Preclinical and 'near-patient' models for the evaluation of experimental therapy in prostate and bladder cancer

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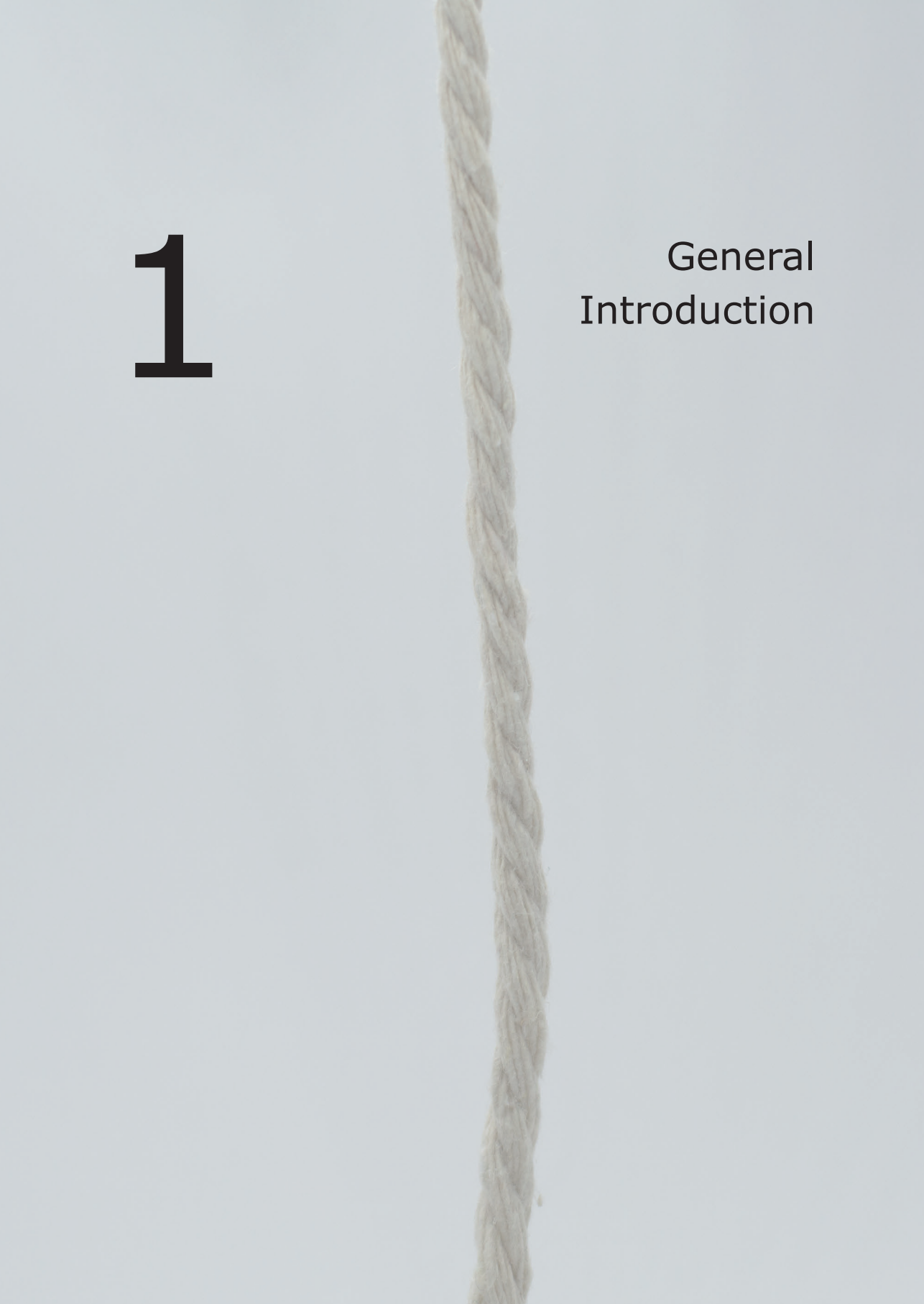
General Introduction



1. Cancer

Cancer is a pathological condition including the uncontrolled growth of cells. In addition, cancer cells have the ability to invade neighboring tissue and spread to distant sites. The word cancer is derived from the Greek 'karkinos' or 'καρκινος' meaning crab and refers to the similarity between the shape of a tumor and veins and the shape of a crab.

Cancer is a major clinical burden and a leading cause of mortality. Every year, 18.1 million of new cancer cases are diagnosed and 9.6 million patients die from cancer worldwide (1). Lung cancer, prostate cancer and colorectal cancer represent the most frequent cancer types in males. In females, breast cancer, colorectal cancer and lung cancer are the most commonly diagnosed cancer types (1). The majority of tumors originates from epithelial cells and is classified as carcinoma (2). The transformation of a normal epithelial cell in a cancer cell is a complex and multistep process and is initiated by the acquisition of (epi)genetic alterations. It has been estimated that only 10% of all cancers is induced by inherited germline mutations whereas 90% of all cancers can be linked to acquired sporadic mutations. Sporadic mutations are acquired during life by environmental exposures (i.e. smoking, infections, exposure to ionizing radiation and life style factors), or develop spontaneously due to errors in DNA replication (2). Genetic alterations in tumor suppressor genes and oncogenes, which in a physiological setting regulate processes like cell division, cell death and DNA repair, can promote the transformation of a normal cell into a malignant tumor cell (3).

Despite its complexity, cancers share common characteristics. These common characteristics include increased cell proliferation and limitless replicative potential, evading growth suppressors, resisting programmed cell death, induction of angiogenesis and tissue invasion and/or metastasis (4). More recently, dysregulation of cellular energetics, evasion of immune destruction, genomic instability and the presence of tumor-promoting inflammation have been identified as common characteristics of cancer (5).

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2. The prostate

2.1. Prostate function and anatomy

The word prostate is derived from the ancient Greek word ''prostátēs'' or προστάτης meaning ''leader'' and ''one standing in front'' (6). The prostate is the largest exocrine gland of the male reproductive system and is located in front of the rectum and inferior to the bladder. The function of the prostate is to secrete seminal fluid that nourishes sperm cells. During ejaculation, smooth muscle cells in the prostate and muscles in the pelvic floor contract, resulting in the expulsion of prostate fluid into the urethra.

The prostate can be anatomically divided into five lobes (i.e. the posterior lobe, the anterior lobe, right and left lateral lobes and median lobe), whereas pathologists divide the prostate in four zones (i.e. the peripheral, central, transitional and fibromuscular zone) (**Figure 1**). The peripheral zone spans 70% of the prostate tissue and includes the posterior part of the prostate. The majority of all prostate cancer cases arises in the peripheral zone. The central zone accounts for 20% of the prostate and spans the ejaculatory ducts. The transitional region spans the proximal urethra and accounts 5% of the prostate cancer gland. In this zone, benign prostate hyperplasia (BPH) primarily develops.

The prostate gland highly depends on testosterone in order to function properly (7, 8). Testosterone is the main male sex hormone and is primarily produced by the Leydig cells, and to a smaller extent in the adrenal gland. The production of testosterone is regulated by the hypothalamic-pituitary-gonadal axis. In the prostate, testosterone is converted in dihydrotestosterone (DHT) by 5-alpha reductase. Both testosterone and DHT can bind to the androgen receptor (AR). As a result, the AR will translocate to the nucleus and induces the expression androgen-responsive genes, including prostate-specific antigen (PSA) and NK3 Homeobox 1 (NKX3-1) (9, 10). Androgen-responsive genes stimulate prostate cell proliferation and inhibit prostate cell apoptosis (9).

Figure 1 Anatomy of the prostate.

The prostate is anatomically divided in the anterior, posterior, lateral and median, and anterior lobes. Pathologists divide the prostate in the peripheral, central, transitional and fibromuscular zone. The majority of all prostate cancers arises in the peripheral zone, whereas benign prostate hyperplasia arises in the transitional zone.

2.2. Histology of the prostate

Epithelial prostate cells are primarily arranged in columnar, acinar structures. In addition, smaller regions of squamous or pseudostratified epithelium can be detected in the prostate (11). The center of the prostate glands often contains corpora amylacea, which are formed after calcification of prostatic secretions (11). The cellular component of the prostatic stroma is composed of smooth muscle cells, fibroblasts and myofibroblasts (8).

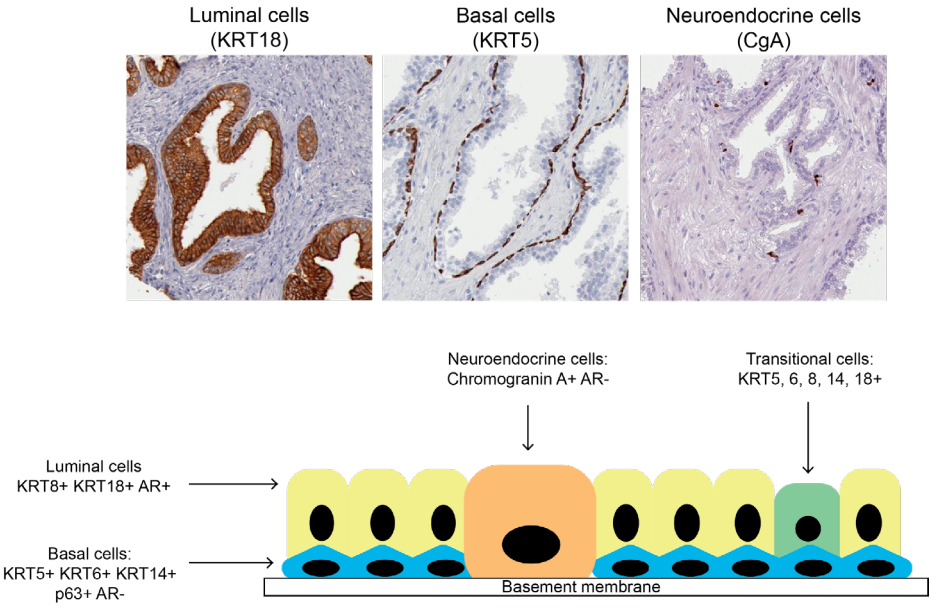

Figure 2 Epithelial subsets in prostate gland.

Epithelial cells in the prostate gland are highly organized and multiple epithelial subsets can be discriminated i.e. basal cells, luminal cells, transit amplifying cells and neuroendocrine cells. Each cell type is characterized by the expression of a unique set of markers. Legend: KRT=keratin, CgA=Chromogranin-A, AR=androgen receptor. Source histological images: Human Protein Atlas www.proteinatlas.org.

Different subsets of epithelial cells can be discriminated in the prostate, including luminal cells, basal cells and neuroendocrine cells (**Figure 2**) (8, 12). Luminal cells represent 60% of cells of the prostate. Luminal cells are differentiated cells that are characterized by a low proliferative index and their dependence on androgens (13). Luminal cells secrete PSA, prostatic acid phosphatase (PAP) and fibrolysin and express high levels of keratin-8 and -18 and the AR (12). Basal cells compose approximately 40% of the prostate epithelium (13). Basal cells rest on the basal membrane, have a flattened shape and are relatively undifferentiated. Basal cells have a high proliferative index, express low levels of AR and are androgen-independent. Basal cells in the prostate gland are characterized by the high expression of keratin-5 and -14 and low levels of keratin-8 and -18 (12). Transit amplifying cells represent an intermediate stage and co-express both basal and luminal markers. In addition to luminal, basal and transit amplifying cells, neuroendocrine cells include a very rare cell type of the prostate epithelium (1%) (14).

Neuroendocrine cells are androgen-insensitive cells and express high levels of specific neuroendocrine markers e.g. chromogranin A (CgA) and synaptophysin (SYP) (8, 12).

3. Prostate cancer

3.1. The clinical problem of prostate cancer

Prostate cancer is the second most commonly diagnosed cancer type in men and represents the fifth cause of cancer-related deaths in men (1). Every year, over 1.2 million men are diagnosed with prostate cancer worldwide (1). Symptoms of early-stage prostate cancer include problems and/or pain during urinating, erectile dysfunction and blood in the semen or urine. The major risk factor of developing prostate cancer is age. Other risk factors include family history, ethnicity and life style factors (15). It has been estimated that only 9% of all prostate cancer cases can be attributed to inherited mutations (16). Hereditary prostate cancer is characterized by an earlier age of onset and has been linked to mutations in *BRCA1* and *BRCA2* genes (17-20). Prostate tumors are generally slowly growing and remain confined to the prostate gland.

Hence, most prostate cancer patients die with prostate cancer rather than from prostate cancer. Patients with organ-confined prostate cancer are closely monitored by active surveillance, or actively treated by prostatectomy and/or radiotherapy. However, approximately 10-20% of all prostate tumors will recur after initial treatments (21-24). Subsequently, these patients are treated with androgen-deprivation therapy (ADT) (including luteinizing-hormone releasing agonists, luteinizing-hormone releasing antagonists, anti-androgens and new androgen pathway targeting agents), chemotherapy (e.g. taxanes including docetaxel and cabazitaxel) and/or radiation. However, the prostate tumors will inevitably become resistant to ADT. This phase is denoted as castration-resistant prostate cancer (CRPC) and as a result, patients will develop incurable distant metastases (21, 22). Once the cancer has spread beyond the prostate towards distant sites, treatments are mainly considered to be palliative (**Figure 3**). Current clinical problems of prostate cancer include the development of therapy resistance resulting in castration-resistant prostate cancer and the formation of distant metastases.

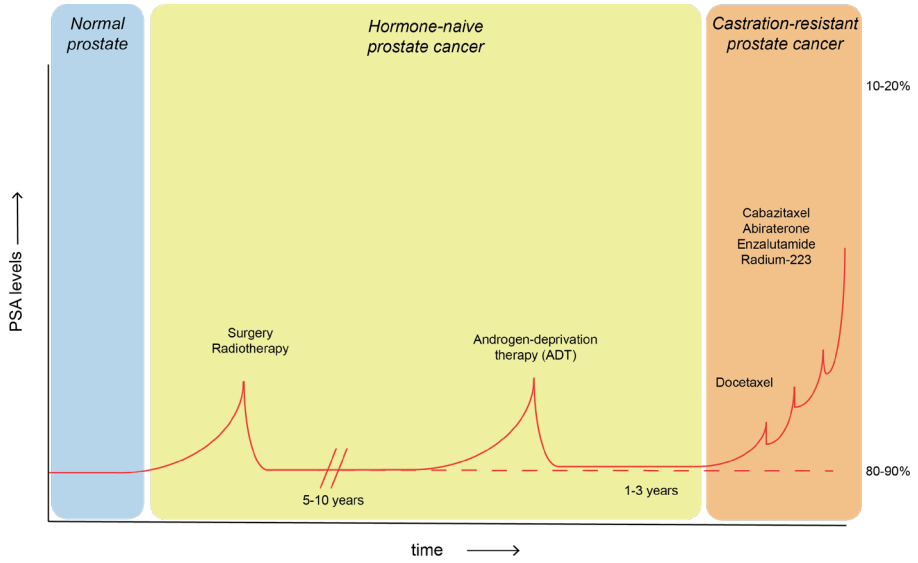

Figure 3 Prostate cancer progression.

Initial therapy options for localized prostate cancer include surgery and/or radiotherapy. The majority of patients (80-90%) will be cured after these initial treatments. However, in 10-20% of all the cases, the prostate cancer will relapse within 5-10 years. These patients are subsequently treated with androgen-deprivation therapy (ADT). Prostate tumors will initially respond to ADT. However, the tumor will eventually become resistant i.e. castration-resistant prostate cancer (CRPC). Despite the treatments with Docetaxel, Cabazitaxel and Enzalutamide, incurable distant metastases will develop.

3.2. Prostate cancer subtypes and genetic alterations

Prostate cancer carcinogenesis is a multistep process. Multiple genetic alterations have been involved in the transformation of a normal prostate cell into a prostate cancer cell. The majority of all prostate tumors arises from epithelial cells and are therefore classified as adenocarcinoma. Genetic alterations that are often detected in prostate adenocarcinoma include PTEN deletions, TMPRSS2:ERG fusion and alterations in the AR-pathway (25). PTEN or phosphatase and tensin homolog is a negative regulator of PI3K-mTOR signaling. It has been estimated that PTEN is deleted in approximately 20% of all primary prostate tumors and 40-60% of all advanced prostate tumors (26). Approximately half of all prostate tumors harbors the TMPRSS2:ERG fusion gene (27). During this genomic translocation, a truncated form of ERG is expressed under control of the promotor of prostate-specific gene TMPRSS2. In addition to PTEN deletions and TMPRSS2:ERG fusion, multiple alterations in the AR signaling axis have been characterized in prostate cancer tumors (9, 28).

Alterations in AR-signaling have been linked to prostate cancer progression and the development of CRPC and include splicing variants of the AR, increased intratumoral steroid production, AR overexpression, gene-amplifications and deregulated expression of AR-coregulators (9, 28). AR-v7 is an alternative splicing variant of the AR and is characterized by a truncated C-terminal ligand-binding domain and an active N-terminal domain (29). As a result, AR-v7 is constitutively active, resulting in a ligand-independent activation of AR-target genes. Research has revealed that the presence of AR-v7 in circulating tumor cells is associated with increased resistance to Abiraterone and Enzalutamide (29).

A small subset (<1%) of the prostate tumors originates from neuroendocrine cells and is classified as neuroendocrine prostate cancer (NEPC) (30, 31). Neuroendocrine prostate cancer is characterized by low PSA levels, the development of visceral metastases and a poor prognosis (32). *De novo* neuroendocrine-prostate cancer is characterized by AR loss, Rb deletion, N-Myc amplification and Aurora kinase A activation (30). Besides *de novo* development of neuroendocrine prostate cancer, neuroendocrine prostate cancer can arise after androgen-deprivation therapy, i.e. treatment-induced NEPC. Although the development of *de novo* NEPC is very rare, it has been estimated that 10-20% of all CRPC patients will develop treatment-induced NEPC (30).

3.3 Prostate cancer classification

In order to determine the most optimal treatment regimen for prostate cancer patients, multiple grading and staging systems are being used. TNM staging is a tool based on the size of the tumor (T), the extent to which the tumor has spread towards the lymph nodes (N) and presence of distant metastasis (M) (**Table 1**). The T stage can be established based on different diagnostic tools including digital rectal examination (clinical T stage(cT)) and histological evaluation of biopsy tissue (pathological T stage (pT)).

Table 1 TNM staging in prostate cancer (33).

Tumor (T): size of the tumor		
TX		Tumor size cannot be determined
T0		No evidence of primary tumor
T1		Tumor is present but is too small to be clinically detected
	T1a	Tumor is present in <5% of tissue resection
	T1b	Tumor is present in >5% of tissue resection
	T1c	Tumor is identified in needle biopsy after rise in PSA levels
T2		Tumor is palpable but confined within the prostate gland
	T2a	Tumor is located in half of one lobe
	T2b	Tumor is located in more than half of one lobe
	T2c	Tumor is located in both lobes
T3		Tumor has invaded the prostatic capsule
	T3a	Tumors has invaded extracapsular
	T3b	Tumor has invaded seminal vesicles
T4		Tumor has spread to other structures that seminal vesicles, e.g. bladder wall

Table 1 NM staging in prostate cancer (33) continued.

Node (N): Regional lymph node involvement		
Nx		Regional lymph nodes cannot be assessed
N0		No regional lymph node involvement
N1		Prostate cancer has spread to regional lymph nodes
Metastasis (M): The presence of distant metastasis		
M0		No distant metastasis
M1		The presence of distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s)

The Gleason grading system was developed by pathologist Donald Gleason and compares the microscopic appearance of a prostate cancer biopsy to normal prostate tissue (34, 35). In this way, the Gleason grading system assesses the aggressiveness of a prostate tumor and provides an estimation of the prognosis of a prostate cancer patient. The Gleason grade ranges from 1 to 5; a grade of 1 is used to describe well-differentiated normal prostate tissue, whereas a grade of 5 is used to describe poorly differentiated prostate cancer tissue. Normal prostate cells are classified as grade 1-2, whereas a Gleason grade of 3 or larger is used to describe malignant prostate cells. The Gleason score is the sum of the primary and the secondary grade, representing the first and second most commonly observed pattern in a prostate cancer biopsy. A higher Gleason score has been associated with aggressive disease and a poor prognosis (34, 36). Research has indicated that the clinical outcome of patients with a Gleason score 7 varies greatly (37, 38). Histological evaluation has revealed that several growth patterns can be discriminated within the Gleason score 7 subgroup, including ill-formed, fused, glomeruloid and cribriform growth (39-41).

Interestingly, especially the presence of cribriform growth within the Gleason score 7 was shown to correlate with a poor prognosis in prostate cancer patients (39, 40). Gleason grading combined with TNM staging represents the golden standard in prostate cancer diagnosis. However, to create a more uniform grading system compared to other cancer types, the International Society of Urological Pathology (ISUP) has developed an alternative grading system based on the Gleason score (**Table 2**) (37, 41).

Table 2 ISUP grading system.

ISUP grade	Gleason score
1	Gleason <6
2	Gleason 3+4 (=7)
3	Gleason 4+3 (=7)
4	Gleason 4+4 (=8)
5	Gleason 9-10

In addition to the TNM stage and the Gleason score/ISUP grade, the measurement of prostate-specific antigen (PSA) levels is a useful tool in the clinical diagnosis of prostate cancer (42). PSA or kallikrein-3 (KLK3) is a serine protease that is produced by prostate epithelial cells. Different conditions including prostatitis, benign prostate hyperplasia and prostate cancer are known to result in increased PSA-levels. Therefore, measuring PSA levels represents a screening tool for the detection of several prostatic diseases, including prostate cancer (43, 44). Moreover, PSA levels are regularly monitored in prostate cancer patients to detect biochemical recurrence after treatment.Based on the TNM stage, the Gleason score/ISUP grade and PSA levels, the European Association of Urology (EAU) has defined different prostate cancer risk groups (**Table 3**) (43, 45). Prostate cancer patients with PSA levels lower than 10 ng/ml, ISUP grade 1 and TNM stage of T1 or T2a are classified as low-risk prostate cancer. Patients with PSA levels of 10-20 ng/ml or ISUP grade 2/3 or TNM stage T2b have an intermediate risk of biochemical recurrence. In contrast, patients with a PSA of higher than 20 ng/ml or ISUP grade 4 or 5 or TNM stage of T2c or prostate cancer patients with a TNM stage of T3/T4 or lymph node involvement (i.e. N+) have a high risk of biochemical recurrence.

Table 3 EAU prostate cancer risk groups.

Risk group	PSA levels (ng/ml)	Gleason score (ISUP)	TNM	Clinical Stage
Low risk	< 10	And Gleason score < 7 (ISUP 1)	And T1-2a	Localized
Intermediate risk	10-20	Or Gleason score 7 (ISUP2/3)	Or T2b	
High risk	PSA >20	Or Gleason score >7 (ISUP4/5)	Or T2c	
	Any PSA	Any Gleason score (any ISUP)	T3-4 or N+	Locally Advanced

3.4. Prostate cancer stages

Besides the measurement of PSA levels, different tools are available for the diagnosis of prostate cancer including digital rectal examination (DRE), tumor biopsies and imaging (e.g. MRI, CT or bone scans). In addition, novel diagnostic tools and biological markers have become available, including detection of the TMPRSS2-ERG fusion gene and measuring PCA3 levels (43, 45). Based on the extent to which the tumor has spread beyond the prostate gland, different prostate cancer stages can be discriminated, including organ-confined, locally advanced and advanced prostate cancer (**Figure 4**).

3.4.1. Organ-confined or localized prostate cancer

In organ-confined or localized prostate cancer, the prostate cancer has not spread beyond the prostatic capsule and is confined to prostate gland. The majority of organ-confined prostate cancers is slowly growing. Treatment options for localized prostate cancer include active surveillance, surgery and radiotherapy (43-45). Active surveillance includes monitoring of a prostate cancer patient based on PSA levels, DRE, prostate biopsies and imaging. By postponing active treatment, active surveillance avoids unnecessary treatment and prevents side effects (43, 45).

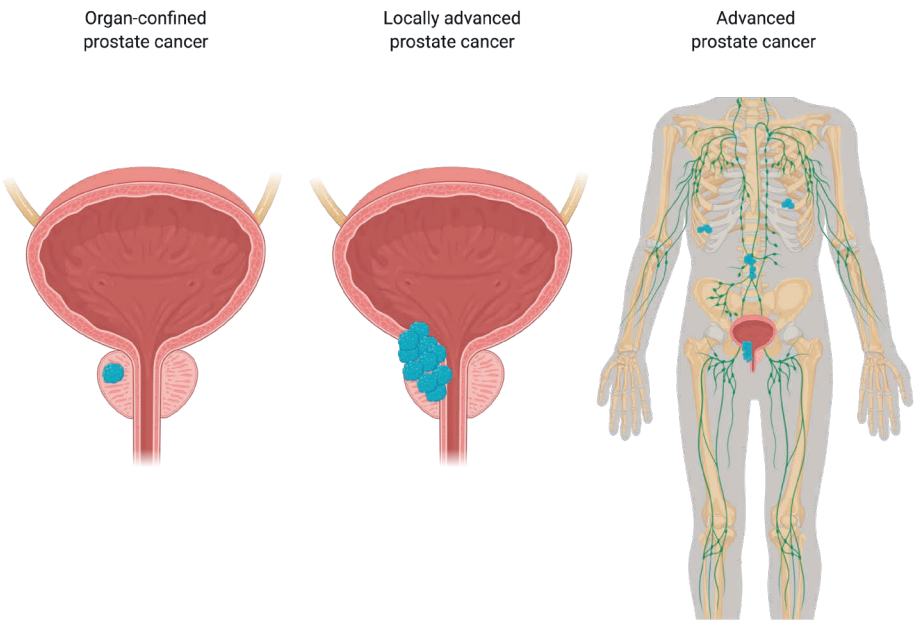

Figure 4 Prostate cancer stages.

Prostate cancer cells are depicted in blue. Depending on the extent to which the prostate cancer has spread, different prostate cancer stages can be discriminated. In organ-confined or localized prostate cancer, the cancer is located within the prostate gland. In locally advanced prostate cancer, the cancer has broken through the prostatic capsule and has invaded neighboring tissues e.g. seminal vesicles, the bladder and/or regional lymph nodes. In advanced or metastatic cancer, the cancer has spread towards loco-regional and distant lymph nodes and other distant organs, e.g. bone/bone marrow compartment. Image created in BioRender.com.

3.4.2. Locally advanced prostate cancer

When the prostate cancer has spread beyond the prostatic capsule to the seminal vesicles, bladder neck and/or local lymph nodes, the prostate cancer is classified as locally advanced prostate cancer. Therapy options for locally advanced prostate cancer include surgery, radiation therapy and ADT (43-45). Since prostate cancer cells are highly depending on AR-signaling for their growth, ADT represents an important treatment modality for locally advanced prostate cancer. Examples of androgen-deprivation therapies include surgical castration, anti-androgens, GnRH agonists, GnRH antagonists and CYP17A1 inhibition by abiraterone (**Figure 5**).

Common side effects of androgen-deprivation therapy include loss of libido, erectile dysfunction, hot flushes, fractures and fatigue. Despite the initial anti-tumor effects of ADT, prostate cancer cells will eventually become resistant to ADT. This will result in the development of castration-resistant prostate cancer (CRPC) and eventually metastatic disease.

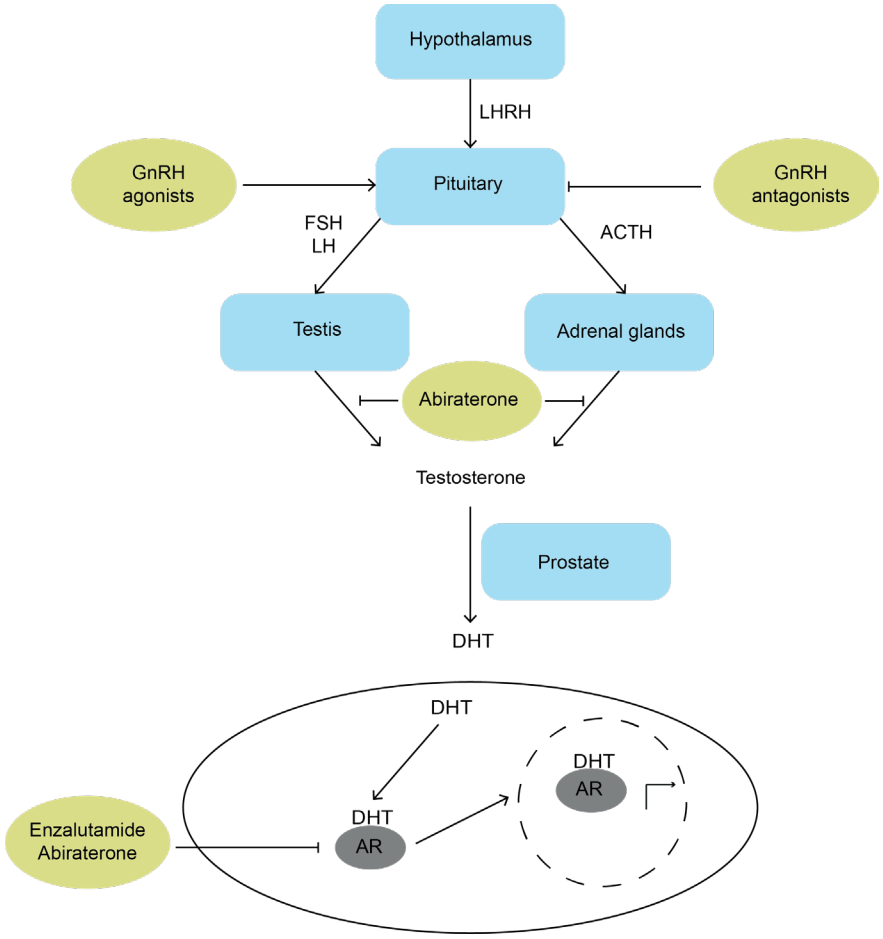

Figure 5 Testosterone synthesis and androgen-deprivation therapy (ADT).

Androgen-deprivation therapy (ADT) can interfere with the testosterone axis via multiple mechanisms, including the surgical blockade of testosterone production by the testis, GnRH agonism, GnRh antagonism, AR blockade and CYP17A1 inhibitor Abiraterone. As a result, serum levels of testosterone and dihydrostestosterone (DHT) will be reduced.

3.4.3. Advanced or metastatic prostate cancer

If the prostate cancer has spread to distant sites of the body, this stage is denoted as advanced or metastatic prostate cancer. The development of metastatic disease results in a dramatical decrease in patient survival. The five year survival for metastatic prostate cancer patients includes 30% and current treatments for metastatic prostate cancer are considered to be palliative (46, 47).

Prostate cancer has the tendency to metastasize towards the lymph nodes and the bones of the axial skeleton. In contrast to other osteotropic tumors such as breast cancer and lung cancer, prostate cancer primarily forms osteoblastic lesions (48, 49). Metastatic prostate cancer causes severe bone pain, pathological fractures and nerve- and spinal cord compression (50). Therapies for metastatic prostate cancer include ADT, radiotherapy and chemotherapy. Chemotherapeutic agents for metastatic prostate cancer include taxanes (docetaxel (first-line) and cabazitaxel (second-line)). Unfortunately, these taxanes are often poorly tolerated by patients and can cause severe of side-effects. In addition, taxane-induced anti-tumor responses are heterogenous in patients and the tumor often develops resistance upon taxane treatment (51). In addition to the use of taxanes, second-line treatment options for metastatic prostate cancer include anti-androgens enzalutamide and abiraterone (43-45). However, 20-40% of all patients do not respond to these anti-androgens or will develop resistance over time (52). In case of prostate tumors with BRCA1/2 mutations, the European Medicines Agency (EMA) has approved the use of PARP-inhibitor Olaparib. Local radiotherapy, bisphosphonates and radium-223 are often prescribed for metastatic prostate cancer patients to reduce pain, to improve the quality of life and to prevent pathological fractures (43, 45, 53).

4. The bladder

4.1. Bladder function and anatomy

The bladder is, together with the kidneys, ureters and urethra, part of the urinary system. The bladder is located at the base of the pelvis and its function is to collect, store and excrete urine. Anatomically, the bladder can be divided into different regions including the fundus, body, apex and neck. The inner wall of the bladder is composed out of multiple folds that expand when the bladder fills with urine. The bladder wall is surrounded by the detrusor muscle.This muscle includes multiple layers of smooth muscle fibers that are arranged in multiple directions.

During urination, the detrusor muscles contract, resulting in the expulsion of urine from the bladder. The bladder wall and detrusor muscles are surrounded by the adventitia (**Figure 6**).

4.2. Histology of the bladder

The bladder wall is composed of multiple layers including an epithelial layer, the basement membrane, a layer of connective tissue with blood vessels and nerves, a muscle layer and a fat layer (11). The epithelium of the bladder, or urothelium, is composed of multiple layers of epithelial cells and is classified as transitional epithelium.

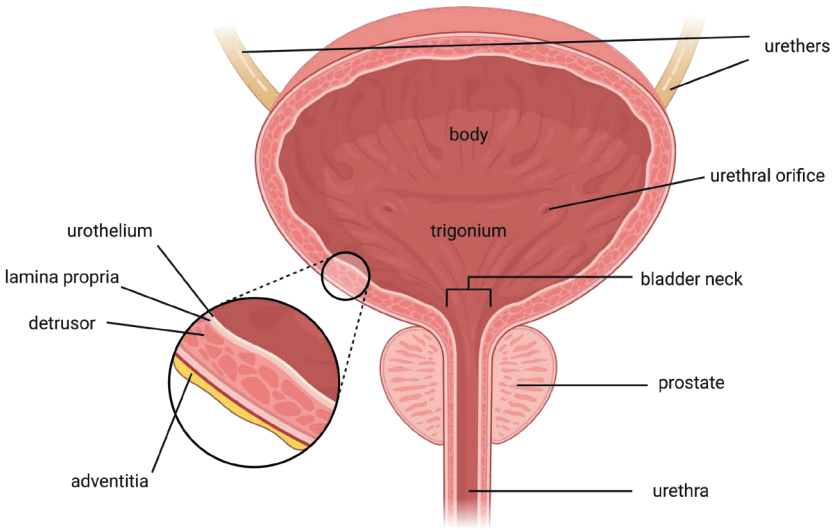

Figure 6 Anatomy of the bladder.

The urinary bladder is located in the pelvis where it collects, stores and excretes urine. The epithelial cells of the bladder (i.e. the urothelium) is surrounded by the detrusor muscle and the adventitia. When the detrusor muscle contracts, this results in the expulsion of urine from the bladder. Image created in BioRender.com.

Different subtypes of epithelial cells can be discriminated in the urothelium including basal cells, intermediate cells and umbrella cells (**Figure 7**) (11). The basal cells are located at the basement membrane and represent the epithelial stem cells of the urinary bladder. Basal cells are characterized by the expression of keratin-5, -10, -14 and p63 (54). The transitional layer of the urothelium consists of rapidly dividing cells that express keratin-10, -14,-18 and p63 (54).

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The outer and most superficial layer of the urothelium is composed of differentiated umbrella cells. Umbrella cells generate a barrier that protects against urinary toxic waste products. Umbrella cells are characterized by the expression of keratin-18 and -20 (54).

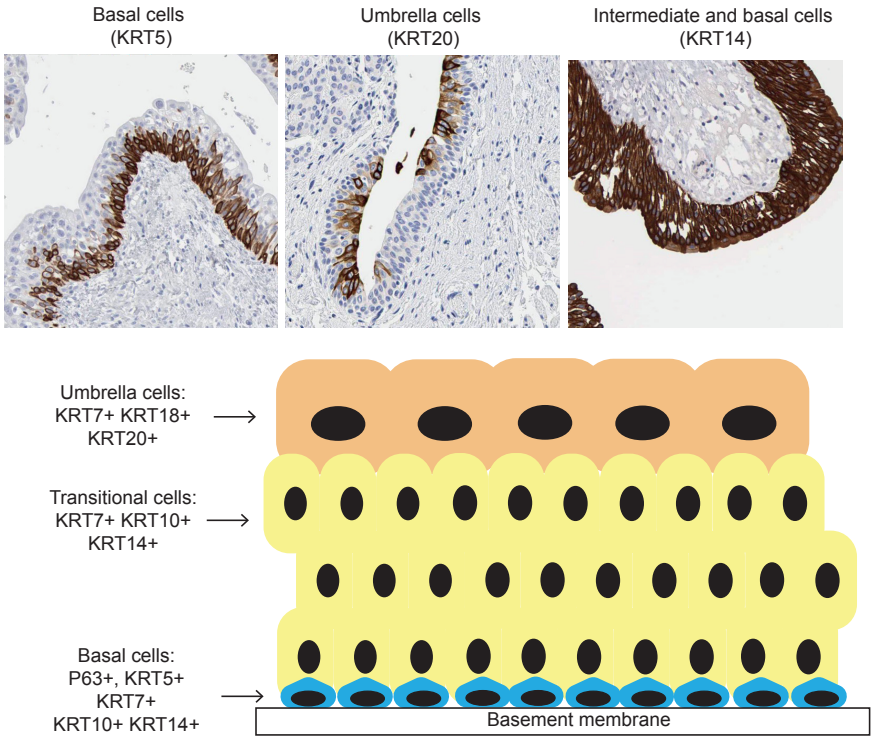

Figure 7 Epithelial subsets in the bladder.

Different epithelial subsets can be discriminated in the urothelium including basal cells, transitional cells and umbrella cells. Each subset is characterized by a unique combination of markers. Legend: KRT=Keratin. Source histological images: Human Protein Atlas www.proteinatlas.org.

5. Bladder cancer

5.1. The clinical problem of bladder cancer

Bladder cancer represents the tenth most common cancer type worldwide (1). Every year, over 500,000 patients are diagnosed with bladder cancer and approximately 200,000 patients die from bladder cancer (1). Bladder cancer is four times more common in men than in women (55). Therefore, bladder cancer represents the sixth most common cancer type and the ninth leading cause of cancer related deaths in men (1). The primary risk factor for developing bladder cancer is smoking and it has been estimated that smoking is responsible for 50% of all bladder cancer cases (56, 57). Moreover, exposure to certain chemicals (e.g. arsenic acid, aromatic amines) is responsible for 25% of all bladder cancer cases (58, 59). The symptoms of bladder cancer include pain during urinating, hematuria and pelvic pain. Due to the high recurrence rates and the frequent disease monitoring and/or treatments, bladder cancer is responsible for the highest costs compared to any other cancer type (60, 61).

5.2. Bladder cancer subtypes and genetic alterations

More than 90% of all bladder tumors are classified as transitional cell carcinoma or urothelial carcinoma. Other subtypes of bladder cancer include squamous cell carcinoma and adenocarcinoma and represent respectively 4% and 2% of all bladder cancers. The minority of all bladder cancer cases is caused by extremely rare subtypes such as sarcoma and small cell bladder cancer.

Genomic profiling of urothelial carcinomas has identified five different molecular subtypes including the urobasal A, genomically unstable, urobasal B, squamous cell carcinoma (SSC)-like and heterogeneous subtypes (62). In addition, six different molecular subtypes have been identified for muscle-invasive bladder tumors and three for non-muscle invasive bladder tumors (63-66).These different subtypes differ in gene expression profile, clinical prognosis and disease outcome (63-65). Genetic alterations that are often detected in bladder tumors include *FGFR3*, *KMD6A*, *TP53* and *EGFR* mutations (62-66). Despite this extensive research on the molecular classification of bladder tumors, these classification systems are not implemented in clinical practice yet.

5.3. Bladder cancer classification

Based on the outcome of different diagnostic tools including imaging, urinary cytology, cystoscopy and histological evaluation, multiple classification systems are being used for the diagnosis of bladder cancer.

TNM staging is based on the size of the tumor and the extent to which the bladder tumor has spread towards lymph nodes and distant sites (**Figure 8** and **Table 4**).

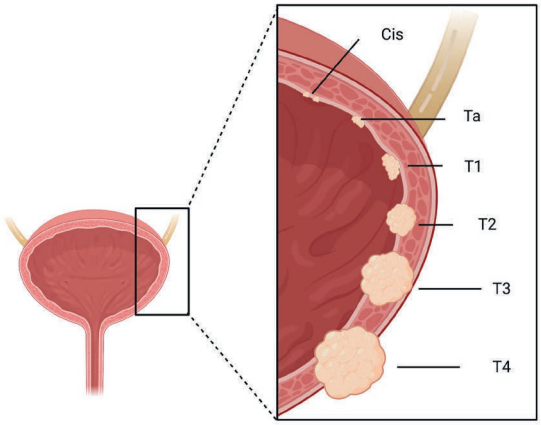

Figure 8 Schematic overview of the different T-stages of bladder cancer. *Depending on the size and location of the bladder tumor, different T-stages can be discriminated (See also **Table 4**). Carcinoma in situ (CIS) includes high-grade tumors that have a high risk of progressing towards muscle-invasive disease. Image created in BioRender.com.*

In addition to TNM-staging, multiple grading systems are used interchangeably in the diagnosis of bladder cancer (57, 67). According to the WHO classification of 1973, papillary urothelial tumors are classified in four categories including papilloma and carcinoma grades 1-3. Grade 1 is used to denote well-differentiated tissue, whereas grade 2 and 3 were applied to describe moderately differentiated and poorly differentiated tissue.

Table 4 TNM staging in bladder cancer (57).

Tumor (T): size of the tumor		
TX		Tumor size cannot be determined
T0		No evidence of primary tumor
Ta		Non-invasive papillary carcinoma
Cis/Tis		Carcinoma *in situ:* ''flat tumor''
T1		Tumor invades subepithelial connective tissue
T2		Tumor invades muscle
	T2a	Tumor invades superficial muscle (inner half)
	T2b	Tumor invades deep muscle (outer half)
T3		Tumor invades perivesical tissue
	T3a	Microscopically
	T3b	Macroscopically (extravesical ass)
	T3a	Tumors has invaded extracapsular
T4		Tumor invades any of the following: prostate stroma, seminal vesicles, uterus, vagina, pelvic wall, abdominal wall
	T4a	Tumor invades prostate stroma, seminal vesicles, uterus or vagina
	T4b	Tumor invades pelvic wall or abdominal wall

Table 4 TNM staging in bladder cancer (57) continued

Node (N): Regional lymph node involvement		
Nx		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastases
N1		Metastases in a single lymph node in the true pelvis (hypogastric, obturator, external iliac or presacral)
N2		Metastases in multiple regional lymph node in the true pelvis (hypogastric, obturator, external iliac or presacral)
N3		Metastasis in common iliac lymph node(s)
Metastasis (M): The presence of distant metastasis		
M0		No distant metastasis
M1		The presence of distant metastasis
	M1a	Non-regional lymph node(s)
	M1b	Other distant metastases

In order to increase the reproducibility, the WHO grading system was updated in 2004 and 2016. The updated grading system discriminates between urothelial papilloma, papillary urothelial neoplasm of low malignant potential (PUNLMP), low-grade (LG) papillary urothelial carcinoma and high-grade (HG) urothelial carcinoma (57, 67).To estimate disease progression and recurrence, bladder cancer patients are stratified in low risk, intermediate risk and high risk bladder cancer (68). This stratification is based on multiple prognostic factors, including gender, age, prior recurrence status, number of tumors, T stage, associated CIS and tumor grade (**Table 5**) (57).

5.4. Bladder cancer stages

Depending on whether the bladder tumor has invaded the muscle layer, non-muscle invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) can be discriminated.

Table 5 Risk group stratification non-muscle invasive bladder cancer (57).

Risk group	Characteristics
Low-risk tumors	Primary, solitary, TaG1 (PUNLMP, LG), <3 cm, no CIS
Intermediate-risk tumors	All tumors not defined in the two adjacent categories (between the category of low- and high-risk)
High-risk tumors	Any of the following: T1 tumor G3 (HG) tumor Carcinoma *in situ* (CIS) Multiple, recurrent and large (> 3 cm) TaG1/G2/LG tumors (all features must be present)
	Subgroup of highest risk tumors:
	T1-G3/HG associated with concurrent bladder CIS, multiple and/or large T1G3/HG and/or recurrent T1G3/HG, T1G3/HG with CIS in prostatic urethra, some forms of variant histology of urothelial carcinoma, lymphovascular invasion

5.4.1. Non-muscle invasive bladder cancer (NMIBC)

Non-muscle invasive bladder cancer (NMIBC) represents 75% of all bladder cancer cases and is characterized by a high recurrence rate (60-70%) and lower mortality compared to muscle invasive bladder cancer (MIBC) (69). The five year survival of Ta and T1 tumors is 100% and 58%-80% respectively (70). According to the EAU, low risk NMIBC tumors should be treated with transurethral resection of the bladder (TURB) in combination with one immediate intravesical administration of chemotherapy (57). In contrast, patients with intermediate risk tumors with a low recurrence rate are to be treated with TURB and intravesical chemotherapy. Subsequently, Bacillus Calmette-Guérin (BCG) or chemotherapy instillations are applied for one year to patients with intermediate risk NMIBC tumors.

The EAU advises that high risk patients should receive intravesical BCG instillations for 1-3 years or cystectomy. Despite the positive responses after BCG administration in the majority of these tumors, 25-45% of the patients do not respond to BCG therapy and 40% of all patients will relapse (71). In addition, the use of BCG instillations is associated with severe side-effects and is often poorly tolerated in patients (72). Tis or CIS (carcinoma *in situ*) represents a unique subtype of NMIBC and denotes flat high-grade tumors located at the inner layer of the bladder wall. Tis tumors are considered to be a precursor of MIBC since 54% of patients with Tis will eventually progress towards muscle invasive disease. Therefore, the presence of Tis is associated with a high risk of progression towards muscle invasive disease and a poor prognosis (73).

5.4.2. Muscle invasive bladder cancer (MIBC) and metastatic bladder cancer

Muscle invasive bladder cancer (MIBC) is responsible for 25% of all bladder tumors and is characterized by the invasion of muscle layer by the bladder tumor. In contrast to NMIBC, MIBC patients have a lower survival ranging from 0-62% depending on the size, location and grade of the tumor (70, 74). The first-line treatment option for MIBC patients includes cystectomy in combination with neoadjuvant chemotherapy. Of all MIBC patients undergoing cystectomy, approximately 50% of these patients will relapse, resulting in the formation of distant metastases (57). Bladder cancer primarily metastasizes to the lungs, liver, lymph nodes, peritoneum and the bone (75, 76). First-line systemic therapy for metastatic bladder cancer includes cisplatin monotherapy, methrotrexate-vinblastine-adriamycin-cisplatin (MVAC) combination therapy and gemcitabine. Patients that are ineligible for cisplatin therapy are treated with immunotherapy (PD-1 and PD-L1 inhibitors) or carboplatin (57). Second-line treatments for MIBC include immune checkpoint inhibitor Pembroluzimab or platinum-based chemotherapy (57). (See section Current status of bladder cancer immunotherapy).

6. Cancer cell metastases

It has been estimated that the formation of distant metastases is responsible for over 90% of all cancer-related deaths (77). As previously described, cancer cell invasion and metastasis formation are included in the hallmarks of cancer (4, 5). The formation of distant metastases is a complex molecular process and encompasses multiple sequential steps: the detachment of cells from primary tumor, invasion and degradation of local stroma, escape into the vasculature or lymphatics, homing and extravasation to distant sites, tumor growth and angiogenesis at the secondary site (47, 78, 79). Each step in the metastatic cascade is rate-limiting and it has been estimated that only 0.01% of circulating tumor cells has the ability to form distant metastasis (**Figure 9**) (80, 81).

6.1. Epithelial-to-mesenchymal transition (EMT)

In order to metastasize towards distant sites, a cancer cell has to detach from neighboring cells and invade the surrounding stroma. In general, epithelial cells have close interactions with neighboring cells and the basement membrane via tight junctions, adherens junctions, desmosomes and gap junctions (82-84). However, tumor cells are able to disrupt these interactions, resulting in the acquisition of migratory and invasive characteristics. Different patterns of cancer cell invasion have been identified including, amoeboid migration, collective cell migration, and epithelial-to-mesenchymal transition (83, 85-87).

Epithelial-to-mesenchymal transition (EMT) is an embryonic process that is reactivated under pathological conditions such as inflammation, wound healing, fibrosis and cancer (88). EMT is characterized by different cellular changes including the loss of apical-basal polarity, modulation of the cytoskeleton and reduction in cell-cell adhesion (82). Depending on the biological setting, three types of EMT can be discriminated (83). Type 1 EMT is involved in the generation of mesenchymal cells during embryonic development and organ development. Type 2 EMT is associated with wound healing, tissue generation and fibrosis. Type 3 EMT takes place during cancer progression and is involved in cancer cell invasion and metastasis formation (83).

At the transcriptional level, increased expression of EMT-transcription factors *ZEB*s, *SNAI*s and *TWIST* will result in the repression of E-cadherin by the recruitment of histone deacetylases at the E-boxes of the E-cadherin promoter (47, 89).

As a consequence, the expression of epithelial markers (e.g. E-cadherin) is reduced, wheras the expression of mesenchymal markers (e.g. Vimentin and N-cadherin) are upregulated (90). EMT is a dynamic and reversible process and switching from an epithelial state towards a mesenchymal state has been associated with an increased drug resistance, the development of a cancer-stem cell phenotype and a poor prognosis (88, 91-93). The opposite process of EMT, mesenchymal-to-epithelial transition (MET), has also been observed in prostate and bladder cancer and is involved in the colonization and outgrowth of disseminated cancer cells at distant sites (**Figure 9**) (82, 94-96).

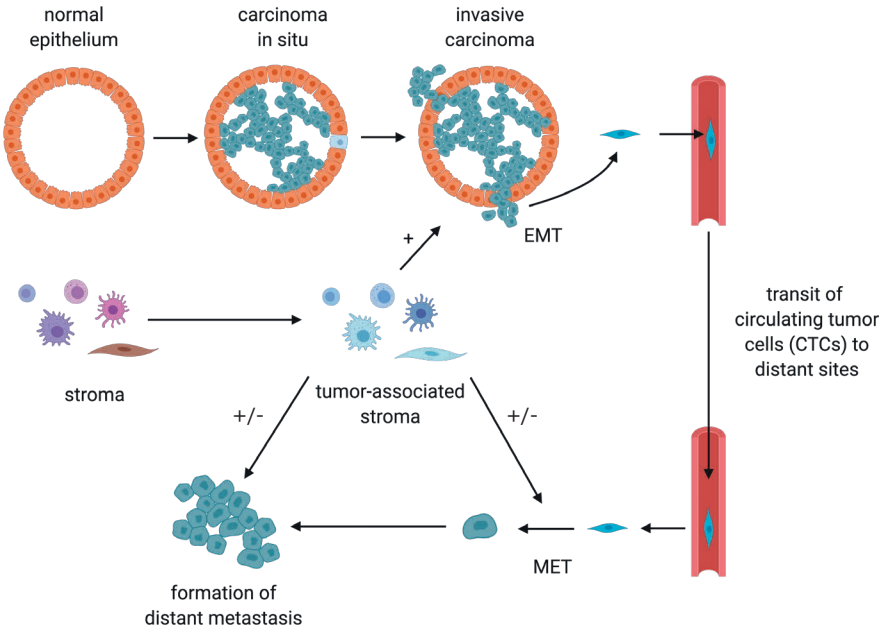

Figure 9 The metastatic cascade.

During the first phase of the metastatic cascade, the tumor cell acquires an invasive phenotype, translocates from the primary tumor and invades the local stroma. One of the processes through which cancer cells acquire an invasive phenotype is by undergoing epithelial-to-mesenchymal transition (EMT). Subsequently, the invasive tumor cell will intravasate in the circulation and circulating tumor cells (CTCs) will travel to distant organs. During the colonization phase, CTCs extravasate from the circulation into distant tissue. Extravasated cells can undergo mesenchymal-to-epithelial transition (MET) and acquire an epithelial phenotype. Only the cells that are capable of surviving and adapt at the secondary site are able to form distant metastasis. Besides the role of the tumor cell, the tumor-associated stroma can positive and negative regulate multiple steps of the metastatic cascade. Image created in BioRender.com.

6.2. Seed and soil hypothesis

Many cancers, including prostate and bladder tumors, show an organ-specific metastasis pattern. Prostate cancer has the tendency to spread towards the lymph nodes and the bone, besides the formation of soft tissue metastases. It has been estimated that approximately 70% of all men that die of prostate cancer have bone involvement (97). Bladder cancer mainly spreads to the lymph nodes, bone, lungs and liver (75). Around one third of all bladder cancer patients will eventually develop skeletal metastases (57, 98).

The presence of positive, loco-regional lymph nodes is indicative of initial lymphogenic (and hematogenous) dissemination in prostate and bladder cancer (99). However, cancer metastasis to distant sites, e.g. to the skeleton in advanced prostate cancer or bladder cancer metastasis to the lungs, cannot be simply explained by anatomical characteristics including the presence blood and/or lymphatic flows (100). Stephen Paget observed that breast cancer cells metastasize in a pattern towards specific sites, which cannot be explained by mechanical blood and lymphatic flow patterns. Therefore, Paget proposed that active crosstalk between tumor cells (i.e. the seed) and the microenvironment (i.e. the soil) will enable the outgrowth of cancer cells at specific secondary sites: ''*When a plant goes to seed, its seeds are carried in all directions; but they can only grow if they fall in congenial soil''* (101-103). This concept has been described as the ''seed and soil hypothesis''. Currently, it is believed that both passive/mechanical dissemination and active crosstalk between the tumor and the stroma are of importance in the formation of distant metastases (104, 105).

7. The immune system and cancer

7.1. The immune system

The immune system includes the system of processes that protects an organism against pathogens such as viruses, bacteria, parasites and fungi. The immune system can be subdivided into the innate immune system and the adaptive immune system. The innate immune system provides the first line of defense and aims to quickly terminate an infection at an early stage. The innate immune system consists of two components: 1) mechanical, chemical and microbiological barriers including epithelial cells and mucosal surfaces and 2) soluble proteins and cell-surface receptors that bind pathogens or their products.

The innate immune system is directed against a general class of pathogens rather than targeting a pathogen specifically. Pathogens are recognized by a fixed number of cell-surface receptors and soluble factors by the innate immune system. Cells of the innate immune system include macrophages, dendritic cells, NK cells, mast cells, neutrophils, eosinophils and basophils (106). The adaptive immune system represents the second-line of defense against a pathogen. In contrast to the innate immune system, the adaptive immune system is tailored to recognize one specific pathogen and consists of an infinite number cell surface receptors. Cells of the adaptive immune system include T- and B-cells. During a primary immune response, foreign antigens are recognized by antigen presenting cells including tissue-resident macrophages and dendritic cells. These antigens are fragmented and presented at the cell surface of antigen presenting cells. Subsequently, dendritic cells will migrate towards to the lymph nodes where they will present the fragmented antigens to lymphocytes. As a result, the lymphocytes will become activated. Some activated T-lymphocytes will leave the lymph node and travel towards the infected tissue where they will help clear the infection. Other T-cells will remain in the lymph node and activate B-cells. These B-cells will differentiate in plasma cells and are responsible for antibody production. Interestingly, some lymphocytes are retained after infection and results in the generation of immunological memory (106).

7.2. The immune system and cancer development

The immune system plays a dual role in the development of cancer and is capable of both facilitating and suppressing tumor growth. This dual role of the immune system in cancer is described by the concept of immunoediting (**Figure 10**) (107-111). Besides the failure of the immune system to recognize and eliminate tumor cells, tumor cells can actively circumvent and suppress immune surveillance. Evasion of immune destruction has been included in the upgraded version of the hallmarks of cancer in 2011 (5). Activation of the immune system by immune therapy has emerged as a promising modality for the treatment of different types of cancer. Based on the number of infiltrated T-cells, immunological hot, cold and excluded tumors can be discriminated (109, 112-115). Immunological hot tumors are characterized by a high number of infiltrated T-cells, a better clinical outcome and a better response to immune checkpoint inhibition, chemotherapy and radiation therapy (116). In contrast, immunological cold/deserted tumors have a low number of infiltrated T-cells. Moreover, infiltrated T-cells in immunological cold tumors have a low effector function. Immunological cold tumors are associated with a poor prognosis and a poor response to immuno therapy (116).

Immunological excluded tumors have a stromal barrier that prevents the infiltration of immune cells (116). Similar to immunological cold tumors, immunological excluded tumors have a poor clinical prognosis and respond poorly to immunotherapy.

7.3. Prostate cancer immune microenvironment

Prostate cancer is classified as an immunological cold tumor (117). In contrast to other tumor types where a high number of infiltrated T-cells (including CD4+ T helper cells and CD8+ cytotoxic T cells) is correlating with a good clinical outcome, the prognostic relevance of T-cell infiltration remains unclear in prostate cancer. However, the presence of a high number of regulatory T-cells (Tregs) is associated with a worse progression-free survival and a poor overall survival in human prostate cancer (117). Prostate cancer has a unique tumor microenvironment which facilitates and reinforces its immunosuppressive state (117).

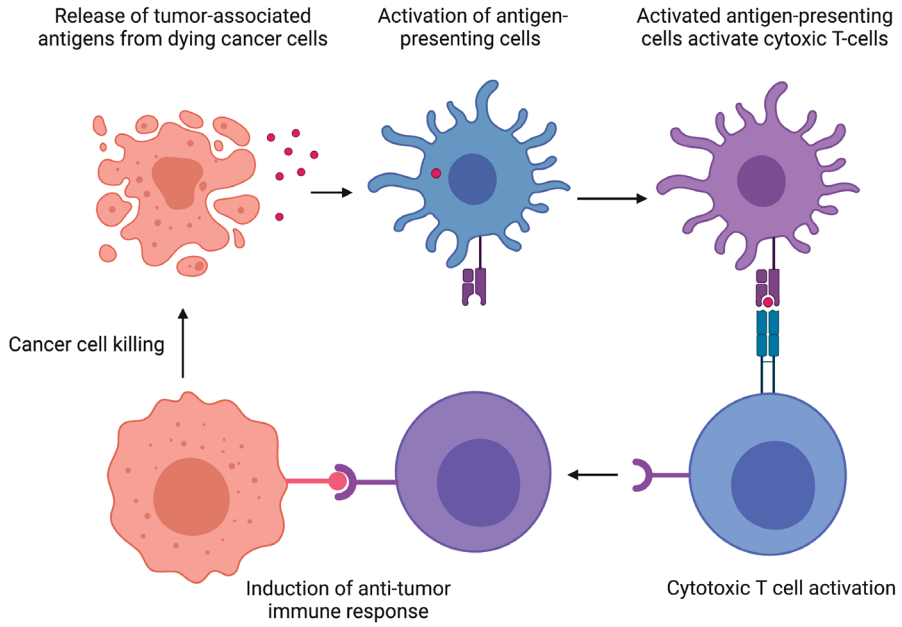

Figure 10 Induction of an anti-tumor immune response.

Dying tumor cells release tumor-associated antigens, which can be recognized by antigen-presenting cells (e.g. dendritic cells). Upon uptake of these tumor-associated antigens, antigen-presenting cells become activated. These activated antigen-presenting cells will travel to distant lymph nodes where cytotoxic T cells become activated. These cytotoxic T cells will travel towards the tumor, where they induce an anti-tumor immune response. Image created in BioRender.com.

Multiple factors have been identified that contribute to the immunological cold phenotype of prostate cancer, including a low tumor mutational burden, a low number of mutations in DNA damage response (DDR) genes, loss of MHC class I expression, PTEN loss and the presence of immunosuppressive cells (117, 118). Besides the intrinsic immune suppressive state, prostate cancer treatments are able to interfere with the prostate cancer immune microenvironment. Preclinical research in prostate cancer xenografts has indicated that treatment with AR-antagonists resulted in the suppression of the immune system by inhibition of T-cell activation (119). However, it has been demonstrated that surgical castration induced immune activation by promoting T-cell proliferation and activation (119, 120). These data suggest opposing immune effects of surgical castration and chemical castration in prostate tumors. In addition, other prostate cancer treatment modalities such as radiotherapy and immunotherapy are known to modulate the immune system by inducing immunogenic cell death (ICD), a form of regulated cell death that involves the induction of a T-cell mediated anti-tumor immune response against antigens that are being released from dying cancer cells (121, 122). Hallmarks of immunogenic cell death includes calreticulin exposure, ATP secretion and HMGB1 release (123-125).

7.4. Current status of prostate cancer immune therapy

Sipuleucel-T is a cell-based immune therapy for the treatment of metastatic prostate cancer (126). Sipuleucel-T is based on the ex vivo activation of peripheral-blood mononuclear cells (PBMCs) with a fusion protein of PAP and granulocyte-macrophage colony-stimulating factor (GM-CSF). Moreover, the use of Pembrolizumab has been approved by the U.S. Food and Drug Administration (FDA) for mismatch repair (MMR)-deficient prostate cancer or instable microsatellite status (MSI-high) prostate cancer. Currently, other immunotherapies are not available for prostate cancer patients (127-136). Multiple clinical studies are currently ongoing that study the effect of immune therapy such as vaccines, immune checkpoint inhibitors and oncolytic viruses in combination with other treatment modalities in prostate cancer patients (a.o. NCT03600350, NCT01706458, NCT02933255, NCT02506114, NCT02649855, NCT01875250, NCT01867333, NCT02985957, NCT03879122, NCT02985957, NCT01498978, NCT04097002).

7.5. Bladder cancer immune microenvironment

Literature has revealed that the immune system is highly active in a subset of bladder tumors (137). Bladder cancer is characterized by the high number of DNA alterations and has the third highest frequency in somatic mutations (138). As a result, the tumor mutational burden (TMB) and neoantigen expression is high in bladder tumors (139).

For these reasons, bladder cancer has been characterized as an immunological hot cancer. Despite this immunological hot phenotype, multiple immunosuppressive immune cells have been detected in human bladder cancer including M2 macrophages, Tregs and myeloid-derived suppressor cells (MDSCs) (137). Multiple immune evasive strategies have been identified in bladder cancer. Beside the accumulation of immunosuppressive cells, the production of immunosuppressive cytokines and the synthesis of anti-apoptotic proteins have been reported in bladder tumors (140). Therefore, it appears that there is a delicate balance between anti-tumorigenic and pro-tumorigenic immune cells and factors in human bladder cancer. This delicate balance is further reflected by the heterogenous responses of bladder cancer patients to immunotherapy, including BCG instillations and immune checkpoint inhibition. It has been reported that 25-45% of NMIBC patients do not respond to BCG therapy (71). Moreover, of all the bladder cancer patients that express PD-L1, only 52% will respond to checkpoint inhibition (141). Research has revealed that infiltration with CD8+ cytotoxic T cells is an important prognosic factor in MIBC and correlates with a better disease-free survival and overall survival (142, 143). In contrast, a high ratio of CD68+ tumor-associated macrophages to CD3+ tumor infiltrating lymphocytes correlated with a poor prognosis in bladder cancer patients (144).

7.6. Current status of bladder cancer immune therapy

BCG instillation represents one of the oldest forms of immunotherapy and includes the standard therapy for intermediate- and high-risk NMIBC (57, 145). Despite the long use of BCG instillations in the treatment of bladder cancer, the exact working mechanism remains unknown (71). It has been proposed that upon instillation with BCG, the mycobacteria are internalized by the bladder cancer cells. This internalization results in an increased antigen presentation and the release of inflammatory cytokines and chemokines, thereby activating the innate and adaptive immune system (71, 72, 146). Besides the use of BCG instillation, several immune checkpoint inhibitors are currently FDA-approved for the treatment of advanced or metastatic bladder cancer, including PD-1/PD-L1 inhibitors and CTLA-4 inhibitors (71).

Multiple clinical trials are currently ongoing studying the use of immunotherapy in combination with other treatment modalities in metastatic bladder cancer, including chemotherapy (a.o. NCT2302807, NCT02807636, NCT02516241, NCT03036098, NCT2853305) and radiotherapy (NCT03601455. NCT03150836) or the use of immunotherapy as neoadjuvant therapy in before cystectomy (NCT03924856, KEYNOTE-905, NCT03732677) (147).

8. Outline and scope of thesis

Urological cancers, including prostate and bladder cancer, represent complex diseases and despite extensive research, the development of therapy resistance and the progression towards metastatic disease represent clinical unmet needs. Therefore, new therapies for prostate and bladder cancer are urgently needed. In this thesis, different preclinical strategies including the use of patient-derived tumor models and screening strategies are exploited to identify novel therapeutic options for the treatment of prostate and bladder cancer. In **Chapter 2**, we provide an overview of the different patient-derived models that are currently being used in uro-oncological research. **Chapter 3** shows the optimization and validation of an *ex vivo* culture model of prostate and bladder cancer tissue slices. In **Chapter 4** we investigated whether repurposing of the class of cationic amphiphilic drugs (CADs), including antipsychotic drug penfluridol, was effective as a novel anti-cancer drug in human bladder cancer. Subsequently in **Chapter 5**, the effect of the FDA-approved antipsychotic drug penfluridol in the treatment of human prostate cancer was assessed. **Chapter 6** focuses on the use of mutant reovirus *jin-3* in human prostate cancer. The direct oncolytic and indirect immune modulatory effects of *jin-3* reovirus were tested in multiple preclinical patient-derived prostate cancer models, including three-dimensional cultures, *ex vivo* cultured tumor tissue slices and patient-derived xenograft models. **Chapter 7** describes a novel screening assay based on E-cadherin (re)induction and inhibition of invasion in order to identify low molecular weight (LMW) compounds that are able to reduce the aggressive phenotype of epithelial tumor cells. **Chapter 8** provides a general conclusion of the findings in this thesis and future perspectives and clinical implications are presented.

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