk and recurrent non-invasive tumors, BCG-resistant Tis and T1G3 tumors. Salvage cystectomy is recommended after failure of conservative treatments. Cystectomy is also used as a palliative intervention including for fistula formation, pain, or recurrent macrohaemoturia.[30] In well selected patients, who are not candidates or refuse definite cystectomy, bladder preservation with TUR-BT followed by radiation therapy with concurrent chemotherapy remains a viable option.[92]

During the cystectomy, urinary diversion is done with a segment of bowel in a non-continent or a continent way.[93] Non-continent ileal conduit according to Bricker is the simplest urinary diversion draining into an external collecting-bag attached to the abdominal wall (urostomy). The ileal conduit is often selected in elderly patients with comorbidities and higher operative risk, whereas the continent diversion is often selected in young, healthy patients.[25] The continent reconstructive procedures involve the creation of an internal reservoir with an antireflux mechanism, which is either brought to the abdominal wall (continent cutaneous or heterotopic neobladder) or sutured to the urethra (orthotopic neobladder).[44] This allows the patient either to self-catheterize or to void in the normal position. From an anatomical standpoint, currently used alternatives after cystectomy are (I) abdominal diversion (such as ureterocutaneostomy and ileal or colonic conduit), (II) urethral diversion (various forms of gastrointestinal pouches to the urethra as a continent, orthotopic urinary diversion), and (III) rectosigmoid diversions (such as uretero-ileo-rectostomy).[30] RC results changes in quality of life by sexual dysfunction and reduction in urinary control. Prostate sparing cystectomy is possible for selected patients. The risks of this operation include prostate cancer, carcinoma in situ of the prostatic urethra, cancer in the prostatic duct/glands, or invasion to the prostate.[94]

The peri-operative mortality associated with RC is 1.2-3.2% at 30 days and 2.3-8.0% at 90 days after the operation.[30] Early complications within three months after surgery occur in 58% of patients, most of being diversion related.[95] The five-year recurrence-free survival after RC is approximately 58-68%. However, the five-year recurrence-free survival in node-positive patients is only 34-43%.[30]

Because the five-year survival after RC is suboptimal, neoadjuvant chemotherapy (NAC, most commonly cisplatin combination chemotherapy) has been used to improve the results of the surgery.[28, 44, 96-100] Distant metastases after RC are more common than local disease, which indicates that micrometastases may be present at the time of the operation. This microscopic dissemination is the target of NAC.[101] NAC responders might reveal a favourable pathological status in RC (pT0, pN0, and negative surgical margins), but in contrast, patients resistant to chemotherapy might compromise the outcome with delayed RC.[102] The downstaging of the tumor increases with NAC, which increases the survival benefits of the treatment.[102] NAC treatment has 5-8% five-year OS benefit compared to surgery only treated patients.[101] The downstaging is a potential surrogate marker for chemosensitivity and survival in BC patients.[102] The challenge is the treatment of non-responders and overtreatment of patients without micrometastatic disease. Only approximately 40% of the patients experience a complete response to NAC and have no residual tumor in RC.[103] A delay in RC might associate with decreased survival and the patients are exposed to

unnecessary toxicity.[104] Persistent invasive BC after NAC is considered chemoresistant.[103] Basal/squamous type tumors have been reported as best potential candidates for NAC, but however, to date, there are no validated biomarkers or tools to indicate which patients will most likely benefit from NAC.[68]

As opposed to neoadjuvant chemotherapy, adjuvant chemotherapy enables improved patient selection to patients with positive nodes or high pathological stage (Table 4). The role of the adjuvant chemotherapy is, however, not clear in the management of BC as the results from randomized trials are limited by poor accrual and conflicting results.[105-107] The disadvantage of the treatment is the delay in the systemic therapy against metastases and poor tolerance in postoperative period, but it does not cause delay in RC and the patient selection is more careful with precise pathological staging compared to NAC treated patients.[25, 101]

Table 4. Pros and cons of the neoadjuvant and adjuvant chemotherapy, adapted from Raghavan *et al.* 2002.[44]

	Neoadjuvant chemotherapy	**Adjuvant chemotherapy**
Pros	Possibility to assess the response. Early treatment of occult metastases. Survival benefit in some randomized trials.	Early systemic impact. Data from other cancers imply survival benefit. Trend in favor of survival from some studies. Improved patient selection.
Cons	Early and increased toxicity. Potential delay of effective therapy. Poor patient selection. May not be needed if clinical understaging.	Enhanced toxicity. No proof that the benefit is from chemotherapy. No proven significant survival benefit. Inability to asses response (potential for continues ineffective therapy)

2.4.3 Metastatic bladder cancer

Metastatic BC is an incurable disease and the survival is poor: only 15% of these patients are alive at 5 years.[108] The initial spread of the disease is typically to the pelvic lymph nodes, but metastases occur via lymphatic and haematogenous channels in other organs, most frequently the lungs and bones.[109] Systemic chemotherapy has been the choice of treatment in metastatic disease.[25] Cispla-tin-based chemotherapy has a 60-70% overall response rate and it leads to median survival of around one year.[110-, 111] However, up to 30-50% of the patients with metastatic BC are ineligible to the cisplatin treatment and until recently, carboplatin-based regimens have been the only treatment option for these patients.[112] Furthermore, the treatment options after failure of the first line chemotherapy have been poor: second-line chemotherapies, including paclitaxel, pemetrexed, docetaxel, and vinflunine, have shown modest efficacy with an overall response rate of 12% and a median OS of 5-7 months.[30, 113] For decades, there has been a lack of any improvements in the treatment of BC. However, recent

advancements in studies of novel immunotherapy agents have led to rapid changes in the treatment of advanced/metastatic BC and immunotherapy is now recognized as the fourth treatment alternative in BC.

Immune checkpoint inhibitors (here referred as CPIs) selectively target pathways whose main target is to remove inhibitory signals or "brakes" on T cells allowing them to work as an effective antitumoral immune response (see Chapter *3.5 Cancer immunotherapy* for the detailed mechanism).[114] The Programmed death 1/programmed death-ligand 1 (PD-1/PD-L1) and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) pathways play important roles in the T-lymphocyte-mediated response and are important drug targets.[115] It has been speculated, that PD-1/PD-L1 blockade drugs will redefine the standard of care for BC. Five PD-1/PD-L1 blockade drugs have been approved by the United States Food and Drug Administration (FDA) in the treatment of BC after the first approval in May 2016: PD-1 inhibitors pembrolizumab and nivolumab (Keytruda and Opdivo as brand names, respectively), and PD-L1 inhibitors atezolizumab, avelumab, and duvalumab (Tecentriq, Bavencio, and Imfinzi, respectively).[116, 117] Pembrolizumab, nivolumab, and atezolizumab have also been approved in Europe.[111] Pembrolizumab is a highly selective, humanized monoclonal IgG4 antibody against PD-1 blocking both PD-L1 and PD-L2. It is the only checkpoint inhibitor that has shown significantly better survival than standard chemotherapy.[115, 118-120] Median survival for pembrolizumab treated patients in a randomized phase III trial was 10.3 months whereas chemotherapy treated patients had 7.4 months median survival. In addition, immunotherapy was better tolerated than chemotherapy.[121] However, the four other CPIs have also been approved for treatment of refractory metastatic BC in patients who have disease progression during the platinum-containing chemotherapy. In addition, pembrolizumab and atezolizumab have been approved as the first-line treatment for cisplatin-ineligible metastatic BC patients. Although new CPIs have shown major improvements for treatment of metastatic BC, there are no tools for predicting which patients will benefit from the treatment. No clear correlation between the PD-L1 positivity on tumor cells and the response rate for the PD-1/PD-L1 inhibitors has been observed.[117] Tumor-mutation burden and neoantigen load, as well as gene signatures, such as interferon γ (IFNγ) and basal molecular subtype, have shown to be potential predictive biomarkers to the CPIs, but further studies are needed to find a reliable biomarker to improve the patient selection.[117] Immunotherapy will continue to shape the treatment alternatives for BC patients. There are ongoing trials to investigate the PD-1/PD-L1 inhibitors further in the treatment of BC, e.g. in the treatment of NMIBC and as a neoadjuvant or adjuvant therapy for MIBC alone or combined with CTLA-4 antibodies or chemotherapy.[122]

Despite the development with the CPIs, new rational targeted therapeutic options to improve outcomes of BC are needed. Most patients will eventually develop resistance to chemotherapy and/or CPIs. Studies have revealed several promising agents that are being developed and studied in advanced BC.[123] Vascular endothelial growth factor (VEGF) has been shown to associate with more aggressive BC. Anti-VEGF receptor antibody combined with docetaxel chemotherapy has shown improvements in progression-free survival compared with docetaxel alone in patients progressed during/after platinum therapy.[124] Other potential therapy targets under investigation are fibroblast growth factor receptor-3 (FGFR3) family proteins, human epidermal growth factor receptors (EGFR and HER), and phosphoinositide 3 kinase (PI3K)/protein kinase B (AKT)/the mammalian target of rapamycin (mTOR) pathway proteins.[123]

2.5 Biomarkers in bladder cancer

There are several scenarios in which biomarkers would have a role in the diagnostics and treatment of BC in clinical practice: (I) in the screening of the high-risk population, (II) in the risk stratifying patients with asymptomatic hematuria, (III) in the surveillance setting to guide the treatment alternatives and follow-up, and (IV) in the prediction of the possible benefit of a specific treatment. A prognostic biomarker predicts the natural history of the disease independent of a particular treatment whereas predictive biomarkers are used to discriminate and determine differences in treatment-specific responses. An ideal biomarker would be accurate, reproducible, easy to use, and it should be validated in multiple datasets. The incidence of BC in population with increased risk of the disease is considered to be too low for screening program with a socioeconomic benefit.[125] On the other hand, patients with asymptomatic gross hematuria have a significant risk of BC, approximately 10%, and thus, need urologic investigations to make or rule out the diagnosis. In this group, urinary biomarkers for BC could limit the need for invasive and expensive procedures and urologic workup. In addition, patients diagnosed with BC need accurate information about their disease and the prognosis to select the right treatment modalities.[126] Long follow-up periods after treatments make BC one of the costliest cancers and patients with very low risk of recurrence suffer from invasive and continual follow-ups.[127] It remains a challenge to predict which patients will progress from non-invasive to invasive disease, to select administration of intravesical therapy (immediate postoperative instillation *vs.* adjuvant chemotherapy) or early RC *vs.* intravesical BCG for high-grade T1 patients, or predict the benefit of the neoadjuvant chemotherapy or immunotherapy. In addition, the investigations of marker molecules and genes provide new information of possible new therapeutic approaches.

Despite several biomarkers being reported to be clinically promising, there are currently no prognostic BC biomarkers in routine clinical practice.[128] The aim of the biomarker studies is to show that adding a molecular marker to existing parameters (with most important clinical and pathologic factors) improves the prognostic or predictive capacity of the current clinical tools. Many promising biomarkers have not been validated, or lack prospective clinical trials to confirm the role of the marker. Also, the reproducibility of biomarker assessment is often understudied.

Urinary biomarkers represent an attractive choice as prognostic biomarkers with their non-invasive approach. Despite the FDA has approved some urinary biomarkers (such as fluorescence in situ hybridization (FISH) assay UroVysion, a combination of urine cytology and immunohistochemical staining uCyt+ assay, and protein based bladder tumor antigen (BTA) and nuclear matrix protein 22 (NMP22) assays) the suboptimal accuracy has limited the clinical use of the biomarkers.[21, 129, 130] The challenges with these biomarkers are the low sensi-tivity (18-43% of the BCs would be missed, approximately 10% in combination with cytologic evaluation) and specificity (false positive results in 12-26% of the patients), and the accuracy is poor especially for low-stage and low-grade tumors.[21, 131]

In several studies, FGFR3 mutation has been shown to protect against progression in NMIBC, while overexpression of the tumor suppressor p53 and the proliferation marker Ki67 are examples of unfavorable molecular pathway in BC.[64] The p53 gene mutation is the most common genetic defect in human cancers. A large number of studies define Ki67 as an independent prognostic marker in BC. Although these markers are widely studied in BC and other cancers, the final prognostic role of these molecules in BC is still debated, and the studies are insufficient to assess the outcome of BC with these markers into standard practice.[128, 132] Several studies have shown that overexpression of EGFR is an independent prognostic factor in patients with advanced BC. The overexpression of HER-2 protein has been shown to correlate with increased tumor grade, cancer-specific survival, and metastatic disease and it has been also an independent prognostic factor is some studies. However, the investigations about EGFR and HER-2 are still heterogeneous and insufficient to prove the prognostic role of these markers.[132] VEGF is also an interesting potential prognostic marker with the possibility of urine measurements, but the prognostic studies of VEGF in BC are still insufficient.[132] Survivin, an antiapoptotic biomarker, has been shown to associate with recurrence, DSS, and OS in a meta-analysis of 14 studies, but it lacks prospective large series to confirm the results.[133]

Next-generation sequencing and gene expression profiling have led to identification of different molecular subtypes of BC (see Chapter *2.3.5 Molecular*

subtypes of bladder cancer). The distinct subtypes show different clinical phenotypes and response rates to different treatment strategies. Tumors with basal subtypes are aggressive and often invasive, but also mostly sensitive to NAC while luminal tumors have best prognoses, but the responses to chemotherapy and CPIs vary.[68, 70] However, the consensus of the subtypes has not been reached and they need further validations before getting into the clinical practice.

New therapy modalities, such as NAC and CPIs, have revolutionized the treatment of BC. There is an urgent need for predictive biomarkers to help guide the therapy options for each patient. Chemotherapy agents, such as platinum-containing compounds, kill cancer cells via DNA damage. Cancer cells that have deficient DNA damage repair mechanisms are unable to fix the damage caused by the chemotherapy and thus, are more susceptible being destroyed by these drugs. Excision repair cross complementing 1 and 2 (ERCC1/2) are key enzymes in the nucleoside excision repair (NER) of DNA damage. ERCC1 have been considered as an attractive biomarker to evaluate the cisplatin sensitivity.[134] Other DNA damage response genes, such as ataxia telangiectasia mutated gene (ATM), retinoblastoma gene (RB1), and Fanconi anemia complementation group C gene (FANCC), have also investigated as predictive markers for neoadjuvant cisplatin-based chemotherapy.[135] However, further prospective evaluations are needed to validate the predictive role of DNA damage response gene alterations in BC. In addition to the systemic chemotherapy, the PD-L1/PD-1 blockade drugs have been approved in the treatment of metastatic BC. PD-L1 is a T-cell regulatory molecule that is found frequently overexpressed in tumors and associates with higher grade, increased pathological stage, and poorer survival in BC.[136, 137] However, there are no biomarkers to select the patients who would benefit most of these therapies either and the studies about the CPI biomarkers are ongoing (see Chapter *2.4 Treatment of bladder cancer*).

3 Tumor immunity

The idea of cancer-related inflammation goes back to the 19th century, when Rudolph Virchow, a German pathologist, provided a potential link between inflammation and cancer.[138] In 2000, Hanahan and Weinberg introduced six hall-marks of cancer that included sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis.[5] A decade later, tumor-promoting inflammation and the capability to avoid immune destruction were added to the list among with the deregulation of cellular energetics and genome instability and mutation as enabling and emerging hallmarks (Figure 2).[139] This proves the evident relationship between cancer and inflammation and describes the dual role of immune cells in cancer: on one hand the elimination of tumor cells and on the other, support for the tumor development. In 2018, the success in the field of cancer immunotherapy has been recognized, when Nobel Prize was awarded to James P. Allison and Tasuku Honjo for their discovery of cancer therapy by inhibition of immunosuppression.

Tumors consist not only of a collection of homogenous cancer cells, but construct a tumor microenvironment during tumorigenesis. This microenvironment includes individual specialized cell types, which contribute to the biology in the tumors and participate in the regulatory signaling of cancer cells. Tumor parenchyma and stroma contain distinct cell types, such as cancer cells and cancer stem cells, cancer-associated fibroblasts, vascular/lymphatic endothelial cells, and pericytes, which all collectively enable tumor growth and progression. In addition to these cells, immune cells are a crucial part of the tumor microenvironment.[139] Immune cells can act in conflicting ways being tumor-antagonizing or tumor-promoting, and can be found in various proportions in the most of the neoplastic lesions.[139] Leukocyte infiltration in tumors contains innate immune cells (including macrophages, neutrophils, dendritic cells, and natural killer cells) and adaptive immune cells (T and B lymphocytes). Inflammatory cells crosstalk with each other by direct contact or cytokine and chemokine production, and act in autocrine and paracrine manners to control tumorigenesis.[140] Immune cells release tumor promoting signaling molecules, such as tumor growth factor EGF, proangiogenic factors (such as VEGF), chemokines and cytokines, and proangiogenic and/or proinvasive matrix-degrading enzymes (e.g. matrix metalloproteinase-9, MMP-9). Via these effectors, inflammatory cells stimulate cell proliferation, induce and help sustain angiogenesis, facilitate tissue invasion, and are involved in the metastatic seeding of cancer cells. In addition, immune cells can release mutagenic chemicals, which can accelerate the genetic evolution of nearby cancer cells toward advanced malignancy.[139, 141]

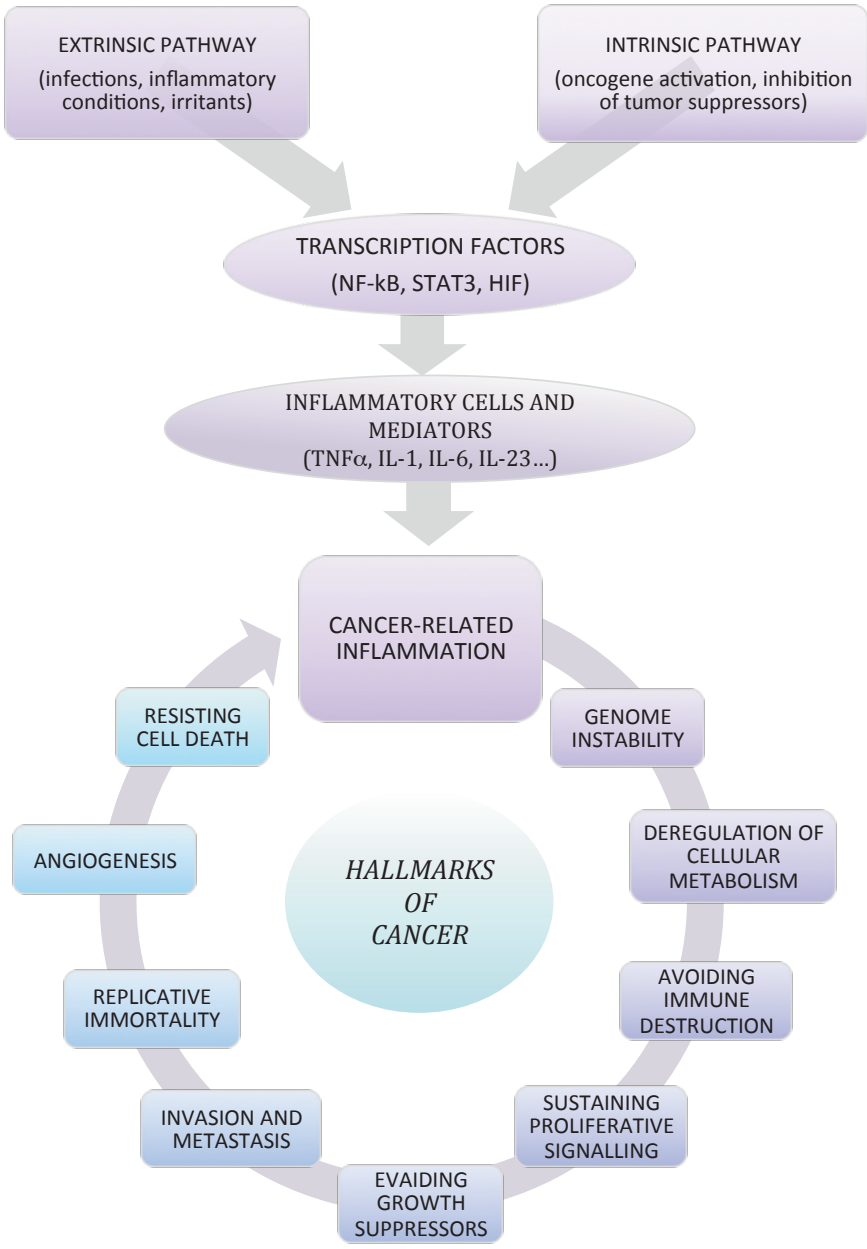

Figure 2. The relationship of inflammation and cancer can be divided into two individual pathways: extrinsic and intrisic. Both pathways lead to activation of transcription factors, such as NF-κB, STAT3 and HIF, which regulate the activation of inflammatory cells and mediators. Resulted cancer-related inflammation is one of the hallmarks of cancer.[139, 141]

3.1 Extrinsic and intrinsic pathway

In some cancers, inflammation is present before malignant change occurs (e.g. chronic inflammation in inflammatory bowel disease and inflammation due to tobacco smoking or obesity; viral/bacterial antigens, such as Helicobacter pylori, hepatitis B/C viruses, or Schistosoma; and cryptogenic inflammations of uncertain origin, such as prostatitis and pancreatitis), while in others, carcinogenesis induces an inflammatory microenvironment which promotes the development of tumors.[140-144] These two origins of inflammation can be viewed as two pathways connecting cancer and inflammation: (I) an extrinsic pathway driven by inflammatory conditions and increased risk of cancer and (II) an intrinsic pathway driven by genomic alterations causing inflammation and neoplasia (Figure 2).[142] The microenvironment-driven extrinsic and the oncogene-driven intrinsic pathways converge and activate transcription factors, e.g. nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), and hypoxia-inducible factor (HIF), which orchestrate the activation of inflammatory cells and mediators, and generate a cancer-related inflammatory microenvironment.[141, 142] However, inflammatory cells and their mediators are present in the microenvironment of most of the cancers despite the origin of the tumorigenesis.

In the intrinsic pathway, different genetic alterations, such as activation of oncogenes or inactivation of tumor suppressors, can trigger the inflammatory cascade.[142] The most frequently mutated oncogenes in human cancer are RAS family oncogenes, which can induce the production of inflammatory mediators, such as CXCL8 to promote angiogenesis and tumor progression.[145] Another oncogene, MYC, promotes cell proliferation, instructs remodeling of the extracellular microenvironment with inflammatory cells and mediators, and recruits mast cells via CC chemokines to sustain angiogenesis and tumor growth.[146] Like oncogenes, tumor suppressors have also capability to regulate the production of inflammatory mediators: Von Hippel Lindau/hypoxia-inducible factor (VHL/HIF) participates for example in the production of chemokine receptor CXCR4 and proinflammatory cytokine TNF-α.[147] Mutation of tumor suppressor phosphatase and tensin homologue (PTEN) in non-small cell lung cancer activates the upregulation of HIF-1 and CXCR4, which promotes metastasis. Ablation of alpha catenin induces for example NF-κB activation, cell proliferation, and wound healing.[148]

3.2 The players of the cancer-related inflammation

3.2.1 Tumor-infiltrated leukocytes

Tumor-promoting inflammation and antitumor immunity coexist in different points of the tumorigenesis.[140] Tumor microenvironment constitutes of several non-immune and immune cells, which impact on tumor growth. Tumor-infiltrated leukocytes (TILs) are a major component of the tumor microenvironment (Table 5): 10% of all cell types infiltrating the tumor consists of T cells.[139] T cells maturate in the thymus. Naïve T cells bearing antigen-specific T cell receptors (TCR) are introduced to antigens by dendritic cells (DCs) in the secondary lymphoid organs.[149]

Table 5. Roles of different inflammatory cells in the tumor microenvironment. Table adjusted from Grivennikov *et al,* 2010.[140]

Cell type	Antitumor immunity	Tumor-promoting inflammation
Macrophages, DCs, MDSCs	Antigen presentation, cytokine production (IL-12, type I IFN)	Immunosuppression, production of cytokines/chemokines/proteases/growth factors/angiogenic factors
Mast cells	---	Cytokine production
B cells	Production of tumor-specific antibodies	Cytokine and antibody production, mast cell activation, immunosuppression
$CD8^+$ CTLs	Direct lysis of cancer cells, cytotoxic cytokine production (perforin, granulysin, granzymes)	---
Th1 cells	Help to CTLs in tumor rejection, proinflammatory cytokine production (IFNγ, TNFα, IL-2, IL-12)	---
Th2 cells	---	Suppression of antitumor immunity, education of macrophages, cytokine production (IL-4, -5, -6, -10, -13), B cell activation
Th17 cells	Activation of CTLs	Production of proinflammatory cytokine (IL-17, -21, -22), promotion of angiogenesis, inhibition of CTL differentiation
Treg cells	---	Suppression of effector T cell functions, production of cytokines (TGFβ, IL-35, IL-10), upregulation of CTLA-4 and PD-1
NK cells	Direct cytotoxicity towards cancer cells, cytotoxic cytokine production	---
Neutrophils	Direct cytotoxicity, regulation of CTL responses	Cytokine/protease/ROS production

CD8+ cytotoxic T cells (CTLs) and natural killer cells (NK cells) are critical mediators of the anti-tumor response by producing IFNγ and directly killing cancer cells (see Figure 3).[150] In addition, NK cells produce several cytokines and chemokines and thus, regulate immune responses and promote the recruitment of DCs into tumors.[151] CTLs recognize tumor-specific neoantigens introduced by APCs and release cytotoxic granules containing perforin, granulysin, and granzymes leading to cancer cell death.[152] CTL infiltration in the tumor tissue is associated with more favourable prognosis in several cancers, e.g. in bladder, prostatic, breast, and colorectal cancers.[153]

While CD8+ CTLs have a well-defined role in preventing cancer development, CD4+ T lymphocytes have more paradoxal functions. CD4+ T cells are a heterogeneous group of cells that develop various functional lineages depending on the cytokine signals during the activation by antigens.[154] Predominant CD4+ cell subset can even vary depending on the stage of the disease.[155] Classically, CD4+ T cells include Th1 and Th2 subgroups (T helper cells) fostered by interleukins 12 and 4 (IL-12 and IL-4), respectively. Th1 restrain cancer development: they regulate CD8+ CTL responses by secreting IFNγ, tumor necrosis factor α (TNFα), IL-2, and IL-12, and can directly kill tumor cells with IFNγ, TNFα and cytolytic granules. In contrast to Th1 cells, Th2 cells associate with cancer progression by expressing high levels of IL-4, IL-5, IL-6, IL-10, and IL-13. They are not directly cytotoxic; they rather modify adaptive immunity by releasing cytokines to activate other immune cell types.[156] Th2 cells induce T cell anergy, inhibit T cell mediated cytotoxicity and foster humoral immune response. The total outcome of Th2 cells to cancer prognosis is controversial: Th2 cells are considered as protumoral in general, but they have been shown to promote anti-tumor activities for example in colorectal cancer, as well.[157]

In addition to Th1 and Th2 cells, CD4+ cells include Th17 subset that is differentiated by IL-6 and transforming growth factor β (TGFβ), and secrete IL-17, IL-21, and IL-22. The role of Th17 cells in cancer development is probably context dependent. Another subgroup of CD4+ cells is T regulatory cells (Tregs, CD4+FoxP3+) that correlate with poor prognosis in several cancers, such as in pancreatic, lung, renal, and breast cancer.[150] They control tumor development by suppressing CD8+ CTLs, NKs, and DCs, increase local levels of immunosuppressive cytokines (e.g. TGFβ, IL-35, and IL-10), and have direct cytolytic effects through production of perforin and granzyme.[158] However, it has been suggested, that there are multiple tissue-specific Treg subpopulations with various bioeffector activities.[159-161]

Neutrophils are the dominant leukocyte subgroup in the peripheral blood. They have a major role in the first line defense against pathogens. With their short life span and fully differentiated phenotype, neutrophils have been considered as neg-

ligible in tumors. However, it has recently been noticed, that tumor-associated neutrophils (TANs) can be polarized towards distinct phenotypes in response to tumor-derived signals and have both anti- and pro-tumoral functions, as well.[162] Neutrophils are involved in carcinogenesis through the release of nitric oxide derivatives and reactive oxygen species (ROS). Neutrophil-derived cytokines and proteins from the granules of neutrophils may also play a dual role in tumor progression.[163]

In addition to other leukocytes, macrophages are a significant part of the tumor microenvironment. Tumor-associated macrophages are covered later in Chapter *3.5 Macrophages*.

3.2.2 Immune mediatoris in cancer-related inflammation

Most of the inflammatory cells in the tumor microenvironment have both pro- and antitumorigenic functions. However, the cytokine and chemokine profile of the microenvironment may be more crucial than its specific immune cell content (Table 6). The key orchestrators in cancer-related inflammation include transcription factors (e.g. NF-κB and STAT3), cytokines (e.g. IL-1β, IL-6, IL-23, and TNF-α), and chemokines. Some of the most essential orchestrators are introduced briefly as follows.

Table 6. Tumor microenvironment includes cytokines and chemokines that act as mediators in the cancer-related inflammation.

Cytokine	**Function**
Interferon γ (IFNγ)	Proinflammatory/Th1
Interleukins 1, 6 (IL-1, -6)	Proinflammatory, prometastatic
Interleukins 4, 5, 10 (IL-4, -5, -10)	Immune regulatory/Th2
Macrophage colony-stimulating factor (M-CSF)	Growth factor
Migration inhibitory factor (MIF)	Proinflammatory
Transforming growth factor β (TGFβ)	Growth factor, immunosuppressive
Tumor necrosis factor (TNF)	Proinflammatory
Vascular endothelial growth factor (VEGF)	Angiogenic, vascular permeability
Chemokine	**Attract**
Eotaxin/CCL11	Eosinophils
B cell attracting chemokine-1 (BCA-1)/CXCL13	B cells
Growth-regulated oncogene-α (gro-α)/CXCL1	Neutrophils
Interleukin 8 (IL-8)/CXCL8	Neutrophils
Macrophage derived chemokine (MDC)/CCL22	Th2 cells
Monocyte chemotactic protein-1 (MCP-1)/CCL2	Polarizes immunity to the Th2 direction
Thymus and activation regulated chemokine (TARC)/CCL17	Th2 cells

NF-κB (Nuclear factor kappa light chain enhancer of activated B cells)

NF-κB is a key player in inflammation. It is crucial for both cancer cells and inflammatory cells.[141, 142, 144] NF-κB is the coordinator of the innate immunity and has been shown to function as an important tumor promoter. As a transcription factor, NF-κB controls cell survival by regulating programmed cell death, proliferation, and growth arrest. It has a dual role in tumor promotion: it prevents the death of malignant cells and stimulates the production of proinflammatory cytokines. Despite the predominantly protumoral role of NF-κB, in some cases and especially in early cancers, NF-κB may be tumor suppressive.[147] NF-κB activates the expression of genes encoding cytokines, adhesion molecules, prostaglandin-synthesis pathway enzymes (such as cyclooxygenase 2, COX2), inducible nitric oxide synthase (iNOS), and angiogenic factors. It also induces the expression of anti-apoptotic genes (such as BCL2), and thus, promotes the cancer cell survival. NF-κB pathway is tightly controlled by activators and inhibitors, such as in intestinal mucosa expressed Toll-interleukin receptor-8 (TIR8) and single immunoglobulin interleukin 1 receptor-related protein (SIGIRR).[142] Deficiency in TIR8 encoding gene is associated with increased risk of intestinal inflammation and carcinoma, as well as with B cell proliferation and autoimmunity. TNF-α produced by neighboring inflammatory cells controls also the NF-κB activation state and localization in the cell. However, NF-κB activation in malignant cells occurs often as a result of genetic mutation rather than in response to signals from surrounding cells.[164, 165] NF-κB has also been shown to be involved in macrophage polarization to M2 macrophages (see Chapter *3.5 Macrophages*).[142]

STAT3 (Signal transducer and activator of transcription 3)

Transcription factor STAT3 is a point of convergence in several oncogenic signaling pathways, and is involved in oncogenesis and in the inhibition of apoptosis. STAT3 is activated both in tumor and immune cells. Its activation has been shown to increase the capacity of cancer cells to evade the immune system by inhibiting the maturation of DCs and suppressing the immune response. STAT3 has a dual role in cancer-related inflammation: it promotes protumoral inflammatory pathways, including NF-κB and IL-6/glycoprotein 130 (gp130)/Janus kinase (JAK) pathways, and opposes STAT1 and NF-κB-mediated Th1 anti-tumor immune responses.[141, 142]

TNF-α (Tumor necrosis factor-α)

TNF-α has a role in the persistence of inflammation and tumor growth and progression.[138] It is a major mediator of inflammation, with actions directed to-wards both tissue destruction and recovery. TNF induces death of diseased cells and

stimulates fibroblast growth. It can destroy blood vessels but also induce angiogenic factors. As its name says, tumor necrosis factor-α causes hemorrhagic necrosis and can stimulate antitumor immunity in high doses. However, cancer cell produced TNF-α acts usually as a tumor promoter.[147] In addition to cancer cells, macrophages and CD4+ cells secrete TNF-α and trigger inflammation. The production of pro-inflammatory TNF-α is associated with increased release of chemokines, IL-6, and MIF-1. Via CXCR4 TNF-α participates in cancer cell spread and tumor cell survival.[142] It also stimulates angiogenesis due to in-duction of CXCL12 and VEGF.

IL-1 (Interleukin-1)

Another soluble mediator of cancer-related inflammation is cytokine IL-1. Patients with increased expression of IL-1 have generally bad prognosis: it promotes tumor growth and metastasis by inducing several pro-metastatic genes. IL-1 can stimulate the expression of endothelial adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). In addition, IL-1 is a potent proangiogenic cytokine and mediates VEGF release. On the other hand, small amounts of IL-1α can be poured out from necrotizing cells and serve as a danger signal for anti-tumoral immune responses.[142]

IL-6 (Interleukin-6)

IL-6 is an inflammatory cytokine promoting tumor growth and antiapoptotic responses. It is one of the effector signals of activated NF-κB and activator of oncogenic STAT3. IL-6 protects cells from apoptosis and promotes proliferation.[142]

TGFβ (Transforming growth factor β)

TGFβ is one of the regulators in the inflammatory reaction in the tumor microenvironment. Specific deletion of tgfbr2 increases tumor progression and metastasis in several cancers, such as breast, pancreatic, intestinal, and head and neck cancers. It is assumed that TGFβ effects are regulated through NF-κB. TGFβ regulates the production of chemokine/chemokine receptors (e.g. CXCL12) important to inflammatory cell recruitment. TGFβ secretion by cancer cells leads to inhibition of DC activation and direct inhibition of T cell and NK cell function, as well.[166]

Chemokines

Chemokines are a major subgroup of cytokines playing a central role in the recruitment of leukocytes to the site of inflammation. Most tumors produce chemokines α (CXC group) and β (CC group). Typically CXC chemokines (such as

IL-8) are active on neutrophils and lymphocytes, whereas CC chemokines (such as eotaxin, macrophage derived chemokine, MDC, or thymus and activation regulated chemokine, TARC) act on several leukocyte subsets including monocytes, eosinophils, DCs, and NK cells (but not on neutrophils). Leukocytes and cancer cells express chemokine receptors (CCR and CXCR) and thus, tumor cells can use chemokine gradients to spread around the body.[138]

3.3 Cancer immunosurveillance and immunoediting

The immune system can prevent tumors in three ways: (I) immune cells can suppress viral infections and thus, protect the host from virus-induced tumors, (II) inflammation can prevent the establishment of a protumoral microenvironment, and (III) immune cells can specifically identify and eliminate malignant cells through tumor-specific antigens by a process called immunosurveillance.[167] The immune system can operate as a barrier to tumor formation and progression which cancer cells need to beat in order to develop a tumor.[139] Follow-up studies of immunosuppressed transplant patients and patients with primary immunodeficiencies have shown that immunosuppressed patients have a significantly higher risk for cancer development.[168]

The innate and adaptive immune systems can protect from the tumor development through immunosurveillance. High numbers of lymphocytes (especially T cells) have been reported to associate with good prognosis in various types of cancer (e.g. in melanoma, breast, ovarian, head and neck, esophagus, bladder and colorectal cancer).[143, 167, 169] The mechanisms of inflammation fighting against cancer include presenting cancer antigens to T-cells via antigen-presenting cells (APCs), priming and activating T cells in lymph nodes, T cell trafficking and infiltration into tumor tissue, recognition of cancer cells by T cells, antigen-specific systemic effector and memory T cell development, and humoral immunity.[170] As the immune system can act as an extrinsic tumor suppressor, it can paradoxically also promote cancer initiation, promotion, and progression. Immunosurveillance and tumor-promoting inflammation can coexist even in the same tumor and thus, the prognostic value of these components needs wide-ranged research.[140, 167]

Cancer immunosurveillance represents only one part of a more complex and dynamic process, cancer immunoediting. The term “immunoediting” contains not only immune system protecting the host against cancer but also sculpting the tumor immunogenicity.[167, 168, 171] At the early stages of tumorigenesis, cancer cells express proinflammatory “danger” signals that initiate the cancer immunoediting. Cells from innate immunity (such as NK cells activated by cytokines released by tumor cells and macrophages and stromal cells surrounding the tumor cells) and

adaptive immunity (mainly tumor antigen specific T lymphocytes) protect the host from tumor formation and eliminate the developing tumor (*immunosurveillance or elimination*, the first phase of immunoediting).[171-173] The strongest evidence for the elimination phase in humans is the phenomenon of spontaneously regressing melanoma lesions accompanied by T cells.[174, 175] Lymphocytes recognize transformed cells and promote IFNγ production. IFNγ initiates a cascade of innate immune response that includes the induction of chemokines, antiproliferative action on the developing tumor, the activation of cytocidal effect in macrophages, and NK cells infiltrating the tumor.[168, 171, 175] Chemokines, such as CXCL10, CXCL9, and CXCL11, block the neovascularization in the tumor and recruit NK cells, DCs, macrophages, and other immune cells. If the sporadic cancer cells survive from this process, they may be maintained chronically or sculpted by immune editors to produce new populations of tumor variants with decreased immunogenity. This is called *equilibrium*, the second phase of immunoediting.[171, 172] In the equilibrium phase, the immune system, especially adaptive immunity, and cancer cells hold a dynamic balance.[167] The correlation between the quality and quantity of intratumoral immune response and patient survival has been revealed in colon and lung cancer.[176, 177] Moreover, the type and density of infiltrating lymphocytes were more powerful prognostic factors than pathological tumor staging. The enhanced survival is associated particularly with subsets of T cells, such as CTLs and Tregs. The term dormancy describes a latent tumor that may eventually, after years or even decades, recur as a local lesion or form distant metastases.[178-180] Evidence of an immune derived equilibrium phase has been seen in the unintentional transplantation of cancer cells from organ donor to immunosuppressed recipient. In these cases the donors had no signs of cancer at the time of organ donation but the recipients undergoing immunosuppression for organ engraftment later developed cancers of donor origin.[181] These variants of tumor cells have evaded the immune system and become macroscopic tumors (*escape*, the last phase of immunoediting).[171] The macroscopic tumor growth is enabled by changes occurring in the tumor cell population (immunoediting), or in the host immune system resulting from cancer-induced immunosuppression or immune system breakdown due to the natural aging process.[167] The escape phase represents the failure of the immune system to eliminate or control the transformed cells allowing tumors to develop in an immunologically unrestricted manner. According to the Darwinian selection, the most fit tumor variants survive and form overt cancer in an immunocompetent host.

Tumor antigens (specific mutant cell antigens consisting of products of mutant genes, abnormal metabolic modifications released by cancer cell death) are the cores of the intrinsic antitumor system. They can be absorbed by DCs and delivered to lymph nodes to introduce the antigen to the lymphocytes (Figure 3). In-

duced B cells differentiate into plasmacytes and produce specific antitumor antibodies, which can direct NK cells to kill tumor cells (antibody-dependent cell cytotoxicity, the ADCC effect) and guide macrophages to phagocytose tumor cells (antibody-dependent cell phagocytosis, the ADCP effect). The killing of the cancer cells releases additional tumor-associated antigens to increase the immune response. DCs can also induce the production of CD8+ CTLs to selectively kill tumor cells after delivered to T cells via TCR. IFN cytokine family can enhance the antitumor responses in several ways: IFNγ increases major histocompatibility complex I (MHC I) expression on tumor cells, making them better targets to CD8+ CTLs.[167] It also plays an important signaling role in host immune and stromal cells. NK cells (having cytoplasm filled with active particles, such as perforin, granzyme and IFNγ, that can be released to destroy cells) and macrophages are natural immune cells that do not need direct antigen stimulation to have a powerful killing effect on cancer cells. In contrast, T cells need to be activated by tumor antigens. However, T cell antigen receptor by itself is insufficient to generate T cell responses. Thus, for proper activation T cells have a combination of TCR signals: costimulatory signals (such as B7/CD28, CD137/CD137L, CD40/CD40L), inhibitory receptor signals (such as CTLA4, PD-1/PD-L1), and environmental factor signals.[140, 182, 183]

The antitumoral immune response can be triggered also with certain bacterial infections. There is evidence of regression and increased survival after bacterial infections. This approach is utilized in bladder cancer treated with *Mycobacterium bovis* BCG. The infection triggers favorable inflammatory response through Toll-like receptors (TLRs), which promotes the M1 phenotype macrophage differentiation and effective adaptive immune response in tumor. However, the mechanism how to trigger the immune response to antitumoral type is still not clear and it is an important field of study to find the optimal stimuli to change the tumor-promoting microenvironment to a tumor-inhibiting one.[141]

To survive from antitumor responses, cancer cells need to escape from NK cells and macrophages by encapsulation/camouflage or formation of natural barrier by tumor stroma.[182] Several pathways in the tumor microenvironment suppress the effective adaptive immune responses.[184] For example, the differentiation of DCs is inhibited by different signals, such as IL-10. In addition, protumorigenic Tregs suppress both adaptive and innate immune cells, and tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs) are potent suppressors of antitumor immunity.[141] Tumor cells can express ligands to suppress T cells (such as PD-L1) and inhibit the activity of effector immune cells by recruiting Tregs and MDSCs. CTL proliferation and activation is suppressed through various mechanisms by tumor cells.[166] In cancer patients, the cycle of cancer immunity does not function optimally: the tumor antigen may not be de-

tected, DCs and T cells can treat the tumor antigens as self rather than foreign, Treg responses may be created rather than effector responses, T cells may not properly home to tumors, or the effector cells might be suppressed by the factors from tumor microenvironment.[185]

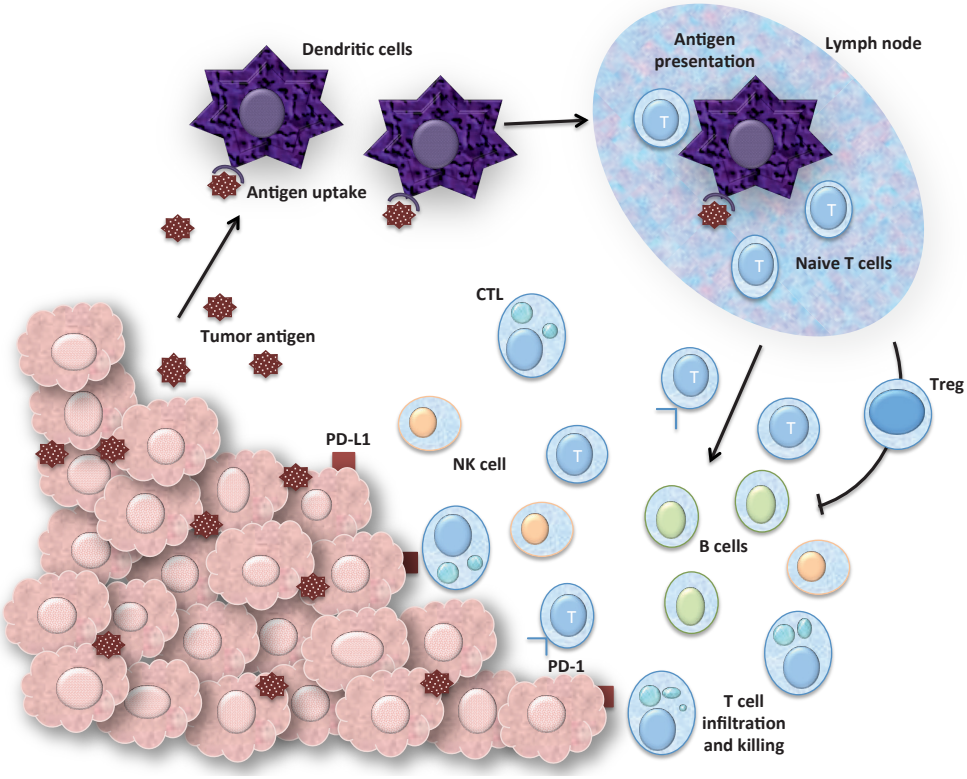

Figure 3. Tumor antigens are released for example from dying tumor cells and presented to naive T cells in the lymph nodes by DCs. T cells are a heterogeneous group of cells that are activated in the draining lymph nodes through antigen presentation. Tregs suppress the function of effector T cells. T cells need several signals for proper function. PD-1/PD-L1 represents an example of an inhibitory signal, which reduces the antitumoral effect of T cells.

3.4 Cancer-related inflammation and metastasis

From a clinical point of view, metastasis is the most critical part of the tumorigenesis, because most of the cancer mortality occurs due to tumor formation at distant sites of the body. Inflammatory components have an essential role in the process of metastasis.[141] Chemokines and cytokines coordinate autocrine and paracrine interactions between cancer cells and infiltrating leukocytes, which

promote cell migration, invasion and survival. They also affect the growth of the tumor and the ability to colonize the metastatic niche. Cancer is a systemic disease and primary tumors produce molecules that influence metastatic outcome at distant sites. However, metastasis as a process is very inefficient in human tumors: only few successful cells make finally metastasis despite the fact that many thousands of cancer cells circulating in the blood stream daily.[186] Tumor cells use the same molecular tools (adhesion molecules, cytokines, chemokines, and chemokine receptors) and pathways as leukocytes when spreading to distal sites during inflammation.[138, 144] Cancer cells start to express chemokine receptors when transformed to invasive and metastatic cells. Chemokine receptors and their ligands direct the movement of the cancer cells and promote metastasis by maintenance of tissue homeostasis and survival at sites distant from the primary tumor.[141] For example, CXCR4 and its ligand CXCL12 are important for cell movement. CXCR4 is the most frequently up-regulated chemokine receptor in tumor cells, and the amount of expression correlates with the extent of metastasis in colorectal, breast, liver, and oesophageal cancer. Expressions of different chemokine receptors in tumor cells are implicated in organ-specific metastases: e.g. CCR7 correlates with lymph node metastasis and CCR9 correlates with small intestine metastasis in melanoma. Chemokine-receptor expression is upregulated by cytokines such as TNF-α, IL-1β, and IL-6. The invasive capacity of cancer cells increases simultaneously with the chemokine-receptor expression.

The process of metastasis can be divided into four stages: (I) epithelial-mesenchymal transition (EMT), (II) intravasation into vascular and lymphatic vessels, (III) trafficking throughout the circulation, and (IV) extravasation from the vessels and proliferation at the metastatic niche. In the first stage, cancer cells acquire fibroblastoid characteristics. This increases the mobility of cancer cells and allows them to invade epithelial lining/basal membrane to reach the vessels. The key event in the EMT is the loss of E-cadherin expression. TGFβ, produced by cancer cells, myeloid cells, and T lymphocytes, regulates the EMT and, thus, metastasis. Increased TGFβ expression in tumors is often associated with poor prognosis. Inflammation promotes the second step, intravasation of the cancer cells, by production of mediators that increase vascular permeability. Intravasation of cancer cells is regulated by prostaglandins, cytokines, and MMPs. Only approximately 0.01% of the cancer cells entering the circulation will survive to give rise to micrometastases. A variety of cytokines, such as TNF-α, IL-6, and epiregulin, can promote the survival of circulating metastatic seeds. However, single metastatic cells in the circulation, away from the immunosuppressive tumor microenvironment, can be targeted by immunosurveillance. At the last stage of metastasis, metastatic seeds arrest on the endothelium and extravasatate into the tissue with the help from e.g. integrins. Several proinflammatory cytokines

elevated in the circulation upregulate the expression of adhesion molecules on the endothelium in the target organ and increase the probability of metastatic cell attachment. In the tissue, single metastatic progenitors interact with inflammatory and stromal cells and start to proliferate resulting a metastatic tumor.[140]

3.5 Macrophages

Macrophages act in innate immunity with multiple functions. Myelomonocytic cells act as the first line defense against pathogens. They activate adaptive responses and are among the first cells to arrive to the wounding or infection site after neutrophils.[187] They produce chemokines and cytokines to recruit other inflammatory cells and promote tissue repair by growth factors, angiogenic factors, and proteases. In addition, macrophages produce reactive oxygen and nitrogen radicals to kill pathogens and function as APCs to T cells.[188] Macrophages originate from bone marrow precursors common hematopoietic stem cells (HSC). In response to M-CSF, they divide and differentiate into monocytes through monoblast and pro-monocyte stages and exit to the circulation from the bone marrow. In response to inflammation, these monocytes integrate into tissues and activate as tissue-specific macrophages, such as osteoclasts in bone, microglial cells in the central nervous system, and Kupffer cells in liver.[189-191]

Plasticity and diversity are the known hallmarks of the multifunctional monocyte-macrophage lineage.[192] They undergo reprogramming of their functional properties in response to signals from microbes, damaged tissues, and lymphocytes. Macrophages constitute a part of the innate immunity, but they have also a built-in adaptive component: they are reshaping their subsequent responses to microbial encounters.[187] In physiological conditions, the main function of macrophages is to clear the interstitial environment from extraneous cellular material by phagocytosis. They remove cellular debris e.g. from necrosis, clear the apoptotic cells, and recycle e.g. iron and hemoglobin by phagocytosis of erythrocytes.[190] Macrophages are the primary sensors of danger in the host. They detect endogenous danger signals present in the debris of necrotic cells. Macrophages are critical components of the wound healing process. In addition, macrophages play a role in development and morphogenesis of many tissues.[188]

3.5.1 Macrophage polarization

Two distinct types of polarized activation of macrophages are recognized: the classically activated M1 macrophage phenotype and the alternatively activated M2 phenotype (Table 7).[189, 192] M1 macrophages are activated by microbial components

such as lipopolysaccharide (LPS), IFNγ from the Th1 cells, CD8+ T cells or NK cells, and TNF from APCs, whereas M2 polarization occurs in response to the cytokines IL-4 (from Th2 immune responses or mast cells, basophils, and granulocytes during tissue injury) and IL-13 (from Th2 immune responses) or in response to stress induced glucocorticoid release.[163, 187, 190, 192-194] The activities of M1 macrophages have been summarized as "fight" activities while M2 macrophages are "fixing" type macrophages.[195] M1 macrophages produce large amounts of proinflammatory cytokines, such as IL-12, IL-23, and TNF, and effector molecules, such as reactive oxygen and nitrogen intermediates (ROI and RNI, respectively). They are characterized by increased expression of MHCII and are efficient in antigen presentation. M1 macrophages have microbicidal and tumoricidal activities, and are key mediators of the immunopathology in several autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease.[163, 187, 188, 190, 192, 193]

M2 macrophages are a part of Th2 immune response, including humoral immunity, and are considered to be involved in intracellular pathogen containment. They have a high expression of immunosuppressive cytokine IL-10 and a low expression of IL-12. They show phagocytic activities and high expressions of scavenging (SR), mannose (MR) and galactose receptors, and are involved in wound healing processes. M2 macrophages help with parasite clearance, dampen inflammation, promote tissue remodeling and tumor progression, and have immunoregulatory functions. In contrast to M1 macrophages, M2 macrophages fail to present antigens to T cells, produce minimal amounts of pro-inflammatory cytokines, and are less efficient at killing intracellular pathogens. They suppress T cell activation and proliferation by prostaglandin, IL-10, and TGFβ production. In addition, M2 macrophages support angiogenesis and lymphangiogenesis by releasing proangiogenic growth factors such as IL-8, VEGF, and EGF.[187, 190, 192, 193]

M1 and M2 macrophages have distinct chemokine profiles: M1 macrophages express chemokines such as CXCL9/monokine induced by IFNγ (MIG) and CXCL10/IFNγ-induced protein-10 (IP-10), whereas M2 macrophages express CCL17/TARC, CCL22/MDC, and CCL24/Eotaxin-2. Chemokines can also affect the polarization of macrophages with CCL2 and CXCL4 driving the polarization toward M2-like phenotype.[187]

Despite the well-known subdivision of macrophages, the M1/M2 polarization is not the whole truth about macrophage diversity and more of an oversimplification of the complex biology of mononuclear phagocytes. M1 and M2 phenotypes can be considered as two opposite states in a broad macrophage polarization spectrum. Especially M2 has been considered as a generic name for a various forms of alternatively activated macrophages that have distinct effects according to the activating factors.[187, 194] M2 macrophages have been suggested to be subdivided

into M2a, M2b, and M2c macrophages.[193] M2a macrophages are polarized by IL-4 and IL-13, and promote Th2 immune responses and are involved in allergic reactions and parasite killing, whereas M2b cells are activated by immune complexes and TLR or IL-1R and promote immune regulation and Th2 responses. M2c macrophages are activated by IL-10 and they function also as immunoregulators, but as matrix deposition and tissue remodeling cells, too. However, this subdivision, too, is probably much simpler than the truth about the wide spectrum of M2-like macrophages. Furthermore, a shift in macrophage phenotypes during the course of several diseases (such as sepsis, cancer, and obesity) has been noticed. Even in the process of wound healing, macrophages undergo dynamic changes switching their phenotype form an M1 to an M2 phenotype.[187, 196]

Table 7. Differences in classically activated (M1) and alternatively activated (M2) macrophages.

	M1 macrophages	**M2 macrophages**
Activators	IFNγ, TNF, LPS	IL-4, IL-13, IL-33, IL-10, glucocorticoids, vitamin D3
Cytokine expression	IL-12, IL-23, TNF, IL-6, IL-1	IL-10, IL-1RA, MSF
Chemokine expression	CXCL9, CXL10 → Th1 cells, NK cells	CCL2, CCL17, CCL18, CCL22, CCL2 → Th2 cells, Tregs, eosinophils, basophils
Receptor expression	MHC, TLR2/4, CD80, CD86, CD16	SR-A, MR, galactose receptor, CD163, CD206, CLEVER-1
Growth factor and effector molecule production	iNOS, ROI	Proangiogenic growth factors (IL-8, VEGFA, VEGFC, EGF), TGF-β, FGF arginase
Functions	Antigen presentation, humoral immunity, killing of the intracellular pathogens	Phagocytosis, scavenging, parasite clearance, tissue remodeling, angiogenesis, allergy, wound healing
Immunity	Th1 response, proinflammatory	Th2 response, anti-inflammatory
Tumor function	Antitumoral	Protumoral

3.5.2 Tumor-associated macropahges

Tumor-associated macrophages (TAMs) are an essential component of the cancer-related inflammation and the tumor microenvironment.[141] Stromal and tumor cells produce chemokines and growth factors to recruit circulating monocytes and differentiate them into macrophages. For example, CCL2/MCP-1, CCL5/regulated upon activation normal T cell expressed and secreted (RANTES), CXCL12/stromal derived factor-1 (SDF-1), and CXC3L1/fractalkine contribute to macrophage recruitment and tumor promotion. In addition, M-CSF/CSF1, VEGF, TGFβ, bFGF,

urokinase plasminogen activator (uPa), and antimicrobial peptide β-defensin-3 are involved in monocyte recruitment and macrophage differentiation in tumors.[141, 192, 193, 197-199]

In cancer, macrophages can have either beneficial or pathological roles depending on the tumor microenvironment.[200] They can prevent the establishment and spread of tumor cells, and simultaneously, support tumor growth and dissemination.[201] TAMs generally have an M2-like phenotype.[202] Th2 cell-derived IL-4 and IL-13 have a key role in M2 activation and protumoral roles of macrophages in tumors.[187] Tumor cells produce IL-10 and CSF1, as well as chemokines CCL17 and CCL22 to promote M2 polarization in tumor tissue.[199] However, variations of TAM polarization have been noted depending on the tumor type.[187] Furthermore, macrophage phenotype can vary according to their location in tumors (sites of initial tumor cell invasion, perivascular areas, stromal regions, and hypoxic/necrotic areas) in response to local signals.[187, 203, 204] As TAMs localized in the hypoxic regions in tumors favor angiogenesis, TAMs at the peritumoral area, at the tumor-stroma interface, help the cancer cells to invade by producing a variety of proteases to breakdown the basement membrane.[198, 204, 205] Moreover, TAMs in the perivascular areas seem to promote the tumor cell intravasation into the blood vessels, and thus promote metastasis.[206] In the tumor tissue, macrophages promote inflammation, suppress immune regulation, and support angiogenesis.[198] In a breast cancer model, M2-like macrophages were found in hypoxic regions, while M1-like phenotypes were seen in normoxic tissues.[207] At the metastatic sites, macrophages prepare the tissue for successful colonization of cancer cells.[198]

TAMs influence almost all steps of carcinogenesis and tumor progression: they contribute to genetic alterations and instability, regulate senescence, promote angiogenesis and lymphangiogenesis, suppress adaptive immunity and CTLs, interact and remodel extracellular matrix (ECM), and promote invasion and metastasis. They promote tumor growth by producing growth factors, such as EGF, VEGF, and bFGF, and suppress immune responses through the release of soluble mediators, such as IL-10, indoleamine dioxigenase (IDO) metabolites, and TGF-β, as well as through cell-to-cell contact mechanisms (e.g. PD-L1).[163, 187, 193, 198] TAMs activate Tregs that strongly suppress effector T cells and other inflammatory cells. In addition being a target to chemokines, TAMs are also a source of chemoattractants, such as CCL2, CCL17, CCL18, and CCL22.[193] For example, TAMs attract naive T cells to induce T cell anergy. TAMs secrete CXCL8, which promotes angiogenesis and reduces CD8+ CTL activity by increasing PD-L1 expression on macrophages.[208]

In the tumor initiation, TAMs are major producers of proinflammatory mediators such as IL-6, TNF and IFNγ, reactive oxygen and nitrogen species, proteases, and growth factors, such as EGF. After tumor progression, TAMs are

recruited continuously into the tumor tissue to have different functions in angiogenesis, invasion, intravasation, and immunosuppression. In the invasion stage, cancer cells require matrix formation and destruction through macrophage produced cathepsin and SPARC (secreted protein, acidic rich in cysteine). Macrophages have been described as "the key that unlocks the gate to allow tumor cells to escape": they induce proteolysis to allow the cancer cells to escape through the basement membrane and to migrate through the stroma.[187, 198] In addition, metastasis-associated macrophages (MAMs) send survival and growth signals to tumor cells and inhibit the functions of effector T cells.[209] MAMs are attracted from bone marrow-derived monocytes through CCL2-CCR2 mechanism and differentiated through CCR1-CCL3 autocrine signaling.[210] They are transcriptionally different from resident macrophages and characterized by the expression of CD11b, VEGF receptor 1 (VEGFR1/FLT1), CXCR3, and CCR2.[209, 211] MAMs support the survival of the metastatic cells interacting physically with them, induce EMT by producing TGFβ, and inhibit CTLs.[212] Tumor cells and macrophages develop a paracrine signaling loop during the initial stages of metastasis, where tumor cells produce CSF1 that stimulates macrophages to produce EGF, a chemoattractant for the tumor cells expressing EGFR, which in turn activates migration of the cancer cells.[213]

Angiogenesis is important to tumor survival and development. In addition, angiogenesis associates strongly with the presence of inflammatory cells. TAMs contribute to tumor angiogenesis, and there are evidence of associations between macrophage infiltration, tumor vascularity, and disease prognosis.[138, 193] Macrophages preferentially accumulate to poorly vascularized regions of tumors.[201] They cooperate with tumor cells to induce a vascular supply of the area by upregulating angiogenic factors and enzymes in response to high levels of adenosine, which is present in the tumor microenvironment during local hypoxia. The angiogenic factors diffuse away from the hypoxic region and stimulate endothelial cells to migrate, proliferate and differentiate into new vessels.[204] Hypoxia induces the metabolic adaptation of TAMs through the activation of hypoxia-inducible transcription factor-1 and 2 (HIF-1 and 2, respectively).[188, 193, 214] HIF-1 and -2 control the transcription of pro-angiogenic genes VEGF, bFGF, and CXCL8 in TAMs.[215] TNF, IL-1, and IL-6 stimulate also the production of angiogenic factors. In addition, TAMs produce TGF-β1, which is angiogenic itself and induces production of VEGF. Some chemokines (e.g. IL-8) are also pro-angiogenic. They have direct effects on microvascular endothelial cells and can stimulate angiogenesis indirectly via TAMs.[138]

The protumoral M2-like phenotype in TAMs is reversible. It has been shown, that, for example, IFNγ can reeducate M2 macrophages *in vitro*.[216] Gradual switching of TAM polarization from M1 phenotype to M2 phenotype is paralleled

by the gradual inhibition of the NF-κB activity and increased STAT3 activity during different stages of tumor progression. Early in the carcinogenesis T cell driven M1 activated macrophages may contribute to elimination of cancer following with regulatory mechanisms of M2 polarized TAMs that orchestrate tumor-promoting inflammation.[199, 201] Transcription factor STAT1 is activated in response to M1 polarization signals (e.g. IFNγ and LPS) and is essential for immune surveillance against cancer.[217] In contrast, STAT3 and STAT6 are activated by M2 cytokines (e.g. IL-10, IL-4, and IL-13). Tumor microenvironment educates macrophages to become protumoral. NF-κB has been considered as the master regulator of TAM transcriptional programs and function.[217] It is an essential transcription factor guiding the inflammatory response in macrophages, and it has an influence on tumor cell proliferation and survival, too. NF-κB activation depends on the stage of the tumor growth. NF-κB is activated in macrophages during early stages of tumorigenesis but is defective in advanced tumors.[203] Defective NF-κB activation in TAMs associates with M2-like macrophage phenotype. On the other hand, full activation of NF-κB in macro-phages inhibits M1-like inflammation and promotes tumorigenesis in the early tumors.[217]

The research of the prognostic role of macrophages in different cancers has shown controversial results. In most of the tumors, a greater amount of TAMs is associated with poor prognosis, e.g. in breast, cervix, bladder, gastric, thyroid, lung, and hepatocellular cancer.[204, 212, 218-221] However, TAMs have been shown to associate with better survival e.g. in osteosarcoma, gastric cancer, and in pancreatic cancer.[222, 223] In colorectal cancer, the prognostic significance is controversial and could depend on distinct macrophage phenotypes on a distinct localization within the tumor.[224, 225] However, differences between studies (antibodies used, localization of macrophages, differences in experimental procedures and techniques) may lead to these controversial results.

3.5.3 TAMs as therapeutic targets

Specific macrophage-targeted therapies are now taking the first steps into the clinical arena e.g. in cancer, asthma, and atherosclerosis.[226] Therapeutic strategies targeting TAMs can be grouped into six mechanisms: (I) blocking the pro-tumoral functions of TAMs, (II) promoting the phagocytosis of tumor cells by TAMs, (III) using inhibitors to allow CTL activity, (IV) reprogramming TAMs into antitumor macrophages to kill tumor cells or activate T cells, (V) inhibiting the tumor suppressive microenvironment, and (VI) inhibiting the recruitment of the TAM progenitors.[212] There are currently several drugs being tested in clinical trials targeting TAM recruitment, survival, and reprogramming as monotherapies or in combination with chemotherapy or immunotherapy.[212]

TAMs can have direct cytotoxicity toward tumor cells (macrophage-mediated tumor cytotoxicity, MTC, or antibody-dependent cellular cytotoxicity, ADCC) or indirect cytotoxicity via the secretion of factors that stimulate the anti-tumor functions of other cell types. MTC is a slow process taking up to three days. In this process, macrophages release toxic factors, such as TNFα, serine proteases and RNIs, into cancer cells resulting in cell lysis. ADCC is dependent on tumor-targeting antibodies. However, the killing mechanism is similar to MTC, although faster. Autologous macrophages have been considered using as a form of adoptive anti-cancer therapy, but the attempts have proved to be largely ineffective so far. There have been no major side effects, but there have been no evidence of an anti-tumor response, either.[197]

Macrophages play an important role in the chemotherapy response and resistance and can be selectively recruited to enhance the response rate for example by CXCR4 blockade.[227, 228] TAMs affect also CPI therapies; TAMs express high levels of PD-L1 and PD-L2, as well as PD-1.[229] CPIs are presented in detail in next Chapter *3.6 Cancer immunotherapy*. Manipulation of TAM functions could improve the efficacy of immunotherapies. PD-L1 expression by TAMs suppresses the T cell response. It has been shown that in the therapy resistant tumors TAMs remain inactive and do not exert antigen-presenting activity.[230] In addition, treatment with PD-L1 antibodies, but not PD-1 antibodies, has been shown to reverse the immunosuppressive macrophage phenotype and trigger macrophage-mediated antitumor-activity.[231] PD-L1-expressing macrophages have been found to be more abundant in several different tumors than PD-L1-expressing cancer cells.[232] Moreover, PD-L1 inhibitor treatment has shown to induce antitumor activity even in models with non-PD-L1-expressing tumor cells suggesting that macrophages may be the key element in the response to PD-L1 inhibitor treatment.[233]

The hypoxia-HIF-1 pathway has a role in TAM recruitment and activation in tumors, and targeting HIF-1 activity could affect TAM accumulation.[193, 215] Inhibitors of monocyte attractants include CSF1 receptor inhibitor (studied in acute myeloid leukemia and melanoma models), CCL2/CCR2 antibodies (prostate and breast cancer), and VEGF inhibitors.[226] CSF1 mediates primarily tumor infiltration of TAMs and is an important regulator of TAM polarization into an M2 phenotype.[234] Clinical trials have shown promising preliminary results from TAM depletion by targeting the CSF1/CSF1R axis especially in tumors overexpressing CSF1, such as synovial giant cell tumors.[195, 212] However, CSF1 inhibitors affect all macrophages, not only M2 typed TAMs, and depleting all macrophages from the body has caused some toxicity problems that have limited dose escalation. There have been attempts to inhibit the CCL2/CCR2 axis to prevent the recruitment of macrophages, but the results from these trials have been disappointing and no effective ways to reduce

the TAM recruitment have been discovered.[212] In bone metastasis models, bisphosphonates have shown anticancer effects by causing apoptosis in osteoclasts.[235]

The reprogramming of TAMs into tumoricidal macrophages is an attractive strategy for novel cancer therapy. This would eliminate the adverse effects of the depletion of all macrophages. Polarized phenotypes are reversible *in vitro* and *in vivo*. Restoration of NF-κB activity in TAMs could restore M1 inflammation and intratumoral cytotoxicity.[193, 202] IFNγ and CD40 agonist antibodies have been found to activate the tumoricidal activity of TAMs and induce high expression of M1 markers in macrophages.[226] CD47 inhibits phagocytosis by macrophages and its antibodies targeting CD47 have been studied in the treatment of acute myeloid leukemia and in pediatric brain tumors.[236, 237] Other molecules studied in switching TAMs into tumoricidal macrophages include different TLR ligands, proinflammatory cytokines (e.g. CSF2 and IL-12), histone deacetylase inhibitors, anti-MARCO antibodies, and phosphoinositide 3-kinase γ (PI3Kγ) inhibitors.[212] However, TLR agonists and cytokines associate with systemic adverse effects.[238] Intratumoral injections and targeted delivery systems have been used to prohibit the side effects and selectively target the tumor lesion. PI3Kγ inhibitor studies have shown that switching the macrophage phenotype into an M1-like state can turn resistant tumors to sensitive to T cell-mediated immune attack.[239] Macrophage targeted therapies is a growing field of study, but currently, there are no macrophage drugs in the clinical practice and further studies are needed to find the holy grail of the field, reorienting and reshaping deranged macrophage polarization.

3.6 Cancer immunotherapy

Classically, cancer treatment consists of surgery, chemotherapy and radiation. These are effective treatments, but they have several limitations, too. Surgical excision of the tumor is effective only in the early stages of the disease and it fails when the cancer becomes metastatic. Chemotherapy is often limited by its severe toxicity and numerous side effects. Radiation therapy causes localized tissue damage in healthy tissue as well as in the tumor tissue. Immunotherapy will revolutionize the standard of care for many types of cancer.[240] At present, cancer immunotherapies mainly include cytokines, cellular therapies, checkpoint blockade, and tumor vaccines.[241] Immunotherapeutic agents lack the side effects associated with traditional cancer treatment by utilizing components of patient's own immune system to selectively target malignant cells. In 2017, there were 26 immunotherapies approved in the treatment of 17 types of cancer. To describe the future of the field, there were 940 immunotherapy agents in clinical development

with another 1064 potential drugs in preclinical phase in 2017, and the numbers are constantly growing.[240]

Immune checkpoints regulate autoimmunity and decrease tissue damage by modulating costimulatory and inhibitory signaling during the immune response. Checkpoint inhibitors induce T cell mediated antitumor responses by selectively blocking the inhibitory signals of T cell activation. These regulatory mechanisms maintain immune responses within desired physiologic range and protect the host from autoimmunity in normal physiology.[116, 242] CPIs include CTLA-4 and PD-1/PD-L1 blockades as well as new CPIs that have generated promising results such as lymphocyte activation gene-3 (LAG-3), B and T lymphocyte attenuator (BTLA), and T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain-3 (TIM-3).

Several research groups have been studied CTLA-4 for many years, notably in 2018 Nobel awarded James P. Allison and his colleagues. It has provided hope for late stage melanoma patients with poor prognosis.[243, 244] CTLA-4 negatively regulates T cell activation and inhibits uncontrolled immune responses. It also prevents chronic autoimmune inflammation.[170] CTLA-4 is recruited to the plasma membrane on T cell activation where it binds to DCs and other APCs via B7 molecules. (CD80, CD86) This prevents the ligation of the co-stimulatory CD28-B7 complex needed for full T cell activation, and thus, inhibits further stimulation and immune response.[116, 183, 185, 243] In cancer, CTLA-4 becomes an inhibitor of antitumoral immune responses.[245] Anti-CTLA-4 antibodies potentiate the antitumor response by blocking CTLA-4 receptors to facilitate T cell activation and unleash the immune system to attack cancer.[173, 242, 244] Ipilimumab, a monoclonal anti-CTLA-4 antibody (approved by the FDA in 2011), was the first checkpoint inhibitor to demonstrate improved overall survival with previously treated metastatic melanoma.[246, 247] The problem with ipilimumab is the significant rate of on-target toxicities: up to 23% of the patients develop serious adverse events such as colitis due to induced inflammation. Also, the stimulation of T cell response with ipilimumab may take several months to occur, while tumors may grow and progress.[242]

In 1992, Tasuko Honjo and co-workers discovered protein called programmed cell death-1 (PD-1), which, along with CTLA-4, led to the Nobel price in Medicine 26 years later. Like CTLA-4, PD-1 belongs to the CD28 family and delivers negative signals.[248] PD-1 is a transmembrane protein expressed upon activation on T cells, B cells and NK cells. It activates by binding to PD-L1 and L2 on APCs and cancer cells, which inhibits TCR-mediated effector functions, decreases cytokine productions and reduces the proliferation of CD8+ T cells. It inhibits directly apoptosis, causes peripheral T effector cell exhaustion, and promotes T effector cells to convert to Tregs. T cell exhaustion is an important mechanism that limits T

cell activity in the chronic antigen stimulation. In normal situation, PD-1 maintains peripheral tolerance and T cell responses within a desired physiological range. The ligands of PD-1 are expressed widely in non-lymphoid tissues and thus, PD-1 acts primarily to dampen T cell activation in the periphery. PD-L1 and PD-L2 (CD274 and CD273, respectively) expressions are induced in response to inflammatory cytokines, such as IFNγ.[116, 170, 185, 248] Under physiological conditions, PD-1 on activated CD8+ T cells binds to PD-L1 ligands on APCs, deactivating the T cell. Many tumor cells express PD-L1 as a survival tactic, while many tumor antigen-specific T cells express PD-1 receptor.[242] PD-1 blockade reinvigorates CD8+ cells leading in increased functional activity and frequency and thus, induce tumor rejection. There is evidence, that PD-L1 expression on macrophages may lead to active eviction of T cells from the tumor microenvironment and regulate T cell trafficking and migration.[116] CPIs targeting PD-1/PD-L1 have demonstrated positive clinical effects on more than 15 cancer types, and after the approval of anti-CTLA-4 ipilimumab for the treatment of metastatic melanoma, five PD-1 blockade drugs have been approved by the FDA in cancer treatment.[116, 249] However, only part of the cancer patients benefit from PD-1 blockade drugs and the reason for this disparity is still unknown.

Immunotherapies are effective treatment modalities, but however, the specificity of the treatment is also a limiting factor.[250] Therefore, combined treatment may be better regulating the immune system to promote antitumor effects. Studies have supported the idea of combination of anti-CTLA-4 and anti-PD-1 to increase response rates. Antibodies to PD-1 have safer toxicity profile than ipilimumab. CTLA-4 and PD-1 regulate distinct inhibitory pathways in T cells.[173, 243] They do not overlap and seem to synergize in eliciting an immunogenic microenvironment.[116, 173, 251, 252] Although they both are CPIs they regulate different phases of the immune response: CTLA-4 blocks early T cell activation in the lymphoid organs, whereas PD-1 inhibits effector T cell activity at later stage immune responses in peripheral tissues and in the tumor microenvironment. CTLA-4 deficient mice develop devastating autoimmune disease and massive lymphoproliferation, and die within five weeks of birth. In contrast, PD-1 deficient mice remain relatively healthy into later stages of life.[248]

Adoptive T cell transfer (ACT) technology is another technique used in immunotherapy. It consists of the reinfusion of autologous anti-tumor T lymphocytes. ACT takes advantage on the reliance of immune cells in the tumor microenvironment, stimulating cells *ex vivo*, and manipulating the immune environment for the introduction of effector cells. Lymphodepletion by chemotherapy or radiation is used to enhance the antitumor effects of transferred lymphocytes. The therapy includes also co-administration of high doses of IL-2 as an immune stimulant for the expansion of the transferred lymphocytes. One form

of transferred lymphocytes is TILs, mononuclear lymphocytes that have propensity to surround and invade tumors. Autologous peripheral blood lymphocytes can also be genetically engineered to recognize specific tumor antigens.[173, 242]

TILs are aimed to kill cancer cells directly, but engineered antibodies can be used to target known molecules within oncogenic pathways to reduce the cancer cell proliferation by activating the immune system. In addition, these biotherapeutics can opsonize tumor cells and trigger their death or removal by antibody-dependent cellular cytotoxicity or phagocytosis. For example, rituximab (anti-CD20 antibody) is the first monovalent antibody (mAb) approved by the FDA for treatment of hematologic cancers. Eight other monoclonal antibodies targeting six cancer-associated proteins (Her2/neu, EGFR, VEGF, CD20, CD52, and CD33) are approved for the treatment of solid and hematological malignancies. However, mAbs can stimulate redundant pathways and have limitations, such as promotion of cancer cell survival. Bispecific antibodies (bsAbs) have dual antigenic specificities and are capable to interact with multiple receptors/ligands. Bispecific T cell engagers (BiTEs) recruit T cell effectors and have become a valid therapeutic option in the treatment of a several cancers. To overcome the issues with rituximab, a tetravalent anti-CD20/CD3 bsAb was developed for treatment of B cell lymphoma. The anti-CD20 is specific for B cells, while the anti-CD3 reacts with T cells. This new antibody have 6 times higher cytotoxicity potential compared to standard rituximab and improved stability, solubility and production yield, but the clinical studies are ongoing.[242, 243]

3.7 Bladder cancer-related inflammation

Inflammation has an important role in bladder carcinogenesis. However, the role of acute and chronic inflammation and tumor immunity is still poorly understood in BC. Chronic infection is a risk factor for BC development.[253, 254] Epidemical studies have shown a correlation between urinary tract infection and risk of BC, especially with three or more infection episodes. In addition, urinary tract infections associate with worse outcome in BC. On the contrary, antibiotics used to treat infrequent infections decrease the risk of BC via cytotoxicity against tumor cells. Higher exposure to nonsteroidal anti-inflammatory drugs (NSAIDs) may also decrease the risk of urothelial malignancy.[253, 254] In addition to common urinary tract infection, a parasitic *Schistosoma* worm infection is strongly associated with BC, particularly with high-grade squamous cell carcinoma of the bladder.[253, 255] *S. Haematobium* is endemic in Africa and the Middle East and it has been classified as group 1 carcinogen.[256]The initiation and progression of schistosomal BC is a complex and multistep process. It has been proposed that *S. Haematobium* eggs give rise to genotoxic factors causing a genomic instability in a host cell, which

leads to a proliferative response to repair tissue damage caused by inflammation. This activates oncogenes or inactivates tumor suppressor genes. *S. Haematobium* has also considered as oncogenic because of the induction of K-RAS mutations.[254, 257] It has been suggested, that chronic irritations of urinary tract (e.g. catheters) are also risk factors of BC via hyperplasia, metaplastic changes, and chronic inflammation in response to local injury of the urothelium.[254, 258]

Despite chronic inflammation being a risk factor in BC, several studies have shown, that lymphocytes in the tumor tissue associate with better prognosis in BC. Inflammatory infiltrate associates with fewer recurrences, better survival, and is an independent prognostic factor in BC.[259, 260] Acute inflammation is associated with anti-tumor response and impairment of acute inflammation at an early stage of tumorigenesis could promote cancer development.[261] However, necrosis and invasive front inflammation have shown to be independent risk factors in high-grade BC.[262] The inflammation studies have several limitations. In meta-analysis 2014 Masson-Lecomte et al. raised the problems in the heterogeneity of the populations and the term inflammatory infiltrate in inflammation studies in BC. They demanded precise characterization of the inflammatory infiltrate in the tumor and establishment of mechanistic hypotheses before drawing any further conclusions about the role of inflammation in BC.[263]

Systemic inflammation markers have also been shown to affect BC survival. Systemic inflammatory marker C-reactive protein (CRP) is associated with survival in BC. CRP is a representative acute-phase reactant and a significant and sensitive inflammatory marker. High CRP (over 5mg/l) associates with higher risk of progression of the disease and has been shown to be an independent prognostic factor in BC.[263-266] The neutrophil-to-lymphocyte ratio (N/L ratio) from peripheral blood samples is also considered to be a systemic inflammatory response marker.[267] Numerous studies have demonstrated, that the N/L ratio is a prognostic factor for survival in BC patients. Decreased lymphocyte count or N/L ratio rising over 2.5-3.0 associates with poor prognosis and DSS, recurrence, and unfavorable treatment outcome.[268-271] However, a clear definition of the cut-off value remains to be determined.[272] Preoperatively N/L ratio predicts the lymph node metastases and survival after RC.[272-274] The ratio predicts poor recurrence-free survival (RFS) in MIBC patients and is correlated with progression and recurrence in NMIBC patients.[271, 275] In NAC treated BC patients high N/L ratio is associated with pathological response.[276]

In addition to the peripheral ratio of neutrophils and lymphocytes, TILs have widely been studied in different cancer subtypes, as well as in BC. Generally, TILs are considered to be a positive prognostic factor in malignancies. They are frequently found in the microenvironment of BC tissue and elicit both tumor-suppressive and tumor-promoting effects.[277] High prevalence of TILs is associated

with more favourable outcome in BC.[277] Especially CD3+ and CD8+ tumor infiltrating lymphocytes have a positive prognostic role in BC.[263, 278] CD4+FoxP3+ Treg cells correlate negatively with OS in the majority of solid tumors, but the prognostic role of these cells varies greatly depending on the cancer type.[279] In BC, Tregs correlate positively with survival, while FoxP3 expression in BC cells associates with decreased survival in MIBC.[278, 280] In cancer cells and macrophages, Tregs suppress MMP2 expression, which is a pro-metastatic molecule associated with poor prognosis in BC.[280] However, FoxP3 positive T cells have also been shown to associate with higher risk for recurrence after TUR-BT.[281] Stimulation of naive CD4+ cells by TGFβ and IL-6 initiates Th17 cell differentiation.[282] In BC, Th17 cell levels are elevated in tumors relative to the peripheral blood levels, whereas Treg levels in the peripheral blood are higher than in the tumor tissue.[258]

In addition to Th17 cell activation, IL-6 is also a major activator of the STAT3 signaling pathway, which implicates in oncogenesis, tumor progression, and metastatic spread.[283] IL-6 is produced under inflammatory conditions. It is the primary pro-inflammatory cytokine in humans and is produced by T cells and macrophages.[258] In BC patients, IL-6 levels in urine and in serum are elevated compared to healthy controls. It has been shown to associate with higher stage and recurrence rate, increased risk for metastasis, and reduced survival in BC.[284, 285] However, tumor-suppressing effects of IL-6 has also been found in BC.[254] In addition, high levels of IL-6 have been found in BC patients responsive to BCG instillations.[283]

STAT3 and NF-κB are major inflammation-promoting transcription factors. They cause an perpetual oncogenic loop: NF-κB activation in immune cells induces expression of cytokines that promote STAT3 activation in both malignant and immune cells, whereas persistently activated STAT3 has been shown to mediate NF-κB activity.[286] NF-κB correlates with histological grade and T category in BC.[254] IL-8 is another cytokine associating with invasiveness and growth of BC.[283] IL-8 has several properties: it is an angiogenic factor stimulating growth and survival of endothelial cells and an inflammatory factor secreted by leukocytes to promote chemotaxis of neutrophils and T cells. It can also avoid cell death. IL-8 activity is mediated by CXCR1 and CXCR2. IL-8 attracts MDSCs, which are critical mediators of cell-associated immune suppression in BC.[253, 287] The urinary concentration of IL-8 has been evaluated as a diagnostic biomarker of BC.[288] IL-2 has also been considered as a prognostic urinary biomarker of BC; low concentrations of IL-2 in the urine correlate with poor prognosis of the disease.[283]

3.7.1 Macrophages in bladder cancer

Macrophages consist a majority of all CD45 positive cells in the mouse bladder.[289] They produce pro-inflammatory cytokines during infections and are the main control of the innate immune response.[290] TAMs associate with the progression of BC.[253] Although several studies have reported that CD68 positive macrophages associate with adverse outcome in BC there were no associations between CD68 positive macrophages and survival in BC in a meta-analysis of 13 studies in 2018.[291-294] However, CD163 positive macrophages were shown to associate with poorer RFS after TUR-BT alone or in combination with BCG treatment.[294] High levels of CD68 positive TAMs have been shown to associate with poorer response to BCG immunotherapy, as well.[291] Furthermore, the polarization status of TAMs can limit the efficacy of BCG therapy; the presence of M2 type macrophages is considered to associate with poorer response for the treatment.[292] TAM count is higher in MIBC tumors than in non-invasive tumors and the localization of TAMs have been shown to be different in NMIBC and MIBC tumors.[293, 295] In NMIBC TAMs tend to localize in the stroma-tumor margin, whereas in MIBC TAMs are mostly infiltrated into the tumor tissue. TAMs have also shown to correlate with increased risk of recurrence in BC.[292, 296]

In vitro co-culture studies have shown, that M1 macrophage levels decreases, while M2 macrophage levels increase during T24 BC cell line proliferation.[297] Mouse studies have shown, that BCG treatment efficiently induces cytotoxic activity in macrophages towards MBT-2 cells (mouse BC cell line).[298] Soluble factors secreted by macrophages, such as IFNγ, TNFα, and IL-6, have been found to account up to 50% of the total killing of the MBT-2 cells. These cytokines are tumoricidal for both human and mouse BC. TNFα is primarily produced by activated macrophages.[258] It stimulates the secretion of MMP-9, which contributes to tumor invasion and metastasis.[254] In BC, TNFα associates with angiogenesis, which strongly associates with poorer prognosis of the disease.[254, 299] The BCG-induced cytotoxicity has been shown to be blocked by IL-10, and the blockade of IL-10 can potentially enhance the effect of BCG in the treatment of BC patients.[300] Th1 simulating cytokines, such as IL-2 and IL-18 enhance the effect of BCG treatment.[298]

3.7.2 Immunotherapy in bladder cancer

BC is one of the few cancer types for which there is long-standing evidence for the efficacy of immunotherapy. Intravesical BCG treatment utilizes the anti-tumor effects of local immune response. The instillation of BCG in NMIBC was firstly reported in 1930s and the first human study of intravesical BCG was conducted by Morales in 1976.[301] In 1990, the FDA approved the use of intravesical BCG for

patients with NMIBC, and it is still recommended as the treatment of choice in NMIBC after TUR-BT.[302] Despite the wide use of the treatment, the mechanism of the action of BCG therapy is still not fully understood. Induction of inflammation and activation of the immune system are the crucial features of the therapeutic response of BCG treatment.[254] BCG is a live attenuated tuberculosis-related bacterium that attaches to the urothelium via extracellular protein fibronectin (FN). FN-BCG complexes induce proinflammatory cytokine release, immune cell recruitment, and direct cell-to-cell cytotoxicity.[258] Neutrophils and macrophages arrive first to the region, followed by CD4+ cells. Multiple BCG instillations result the accumulation of NK cells, CD8+ cells, and macrophages in the suburothelial stroma. A wide range of cytokines are involved. Tumor destruction is considered to associate with cellular immunity by high proportion of T helper cells and in some degree with the direct tumoricidal action of BCG itself. Macrophages act as a first line defense of innate immunity against BCG infection. They have been considered to initiate BCG-induced anti-cancer responses and kill tumor cells.[303] Approximately 25% of the NMIBC patients do not respond to BCG.[254] The reason for the unresponsiveness of the treatment is unclear. Patients responding to BCG commonly release large amounts of Th1 cytokines (e.g. IL-2 and IFNγ) whereas high amounts of Th2 cytokines (e.g. IL-4, IL-5, and IL-10) relate to BCG failure.[304] However, there is no biomarker in clinical use to predict the response to BCG.

In addition to intravesical BCG treatment, new immunotherapy approaches have come through as the treatment options in BC. PD-L1 expression levels on BC cells have been shown to correlate with severity of the disease, as well as with the patient outcome.[263, 305] Tumors with high levels of PD-L1 on cancer cells are more likely to be high-grade, more prone to recur, and the patient survival is poorer.[136, 137, 306] In addition, PD-L1 expression on tumor cells associates with increased resistance to BCG therapy, which requires a fully functioning immune system.[306] The presence of M2 macrophages in BC tumor has been shown to associate with poor response to immunotherapy.[304]

A T-cell-based adoptive immunotherapy is another feaseble novel treatment modality for advanced BC.[307, 308] It has already been shown to induce tumor regression in melanoma patients.[309] In this method, autologous T lymphocytes from tumor-draining lymph nodes are utilized to result a robust response against tumor cells.[310] The adoptive immunotherapy has potential to increase the OS for advanced BC and even result in a vaccination effect against cancer, but it still has technical problems to conquer to reach the benefits in the clinical world.

4 Markers

4.1 CD68

CD68 (also known as gp110 or macrosialin) is a 110 Kd transmembrane lysosomal glycoprotein expressed on tissue macrophages and blood monocytes. It is considered to be a pan-macrophage marker that recognizes both M1 and M2 phenotypes of macrophages.[311] The function of CD68 is unknown, but it has been considered to play a role in phagocytic activities of tissue macrophages.[312] In tissues, the infiltration of CD68 positive macrophages is observed most in papillary axes and stroma, and in lymphoid aggregates. CD68 expression can be also observed in some tumor cells.[296]

CD68 positive macrophage counts have been shown to be higher in MIBC than in NMIBC.[293] High levels of CD68 positive macrophages have also been shown to associate with higher stage, grade and tumor size, and with tumor recurrence and progression.[291-293, 295, 304, 313] CD68+ macrophage infiltrates associate with unfavorable prognosis and are shown to be an independent prognostic factor in BC in certain studies.[292, 293, 296, 314] However, in a meta-analysis in 2018 CD68 did not associate with BC survival.[294] Increased number of CD68 positive macrophages associate with BCG failure and greater risk of recurrence after BCG therapy.[291, 315]

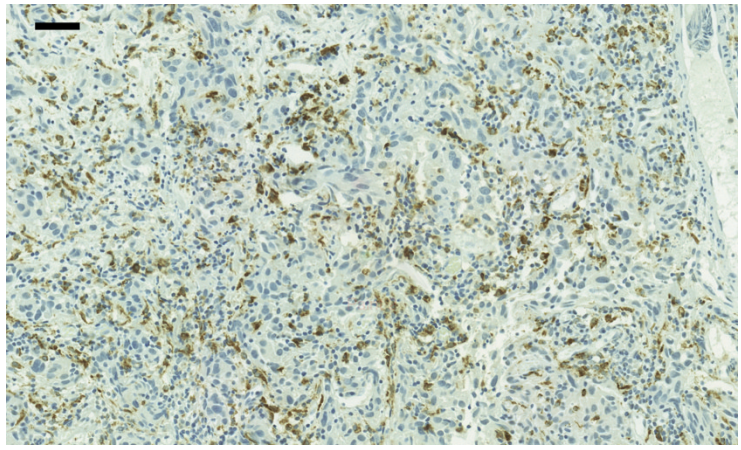

Figure 4. CD68 staining in bladder cancer tissue. CD68 positivity is seen as brown color. Scale bar 50μm.

4.2 MAC387

MAC387 is a monoclonal antibody recognizing the L1 protein, a 36 Kd myelomonocytic antigen, which is a member of the cytoplasmic calcium binding proteins known as calgranulins. It is expressed on tissue macrophages, monocytes, and granulocytes.[316-318] The L1-light chain shows sequence homology with the cystic fibrosis antigen, and the two L1 chains are identical with myeloid-related proteins (MRP) 8 and 14 (S100A8 and S100A9, also known as calgranulin A and B, respectively).[319] S100 proteins are involved in cell differentiation, cytoskeletal organization, and cell cycle regulation.[320, 321] MAC387 has been also shown to recognize in a lesser extent the MRP8/MRP14 heterocomplex, also called calprotectin.[322, 323] These intracellular MRPs have been used to define macrophage differentiation and different stages of inflammatory lesions in the central nervous system (early acute, late acute, chronic).[324] Calprotectin is involved in innate immunity, leukocyte adhesion, and endothelial transmigration.[325, 326] It is expressed by neutrophils, activated monocytes, and macrophages, and acts as a proinflammatory mediator in acute and chronic inflammation.[321] MRP14 is expressed on recently infiltrated monocytes/macrophages during early acute inflammation, and thus, MAC387 is considered to be a marker of recently infiltrated monocytes/macrophages and might be the earliest macrophage marker expressed on such cells as they enter the tissu.[324] Monocytes lose calprotectin gradually after migration from blood to tissues.[327] High endothelial venules (HEVs) have been considered to be the entry site of MAC387 positive macrophages, because they are seen surrounding these venules.[328] MAC387 is also expressed in some epithelial cells, and it has been used as a marker of squamous differentiation in BC (sensitivity of 99% and specificity of 70%).[316, 329, 330] The presence of squamous cell differentiation is considered to be a predictor for local recurrence and worse survival in BC.[331] MAC387 is expressed also on other cancer types, e.g. on breast cancer cells.[332]

MPR8 and MPR14 are up-regulated in breast, lung, gastric, colorectal, pancreatic and prostate cancer.[326] In breast cancer, MPR8 and MPR14 have been found to associate with poor tumor differentiation, vessel invasion, positive lymph nodes, and cancer progression.[325, 332, 337] The association with poor differentiation has also been reported in lung and thyroid glad cancers, and high MAC387 positive macrophage count has been shown to associate with poor survival in cholangiocarcinoma.[326, 334] In BC, MAC387 positive macrophages have not been studied. However, calgranulin A (S100A8) is overexpressed in BC tumor cells, especially in a metastatic disease.[335, 336] Furthermore, higher urinary calprotectin concentrations have been detected in BC patients compared to healthy controls and S100A8 gene expression levels significantly predict the progression of MIBC.[337, 338]

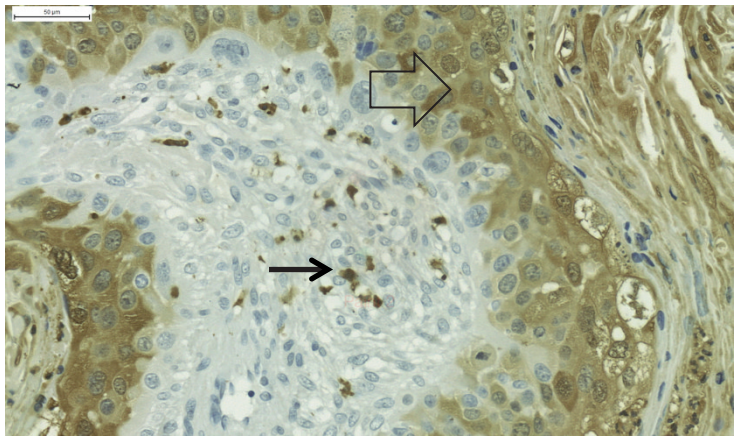

Figure 5. MAC387 staining in bladder cancer tissue. Little black arrow shows macrophage staining, big open arrow shows squamous differentiation. Scale bar 50μm.

4.3 CLEVER-1

CLEVER-1 (common lymphatic endothelial and vascular endothelial receptor-1) is a large 270-300kD type I transmembrane protein, a multifunctional scavenger receptor.[339] It is also known as Stabilin-1 by Goerdt *et al.* and FEEL-1 (fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1) by Adachi *et al.*[340-342] CLEVER-1 is encoded by the *Stab1* gene and it is expressed primarily in two cell types: tissue macrophages and endothelial cells.[341] It is present in HEVs in lymphoid organs and in lymphatic vessels in all lymphoid and non-lymphoid tissues except cerebellum, but absent from all blood vessel endothelium in normal non-lymphoid tissues.[343] However, CLEVER-1 is upregulated on HEV-like vessels at sites of inflammation. It is expressed in a population of immunosuppressive tissue macrophages, but absent in blood leukocytes.[339] CLEVER-1 has multiple ligands, such as modified low-density lipoprotein (LDL), phosphotidylserine expressed apoptotic cells, SPARC, placental lactogen, and bacteria.[344]

CLEVER-1 is a class H scavenger receptor.[345] Scavenger receptors are structurally very heterogeneous, but share functional properties as they identify and remove unwanted entities (modified self molecules, such as apoptotic cells or damaged proteins, or non-self molecules, such as microorganisms) by endocytosis, macropinocytosis, or phagocytosis. They also play role in cellular adhesion and antigen presentation, and are involved in the maintenance of homeostasis and in the pathogenesis of various diseases. Scavenger receptors are more prominently

expressed by M2 macrophages, but are not exclusive to this macrophage population.[346]

CLEVER-1 is used as a specific marker for alternatively activated M2 macrophages. In vivo CLEVER-1 expression requires stimulation e.g. by glucocorticoids; in *in vitro* human monocyte-derived macrophage cultures CLEVER-1 can be induced by stimulation of dexamethasone alone or with IL-4. IFNγ has a negative effect on CLEVER-1 expression. Expressed by M2 macrophages, CLEVER-1 mediates the uptake and clearance of acetylated LDL and SPARC. SPARC is a universal regulator of tissue remodeling. It is involved in developmental processes, tissue remodeling, angiogenesis and wound healing by modulation of extracellular matrix organization, binding of growth factors, and induction of an anti-adhesive state.[341, 347] In lymphoid organs, CLEVER-1 mediates lymphocyte binding and entrance to HEVs and to lymphatic endothelium. It also mediates the adhesion of lymphocytes, monocytes and granulocytes to HEV-like venules at sites of inflammation in non-lymphoid tissues.[339, 348]

The prognostic significance of CLEVER-1 in bladder cancer is unknown. In other cancers, such as breast cancer, melanoma, and lymphoma, CLEVER-1 has been shown to contribute tumor progression.[345, 349, 350] In colorectal cancer, high CLEVER-1 positive macrophage count associates with more favourable DSS and decreased risk of recurrence in stage II and III disease, but in contrast, with poorer DSS in higher stage disease.[224] Karikoski *et al.* demonstrated with CLEVER-1 deficient mice, that the absence of CLEVER-1 and, moreover, anti-CLEVER-1 treatment inhibited melanoma progression.[350] Furthermore, CLEVER-1 mediates binding of malignant cells to the lymphatic endothelium, and thus, has a role in tumor metastasis.[351] In breast cancer, CLEVER-1 expression in lymph vessels has been shown to correlate with lymph node metastasis, and that high macrophage index correlate with a worse prognosis.[352, 353] CLEVER-1/Stabilin-1 has been shown to mark a subpopulation of CD68 low/negative TAM in human breast cancer.[349, 354] In addition, Riabov *et al.* showed in 2016 that breast cancer growth is suppressed in Stabilin-1 knockout mice. Stabilin-1 expression was most intensive in stage I and IV disease, which was speculated to result from its crucial role for early primary tumor growth and progression.[349] The reduction in the expression of Stabilin-1 in the tumor microenvironment during the tumor progression has been shown also in hepatocellular carcinoma and glioblastoma mouse models.[355, 356] However, David *et al.* speculated TAMs to stop expressing Stabilin-1 as they switch phenotypes to one that promotes tissue remodeling and angiogenesis.[356] The role of SPARC, the endocytic ligand of CLEVER-1, is debated and seems to vary in different cancer types. It has been shown to inhibit cancer growth in lung carcinoma, neuroblastoma and beast cancer, and the reduced expression of SPARC has been shown to correlate with poor prognosis in breast cancer patients.[357, 361]

However, it associates with increased tumor metastasis and proliferation in glioblastoma.[356, 362]

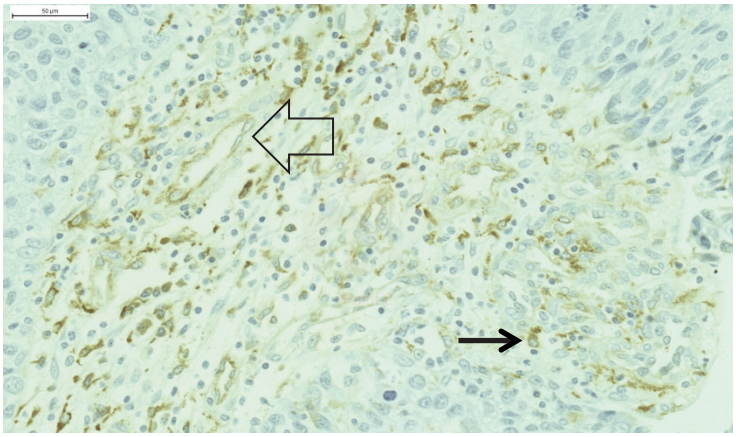

Figure 6. CLEVER-1 (antibody 2-7) staining in bladder cancer tissue. Little black arrow points positive macrophage staining as an example and big open arrow points vessel staining. Scale bar 50μm.

4.4 CD73

CD73 is a plasma membrane-bound ecto-5'-nucleotidase (ecto-5'-NT), a 70 kD glycosyl-phosphatidylinositol (GPI)-linked molecule detected in several different tissues and cell types.[363-365] It is also known as L-VAP-2.[366] CD73 functions in the extracellular nucleotide turnover hydrolyzing extracellular 5'-mononucleotides (AMP) to the corresponding bioactive nucleosides (adenosine) (Figure 7).[364] Extracellular purines regulate cellular hemostasis and thrombosis via activation and aggregation of platelets and mediate several inflammatory responses, including the release of the cytokines, TNFα and IL-1β from monocytes/macrophages, and facilitate the leukocyte adhesion to the endothelium.[367, 368] Intracellular adenosine triphosphate (ATP) is primarily utilized in energy-requiring processes (such as active transport and biosynthesis), whereas extracellular ATP is a powerful signaling molecule.

Adenosine is a highly anti-inflammatory molecule that has a role in regulation of endothelial permeability and vasodilatation, neutrophil adhesion, and stimulation of chloride secretion.[269-374] It binds to specific cell surface receptors that are expressed on a wide variety of cells (A1R, A2aR, A2bR, and A3R) and its effects are often opposite to inflammatory ATP.[372, 375-377] Adenosine has a short half-life, only under 10 seconds and, thus, can be highly regulated.[376] It accumulates in response to stress (such as hypoxia or tissue injury); increased adenosine levels are

detected during inflammation and wound healing.[372, 378] Extracellular adenosine signals tissue injury to surrounding tissues in an autocrine and paracrine manner. Increased levels of adenosine leads to decreased production of cytokines and decreased expression of adhesion molecules on endothelium and leukocytes, such as E-selectin, ICAM-1, and beta 2 integrins.[379] As a negative regulator of immune cells, it protects normal tissue from inflammatory damage, but also cancer cells from the antitumoral T cells.[380]

CD73 is expressed in a variable extent on different tissues. It is expressed on a subpopulation of peripheral blood lymphocytes: on the majority of B cells (70%) and CD8+ T cells (50%), but only on approximately 10% of CD4+ T cells.[365, 381] These cells form 15-25% of peripheral blood lymphocytes. Neutrophils, erythrocytes, platelets, and other blood cells express little or no CD73. CD73 is present on some epithelial cells of various origins and on vascular endothelial cells, predominantly on large vessels such as aorta, carotid and coronary artery.[383, 383] It has been detected in colon, kidney, brain, liver, heart and lung.[363] CD73 expression is upregulated by HIF-1α activation and exposure to type I interferons, and has been associated with Wnt signaling, which is deregulated in several cancers.[384-387] IL-1β, TNFα and prostaglandin E2 also enhances the activity of CD73, whereas IFNγ and IL-4 downregulate CD73.[388, 389]

CD73 is a multifunctional molecule and its physiological role varies between cells and organs.[382] Although the primary physiological role of CD73 is its regulatory function in the purinergic cascade (Figure 7), other non-enzymatic functions have also been proposed.[390] It mediates lymphocyte binding and maintains the barrier function as a gatekeeper in epithelium and endothelium in various tissues, especially during hypoxia enhancing the generation of extracellular adenosine.[366, 369, 371, 374, 386, 391-397] Leukocyte binding to CD73 on endothelium causes the inhibition of CD73 and, thus, decreased adenosine production.[392] This increases the endothelial permeability and allows leukocytes to transmigrate through the vascular endothelium. *Cd73*$^{-/-}$ mice are viable, but have shown to have significant vascular leakage in multiple organs, enhanced leukocyte adhesion to vascular endothelium, and increased leukocyte migration to inflammatory sites.[371, 385] It is a signal-transducing molecule in the immune system and it has a costimulatory role in T cell activation through the CD3/TCR signaling pathway.[285] It is also used as a lymphocyte maturation marker, as it is absent from the surface of immature B and T cells.[365, 381, 398-400]

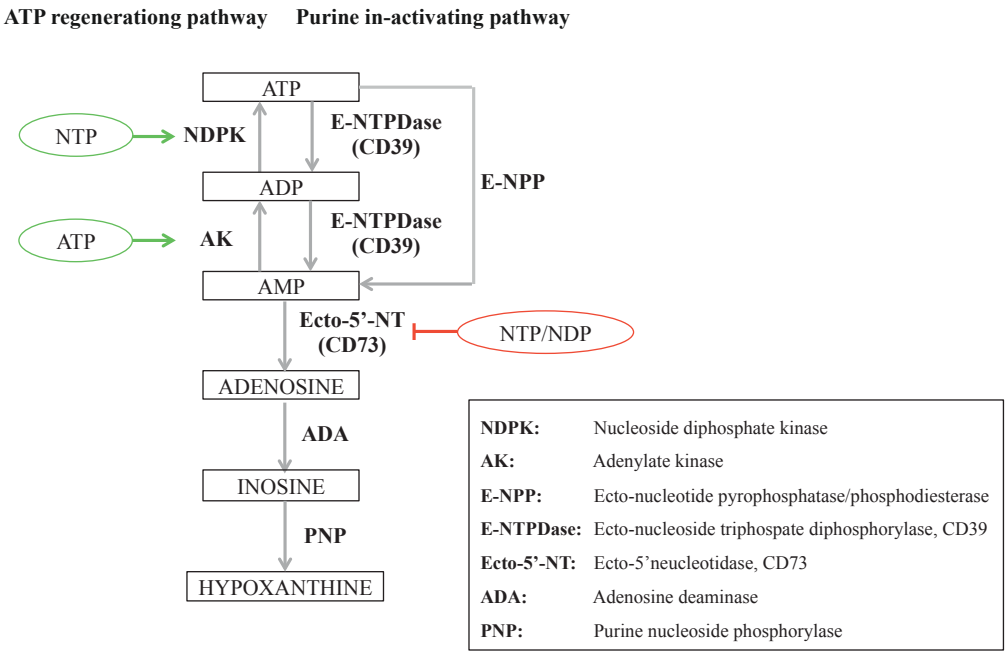

Figure 7. Purine in-activating pathway and ATP regenerating pathway. Green arrow depicts activating and red blunted line inhibitory regulatory mechanisms. Adapted from Yegutkin *et al.* 2002 and Yegutkin 2008.[363, 390]

4.4.1 CD73 in cancer

CD73 has been considered as an important player of tumor progression, but its true role in cancer is still widely unknown. CD73 is expressed in several cancer types, such as ovarian, esophageal, gastric, colon, prostate and breast cancer.[401-404] CD73 associates with poor prognosis in certain cancer types, such as leukemia and breast cancer and the lack of CD73 leads to diminished tumor growth in colon cancer, lymphoma, breast cancer and melanoma mouse models.[404-407] In breast cancer, CD73 supports tumor growth and enhances the migration, invasion and neovascularization of the tumor cells.[408-410] It has also been suggested to act as a prognostic marker in breast cancer.[409-410] Yegutkin *et al.* have shown with a CD73-deficient mouse model, that CD73 activity favors tumor progression also in melanoma.[370] Furthermore, they showed that it is amenable to therapeutic interventions, as it has also been shown in breast cancer.[408]

Overexpression of CD73 has been shown to associate with resistance to anti-tumor drugs, such as doxorubicin in breast cancer mouse models.[404] High

concentrations of adenosine in tumors may lead to a failure of an effective anti-tumor immune response. Thus, it has been suggested that CD73 could serve as a novel target for cancer treatment.[411] Anti-CD73 treatment and knockdown of CD73 on tumor cells by siRNA (small interfering RNA) increase the tumor free survival and reduce tumor growth and metastasis in mouse models.[403, 408, 411, 412] has been proposed, that CD73 enhances tumor growth in a T cell-dependent manner, because the blockade of CD73 has no impact on tumor growth in T cell-deficient mice.[403] The altered purinergic balance with the absence of CD73 causes a specific decrease in the numbers of intratumoral Tregs and M2 MR positive macrophages, and increase in IFNγ and NOS2 synthesis by TILs.[370] Tregs and M2 macrophages are key players in diverting inflammatory reaction in the tumor microenvironment in a way that enhances the tumor growth.[198, 413, 414] IFNγ, on the other hand, inhibits tumor formation and drives macrophage polarization into anti-tumoral M1 macrophages.[415] Inducible NOS is one of the best marker of M1 macrophages, and its synthesis is driven by IFNγ.[190] Many of CD73 effects consequences the production of adenosine. Elevated levels of adenosine in the mouse tumor microenvironment have been described.[416] Adenosine regulates macrophage activation; it inhibits the antitumoral M1 activation and enhances the activation towards protumoral M2 macrophages.[417, 418] It accumulates in solid tumors and stimulates tumor growth and angiogenesis and inhibits cytokine synthesis, immune cell adhesion to the endothelium, and T cell and NK cell function, and thus, have tumor-promoting activities.[402] CD73 expression on tumor cells has been shown to enhances resistance to new immunotherapeutic anti-PD-1 agents, as PD-1 blockade and the activation of T cells upregulate the expression of adenosine receptor A2aR making these cells more susceptible to adenosine-mediated suppression.[419] Hence, the inhibition of CD73 in combination with anti-PD-1 drugs has been shown to increase the anti-tumor T cell responses. The blocking of CD73 also enhances chemotherapy responses e.g. in breast cancer.[420]

BC cells express CD73 in vitro in grade 1 RT4 and in grade 3 T24 cell lines. However, RT4 cells seem to hydrolyze more efficiently tri- and diphosphonucleosides via CD39, while the less differentiated, more invasive T24 cells show higher levels of AMP hydrolysis via CD73.[421] In a BC mouse model, an increased CD73 expression in tumor cells has been detected.[422] In human BC cells, CD73 status is comparable with non-cancerous urothelial cells.[423] However, in contrary to many other cancers, Wettstein *et al.* have shown in 2015 that CD73 positive human BC cells associate with lower stage, lower grade, and with lower risk for progression.[424] However, in this study CD73 failed to be an independent prognostic marker in multivariate analyses. Similar to BC, CD73 associates with more favourable prognosis also in ovarian and breast cancers.[425-427] Hence, the anti-CD73 treatment is not that simple in cancer therapy. Further investigations translating the

mouse model findings and preclinical studies into the clinic are needed. Extensive evidence of the CD73 expression in different cancer types and in different cell and tissue types need to be accomplished.

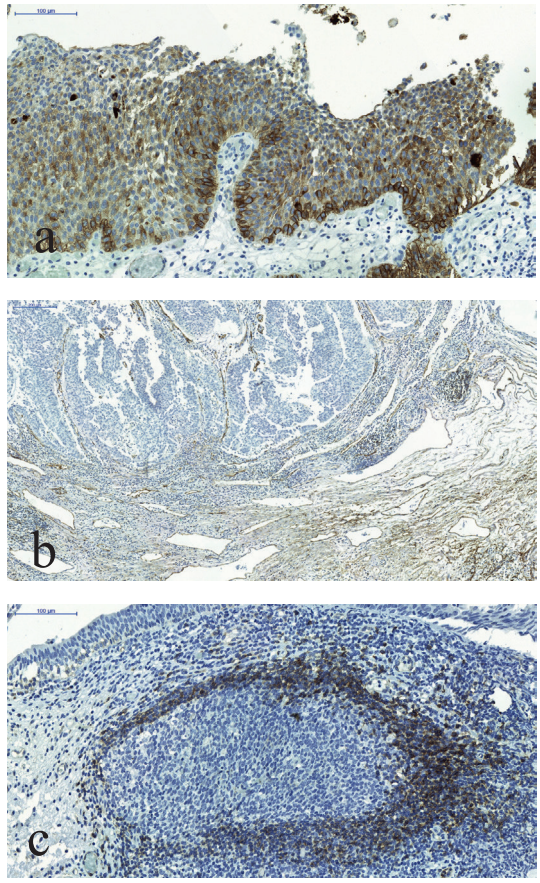

Figure 8. CD73 staining in bladder cancer tissue: a. CD73 on urothelial cancer cells (magnification x20, scale bar 100μm), b. CD73 on stroma and vessels (magnification x10, scale bar 200μm), c. CD73 on lymphocytes (magnification x20, scale bar 100μm).

Aims of the Study

Bladder cancer is a heterogeneous disease. Non-invasive and especially well differentiated tumors have often relatively indolent natural history but poorly differentiated tumors are prone to invade, recur after treatment, and metastasize. There is a lack of biomarkers in clinical use to predict the outcome of the disease and to select the most effective treatment for the patient.

Inflammation is a critical feature of carcinogenesis. Tumor-associated macrophages associate with poor prognosis in several cancers. However, there is evidence of macrophages associating with more favourable outcome, for example, in distinct groups of colorectal cancer. Current knowledge regarding the prognostic significance of TAMs in BC is limited.

The aim of the study was to investigate immunological prognostic immunological factors in human BC after TUR-BT and radical cystectomy, and the predictive functions of these factors in patients treated with neoadjuvant chemotherapy.

The specific aims of the study were:

1. To examine the prognostic value of TAMs in human bladder cancer with antibodies CD68, MAC387, and CLEVER-1.

2. To examine the predictive value of TAMs (CD68, MAC387, and CLEVER-1) in bladder cancer patients treated with neoadjuvant chemotherapy.

3. To examine the immunohistochemical expression and prognostic value of CD73 in human bladder cancer.

Materials and methods

1 Patients

The patient material and the immunostaining methods are summarized in Table 8. Non-urothelial BCs and cases with insufficient tissue material available for histological re-review and immunohistochemistry or unavailable clinical data were excluded from the studies. In studies I and III, patients with intravesical instillations (BCG or chemotherapy) or systemic chemotherapy prior the study inclusion were excluded. In the study II, inadequate NAC treatment (under 2 cycles) was included in the exclusion criteria. After exclusions, 184 BC patients were included in the study I, 68 patients in the study II, and 166 NMIBC and 104 MIBC patients were included in the study III.

Table 8. Patient material and immunostaining methods in the studies.

	Study I	**Study II**	**Study III**
Number of patients	184	68	270
Hospital	Turku University Hospital	Turku and Helsinki University Hospitals	Turku University Hospital
Study period	1985-2004	2007-2013	1985-2004
Treatment modalities	TUR-BT/RC	NAC + RC	TUR-BT/RC
Tissue samples	Whole sections	TMA	Whole sections (TUR-BT), TMA (RC)
Antibodies	CD68, MAC387, CLEVER-1	CD68, MAC387, CLEVER-1	CD73
Immunostaining method	Vectastain Elite ABC Kit (Vector Laboratories)	Vectastain Elite ABC Kit (Vector Laboratories)	Vectastain Elite ABC Kit (Vector Laboratories)
Immunoscoring	Manual	Manual + digital	Manual

TUR-BT was performed using standard techniques in all studies. In the study II, patients received 2-6 cycles (median 4 cycles) either cisplatin-gemcitabine (64/68 patients) or carboplatin-gemcitabine (4/68 patients) prior to the RC. After RC, patients were followed every 3 months for the first year and semiannually thereafter A detailed clinicopathological database was assembled retrospectively. Histological samples were re-reviewed for histology, differentiation grade, and stage by expert uropathologists. The tumors were graded according to both WHO 1973 and the WHO/ISUP 2004 classifications and staged according to the 2010 TNM staging.[47, 428]

2 Antibodies

The antibodies used in the studies are shown in Table 9.

Table 9. Antibodies used in the studies.

Antibody	Antigen	Isotype	Dilution	Antigen retrieval	Source
Primary antibodies					
CD68 (KP1) (ab845)	Human CD68	Mouse IgG_1	1:5	none	Abcam, U.K.
MAC387 (ab22506)	S100A9 + Calprotectin (S100A8/A9)	Mouse IgG_1	1:500	none	Abcam, U.K.
2-7	CLEVER-1	Rat IgG	1:5	Proteinase K (Dako)	Palani *et al,* 2011[329]
D7F9A	NT5E/CD73	Rabbit IgG_1	1:500	Aptum 2000 epitope recovery unit (Aptum biologics ltd)	Cell Signaling Technology Inc.
Negative controls					
3G6	Chicken T cells	Mouse IgG_1	5μg/ml	none	Salmi &Jalkanen, 1992 [348]
MEL-14	Mouse L-selectin (CD62L)	Rat IgG_{2a}	5μg/ml	Proteinase K (Dako)	Exbio, Czech Republic
Rabbit IgG_1 isotype control	Unknown	Rabbit IgG_1	5μg/ml	Aptum 2000 epitope recovery unit (Aptum biologics ltd)	BD Pharmingen

3 Scoring

In study I, the whole tumor area was screened and three hotspot areas per block with most CD68+ macrophages, MAC387+ macrophages, and CLEVER-1+ macrophages and vessels by eye were scored. A 0.0625 mm grid was used with 40x magnification with macrophages and 20x with vessels. The numbers of macrophages/vessels were calculated and scored semiquantitatively (from -/negative to +++/abundant) within one high-power field independently by two observers blinded to the clinical information. MAC387+ tumor cells were also detected and scored with semiquantitative scoring. The mean numbers of the hotspots were calculated to form the macrophage/vessel count for each patient.

In study II the scoring was performed manually and digitally. The manual scoring was performed similarly as in study I. For digital scoring, the hotspots were scanned and analyzed with Fiji-ImageJ 2.0.0. The macrophage-positive areas were extracted by color deconvolution and the resulting image was thresholded. A minimal size limit was applied to exclude artefacts and macrophage-positive areas were calculated. For MAC387 analyses, the images were watershed and a size limit was applied to exclude larger positive tumor cells from macrophages. The mean percentages of hotspots were calculated and used in analyses. CLEVER-1+ vessels were calculated manually only.

In study III, the CD73 immunoreactivity was assessed by two independent readers in a blinded fashion using a dichotomous scoring. CD73 expression was analyzed separately for the different cell types in the samples. Basal cell layer of the epithelium (BCL), suprabasal epithelial cells (SEC), tumor stroma fibroblasts, peritumoral endothelial cells (lymphatic and vascular endothelia), and lymphocyte infiltrates were scored as negative or positive.

4 Statistical analyses

Statistical analyses were performed with SPSS Statistics (versions 20, 21, and 24, IBM) and SAS System for Windows (version 9.3, SAS Institute Inc. Cary, NC, USA). The relevant statistical tests used in the studies are shown in Table 10. Outcome measures included disease-specific survival (DSS), overall survival (OS), recurrence-free survival (RFS), and progression-free survival (PFS). NAC response was categorized as follows: complete response (pT0N0), partial response (pT1/pTa/pTisN0), no response (pT2N0), and progression (pT3 and/or N+). In studies I and II, the macrophage and vessel counts were dichotomized according to the mean number (low vs. high). In study I combination variables from macrophage markers were generated: CD68/MAC387, CD68/CLEVER-1, and MAC387/CLEVER-1. Each of he combination variable included low-low, low-high, and high-high groups. In study II, MAC387+ tumor cells were divided into two

groups according to the density of positive cells (0-2 vs. 3). In study III, the variables were scored as dichotomized (negative vs. positive), disregarding CD73+ lymphocytes, which were dichotomized as <1% vs. ≥1%. All statistical tests were two-sided, and P values of <0.05 were considered as statistically significant.

Table 10. Statistical tests used in the studies

The association studied	Statistical test used
The markers *vs.* clinicopathological variables	Pearson's Chi-Square test Fisher's exact test Spearman rank-order correlation coefficient test Mann-Whitney U test Kruskal-Wallis test
The markers *vs.* outcome	The Kaplan-Meier method Cox proportional hazards regression models
The NAC response *vs.* clinicopathological characteristics	Pearson Chi-square test Fisher's exact test
The markers *vs.* the NAC response	Regression analyses Mann-Whitney U test Pearson's Chi-Square test Fisher's exact test

5 Ethics

The study protocols were approved by the Research Ethics board of the Hospital District of Southwest Finland (1.8.2006/301). Written consents were obtained from the patients participating the studies. The studies did not affect the patients or their further treatment of follow-up in any way. All the sample collections were done on already existing tissue specimens received during the diagnosis and treatment of the patients. All studies were performed in accordance with the Declaration of Helsinki.

Results

1 The study populations

Table 11. Clinicopathological characteristics of the study populations.

		Study I		Study II	Study III	
		TUR-BT n=92 (%)	RC n=92 (%)	n=68 (%)	NMIBC n=166 (%)	MIBC n=104 (%)
Gender	Male	69 (75)	74 (80)	58 (85)	137 (83)	78 (75)
Age (y)	Median	71	65	65	67	66
	(range)	(34-92)	(38-78)	(47-76)	(34-92)	(35-84)
Smoking	No	34 (37)	43 (47)	9 (13)	96 (58)	46 (44)
	Yes	35 (38)	54 (59)	52 (76)	70 (42)	58 (56)
Grade, WHO 1973	G1	37 (40)	2 (2)	0	45 (27)	0
	G2	17 (19)	26 (28)	1 (1)	58 (35)	23 (22)
	G3	30 (33)	64 (70)	67 (99)	56 (34)	81 (78)
Grade, WHO/ISUP 2004	PUNLMP	0	0	0	33 (20)	0
	Low	60 (55)	19 (21)	17 (25)	50 (30)	6 (6)
	High	32 (35)	73 (79)	51 (75)	83 (50)	98 (94)
pT category	pTa	81 (88)	33 (36)	0	83 (50)	0
	pTcis			0	66 (40)	0
	pT1			0	17 (10	0
	pT2	11(12)	24 (26)	48 (71)	0	46 (44)
	pT3	0	23 (25)	18 (27)	0	44 (42)
	pT4	0	12 (13)	2 (3)	0	14 (14)
CIS	Yes	NA	NA	15 (22)	40 (24)	24 (23)
LVI	Yes	NA	NA	17 (25)	6 (4)	60 (58)
pN category	Positive	NA	NA	7 (11)	0	13 (13)
	Negative	NA	NA	58 (89)	166 (100)	101 (87)
Outcome	Alive	33 (36)	26 (28)	53 (78)	65 (39)	15 (14)
	Dead, BC	22 (24)	39 (42)	13 (19)	27 (16)	62 (40)
	Dead, other	37 (40)	17 (19)	2 (3)	63 (38)	22 (21)
Recurrence	Yes	47 (51)	NA	13 (19)	61 (37)	58 (56)
Progression	Yes	34 (37)	NA	NA	24 (15)	9 (9)
Follow-up time (mo)	Median	6.9	4.2	3.6	6.8	1.5
	(range)	(0-14.3)	(0-17.8)	(0.3-7.7)	(0-22.0)	(0-20.5)

2 TAMs in bladder cancer (Study I and II)

To study TAMs in BC, the prognostic and predictive role of three macrophage markers were evaluated by immunohistochemistry in TUR-BT and RC samples. CD68 was analyzed as a pan-macrophage marker, MAC387+ macrophages represented a subgroup of recently infiltrated macrophages, and CLEVER-1 was expressed on M2 macrophages, as well as on endothelial cells. MAC387 expression on cancer cells was studied, as well.

2.1 CD68, MAC387 and CLEVER-1 expression in BC

CD68, MAC387, and CLEVER-1 expressions were analyzed in TUR-BT and RC samples in study I. In study II markers were analyzed in TUR-BT samples from patients treated with neoadjuvant chemotherapy and RC after sample taking. Macrophage and vessel counts were dichotomized according to the mean value into low/high groups and the results are represented in Table 12.

Table 12. Markers analyzed in study I and II. The macrophage and vessel counts were dichotomized according to the mean value.

		Study I		**Study II**
Marker	Score	TUR-BT n=92 (%)	RC n=92 (%)	NAC n=68 (%)
$CD68^+$ macrophages	Mean number (range)	18 (0-81)	30 (0-102)	59 (0-175)
	Low	61 (66)	34 (60)	30 (44)
	High	31 (34)	31 (34)	38 (56)
$MAC387^+$ macrophages	Mean number (range)	19 (0-135)	34 (0-197)	78 (8-240)
	Low	62 (67)	41 (65)	37 (54)
	High	30 (32)	22 (35)	31 (46)
$MAC387^+$ tumor cells	0	NA	NA	9 (14)
	1	NA	NA	29 (44)
	2	NA	NA	15 (23)
	3	NA	NA	12 (18)
CLEVER-1^+ macrophages	Mean number (range)	26 (0-73)	11 (0-41)	53 (0-209)
	Low	56 (61)	43 (60)	34 (50)
	High	36 (39)	29 (40)	34 (50)
CLEVER-1^+ vessels	Mean number (range)	8 (0-22)	0 (0-6)	4 (0-27)
	Low	56 (61)	42 (65)	39 (57)
	High	36 (39)	23 (35)	29 (43)

In study II, different immunohistochemical methods analyzing macrophages immunohistochemically in tissues were tested. Macrophages were counted from whole sections and TMAs to study if TMA could be utilized when investigating macrophages. TMAs correlated with whole sections in CD68 and MAC387 stainings ($p=0.002$ and $p<0.001$, respectively), but not in CLEVER-1 stainings ($p=0.41$). Macrophages were also counted digitally and it was shown, that the digital and manual countings correlated with each other in all markers studied (p-values in CD68 0.008, MAC387 <0.001, and CLEVER-1 <0.001, respectively). All further correlation and survival analyses were tested with the manually counted macrophages from whole sections.

2.2 The prognostic role of CD68, MAC387 and CLEVER-1

The associations between immunohistological markers and clinicopathological variables of the BC patients were analyzed in the studies. In study I high numbers of CD68+ macrophages and MAC387+ macrophages associated with higher pT category and tumor grade. In contrast, lower CLEVER-1+ macrophage and vessel counts associated with more adverse T-category and grade of the tumor. In study II, only an association between high CD68+ macrophage count and the presence of LVI was noted. The markers did not associate with any other clinicopathological variables in study II.

An overview of survival analyses in studies I and II is shown in Table 13. The Kaplan-Meier analyses and the Cox proportional hazards regression analyses affecting OS in detail in studies I and II are presented in Figure 9 and Table 14. High counts of CD68+ and MAC387+ macrophages associated significantly with higher risk of progression, as well as with poorer DSS and OS after TUR-BT. CLEVER-1+ macrophages did not associate with survival, but in contrast, high CLEVER-1+ vessel count associated with lower risk of progression and favourable DSS in the univariate analyses. However, any of these associations did not remain significant in multivariate analyses. After RC, only MAC387+ macrophages associated with greater risk of death due to the BC in univariate, but not in multivariate analyses.

In study II high counts of CD68+ macrophages associated with shorter OS after NAC and RC in multivariate analysis with 3.97 risk of death in the high *vs.* low macrophage group (95% confidence interval, CI, 1.11-14.12). High counts of CLEVER-1+ macrophages associated with poorer OS in univariate analysis, as well, but failed to stay significant in multivariate tests. MAC387+ macrophages/tumor cells or CLEVER-1+ vessels did not affect the survival in study II.

Table 13. Overview of the survival analyses (univariate and multivariate Cox proportional hazards regression model) in studies I and II. P-values and hazard ratios of the Cox models are shown (HR; p).

Test (HR; p)	**Study I TUR-BT**				**Study I RC**		**Study II NAC**
	OS	DSS	PFS	RFS	OS	DSS	OS
CD68$^+$ macrophages							
Univariate	1.031; <0.001*	1.043; <0.001*	1.031; <0.001*	1.005; 0.68	1.004; 0.51	1.004; 0.57	3.5; 0.053
Multivariate	1.012; 0.19	1.019; 0.075	1.005; 0.64	0.996; 0.77	0.997; 0.72	0.994; 0.51	3.97; 0.033*
MAC387$^+$ macrophages							
Univariate	1.016; 0.002*	1.029; <0.001*	1.022; <0.001*	0.998; 0.85	1.006; 0.070	1.008; 0.032*	1.48; 0.45
Multivariate	1.003; 0.65	1.011; 0.16	1.005; 0.55	0.978; 0.046*	1.002; 0.56	1.003; 0.59	1.57; 0.39
CLEVER-1$^+$ macrophages							
Univariate	1.011; 0.30	1.008; 0.62	1.010; 0.48	1.007; 0.61	1.017; 0.28	1.020; 0.29	3.17; 0.048*
Multivariate	1.005; 0.62	0.955; 0.73	0.999; 0.93	1.002; 0.87	1.017; 0.27	1.017; 0.33	2.94; 0.066
CLEVER-1$^+$ vessels							
Univariate	0.958; 0.17	0.898; 0.046*	0.910; 0.030*	0.997; 0.94	1.052; 0.75	1.105; 0.60	0.64; 0.42
Multivariate	1.015; 0.65	0.989; 0.83	0.971; 0.53	0.998; 0.95	1.149; 0.39	1.222; 0.27	0.65; 0.43

* Significant p-value

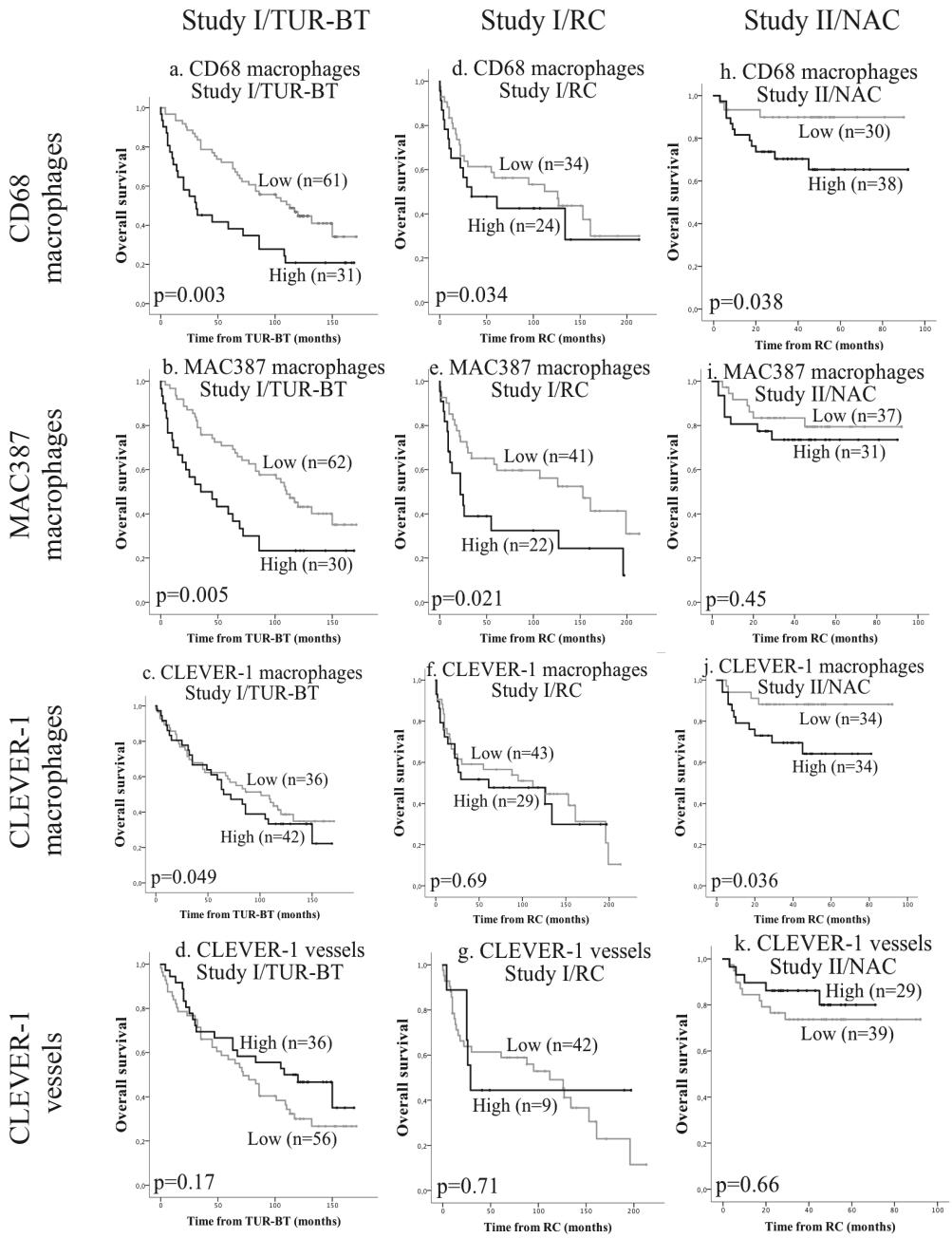

Figure 9. Kaplan-Meier estimates of the markers for OS in study cohorts: a-d. study I, TUR-BT cohort; d-g. study I, RC cohort; and h-k study II, NAC cohort.

Table 14. Univariate and multivariate Cox proportional hazards regression analyses of CD68$^+$ macrophages, MAC387$^+$ macrophages, CLEVER-1$^+$ macrophages, and CLEVER-1$^+$ vessels affecting OS. In study I the markers were analyzed as continuous variables in Cox analyses and in study II the markers were analyzed as continuous and dichotomized variables.

	Univariate			Multivariate		
Marker	HR	95% CI	p-value	HR	95% CI	p-value
Study I TUR-BT						
CD68$^+$ macrophages	1.031	1.016-1.046	<0.001*	1.012[a]	0.994-1.013	0.19
MAC387$^+$ macrophages	1.016	1.006-1.027	0.002*	1.003[a]	0.989-1.018	0.65
CLEVER-1$^+$ macrophages	1.011	0.990-1.032	0.30	1.005[a]	0.985-1.026	0.62
CLEVER-1$^+$ vessels	0.958	0.901-1.019	0.17	1.015[a]	0.950-1.086	0.65
Study I RC						
CD68$^+$ macrophages	1.004	0.993-1.015	0.51	0.997[b]	0.983-1.012	0.72
MAC387$^+$ macrophages	1.006	0.999-1.013	0.070	1.002[b]	0.994-1.010	0.56
CLEVER-1$^+$ macrophages	1.017	0.986-1.049	0.28	1.017[b]	0.987-1.049	0.27
CLEVER-1$^+$ vessels	1.052	0.768-1.442	0.75	1.149[b]	0.839-1.573	0.39
Study II NAC						
CD68$^+$ macrophages	1.009/ 3.50	0.994-1.023/ 0.99-12.44	0.024*/ 0.053	1.008/ 3.97[c]	0.995-1.021/ 1.11-14.12	0.23/ 0.033*
MAC387$^+$ macrophages	1.002/ 1.48	0.990-1.014/ 0.54-4.08	0.80/ 0.45	1.002/ 1.57[c]	0.991-1.013/ 0.57-4.32	0.73/ 0.39
CLEVER-1$^+$ macrophages	0.999/ 3.17	0.981-1.018/ 1.01-9.97	0.95/ 0.048*	0.998/ 2.94[c]	0.982-1.015/ 0.93-0.27	0.82/ 0.066
CLEVER-1$^+$ vessels	0.873/ 0.64	0.718-1.062/ 0.22-1.87	0.17/ 0.42	0.901/ 0.65[c]	0.748-1.086/ 0.22-1.90	0.28/ 0.43

[a] Analyses adjusted with grade, T-category, age, and tumor number.
[b] Analyses adjusted with grade, T-category, and age.
[c] Analyses adjusted with T-category.
* Significant p-value

In study I, the prognostic roles of combination variables generated from macrophage markers (CD68/MAC387, CD68/CLEVER-1, and MAC387/CLEVER-1) were analyzed. The results according to the OS are presented in Figure 10 and Table 15. All groups with "double-high" macrophage counts (CD68high/MAC387high, CD68high/CLEVER-1high, and MAC387high/CLEVER-1high) associated with shorter PFS and worse survival compared to other groups in TUR-BT samples. When CD68/MAC387 groups were tested, the "double-low" group had the longest PFS. In CD68/CLEVER-1 or in MAC387/CLEVER-1 groups there were no differences between the "double-low" group and CD68high/CLEVER-1low or MAC387high/CLEVER-1low groups. Moreover, CD68/MAC387 "double-high" and CD68/CLEVER-1 "double-high" groups were independent prognostic factors for OS with HR 3.5 (95% CI 1.1-11) and 3.8 (95% CI 1.4-10), respectively. When the combination data was analyzed from RC samples, the "double-high" groups from CD68/MAC387 and CD68/CLEVER-1 associated with worse OS in Kaplan-Meier and univariate analyses, but did not remain as independent prognostic factors in multivariate Cox proportional hazards regression model.

Table 15. Univariate and multivariate Cox proportional hazards regression analyses of macrophage combination groups affecting OS.

	Univariate			Multivariate		
Marker	HR	95% CI	p-value	HR	95% CI	p-value
Study I TUR-BT						
CD68/MAC387$^{-/-}$		*REF*			*REF*	
CD68/MAC387$^{-/+}$	1.8	0.99-3.3	0.054	1.6[a]	0.72-3.4	0.26
CD68/MAC387$^{+/+}$	6.9	3.4-14	<0.001*	3.5[a]	1.1-11	0.036*
CD68/CLEVER-1$^{-/-}$		*REF*			*REF*	
CD68/CLEVER-1$^{-/+}$	0.89	0.47-1.7	0.71	1.1[a]	0.55-2.3	0.77
CD68/CLEVER-1$^{+/+}$	3.1	3.4-14	<0.001*	3.8[a]	1.4-10	0.008*
MAC387/CLEVER-1$^{-/-}$		*REF*			*REF*	
MAC387/CLEVER-1$^{-/+}$	0.94	0.50-1.8	0.85	1.4[a]	0.71-2.8	0.34
MAC387/CLEVER-1$^{+/+}$	2.9	1.4-5.9	0.004*	1.6[a]	0.66-3.7	0.32
Study I RC						
CD68/MAC387$^{-/-}$		*REF*			*REF*	
CD68/MAC387$^{-/+}$	1.2	0.48-3.0	0.70	1.1[b]	0.34-3.5	0.88
CD68/MAC387$^{+/+}$	2.9	1.2-7.2	0.020*	2.1[b]	0.79-5.7	0.13
CD68/CLEVER-1$^{-/-}$		*REF*			*REF*	
CD68/CLEVER-1$^{-/+}$	2.0	0.89-4.5	0.095	1.3[b]	0.53-3.0	0.60
CD68/CLEVER-1$^{+/+}$	3.4	1.2-10	0.027*	2.2[b]	0.69-6.8	0.18
MAC387/CLEVER-1$^{-/-}$		*REF*			*REF*	
MAC387/CLEVER-1$^{-/+}$	1.8	0.84-3.8	0.14	1.0[b]	0.44-2.4	0.95
MAC387/CLEVER-1$^{+/+}$	2.0	0.63-6.4	0.24	2.6[b]	0.72-9.1	0.15

[a] Analyses adjusted with grade, T-category, age, and tumor number.
[b] Analyses adjusted with grade, T-category, and age.
* Significant p-value

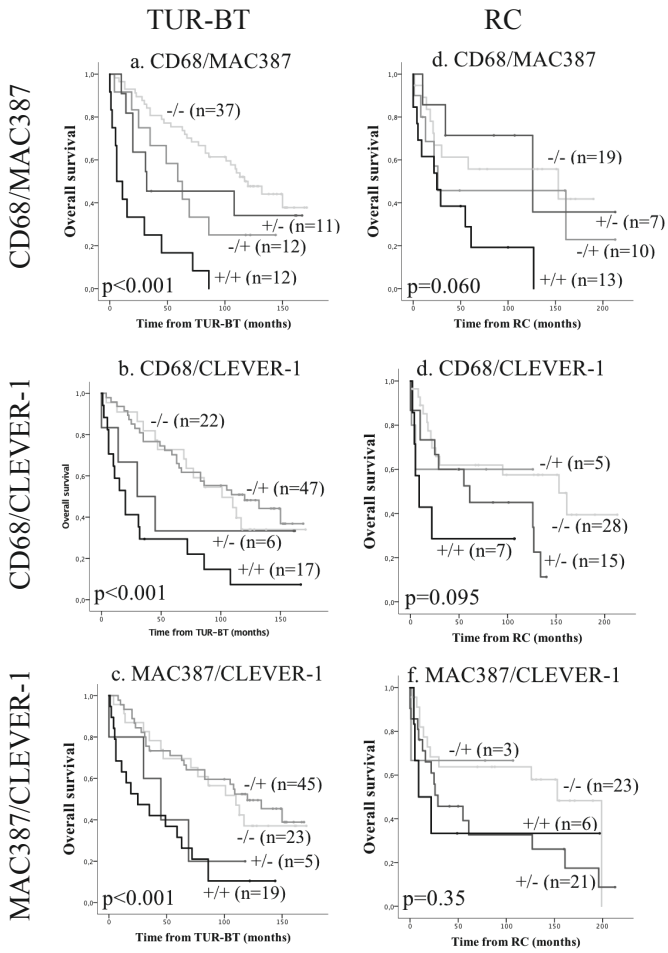

Figure 10. Kaplan-Meier estimates of the combination groups for OS in TUR-BT cohort (a.-c.) and RC cohort (d.-f.) in study I.

2.3 The predictive role of CD68, MAC387 and CLEVER-1

In study II the predictive roles of CD68, MAC387, and CLEVER-1 were investigated in NAC treated BC patients. No associations between known clinico-pathological characteristics (gender, smoking, type of chemotherapy, tumor grade, or presence of CIS, or LVI) and response to NAC were noticed. Associations between chemotherapy response and studied markers are shown in Table 16. The overall response rate for complete response in the study was 41%. 21% of the

patients received a partial response and 25% of the tumors progressed during NAC. CD68+ or MAC387+ macrophages did not associate with the chemotherapy response. However, a high MAC387+ tumor cell density associated with progression following NAC with risk of 3.76 (95% CI 1.10-12.82). Of the patients with high MAC387+ tumor cell density, 47% progressed during the follow-up, while 20% received a complete response (pT0N0). In contrast, 19% of the patients with low amounts of MAC387+ tumor cells progressed during the treatment, while 47% of these patients had no tumors at the time of RC (Table 17.). High CLEVER-1+ macrophage count associated with poorer response to NAC (HR 2.78, 95% CI 1.00-7.67) (Figure 11).

Table 16. Associations between markers and chemotherapy response. Regression analyses were used to evaluate the associations between the chemotherapy response and marker groups dichotomized according to the mean value. Mann-Whitney U test was used to evaluate the association between the continuous variables and chemotherapy response, a figure is provided for the significant findings (Figure 11). Pearson Chi-square/Fisher's exact test was used to evaluate the association between MAC387$^+$ tumor cells and chemotherapy response (low/high 0-2 *vs.* 3; all groups 0-3).

Variable		Complete *vs.* other			Complete/Partial *vs.* other			Other *vs.* progression		
		HR	95% CI	p-value	HR	95% CI	p-value	HR	95% CI	p-value
CD68$^+$ macroph.	Low/high	0.72	0.27-1.91	0.50	1.13	0.42-3.023	0.81	1.63	0.52-5.078	0.40
	Continuous			0.33			0.58			0.97
MAC387$^+$ macroph.	Low/high	0.94	0.36-2.49	0.91	1.038	0.39-2.77	0.94	1.48	0.49-4.46	0.48
	Continuous			0.42			0.40			0.53
MAC387$^+$ tumor cells	Low/high	3.57	0.90-14.13	0.070	3.18	0.97-10.37	0.056	3.76	1.10-12.82	0.034*
	All groups			0.41			0.36			0.40
CLEVER-1$^+$ macroph.	Low/high	1.63	0.62-4.32	0.33	2.78	1.006-7.67	0.049*	1.61	0.53-4.88	0.40
	Continuous			0.49			0.21			0.88
CLEVER-1$^+$ vessels	Low/high	0.99	0.37-2.62	0.98	0.76	0.28-2.050	0.58	0.47	0.14-1.52	0.21
	Continuous			0.26			0.10			0.012*

* Significant p-value

Table 17. Dichotomized marker counts, n (%), in different chemotherapy response groups.

Variable n (%)		Complete response	Partial response	No response	Progression
CD68$^+$ macrophages	Low, n=75	11 (37)	8 (27)	5 (17)	6 (20)
	High, n=38	17 (45)	6 (16)	4 (11)	11 (29)
MAC387$^+$ macrophages	Low, n=37	15 (41)	8 (22)	6 (16)	8 (22)
	High, n=31	13 (42)	6 (19)	3 (10)	9 (29)
MAC387$^+$ tumor cells	Low, n=62	25 (47)	11 (21)	7 (13)	10 (19)
	High, n=15	3 (20)	3 (30)	2 (13)	7 (47)
CLEVER-1$^+$ macrophages	Low, n=34	16 (47)	9 (27)	2 (6)	7 (21)
	High, n=34	12 (35)	5 (15)	7 (21)	10 (29)
CLEVER-1$^+$ vessels	Low, n=39	16 (41)	7 (18)	4 (10)	12 (31)
	High, n=29	12 (41)	7 (24)	5 (17)	5 (17)

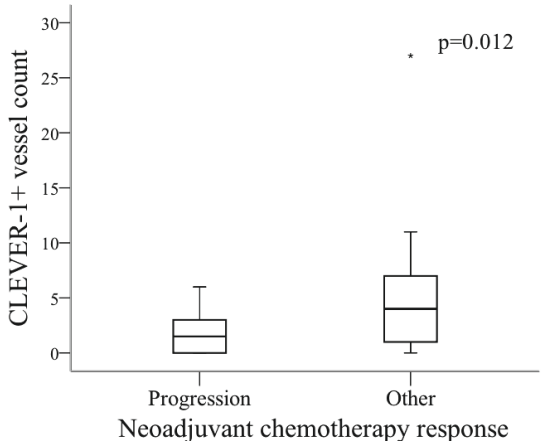

Figure 11. Association between neoadjuvant chemotherapy response (progression vs. Other) and CLEVER-1$^+$ vessels.

3 CD73 in bladder cancer (Study III)

3.1 CD73 expression in BC

The expression patterns of CD73 were analyzed in different cellular types in BC tumors: epithelium, endothelium, stromal fibroblasts, and lymphocytes. The distribution of CD73 expression in the different cellular compartments is represented in Table 18. The expression of CD73 between different cell types in BC tumors varied highly. CD73 stained differently BCL and SEC in BC tumors. When identified, BCL was positive approximately in 2/3 tumors in NMIBC and MIBC cohorts, whereas SEC remained negative in approximately 70% of the tumors. However, BCL could be identified only in 12% of poorly differentiated MIBC tumors, while in NMIBC samples BCL was seen in 62% of the samples. In the NMIBC cohort, only 22% of the patients had CD73 positive stromal fibroblasts, whereas in the MIBC cohort almost half of the patients had CD73 positive fibroblasts. The majority of the tumors in both cohorts had CD73 positive vessels. Approximately half of he patients had only <1% of CD73 positive tumor-infiltrating lymphocytes.

Table 18. Cell type specific expression of CD73 in BC.

Cell type	Score	NMIBC n=166 (%)	MIBC n=104 (%)
Basal cell layer of epithelium (BCL)	Negative	30 (18)	4 (4)
	Positive	67 (40)	8 (8)
	Not applicable	63 (38)	91 (88)
Suprabasal epithelial cells (SEC)	Negative	117 (71)	78 (75)
	Positive	42 (25)	25 (24)
Epithelium (total)	Negative	79 (48)	74 (71)
	Positive	80 (48)	29 (28)
Stromal fibroblasts	Negative	129 (78)	53 (51)
	Positive	37 (22)	46 (48)
Endothelial cells	Negative	49 (30)	23 (22)
	Positive	117 (70)	78 (75)
Lymphocytes	<1% positive	74 (45)	51 (49)
	≥1% positive	88 (53)	52 (50)

3.2 The prognostic role of CD73

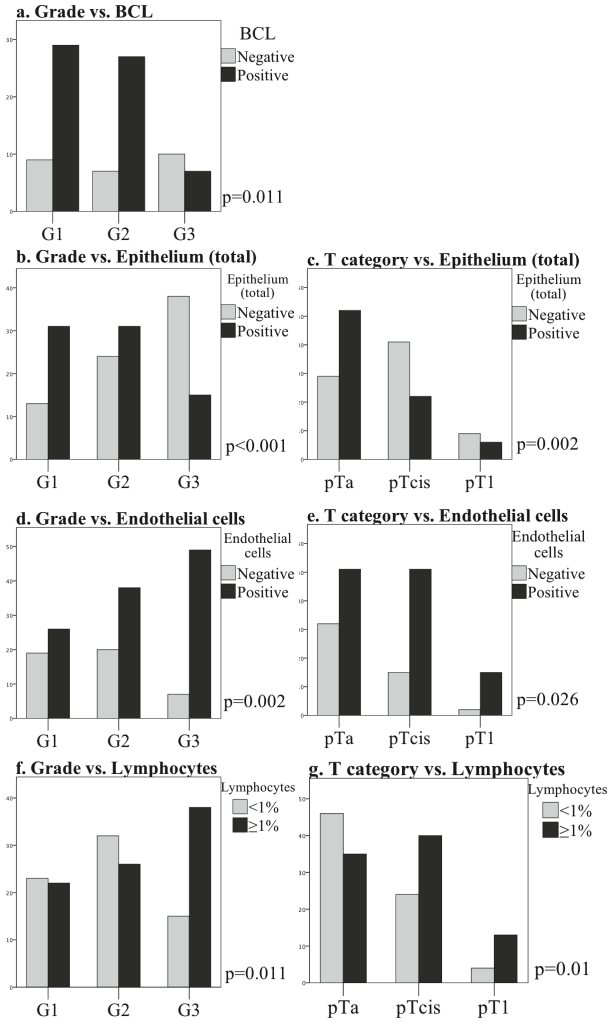

Figure 12. Correlations between the grade of the tumor and T category to CD73 positivity on different cellular components in NMIBC cohort in study III.

The correlations between CD73 positivity in BC and the clinicopathological characteristics were evaluated in Study III. In the NMIBC cohort, CD73 positive epithelium correlated significantly with grade of the tumor and T category. The majority of grade 3 tumors had lost CD73 from the epithelium, while tumors with lower grade had more CD73 positive epithelial cells. Most of the Ta tumors had positive total epithelium in contrast to Tcis and T1 tumors. CD73 positive BCL correlated also with grade of the tumor but not with T category. However, SEC did

not have any significant correlations with any parameters studied. Tumor grade and T category correlated also with CD73 positive endothelial cells and lymphocytes. However, the frequency of CD73 positive endothelial cells was increased in more advanced tumors compared to more indolent ones. There were also more CD73 positive lymphocytes in Tcis and T1 tumors as in Ta tumors. CD73 positivity among stromal fibroblasts did not correlate with any of the clinicopathological parameters in the NMIBC cohort. The significant correlations between grade and T category and the CD73 positive cell types are shown in Figure 12.

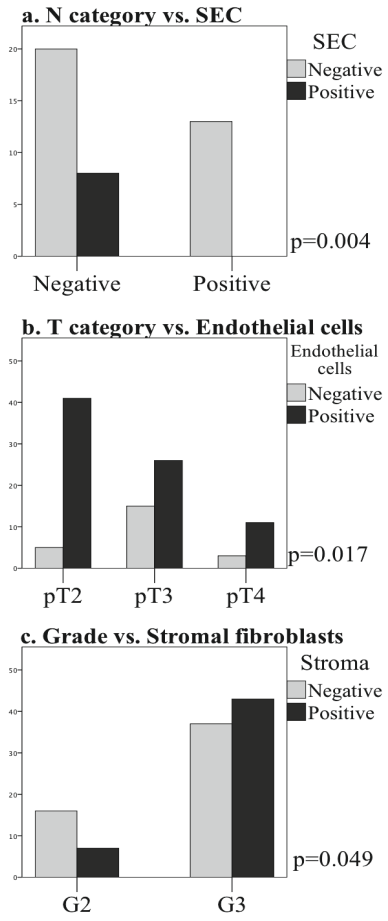

Figure 13. Significant correlations between the clinicopathological variables and CD73 positivity on different cellular components in MIBC cohort in study III: a. N category vs. SEC, b. T category vs. Endothelial cells, and c. Grade vs. Stromal fibroblasts.

In MIBC cohort, CD73 positive epithelium correlated significantly with N category, but not with tumor grade or T category. All MIBC patients with lymph node metastases had CD73 negative epithelial cells. CD73 positive endothelial cells correlated with T category in MIBC cohort. In contrast to NMIBC cohort, there were more CD73 positive vessels in tumors with lower T category. Stromal fibroblasts, in contrast, were more frequently CD73 positive in grade 3 tumors than in grade 2. The significant correlations in the MIBC cohort are shown in Figure 11.

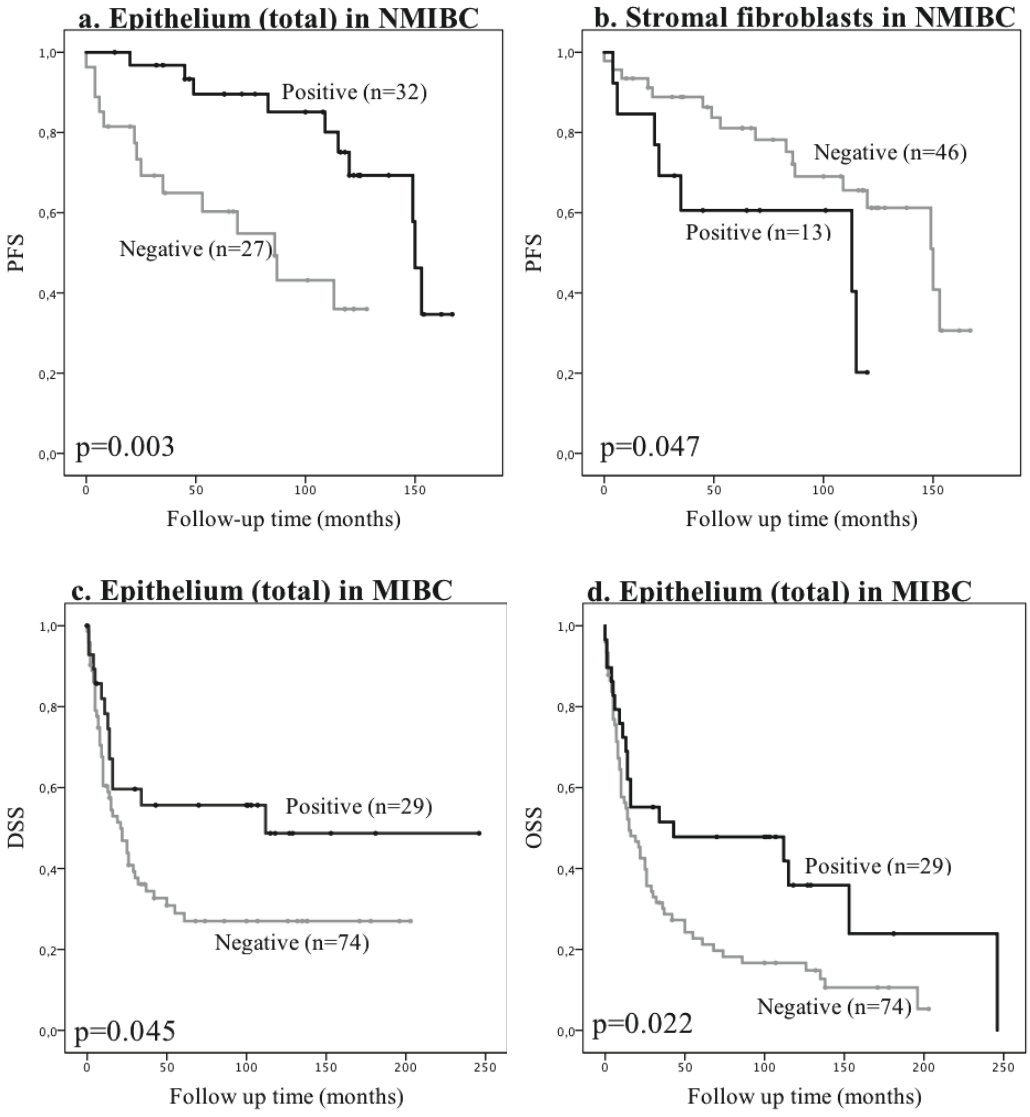

Figure 14. A. Association of CD73 positivity in total epithelium and PFS in NMIBC cohort. B. Association of CD73 positivity in stromal fibroblasts and PFS in NMIBC cohort. C. Association of CD73 positivity in total epithelium and OSS in MIBC cohort. D. Association of CD73 positivity in total epithelium and OSS in MIBC cohort.

The prognostic value of CD73 positivity in different cellular components in NMIBC according to RFS and PFS and in MIBC to DSS and OS was analyzed with Kaplan-Meier method and Cox proportional hazards regression model. In the NMIBC cohort, CD73 positive total epithelium associated with more favourable PFS, whereas CD73 positive stromal fibroblasts associated with poorer PFS in Kaplan-Meier analyses (Figure 12a-b.). In MIBC cohort, CD73 positive epithelium (total and SEC) associated with better DSS and OS (Figure 12c-d.). The results on total epithelium and SEC were similar to each other, because BCL could not be identified in most of the MIBC tumors.

Table 19. Univariate and multivariate Cox proportional hazards regression analyses of cell-type-specific CD73 expression affecting RFS, PFS, DSS, and OS in NMIBC cohort.

Variable		Univariate			Multivariate		
NMIBC		HR	95% CI	p-value	HR	95% CI	p-value
BCL	RFS	0.68	0.34-1.35	0.27	0.46	0.11-1.92	0.28
Neg. *vs.* Pos.	PFS	0.43	0.13-1.43	0.17	0.45	0.12-1.64	0.23
	DSS	0.65	0.36-1.18	0.16	1.54	0.45-5.35	0.49
	OS	0.29	0.092-0.92	0.035*	0.082	0.007-0.96	0.047*
SEC	RFS	0.80	0.45-1.40	0.43	0.37	0.10-1.39	0.14
Neg. *vs.* Pos.	PFS	0.72	0.27-1.90	0.51	0.92	0.33-2.54	0.87
	DSS	0.76	0.47-1.22	0.28	0.52	0.21-1.31	0.17
	OS	0.54	0.21-1.43	0.22	0.41	0.087-1.94	0.26
Epithelium	RFS	0.74	0.44-1.23	0.25	0.36	0.12-1.11	0.076
(total)	PFS	0.27	0.11-0.67	0.005*	0.36	0.13-1.00	0.049*
Neg. *vs.* Pos.	DSS	0.56	0.37-0.87	0.01*	0.62	0.29-1.32	0.21
	OS	0.26	0.11-0.63	0.002*	0.18	0.035-0.93	0.041*
Endothelial	RFS	0.78	0.46-1.31	0.35	0.55	0.19-1.57	0.26
cells	PFS	1.75	0.72-4.23	0.21	1.18	0.45-3.14	0.74
Neg. *vs.* Pos.	DSS	0.82	0.53-1.27	0.37	0.76	0.33-1.76	0.52
	OS	0.85	0.38-1.89	0.69	0.26	0.057-1.17	0.079
Stromal	RFS	1.69	0.93-3.10	0.086	1.04	0.29-3.78	0.95
fibroblasts	PFS	2.46	0.98-6.17	0.055	2.54	1.00-6.55	0.053
Neg. *vs.* Pos.	DSS	1.73	1.07-2.79	0.025*	1.28	0.56-2.95	0.57
	OS	1.81	0.79-4.14	0.16	1.03	0.29-3.65	0.96
Lymphocytes	RFS	0.8	0.48-1.34	0.40	0.53	0.18-1.57	0.25
Neg. *vs.* Pos.	PFS	2.19	0.90-5.31	0.083	2.19	0.90-5.32	0.084
	DSS	0.89	0.58-1.37	0.60	1.23	0.55-2.75	0.61
	OS	0.75	0.34-1.63	0.46	0.24	0.053-1.06	0.06

*Significant p-value

In Cox proportional hazards regression model CD73 positivity in total epithelium associated significantly with longer PFS and better DSS and OS in the NMIBC cohort. Moreover, in multivariate analyses, CD73 positive epithelium associated independently with longer PFS and better OS. BCL associated also with better OS both in univariate and multivariate analyses, but SEC did not affect the survival in

NMIBC cohort. In contrast to the CD73 positive epithelium, CD73 positive stromal fibroblasts associated with poorer DSS in NMIBC cohort. However, this result did not remain significant in multivariate analysis. CD73 positive endothelial cells or lymphocytes did not associate with outcome in the NMIBC cohort. The Cox proportional hazards regression analyses in the NMIBC cohort are shown in Table 19.

In MIBC cohort, CD73 positivity on SEC associated significantly with better RFS, DSS, and OS. CD73 positive SEC predicted also independently longer DSS in multivariate analysis. In addition, CD73 positivity on total epithelium associated with better PFS and DSS, and predicted DSS independently in multivariate analysis. Because of the lack of BCL in the samples, CD73 positivity on BCL could not be analyzed in multivariate analyses. CD73 expression on endothelial cells, stromal fibroblasts, or lymphocytes did not associate with outcome in univariate models. However, CD73 positive endothelial cells associated independently with more favourable PFS, DSS, and OS. The Cox proportional hazards regression analyses in the MIBC cohort are shown in Table 20.

Table 20. Univariate and multivariate Cox proportional hazards regression analyses of cell-type-specific CD73 expression affecting PFS, DSS, and OS in MIBC cohort.

Variable		Univariate			Multivariate		
MIBC		HR	95% CI	p-value	HR	95% CI	p-value
BCL	RFS	1.78	0.20-15.97	0.61	-	-	-
Neg. *vs.* Pos.	DSS	0.82	0.20-3.30	0.78	-	-	-
	OS	0.88	0.16-4.82	0.88	-	-	-
SEC	RFS	0.39	0.19-0.83	0.014*	0.24	0.049-1.14	0.072
Neg. *vs.* Pos.	DSS	0.48	0.27-0.85	0.012*	0.16	0.033-0.74	0.019*
	OS	0.48	0.24-0.94	0.032*	0.23	0.047-1.10	0.065
Epithelium	RFS	0.47	0.25-0.92	0.026*	0.24	0.049-1.14	0.072
(total)	DSS	0.55	0.32-0.93	0.025*	0.16	0.033-0.76	0.019*
Neg. *vs.* Pos.	OS	0.54	0.29-1.00	0.051	0.30	0.047-1.10	0.065
Endothelial	RFS	0.67	0.38-1.20	0.18	0.14	0.04-0.48	0.002*
cells	DSS	0.89	0.52-1.51	0.66	0.17	0.054-0.56	0.004*
Neg. *vs.* Pos.	OS	0.69	0.39-1.21	0.19	0.14	0.041-0.50	0.002*
Stromal	RFS	0.84	0.50-1.43	0.53	1.22	0.47-3.16	0.68
fibroblasts	DSS	0.95	0.62-1.47	0.83	0.92	0.37-2.29	0.86
Neg. *vs.* Pos.	OS	0.97	0.58-1.60	0.89	1.20	0.46-3.10	0.71
Lymphocytes	RFS	0.75	0.44-1.27	0.28	0.67	0.29-1.53	0.34
Neg. *vs.* Pos.	DSS	0.97	0.63-1.50	0.91	0.81	0.33-1.73	0.59
	OS	0.87	0.52-1.43	0.58	0.68	0.30-1.56	0.36

*Significant p-value

Discussion

1 CD68, MAC387, and CLEVER-1 in bladder cancer

BC is a heterogenous disease with a broad diversity in survival between superficial non-invasive disease and invasive, metastatic disease. Despite the good prognosis of many NMIBC patients, over half of the patients receive recurrence and the progression to MIBC is not uncommon. Because BC tumors are often nearly symptomless, frequent follow-up is needed. This causes inconvenience to the patient and major social costs. There is a critical need for biomarkers to assess accurately the outcome of BC and help guide the decision-making between treatment alternatives and follow-up protocols. In this study, CD68, MAC387, and CLEVER-1 were investigated as prognostic biomarkers in BC. The results demonstrate that intratumoral macrophage density is significantly associated with conventional high-risk features including high grade and advanced T category in BC. Furthermore, high macrophage count associates with poorer survival and higher risk for progression after TUR-BT. CD68 positive macrophage count is an independent prognostic factor for overall death in BC patients treated with NAC and RC.

As in many cancers, several studies have shown that CD68 positive macrophages associate with more advanced disease, greater risk for recurrence and progression, and poorer survival in BC.[291-293, 295, 296, 304, 313, 314] However, in a meta-analysis in 2018 consisting of 13 studies, Wu and colleagues did not find any prognostic value in CD68 positive TAMs with regard to OS in BC patients.[294] Our study from Plos One in 2015 was included in this meta-analysis with the most patients and the highest Newcastle-Ottawa scale score (NOS score 9). The meta-analysis was in line with our study, as we did not find any association between CD68 positive macrophages and BC survival in TUR-BT and RC treated patients. However, we found an independent association between CD68 positive macrophages and survival in NAC treated BC. CD68 is classically considered as a pan-macrophage marker, which does not distinguish the polarization of the macrophages. Therefore, we investigated two different macrophage populations in addition to CD68 positive macrophages: MAC387 and CLEVER-1 positive macrophages. MAC387 positive macrophages are a largely unknown subgroup of macrophages, which are considered as recently infiltrated, possibly the earliest macrophage group in the tissue during

early acute inflammation.[324] MAC387 positive macrophages have not been studied in BC earlier and our work is the first to detect a relationship between MAC387 positive macrophage density and poor outcome in BC in univariate analyses. In line with our results, S100A8 protein, one of the ligands of MAC387, is overexpressed in advanced BC and predicts the progression of the disease.[335-338] MAC387 positive macrophages associate with poor survival with several other cancers, too, e.g. with breast cancer and cholangiocarcinoma.[332, 334, 430] Soulas and co-workers speculate in their encephalitis study, that MAC387 positive macrophages would be M1-polarized, because of the proinflammatory activities of MRP14 and the absence of CD163 and CCR expression on MAC387 positive cells.[324] However, in our study MAC387 positive macrophages did not show antitumoral functions typical to M1 macrophages, but associated with more aggressive disease similar to CD68 positive macrophages. There is still a lack of a definitive surface molecule as a biomarker for proinflammatory M1 macrophages.

In our study, M2-polarized macrophages were investigated with CLEVER-1 antibody, a macrophage marker not investigated in BC earlier. CLEVER-1 is a large scavenger receptor expressed on afferent and efferent lymphatic and sinusoidal endothelial cells, as well as on a subset of immunosuppressive and pro-tumoral M2 macrophages with multiple functions.[342, 435] Another M2 macrophage marker, CD163, has been shown to act as a prognostic biomarker in BC in the meta-analysis in 2018.[294] However, our study showed incoherent results when studied CLEVER-1 positive M2 macrophages in BC patients treated with TUR-BT, RC alone, or NAC before RC: CLEVER-1 positive macrophages did not associate with survival after TUR-BT or RC alone, but had an association with poorer survival in univariate analyses in patients treated with NAC and RC. CLEVER-1 positive macrophages have also shown opposite prognostic results in different stages of cancer, e.g. in colorectal cancer.[224] It has been speculated, that macrophages loose the expression of CLEVER-1 when switching to phenotypes promoting tissue remodeling and angiogenesis.[356] Surprisingly, however, CLEVER-1 positive vessels seem to associate with lower risk of progression and more favourable DSS in BC. This result was disparate to earlier findings e.g. in colorectal and breast cancers.[224, 352] Thus, the prognostic role of CLEVER-1 in cancer cannot be oversimplified. The reason for CLEVER-1 positive vessels acting as protective factors in BC is unclear and need further investigations. However, a trend towards this result was seen in both of our studies about CLEVER-1 in BC. CLEVER-1 is expressed in HEVs in lymphoid organs and in afferent lymphatic vessels in non-lymphoid tissues.[343] It is also upregulated on HEV-like vessels at sites of inflammation.[339] In our study, the vessels studied were not distinguished as vascular or lymphatic vessels. BC is a highly immunogenic cancer, and one can speculate, that CLEVER-1 positive vessels could

act as a gate for antitumoral inflammatory cells to the tumor tissue. However, as stated, further studies to define this finding are warranted.

Although single macrophage markers were not significant prognostic factors in TUR-BT or RC treated BC in multivariate analyses, combinations of two markers provided independent prognostic information in multivariate models in the TUR-BT cohort. Combination groups with CD68 and MAC387, CD68 and CLEVER-1, and MAC387 and CLEVER-1 were generated. The relationship between CD68, MAC387, and CLEVER-1 positive macrophages is not known. It is unclear, whether these macrophage groups are subgroups of each other, different phenotype variations from same macrophages, or different macrophage groups activated and recruited by different factors. As these markers detect different populations of TAMs, the combination groups may better identify tumors that are skewed either towards M1 phenotype (CD68high/MAC387high and MAC387high/CLEVER-1low) or M2 type (CD68high/CLEVER-1high and MAC387low/CLEVER-1high). However, these groups were generated statistically, and thus, these groups do no represent macrophages with distinct marker expressions, but tumors with distinct ratios of different macrophage subtypes. It would be interesting to investigate BC tumors with double-stainings to find more distinctive macrophage subgroups. The results from our study showed, that despite the studied macrophage subtypes, higher macrophage count in general associated with poorer prognosis in BC. The combinations of the macrophage groups provided an auxiliary tool for prognostic factors in BC. CD68/MAC387 and CD68/CLEVER-1 groups could represent opposite macrophage polarizations, but the results were nearly similar. In CD68/CLEVER-1 combinations CD68 positive macrophages seemed to be critical for the survival as CD68high/CLEVER-1low/high groups associated with poorer survival than CD68low/CLEVER-1low/high groups and there were no differences whether the macrophages were M2 polarized CLEVER-1 positive cells or not. This phenomenon was also seen in MAC387/CLEVER-1 groups, where MAC387high/CLEVER-1low/high patients associated with poorer survival than MAC387low/CLEVER-1low/high patients.

This study has the known limitations of retrospective studies. Some clinical practices, e.g. performing the lymphadenectomy, might have changed during the long follow-up. In addition, the grading system was changed and the 2004 WHO/ISUP classification was defined after the 1973 WHO classification. However, the 2004 WHO/ISUP classification was utilized in the entire cohort after re-review of all cases by an expert genitourinary pathologist. In addition, there are several challenges when macrophages are investigated immunohistochemically. First of all, the heterogeneity of macrophages complicates the research: CD68 positive macrophages are a wide group of diffuse polarization orientations and do not explain the different roles between antitumoral/proinflammatory and pro-tumoral/anti-inflammatory

macrophages in the tumor. Macrophages may switch their phenotype during the process or tumorigenesis and thus, the stage of the disease is crucial in macrophage research.[201, 215] Even more, macrophage phenotype can vary according to their location in tumors, such as sites of initial tumor cell invasion, perivascular area, stromal region, and hypoxic or necrotic areas.[187, 203, 204] Hence, it would be important to investigate the role of different macrophages at different sites of the tumor. Macrophages tend to locate in clusters, which make the scoring of the TAMs in the tumor tissue more challenging and diverse especially in small samples, such as in TMA. In this study, macrophages were analyzed from whole histological immunosections and from TMA. The results demonstrated, that these analyzes may vary and it needs to be considered when studying TAMs.

Previous intravesical treatments were considered as the exclusion criteria in this study, because these treatments change the inflammatory microenvironment in the tumor. However, this complicates the interpretation of the results with a common BC patient receiving, for example, BCG treatments. Thus, it would be important to investigate the biomarkers in BCG treated BC, too. BCG is an important treatment modality in NMIBC, which utilizes the antitumor effect of local immune response. Macrophages act as a first line defense of innate immunity against BCG infection and have been considered to initiate BCG-induced anti-cancer responses.[303] Cells of the urothelium respond to the treatment with an inflammatory cascade and release cytokines, such as IL-8 and TNF, and recruit neutrophils to destroy malignant cells.[431] However, tumors with high TAM counts have also been shown to have worse outcome and to be less responsive to BCG treatment.[291, 292, 432] CD68 positive and CD163 positive macrophages associate with BCG failure.[315] It would be interesting to investigate further the role of different macrophage subtypes in the response to BCG treatment and even the possibility to enhance the effect of BCG treatment by manipulating the macrophage polarization in the bladder.

BCG is one of the oldest cancer immunotherapies. However, the treatment of BC is transforming due to new checkpoint inhibitor immunotherapies. The expectations for these drugs are high. Clinicians are, however, challenged to select the patients who will benefit from these treatments. Some patients receive poor responses to the new PD-1/PD-L1 therapies, but the reason for this is still unknown. No correlation between the PD-L1 positivity and the response rate for the inhibitors has been observed.[117] TAMs express high levels of PD-1 and its ligands.[229] Because the PD-L1 inhibitor treatment has shown antitumor activity even with non-PD-L1-expressing tumors, macrophages have been suggested to be the key element in the response to PD-L1 inhibitors.[233] TAMs remain inactive and do not exert antigen-presenting activity in CPI resistant tumors.[230] In some tumors, macrophages neutralize efforts to reactivate CD8+ T cells. (216) Manipulation of TAM phenotype could improve the efficacy of the immunotherapy. On the other hand, PD-L1

treatment has shown to reverse the immunosuppressive macrophage phenotype and trigger the macrophage-mediated antitumoral activity.[231] Thus, it would be extremely interesting to be able to expand our studies about CD68, MAC387, and CLEVER-1 into CPI treated patients.

The clinicians are challenged not only with selecting patients benefiting from CPI treatment but also from chemotherapy, and especially from neoadjuvant chemotherapy. Approximately 30-40% of the patients receiving NAC respond with no tumor (pT0N0) in cystectomy. The non-responders suffer from the toxicity of the chemotherapy agents and delay of the surgery. On the other hand, patients without micrometastatic disease are overtreated with toxic therapy. Among multiple different molecules, markers indicating DNA damage have been investigated as predictive factors for chemotherapy response. However, to date, there are no biomarkers in clinical use to indicate which patients will benefit from NAC in the treatment of BC. This is the first study investigating CD68, MAC387, and CLEVER-1 in BC patients treated with NAC. We reported, that high CD68 positive macrophage count is an independent prognostic factor for overall death in patients treated with NAC. When the markers were studied as predictive factors in NAC treated patients, neither CD68 nor MAC387 positive macrophages did affect the response to NAC. Instead, high MAC387 positive tumor cell count associated with progression following NAC while most of the patients with low MAC387 positive tumor cell count received a complete response for NAC and had no tumors at the time of RC. In addition, high CLEVER-1 positive macrophage count associated with poorer response to NAC, whereas low count of CLEVER-1 positive vessels associated with progression after NAC.

MAC387 expression on tumor cells has been used as a marker of squamous differentiation in BC, which is considered as a risk factor for local recurrence and poorer survival.[316, 329-331] Squamous cell carcinoma is the most commonly found non-urothelial histological subtype. Squamous cell differentiation has been observed also when dividing BC into molecular subtypes. However, BC is often a molecularly heterogenous disease and different molecular subtypes may emerge in the same tumor. Basal squamous tumors are aggressive tumors with frequent invasive and metastatic features.[60, 64, 433] However, in contrast to luminal tumors, they tend to respond to NAC and they have been reported as the best candidates for NAC.[67, 68] MAC387 positive tumor cells indicate the squamous metaplasia within the bladder tumor, and thus, act as predictive markers for tumors most suitable for NAC. In my knowledge, TAM expression in different molecular subtypes in BC has not been studied. The subtypes display prognostic significance and associate with different benefit from systemic therapies. It would be interesting to investigate what kind of differences there are in the TAM appearance in different BC subtypes with different kind of characteristics and survival prognosis.

Macrophages play an important role in the chemotherapy response and resistance. They can synergize with therapy or induce protumoral functions through the activation of tissue repair mechanisms.[212] They increase tumor progression by promotion of angiogenesis, maintenance of stem cells and inhibition of immune responses.[434] On the other had, chemotherapy agents can inhibit or activate macrophage mediated antitumor responses and the modulation of tumor responses to chemotherapy can vary between different cytotoxic factors and tumors.[435, 356] CLEVER-1 is expressed on M2 type macrophages considered as protumoral and anti-inflammatory. This subtype of TAMs seems to associate with poorer NAC response and progression on the tumor in BC patients. However, low counts of CLEVER-1 positive vessels associated also with progression of the disease after NAC. This is in line with our results from TUR-BT and RC treated BC patients, where CLEVER-1 positive vessels seemed to act as protective factors. These results demonstrate, that CLEVER-1 treatment for enhancing NAC in BC patients or for BC in the first place, either, would not be appropriate.

In addition to affecting other cancer therapies, TAMs are considered and investigated as treatment targets themselves in several diseases, such as asthma, atherosclerosis, and cancer, as well.[226] There are currently several drugs being tested in clinical trials targeting TAM recruitment, survival, and reprogramming as monotherapies or in combination with chemotherapy or immunotherapy.[212] Our study demonstrates, that CD68 and MAC387 positive TAMs associate with more advanced BC and poorer survival of the disease. Further investigations of these molecules as therapeutic targets in BC are in place. Anti-CLEVER-1 treatment has been shown to inhibit disease progression in malignant melanoma model.[350] However, our study shows, that CLEVER-1 is not only a protumoral factor in BC, but CLEVER-1 vessels also act as a protective fashion in these tumors, which naturally weakens CLEVER-1 as a therapeutic target in BC. Blocking the protumoral role of TAMs and reprogramming TAMs into antitumoral M1-like macrophages is a fascinating therapeutic approach in cancer. However, because of the heterogeneity of the macrophages, further studies are needed to distinguish the functions of the polarization subtypes in tumors.

2 CD73 in bladder cancer

CD73 is a multifunctional ecto-5'nucleotidase, which functions in the extracellular nucleotide turnover hydrolyzing extracellular AMP to its bioactive nucleoside, adenosine.[363, 364] Adenosine is a highly anti-inflammatory molecule with role e.g. in regulation of endothelial permeability and neutrophil adhesion.[369-374] The physiological function of CD73 varies between different cells.[382] In cancer, CD73 has been considered as an important player of tumor progression, but its true role in cancer is still widely unknown. CD73 has been shown to associate with poor prognosis in several cancers, such as leukemia and breast cancer.[404, 406] Furthermore, CD73 have been considered as possible prognostic biomarker and therapy target e.g. in melanoma and breast cancer.[401, 409, 410]

In contrast to many other cancer types, CD73 expression on BC cells has been shown to associate with lower stage and grade and lower risk for progression.[424] However, there are only few studies about CD73 in BC and further investigations are needed. In our study, CD73 was studied in different cell components in BC. The expression pattern of CD73 was analyzed in epithelial cells, endothelial cells, stromal fibroblasts, and lymphocytes in BC tumors. The expression of CD73 on these cell types varied highly.

CD73 positive epithelium correlated significantly with lower grade and T category in NMIBC and lower lymph node metastasis risk in MIBC tumors. In addition, CD73 positivity on epithelial cells associated with lower risk for progression in NMIBC patients and lower recurrence rate and better survival in MIBC. Moreover, CD73 positive epithelium was an independent prognostic factor both in NMIBC and MIBC. The CD73 expression varied substantially between the basal cell layer of the epithelium (BCL) and the rest of the epithelial/cancer cells (suprabasal epithelial cells, SEC): BCL was CD73 positive in the majority of the samples, whereas the majority of SEC remained CD73 negative. However, BCL was noticed only in better-differentiated tumors (12% in MIBC vs. 62% in NMIBC).

In contrast, the results showed CD73 positivity on stromal fibroblasts associating with poorer PFS and DSS in NMIBC tumors. However, this result could not been seen in MIBC. On the other hand, the frequency of CD73 positive endothelial cells was increased in more advanced NMIBC tumors, whereas MIBC patients with lower T category had more CD73 positive vessels than patients with higher T category. In addition, CD73 positive endothelial cells predicted more favourable survival in multivariate analyses in the MIBC cohort. CD73 was expressed at very low frequencies on TILs in most BC patients. Higher CD73 expression on lymphocytes correlated with more aggressive disease in NMIBC. However, CD73 positive lymphocytes did not predict disease survival.

Our results are in line with a previous study by Wettstein and colleagues.[424] They showed an association between CD73 positive BC cells and lower stage, grade and

risk for progression. However, in their study CD73 positivity on cancer cells was not an independent prognostic biomarker in BC. Our results of the favourable effect of CD73 positivity on epithelium is also in line with earlier studies suggesting that the CD73 negativity is associated with the malignant transformation of the urothelium.[421, 422] Our study is the first to investigate CD73 in different cell types in BC. The results point out, that the identity of the cell type expressing CD73 is important when interpreting the association of CD73 expression and survival. Moreover, our data imply that CD73 is unlikely to be a successful therapeutic target in BC. The systemic approaches would simultaneously target multiple different cell types, in which biological functions of CD73 varies. Interestingly, CD73 negativity on endothelial cells predicted poor outcome in the MIBC cohort in multivariate models. However, CD73 positivity on endothelial cells correlated with more advanced disease in the NMIBC cohort. Endothelial CD73 is involved in controlling the permeability of the blood vessels as well as in the regulation of the leukocyte extravasation.[437] CD73 is also expressed on lymphatic endothelium, in which its functions remain unknown.[363, 438] The blood and lymphatic vessels were not distinguished in our study. It is possible, that endothelial CD73 regulates the permeability and antitumor immunity in BC in a manner, which remains independent of the tumor grade and stage. Cancer-associated fibroblasts are known to express CD73.[439] CD73 is also present on mesenchymal stem cells, which may be recruited to the stroma during the tumor progression.[440] EMT may contribute also in generating CD73 positive fibroblast-like stromal cells. Different pathogenesis, such as cell-type origin, epigenetic regulators, driver mutations, and overall mutational load, and invasive potential of NMIBC and MIBC may affect the biological role of endothelial CD73 in cancer progression. It would be also interesting to investigate CD73 expression not only in different cell types in BC, but also in different molecular subtypes of BC. Because the expression of CD73 varies highly between different cellular types in tumors, the expression pattern may be different in different subtypes of same cancer type, too.

The present study is a retrospective, single institutional study with a limited number of patients and thus, limited number of disease specific deaths. Furthermore, we only studied CD73 protein expression and not the enzymatic activity of CD73, which is crucial for the production of immunosuppressing adenosine. However, the enzymatic activity of CD73 usually usually well with the protein expression.

Conclusions and the future

The recurrence rate of NMIBC is high, and frequent procedures and intense follow-up results in BC being one of the most expensive cancers to treat. Treatment options for advanced BC are limited and novel therapies are needed. There is a lack of new biomarkers to help diagnose aggressive cases that require more intensive treatment. In this study CD68, MAC387, CLEVER-1, and CD73 were studied as prognostic and predictive factors in BC. The specific conclusions of the studies are summarized in Table 21. Further questions and new possible study objectives arise from these studies (Table 22).

In this study, we found that high TAM counts associate with more advanced disease and poorer survival in BC patients. CD68 positive macrophage count is an independent prognostic biomarker in NAC treated BC. The role of CLEVER-1 positive macrophages in BC remains unknown, but surprisingly, CLEVER-1 positive vessels seem to be a protective factor in BC. When used in combinations, "double-high" macrophage groups have also independent prognostic value in BC and could potentially be used to identify aggressive cases among BC patients.

TAMs were studied in NAC treated BC patients as predictive biomarkers. We found, that MAC387 positive tumor cells and CLEVER-1 positive macrophages and vessels associate with the response for NAC in BC patients. High MAC387 tumor cell density associates with disease progression after NAC, whereas majority the patients with lower amount of MAC387 positive tumor cells receive a complete response for the treatment. Patients with higher amounts of CLEVER-1 positive vessels associate with more favourable response after NAC, whereas CLEVER-1 positive macrophages associated with poorer response to NAC. Our results demonstrate, that MAC387 and CLEVER-1 are possible predictive biomarkers to predict which BC patients are more prone to benefit from NAC. However, these results need validation with larger patient cohorts from multicenter studies.

Our study is the first to show, that CD73 expression varies highly between different cell types even in the same tumor samples. CD73 positive epithelium independently predicts better survival and lower risk for progression and recurrence in BC. In contrast, CD73 positivity on stromal fibroblasts associates with poorer

survival in NMIBC. The frequency of CD73 positive endothelial cells associate with more advanced NMIBC tumors, whereas in muscle-invasive disease CD73 positive vessels associate with lower T category. In addition, CD73 positive endothelial cells predicted more favourable survival in multivariate analyses in MIBC patients. CD73 was expressed at very low frequencies on TILs in most BC patients. Higher CD73 expression on lymphocytes correlates with more aggressive disease in NMIBC. Our results show, that the identity of the cell type expressing CD73 is important when interpreting the association of CD73 expression and survival. Moreover, our data imply that CD73 is unlikely to be a successful therapeutic target in BC due to the differences in the expression pattern and functions in different cell types.

Table 21. Specific conclusions of the studies.

CD68, MAC387, and CLEVER-1 as prognostic biomarkers in BC	
I	Intratumoral TAM density (CD68 and MAC387 positive macrophages) associate with conventional high-risk features, including high grade and advanced T category, and poorer survival in univariate analyses in BC.
II	CD68 positive macrophage count is an independent prognostic factor for overall death in BC patients treated with NAC and RC.
III	In contrast to earlier findings in other cancer types, CLEVER-1 positive vessels associate with lower risk of progression and more favourable DSS in BC. Thus, CLEVER-1 treatment would not be reasonable in BC.
IV	The combinations of the macrophage groups provide an auxiliary tool to predict the prognosis of BC: “double-high” macrophage groups have an independent prognostic value in BC and could potentially be used to identify aggressive cases among BC patients.
CD68, MAC387, and CLEVER-1 as predictive biomarkers in NAC treated BC	
I	MAC387 positivity on BC cells is a potential predictive marker for NAC treatment in BC.
II	High CLEVER-1 positive macrophage count and low CLEVER-1 vessel count associate with poorer response to NAC.
CD73 as prognostic biomarker in BC	
I	The expression of CD73 on different cell types in BC tumors varies highly and the identity of the cell type expressing CD73 is important when interpreting the association of CD73 expression and survival. - CD73 positive epithelium is an independent positive prognostic factor both in NMIBC and MIBC. - CD73 positivity on stromal fibroblasts associates with poorer PFS and DSS in NMIBC. - The frequency of CD73 positive endothelial cells is increased in more advanced NMIBC tumors, whereas in MIBC it predicts more favourable survival in multivariate analyses. - Higher CD73 positive lymphocyte count correlates with more aggressive disease in NMIBC.
II	CD73 is unlikely to be a successful therapeutic target in BC.

Table 22. The future study objectives.

I	Investigation of more detailed macrophage groups in BC with double-stainings and other macrophage markers (a) in different stages of BC, (b) in different sites in BC tumors, such as initial tumor cell invasion areas, perivascular area, stromal region, and hypoxic or necrotic areas, as well as in the metastatic foci, and (c) in different molecular subtypes of BC.
II	Detailed investigation of the characteristics and function of MAC387 macrophages.
III	The validation of the protective role of CLEVER-1 vessels in BC and further studies about the mechanism behind this.
IV	Investigation of the relationship between CD68, MAC387, and CLEVER-1 positive macrophages: Are they subgroups of each other, different phenotype variations from same macrophages, or different macrophage groups activated and recruited by different factors?
V	The validation of the predictive role of MAC387 and CLEVER-1 in NAC treated BC patients.
VI	CD68, MAC387, and CLEVER-1 reaction in the BCG treatment.
VII	Investigation of CD68, MAC387, and CLEVER-1 as predictive factors in checkpoint inhibitor treatments.
VIII	Investigation of TAMs (CD68 and MAC387) as treatment targets in BC.
IX	Investigation of CD73 expression on different cell types in other cancers.
X	Investigation of the role of CD73 subtypes in different molecular subtypes of BC.

Acknowledgements

The study was carried out at the Department of Medical Microbiology and Immunology, MediCity Research Laboratory, and the Department of Urology, University of Turku and Turku University Hospital during years 2008-2019. My sincere gratitude goes out to the heads of these institutions for providing excellent facilities for scientific work. I am also grateful to the Turku Doctoral Programme of Molecular Medicine (TuDMM) for financial and educational support, as well as to the Research Track in the Faculty of Medicine in University of Turku for introducing the scientific world to a rookie scientist.

I am deeply grateful to my supervisor Professor Sirpa Jalkanen for her support and guidance. I thank her for being the greatest role model as a scientist and showing that women – and mothers – definitely can rule the world. She has shown what can be accomplished with never-ending optimism and enthusiasm. I admire her knowledge in the field of science and I thank her for introducing me to her laboratory and giving me the chance to work there throughout these years.

Words cannot describe my gratefulness to my other supervisor, and brother, Docent Peter J. Boström. I thank him for giving me the opportunity work on this project and teaching me the scientific way of thinking. I am truly grateful for his endless support and time, whether it was a phone call or a message, a meeting in one of ours work places, or a Sunday night with the family. He believed me and this project even when I could not. Without him, this project would not have been done and I would not be the scientist and the clinician I am today.

I wish to express my sincerest gratitude to the excellent reviewers of the thesis, Professor Risto Renkonen and Docent Amir Sherif, who took the time to provide comments for the work. I thank them both for the kind words and for showing interest in my work.

I thank the members of my supervisory committee: my supervisors, Professor Matti Laato, and Professor Jukka Westermarck for their time, support, and advice. The meetings always gave me more ideas, inspiration and motivation to continue with the project.

I express my warmest thanks to all my co-authors. Their professional contribution to this work has been invaluable. I thank Pekka Taimen and Tuomas Mirtti for

the advice and support. Their enormous knowledge in the field of urologic pathology has been irreplaceable for the project. I thank Heikki Irjala for the extensive microscope analyses and Annika Ålgars for the advice and support in the laboratory. I am thankful to Tommi Kauko for helping me with the statistical part of the project. I thank Ilmari Koskinen and Thorsten Ecke for their contributions in the neoadjuvant chemotherapy project, and István Kenessey for his contributions in the CD73 project. I am grateful for Maarit Koivisto and professor Marko Salmi for giving me the opportunity to work with them in the CD73 project. The collaboration was always pleasant and fluent.

My warmest thanks to the skilled laboratory personnel at MediCity, Sari Mäki, Etta-Liisa Väänänen, Riikka Sjöroos, Maritta Pohjansalo, Teija Kanasuo, and Mari Parsama. From the first day in the lab the atmosphere was easy and supportive and every tiniest question was answered friendly and patiently. Sinikka Kollanus is acknowledged for her excellent help with the tissue samples, Tero Vahlberg for his expertise in statistical questions, Jouko Sandholm and Marko Saari for teaching me the ways of computed microscope analyses, and Ioan "John" Iagar and Antti-Pekka Laine for technical help in the laboratory.

I have been privileged to work with talented and hard-working students and post-docs in MediCity: Kaisa Auvinen, Johannes Dunkel, Dominik Eichin, Heli Elovaara, Kati Elima, Ruth Fair-Mäkelä, Cecilia Hagert, Maija Hollmen, Heikki Irjala, Norma Jäppinen, Sam Kaitaniemi, Marika Karikoski, Imtiaz Iftakhar-E-Khuda, Johannes Keuschigg, Juha Laurila, Fumiko Marttila, Mikael Maximov, Senthil Palani, Pia Rantakari, Kristiina Santalahti, Outi Sareila, Tibor Veres, Reetta Virtakoivu, and Gennady Yegutkin are thanked for the friendship and support during these many years in the laboratory. A special thanks to Mikko Koivisto at Lohja Hospital for helping with the page numbers.

My deepest thanks to my friends from Hanko, Kaisa Heinonen, Robert Nordlund, Vera and Kristian Fast, Janita Rantanen, Krista and Johannes Cederberg − I love you guys. I am grateful to them for always believing me and for just being there. Whether it was a long summer night in Hanko or a day of laziness and laughter in Helsinki, it has always been a pleasure and joy. I thank them for making me so happy. I also thank all my friends form medical school, especially Tiia Salminen, Noora Pienimaa-Kokko, and Salla Lempiäinen for the laughter and endless discussions that make the work, relationships and the whole world feel a lot easier.

I am deeply grateful to my parents Kaarina and Rune for the unconditional love and support. They have given me more than anyone could ask for. I am always looking forward to spend time with the family. The warmest thanks to my brother Janne and his wife Laura and the kids, Joel, Venla, and Eemeli for the funny and happy times in Hanko. Thanks to Pepe and Jutta and the kids, Elsa, Aapo, and Elias

for the countless dinners and happy memories in Turku. Jutta, thank you for your patience, I am sorry for bringing the work to your home on days off.

Last, but certainly not least, I owe my deepest gratitude to my beloved family. I want to thank my husband and best friend, Marko, for putting up with me. This work could not have been finished without his never-ending patience and love. I thank him for being the most incredible father to our kids. I want also thank the girls, Astrid and Louna. Thank you for being you: the funniest, liveliest, noisiest, most lovely and stubborn, and the most adorable kids in the world. I am sorry for the time that this work has taken away from us. You are still young but I hope that you will some day consider this book as a reminder of that with hard work you can do anything you want in the whole wide world.

This work was supported financially by Orion-Farmos, Turku Medical Foundation Duodecim, the Instrumentarium Science Foundation, Turku University Foundation, Oskar Öflund Foundation, Turku Doctoral Programme of Molecular Medicine (TuDMM), Emil Aaltonen Foundation, the Maud Kuistila Memorial Foundation, and Helsinki Medical Society.

Helsinki, 13.7.2019
Minna Tervahartiala

Abbreviations

ACT	Adoptive T cell transfer
ADCC	Antibody-dependent cell cytotoxicity
ADCP	Antibody-dependent cell phagocytosis
ADP	Adenosine diphosphate
AKT	Protein kinase B
APC	Antigen presenting cell
ATM	Ataxia telangiectasia mutated gene
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
AUA	American Urological Association
BC	Bladder cancer
BCA-1	B cell attracting chemokine
BCG	Bacillus Calmette-Guérin
BCL	Basal cell layer of the epithelium
BiTE	Bispecific T cell engager
bsAb	Bispecific antibody
BTA	Bladder tumor antigen
BTLA	B and T lymphocyte attenuator
CI	Confidence interval
CIS	Carcinoma *in situ*
CLEVER-1	Common lymphatic endothelial and vascular endothelial receptor-1
COX2	Cyclooxygenase 2
CPI	Immune checkpoint inhibitor
CTL	Cytotoxic T cell
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CT	Computed tomography
cT	Clinical T category
CRP	C-reactive protein

CXC/CC	C-X-C/C-C motif chemokine
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticized xylene
DSS	Disease-specific survival
EAU	European Association of Urology
ECM	Extracellular matrix
ecto-5'-NT	Ecto-5'-nucleotidase
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERCC1/2	Excision repair cross complementing 1 and 2
FANCC	Fanconi anemia complementation group C gene
FDA	The United States Food and Drug Administration
FEEL-1	Fasciclin, EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1
FGFR3	Fibroblast growth factor receptor-3
FISH	Fluorescence *in situ* hybridization
FN	Fibronectin
FoxP3	Forkhead box P3
GPI	Glycosyl-phosphatidylinositol
gp130	Glycoprotein 130
gro-α	Growth-regulated oncogene α
GSTM1	Gluthathione S-transferase-μ1
HER-2	Human epidermal growth factor receptor-2
HEV	High endothelial venule
HIF	Hypoxia-inducible transcription factor
HR	Hazard ratio
HSC	Hematopoietic stem cell
ICAM-1	Intercellular adhesion molecule-1
IDO	Indoleamine dioxigenase
IFNγ	Interferon γ
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP-10	IFNγ-induced protein-10
ISUP	International Society of Urological Pathology
JAK	Janus kinase
LAG-3	Lymphocyte activation gene-3
LDL	Low-density lipoprotein

LPS	Lipopolysaccharide
LVI	Lymphovascular invasion
mAb	Monovalent antibody
MCP-1	Monocyte chemotactic protein-1
M-CSF	Macrophage colony-stimulating factor
MDC	Macrophage derived chemokine
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MIBC	Muscle invasive bladder cancer
MIF	Migration inhibitory factor
MIG	Monokine induced by gamma interferon
MMP-9	Matrix metalloproteinase 9
MR	Mannose receptor
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MRP	Myeloid-related protein
MTC	Macrophage-mediated tumor cytotoxicity
mTOR	The mammalian target of rapamycin
NAC	Neoadjuvant chemotherapy
NAT2	N-acetyltransferase 2
NCCN	National Comprehensive Cancer Network
NER	The nucleoside excision repair
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NK cells	Natural killer cells
N/L ratio	Neutrophil-to-lymphocyte ratio
NMIBC	Non-muscle invasive bladder cancer
NMP22	Nuclear matrix protein 22
NOS	Newcastle-Ottawa scale
NSAID	Nonsteroidal anti-inflammatory drug
OS	Overall survival
PDD	Photodynamic diagnosis
PD-1/PD-L1	Programmed death 1/programmed death-ligand 1
PFS	Progression-free survival
PI3K	Phosphoinositide 3 kinase
pT	Pathological T category
PTEN	Phosphatase and tensin homologue
PUNMLP	Papillary urothelial neoplasm of low malignant potential
p53	Tumor protein 53

RANTES	Regulated upon activation normal T cell expressed and secreted
RB1	Retinoblastoma gene
RC	Radical cystectomy
RFS	Recurrence-free survival
RNI	Reactive nitrogen intermediate
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
SDF-1	Stromal derived factor-1
SEC	Suprabasal epithelial cells
SIG1RR	Single immunoglobulin interleukin 1 receptor-related protein
siRNA	Small interfering ribonucleic acid
SPARC	Secreted protein, acidic rich in cysteine
SR	Scavenging receptor
STAT3	Signal transducer and activator of transcription
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophil
TARC	Thymus and activation regulated chemokine
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th cell	T helper cell
TIL	Tumor-infiltrating leukocytes
TIM-3	T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain-3
TIR8	Toll-interleukin receptor-8
Tis, CIS	Carcinoma *in situ*
TLR	Toll-like receptor
TMA	Tissue microarray
TNF	Tumor necrosis factor
TNM	Tumor, Node, Metastasis classification system
Treg cell	T regulatory cell
TUR-BT	Transurethral resection of bladder tumor
UICC	Union for International Cancer Control
uPa	Urokinase plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VHL/HIF	Von Hippel Lindau/Hypoxia-inducible factor
WHO	World Health Organization

List of references

1. National Cancer Institute (2015). What Is Cancer? *www.cancer.gov*

2. GBD 2015 Disease and Injury Incidence and Prevalnce Collaborators (2016). Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet.* 388(10053): 1545-602

3. Finnish Cancer Registry, *www.cancerregistry.fi*

4. Parkin DM, Boyd L, and Walker LC (2011). The fraction of cancer attributable to lifestyle and environmental factors in the UK in 2010. *Br J Cancer.* 105 Suppl 2: S77-81.

5. Hanahan D and Weinberg RA (2000). The hallmarks of cancer. *Cell.* 100(1): 57-70.

6. GBD 2015 Mortality and Causes Of Death Collaborators (2016). Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet.* 388(10053): 1459-544.

7. WHO, International Agency for Research on Cancer. GLOBOCAN 2012, Estimated cancer incidence, mortality and prevalence worldwide. *www.globocan.iarc.fr*

8. Miyazaki J and Nishiyama H (2017). Epidemiology of urothelial carcinoma. *Int J Urol.* 24 /19: 730-734.

9. Antoni S, Ferlay J, Soerjomataram I, *et al* (2017). Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. *Eur Urol.* 71(1): 96-108.

10. Scosyrev E, Noyes K, Feng C, *et al* (2009). Sex and racial differences in bladder cancer presentation and mortality in the US. *Cancer.* 115(1): 68-74.

11. Burger M, Catto JW, Dalbagni G, *et al* (2013). Epidemiology and risk factors of urothelial bladder cancer. *Eur Urol.* 63(2): 234-41.

12. Freedman ND, Silverman DT, Hollenbeck AR, *et al* (2011). Association between smoking and risk of bladder cancer among men and women. *JAMA.* 306(7): 737-45.

13. Parkin DM (2006). The global health burden of infection-associated cancers in the year 2002. *Int J Cancer.* 118(12): 3030-44.

14. Aben KK, Witjes JA, Schoenberg MP, *et al* (2002). Familial aggregation of urothelial cell carcinoma. *Int J Cancer.* 98(2):2 74-8.

15. Mishriki SF, Grimsley SJ, and Nabi G (2009). Incidence of recurrent frank hematuria and urological cancers: prospective 6.9 years of followup. *J Urol.* 182(4): 1294-8

16. Lamm D, Herr H, Jakse G, *et al* (1998). Updated concepts and treatment of carcinoma in situ. *Urol Oncol.* 4(4-5): 130-8.

17. Yafi FA, Brimo F, Steinberg J, *et al* (2015). Prospective analysis of sensitivity and specificity of urinary cytology and other urinary biomarkers for bladder cancer. *Urol Oncol.* 33(2): 66.e25-31.

18. van Rhijn BW, van der Poel HG, and van der Kwast TH (2005). Urine markers for bladder cancer surveillance: a systematic review. *Eur Urol.* 47(6): 736-48.

19. Mowatt G, N'Dow J, Vale L, et al (2011). Photodynamic diagnosis of bladder cancer compared with white light cystoscopy: Systematic review and meta-analysis. *Int J Technol Assess Health Care.* 27(1): 3-10.

20. Burger M, Grossman HB, Droller M, *et al* (2013). Photodynamic diagnosis of non-muscle-invasive bladder cancer with hexaminolevulinate cystoscopy: a meta-analysis of detection and recurrence based on raw data. *Eur Urol.* 64(5): 846-54.

21. Woldu SL, Bagrodia A, and Lotan Y (2017). Guideline of guidelines: non-muscle-invasive bladder cancer. *BJU Int.* 119(3): 371-80.

22. Jakse G, Algaba F, Malmström PU, et al (2004). A second-look TUR in T1 transitional cell carcinoma: why? *Eur Urol.* 45(5): 539-46; discussion 46.

23. Brausi M, Witjes JA, Lamm D, et al (2011). A review of current guidelines and best practice recommendations for the management of nonmuscle invasive bladder cancer by the International Bladder Cancer Group. *J Urol.* 186(6): 2158-67.

24. Herr HW (1999). The value of a second transurethral resection in evaluating patients with bladder tumors. *J Urol.* 162(1): 74-6.

25. Kaufman DS, Shipley WU, and Feldman AS (2009). Bladder cancer. *Lancet.* 374(9685): 239-49

26. Braendengen M, Winderen M, Fosså SD (1996). Clinical significance of routine pre-cystectomy bone scans in patients with muscle-invasive bladder cancer. *Br J Urol.* 77(1): 36-40.

27. van der Horst G, Bos L, and van der Pluijm G (2012). Epithelial plasticity, cancer stem cells, and the tumor-supportive stroma in bladder carcinoma. *Mol Cancer Res.* 10(8): 995-1009.

28. Kamat AM, Hahn NM, Efstathiou JA, *et al* (2016). Bladder cancer. *Lancet.* 388(10061): 2796-810.

29. Black PC, Brown GA, and Dinney CP (2009). The impact of variant histology on the outcome of bladder cancer treated with curative intent. *Urol Oncol.* 27(1): 3-7.

30. Witjes A, Compérat E, Cowan NC, et al (2017). EAU guidelines on Muscle-invasice and Metastatic Bladder Cancer, http://www.uroweb.org/guidelines/online-guidelines/

31. Liu Y, Bui MM, and Xu B (2017). Urothelial carcinoma with squamous differentiation is associated with high tumor stage and pelvic lymph-node metastasis. *Cancer Control.* 24(1): 78-82.

32. Minato A, Noguchi H, Tomisaki I, et al (2018). Clinical significance of squamous differentiation in urothelial carcinoma of the bladder. *Cancer Control.* 25(1): 1073274818800269.

33. Erdemir F, Tunc M, Ozcan F, *et al* (2007). The effect of squamous and/or glandular differentiation on recurrence, progression and survival in urothelial carcinoma of bladder. *Int Urol Nephrol.* 39(3): 803-7.

34. Izard JP, Siemens DR, Mackillop WJ, *et al* (2015). Outcomes of squamous histology in bladder cancer: a population-based study. *Urol Oncol.* 233(10): 425.e7-13.

35. Mitra AP, Bartsch CC, Bartsch G, *et al* (2014). Does presence of squamous and glandular differentiation in urothelial carcinoma of the bladder at cystectomy portend poor prognosis? An intensive case-control analysis. *Urol Oncol.* 32(2): 117-27.

36. Sobin L, Gospodarowicz M, and Wittekind C (eds). TNM classification of malignant tumors. UICC International Union Against Cancer, 7th ed, Wiley-Blackwell (2009).

37. Cao D, Vollmer RT, Luly J, *et al* (2010). Comparison of 2004 and 1973 World Health Organization grading systems and their relationship to pathologic staging for predicting long-term prognosis in patients with urothelial carcinoma. *Urology.* 76(3): 593-9.

38. Hansel DE, Amin MB, Comperat E, *et al* (2013). A contemporary update on pathology standards for bladder cancer: transurethral resection and radical cystectomy specimens. *Eur Urol.* 63(2): 321-32.

39. Reuter VE (2006). The pathology of bladder cancer. *Urology.* 67: 7-11.

40. Shariat SF, Palapattu GS, Karakiewicz PI, *et al* (2007). Discrepancy between clinical and pathologic stage: impact on prognosis after radical cystectomy. *Eur Urol.* 51(1): 137-51.

41. Humphrey PA, Moch H, Cubilla AL, *et al* (2016). The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part B: Prostate and Bladder Tumours. *Eur Urol.* 70(1): 106-19.

42. Sylvester RJ, van der Meijden A, Witjes JA, *et al* (2005). High-grade Ta urothelial carcinoma and carcinoma in situ of the bladder. *Urology.* 66: 90-107.

43. Rübben H, Lutzeyer W, Fischer N, *et al* (1988). Natural history and treatment of low and high risk superficial bladder tumors. *J Urol.* 139(2): 283-5.

44. Raghavan D, Quinn D, Skinner DG, *et al* (2002). Surgery and adjunctive chemotherapy for invasive bladder cancer. *Surg Oncol.* 11(1-2): 55-63.

45. Dong F, Shen Y, Gao F, *et al* (2017). Prognostic value of site-specific metastases and therapeutic roles of surgery for patients with metastatic bladder cancer: a population-based study. *Cancer Manag Res.* 9: 611-26.

46. Mostofi F, Sobin K, and Torloni H (1973). Histologic Typing of Urinary Bladder Tumors: International Histological Classification of Tumors. World Health Organization, Geneva.

47. Eble J, Sauter G, Epstein J, et al (eds.). World health organization classification of tumors. Pathology and genetics: tumours of the urinary system and male genital organs, IARCC Publishing, Lyon, France (2004).

48. Babjuk M, Bohle A, Burger M, *et al* (2016). EAU guidelines on non–muscle-invasive urothelial carcinoma of the bladder. *www.uroweb.org/guidelines/online-guidelines/*

49. MacLennan GT, Kirkali Z, and Cheng L (2007). Histologic grading of noninvasive papillary urothelial neoplasms. *Eur Urol.* 51(4): 889-98,

50. Lamm DL (1992). Carcinoma in situ. *Urol Clin North Am.* 19(3): 499-508.

51. Sylvester RJ (2006). Natural history, recurrence, and progression in superficial

bladder cancer. *Scientific World Journal*. 6: 2617-25.

52. Kimura S, Mari A, Foerster B, *et al* (2018). Prognostic value of concomitant carcinoma in situ in the radical cystectomy specimen: A systematic review and meta-analysis. *J Urol*. 201(1): 46-53

53. Blaveri E, Simko JP, Korkola JE, *et al* (2005). Bladder cancer outcome and subtype classification by gene expression. *Clin Cancer Res*. 11(11): 4044-55.

54. Dyrskjøt L, Thykjaer T, Kruhøffer M, *et al* (2003). Identifying distinct classes of bladder carcinoma using microarrays. *Nat Genet*. 33(1): 90-6.

55. Sanchez-Carbayo M, Socci ND, Lozano J, *et al* (2006). Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. *J Clin Oncol*. 24(5): 778-89.

56. Lindgren D, Frigyesi A, Gudjonsson S, *et al* (2010). Combined gene expression and genomic profiling define two intrinsic molecular subtypes of urothelial carcinoma and gene signatures for molecular grading and outcome. *Cancer Res*. 70(9): 3463-72.

57. Alfred Witjes J, Lebret T, Compérat EM, *et al* (2017). Updated 2016 EAU Guidelines on Muscle-invasive and Metastatic Bladder Cancer. *Eur Urol*. 71(3): 462-75.

58. McConkey DJ and Choi W (2018). Molecular Subtypes of Bladder Cancer. *Curr Oncol Rep*. 20(10): 77.

59. Sjödahl G, Lauss M, Lövgren K, *et al* (2012). A molecular taxonomy for urothelial carcinoma. *Clin Cancer Res*. 18(12): 3377-86.

60. Sjödahl G, Eriksson P, Liedberg F, *et al* (2017). Molecular classification of urothelial carcinoma: global mRNA classification versus tumour-cell phenotype classification. *J Pathol*. 2242(1): 113-25.

61. Aine M, Eriksson P, Liedberg F, *et al* (2015). Biological determinants of bladder cancer gene expression subtypes. *Sci Rep*. 5: 10957.

62. Damrauer JS, Hoadley KA, Chism DD, *et al* (2014). Intrinsic subtypes of high-grade bladder cancer reflect the hallmarks of breast cancer biology. *Proc Natl Acad Sci USA*. 111(8): 3110-5.

63. Choi W, Porten S, Kim S, Willis D, *et al* (2014). Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. *Cancer Cell*. 25(2): 152-65.

64. Network CGAR (2014). Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature*. 507(7492): 315-22.

65. Tan TZ, Rouanne M, Tan KT, et al (2018) Molecular subtypes of urothelial bladder cancer: results from a meta-cohort analysis of 2411 tumors. *Eur Urol*.75(3): 423-32.

66. Warrick JI, Sjödahl G, Kaag M, *et al* (2018). Intratumoral Heterogeneity of Bladder Cancer by Molecular Subtypes and Histologic Variants. *Eur Urol*. 75(1): 18-22.

67. McConkey DJ, Choi W, Shen Y, *et al* (2016). A prognostic gene expression signature in the molecular classification of chemotherapy-naïve urothelial cancer is predictive of clinical outcomes from neoadjuvant chemotherapy: A phase 2 trial of dose-dense methotrexate, vinblastine, doxorubicin, and cisplatin with bevacizumab in urothelial cancer. *Eur Urol*. 69(5): 855-62.

68. Seiler R, Ashab HAD, Erho N, *et al* (2017). Impact of molecular subtypes in muscle-invasive bladder cancer on predicting response and survival after neoadjuvant chemotherapy. *Eur Urol.* 72(4): 544-54.

69. Robertson AG, Kim J, Al-Ahmadie H, *et al* (2017). Comprehensive molecular characterization of muscle-Invasive bladder cancer. *Cell.* 171(3): 540-56.e25.

70. Rosenberg JE, Hoffman-Censits J, Powles T, *et al* (2016). Atezolizumab in patients with locally advanced and metastatic urothelial carcinoma who have progressed following treatment with platinum-based chemotherapy: a single-arm, multicentre, phase 2 trial. *Lancet.* 387(10031): 1909-20.

71. Mariathasan S, Turley SJ, Nickles D, *et al* (2018). TGFβ attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature.* 554(7693): 544-8.

72. Herr HW (2006). Early history of endoscopic treatment of bladder tumors from Grunfeld's polypenkneipe to the Stern-McCarthy resectoscope. *J Endourol.* 20(2): 85-91.

73. Herr HW (2005). Legacy of Edwin Beer: fulguration of papillary bladder tumors. *J Urol.* 173(4): 1087-9.

74. Tan WS, Rodney S, Lamb B, *et al* (2016). Management of non-muscle invasive bladder cancer: A comprehensive analysis of guidelines from the United States, Europe and Asia. *Cancer Treat Rev.* 47: 22-31.

75. Solsona E, Iborra I, Ricós JV, *et al* (1999). Effectiveness of a single immediate mitomycin C instillation in patients with low risk superficial bladder cancer: short and long-term followup. *J Urol.* 161(4): 1120-3.

76. Sylvester RJ, Oosterlinck W, and van der Meijden AP (2004). A single immediate postoperative instillation of chemotherapy decreases the risk of recurrence in patients with stage Ta T1 bladder cancer: a meta-analysis of published results of randomized clinical trials. *J Urol.* 171: 2186-90.

77. Perlis N, Zlotta AR, Beyene J, *et al* (2013). Immediate post-transurethral resection of bladder tumor intravesical chemotherapy prevents non-muscle-invasive bladder cancer recurrences: an updated meta-analysis on 2548 patients and quality-of-evidence review. *Eur Urol.* 64(3): 421-30.

78. Brocks CP, Büttner H, and Böhle A (2005). Inhibition of tumor implantation by intravesical gemcitabine in a murine model of superficial bladder cancer. *J Urol.* 174(3): 1115-8.

79. Sylvester RJ, Oosterlinck W, Holmang S, *et al* (2016). Systematic review and individual patient data meta-analysis of randomized trials comparing a single immediate instillation of chemotherapy after transurethral resection with transurethral resection alone in patients with stage pTa-pT1 urothelial carcinoma of the bladder: which patients benefit from the instillation? *Eur Urol.* 69(2): 231-44.

80. Sylvester RJ, van der Meijden AP, Witjes JA, *et al* (2005). Bacillus calmette-guerin versus chemotherapy for the intravesical treatment of patients with carcinoma in situ of the bladder: a meta-analysis of the published results of randomized clinical trials. *J Urol.* 174(1): 86-92.

81. Böhle A, Bock PR. Intravesical bacille Calmette-Guérin versus mitomycin C in superficial bladder cancer: formal meta-analysis of comparative studies on tumor progression. Urology. 2004;63(4):682-6; discussion 6-7.

82. Han RF and Pan JG (2006). Can intravesical bacillus Calmette-Guérin reduce recurrence in patients with superficial bladder cancer? A meta-analysis of randomized trials. *Urology.* 67(6): 1216-23.

83. Herr HW and Sogani PC (2001). Does early cystectomy improve the survival of patients with high risk superficial bladder tumors? *J Urol.* 166(4): 1296-9.

84. van den Bosch S and Alfred Witjes J (2011). Long-term cancer-specific survival in patients with high-risk, non-muscle-invasive bladder cancer and tumour progression: a systematic review. *Eur Urol.* 60(3): 493-500

85. Shen PL, Lin ME, Hong YK, *et al* (2018). Bladder preservation approach versus radical cystectomy for high-grade non-muscle-invasive bladder cancer: a meta-analysis of cohort studies. *World J Surg Oncol.* 16(1): 197.

86. Cookson MS, Herr HW, Zhang ZF, *et al* (1997). The treated natural history of high risk superficial bladder cancer: 15-year outcome. *J Urol.* 158(1): 62-7.

87. Svatek RS, Hollenbeck BK, Holmäng S, *et al* (2014). The economics of bladder cancer: costs and considerations of caring for this disease. *Eur Urol.* 66(2): 253-62.

88. Palou J, Rodríguez-Rubio F, Millán F, *et al* (2009). Recurrence at three months and high-grade recurrence as prognostic factor of progression in multivariate analysis of T1G2 bladder tumors. *Urology.* 73(6): 1313-7.

89. Babjuk M, Oosterlinck W, Sylvester R, *et al* (2011). EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder, the 2011 update. *Eur Urol.* 59(6): 997-1008.

90. Stenzl A, Nagele U, Kuczyk M, *et al* (2015). Cystectomy – Technical considerations in male and female patients. *EAU Update Series* p. 138–46.

91. Dorin RP, Daneshmand S, Eisenberg MS, *et al* (2011). Lymph node dissection technique is more important than lymph node count in identifying nodal metastases in radical cystectomy patients: a comparative mapping study. *Eur Urol.* 60(5): 946-52.

92. Chang SS, Bochner BH, Chou R, *et al* (2017). Treatment of Non-Metastatic Muscle-Invasive Bladder Cancer: AUA/ASCO/ ASTRO/SUO Guideline. *J Urol.* 198(3): 552-9.

93. Hautmann RE, Abol-Enein H, Hafez K, *et al* (2007). Urinary diversion. *Urology.* 69: 17-49.

94. Vallancien G, Abou El Fettouh H, Cathelineau X, *et al* (2002). Cystectomy with prostate sparing for bladder cancer in 100 patients: 10-year experience. *J Urol.* 168(6): 2413-7.

95. Hautmann RE, de Petriconi RC, and Volkmer BG (2010). Lessons learned from 1,000 neobladders: the 90-day complication rate. *J Urol.* 184(3): 990-4.

96. Ghoneim MA, El-Mekresh MM, Mokhtar AA, *et al* (2000). A predictive model of survival after radical cystectomy for carcinoma of the bladder. *BJU Int.* 85(7): 811-6.

97. Grossman HB, Natale RB, Tangen CM, *et al* (2003). Neoadjuvant chemotherapy plus cystectomy compared with cystectomy alone for locally advanced bladder cancer. *N Engl J Med.* 349(9): 859-66.

98. Vale CL (2005). Neoadjuvant Cchemotherapy in invasive bladder cancer: update of a systematic review and meta-analysis of individual patient data:

advanced bladder cancer (ABC) meta-analysis collaboration. *Eur Urol.* 48(2): 202-5.

99. Sherif A, Holmberg L, Rintala E, *et al* (2004). Neoadjuvant cisplatinum based combination chemotherapy in patients with invasive bladder cancer: a combined analysis of two Nordic studies. *Eur Urol.* 45(3): 297-303.

100. Nguyen TT, Huillard O, Dabi Y, *et al* (2018). Neoadjuvant chemotherapy in patients with muscle-invasive bladder cancer and its impact on surgical morbidity and oncological outcomes: a real-world experience. *Front Surg.* 5: 58.

101. Black PC, Brown GA, Grossman HB, *et al* (2006). Neoadjuvant chemotherapy for bladder cancer. *World J Urol.* 24(5): 531-42.

102. Rosenblatt R, Sherif A, Rintala e, *et al* (2012). Pathologic downstaging is a surrogate marker for efficacy and increased survival following neoadjuvant chemotherapy and radical cystectomy for muscle-invasice urothelial bladdr cancer. *Eur Urol.* 61(6): 1229-38.

103. Zargar H, Espiritu PN, Fairey AS, *et al* (2015). Multicenter assessment of neoadjuvant chemotherapy for muscle-invasive bladder cancer. *Eur Urol.* 67(2): 241-9

104. Sánchez-Ortiz RF, Huang WC, Mick R, *et al* (2003). An interval longer than 12 weeks between the diagnosis of muscle invasion and cystectomy is associated with worse outcome in bladder carcinoma. *J Urol.* 169(1): 110-5.

105. Stöckle M, Meyenburg W, Wellek S, *et al* (1992). Advanced bladder cancer (stages pT3b, pT4a, pN1 and pN2): improved survival after radical cystectomy and 3 adjuvant cycles of chemotherapy. Results of a controlled prospective study. *J Urol.* 148: 302-7.

106. Cognetti F, Ruggeri EM, Felici A, *et al* (2012). Adjuvant chemotherapy with cisplatin and gemcitabine versus chemotherapy at relapse in patients with muscle-invasive bladder cancer submitted to radical cystectomy: an Italian, multicenter, randomized phase III trial. *Ann Oncol.* 23(3): 695-700.

107. Skinner DG, Daniels JR, Russell CA, *et al* (1991). The role of adjuvant chemotherapy following cystectomy for invasive bladder cancer: a prospective comparative trial. *J Urol.* 145(3): 459-67.

108. von der Maase H, Sengelov L, Roberts JT, *et al* (2005). Long-term survival results of a randomized trial comparing gemcitabine plus cisplatin, with methotrexate, vinblastine, doxorubicin, plus cisplatin in patients with bladder cancer. *J Clin Oncol.* 23(21): 4602-8.

109. Prout GR, Griffin PP, and Shipley WU (1979). Bladder carcinoma as a systemic disease. *Cancer.* 43(6): 2532-9.

110. Sternberg CN, de Mulder PH, Schornagel JH, *et al* (2001). Randomized phase III trial of high-dose-intensity methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) chemotherapy and recombinant human granulocyte colony-stimulating factor versus classic MVAC in advanced urothelial tract tumors: European Organization for Research and Treatment of Cancer Protocol no. 30924. *J Clin Oncol.* 19(10): 2638-46.

111. Merseburger AS, Apolo AB, Chowdhury S, *et al* (2018). SIU-ICUD recommendations on bladder cancer: systemic therapy for metastatic bladder cancer. *World J Urol.* 37(1): 95-105.

112. Bellmunt J, Mottet N, and De Santis M (2016). Urothelial carcinoma management in elderly or unfit patients. *EJC Suppl.* 14(1): 1-20.

113. Galsky MD, Pal SK, Lin SW, et al (2018). Real-world effectiveness of chemotherapy in elderly patients with metastatic bladder cancer in the united states. *Bladder Cancer.* 4(2): 227-38.

114. Sharma P and Allison JP (2015). Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell.* 161(2): 205-14.

115. Farina MS, Lundgren KT, and Bellmunt J (2017). Immunotherapy in urothelial cancer: recent results and future perspectives. 77(10): 1077-89.

116. Wei SC, Duffy CR, and Allison JP (2018). Fundamental Mechanisms of Immune Checkpoint Blockade Therapy. *Cancer Discov.* 8(9): 1069-86.

117. Stenehjem DD, Tran D, Nkrumah MA, *et al* (2018). PD1/PDL1 inhibitors for the treatment of advanced urothelial bladder cancer. *Onco Targets Ther.* 11: 5973-89.

118. Pinto IG (2017). Systemic therapy in bladder cancer. *Indian J Urol.* 33(2): 118-26.

119. Zibelman M, Ramamurthy C, and Plimack ER (2016). Emerging role of immunotherapy in urothelial carcinoma-Advanced disease. *Urol Oncol.* 34(12): 538-47.

120. Kim J (2016). Immune checkpoint blockade therapy for bladder cancer treatment. *Investig Clin Urol.* 57: 98-105.

121. Bellmunt J, de Wit R, Vaughn DJ, *et al* (2017). Pembrolizumab as second-line therapy for advanced urothelial carcinoma. *N Engl J Med.* 376(11): 1015-26.

122. Kim HS and Seo HK (2018). Immune checkpoint inhibitors for urothelial carcinoma. *Investig Clin Urol.* 59(5): 285-96.

123. Mendiratta P and Grivas P (2018). Emerging biomarkers and targeted therapies in urothelial carcinoma. *Ann Transl Med.* 6(12): 250.

124. Bellmunt J (2017). Antiangiogenesis to curb urothelial cancer. *Lancet.* 390(10109): 2220-1.

125. Soria F, Krabbe LM, Todenhöfer T, *et al* (2018). Molecular markers in bladder cancer. *World J Urol.* 37(1) 31-40.

126. Amin MB, Trpkov K, Lopez-Beltran A, *et al* (2014). Best practices recommendations in the application of immunohistochemistry in the bladder lesions: report from the International Society of Urologic Pathology consensus conference. *Am J Surg Pathol.* 38(8): e20-34.

127. Botteman MF, Pashos CL, Hauser RS, *et al* (2003). Quality of life aspects of bladder cancer: a review of the literature. *Qual Life Res.* 12(6): 675-88

128. van Rhijn BW, Catto JW, Goebell PJ, *et al* (2014). Molecular markers for urothelial bladder cancer prognosis: toward implementation in clinical practice. *Urol Oncol.* 32(7): 1078-87.

129. Chou R, Gore JL, Buckley D, *et al* (2015). Urinary biomarkers for diagnosis of bladder cancer: a systematic review and meta-analysis. *Ann Intern Med.* 163(12): 922-31.

130. Kojima T, Kawai K, Miyazaki J, *et al* (2017) Biomarkers for precision medicine in bladder cancer. *Int J Clin Oncol.* 22(2): 207-13.

131. Brismar J and Gustafson T (1988). Bone scintigraphy in staging of bladder carcinoma. *Acta Radiol.* 29(2): 251-2.

132. Habuchi T, Marberger M, Droller MJ, *et al* (2005) Prognostic markers for bladder cancer: International Consensus

Panel on bladder tumor markers. *Urology*. 66: 64-74.

133. Jeon C, Kim M, Kwak C, *et al* (2013). Prognostic role of survivin in bladder cancer: a systematic review and meta-analysis. PLoS One. 8(10):e76719.

134. Bellmunt J, Paz-Ares L, Cuello M, *et al* (2007). Gene expression of ERCC1 as a novel prognostic marker in advanced bladder cancer patients receiving cisplatin-based chemotherapy. *Ann Oncol.* 18(3) :522-8.

135. Plimack ER, Dunbrack RL, Brennan TA, *et al* (2015) Defects in DNA repair genes predict response to neoadjuvant cisplatin-based chemotherapy in muscle-invasive bladder cancer. *Eur Urol.* 68(6): 959-67.

136. Nakanishi J, Wada Y, Matsumoto K, *et al* (2007). Overexpression of B7-H1 (PD-L1) significantly associates with tumor grade and postoperative prognosis in human urothelial cancers. *Cancer Immunol Immunother.* 56(8): 1173-82.

137. Boorjian SA, Sheinin Y, Crispen PL, *et al* (2008). T-cell coregulatory molecule expression in urothelial cell carcinoma: clinicopathologic correlations and association with survival. *Clin Cancer Res.* 14(15): 4800-8.

138. Balkwill F and Mantovani A (2001). Inflammation and cancer: back to Virchow? *Lancet.* 357(9255): 539-45.

139. Hanahan D and Weinberg RA (2011). Hallmarks of cancer: the next generation. *Cell.* 144(5): 646-74.

140. Grivennikov SI, Greten FR, and Karin M (2010). Immunity, inflammation, and cancer. *Cell.* 140(6): 883-99.

141. Mantovani A, Allavena P, Sica A, *et al.* (2008). Cancer-related inflammation. *Nature.* 454(7203): 436-44.

142. Del Prete A, Allavena P, Santoro G, F *et al* (2011). Molecular pathways in cancer-related inflammation. *Biochem Med*. 21(3): 264-75.

143. Bindea G, Mlecnik B, Fridman WH, *et al* (2010). Natural immunity to cancer in humans. *Curr Opin Immunol.* 22(2): 215-22.

144. Coussens LM and Werb Z (2002). Inflammation and cancer. *Nature*. 420(6917): 860-7.

145. Wang D, Wang H, Brown J, *et al* (2006). CXCL1 induced by prostaglandin E2 promotes angiogenesis in colorectal cancer. *J Exp Med*. 203(4): 941-51.

146. Soucek L, Lawlor ER, Soto D, *et al* (2007). Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. *Nat Med.* 13(10): 1211-8.

147. Balkwill F, Charles KA, and Mantovani A (2005). Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell.* 7(3): 211-7.

148. Kobielak A and Fuchs E (2006). Links between alpha-catenin, NF-kappaB, and squamous cell carcinoma in skin. *Proc Natl Acad Sci USA.* 103(7): 2322-7.

149. Reiner SL (2007). Development in motion: helper T cells at work. *Cell.* 129(1): 33-6.

150. Ruffell B, DeNardo DG, Affara NI, *et al* (2010). Lymphocytes in cancer development: polarization towards pro-tumor immunity. *Cytokine Growth Factor Rev*. 21(1): 3-10.

151. Chiossone L, Dumas PY, Vienne M, *et al* (2018). Natural killer cells and other innate lymphoid cells in cancer. *Nat Rev Immunol.* 18(11): 671-88

152. Lieberman J (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol.* 3(5): 361-70.

153. Fridman WH, Pagès F, Sautès-Fridman C, *et al* (2012). The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer.* 12(4):298-306.

154. Zhou L, Chong MM, and Littman DR (2009). Plasticity of CD4+ T cell lineage differentiation. *Immunity.* 30(5): 646-55.

155. Huang Y, Ma C, Zhang Q, *et al* (2015). CD4+ and CD8+ T cells have opposing roles in breast cancer progression and outcome. *Oncotarget.* 6(19): 17462-78.

156. Lorvik KB, Hammarström C, Fauskanger M, *et al* (2016) Adoptive Transfer of Tumor-Specific Th2 Cells Eradicates Tumors by Triggering an In Situ Inflammatory Immune Response. *Cancer Res.* 76(23): 6864-76.

157. Chraa D, Naim A, Olive D, *et al* (2018) T lymphocyte subsets in cancer immunity: Friends or foes. *J Leukoc Biol.* 105(2): 243-55.

158. DeNardo DG, Andreu P, and Coussens LM (2010). Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. *Cancer Metastasis Rev.* 29(2): 309-16.

159. Fehérvari Z and Sakaguchi S (2004). CD4+ Tregs and immune control. *J Clin Invest.* 114(9): 1209-17.

160. Feuerer M, Hill JA, Mathis D, *et al* (2009). Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. Nat Immunol.10(7): 689-95.

161. Cao X, Cai SF, Fehniger TA, *et al* (2007). Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance. *Immunity.* 27(4): 635-46.

162. Stephen B and Hajjar J (2018). Overview of Basic Immunology and Translational Relevance for Clinical Investigators. *Adv Exp Med Biol.* 995:1-41.

163. Galdiero MR, Bonavita E, Barajon I, *et al* (2013). Tumor associated macrophages and neutrophils in cancer. *Immunobiology.* 218(11): 1402-10.

164. Pikarsky E, Porat RM, Stein I, *et al* (2004). NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature.* 431(7007): 461-6.

165. Greten FR, Eckmann L, Greten TF, *et al* (2004). IKKbeta links inflammation and tumorigenesis in a mouse model of colitis- associated cancer. *Cell.* 118(3): 285-96.

166. Yang L (2010). TGFbeta and cancer metastasis: an inflammation link. *Cancer Metastasis Rev.* 29(2): 263-71.

167. Vesely MD, Kershaw MH, Schreiber RD, *et al* (2011). Natural innate and adaptive immunity to cancer. *Annu Rev Immunol.* 29: 235-71.

168. Dunn GP, Bruce AT, Ikeda H, *et al* (2002). Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol.* 3(11): 991-8.

169. Pagès F, Galon J, Dieu-Nosjean MC, *et al* (2010). Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene.* 29(8): 1093-102.

170. Sukari A, Nagasaka M, Al-Hadidi A, *et al* (2016). Cancer Immunology and Immunotherapy. *Anticancer Res.* 36(11): 5593-606.

171. Dunn GP, Old LJ, and Schreiber RD (2004). The immunobiology of cancer

immunosurveillance and immunoediting. *Immunity*. 21(2): 137-48.

172. Kim R, Emi M, and Tanabe K (2007). Cancer immunoediting from immune surveillance to immune escape. *Immunology*. 121(1): 1-14.

173. Voena C and Chiarle R (2016). Advances in cancer immunology and cancer immunotherapy. *Discov Med.* 21(114): 125-33.

174. Ferradini L, Mackensen A, Genevée C, *et al* (1993). Analysis of T cell receptor variability in tumor-infiltrating lymphocytes from a human regressive melanoma. Evidence for in situ T cell clonal expansion. *J Clin Invest.* 91(3): 1183-90.

175. Zorn E and Hercend T (1999). A natural cytotoxic T cell response in a spontaneously regressing human melanoma targets a neoantigen resulting from a somatic point mutation. *Eur J Immunol.* 29(2): 592-601.

176. Pagès F, Berger A, Camus M, *et al* (2005). Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med*. 353(25): 2654-66.

177. Galon J, Costes A, Sanchez-Cabo F, *et al* (2006). Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* 313(5795): 1960-4.

178. Weckermann D, Müller P, Wawroschek F, *et al* (2001) Disseminated cytokeratin positive tumor cells in the bone marrow of patients with prostate cancer: detection and prognostic value. *J Urol.* 166(2): 699-703.

179. Karrison TG, Ferguson DJ, and Meier P (1999). Dormancy of mammary carcinoma after mastectomy. *J Natl Cancer Inst.* 91(1): 80-5.

180. Callaway MP and Briggs JC (1989). The incidence of late recurrence (greater than 10 years); an analysis of 536 consecutive cases of cutaneous melanoma. *Br J Plast Surg.* 42(1): 46-9.

181. Kauffman HM, McBride MA, Cherikh WS (2002). Transplant tumor registry: donors with central nervous system tumors. *Transplantation.* 73(4): 579-82.

182. Duan H (2018). Novel Therapeutic Strategies for Solid Tumor Based on Body's Intrinsic Antitumor Immune System. *Cell Physiol Biochem.* 47(2): 441-57.

183. Chambers CA, Kuhns MS, Egen JG, *et al* (2001). CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. *Annu Rev Immunol.* 19: 565-94.

184. Allard B, Aspeslagh S, Garaud S, *et al* (2018). Immuno-oncology-101: overview of major concepts and translational perspectives. *Semin Cancer Biol.* 52: 1-11.

185. Chen DS and Mellman I (2013). Oncology meets immunology: the cancer-immunity cycle. *Immunity.* 39(1): 1-10.

186. Joyce JA and Pollard JW (2009). Microenvironmental regulation of metastasis. *Nat Rev Cancer.* 9(4): 239-52.

187. Biswas SK and Mantovani A (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol.* 11(10): 889-96.

188. Pollard JW (2004). Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer.* 4(1): 71-8.

189. Gordon S and Taylor PR (2005). Monocyte and macrophage heterogeneity. *Nat Rev Immunol.* 5(12): 953-64.

190. Mosser DM and Edwards JP (2008). Exploring the full spectrum of macrophage activation. *Nat Rev Immunol.* 8(12): 958-69.

191. Gordon S, Plüddemann A, Martinez *et al* (2014). Macrophage heterogeneity in tissues: phenotypic diversity and functions. *Immunol Rev.* 262(1): 36-55.

192. Mantovani A, Sozzani S, Locati M, *et al* (2002). Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol.* 23(11): 549-55.

193. Sica A, Schioppa T, Mantovani A, *et al* (2006). Tumour-associated macrophages are a distinct M2 polarised population promoting tumour progression: potential targets of anti-cancer therapy. *Eur J Cancer.* 42(6): 717-27.

194. Gordon S (2003). Alternative activation of macrophages. *Nat Rev Immunol.* 3(1): 23-35.

195. Ponzoni M, Pastorino F, Di Paolo D, *et al* (2018) Targeting Macrophages as a potential therapeutic intervention: impact on inflammatory diseases and cancer. *Int J Mol Sci.* 19(7).

196. Sica A, Erreni M, Allavena P, *et al* (2015). Macrophage polarization in pathology. *Cell Mol Life Sci.* 72(21): 4111-26.

197. Bingle L, Brown NJ, and Lewis CE (2002). The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol.* 196(3): 254-65.

198. Qian BZ and Pollard JW (2010). Macrophage diversity enhances tumor progression and metastasis. *Cell.* 141(1): 39-51.

199. Mantovani A and Sica A (2010). Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Curr Opin Immunol.* 22(2): 231-7.

200. Allavena P, Sica A, Garlanda C, *et al* (2008). The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunol Rev.* 222: 155-61.

201. Sica A, Larghi P, Mancino A, *et al* (2008). Macrophage polarization in tumour progression. *Semin Cancer Biol.* 18(5): 349-55.

202. Biswas SK, Gangi L, Paul S, *et al* (2006). A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood.* 107(5): 2112-22.

203. Biswas SK, Sica A, and Lewis CE (2008). Plasticity of macrophage function during tumor progression: regulation by distinct molecular mechanisms. *J Immunol.* 180(4): 2011-7.

204. Lewis CE and Pollard JW (2006). Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 66(2): 605-12.

205. Pollard JW (2008). Macrophages define the invasive microenvironment in breast cancer. *J Leukoc Biol.* 84(3): 623-30.

206. Wyckoff JB, Wang Y, Lin EY, *et al* (2007). Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res.* 67(6): 2649-56.

207. Movahedi K, Laoui D, Gysemans C, *et al* (2010). Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Res.* 70(14): 5728-39.

208. Lin C, He H, Liu H, *et al* (2019). Tumour-associated macrophages-derived CXCL8 determines immune evasion through autonomous PD-L1 expression in gastric cancer. *Gut*. Epub ahead of print.

209. Qian B, Deng Y, Im JH, *et al* (2009). A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS One*. 4(8): e6562

210. Argyle D and Kitamura T (2018). Targeting macrophage-recruiting chemokines as a novel therapeutic strategy to prevent the progression of solid tumors. *Front Immunol*. 9: 2629.

211. Kitamura T, Qian BZ, Soong D, *et al* (2015). CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. *J Exp Med*. 212(7): 1043-59.

212. Cassetta L and Pollard JW (2018). Targeting macrophages: therapeutic approaches in cancer. *Nat Rev Drug Discov*. Epub ahead of print.

213. Wyckoff J, Wang W, Lin EY, *et al* (2004). A paracrine loop between tumor cells and macrophages is required for tumor cell migration in mammary tumors. *Cancer Res*. 64(19): 7022-9.

214. Leek RD, Talks KL, Pezzella F, *et al* (2002). Relation of hypoxia-inducible factor-2 alpha (HIF-2 alpha) expression in tumor-infiltrative macrophages to tumor angiogenesis and the oxidative thymidine phosphorylase pathway in Human breast cancer. *Cancer Res*. 62(5): 1326-9.

215. Mantovani A, Schioppa T, Porta C, *et al* (2006). Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev*. 25(3): 315-22.

216. Duluc D, Corvaisier M, Blanchard S, *et al* (2009). Interferon-gamma reverses the immunosuppressive and protumoral properties and prevents the generation of human tumor-associated macrophages. *Int J Cancer*. 125(2): 367-73.

217. Sica A and Bronte V (2007). Altered macrophage differentiation and immune dysfunction in tumor development. *J Clin Invest*. 117(5): 1155-66.

218. Chen JJ, Lin YC, Yao PL, *et al* (2005). Tumor-associated macrophages: the double-edged sword in cancer progression. *J Clin Oncol*. 23(5): 953-64.

219. Ryder M, Ghossein RA, Ricarte-Filho JC, *et al* (2008). Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer. *Endocr Relat Cancer*. 15(4): 1069-74.

220. Zhu XD, Zhang JB, Zhuang PY, *et al* (2008). High expression of macrophage colony-stimulating factor in peritumoral liver tissue is associated with poor survival after curative resection of hepatocellular carcinoma. *J Clin Oncol*. 26(16): 2707-16.

221. Murdoch C, Muthana M, Coffelt SB, *et al* (2008). The role of myeloid cells in the promotion of tumour angiogenesis. *Nat Rev Cancer*. 8(8): 618-31.

222. Buddingh EP, Kuijjer ML, Duim RA, *et al* (2011). Tumor-infiltrating macrophages are associated with metastasis suppression in high-grade osteosarcoma: a rationale for treatment with macrophage activating agents. *Clin Cancer Res*. 17(8): 2110-9.

223. Ohno S, Inagawa H, Dhar DK, *et al* (2003). The degree of macrophage infiltration into the cancer cell nest is a significant predictor of survival in gastric cancer patients. *Anticancer Res*. 23(6D): 5015-22.

224. Algars A, Irjala H, Vaittinen *et al* (2012). Type and location of tumor-infiltrating macrophages and lymphatic vessels predict survival of colorectal cancer patients. *Int J Cancer*. 131(4): 864-73.

225. Erreni M, Mantovani A, and Allavena P (2011). Tumor-associated Macrophages (TAM) and Inflammation in Colorectal Cancer. *Cancer Microenviron*. 4(2): 141-54.

226. Sica A and Mantovani A (2012). Macrophage plasticity and polarization: in vivo veritas. *J Clin Invest*. 122(3): 787-95.

227. Correia AL and Bissell MJ (2012). The tumor microenvironment is a dominant force in multidrug resistance. *Drug Resist Updat*. 15(1-2): 39-49.

228. Hughes R, Qian BZ, Rowan C, *et al* (2015). Perivascular M2 Macrophages Stimulate Tumor Relapse after Chemotherapy. *Cancer Res*. 75(17): 3479-91.

229. Gordon SR, Maute RL, Dulken BW, *et al* (2017). PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature*. 545(7655): 495-9.

230. Muraoka D, Seo N, Hayashi T, *et al* (2019). Antigen delivery targeted to tumor-associated macrophages overcomes tumor immune resistance. *J Clin Invest*. 1(129): 1278-1294.

231. Hartley GP, Chow L, Ammons DT, *et al* (2018). Programmed Cell Death Ligand 1 (PD-L1) Signaling Regulates Macrophage Proliferation and Activation. *Cancer Immunol Res*. 6(10): 1260-73.

232. Hartley G, Regan D, Guth A, *et al* (2017). Regulation of PD-L1 expression on murine tumor-associated monocytes and macrophages by locally produced TNF-α. *Cancer Immunol Immunother*. 66(4): 523-35.

233. Herbst RS, Soria JC, Kowanetz M, *et al* (2014). Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature*. 515(7528): 563-7.

234. Hamilton JA (2008). Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol*. 8(7): 533-44.

235. Van Acker HH, Anguille S, Willemen Y, *et al* (2016). Bisphosphonates for cancer treatment: Mechanisms of action and lessons from clinical trials. *Pharmacol Ther*. 158: 24-40.

236. Liu J, Wang L, Zhao F, *et al* (2015). Pre-Clinical Development of a Humanized Anti-CD47 Antibody with Anti-Cancer Therapeutic Potential. *PLoS One*. 10(9): e0137345.

237. Gholamin S, Mitra SS, Feroze AH, *et al* (2017). Disrupting the CD47-SIRPα anti-phagocytic axis by a humanized anti-CD47 antibody is an efficacious treatment for malignant pediatric brain tumors. *Sci Transl Med*. 9(381).

238. Wang Y, Lin YX, Qiao SL, *et al* (2017). Polymeric nanoparticles promote macrophage reversal from M2 to M1 phenotypes in the tumor microenvironment. *Biomaterials*. 112: 153-63.

239. Kaneda MM, Messer KS, Ralainirina N, *et al* (2016). PI3Kγ is a molecular switch that controls immune suppression. *Nature*. 539(7629): 437-42.

240. Tang J, Shalabi A, and Hubbard-Lucey VM (2018). Comprehensive analysis of the clinical immuno-oncology landscape. *Ann Oncol*. 29(1): 84-91.

241. Sun L, Chen L, and Li H (2019). Checkpoint-modulating immunotherapies in tumor treatment: Targets, drugs, and

mechanisms. *Int Immunopharmacol.* 67: 160-75.

242. Koury J, Lucero M, Cato C, *et al* (2018). Immunotherapies: exploiting the immune system for cancer treatment. *J Immunol Res.* 2018: 9585614.

243. Mellman I, Coukos G, and Dranoff G (2011). Cancer immunotherapy comes of age. *Nature.* 480(7378): 480-9.

244. Allison JP (2015). Checkpoints. *Cell.* 162(6): 1202-5.

245. Tivol EA, Borriello F, Schweitzer AN, *et al* (1995). Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity.* 3(5): 541-7.

246. Hodi FS, O'Day SJ, McDermott DF, *et al* (2010). Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med.* 363(8): 711-23.

247. Maker AV, Phan GQ, Attia P, *et al* (2005). Tumor regression and autoimmunity in patients treated with cytotoxic T lymphocyte-associated antigen 4 blockade and interleukin 2: a phase I/II study. *Ann Surg Oncol.* 12(12): 1005-16.

248. Iwai Y, Hamanishi J, Chamoto K, *et al* (2017). Cancer immunotherapies targeting the PD-1 signaling pathway. *J Biomed Sci.* 24(1): 26.

249. Chen L and Han X (2015). Anti-PD-1/PD-L1 therapy of human cancer: past, present, and future. *J Clin Invest.* 125(9): 3384-91.

250. Marin-Acevedo JA, Soyano AE, Dholaria B, *et al* (2018). Cancer immunotherapy beyond immune checkpoint inhibitors. *J Hematol Oncol.* 11(1): 8.

251. Callahan MK, Postow MA, and Wolchok JD (2014). CTLA-4 and PD-1 Pathway Blockade: Combinations in the Clinic. *Front Oncol.* 4: 385.

252. Pardoll DM (2012). The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer.* 12(4): 252-64.

253. Sui X, Lei L, Chen L, *et al* (2017). Inflammatory microenvironment in the initiation and progression of bladder cancer. *Oncotarget.* 8(54): 93279-94.

254. Nesi G, Nobili S, Cai T, *et al* (2015). Chronic inflammation in urothelial bladder cancer. *Virchows Arch.* 467(6): 623-33.

255. Bernardo C, Cunha MC, Santos JH, *et al* (2016). Insight into the molecular basis of Schistosoma haematobium-induced bladder cancer through urine proteomics. *Tumour Biol.* 37(8): 11279-87.

256. Humans IWGotEoCRt (2012). Biological agents. Volume 100 B. A review of human carcinogens. *IARC Monogr Eval Carcinog Risks Hum.* 100(Pt B): 1-441.

257. Honeycutt J, Hammam O, Hsieh MH (2015). Schistosoma haematobium egg-induced bladder urothelial abnormalities dependent on p53 are modulated by host sex. *Exp Parasitol.* 158: 55-60.

258. Thompson DB, Siref LE, Feloney MP, *et al* (2015). Immunological basis in the pathogenesis and treatment of bladder cancer. *Expert Rev Clin Immunol.*11(2): 265-79.

259. Cai T, Nesi G, Boddi V, *et al* (2006). Prognostic role of the tumor-associated tissue inflammatory reaction in transitional bladder cell carcinoma. *Oncol Rep.* 16(2): 329-34.

260. Offersen BV, Knap MM, Marcussen N, *et al* (2002). Intense inflammation in bladder carcinoma is associated with angiogenesis and indicates

good prognosis. *Br J Cancer*. 87(12): 1422-30.

261. Foth M, Ismail NFB, Kung JSC, *et al* (2018). FGFR3 mutation increases bladder tumourigenesis by suppressing acute inflammation. *J Pathol*. 246(3): 331-43.

262. Hodgson A, Xu B, Satkunasivam R (2018). Tumour front inflammation and necrosis are independent prognostic predictors in high-grade urothelial carcinoma of the bladder. *J Clin Pathol*. 71(2): 154-60.

263. Masson-Lecomte A, Rava M, Real FX, *et al* (2014). Inflammatory biomarkers and bladder cancer prognosis: a systematic review. *Eur Urol*. 66(6): 1078-91.

264. Dai J, Tang K, Xiao W, *et al* (2014). Prognostic significance of C-reactive protein in urological cancers: a systematic review and meta-analysis. *Asian Pac J Cancer Prev*. 15(8): 3369-75.

265. Mbeutcha A, Shariat SF, Rieken M, *et al* (2016). Prognostic significance of markers of systemic inflammatory response in patients with non-muscle-invasive bladder cancer. *Urol Oncol*. 34(11): 483.e17-.e24.

266. Saito K and Kihara K (2013). Role of C-reactive protein in urological cancers: a useful biomarker for predicting outcomes. *Int J Urol*. 20(2): 161-71.

267. Bugan B and Onar LC (2013). Is the neutrophil-to-lymphocyte ratio a crucial indicator of systemic inflammation, coronary artery ectasia, and atherogenesis? *Angiology*. 64(8): 636.

268. Wei Y, Jiang YZ, and Qian WH (2014). Prognostic role of NLR in urinary cancers: a meta-analysis. *PLoS One*. 9(3): e92079.

269. Yakovlev P and Klyushin D (2018). Lymphocyte count in peripheral blood is a sensitive tool in pretreatment assessment of patients with urological cancer. *Exp Oncol*. 40(2): 119-23.

270. Kaiser J, Li H, North SA, *et al* (2018). The Prognostic role of the change in neutrophil-to-lymphocyte ratio during neoadjuvant chemotherapy in patients with muscle-invasive bladder cancer: a retrospective, multi-institutional study. *Bladder Cancer*. 4(2): 185-94.

271. Mano R, Baniel J, Shoshany O, *et al* (2015). Neutrophil-to-lymphocyte ratio predicts progression and recurrence of non-muscle-invasive bladder cancer. *Urol Oncol*. 33(2): 67.e1-7.

272. Marchioni M, Primiceri G, Ingrosso M, *et al* (2016). The clinical use of the neutrophil to lymphocyte ratio (NLR) in urothelial cancer: a systematic review. *Clin Genitourin Cancer*. 14(6): 473-84.

273. Potretzke A, Hillman L, Wong K, *et al* (2014). NLR is predictive of upstaging at the time of radical cystectomy for patients with urothelial carcinoma of the bladder. *Urol Oncol*. 32(5): 631-6.

274. D'Andrea D, Moschini M, Gust KM, *et al* (2017). Lymphocyte-to-monocyte ratio and neutrophil-to-lymphocyte ratio as biomarkers for predicting lymph node metastasis and survival in patients treated with radical cystectomy. *J Surg Oncol*. 115(4): 455-61.

275. Bhindi B, Hermanns T, Wei Y, *et al* (2016). Identification of the best complete blood count-based predictors for bladder cancer outcomes in patients undergoing radical cystectomy. *Br J Cancer*. 114(2): 207-12.

276. Seah JA, Leibowitz-Amit R, Atenafu EG, *et al* (2015). Neutrophil-lymphocyte ratio and pathological response to neoadjuvant chemotherapy in patients with muscle-invasive bladder

cancer. *Clin Genitourin Cancer.* 13(4): e229-33.

277. Liakou CI, Narayanan S, Ng Tang D, *et al* (2007). Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human bladder cancer. *Cancer Immun.* 7: 10.

278. Winerdal ME, Marits P, Winerdal M, *et al* (2011). FOXP3 and survival in urinary bladder cancer. *BJU Int.* 108(10): 1672-8.

279. Shang B, Liu Y, and Jiang SJ (2015). Prognostic value of tumor-infiltrating FoxP3+ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep.* 5: 15179.

280. Winerdal ME, Krantz D, Hartana CA, *et al* (2018). Urinary bladder cancer Tregs suppress MMP2 and potentially regulate invasiveness. *Cancer Immunol Res.* 6(5): 528-38.

281. Murai R, Itoh Y, Kageyama S, *et al* (2018). Prediction of intravesical recurrence of non-muscle-invasive bladder cancer by evaluation of intratumoral Foxp3+ T cells in the primary transurethral resection of bladder tumor specimens. *PLoS One.* 13(9): e0204745.

282. Bettelli E, Carrier Y, Gao W, *et al* (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* 441(7090): 235-8.

283. Schepisi G, Santoni M, Massari F, *et al* (2016). Urothelial cancer: inflammatory mediators and implications for immunotherapy. *BioDrugs.* 30(4): 263-73.

284. Chen MF, Lin PY, Wu CF, *et al* (2013). IL-6 expression regulates tumorigenicity and correlates with prognosis in bladder cancer. *PLoS One.* 8(4): e61901.

285. Thompson LF, Ruedi JM, Glass A, *et al* (1989). Antibodies to 5'-nucleotidase (CD73), a glycosyl-phosphatidylinositol-anchored protein, cause human peripheral blood T cells to proliferate. *J Immunol.* 143(6): 1815-21.

286. Degoricija M, Situm M, Korać J, *et al* (2014). High NF-κB and STAT3 activity in human urothelial carcinoma: a pilot study. *World J Urol.* 32(6): 1469-75.

287. Muthuswamy R, Wang L, Pitteroff J, *et al* (2015). Combination of IFNα and poly-I:C reprograms bladder cancer microenvironment for enhanced CTL attraction. *J Immunother Cancer.* 3: 6.

288. Urquidi V, Chang M, Dai Y, *et al* (2012). IL-8 as a urinary biomarker for the detection of bladder cancer. *BMC Urol.* 12: 12.

289. Mora-Bau G, Platt AM, van Rooijen N, *et al* (2015). Macrophages subvert adaptive immunity to urinary tract infection. *PLoS Pathog.* 11(7): e1005044.

290. Schiwon M, Weisheit C, Franken L, *et al* (2014). Crosstalk between sentinel and helper macrophages permits neutrophil migration into infected uroepithelium. *Cell.* 156(3): 456-68.

291. Ayari C, LaRue H, Hovington H, *et al* (2009). Bladder tumor infiltrating mature dendritic cells and macrophages as predictors of response to bacillus Calmette-Guérin immunotherapy. *Eur Urol.* 55(6): 1386-95.

292. Takayama H, Nishimura K, Tsujimura A, *et al* (2009). Increased infiltration of tumor associated macrophages is associated with poor prognosis of bladder carcinoma in situ after intravesical bacillus Calmette-Guerin instillation. *J Urol.* 181(4): 1894-900.

293. Hanada T, Nakagawa M, Emoto A, *et al* (2000). Prognostic value of tumor-

associated macrophage count in human bladder cancer. *Int J Urol.* 7(7): 263-9.

294. Wu SQ, Xu R, Li XF, *et al* (2018). Prognostic roles of tumor associated macrophages in bladder cancer: a system review and meta-analysis. *Oncotarget.* 9(38): 25294-303.

295. Sjödahl G, Lövgren K, Lauss M, *et al* (2014). Infiltration of $CD3^+$ and $CD68^+$ cells in bladder cancer is subtype specific and affects the outcome of patients with muscle-invasive tumors. *Urol Oncol.* 32(6): 791-7.

296. Ayari C, LaRue H, Hovington H, *et al* (2013). High level of mature tumor-infiltrating dendritic cells predicts progression to muscle invasion in bladder cancer. *Hum Pathol.* 44(8): 1630-7.

297. Dufresne M, Dumas G, Asselin E, *et al* (2011). Pro-inflammatory type-1 and anti-inflammatory type-2 macrophages differentially modulate cell survival and invasion of human bladder carcinoma T24 cells. *Mol Immunol.* 48(12-13): 1556-67.

298. Luo Y, Yamada H, Evanoff DP, *et al* (2006). Role of Th1-stimulating cytokines in bacillus Calmette-Guérin (BCG)-induced macrophage cytotoxicity against mouse bladder cancer MBT-2 cells. *Clin Exp Immunol.* 146(1): 181-8.

299. Bartoletti R, Cai T, Nesi G, *et al* (2005). Qualitative and quantitative analysis of angiogenetic factors in transitional cell bladder carcinoma: relationship with clinical course at 10 years follow-up. *Oncol Rep.* 14(1): 251-5.

300. Luo Y, Han R, Evanoff DP, *et al* (2010). Interleukin-10 inhibits Mycobacterium bovis bacillus Calmette-Guérin (BCG)-induced macrophage cytotoxicity against bladder cancer cells. *Clin Exp Immunol.* 160(3): 359-68.

301. Morales A, Eidinger D, and Bruce AW (1976). Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. *J Urol.* 116(2): 180-3.

302. Herr HW and Morales A (2008). History of bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story. *J Urol.* 179(1): 53-6.

303. Hamerman JA and Aderem A (2001). Functional transitions in macrophages during in vivo infection with Mycobacterium bovis bacillus Calmette-Guérin. *J Immunol.* 167(4): 2227-33.

304. Lima L, Oliveira D, Tavares A, *et al* (2014). The predominance of M2-polarized macrophages in the stroma of low-hypoxic bladder tumors is associated with BCG immunotherapy failure. *Urol Oncol.* 32(4): 449-57.

305. Bellmunt J, Powles T, and Vogelzang NJ (2017). A review on the evolution of PD-1/PD-L1 immunotherapy for bladder cancer: The future is now. *Cancer Treat Rev.* 54: 58-67.

306. Inman BA, Sebo TJ, Frigola X, *et al* (2007). PD-L1 (B7-H1) expression by urothelial carcinoma of the bladder and BCG-induced granulomata: associations with localized stage progression. *Cancer.* 109(8): 1499-505.

307. Sherif A, Hasan MN, Marits P, *et al* (2010). Feasibility of T-cell-based adoptive immunotherapy in the first 12 patients with advanced urothelial urinary bladder cancer. Preliminary data on a new immunologic treatment based on the sentinel node concept. *Eur Urol.* 58(1): 105-11.

308. Sherif A, Hasan MN, Redecka E, *et al* (2015). Pilot study of adoptive immunotherapy with sentinel node-derived T cells in muscle-invasive urinary

bladder cancer. *Scand J Urol.* 49(6): 453-62.

309. Dudley ME, Wunderlich JR, Robbins PF, *et al* (2002). Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science.*298(5594): 850-4.

310. Antony PA, Piccirillo CA, Akpinarli A, *et al* (2005). CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells. *J Immunol.* 174(5): 2591-601.

311. Tang X (2013). Tumor-associated macrophages as potential diagnostic and prognostic biomarkers in breast cancer. *Cancer Lett.* 332(1): 3-10.

312. Holness CL and Simmons DL (1993). Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. *Blood.* 81(6): 1607-13.

313. Krpina K, Babarović E, Španjol J, *et al* (2016). Correlation of tumor-associated macrophages and NK cells with bladder cancer size and T stage in patients with solitary low-grade urothelial carcinoma. *Wien Klin Wochenschr.* 128(7-8): 248-52.

314. Wang B, Liu H, Dong X, *et al* (2015). High CD204+ tumor-infiltrating macrophage density predicts a poor prognosis in patients with urothelial cell carcinoma of the bladder. *Oncotarget.* 6(24): 20204-14.

315. Pichler R, Fritz J, Zavadil C, *et al* (2016). Tumor-infiltrating immune cell subpopulations influence the oncologic outcome after intravesical Bacillus Calmette-Guérin therapy in bladder cancer. *Oncotarget.* 7(26): 39916-30.

316. Tungekar MF, Heryet A, and Gatter KC (1991). The L1 antigen and squamous metaplasia in the bladder. *Histopathology.* 19(3): 245-50.

317. Loftus B, Loh LC, Curran B, *et al* (1991). Mac387: its non-specificity as a tumour marker or marker of histiocytes. *Histopathology.* 19(3): 251-5.

318. Flavell DJ, Jones DB, and Wright DH (1987). Identification of tissue histiocytes on paraffin sections by a new monoclonal antibody. *J Histochem Cytochem.* 35(11): 1217-26.

319. Brandtzaeg P, Jones DB, Flavell DJ, *et al* (1988). Mac 387 antibody and detection of formalin resistant myelomonocytic L1 antigen. *J Clin Pathol.* 41(9): 963-70.

320. Donato R (2003). Intracellular and extracellular roles of S100 proteins. *Microsc Res Tech.* 60(6): 540-51.

321. Yao R, Davidson DD, Lopez-Beltran A, *et al* (2007). The S100 proteins for screening and prognostic grading of bladder cancer. *Histol Histopathol.* 22(9): 1025-32.

322. Goebeler M, Roth J, Teigelkamp S, *et al* (1994). The monoclonal antibody MAC387 detects an epitope on the calcium-binding protein MRP14. *J Leukoc Biol.* 55(2): 259-61.

323. Hessian PA and Fisher L (2001). The heterodimeric complex of MRP-8 (S100A8) and MRP-14 (S100A9). Antibody recognition, epitope definition and the implications for structure. *Eur J Biochem.* 268(2): 353-63.

324. Soulas C, Conerly C, Kim WK, *et al* (2011). Recently infiltrating MAC387(+) monocytes/macrophages a third macrophage population involved in SIV and HIV encephalitic lesion formation. *Am J Pathol.* 178(5): 2121-35.

325. Arai K, Takano S, Teratani T, *et al* (2008). S100A8 and S100A9

overexpression is associated with poor pathological parameters in invasive ductal carcinoma of the breast. *Curr Cancer Drug Targets.* 8(4): 243-52.

326. Gebhardt C, Németh J, Angel P, *et al* (2006). S100A8 and S100A9 in inflammation and cancer. *Biochem Pharmacol.* 72(11): 1622-31.

327. Poston RN and Hussain IF (1993). The immunohistochemical heterogeneity of atheroma macrophages: comparison with lymphoid tissues suggests that recently blood-derived macrophages can be distinguished from longer-resident cells. *J Histochem Cytochem.* 41(10): 1503-12.

328. Otani I, Mori K, Sata T, *et al* (1999). Accumulation of MAC387+ macrophages in paracortical areas of lymph nodes in rhesus monkeys acutely infected with simian immunodeficiency virus. *Microbes Infect.* 1(12): 977-85.

329. Huang W, Williamson SR, Rao Q, *et al* (2013). Novel markers of squamous differentiation in the urinary bladder. *Hum Pathol.* 44(10): 1989-97.

330. Lopez-Beltran A, Requena MJ, Alvarez-Kindelan J, *et al* (2007). Squamous differentiation in primary urothelial carcinoma of the urinary tract as seen by MAC387 immunohistochemistry. *J Clin Pathol.* 60(3): 332-5.

331. Ehdaie B, Maschino A, Shariat SF, *et al* (2012). Comparative outcomes of pure squamous cell carcinoma and urothelial carcinoma with squamous differentiation in patients treated with radical cystectomy. *J Urol.* 187(1): 74-9.

332. Mukhtar RA, Moore AP, Tandon VJ, *et al* (2012). Elevated levels of proliferating and recently migrated tumor-associated macrophages confer increased aggressiveness and worse outcomes in breast cancer. *Ann Surg Oncol.* 19(12): 3979-86.

333. McKiernan E, McDermott EW, Evoy D, *et al* (2011). The role of S100 genes in breast cancer progression. *Tumour Biol.* 32(3): 441-50.

334. Subimerb C, Pinlaor S, Khuntikeo N, *et al* (2010). Tissue invasive macrophage density is correlated with prognosis in cholangiocarcinoma. *Mol Med Rep.* 3(4): 597-605.

335. Tolson JP, Flad T, Gnau V, *et al* (2006). Differential detection of S100A8 in transitional cell carcinoma of the bladder by pair wise tissue proteomic and immunohistochemical analysis. *Proteomics.* 6(2): 697-708.

336. Pilchowski R, Stöhr R, von Eggeling F, *et al* (2011). Specific protein patterns characterize metastatic potential of advanced bladder cancer. *J Urol.* 186(2): 713-20.

337. Ebbing J, Mathia S, Seibert FS, *et al* (2014). Urinary calprotectin: a new diagnostic marker in urothelial carcinoma of the bladder. *World J Urol.* 32(6): 1485-92.

338. Kim WJ, Kim SK, Jeong P, *et al* (2011). A four-gene signature predicts disease progression in muscle invasive bladder cancer. *Mol Med.* 17(5-6): 478-85.

339. Irjala H, Elima K, Johansson EL, *et al* (2003). The same endothelial receptor controls lymphocyte traffic both in vascular and lymphatic vessels. *Eur J Immunol.* 33(3): 815-24.

340. Goerdt S, Walsh LJ, Murphy GF, *et al* (1991). Identification of a novel high molecular weight protein preferentially expressed by sinusoidal endothelial cells in normal human tissues. *J Cell Biol.* 113(6): 1425-37.

341. Kzhyshkowska J, Gratchev A, and Goerdt S (2006). Stabilin-1, a homeostatic scavenger receptor with multiple functions. *J Cell Mol Med.* 10(3): 635-49.

342. Adachi H and Tsujimoto M (2002). FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities. *J Biol Chem.* 277(37): 34264-70.

343. Salmi M, Koskinen K, Henttinen T, *et al* (2004). CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. *Blood.* 104(13): 3849-57.

344. Patten DA and Shetty S (2018). More than just a removal service: scavenger receptors in leukocyte trafficking. *Front Immunol.* 9:2904.

345. Kzhyshkowska J (2010). Multifunctional receptor stabilin-1 in homeostasis and disease. *Scientific World Journal.* 10: 2039-53.

346. Canton J, Neculai D, and Grinstein S (2013). Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol.* 13(9): 621-34.

347. Kzhyshkowska J, Workman G, Cardó-Vila M, *et al* (2006). Novel function of alternatively activated macrophages: stabilin-1-mediated clearance of SPARC. *J Immunol.* 176(10): 5825-32.

348. Salmi M and Jalkanen S (1992). A 90-kilodalton endothelial cell molecule mediating lymphocyte binding in humans. *Science.* 257(5075): 1407-9.

349. Riabov V, Yin S, Song B, *et al* (2016). Stabilin-1 is expressed in human breast cancer and supports tumor growth in mammary adenocarcinoma mouse model. *Oncotarget.* 7(21): 31097-110.

350. Karikoski M, Marttila-Ichihara F, Elima K, *et al* (2014). Clever-1/stabilin-1 controls cancer growth and metastasis. *Clin Cancer Res.* 20(24): 6452-64.

351. Irjala H, Alanen K, Grénman R, *et al* (2003). Mannose receptor (MR) and common lymphatic endothelial and vascular endothelial receptor (CLEVER)-1 direct the binding of cancer cells to the lymph vessel endothelium. *Cancer Res.* 63(15): 4671-6.

352. Ammar A, Mohammed RA, Salmi M, *et al* (2011). Lymphatic expression of CLEVER-1 in breast cancer and its relationship with lymph node metastasis. *Anal Cell Pathol (Amst).* 34(1-2):67-78.

353. Mitrofanova I, Zavyalova M, Telegina N, *et al* (2017). Tumor-associated macrophages in human breast cancer parenchyma negatively correlate with lymphatic metastasis after neoadjuvant chemotherapy. *Immunobiology.* 222(1): 101-9.

354. Buldakov M, Zavyalova M, Krakhmal N, *et al* (2017). CD68+, but not stabilin-1+ tumor associated macrophages in gaps of ductal tumor structures negatively correlate with the lymphatic metastasis in human breast cancer. *Immunobiology.* 222(1): 31-8.

355. Géraud C, Mogler C, Runge A, E *et al* (2013). Endothelial transdifferentiation in hepatocellular carcinoma: loss of Stabilin-2 expression in peri-tumourous liver correlates with increased survival. *Liver Int.* 33(9): 1428-40.

356. David C, Nance JP, Hubbard J, *et al* (2012). Stabilin-1 expression in tumor associated macrophages. *Brain Res.* 1481: 71-8.

357. Brekken RA, Puolakkainen P, Graves DC, *et al* (2003). Enhanced growth of tumors in SPARC null mice is associated with changes in the ECM. *J Clin Invest.* 111(4): 487-95.

358. Chlenski A, Guerrero LJ, Peddinti R, *et al* (2010). Anti-angiogenic SPARC peptides inhibit progression of neuroblastoma tumors. *Mol Cancer.* 9: 138.

359. Dhanesuan N, Sharp JA, Blick T, *et al* (2002). Doxycycline-inducible expression of SPARC/Osteonectin/BM40 in MDA-MB-231 human breast cancer cells results in growth inhibition. *Breast Cancer Res Treat.* 75(1): 73-85.

360. Koblinski JE, Kaplan-Singer BR, VanOsdol SJ, *et al* (2005). Endogenous osteonectin/SPARC/BM-40 expression inhibits MDA-MB-231 breast cancer cell metastasis. *Cancer Res.* 65(16): 7370-7.

361. Nagai MA, Gerhard R, Fregnani JH, *et al* (2011). Prognostic value of NDRG1 and SPARC protein expression in breast cancer patients. *Breast Cancer Res Treat.* 126(1): 1-14.

362. Golembieski WA, Ge S, Nelson K, *et al* (1999). Increased SPARC expression promotes U87 glioblastoma invasion in vitro. *Int J Dev Neurosci.* 17(5-6): 463-72.

363. Yegutkin GG (2008). Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta.* 1783(5): 673-94.

364. Zimmermann H (1992). 5'-Nucleotidase: molecular structure and functional aspects. *Biochem J.* 285(Pt 2): 345-65.

365. Thomson LF, Ruedi JM, Glass A, *et al* (1990). Production and characterization of monoclonal antibodies to the glycosyl phosphatidylinositol-anchored lymphocyte differentiation antigen ecto-5'-nucleotidase (CD73). *Tissue Antigens.* 35(1): 9-19.

366. Airas L, Hellman J, Salmi M, *et al.* (1995). CD73 is involved in lymphocyte binding to the endothelium: characterization of lymphocyte-vascular adhesion protein 2 identifies it as CD73. *J Exp Med.* 182(5): 1603-8.

367. Faas MM, Sáez T, and de Vos P (2017) Extracellular ATP and adenosine: The Yin and Yang in immune responses? *Mol Aspects Med.* 55:9-19.

368. Hechler B and Gachet C (2015). Purinergic Receptors in Thrombosis and Inflammation. *Arterioscler Thromb Vasc Biol.* 35(11): 2307-15.

369. Eltzschig HK, Thompson LF, Karhausen J, *et al* (2004). Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination by extracellular nucleotide metabolism. *Blood.* 104(13): 3986-92.

370. Yegutkin GG, Marttila-Ichihara F, Karikoski M, *et al* (2011). Altered purinergic signaling in CD73-deficient mice inhibits tumor progression. *Eur J Immunol.* 41(5): 1231-41.

371. Thompson LF, Eltzschig HK, Ibla JC, *et al* (2004). Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med.* 200(11): 1395-405.

372. Linden J (2001). Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol.* 41: 775-87.

373. Madara JL, Patapoff TW, Gillece-Castro B, *et al* (1993) 5'-adenosine monophosphate is the neutrophil-derived paracrine factor that elicits chloride secretion from T84 intestinal epithelial cell monolayers. *J Clin Invest.* 91(5): 2320-5.

374. Lennon PF, Taylor CT, Stahl GL, *et al* (1998) Neutrophil-derived 5'-adenosine monophosphate promotes endothelial barrier function via CD73-mediated conversion to adenosine and endothelial

A2B receptor activation. *J Exp Med.* 188(8): 1433-43.

375. Olah ME and Stiles GL (1995). Adenosine receptor subtypes: characterization and therapeutic regulation. *Annu Rev Pharmacol Toxicol.* 35: 581-606.

376. Sitkovsky MV and Ohta A (2005). The 'danger' sensors that STOP the immune response: the A2 adenosine receptors? *Trends Immunol.* 26(6): 299-304.

377. Jacobson KA and Gao ZG (2006). Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov.* 5(3): 247-64.

378. Sitkovsky M and Lukashev D (2005). Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. *Nat Rev Immunol.* 5(9): 712-21.

379. Salmi M and Jalkanen S (2011). Homing-associated molecules CD73 and VAP-1 as targets to prevent harmful inflammations and cancer spread. *FEBS Lett.* 585(11): 1543-50.

380. Ohta A, Gorelik E, Prasad SJ, *et al* (2006). A2A adenosine receptor protects tumors from antitumor T cells. *Proc Natl Acad Sci USA*. 103(35): 13132-7.

381. Yamashita Y, Hooker SW, Jiang H, *et al.* (1998). CD73 expression and fyn-dependent signaling on murine lymphocytes. *Eur J Immunol.* 28(10): 2981-90.

382. Airas L, Niemelä J, Salmi M, *et al* (1997). Differential regulation and function of CD73, a glycosyl-phosphatidylinositol-linked 70-kD adhesion molecule, on lymphocytes and endothelial cells. *J Cell Biol.* 136(2): 421-31.

383. Koszalka P, Ozüyaman B, Huo Y, *et al* (2004). Targeted disruption of cd73/ecto-5'-nucleotidase alters thromboregulation and augments vascular inflammatory response. *Circ Res.* 95(8): 814-21.

384. Niemelä J, Henttinen T, Yegutkin GG, *et al* (2004). IFN-alpha induced adenosine production on the endothelium: a mechanism mediated by CD73 (ecto-5'-nucleotidase) up-regulation. *J Immunol.* 172(3): 1646-53.

385. Kiss J, Yegutkin GG, Koskinen K, *et al* (2007). IFN-beta protects from vascular leakage via up-regulation of CD73. *Eur J Immunol.* 37(12): 3334-8.

386. Synnestvedt K, Furuta GT, Comerford KM, *et al* (2002). Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. *J Clin Invest.* 110(7): 993-1002.

387. Spychala J and Kitajewski J (2004). Wnt and beta-catenin signaling target the expression of ecto-5'-nucleotidase and increase extracellular adenosine generation. *Exp Cell Res.* 296(2): 99-108.

388. Savic V, Stefanovic V, Ardaillou N, *et al* (1990). Induction of ecto-5'-nucleotidase of rat cultured mesangial cells by interleukin-1 beta and tumour necrosis factor-alpha. *Immunology.* 70(3): 321-6.

389. Christensen LD, Andersen V, Nygaard P, *et al* (1992). Effects of immunomodulators on ecto-5'-nucleotidase activity on blood mononuclear cells in vitro. *Scand J Immunol.* 35(4): 407-13.

390. Yegutkin GG, Henttinen T, Samburski SS, *et al.* (2002). The evidence for two opposite, ATP-generating and ATP-consuming, extracellular pathways on endothelial and lymphoid cells. *Biochem J.* 367(Pt1): 121-8.

391. Airas L, Niemelä J, and Jalkanen S (2000). CD73 engagement promotes

lymphocyte binding to endothelial cells via a lymphocyte function-associated antigen-1-dependent mechanism. *J Immunol.* 165(10): 5411-7.

392. Henttinen T, Jalkanen S, and Yegutkin GG (2003). Adherent leukocytes prevent adenosine formation and impair endothelial barrier function by Ecto-5'-nucleotidase/CD73-dependent mechanism. *J Biol Chem.* 278(27): 24888-95.

393. Takedachi M, Qu D, Ebisuno Y, *et al* (2008). CD73-generated adenosine restricts lymphocyte migration into draining lymph nodes. *J Immunol.* 180(9): 6288-96.

394. Airas L, Salmi M, and Jalkanen S (1993). Lymphocyte-vascular adhesion protein-2 is a novel 70-kDa molecule involved in lymphocyte adhesion to vascular endothelium. *J Immunol.* 151(8): 4228-38.

395. Arvilommi AM, Salmi M, Airas L, *et al* (1997). CD73 mediates lymphocyte binding to vascular endothelium in inflamed human skin. *Eur J Immunol.* 27(1): 248-54.

396. Zernecke A, Bidzhekov K, Ozüyaman B, *et al* (2006). CD73/ecto-5'-nucleotidase protects against vascular inflammation and neointima formation. *Circulation.* 113(17): 2120-7.

397. Colgan SP, Eltzschig HK, Eckle T, *et al* (2006). Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal.* 2(2): 351-60.

398. Thompson LF, Ruedi JM, O'Connor RD, *et al* (1986). Ecto-5'-nucleotidase expression during human B cell development. An explanation for the heterogeneity in B lymphocyte ecto-5'-nucleotidase activity in patients with hypogammaglobulinemia. *J Immunol.* 137(8): 2496-500.

399. Bastian JF, Ruedi JM, MacPherson GA, *et al* (1984). Lymphocyte ecto-5'-nucleotidase activity in infancy: increasing activity in peripheral blood B cells precedes their ability to synthesize IgG in vitro. *J Immunol.* 132(4): 1767-72.

400. Edwards NL, Gelfand EW, Burk L, *et al* (1979). Distribution of 5'-nucleotidase in human lymphoid tissues. *Proc Natl Acad Sci USA.* 76(7): 3474-6.

401. Stagg J and Smyth MJ (2010). Extracellular adenosine triphosphate and adenosine in cancer. *Oncogene.* 29(39): 5346-58.

402. Spychala J (2000). Tumor-promoting functions of adenosine. *Pharmacol Ther.* 87(2-3): 161-73.

403. Jin D, Fan J, Wang L, *et al* (2010). CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression. *Cancer Res.* 70(6): 2245-55.

404. Gao ZW, Dong K, and Zhang HZ (2014). The roles of CD73 in cancer. *Biomed Res Int.* 2014: 460654.

405. Gutensohn W and Thiel E (1990). Prognostic implication of ecto-5'-nucleotidase activity in acute lymphoblastic leukemia. *Cancer.* 66(8): 1755-8.

406. Leth-Larsen R, Lund R, Hansen HV, *et al* (2009). Metastasis-related plasma membrane proteins of human breast cancer cells identified by comparative quantitative mass spectrometry. *Mol Cell Proteomics.* 8(6): 1436-49.

407. Stagg J, Divisekera U, Duret H, *et al* (2011). CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. *Cancer Res.* 71(8): 2892-900.

408. Stagg J, Divisekera U, McLaughlin N, *et al* (2010). Anti-CD73 antibody

therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci USA.* 107(4): 1547-52.

409. Zhou X, Zhi X, Zhou P, *et al* (2007). Effects of ecto-5'-nucleotidase on human breast cancer cell growth in vitro and in vivo. *Oncol Rep.* 17(6): 1341-6.

410. Wang L, Zhou X, Zhou T, *et al* (2008). Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. *J Cancer Res Clin Oncol.* 134(3): 365-72.

411. Zhang B (2010). CD73: a novel target for cancer immunotherapy. *Cancer Res.* 70(16): 6407-11.

412. Zhang B (2012). Opportunities and challenges for anti-CD73 cancer therapy. *Immunotherapy.* 4(9): 861-5.

413. Gordon S and Martinez FO (2010). Alternative activation of macrophages: mechanism and functions. *Immunity.* 32(5): 593-604.

414. Nishikawa H and Sakaguchi S (2010). Regulatory T cells in tumor immunity. *Int J Cancer.* 127(4): 759-67.

415. Shankaran V, Ikeda H, Bruce AT, *et al* (2001) IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature.* 410(6832): 1107-11.

416. Blay J, White TD, and Hoskin DW (1997). The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. *Cancer Res.* 57(13): 2602-5.

417. Haskó G, Pacher P, Deitch EA, *et al* (2007) Shaping of monocyte and macrophage function by adenosine receptors. *Pharmacol Ther.* 113(2): 264-75.

418. Csóka B, Selmeczy Z, Koscsó B, *et al* (2012). Adenosine promotes alternative macrophage activation via A2A and A2B receptors. *FASEB J.* 26(1): 376-86.

419. Beavis PA, Milenkovski N, Henderson MA, *et al* (2015) Adenosine Receptor 2A Blockade Increases the Efficacy of Anti-PD-1 through Enhanced Antitumor T-cell Responses. *Cancer Immunol Res.* 3(5): 506-17.

420. Loi S, Pommey S, Haibe-Kains B, *et al* (2013). CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. *Proc Natl Acad Sci USA.* 110(27): 11091-6.

421. Stella J, Bavaresco L, Braganhol E, *et al* (2010). Differential ectonucleotidase expression in human bladder cancer cell lines. *Urol Oncol.* 28(3):260-7.

422. Rockenbach L, Braganhol E, Dietrich F, *et al* (2014). NTPDase3 and ecto-5'-nucleotidase/CD73 are differentially expressed during mouse bladder cancer progression. *Purinergic Signal.* 10(3): 421-30.

423. Durak I, Perk H, Kavutçu M, *et al* (1994). Adenosine deaminase, 5'nucleotidase, xanthine oxidase, superoxide dismutase, and catalase activities in cancerous and noncancerous human bladder tissues. *Free Radic Biol Med.* 16(6): 825-31.

424. Wettstein MS, Buser L, Hermanns T, *et al* (2015). CD73 predicts favorable prognosis in patients with nonmuscle-Invasive urothelial bladder cancer. *Dis Markers.* 2015: 785461.

425. Oh HK, Sin JI, Choi J, *et al* (2012) Overexpression of CD73 in epithelial ovarian carcinoma is associated with better prognosis, lower stage, better differentiation and lower regulatory T cell infiltration. *J Gynecol Oncol.* 23(4): 274-81.

426. Supernat A, Markiewicz A, Welnicka-Jaskiewicz M, *et al* (2012). CD73

expression as a potential marker of good prognosis in breast carcinoma. *Appl Immunohistochem Mol Morphol.* 20(2): 103-7.

427. Soleimani A, Taghizadeh E, Shahsavari S, *et al* (2019) CD73; a key ectonucleotidase in the development of breast cancer: Recent advances and perspectives. *J Cell Physiol.* Epub ahead of print.

428. Sobin H, Gospodariwicz MK, Wittekind C (eds). TNM classification of malignant tumors. UICC International Union Against Cancer, 7th ed, Wiley-Blackwell (2009).

429. Palani S, Maksimow M, Miiluniemi M, *et al* (2011). Stabilin-1/CLEVER-1, a type 2 macrophage marker, is an adhesion and scavenging molecule on human placental macrophages. *Eur J Immunol.* 41(7): 2052-63.

430. Shabo I, Stål O, Olsson H, *et al* (2008). Breast cancer expression of CD163, a macrophage scavenger receptor, is related to early distant recurrence and reduced patient survival. *Int J Cancer.* 123(4): 780-6.

431. Rosevear HM, Lightfoot AJ, O'Donnell MA, *et al* (2009). The role of neutrophils and TNF-related apoptosis-inducing ligand (TRAIL) in bacillus Calmette-Guérin (BCG) immunotherapy for urothelial carcinoma of the bladder. *Cancer Metastasis Rev.* 28(3-4): 345-53.

432. Lima L, Dinis-Ribeiro M, Longatto-Filho A, *et al* (2012). Predictive biomarkers of bacillus calmette-guérin immunotherapy response in bladder cancer: where are we now? *Adv Urol.* 2012: 232609.

433. Choi W, Czerniak B, Ochoa A, *et al* (2014). Intrinsic basal and luminal subtypes of muscle-invasive bladder cancer. *Nat Rev Urol.* 11(7): 400-10.

434. Ruffell B and Coussens LM (2015). Macrophages and therapeutic resistance in cancer. *Cancer Cell.* 27(4): 462-72.

435. Mantovani A and Allavena P (2015). The interaction of anticancer therapies with tumor-associated macrophages. *J Exp Med.* 212(4): 435-45.

436. De Palma M and Lewis CE (2013). Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell.* 23(3): 277-86.

437. Idzko M, Ferrari D, and Eltzschig HK (2014). Nucleotide signalling during inflammation. *Nature.* 509(7500): 310-7.

438. Ålgars A, Karikoski M, Yegutkin GG, *et al* (2011). Different role of CD73 in leukocyte trafficking via blood and lymph vessels. *Blood.* 117(16): 4387-93.

439. Leclerc BG, Charlebois R, Chouinard G, *et al* (2016). CD73 expression is an independent prognostic factor in prostate cancer. *Clin Cancer Res.* 22(1): 158-66.

440. de Oliveira Bravo M, Carvalho JL, and Saldanha-Araujo F (2016). Adenosine production: a common path for mesenchymal stem-cell and regulatory T-cell-mediated immunosuppression. *Purinergic Signal.* 12(4): 595-609.

UNIVERSITY OF TURKU

ISBN 978-951-29-7742-0 (PRINT)
ISBN 978-951-29-7743-7 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)

Grano Oy - Turku, Finland 2019