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Faculty of Health Sciences

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Immunological biomarkers in prostate cancer

A retrospective cohort study utilizing immunohistochemistry on tissue microarrays for evaluation of immune biomarker expression and experimental *in vitro* assays

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LIST OF ABBREVIATIONS

ADT = Androgen deprivation therapy
AJCC = American Joint Committee on Cancer
AR = Androgen receptor
ATM = Ataxia-Telangiectasia Mutated
AUA = American Urological Association
BF = Biochemical failure
BFFS = Biochemical failure-free survival
BPH = Benign prostatic hyperplasia
BRCA = Breast cancer gene
CAB = Combined androgen blockade
CAF = Cancer associated fibroblast
CF = Clinical failure
CFFS = Clinical failure-free survival
CRPC = Castrate-resistant prostate cancer
mCRPC metastatic castrate-resistant prostate cancer
CTLA-4 = Cytotoxic T lymphocyte antigen 4
DC = Dendritic cell
DRE = Digital rectal examination
EAU = European Association of Urology
ECM = Extracellular matrix
EPE = Extraprostatic extension
ePLND = Extended pelvic lymph node dissection
ESMO = European Society for Medical Oncology
FDA = U.S. Food and Drug Administration
FFPE = Formalin-fixed and paraffin-embedded
HDI = Human Development Index
HLA = Human leukocyte antigen
HPC = Hereditary prostate cancer
HPV = Human papillomavirus
IHC = Immunohistochemistry
ISUP = International Society of Urological Pathology
LHRH = Luteinizing-hormone-releasing hormone
LUTS = Lower urinary tract symptoms
LVI = Lymphovascular invasion
mAbs = Monoclonal antibodies
MDSC = Myeloid-derived suppressor cell
MMR = Miss-match repair
dMMR = mismatch repair deficiency
MRI = Magnetic resonance imaging
MSI-H = High microsatellite instability
NCCN = National Comprehensive Cancer Network
NK cell = Natural killer cell
NKT = Natural killer T cell
NLSH = Nordland Hospital
PALB2 = Partner and localizer of BRCA2
PAP = Prostate-specific acid phosphatase
PARP = Poly (ADP-ribose) polymerase
PCa = Prostate cancer
PCD = Prostate cancer-specific death
PCDFS = Prostate cancer death-free survival
PD-1 = Programmed cell death 1
PET = Positron emission tomography
PIA = Proliferative inflammatory atrophy
PIN = Prostatic intraepithelial neoplasia
HGPIN = High-grade prostatic intraepithelial neoplasia
LGPIN = Low.grade prostatic intraepithelial neoplasia
PNI = Perineural invasion
PSA = Prostate-specific antigen
PSM = Positive surgical margin
PSMA = Prostate-specific membrane antigen
PTEN = Phosphatase and tensin homolog
RALP = Robotic-assisted laparoscopic radical prostatectomy
RP = Radical prostatectomy
RT = Radiation therapy
EBRT = External beam radiation therapy
SVI = Seminal vesicle invasion
TAA = Tumor-associated antigen
TAM = Tumor associated macrophage
TAN = Tumor associated neutrophiles
TCR = T cell receptor
TIL = Tumor infiltrating lymphocytes
TMA = Tissue microarray
TME = Tumor microenvironment
Treg = Regulatory T cell
TRUS = Transrectal ultrasound
TSA = Tumor-specific antigen
TUR-P = Transurethral resections of the prostate
UICC = International Union Against Cancer
UNN = University Hospital of Northern Norway
VISTA = V-domain Ig suppressor of T-cell activation
WHO = World Health Organization

LIST OF PAPERS

Paper I.

Ness N, Andersen S, Valkov A, Nordby Y, Donnem T, Al-Saad S, Busund LT, Bremnes RM, Richardsen E. Infiltration of CD8+ lymphocytes is an independent prognostic factor of biochemical failure-free survival in prostate cancer. *Prostate.* 2014. 74(14):1452-61. doi: 10.1002/pros.22862.

Paper II.

Ness N, Andersen S, Nordbakken CV, Valkov A, Khanehkenari MR, Paulsen EE, Nordby Y, Bremnes RM, Donnem T, Busund LT, Richardsen E. The prognostic role of immune checkpoint markers Programmed cell death protein 1 (PD-1) and Programmed death ligand 1 (PD-L1) in a large, multicenter prostate cancer cohort. *Oncotarget*. 2017. 18;8(16):26789-26801. doi: 10.18632/oncotarget.15817.

Paper III.

Richardsen E, Ness N, Melbø-Jørgensen C, Johannesen C, Grindstad T, Nordbakken C, Al-Saad S, Andersen S, Dønnem T, Nordby Y, Busund LT, Bremnes RM. The prognostic significance of CXCL16 and its receptor CXCR6 in prostate cancer.
Am J Pathology. 2015. 185(10):2722-30. doi: 10.1016/j.ajpath.2015.06.013.

ABSTRACT

Introduction: Prostate cancer (PCa) is a very common, but particularly heterogenic cancer form. Whilst some patients have an aggressive course with a fatal outcome, the majority of patients have slow-growing disease with low risk of death or significant symptoms. Unfortunately, doctors do not possess sufficient prognostic tools, leading to a high risk of overtreatment with unnecessary side-effects. We set out to find new biomarkers for prognostic stratification of PCa patients, and possibly at the same time generate hypotheses for potential therapeutic targets. We chose to analyze different immune system markers as the immune system is known to be a key player in cancer development. **Materials and methods:** Patient data and prostatectomy specimens from 535 Norwegian patients with primary PCa was collected retrospectively. Tissue microarrays were constructed from representative tumor areas as well as surrounding non-malignant areas. Immunohistochemistry was used to evaluate proportions of intratumoral CD3+, CD4+, CD8+, CD20+ and PD-1+ lymphocytes as well as expression of PD-L1, CXCR6 and CXCL16 on both tumor epithelial cells and tumor stromal cells. Further, we investigated the independent prognostic impact of each biomarker, as well as their correlation with each other, and well-known clinical- and histopathological parameters. We also performed experimental assays to explore pro-tumorigenic properties of CXCL16 in PCa cell lines. **Results:** A high density of intratumoral CD8+ lymphocytes independently predicted a shorter time to disease relapse in form of biochemical failure. A high density of intratumoral PD-1+ lymphocytes independently predicted a shorter time to disease relapse in form of clinical failure, and a high density was also associated with a worse prognosis in most subgroups related to poor prognosis. PD-L1 expression was commonly seen in tumor epithelial cells and was consistently correlated to worse prognosis, although was not statistically significant. A high expression of CXCR6 by tumor epithelial cells or when analyzed in tumor tissue as a whole, independently predicted a shorter time to both biochemical- and clinical failure. A high expression of CXCL16 by tumor epithelial cells, and CXCR6/CXCL16 co-expression in tumor tissue as a whole, independently predicted a shorter time to clinical failure. **Conclusions:** Based on our observations, we propose further investigation of the biological mechanisms and prognostic effect of CD8+, PD-1+, PD-L1+ cells and CXCR6 and its ligand CXCL16 in PCa. Hopefully one or more of these markers may improve risk stratification of PCa patients and may even be of value as targets of therapy in future PCa treatment.

1. INTRODUCTION

When we started our research approximately ten years ago, our general aim was to contribute to reduce challenges in prostate cancer (PCa) clinical decision making, by improving prognostication, as well as generate hypotheses on PCa biology and possible therapeutic targets.

PCa represents an especially heterogenous group of cancerous disease. Some individuals will have a very aggressive course with a fatal outcome, whilst the majority of patients have indolent disease and succumb to other conditions (1). In the last few decades, the detection rate of indolent cases has increased, caused by uncritical PSA-testing in what we call the "PSA Era". Unfortunately, available prognostic tools are inadequate to precisely predict the fate of each individual patient. This leads to both under- (2), but mainly, overtreatment (3) with subsequent side-effects. This situation, combined with the relatively high incidence and low mortality of PCa (4) generates a substantial group of men who have undergone radical PCa treatment, are under surveillance, or are long-term survivors of metastatic PCa. At a societal level, this causes socioeconomic concerns, with decreased revenue from income taxes and increased pension- and sick leave expenses, as well as high health system expenses. But, even more important, at a personal level, the diagnosis and its consequences create economic-, health-, social- and sexual challenges for the affected individuals.

In oncology today, there is a shift towards precision medicine (also referred to as personalized medicine), where the goal is to optimize treatment response based on the individual tumor biology and host characteristics rather than organ origin, whilst at the same time decreasing side-effects. This is of utmost importance in cancer types with heterogenic behaviour such as PCa. Complementary to precision medicine is immunotherapy, in which the individual's immune system is manipulated to exercise a significant anti-tumor immune response. However, the biological mechanisms of immuno-oncology are complex, as different immune components can have opposite roles in tumorigenesis. Namely, immune cells can eliminate cancer cells (5–8), but inflammatory components are also known promotors of cancer development and progression (9). In addition, for some cancers the immune system seems to not play an important role at all.

Biomarker studies are a way of quantifying biological differences between tumor samples. If put together with patient clinical outcome data, biomarker expression may possibly be translated into clinically relevant information. The goal can be to find prognostic and/or predictive markers, as well as therapeutic targets. Compared to other high-incidence cancer types, such as breast cancer (HER2, estrogen- and progesterone receptor etc.), colon cancer (NRAS, KRAS, BRAF, MSI etc.) and lung cancer (EFGR, PD-1/PD-L1, ALK, ROS1 etc.), PCa biomarker studies have so far unfortunately yielded few clinically relevant results. Thus, we set out to contribute to finding prognostic markers in a cancer type in a great need of such.

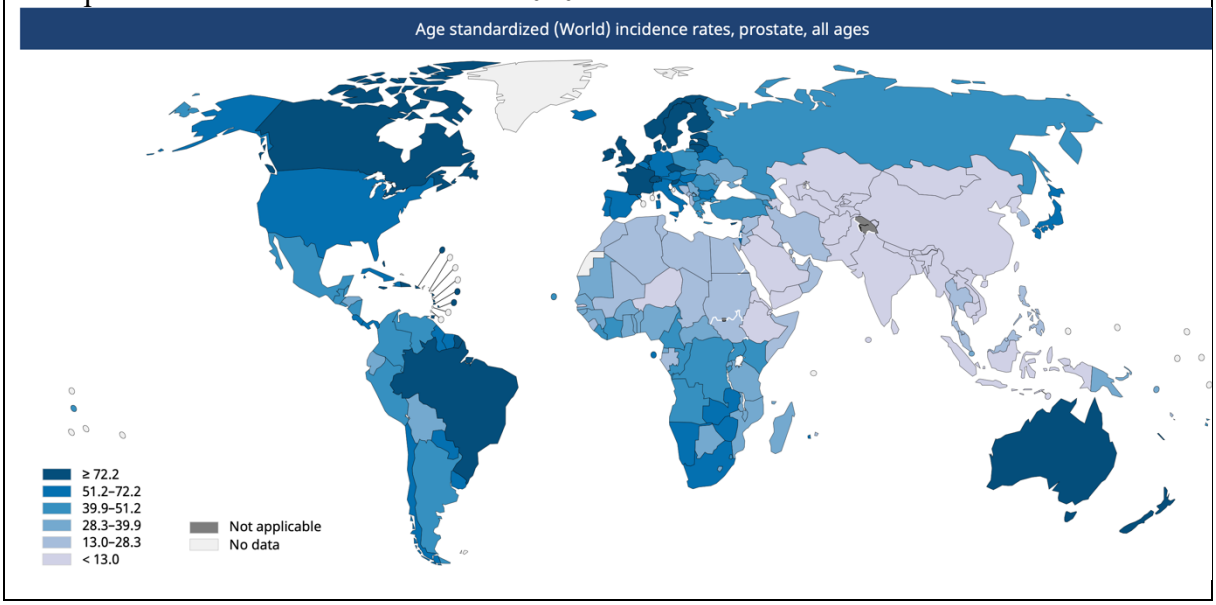
1.1. PROSTATE CANCER

1.1.1. Epidemiology

Incidence and prevalence

PCa is the fourth most common cancer form worldwide, with an estimated 1.3 million cases in 2018 (10). For men only, PCa is the second most common cancer form worldwide, but the most common in majority of countries in the Americas, Northern- and Western Europe, Oceania and much of Sub-Saharan Africa (Figure 1). The incidence is relatively low in Northern Africa and Eastern- and South-Central Asia, where cancers related to viral infections are more common, and sometimes endemic. In Norway, PCa is the most commonly diagnosed cancer, accounting for approximately 30% of cancer cases in men. Almost 1 in 8 men will be diagnosed with the disease before the age of 75 (4).

Figure 1. Age-standardized incidence rates for PCa, all ages, world, 2020 (11). Reprinted with permission from GLOBOCAN© 2020



In high/very high Human Development Index (HDI) countries, incidence rates have increased steadily in the 1970'ties and -80'ties. Some of these cases were possibly diagnosed incidentally by finding PCa in tissue material from transurethral resections of the prostate (TUR-P), performed to relieve symptoms related to enlargement of the prostate gland (Figure 2). A dramatic increase was seen in the early 1990'ties, mainly because of prostate-specific antigen (PSA) testing becoming available (12), and leading to massive, unorganized screening. The dramatic increase in incidence has mainly been explained by higher life-expectancy causing an aging population, as well as overdiagnosis of indolent cancers and *"pulling diagnosis forward in time"* caused by PSA-testing. Rates are now stable or declining in high/very high HDI countries, partly because the pool of prevalent cases has diminished because of earlier detection by PSA-testing (4,10).

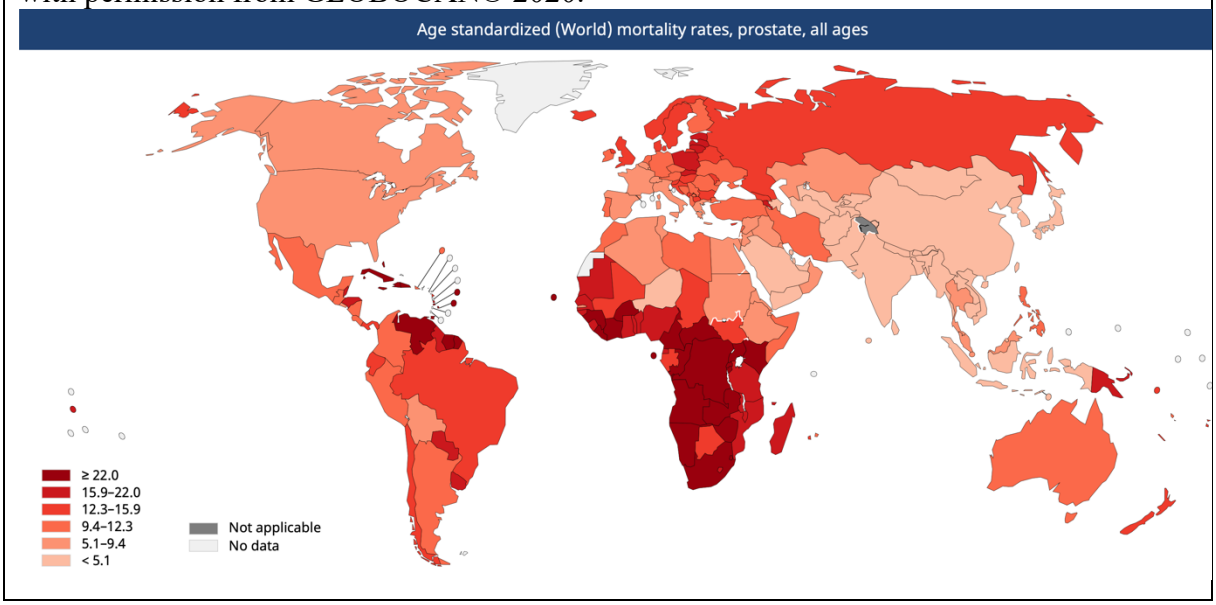
Figure 2. Age-standardized incidence and mortality rates of PCa in Norway (left) and the Nordic countries (right) from 1954 to 2014. Incidence (red) and mortality (green). All ages. Curves are smoothed with use of 3-year average. Curves made by a tool in the NORDCAN database (13).

Mortality

PCa in general has a low mortality, but affected by the high incidence rate, it is still a huge contributor to cancer-related death. In 2018, PCa was the fifth leading cause of cancer-related death in men, with a worldwide total of 359 000 deaths (10). In 46 countries, mainly in Sub-Saharan Africa and the Caribbean, PCa mortality rates are higher compared to other high-incidence countries, and it is the leading cause of cancer death in males (Figure 3). In Norway, PCa is the third most common cause of cancer mortality (8% of all cancer related deaths, both sexes) (4).

In high/very high HDI countries, mortality rates are now decreasing (Figure 2), which can be due to diagnosis in early stages of disease because of PSA-testing, as well as improvements in treatment. Some of the explanation may also be due to detection of a considerable proportion of indolent tumors through PSA-testing that otherwise would not have been detected (length-time bias). In low/medium HDI countries, mortality rates are increasing, which may possibly be explained by an increasing incidence due to exposure to risk factors such as a more Westernized lifestyle, in combination with limited access to effective treatment (14,15).

Figure 3. Age-standardized mortality rates for PCa, all ages, world, 2020 (11). Reprinted with permission from GLOBOCAN© 2020.



In Norway, a PCa diagnosis is usually associated with a good prognosis; The overall 5-, 10- and 15-year relative survival is approximately 95%, 90% and 80% respectively. For men with distant metastases at time of diagnosis, the 5-year survival is only around 40% (4), and average survival time is 3 years. However, some patients with distant metastases are long-term survivors and may not die from the disease – a concept known as *"statistical curation"* (16,17).

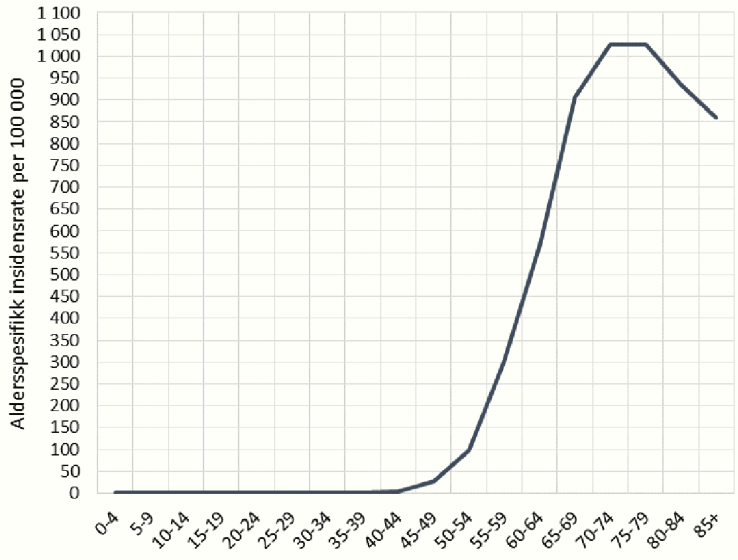
1.1.2. Etiology and risk factors

The development of PCa is most likely complex and multifactorial, caused by both hereditary and environmental factors. Age, ethnicity and familial accumulation are the most established risk factors. Unfortunately, we yet do not know enough about the causalities of the disease to prevent it from occurring.

Age

The most well-known risk factor for developing PCa, is a high age. By many, PCa is almost considered a normal aging process. Diagnosis under the age of 40 is extremely rare, with a progressively increasing incidence after the age of 55 (Figure 4) (4). In Norway, over 70% of patients are 65 years or older at the time of diagnosis. Autopsy studies have confirmed this pattern, with cancer prevalent in only 5% of men under 30 years, and in approximately 60% of men over 79 years (18).

Figure 4. PCa incidence rates (per 100 000 person years) in different age-groups in Norway. Reprinted with permission from Oslo: Kreftregisteret, 2016 (19)



Race and ethnicity

Migration studies have shown that when men move from low-incidence- to high-incidence areas, their risk of PCa diagnosis increase considerable (20,21). One of the most well studied groups in this aspect are Japanese Americans, who have an incidence rate 43 times higher compared to their counterparts in Japan (22,23). This variations in incidence rates may be explained by environmental factors and different strategies in diagnostics and screening. However, within the USA, the incidence rates are very different between different ethnic groups (24), which does indicate inheritable, genetic variation (25). Worldwide, the cumulative risk of being diagnosed with PCa is lowest in South-Central and Eastern Asia, and Northern Africa (between 0.6-1.7%), and highest in Australia, New Zealand, Northern Europe and North America (between 9.5-10.9%) (Figure 1) (11). The rates are highest among men of

African descent in the Caribbean and in the US. PCa in these individuals often occurs at a younger age and have a more aggressive behavior compared to other ethnic groups (10,26).

Inheritance

A positive family history of PCa is established as one of the most important risk factors for disease development. Men who have a first-degree relative with PCa have at least a 2-fold increased risk of disease compared to the general population. The risk increases with increasing number of affected relatives, and also with low age at the time of their diagnosis (27–29). Twin studies in a Nordic population have indicated that in patients affected with PCa, the heritability, which means proportion of PCa variation attributed to germline genetics, is as much as 58% (30). This makes it one of the most heritable cancers we know. A positive family history of PCa is not necessarily enough to meet the proposed criteria of hereditary PCa (HPC), which are (a) nuclear family with three (or more) cases of PCa, (b) PCa in three successive generations, or (c) at least two men in a pedigree diagnosed with the disease before the age of 55 years (31). Familial clustering of cases that do not fulfill these criteria are defined as familial PCa. Thus, HPC and familial cancer is most likely a heterogenic group, with different mutations and inheritance patterns accounting for the increased risk of disease. It is disputed whether HPC is more aggressive than sporadic PCa (32,33). The worse prognosis observed in PCa families may be due to an earlier onset of disease, combined with a delayed diagnosis (34,35). In Norway, diagnostic monitoring in terms of PSA-testing is recommended in PCa families. These men should also be offered genetic counselling, and genetic germline testing (*for further information on genetics, see section «1.1.4. Biological characteristics of prostate cancer»)*. Additionally, also men with PCa with Gleason grade ≥ 7 if 60 years old or younger, men with high-risk disease, men with metastatic PCa, men with relapse after radical treatment, and men with both PCa and another form of cancer before 60 years old should be presented with this offer (36).

Lifestyle and diet

Multiple lifestyle choices have been proposed as risk factors for PCa development. Factors associated with a possible decreased risk are exercise (37), a high intake of lycopene (38), and vitamin D (39). Suggested risk-increasers are high intake of dairy products/calcium (40), high calorie diet (41), alcohol (42), cigarette smoking (43) and obesity (44). However, though there is some evidence for these factors in the literature, they appear to be limited. Comparable studies have not been able to reproduce similar correlations, or in some cases have produced

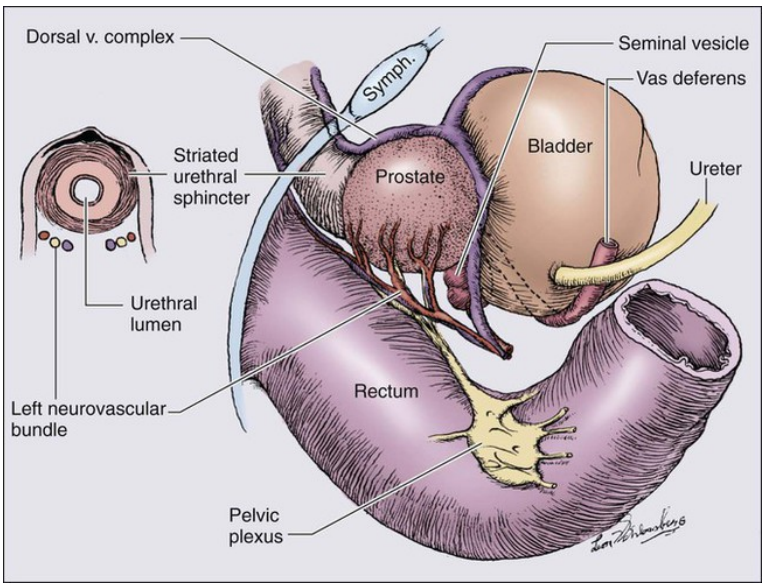
conflicting results. In Norway, the conclusion is that there is no evidence for giving lifestyle advices beyond the general national advices for diet, exercise, smoking and alcohol (36).

1.1.3. The normal prostate gland

Anatomy

The prostate gland is an exocrine organ lying in the human midline, between the pelvic floor and the base of the bladder, surrounding the bladder neck and the first part of the urethra where it is joint with the ejaculatory ducts. The proximal part is referred to as the base, and the distal part as the apex (45). Of importance in the surgical field are the location of the dorsal vein complex (46) and neurovascular bundle (47), both necessary for penile erectile function (Figure 5). In women, Skene's gland is an anatomical homolog, but is a rare location of pathology in comparison to its male counterpart (48).

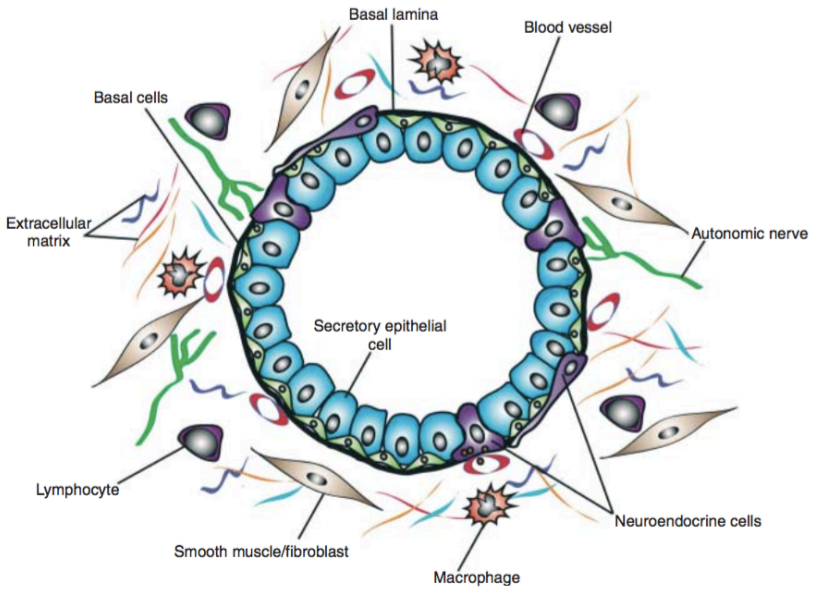
Figure 5. The anatomy of the prostate in relations to surrounding organs (49). Reprinted with permission from Elsevier© 2014.



Histology

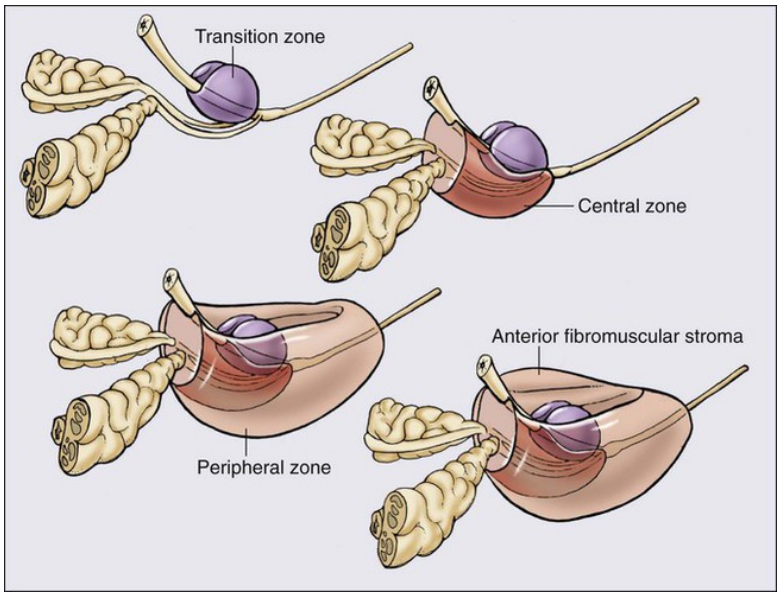
The normal prostate gland consists of glandular tissue (parenchyma) and non-glandular stromal tissue fused together inside a common fibromuscular, capsular structure. In addition, the anterior part of the gland consists of a purely stromal compartment known as the anterior fibromuscular stroma, which is not covered by the capsular structure. The glandular component is composed of multiple secretory acini, connected to a draining system of branching epithelial ducts and tubular structures, that eventually end in the prostatic urethra. Each acinus is organized as a lumen, surrounded by a simple columnar-, and regions of pseudostratified columnar, epithelium. The epithelium is lined by a layer of basal cells, and a small number of neuroendocrine cells, resting on the basal lamina separating the acini from the surrounding stromal tissue (Figure 6). The surrounding stromal tissue is composed of fibroblasts, smooth muscle cells, endothelial cells, autonomic nerve cells, immune cells and extracellular matrix (ECM) (Figure 6) (50,51).

Figure 6. Cellular components of the normal human prostate gland (51). Reprinted with permission from BioScientifica Limited© 2012.



The prostate is usually divided into four distinct zones (Figure 7); (a) The transition zone surrounds the proximal urethra and is site of origin for benign prostatic hyperplasia (BPH) as well as 20% of PCa cases, (b) the central zone encloses the ejaculatory ducts, (c) the peripheral zone account for 70% of the gland, and is where 70-80% of cancers origin, and (d) the anterior fibromuscular stroma zone which contain no glandular tissue.

Figure 7. Zones of the prostate (49). Reprinted with permission from Elsevier© 2014.



Physiology

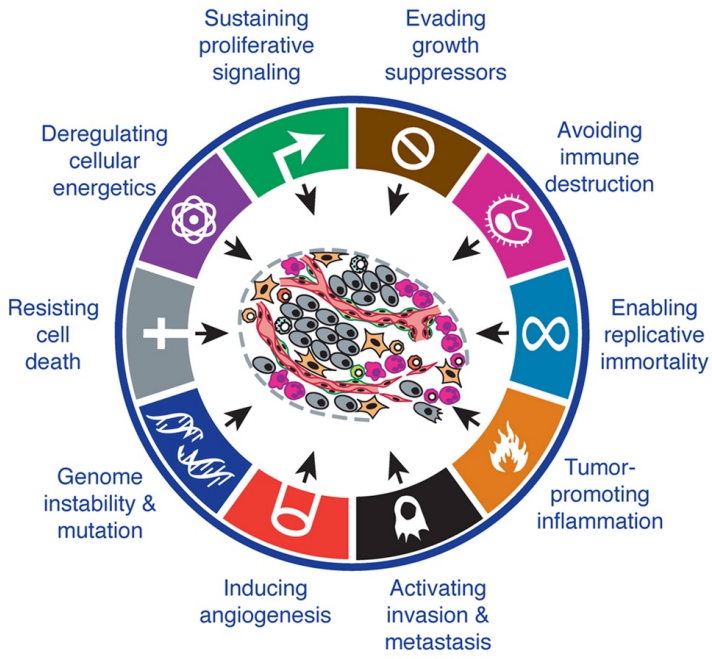
The main biological role of the prostate is to produce, temporary store and secrete prostatic fluid during ejaculation. The fluid contains high levels of zinc and citrate in addition to other electrolytes, free amino acids, and numerous different proteolytic enzymes all important for the function and transportation of spermatozoa (52). Through activation of androgen receptors (ARs), androgens such as testosterone and its more potent metabolite 5α-dihydrotestosterone, are the most important regulators of the glands' development, growth and function (45).

1.1.4. Biological characteristics of prostate cancer

The development of solid tumors is generally thought to be a multistep process, whereby successive genetic events occur in a normal cell, rendering it with increasingly malignant characteristics. The collection of traits a cell must acquire to become a successful cancer cell are known as "the hallmarks of cancer" (Figure 8). The term became wildly known by a seminal article by D. Hanahan and R. Weinberg published in 2000 (53) and its update in 2011

(54). It has since been proposed expanded and revised by others (55–57). Development of PCa has been suggested to depend on the classic hallmarks, in addition to some PCa-unique ones, especially related to androgen signaling (58).

Figure 8. Hallmarks of cancer (54). Reprinted with permission from Elsevier© 2011.



Heterogeneity and multifocality

One typical trait of PCa is multifocality. Somewhere between 56-87% of cases presents with more than one tumor foci in the gland at time of diagnosis (59,60). Recent studies have found that multifocal PCa represents different clones without a shared mutational profile, and thus each focus is thought to have independent origin (61–64). With respect to metastatic potential, studies have shown that different foci in the prostate gland have different degrees of aggressiveness, and that metastatic disease do not necessarily develop from the index tumor (65). This creates a lot of possible clinical issues. One the one hand, tumor tissue sampled by biopsy and used to decide treatment strategy may not be representative for biological aggressiveness. On the other, the heterogeneity makes the tumor able to develop treatment resistance through evolution. In addition, heterogeneity and multifocality are also huge obstacles for precision treatment, as well as focal treatment. A more well-known, but nonetheless problematic, observation is the heterogeneity in tumor aggressiveness from patient to patient, as commented in the introduction chapter.

Androgen dependence

The growth and maintenance of both normal and cancerous prostatic cells is stimulated by androgens (66), mainly testosterone and 5α-dihydrotestosterone. Most androgens are produced in the testicles and additionally a smaller amount in the adrenal glands. Further, PCa cells and stromal cells in the tumor microenvironment (TME) may acquire the ability to make androgens from cholesterol (67). PCa cells natural androgen dependence is exploited in cancer treatment *(see further information in section "1.1.7. Disease management and treatment principals").*

Genetic alterations

Somatic genomic alterations

As earlier stated, PCa is a very heterogenic disease group, and thus few somatic genomic alterations are recurrent. However, recurrent alterations include loss of function mutations on tumor suppressor genes such as TP53 (68), phosphatase and tensin homolog (PTEN) (which leads to activation of PI3K/AKT/mTOR pathway which is an important signaling pathway in PCa pathogenesis) (68), NKX3.1 (69), and/or retinoblastoma protein gene (RB1) (68). Mutations in oncogene C-MYC is also relative common, and more so in metastatic than in primary PCa tumors (70). One of the most recurrent genomic alteration, are structural rearrangements that fuse androgen-regulated promoters with ERG and other members of the erythroblast transformation-specific/ETS family of transcription factors. One example is ERG:TMPRSS which is present in approximately 50% of PCa patients, and associated with protein expression of the oncogene ERG (71,72). Mutations related to the AR and its pathway are also common, mainly in castrate-resistant PCa (CRPC) where it is a mechanism of resistance to hormone therapy (68). Missense-mutations in SPOP are the most common point mutations in primary PCa, occurring in about 10% of both clinically localized and metastatic CRPC. PCa with SPOP mutations often have characteristic genomic alterations, defining them as a distinct subclass of PCa (73).

Germline genomic alterations

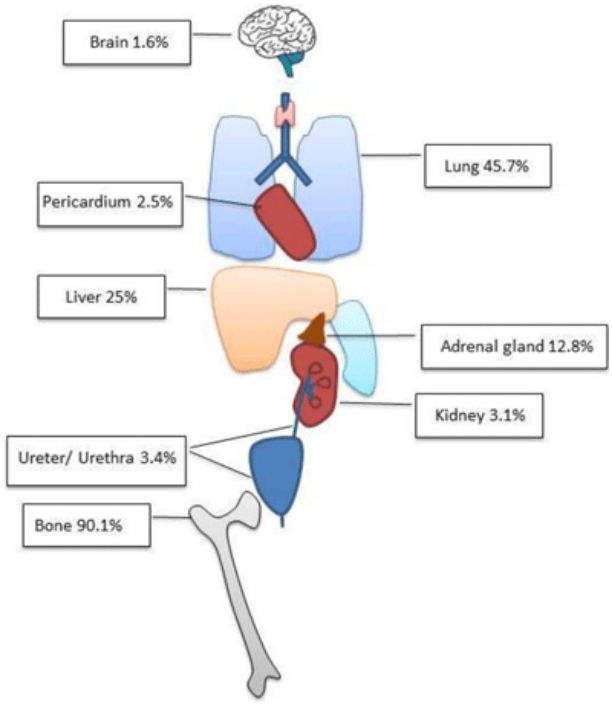
Multiple inherited germline mutations have been reported to be associated with HPC and familial cancer *(see further information in section "1.1.2. Etiology and risk factors").* Familial cancers may be due to inheritance of multiple, common low-to-moderate-penetrance risk alleles that in combination causes a predisposition for developing cancer (74,75). Contrary, it is proposed that HPC is probably caused by rare mutations in different genes,

often involved DNA-repair, which have an autosomal, dominant hereditary pattern, with a high penetrance and early onset of disease. Proposed examples are breast cancer gene (BRCA) 1 or 2 (76,77), Partner and localizer of BRCA2 (PALB2) (78), miss-match repair (MMR) genes (MLH1, MSH2, MSH6, and PMS2) (79), homeobox B13/HOXB13 (80), Ataxia-Telangiectasia Mutated (ATM) (81), checkpoint kinase 2/CHEK2 (82), RAD51 paralog D/RAD51D (83), elaC homolog 2/ELAC2 (84), Ribonuclease L/RNASEL (85), Nibrin/NBN (also known as Nijmegen breakage syndrome 1 (NBS1)) (86), and macrophage scavenger receptor 1/MSR1 (87). In men with metastatic CRPC (mCRPC), there is an approximately 12% incidence of germline mutations in DNA-repair genes (83). In Norway, proposed genetic germline testing for the time being focuses on BRCA1/2, miss-match repair genes and HOXB13 (36).

Metastatic disease

PCa dissemination can be lymphogenic to lymph nodes, or hematogenic to distant organs. The most common place for hematogenic metastases is red bone marrow in the axial skeleton, and bone lesions are primarily osteosclerotic (also known as osteoblastic) (Figure 9) (88). One study found that 72% of PCa patients had disseminated cancer cells in the bone marrow prior to radical prostatectomy (RP) (89), which suggest that PCa cells disseminate early on from the primary tumor but does not necessarily cause clinically relevant disease. Visceral metastases are less common, and mainly to lung/pleura, liver and adrenal glands (90). The biology differs from that of bone metastases, and patients with visceral metastases (especially liver metastases) have a worse prognosis than patients with bone-only metastases (91,92). In one study on primary metastatic patients, median cancer-specific survival time was 43 months for lymph node metastasis, 24 months for bone-only metastases, and 16 months for visceral metastases (93).

Figure 9. Hematogenic, metastatic pattern of PCa (90). Of patients with hematogenous metastases, 90% had bone-, 46% had lung-, 25% had liver-, 21% had pleura-, and 13% had adrenal gland metastases. Reprinted with permission from Elsevier© 2000.



1.1.5. Clinical presentation and diagnostics

The first step in diagnosing PCa is usually an elevated PSA and/or a suspect finding with digital rectal examination (DRE). Such examinations may be performed because of lower urinary tract symptoms (LUTS) or based on the individual patients wishes and perhaps concerns. The definite diagnosis is based on histopathological evaluation of prostate tissue, usually sampled from biopsies or in some cases TUR-P or prostatectomy performed in suspicion of BPH.

Symptoms

From US-based data, 77% of patients have localized cancer at time of diagnosis, 13% have lymph node metastases, 6% have distant metastatic spread, and 4% unknown metastatic status (94). With localized disease, some patients experience nonspecific LUTS with problems related to storage of urine, voiding and/or post-micturition symptoms (95). However, most patients in early stages are usually asymptomatic, and LUTS are often due to nonmalignant conditions such as BPH. If the primary tumor or lymph node metastases infiltrate or compress nearby structures, related symptoms such as hematospermia, hematuria, pain, and urine

retention with the possibility for post-renal kidney failure, may occur. Bone metastases usually leads to pain due to the pressure on the periosteum from metastatic volume, or pathologic fractures. A feared complication is spinal cord compression caused by bone metastases in the spine, potentially causing permanent neurological paresis. General symptoms can include weight loss and fatigue.

Prostate-specific antigen testing in diagnosing prostate cancer

PSA is a proteolytic enzyme produced almost exclusively by prostatic epithelial cells as a part of the seminal fluid. It is present in small quantities in the serum of healthy men, but the quantity increases with cellular disruption of the prostate epithelium and/or an increased number of epithelial cells. Thus, an elevated PSA-level in serum can be present in both benign and malignant conditions of the prostate but can also have less sinister causes such as moderate perineal trauma from a long bike ride. Since the first article on its use as a diagnostic test was published in 1987 (12), it has become the most used and valuable test for early detection of PCa. Still, PSA-testing is not without challenge. Specificity is decreased because benign conditions such as BPH, prostatitis, urinary tract infections and urinary retention are frequent reasons for PSA elevation. Sensitivity is challenged by the fact that in cases with high grade PCa (typically Gleason 5+5) where cellular differentiation is low and/or in cases with neuroendocrine trans-differentiation *(see further information in section on "1.1.7. Disease management and treatment principals"),* cancer cells do often not produce PSA. Thus, serum levels may be normal despite a severe condition. Moreover, local forms of PCa may not elevate systemic PSA levels and 5α-reductase inhibitors used for BPH decrease the PSA-level. An additional challenge is the fact that an elevated PSA may help detect a large group of cancers that are clinically irrelevant (96).

Studies have not conclusively identified one single threshold for defining an abnormal PSA-value applicable to every patient. Different approaches can be used in each case; the PSA-value can be compared to what is considered normal in a given age group (Table 1), the value can be compared to the patients' prior values, for example if the PSA-increase of more than 0.75 ng/mL in one year. The generally accepted cut-off value for all age groups is 4 ng/mL. Still, with a PSA value under 10 ng/mL, BPH is statistically more likely than cancer. For a PSA value in a range between 10 to 30 ng/mL PCa is the most likely reason, and a PSA value over 100 ng/mL is almost always due to disseminated PCa (36). PSA density refers to the level of serum-PSA divided by the transrectal ultrasound (TRUS)-determined volume and can

be used to evaluate how likely it is that PCa is the cause of an elevated PSA-value (97). An elevated PSA-test needs to be verified with a new test after 2-3 weeks. In addition to diagnostic value, PSA-testing is used in risk stratification of PCa patients *(see further information in section "1.1.6. Prognostication")*, and monitoring of patients treated for, under active treatment or under active surveillance for PCa *(see further information in section on "1.1.7. Disease management and treatment principals")*.

Table 1. Proposed cut-off values for PSA. Adapted from the Norwegian Guidelines for PCa (36)

Age	PSA value
< 50 years	3.0 ng/mL
50 – 59 years	3.5 ng/mL
60 – 69 years	4.5 ng/mL
> 69 years	6.5 ng/mL

Digital rectal examination

DRE is used to identify possible changes in consistency or volume on the posterior and lateral surface of the prostate gland. Not all tumors can be detected with DRE, as some are T1 tumors who by definition are not palpable, and others are in regions not reachable via the rectum (98). Depending on the level of fitness of the individual patient, suspicious findings for PCa such as nodules, induration, or asymmetry, are almost always indications of further examinations such as biopsies, regardless of the PSA-level.

New diagnostic tools

It is of utmost importance to avoid overdiagnosis of clinically irrelevant PCa cases. To help decide if biopsies should be conducted, tests (Table 2), and risk calculators such as the European Randomized Study of Prostate Cancer risk calculators/ERSPC-RCs (99), the Prostate Cancer Prevention Trial calculator (100) or the Montreal model has been developed (101).

Table 2. New diagnostic tools in PCa

Test	Description
Prostate cancer antigen 3 (PCA3) (urine)	A non-coding mRNA highly overexpressed in PCa tissue, that can be detected in urine obtained after DRE. Indicates cancer risk before biopsy and after a negative biopsy, but has low correlation to clinically relevant cancer (102).
TMPRSS2:ERG fusion (urine)	Gene fusion present in approximately 50% of PCa cases *(see section "1.1.4 Biological characteristics of prostate cancer")*. mRNA can be measured in urine (103).
Kallikrein panel (4k-panel serum)	A panel of total PSA, free PSA, intact PSA, and human kallikrein 2 in serum (102).
Prostate Health Index (PHI) (serum)	Serum measurement of total PSA, free PSA and p2PSA, combining them into a total score (104).

Biopsies

After a magnetic resonance imaging (MRI) of the prostate is performed, biopsies are the next diagnostic step. Biopsies are usually performed transrectal under guidance of TRUS and the standard is a total of 10-12 systematic biopsies from the gland (105). Additional biopsy cores should be sampled from areas deemed suspicious by DRE, TRUS and/or MRI. The patient receives prophylactic antibiotics to minimize the risk of infection, but still approximately 1-3.5% of patients still need treatment for sepsis (106). Other side-effects include haematospermia, haematuria, rectal bleeding, prostatitis, epididymitis, and urinary retention. It is recommended to do re-biopsies in patients with initial negative biopsies if DRE, MRI and/or PSA leves are suspicious for cancer, and/or if the histopathological pattern is suggestive but not conclusive of malignancy at initial biopsy.

Histopathological assessment for diagnosis

The prostate is place for both cancerous and non-cancerous conditions (Table 3), and neoplastic conditions are classified according to the 2016 World Health Organization (WHO) Classification of Tumours (107).

Table 3. Disease categories and histopathological classification of prostatic disease. Based on a table from the 2016 WHO Classification of Tumours (107)

Disease category	Disease	Subtypes
(A) Prostatitis	Acute bacterial prostatitis Chronic bacterial prostatitis Chronic pelvic pain syndrome (CPPS)/ (also known as) Chronic nonbacterial prostatitis Asymptomatic inflammatory prostatitis	
(B) Benign lesions, precancerous neoplasia and neoplasia with uncertain malignant potential	Benign prostatic hyperplasia Atypical adenomatous hyperplasia (adenosis) Low grade intraepithelial neoplasia High grade intraepithelial neoplasia of the prostate Intraductal carcinoma (without associated invasive adenocarcinoma) Atypical small acinar proliferation Atrophic lesions	
(C) Malignant neoplasia	Epithelial tumors	Glandular neoplasms - Acinar adenocarcinoma (most common) *(atrophic, pseudohyperplastic, microcystic, foamy gland, mucinous, signet ring-like cell, pleomorphic giant cell, sarcomatoid)* - Ductal adenocarcinoma *(cribriform, papillary, solid)* - Intraductal carcinoma (acinar or ductal) - Urothelial carcinoma (Transitional cell cancer) Squamous neoplasms - Adenosquamous carcinoma - Squamous cell carcinoma Basal cell carcinoma
	Neuroendocrine tumors	Adenocarcinoma with neuroendocrine differentiation Well-differentiated neuroendocrine tumor (carcinoid) Small cell neuroendocrine tumor Large cell neuroendocrine tumor
	Mesenchymal tumors	Different sarcomas etc.
	Haematolymphoid tumors	Different lymphomas/leukemias

Precancerous neoplasia and neoplasia with uncertain malignant potential

Prostatic intraepithelial neoplasia (PIN) is defined as a noninvasive, neoplastic proliferation of glandular epithelial cells confined to preexisting acinar structures and, contrary to cancer, contains basal cells. PIN is commonly divided into low-grade (LGPIN) and high-grade (HGPIN), with HGPIN showing cytological atypia in addition to abnormal proliferation. HGPIN is a known precursor of cancer, and if found in biopsies, further clinical follow-up and possibly repeat biopsies may be indicated due to its association with invasive PCa. LGPIN has no clinical significance. Other intraepithelial neoplasia includes *intraductal carcinoma/IDCP* (Table 3) which consist of neoplastic epithelial cells with architectural and cytological atypia, present intra-acinar and/or intraductal (107). It is thought to mainly represent intraductal spread of aggressive carcinoma into preexisting ducts and acini, but in approximately 10% of cases the condition is found without associated invasive adenocarcinoma (108). *Atypical small acinar proliferation/ASAP* is another intraepithelial neoplasia, which is a diagnostic term, rather than a biological entity or a premalignant lesion. It refers to small focus of atypical glands falling short of the threshold for the diagnosis of PCa. If found in biopsies, repeat biopsies are usually recommended as the risk of finding adenocarcinoma is 40-50% (109). Sometimes, *atrophic lesions* can be identified microscopically. Atrophy is identified as a reduction in the volume of preexisting glands and stroma and can be classified into diffuse (affecting the whole gland) or focal. Diffuse atrophy results from a decrease in circulating androgens, whereas focal atrophy does not. Focal atrophy may be further divided into sclerotic-, simple-, or postatrophic hyperplasia atrophy (110). If there is inflammation in addition to focal simple or focal postatrophic hyperplasia, the term "proliferative inflammatory atrophy" (PIA) is used (111). Most atrophic lesions are considered benign conditions, but PIA is suggested as a precursor of HGPIN and/or adenocarcinoma *(see further information in section "1.2.4. The immune system in prostate cancer").*

Prostate malignancy

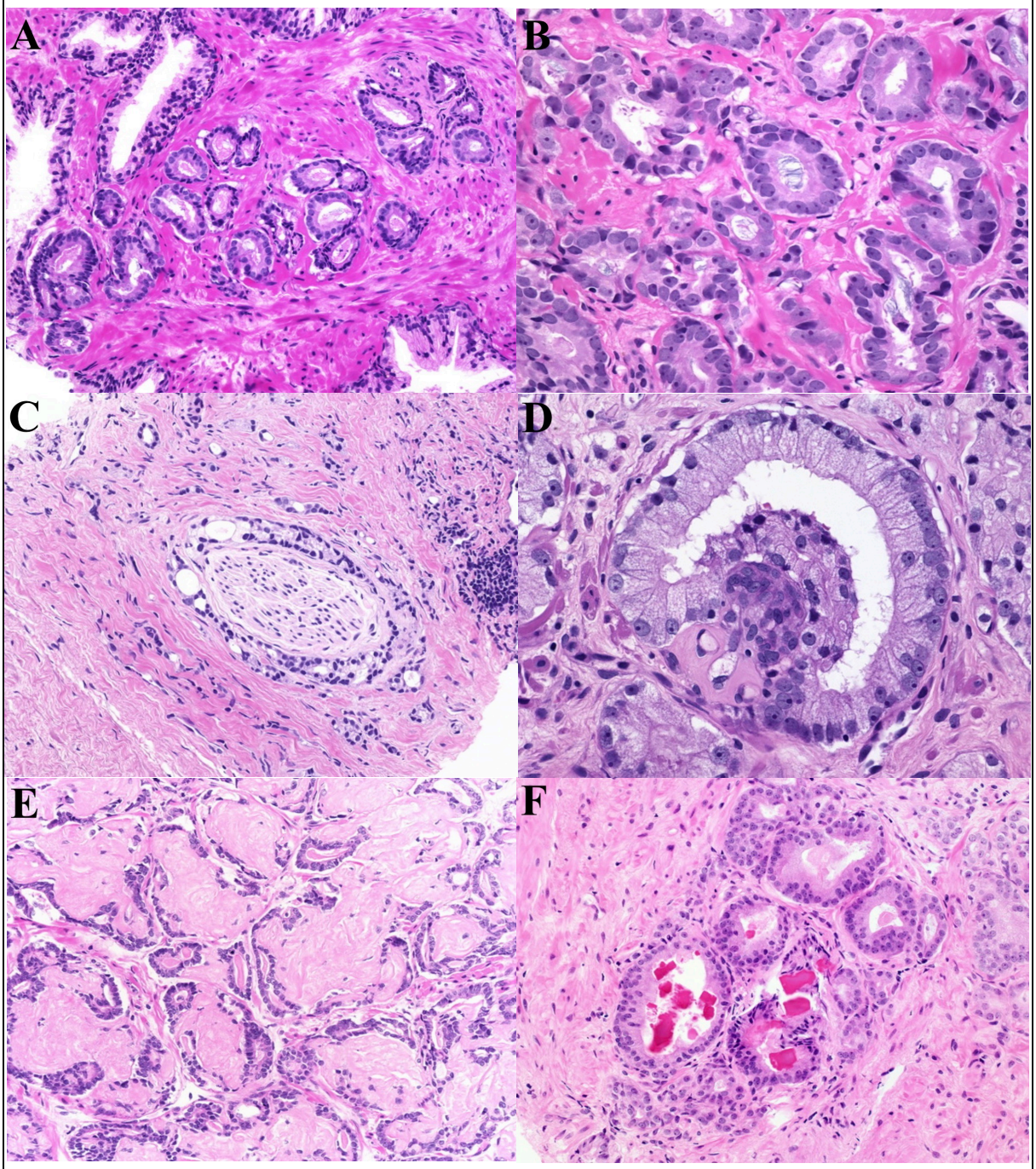
The diagnosis of PCa is ultimately based on histopathological examination of prostate tissue. Adenocarcinoma is the most common type of PCa, comprising 95-99% of PCa cases (Table 3). In the adenocarcinoma group, acinar adenocarcinoma is the most prevalent, representing 95-99% of adenocarcinoma cases, and is what is usually referred to as "prostate cancer".

The histopathological diagnosis of PCa relies on a combination of *architectural* (structural changes in the tissue) and *cytological* (altered cell morphology) features (Figure 10). *Architectural features* (Figure 10 and 11) include an infiltrative growth pattern with disorganized arrangement of clusters, single atypical glands or single cells variably scattered between more complex benign glands. Malignant glands can be ill-formed, fused, glomeruloid or cribriform. They can be closely packed and organized in a crowded linear arrangement spanning the whole width of the biopsy core, or as cords of cells (112,113). *Cytological features* consist of nuclear and nucleolar atypia and altered cytoplasm. The nucleolus is located inside the nucleus and is an important site in making ribosomes that are necessary for protein synthesis. Prominent nucleolus, or two or more nucleoli, is a typically visible in cancer. However, it is important to mention that foamy carcinoma is characterized by bland nuclear features without prominent nucleoli, and that HGPIN and benign conditions may also have prominent nucleoli. The cytoplasm of malignant cells often has a more amphiphilic cytoplasm compared to paler color in benign glands. In addition, there are different intraluminal contents typical, but not exclusively, for PCa (Figure 10, Table 4) (112,113). The histopathological diagnostic criteria for PCa can be divided into major traits, minor traits and pathognomonic features (Table 4).

Table 4. Major and minor criteria and pathognomonic histopathological features of PCa. Based on a table from (112,114)

Criteria	
Major criteria	Infiltrative growth pattern: Infiltrative small glands or cribriform glands too large or irregular to represent high-grade prostatic intraepithelial neoplasia.
	Single layer of epithelium (absence of basal cells).
	Nuclear atypia: Nuclear and nucleolar enlargement
Minor criteria	Intraluminal contents: - Wispy blue mucin (blue-tinged mucinous secretions) - Pink amorphous secretions - Intraluminal crystalloids
	Mitotic figures
	Adjacent HGPIN
	Amphiphilic cytoplasm
	Nuclear hyperchromasia
Pathognomonic features	Perineural invasion (PNI)
	Extraprostatic extensions
	Invasion of seminal vesicles
	Glomerulation: Glands with a cribiform proliferation attached to only one edge of the gland, resulting in a structure resembling the glomerulus of the kidneys.
	Mucinous fibroplasia/collagenous micronodule: Nodules of hypocellular eosinophilic stromal tissue that are present within and around malignant glands, often causing considerable distortion of their shape.

Figure 10. Histopathological features of acinar adenocarcinoma. Pictures reprinted with permission from WebPathology.com© 2021.



(A) Architectural features with small, crowded glands and round or oval lumens. **(B)** Cytological features with nuclear enlargement, prominent nucleoli, amphiphilic cytoplasm, and intraluminal blue mucin. **(C)** Perineural invasion (PNI). **(D)** Glomerulation. **(E)** Mucinous fibroplasia/collagenous micronodule. **(F)** Intraluminal crystalloids.

Immunohistochemistry in diagnostics

Immunohistochemical (IHC) staining is used to some degree in PCa adenocarcinoma diagnostics, but so far not for prognostic purposes. IHC can be used if uncertainty concerning presence of basal cells (major cancer criteria, Table 4). To examine if basal cells are present, IHC for high molecular weight cytokeratin (detected by 34βE12 antibody binding) and p63 can be used. Absence of basal cell antibodies in combination with a with positivity for α-Metylacyl Coenzyme A racemase/AMACR (also known as p504s enzyme) increase cancer suspicion. PSA, prostate-specific acid phosphatase (PAP), prostein/P501S, or NKX3.1 can be helpful in diagnosing a prostatic acinar cell origin on metastatic tissue with unknown origin. Other options for this purpose are ERG:TMPRSS fusion protein which is relatively specific for PCa, but only present in about 50% of cases *(see further information in section 1.1.4. Biological characteristics of prostate cancer)* (115).

Image diagnostics

Magnetic resonance imaging

In Norway, MRI is the first step for patients referred to a urologist with suspicion of PCa. It is used for detection of suspicious lesions (116), localization of suspicious lesions which can guide targeted biopsies (117), and mapping pelvic extent of disease for TNM-staging. A negative MRI is not sufficient to rule out PCa and therefore is not advised as an initial screening tool (36,118,119). Diffusion-weighted whole-body and axial MRI is also more sensitive and specific than most other imaging techniques for bone metastasis, with the probable exception of Prostate-specific membrane antigen-Positron emission tomography (PSMA-PET) (119,120).

Transrectal Ultrasound

Grayscale TRUS is not reliable in detecting PCa but used in guiding biopsies and calculating size (121). Sonographic modalities such as contrast-enhanced ultrasound (122) and sonoelastography (123) for detection and use in ultrasound-targeted biopsies is under investigation.

Radionuclide bone scan

A radionuclide bone scan (also known as bone scintigraphy) has been the preferred technique for identifying bone metastases in newly diagnosed PCa patients, however PSMA-PET is increasingly used for this purpose (see below). A radionuclide bone scan has a 78%

sensitivity and an 85% specificity for bone metastases (124). Conditions such as arthritis and bone trauma can cause false positive results. Adding single-photon emission computed tomography/SPECT to plain bone scan has been shown to reduce the number of equivocal lesions (125).

Positron emission tomography

PET, usually in combination with a computed tomography/CT unit, can produce images that locate cancer-suspicious areas. To achieve this, the patient is given a radiotracer designed to accumulate in cancerous tissue. Common tracers, such as 11C- or 18F-choline which detects tissues with a high metabolic activity, has low sensitivity in PCa probably because of its slow-growing nature. In addition, accumulation in the bladder can disturb visualization of the prostate gland and/or regional lymph nodes (126). PSMA is a receptor on PCa cells expressed in 90-95% of cases. Thus, a ligand for PSMA labelled with 68Ga or 18F has a relatively high detection rate for PCa cells (127,128). PSMA-PET is increasingly used as a staging-tool for treatment strategy. It is also important as a diagnostic tool in cases with biochemical recurrence after radical treatment, but only if the patient may be candidate for radical locoregional treatment and has a life-expectancy > 10 years. 18F-Fluciclovine is another relevant tracer for PCa with the same purpose as PSMA. The radiotracer 18F-sodium fluoride (18F-NaF) can be used for detecting bone metastases but does not detect nodal or soft tissue metastases (129).

1.1.6. Prognostication

Because of disease heterogeneity, it is of utmost significance to risk-stratify cases to avoid over- or undertreatment.

Staging

PCa is staged using the TMA-system, which describes the anatomical extent of disease (Table 5). The letters stand for what they describe; primary tumor (T), dissemination to regional lymph nodes (N), and distant metastases (M). The initial staging is a clinical staging where T stage is based on DRE (MRI and other imaging techniques should not be used according to the 8th AJCC edition (130), while other guidelines open up for MRI for this use (36,119,131,132). Patients who undergo RP as cancer treatment are in addition assigned a pathological T stage (pT) (Table 5) which is considered more accurate. N and M-stage is decided through clinical examination and/or image diagnostics.

Table 5. The TNM classification system. Developed jointly by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC). Adapted from (130)

Primary tumor (T)	
Clinical T (cT)	
T category	**T criteria**
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically inapparent tumor that is not palpable
T1a	Tumor incidental histologic finding in 5% or less of tissue resected
T1b	Tumor incidental histologic finding in more than 5% of tissue resected
T1c	Tumor identified by needle biopsy found in one or both sides, but not palpable
T2	Tumor is palpable and confined within prostate
T2a	Tumor involves one-half of one side or less
T2b	Tumor involves more than one-half of one side but not both sides
T2c	Tumor involves both sides
T3	Extraprostatic tumor that is not fixed or does not invade adjacent structures.
T3a	Extraprostatic extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall.
Pathological T (pT)	
T category	**T criteria**
T2	Organ confined
T3	Extraprostatic extension
T3a	Extraprostatic extension (unilateral or bilateral) or microscopic invasion of bladder neck
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall
Regional lymph nodes (N)	
N category	**N criteria**
NX	Regional nodes were not assessed
N0	No positive regional nodes
N1	Metastases in regional node(s). Regional lymph nodes include pelvic nodes located below the bifurcation of the common iliac arteries and can be uni- or bilateral.
Distant metastasis (M)	
M category	**M criteria**
M0	No distant metastasis
M1	Distant metastasis
M1a	Nonregional lymph node(s)
M1b	Bone(s)
M1c	Other site(s) with or without bone disease
NOTE: When more than one site of metastasis is present, the most advanced category (M1c) is used.	

Prostate-specific antigen in prognostication of prostate cancer

In addition to being used for screening and diagnosing PCa, PSA-testing is an important part of prognostic assessment. Two methods of measuring PSA kinetics, namely PSA velocity and -doubling time, have limited diagnostic use, but may guide treatment strategy (133). PSA velocity measures the absolute annual increase in serum PSA (ng/mL/year) and tells us how quick PSA is rising (134). PSA doubling time measures the exponential increase in serum PSA over time and estimate how much time it will take for the PSA-value to double (135).

Histopathological assessment for prognostication

Gleason grading and ISUP Grade Groups

The Gleason grading system was developed between 1966 and 1977 by American pathologist Dr. D. Gleason and his colleagues at the Veterans Administration Cooperative Urologic Research Group (136–140). It is still regarded as one of the most important prognostic tools for PCa. The system has been revised multiple times since it was first reported (141), and today the most used version is the "2005 International Society of Urological Pathology (ISUP) Modified Gleason system" (142) in combination with the "2014 ISUP Contemporary Gleason Grading system" (143–145). The Contemporary Gleason Grade Group system have been incorporated into the 8th edition TNM PCa staging (Table 5) (130) as well as the 2016 WHO classification of genitourinary tumours (Table 3) (107). The Gleason system is based solely on *architectural pattern* of cancerous glands, and the patterns are graded 1-5, where 5 refers to the most dedifferentiated glandular tissue (Figure 11). A tumor is always assigned with two grades that can be summed up to a Gleason score and categorized in a designated ISUP Grade Group (Table 6).

Both biopsies and RP specimens, but not metastatic tissue, are graded according to the Gleason system. When grading tumor material from needle biopsies, the Gleason score is the summation of *the most common* plus *the highest grade* pattern, regardless the amount of the latter. The reason for this is that any amount of high-grade tumor sampled on needle biopsy is likely an indication of a more significant amount of high-grade tumor present within the prostate. In addition, Gleason scores 2-4 should not be assigned to cancer on needle biopsy, mainly because there is likely more high-grade cancer present. Thus, most of the lesions that appear to be very low grade on needle biopsies are currently diagnosed by uropathologists as Gleason score 3+3 = 6 (146). Grading tumor material from RPs is different. As a general rule, *the most- and second-most common* grade pattern is reported. However, if grade pattern 4 is

present to a lesser degree, even if not second-most common, this shall be reported as the second-grade pattern anyways. A comment should be added if the percentage is under 5%. If there is a grade pattern 5 present as the third most common pattern, and the amount is under 5% of the tumor, this should be reported as "a minor high-grade pattern" (also known as a tertiary pattern). If there is more than 5% grade pattern 5 present, this should be reported as the second-grade pattern (141,147). The minor high-grade pattern is not yet incorporated with the ISUP Grade Groups-system.

Figure 11. Gleason patterns. Figure (148) by Dr. D. Grignon reprinted with permission from Indiana University School of Medicine© 2015

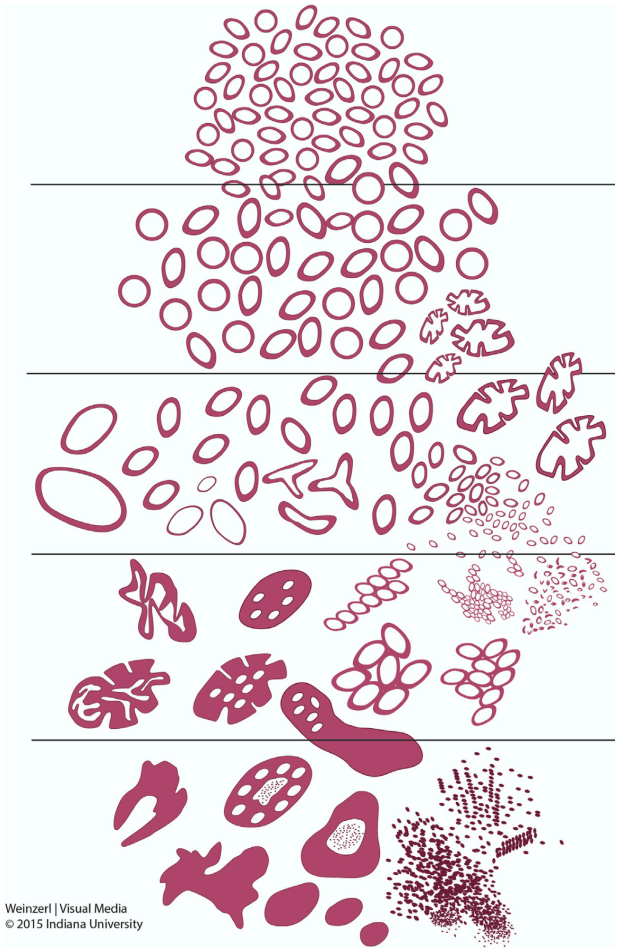
Gleason grade	Gleason pattern	Visuals
Grade 1	Circumscribed nodule of closely packed but separate, uniform, rounded to oval, medium-sized acini (larger glands than pattern 3).	
Grade 2	Like pattern 1, fairly circumscribed, yet at the edge of the tumor nodule there may be minimal infiltration. Glands are more loosely arranged and not quite as uniform as Gleason pattern 1.	
Grade 3	Discrete glandular units. Smaller glands than seen in Gleason pattern 1 or 2. Infiltrates in and among non-neoplastic prostate acini. Marked variation in size and shape.	
Grade 4	Fused microacinar glands. Ill-defined glands with poorly formed glandular lumina. Large cribriform glands. Cribriform glands. Glomeruloid glands.	
Grade 5	Essentially no glandular differentiation, composed of solid sheets, cords, or single cells. Comedocarcinoma with central necrosis surrounded by papillary, cribriform, or solid masses.	

Table 6. Histologic grade groups. Adapted from (145)

ISUP Grade Group	Gleason score	Gleason grade
1	≤6	≤3+3
2	7	3+4
3	7	4+3
4	8	4+4, 3+5, 5+3
5	9 or 10	4+5, 5+4, or 5+5

Both Gleason scores (139,149) and ISUP Grade Groups (143,144) are strongly associated with clinical outcome in terms of a greater likelihood of having disseminated disease, as well as a worse outcome after treatment of localized disease. The higher the score/group, the worse the prognosis.

Other histopathological assessments of prognostic value

The histopathological evaluation of biopsies should include an *estimate of tumor volume*. Both the number of involved biopsy cores, and the extent of tumor tissue within each biopsy core should be reported. A high estimated tumor volume in biopsies, have been linked to a worse prognosis after primary curative treatment, especially in the intermediate risk group (Table 8) (150,151) and is a predictor of other negative prognostic histopathological variables (152,153). More controversial however, is the independent prognostic value of *PCa volume in RP specimens* (154,155). Some report that the largest diameter of the index tumor is highly correlated to total PCa volume (156) and is an important prognostic factor (154,157).

Perineural invasion (PNI) (Figure 10) can be defined in slightly different ways (158,159) but referrers to circumferential growth of cancer cells within the perineural space. It is believed to be a major mechanism for local advancement of PCa, with cancer cells growing along the nerves and using them as a bridge from the inside to the outside of the prostate capsular structure (160). If extensive, the likelihood of finding PNI in a biopsy core increases. Thus, this is thought to explain why PNI found in biopsies is a pre-operative predictor of adverse pathologic features, such as extraprostatic extension (EPE), seminal vesicle invasion, and positive surgical margins (161,162). To the contrary, the prognostic impact of PNI in prostatectomy specimens is disputed (159,163).

Lymphovascular invasion (LVI) is defined as the presence of cancer cells within a vascular or lymphatic, endothelium-lined space, and if present, should be reported in pathology reports on both biopsies and RP specimens (164) as it is stated to be an independent negative prognostic

factor (165,166). The most likely biological explanation for this is that LVI is an indicator of lymphatic or hematogenous dissemination of disease.

Extraprostatic extension (EPE), and thus a pT3a tumor, is a known negative prognostic factor for PCa (167,168). It can sometimes be found in biopsy material; however, it is usually diagnosed on RP specimens. It is defined as cancer cells beyond the confines of the prostatic capsular structure, and into periprostatic adipose tissue, and/or extending into/around the neurovascular bundle, and/or beyond the anterior prostate, as well as microscopic invasion of smooth muscle fibers in bladder neck (it is a pT4 if the tumor invades the bladder muscle wall macroscopically). At the apex of the prostate, tumor mixed with skeletal muscle does not constitute EPE (164).

Seminal vesicle invasion (SVI), and thus a pT3b tumor, can sometimes be diagnosed in biopsy material, and should always be addressed in RP reports. It is predictive of both local relapse and disseminated disease (168,169).

A positive surgical margin (PSM) is defined as cancer cells present at the edge of the RP resection specimen (170). In PCa, the location of the PSM is of relevance in relation to prognosis, with PSM in the apical parts of the prostate being a better prognostic feature than other locations (159,171,172).

Some *histological classifications* of acinar adenocarcinoma are correlated with a worse prognosis, such as signet ring-like, sarcomatoid and pleomorphic giant cell acinar adenocarcinoma (Table 3) (173). Also of prognostic relevance is neuroendocrine trans-differentiation *(see further information in section on "1.1.7. Disease management and treatment principals"),* which have a significantly poorer prognosis.

Genetic analyses of tumors

In PCa patients, as for most other cancers, there are increasing recommendations and possibilities to do genomic profiling (36,119,131,132). Testing of both germline mutations and tumor genomics are probably necessary for precision treatment, as different mutations predict sensitivity or resistance towards certain therapies. For example, mismatch repair deficiency (dMMR) which can lead to high microsatellite instability (MSI-H) may indicate eligibility for immune checkpoint-inhibitors, and mutations in BRCA1, BRCA2, ATM,

PALB2 or FANCA may imply that treatment with a poly (ADP-ribose) polymerase (PARP) inhibitor can be effective *(see further information in section "1.1.7. Disease management and treatment principals")* (174). The European Society for Medical Oncology (ESMO) recommend next generation sequencing of PCa tissue for metastatic PCa patients, and as a minimum testing for somatic mutations in BRCA1/2 and MSI-status (175).

Summary prognostication

In combination, prognostic data allow for stage-grouping (Table 7) and stratification of patients into risk categories (Table 8), in order to guide initial treatment strategy. Different risk classification systems have been developed by various cancer- and urological organizations (176), including the National Institute for Health and Clinical Excellence (NICE, UK), ESMO, National Comprehensive Cancer Network (NCCN, USA), American Urological Association (AUA, USA), the European Association of Urology (EAU) – the last two as adaptions of D'Amico's classification system (177). Additionally, the patients' overall fitness, life-expectancy and individual preferences are also important in guiding treatment.

Table 7. Prognostic Stage Groups constructed by AJCC. Adapted from (130)

When T is	And N is	And M is	And PSA is	And ISUP Grade Group is	The stage is
cT1a-c, cT2a	N0	M0	< 10	1	**I**
pT2	N0	M0	< 10	1	**I**
cT1a-c, cT2a, pT2	N0	M0	≥ 10 < 20	1	**IIA**
cT2b-c	N0	M0	< 20	1	**IIA**
T1-2	N0	M0	< 20	2	**IIB**
T1-2	N0	M0	< 20	3	**IIC**
T1-2	N0	M0	< 20	4	**IIC**
T1-2	N0	M0	≥ 20	1-4	**IIIA**
T3-4	N0	M0	Any	1-4	**IIIB**
Any T	N0	M0	Any	5	**IIIC**
Any T	N1	M0	Any	Any	**IVA**
Any T	Any	M1	Any	Any	**IVB**
Note: When neither PSA nor ISUP Grade Group is available, grouping should be determined by T category and/or either PSA or ISUP Grade Group as available.					

Table 8. Risk Stratification for clinically localized disease. Based on parts of the NCCN Guidelines 4.2019 PROS-2 (178)

Risk group	Clinical/pathologic features
Very low	T1c AND Gleason score ≤ 6/ISUP Grade Group 1 AND PSA < 10 ng/mL AND Fewer than 3 prostate biopsy cores positive, ≤ 50% cancer in each core AND PSA density < 0.15 ng/mL/g
Low	T1-T2a AND Gleason score ≤ 6/ ISUP Grade Group 1 AND PSA < 10 ng/mL
Favorable intermediate	T2b-c OR Gleason score 3+4=7/ ISUP Grade Group 2 OR PSA 10-20 ng/mL AND Percentage of positive biopsy cores < 50%
Unfavorable intermediate	T2b-c OR Gleason score 3+4=7/ ISUP Grade Group 2 or Gleason score 4+3=7/Grade Group 3 OR PSA 10-20 ng/mL
High	T3a OR Gleason score 8/ ISUP Grade Group 4 or Gleason score 4+5=9/ ISUP Grade Group 5 OR > 4 cores with Gleason score 8-10/ ISUP Grade Group 4 or 5
Regional	Any T, N1, M0
Metastatic	Any T, any N, M1

1.1.7. Disease management and treatment principals

As cancer treatment is an ever-evolving field, and treatment guidelines vary between countries, this chapter aims to give an overview of treatment strategies, without being too specific.

Deferred treatment

Because of the frequent indolent nature of PCa, many patients will not benefit from curative PCa treatment (1). For the purpose of avoiding unnecessary treatment and treatment-related side-effects and costs, two conservative regimes with different intentions are used. *Active surveillance* is a structured monitoring of patients using PSA, DRE, re-biopsies and MRI (179–182). If progression occurs, or sometimes on a patient's request, active, curative treatment may be initiated. The strategy may be suitable for patients with very low, low, or subgroups of favorable intermediate risk PCa. Contrary, *watchful waiting* is used for patients who are not willing to, or suitable for, receiving treatment with curative intent (180,183,184). It is a palliative strategy where the aim is to only give treatment to relieve cancer related symptoms.

Surgical procedures

Curative surgery

Radical prostatectomy (RP) consists of surgical removal of the whole prostate gland and partial or total excision of the seminal vesicles. RP is a primary, curative treatment option applicable for patients in the low/intermediate risk group, especially for men with urinary obstruction or relative contraindications for radiation, such as inflammatory bowel disease, very aggressive but localized disease, a large prostate volume, low age with a risk of developing secondary cancer if radiated etc. In addition, cT3 tumors can in some cases be suitable for RP instead of radiation therapy (often together with extended lymph node dissection) (185). Life-expectancy should be over 10 years. Although RP is considered standard care for localized PCa in fit patients, its documented survival benefit is based on surprisingly few high-quality studies (179,180,184), and number needed to treat is relatively high for many subgroups of prostate cancer patients (184,186,187). In addition to low peri- and postoperative risks (0-1,5% mortality), the most important long-term side-effects include urinary stress incontinence, erectile dysfunction and stricture of the vesico-urethral anastomosis (188). Hence, unilateral or bilateral nerve-saving surgery to reduce risk for side-effects should be offered if oncological safety can be preserved (36). RP can be performed by open or by minimal invasive techniques such as traditional laparoscopy or robot-assisted laparoscopy. Open surgery is done through a retropubic, or more rarely, a perineal access. For many centers in high/very high HDI countries, robotic-assisted laparoscopic radical prostatectomy (RALP) is now the most commonly used method. In materials with a short follow-up, open RP and RALP have been reported to give equivalent early oncological outcomes (189,190). Some studies report the rate of PSM is to be lower for RALP patients compared to open RP (191,192) which may have a long-term oncological effect, while others have not found such correlations (190,193). RALP and open RP have approximately the same rate of long-term side effects such as stress incontinence and erectile dysfunction, however, RALP is reported to be superior when it comes to perioperative side-effects such as shortened hospitalizations and reduced risk for transfusions and strictures (189,190).

Extended pelvic lymph node dissection (ePLND) should be performed in addition to RP for patients with T3 tumors, if high-risk cancer, or if intermediary-risk cancer with > 5% risk of lymph node metastases assessed pre-operatively by nomograms such Briganti (194,195). There is no proven oncological effect of ePNLD (196), but node-positive disease can guide adjuvant androgen treatment. Side-effects include lymphocele and lymphedema (197).

Palliative surgery

Palliative surgery options focus on symptomatic relieve in locally advanced disease. Alternatives are adenectomy procedures designed for BPH which can be performed transurethral (e.g. TUR-P) or open (e.g. Millin's technique), and sometimes palliative RP or palliative radical cystoprostatectomy (198).

Radiation therapy

Primary curative radiation therapy (RT)

External beam radiation therapy (EBRT) is a primary, curative treatment option applicable for all risk groups of non-metastatic PCa and life-expectancy over 10 years. It is standard treatment for locally advanced tumors (T3N0M0), but there are ongoing clinical trials for RP versus RT for these patients (185). Standard fractions are 1.8-2.2 Gray up to 78 Gray in intermediate and high-risk patients, but hypofractionated regimes seems to be equally effective (199,200). For patients in the intermediate- and high-risk group, or patients with locally advanced disease treated with EBRT, hormone therapy is added neoadjuvant, concomitantly and/or adjuvant to RT. The treatment time for hormone therpy should be 6 months for the intermediate risk group, and up to 2-3 years for the high-risk group and for patients with locally advanced tumors (201–203). Most prominent long-term side-effects of EBRT include gastrointestinal complications such as fecal incontinence, fecal urge, rectal bleeding (204), urinary incontinence (205) and erectile dysfunction (though more rarely than with RP) (206). However, it is important to be aware of an increased risk of development of secondary cancers, mainly bladder and colorectal cancers (207).

Brachytherapy is a form of "internal" radiotherapy where a sealed radiation source is placed inside, or next to, the area requiring treatment. This gives the benefit of a higher radiation dosage to the central parts of the prostate, and lower radiation to surrounding structures compared to EBRT. There are two types of brachytherapy used in PCa patients. The *low dose brachytherapy/LDR-BT* procedure is done by TRUS-assisted placement of permanent radioactive seeds in the prostate gland where the radiation is delivered over weeks or months (208). In *high dose brachytherapy/HDR-BT* a temporary placement of a radioactive source is placed in the prostate gland, delivering the radiation over a few minutes. It is often combined with EBRT (209). In Norway, brachytherapy is a relatively new treatment modality, and at the time only offered as HDR-BT at The Norwegian Radium Hospital in Oslo.

Adjuvant and salvage radiation therapy

Patients who have undergone RP as primary curative treatment, but have a pT3 cancer and/or PSM, can be offered adjuvant EBRT against the prostate bed (210,211). Alternatively, one can wait for biochemical failure with assumed local recurrence, and then give salvage EBRT with a possibility for curation (212,213). Salvage RT should start early (before the PSA-levels increase to > 0.4 ng/mL) (214).

Radiation therapy in metastatic disease

For men with regional lymph node metastases (N1M0), RT of pelvic lymph nodes (and prostate) can increase survival in comparison to hormone therapy alone (17,215). In cases with de novo hormone-naïve metastatic PCa, life-long castration-treatment is initiated as first line treatment, followed by consideration for chemotherapy (see section below). Patients with low metastatic burden (< 3-5 bone metastases and no visceral metastases), should additionally be considered for RT to the prostate to achieve local control of the primary tumor, as this has shown improved overall survival (216,217). In patients diagnosed with oligometastatic disease after initial curative local treatment, metastases-targeting RT as a mean to delay systemic treatment can be considered but is still regarded as experimental (218). In patients with painful bone metastases, or metastatic spinal cord compression, palliative RT can give symptomatic relief (219).

Hormone therapy

In 1966 Dr. C. Huggins received the Nobel Prize in Physiology or Medicine for his discoveries concerning hormone therapy for PCa (220). Namely, that decreasing the effect of androgens on PCa cells, inhibits their growth and proliferation by inducing androgen deprivation-induced senescence. This is exploited therapeutically in different ways.

Castration is suppression of the testicular production of androgens, and may be achieved by surgical bilateral orchiectomy, or by drugs such as estrogens, luteinizing-hormone-releasing hormone (LHRH) agonists, or LHRH antagonists. Definition is serum testosterone < 1.7 nmol/L. In time, usually 2-3 years, the cancer may progress despite effective castration into a phase called CRPC (221). Even though CRPC proliferate despite castration-level androgens in the blood, a large proportion of cases are still androgen-dependent. Thus, CRPC cases can for simplicity be divided into AR-independent and AR-dependent (66,222). For AR-dependent cases, it can be effective to add further hormone therapy.

Antiandrogens inhibit the effect of circulating androgens at the level of their receptor. They can be steroidal and non-steroidal. First-generation, non-steroidal antiandrogens include for example flutamide and bicalutamide. Second-generations antiandrogens have a higher affinity for the AR, block the transfer of the AR to the cell nucleus (thus serve an exclusively antagonist effect), and have an increased specificity to the AR over other steroid receptors. Examples are apalutamide, enzalutamide and darolutamid (223,224).

Androgen synthesis inhibitors suppresses testosterone synthesis in the adrenal glands, testis and inside cancer cells, resulting in a significant decrease in endogenous androgen levels, thus inhibiting transcription of AR-targeted genes. One example is abiraterone which inhibit CYP17 (225). Because abiraterone also inhibits cortisol synthesis, it must be used together with a corticosteroid to prevent negative feedback-related rice in adrenocorticotropic hormone/ACTH-levels and subsequent hyperaldosteronism.

Traditionally, androgen deprivation therapy (ADT) has referred to castration and/or use of non-steroidal, first-generation antiandrogens such as bicalutamide. When used in combination, treatment has been known as combined androgen blockade (CAB). Newer hormonal therapies such as abiraterone, apalutamide and enzalutamide in combination with castration have thus far mostly been used in a mCRPC-setting but are increasingly being introduced in earlier treatment lines.

For mCRPC, adding a low dose of dexamethasone which decrease adrenal production of androgens (226), or a non-steroid, first-line antiandrogen to the castration treatment (227), can give a PSA-response and symptomatic effect in approximately 20-30%. However, there is only a marginal or no additional benefit on overall survival. Non-steroid, first-line antiandrogens should always be discontinued if disease progression because of a potential of agonistic effect. In general, adding second-generations antiandrogens to castration should be the preferred hormonal therapy in this setting (36).

Side-effects of hormone therapy include fatigue, cardiovascular risk, hot flashes, sexual dysfunction, liver failure and osteoporosis. Regular exercise can reduce fatigue and improve quality of life (228).

In the case of CRPC independent of AR-signaling, increasing evidence suggest that neuroendocrine trans-differentiation represents one of the major mechanisms (229). This is a process whereby adenocarcinoma cells trans-differentiate into neuroendocrine-like cancerous cells. As such, they secrete hormones, lacks expression of AR and PSA, and is thus resistant to traditional PCa treatment. There is evidence that neuroendocrine trans-differentiation happens in response to PCa treatment such as hormone therapy, chemotherapy and radiotherapy (230). It is important to mention, that in addition to being considered a dedifferentiated form of adenocarcinoma, pure neuroendocrine small-cell carcinoma can also occur as a primary cancer in the prostate (Table 3) (173). Adenocarcinoma with neuroendocrine trans-differentiation should be suspected in patients with progressing, disseminated disease combined with low PSA values. Neuroendocrine tumor markers chromogranin A/CgA and neuron-specific enolase/NSE are often elevated in serum or present in tissue. These patients have a significantly poorer prognosis compared to "regular" PCa patients and may be considered for platinum-based chemotherapy regimens similar to regimens for treating other neuroendocrine carcinomas (132).

Chemotherapy

PCa is not a typically chemo-sensitive disease, but the taxane *docetaxel* has several indications. It is recommended for patients with de novo hormone-naïve metastatic PCa. For this group an initial treatment of six, three-weekly courses of docetaxel, in addition to initiation of life-long castration, has shown improved overall survival compared to castration alone (231). The biological explanation may be that early chemotherapy can potentially eradicate present castration-resistant subpopulations, and delay CRPC-transition (232). In addition, docetaxel is commonly used in a palliative setting for patients with progressive mCRPC in combination with prednisolone and castration. It has shown significant increased survival and can be used continuously until progression, or intolerable toxicity (233). *Cabazitaxel* is a novel taxane with effect for some patients with docetaxel-resistant cancers in terms of overall survival (234).

Immunotherapy

Sipuleucel-T is an U.S. Food and Drug Administration (FDA)-approved, autologous, cancer treatment vaccine. It is made by harvesting autologous peripheral-blood mononuclear cells (mainly antigen-presenting dendritic cells) from the patient. These cells are further activated *ex vivo* with prostatic acid phosphatase (PAP) antigen which is present in 95% of PCa cells,

and matured with granulocyte-macrophage colony stimulating factor/GM-CSF. The vaccine is then reinfused into the patients' blood stream where it is designated to stimulate an immune response against PAP-expressing PCa cells. It has shown a 3,3 – 4,5 months increased overall survival in patients with mCRCP, but no change in progression-free survival, and no significant impacts on PSA, tumor burden, or symptoms (235).

The last decade, *immune checkpoint inhibitors (see further information in section 1.2.5. Clinical use of immuno-oncology)* has proven revolutionary for multiple cancer types, however, PCa is not one of them. Nonetheless, it is worth noting that there are reported cases which do show response to immune checkpoint-inhibitors; hence it may be effective treatment for subgroups of PCa patients (236–240). One such subgroup is patients with dMMR/MSI-H, which applies to approximately 3-12% of prostate cancer patients, and where immune checkpoint-inhibitor Pembrolizumab is FDA approved (241,242). Damage in DNA repair genes (such as BRCA1/2 or ATM), PD-L1 (236,237) and loss-of-function CDK12 mutations (243,244) are other suggested predictive markers for responsiveness to immune checkpoint-inhibitors. In addition, there is ongoing research into targeting lesser-known immune checkpoint V-domain Ig suppressor of T-cell activation (VISTA) in PCa patients (245).

Other treatment modalities

Ablative procedures using heating or freezing

High Intensity Focused Ultrasound/HiFU consists of a rectal probe sending focused ultrasound waves into the prostate, generating high temperatures which destroys tissue. *Cryoablation* is a procedure where cryoneedles with circulating Argon gas is placed in the prostate through the perineum. The gas generates a low temperature that destroys tissue through freezing. Treatments can be focal, or alternatively the whole gland can be treated (246). However, improved outcomes with these intriguing therapies are not sufficiently proved to mandate a role in standardized localized treatment.

Radionuclide therapy

Radium-223 is a radionuclide therapy which has shown survival benefit and can be used in patients with symptomatic bone metastases, and no additional visceral metastases (247). It is a calcium mimetic, targeting areas with increased bone turnover. As reviewed in section *1.1.5. clinical presentation and diagnostics*, PSMA is commonly expressed by PCa cells, and this can be utilized in *PSMA-targeted radionuclide therapy*. Namely, molecules with a high

affinity for the PSMA-receptor can be labelled with radioisotope molecules such as Lutetium, which through binding and endocytosis leads to local radiation of the PSMA-expressing cell (248). PSMA-targeted radionuclide therapy it is not yet implemented as a treatment option in Norway.

Poly-ADP ribose polymerase inhibitors

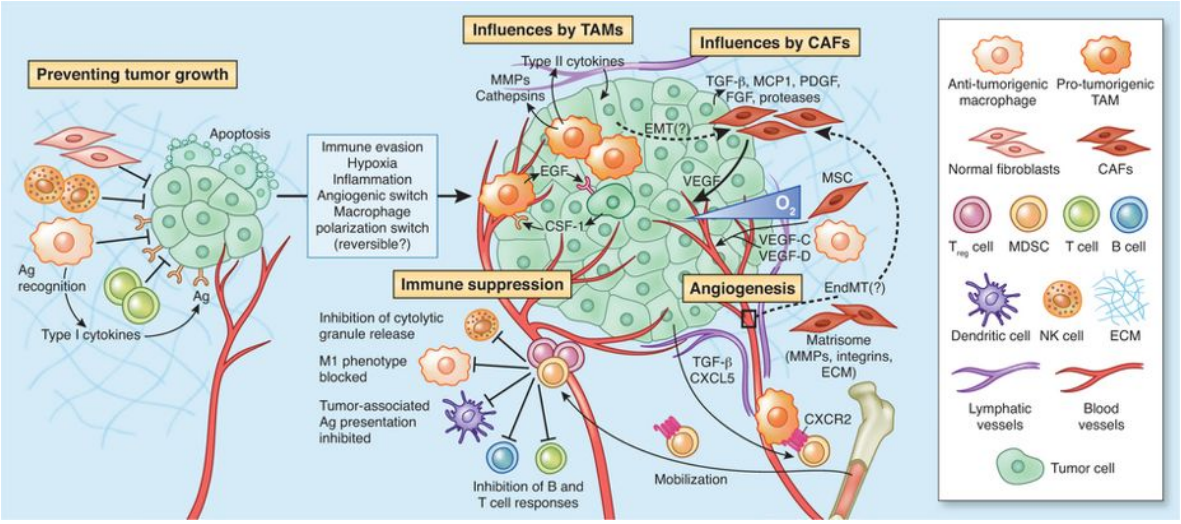
PARP inhibitors are currently evaluated in patients with mCRPC. Promise is shown for patients with either germline or somatic tumor mutations in DNA repair genes such as BRCA1, BRCA2 and/or PALB2. PARP1 and -2 are proteins important for repairing single-strand breaks in DNA. If they are inhibited, double-strand breaks will accumulate with time. Cancer cells with BRCA1, BRCA2 and/or PALB2 mutations, cannot use homologous recombination to repair these breaks, thus leading to death of the affected cancer cell (174).

1.2. IMMUNO-ONCOLOGY

1.2.1. The tumor microenvironment

Cancer is more than just the cancerous cells themselves. In recent decades, the cancer research field has shifted from studying the nature of the malignantly transformed parenchymal cells in a tumor, to investigating all of the different components of the TME. In addition to cancer cells, the TME consist of a stromal compartment of ECM and stromal cells such as cancer associated fibroblasts (CAFs), different subsets of immune cells, and the cells that constitute lymph- and blood vessels (Figure 12) (249). In many ways, the tumors have structures as if they were an organ on their own, with chronic tissue alterations resembling those accompanying normal wound healing (250). Tumor stromal cells does not themselves contain genetic mutations but are in many TMEs skewed into a subtype of cells not seen in healthy tissues. Through interaction with the cancer cells, and amongst themselves, they function as important contributors to tumorigenesis. Contrary, some tumor stromal cells are also involved in suppression and elimination of cancer cells. Knowledge of the tumor stromal compartment have led to multiple anti-cancer therapies which target tumor stromal cells rather than the cancer cells directly (251).

Figure 12: The tumor microenvironment. Multiple stromal cell types converge to support a tumorigenic primary niche (252). Reprinted with permission from Springer Nature© 2014.



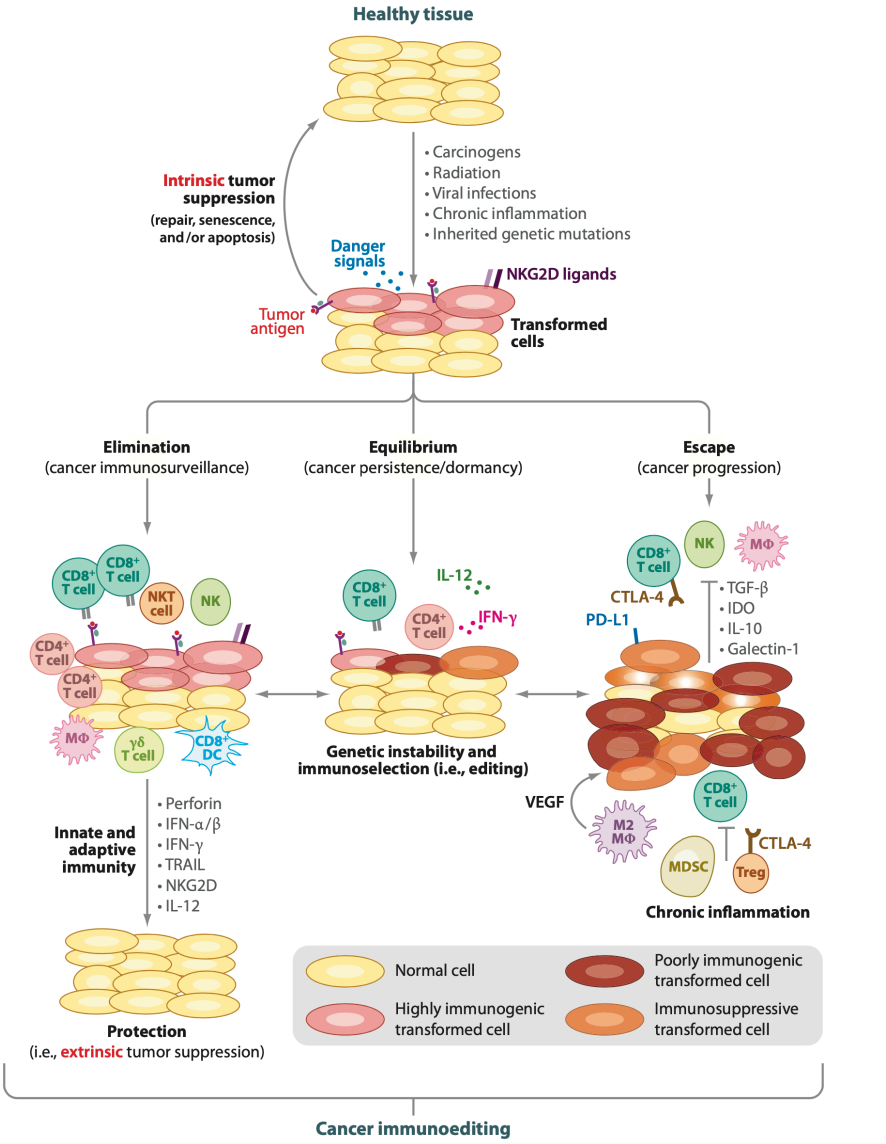
Abbreviations: Ag = Antigen; CAFs = Cancer associated fibroblasts; ECM = Extracellular matrix; FGF = Fibroblast growth factor; MSC = Mesenchymal stem cell; MMP = Matrix metalloproteinase; NK cell = Natural killer cell; PDGF = Platelet derived growth factor; TAM = tumor associated macrophage; TGF-β = Tissue growth factor β; Treg cell = regulatory T-cell; VEGF = Vascular endothelial growth factor

1.2.2. Cancer immunoediting

In 1909 Dr. P. Ehrlich laid the grounds for modern immunology when he defined the immune system as the individual's defense mechanism towards "not-self", in which he also included cancerous cells (253). Since then, it has been heavily debated if, and how, the immune system is able to eliminate pre-malignant and malignantly transformed cells in a similar fashion they do infectious agents. In the 50ties and 60ties Dr. L. Thomas and Sir F. Macfarlane Burnet formed the hypothesis of "Cancer immunosurveillance", in which they postulated that malignant transformation of cells occurs in different tissues at a regular basis, but that the immune system actively patrols, and in most cases, recognize and eliminate such neoplasms (254–256). The hypothesis was later refuted and contradicted by many throughout the years, before a Renaissance in the 1990ties where multiple studies linked various forms of immunosuppression with an increased risk of developing cancer, especially virally-, radiation- and carcinogen associated cancers (257–261). Today, most research communities recognize avoiding immune destruction as a hallmark of cancer (Figure 8) (54), and this knowledge is utilized in what is perhaps the greatest progress in cancer research in recent years; namely immune checkpoint-inhibitors *(see further information in section 1.2.5. Clinical use of immuno-oncology).* In the early 2000s, Prof. R. Schreiber with collogues further developed the "Cancer immunosurveillance"-hypothesis to the cancer immunoediting-theory which

gives a more wholesome picture of the relationship between cancer cells and the immune system (5–8). Cancer immunoediting is described as a process of three phases: Elimination, equilibrium and escape, often referred to as “The three E’s of cancer immunoediting” (Figure 13).

Figure 13. The three E’s of cancer immunoediting (262). Reprinted with permission from ANNUAL REVIEWS© *2011.*



Elimination

The term “immune elimination” refers to the same process as immunosurveillance, where the immune system (Figure 14) identifies transformed cells that have escaped cell-intrinsic tumor-suppressor mechanisms and eliminates them before they can establish a clinically relevant malignant tumor.

The growing tumor disturbs tissue-homeostasis and damages surrounding tissue, leading to releasement of proinflammatory factors by tissue-resident macrophages and mast cells. This subsequently attract additional innate immune cells (Figure 14). Natural killer (NK)-cells can recognize and cause apoptosis of cancer cells through multiple mechanisms, such as (a) by detecting downregulation of human leukocyte antigen (HLA)-I-molecules, or (b) by recognizing stress-molecules (e.g MICA7B, ULBP and Letal) on cancer cells via binding to their NKG2D-receptors (263).

Furthermore, a more specific anti-cancer immune response can be put in place through activation of the adaptive immune system (Figure 14). In order for the adaptive immune system to be able to generate an anti-cancer immune response, cancer cells must express antigens so different from normal cells, that the individual's immune system has not developed immunotolerance for them during development. Tumor antigens recognized can be (a) tumor-specific antigens (TSAs) which are neoantigens not expressed by normal cells, e.g. oncogenes (such as fusion protein BCL-ABL in chronic myelogenous leukemia) or products of oncoviruses. Or they can be (b) tumor-associated antigens (TAAs) which are structures not limited to cancer cells, but e.g. overexpressed by the cancer cell (HER2 in breast- and gastric cancer), or normally only expressed on cells during stages of fertilization and embryonal development (for example carcinoembryonic antigen/CEA occurring in ovarian- and colon cancer) (264).

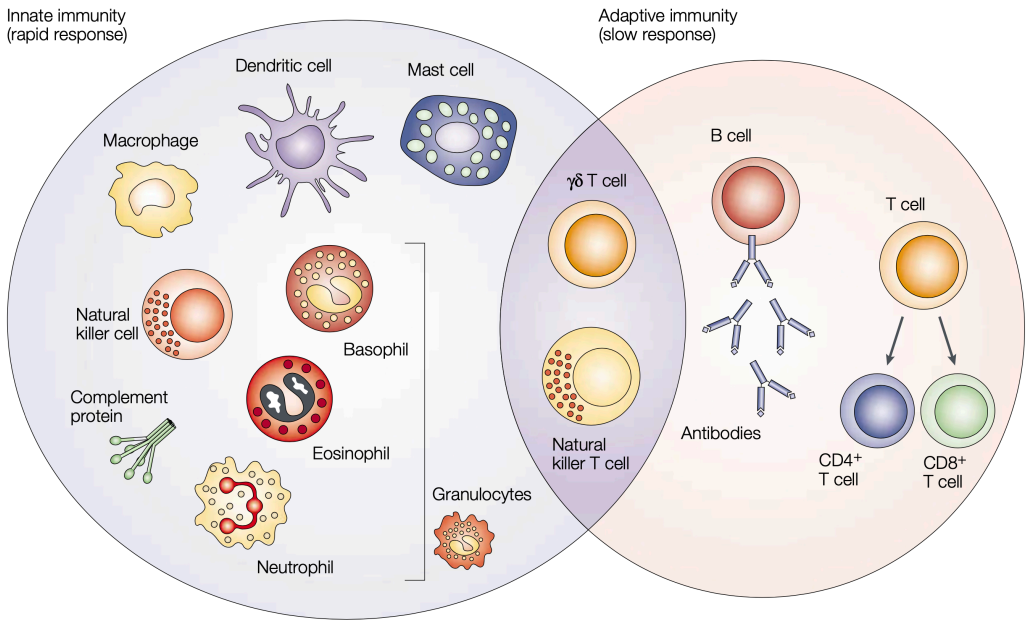
The T cell receptor (TCR) of naïve CD4+ and CD8+ T lymphocytes, cannot recognize natural forms of antigens, and depend on antigen presentation by dendritic cells (DCs) arriving in secondary lymphoid tissue. Antigens can be presented on either HLA-I or HLA-II molecules. Intracellular antigens (degraded in the cytosol) are presented on HLA-I which all cells in the body express, and extracellular antigens (degraded in phagosomes) is presented on HLA-II which is only expressed by phagocytic cells (265).

In adaptive, cellular anti-cancer immune response, DCs ingest tumor debris through the process of phagocytosis, and migrate to secondary lymphoid tissues where they serve as specialized antigen presenting cells. To become activated, the TCR of naïve CD8+ cytotoxic T lymphocytes must specifically recognize and bind to the tumor antigen presented on HLA-I on the DC. In addition, CD28 on the T lymphocyte bind to B7 on the DC, which provide a necessary costimulatory signal. Activated CD8+ T lymphocytes home to the tumor site, and cytotoxic mediated cancer cell murder is activated upon reencounter with cancer cells expressing the same HLA-I-tumor-antigen-complex as it was presented with in lymphoid tissue. Naïve CD4+ T lymphocytes are activated in secondary lymphoid tissue by DCs presenting HLA-II-tumor antigen complexes, and develop into Th1 lymphocytes, which main job is to increase effectiveness of CD8+ cytotoxic T lymphocytes and macrophages at the tumor site (265,266).

Relative to the above described cellular anti-cancer immune response driven by CD8+ cytotoxic T lymphocytes, the role of adaptive humoral immune response generated by B lymphocytes and CD4+ Th2-lymphocytes in cancer is still not fully understood. However, there are multiple indications it plays a role in cancer immune elimination. Tumor-specific antibodies are found in multiple cases, and presumably, Fc receptor-bearing effector cells such as NK cells and macrophages can recognize and kill antibody-coated cancer cells by phagocytosis. Furthermore, B lymphocytes and plasma B cells can in some cases be found in tumors and tumor-draining lymph nodes where they are thought to interact with both T lymphocytes and innate immune cells e.g., by HLA-II-antigen presentation and cytokine production (263,267).

Other cancer immune elimination mechanisms include upregulation of death ligands such as TRAIL, FasL or membrane-displayed TNF-α on cytotoxic immune cells such as CD8+ T lymphocytes and NK cells, which binds to, and activate apoptosis of cancer cells expressing DR4/DR5, Fas or TNFR1 respectively (268). In addition, natural killer T (NKT) cells and γδ T lymphocytes are contributing cytotoxic T lymphocytes with both innate and adaptive qualities which may be contributors to cancer immune elimination (Figure 14) (269).

Figure 14. Components of the innate and adaptive immune system (269). Reprinted with permission from Springer Nature© 2004.



Equilibrium

Tumor dormancy is a term relating to malignant tumors in a latent phase where they do not grow in size. It has been proposed that this, in a non-treatment setting, can be due to the immune system and the cancer cells joining in a dynamic balance where the cancer cell proliferation is equal to the immunological elimination (262). According to the theory of cancer immunoediting, there are three different outcomes of this phase, namely (a) elimination of the malignant neoplasm, (b) permanent equilibrium-phase, or (c) immune escape (6).

Escape

Cancer cells can evade immune elimination by going through biological changes. One main mechanism is developing decreased immunogenicity. Because cancer cells are genetically unstable (54), new mutations are bound to happen in a proliferating tumor, causing a heterogenic cell population. This gives a basis for a Darwinist TME, where immune elimination may select for a non-immunogenic cancer cell population, able to escape the immune system (7,8). Another important mechanism of immune escape is the establishment of an immune privileged TME (270). This can happen partly by exploiting normal, physiological mechanisms involved in inducing immunotolerance to avoid development of

autoimmune disease, and/or activation of systems already in place at immune privileged sites (e.g placenta, testicles) (265).

To avoid NK cell mediated elimination, some cancer cells produce proteases, cutting of their stress-induced molecules normally recognized by NKG2D-reseptors. Also, soluble forms of stress-molecules such as MICA can bind to NKG2D-receptors thus inhibiting its function (271). Cancer cells can also inhibit NK cells by expressing ligands which stimulate the NK cells' inhibiting receptor, killer-cell immunoglobulin-like receptor/KIR (272).

CD8+ cytotoxic T lymphocytes are conceivably the most important protagonist in cancer immune elimination, and thus mechanisms preventing their activation, as well as evading their attack, are crucial parts of the immune escape process. Mechanisms for halting adaptive cellular anti-cancer immune response, include preventing successful antigen presentation. A decreased level of functional DCs have been observed in multiple cancers (273). Also, phagocytosis of apoptotic cancer cells by DCs in the absence of the right danger signals, generally leads to development of immune tolerance instead of activation of immune response. Further, impairment of antigen presentation can be elicited through inactivating mutations or downregulation of HLA-molecules, or defects in other parts of the antigen-presentation machinery in the cancer cell (274).

Another way cancer cells can evade immune mediated killing, is to recruit immune cells with immunosuppressive qualities. The most well-known is perhaps regulatory T cells (Tregs, characterized as CD4+CD25+FoxP3+ cells), assumed essential for maintaining tolerance to self-antigens under normal physiological conditions. Tregs are able to inhibit activation of naïve T lymphocytes and suppress activated T lymphocytes (275). Studies have implicated that a high density of Tregs in the TME in most, but not all, cancers is indicative of a poor prognosis, probably through suppressing cancer immune elimination (276). Additionally, different tumor associated immune cells of myeloid origin are described to elicit immunosuppressive qualities on both innate and adaptive cancer-eliminating immune cells. Examples are myeloid-derived suppressor cells (MDSCs), a heterogenous population of immature cells of myeloid lineage (277), tumor associated neutrophils (TANs) (278) and tumor associated macrophages (TAMs). Though under further investigations, TAMs which are skewed towards an immunosuppressive phenotype is often referred to as M2-like, contrary to M1-like TAMs which is considered enhancing cancer immune elimination (279).

Some studies have also proposed subsets of CD8+ T lymphocytes (280–282) and B lymphocytes (283) to have immunosuppressive properties in cancer, and the picture is probably both complex and diverse for different TMEs.

Another important tactic cancer cells use to avoiding T lymphocyte mediated cancer elimination, is through exploitation of their immune checkpoint pathways. During the physiological activation phase of naïve T lymphocytes in secondary lymphoid tissues, the T lymphocyte will after a while start expressing Cytotoxic T lymphocyte antigen 4 (CTLA-4) instead of CD28, which can bind to B7 on the DC with a higher affinity. In contrast to CD28/B7-binding, CTLA-4/B7-binding leads to immunosuppressive signals in the T lymphocytes, preventing immune hyperactivation (284). For activated T lymphocytes, the Programmed cell death 1 (PD-1) pathway is the most well-known immune checkpoint receptor. PD-1 (also referred to CD279) is expressed on activated forms of T lymphocytes, as well as B lymphocytes, NK cells and MDSCs. Binding of ligands PD-L1 (also referred to B7-H1 or CD274) or PD-2 (B7-DC), to PD-1 on cytotoxic CD8+ T lymphocytes leads to apoptosis, anergy and exhaustion. Contrary, ligand binding to PD-1 on Tregs leads to stimulatory signals. PD-L1 is expressed on the surface of hematopoietic cells, healthy cells of certain organs, and some cancer cells, whilst PD-L2 seems more restricted to hematopoietic cells (285,286). Other immune checkpoint receptors on T lymphocytes include T-cell immunoglobulin and mucin-domain containing-3/Tim3, Lymphocyte-activation gene 3/LAG3 and VISTA. Cancer cells can utilize these physiological checkpoint mechanisms and drive cytotoxic CD8+ T lymphocytes into a state of anergy, exhaustion, or senescence, whilst having the opposite effect on Tregs (turning them off) (287).

Cancer cells can also directly produce different immune-suppressive molecules, such as indoleamine 2,3-dioxygenase/IDO, an enzyme necessary for T lymphocyte proliferation (288), Fas ligand which stimulate apoptosis in immune cells (289), as well as immunosuppressive cytokines such as tumor growth factor β/TGF-β (290) and interleukin-10/IL-10 (273).

1.2.3. Inflammation and tumorigenesis

Even though some immune cells fight cancer development, others have the opposite effect (9). As early as in 1863, pathologist Dr. R. Virchow discovered malignant tumors to have a high rate of immune cells compared to normal tissue and proposed chronic irritation as a driver of tumorigenesis (291,292). Another indication for cancer promoting inflammation, is that approximately 15% of the cancer cases worldwide are caused by commensal or infectious microbes or viruses (293). A considerable fraction of cases is caused by oncogenic viruses. A well-known example is human papillomavirus (HPV) 16 and 18, containing oncogenes E6 and E7 in their genome, which inactivate tumor suppressor proteins p53 and pRb respectively, causing cancers of the cervix, vagina, penis, anus and certain head and neck tumors (294). However, not all microbes and viruses associated with cancer development have equally unequivocal oncogenic properties, and thus their development of chronic inflammation is assumed to be a main mechanism for driving cancer development. Common examples are hepatitis virus B and C in liver cancer (295,296), Helicobacter pylori in gastric cancer (297), Schistosoma haematobium in bladder cancer (293), and Epstein Barr virus which is linked to multiple cancers for example Burkitt's lymphoma and gastric cancer (298). In addition, the microbiota is probably involved in supporting colon cancer development (299). In general, whilst the ability to generate acute inflammation is essential both in fighting infection and cancer, chronic inflammation is a driving factor in multiple diseases, including cancer, where it is acknowledged as an enabling hallmark (54).

Cancer-promoting inflammation differs from cancer-eliminating inflammation in terms of immune content. In a tumor where immune elimination dominates, the typical immune cell types present are those of acute inflammation such as NK cells, DCs, CD4+ Th1 T lymphocytes, CD8+ T lymphocytes, and TAMs of M1-subset. Contrary, in a pro-malignant TME, immune cells typical of chronic inflammation dominate, such as Tregs, MDSCs, chronically activated B lymphocytes, CD4+ Th2 lymphocytes, and TAMs of M2 subset (300,301). However, every TME is unique, and other stromal cells with pro-tumorigenic, and often pro-inflammatory properties, can reside, such as TANs (278), mast cells (302), and CAFs. CAFs differs from normal fibroblasts, and a high tumor density of CAFs in the TME usually indicates a poor prognosis (303).

Immune cell mechanisms for promoting cancer

Immune cells are vital helpers in tumorigenesis through multiple mechanisms. An inflammatory TME will generate reactive oxygen- and nitrogen species that can drive mutations through DNA damage (292), and lead to epigenetic changes such as DNA methylation and transcriptional silencing of certain gene promotors (304).

In addition, chronic inflammation leads to apoptosis and necrosis, and in the quest of replacement of lost tissue, cell proliferation-rate increase. Since most spontaneous mutations occurs in the DNA replication phase, an escalated proliferation rate increases risk of potentially oncogenic mutations. Additionally, it gives the DNA-repair system less time to repair DNA-damage, thus elevating the risk of malignant mutations being passed on to daughter cells (305). Immune cells, together with CAFs, can also in a more direct way support tumorigenesis by supporting most of the hallmarks of cancer (Figure 8) (54). They are able to produce mitogens, growth factors, survival signals, hormones and cytokines that stimulate proliferation as well as inhibits apoptosis of the cancer cells. They can mediate immunosuppression, enhance the migration of tumor promoting immune cells into the tumor, and activate transcription factors such as NF-κB and STAT3 which act as further drivers of inflammation and tumorigenesis. They contribute to invasive growth, motility and metastasis by stimulate angiogenesis and lymphangiogenesis, remodeling of the ECM (which additionally will release mitogens and growth factors who previously have been inactivated by binding here), and supporting cancer stemness and/or activation of epithelial-mesenchymal transition/EMT-programs in the cancer cells (249,300,306–308).

Chemokines in cancer development and metastasis

Chemokines are a group of cytokines that regulate cell trafficking through chemotaxis. They are required in (a) guiding immune cells throughout the body as part of immunosurveillance, (b) stimulating migration of immune cells to sites of tissue injury, (c) generation of immune response, (d) formation of immunological memory, and (e) homeostatic homing causing accumulation of immune cells in specific organs and lymphoid tissues (309). Chemokines are subclassified into four main subfamilies, namely CC, CXC, C, CX3C, and bind to chemokine receptors – a large family of seven-transmembrane G-coupled proteins (310).

In cancer, chemokines and their receptors can be expressed by both cancer cells and tumor stromal cells. They can induce a tumor-friendly environment by recruiting certain types of

immune cells into the TME, modifying their phenotypes and shaping their biologic functions, thus making them pro-tumorigenic. Chemokines can stimulate angiogenesis and lymphangiogenesis. They are important mediators in cancer cell migration, invasion, adhesion and metastasis, and may be part of the explanation as to why different cancers types seems to prefer certain organs as their metastatic destination (metastatic homing). Additionally, they can activate cell proliferation, and anti-apoptotic pathways. However, chemokines are also necessary in attracting immune cells that generate an anti-cancer immune response, and thus can suppress tumorigenesis and metastasis (310–312).

1.2.4. The immune system in prostate cancer

Based on its position, the prostate is proposed to inhibit the urinary microbiome and pathogens from the bladder or distal urethra entering the male reproductive system. As the aging prostate inherently develop pathology, glands without pathology are rarely studied. However, autopsy studies, as well as normal areas in prostates with pathology, indicate that healthy prostate tissue contains both stromal and intraepithelial lymphocytes, mainly T lymphocytes (313,314). Resident innate immune cells such as mast cells and macrophages are also present, mainly in the stromal compartment. Other innate immune cells such as neutrophils, basophils and eosinophils are rarely seen in the normal prostate (315,316).

Studies have concluded that the TME in PCa is heterogenic in composition, but typically characterized by the presence of CAFs (317) and immune cells such as CD3+ T lymphocytes (mainly of CD4+ subset), CD20+ B lymphocytes and TAMs (318). ARs are found on both tumor epithelial cells and tumor stromal cells, depicting that androgens exerts stimulating and possible pro-tumorigenic effects on both compartments (319). The contribution of the immune system in PCa tumorigenesis – either in a positive or a negative direction – is however controversial.

By mechanisms described previously, longstanding, chronic inflammation is an enabling hallmark for cancer development (54). As areas of inflammation is relatively common in the aging prostate, its relation to tumorigenesis is difficult to examine without bias. However, there are multiple circumstantial evidence on inflammation as a driver for prostate tumorigenesis. One study sampled biopsies form presumably healthy prostates, and after follow-up concluded that inflammation in these benign glands was positively associated with later development of PCa (320). Another example is the fact that the long-term use of

nonsteroidal anti-inflammatory drugs/NSAIDs may reduce risk of PCa development (321), as also seen for colon cancer. In addition, PIA is suggested as a precursor of HGPIN and/or adenocarcinoma. PIA is a condition which consist of atrophic prostate epithelial cells with increased proliferative rate and associated inflammation. The epithelial cells in PIA have genetic changes theoretically making them susceptible for tumorigenesis (111,322). Their increased proliferation rate is proposed as a reaction to cell loss caused by prostate epithelial injury and inflammatory stress (315). There are multiple different proposed causes of prostate epithelial injury and subsequent inflammation, e.g., urine reflux inducing chemical and physical trauma, environmental toxins (for example dietary factors), hormonal imbalances, corpora amylacea and bacteria. When it comes to the latter, both sexually transmitted bacteria (e.g Neisseria gonorrhoeae, Chlamydia trachomatis, Trichomonas vaginalis, Treponema pallidum), urinary infections (Escherichia coli and other species of Enterobacteriaceae) (323,324) and/or certain compositions of the urinary microbiome (such as Propionibacterium) has been suggested (325). Infections with viruses such as HPV, human herpes simplex virus type 2/HSV2, cytomegalovirus/CMV and human herpes virus type 8/HHV8 is also observed in the prostate, but it is not clear if they cause inflammation and/or cancer (323,324).

Even more controversial than the role of inflammation as an enabling hallmark for PCa development, is the role of the immune system in PCa elimination. In general, PCa is viewed as a non-immunogenic cancer.

1.2.5. Clinical use of immuno-oncology

Immune markers in prognostics

Today, prognostic evaluation for guiding treatment strategy for most solid cancers, relay on clinical- and radiological examination and histopathological qualities of the biopsy or surgically removed tumor tissue. Almost all solid tumors are staged using the TNM-system, giving information on invasiveness and metastatic status. The main histopathological features evaluated are tumor size, differentiation grade, grade of atypia, number of lymph node metastases/perinodal growth, resection margins, PNI, vascularization and for some cancer types; abnormal expression of specific proteins and/or genetic markers (tumor biomarkers). However, these tools often do not give enough information to fully stratify patients into the right risk group, hence patients within the same group often have different clinical outcomes. One reason may be that histopathological evaluation systems mainly concentrate on the cancer cells and does not adequately assess the stromal compartment of the tumor (326,327).

Multiple scientists have reported that immune cells in the TME is correlated to clinical outcome (326). Some immune cells are found to be good prognostic factors, while others are associated with a worse prognosis. These correlations vary in different cancer types. Over a decade ago, Dr. J. Galon and collegues, proposed using a prognostic scoring system based on the arrangement and density of tumor infiltrating lymphocytes (TILs) in colon cancer, simply referred to as Immunoscore® (328). For colon cancer, where high densities of CD3+ T lymphocytes, CD8+ cytotoxic T lymphocytes, and CD45RO+ memory T lymphocytes are associated with improved prognosis after surgical resection of the primary tumor, Immunoscore® have proven better than the TNM-system in predicting prognosis (329). A global initiative called The Society for Immunotherapy of Cancer Immunoscore® Validation Project aims to evaluate Immunoscore® for all solid cancer types (330).

Cancer immunotherapy

The traditional cancer therapies include surgery, radiation and chemotherapy (also known as "slash, burn and poison") and are obviously not sufficient to cure every cancer case. In addition, especially radiation and chemotherapy, are non-specific and often damage healthy tissue in addition to cancer cells, causing harmful side effects. To solve this, recent years of cancer research have focused on finding more specific and individual forms of cancer treatment. Some of the treatment modalities investigated, utilizes the knowledge of immunological mechanisms in cancer. Cancer immunotherapy refers to treatment that manipulates and/or enhances the patient's own immune system in elimination of cancer cells (331). Compared to traditional cancer treatment, immunotherapy depend on both tumor biology, and the immune system of the individual. Advantages includes specificity, systemic reach and creation of memory preventing relapse. Disadvantages are autoimmune side-effects, high costs, and that at least so far, immunotherapy only seems beneficial for some cancer forms and subsets of patients. In addition, therapeutic resistance can evolve because of the possibility for immune escape.

<u>Monoclonal antibodies</u>

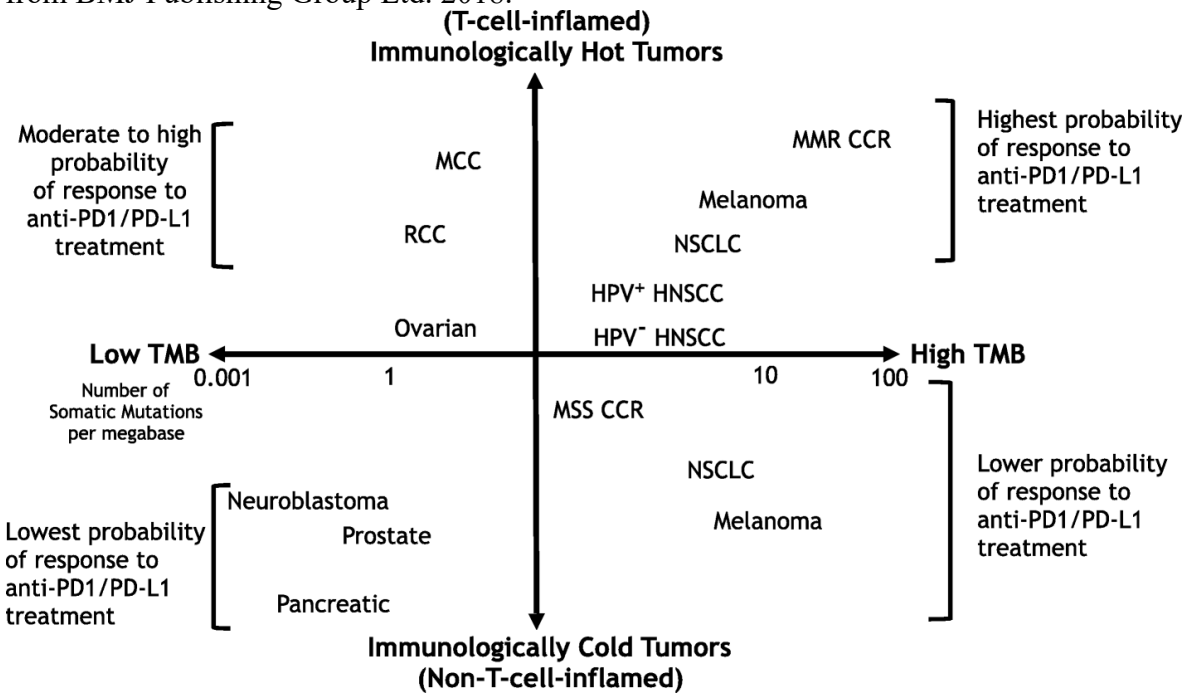
Monoclonal antibodies (mAbs) in cancer treatment, are lab-produced antibodies designed to attach to specific tumor antigens (TSAs or TAAs), or on targets in the TME. Some mAbs act through the mechanism of passive immunization, inducing an immune mediated killing of cancer cells through activation of the complement cascade and facilitating antibody dependent cell cytotoxicity. One example is rituximab which binds to CD20 on B lymphocytes and used

in Non-Hodgkin B cell lymphoma. However, most mAbs used in cancer treatment does not have a predominantly immunologic effect, but rather by blocking signaling pathways, and are thus considered to be target treatment rather than immunotherapy (332). Well-known examples of the latter are trastuzumab which binds to HER2 on cancer cells, or bevacizumab binding to VEGF-receptors on endothelial cells (thus inhibiting angiogenic effect).

Immune checkpoint-inhibitors

As described earlier, cancer cells can activate immune checkpoint-pathways, turning off T lymphocyte-mediated elimination. Immune checkpoint-inhibitors are mAbs blocking this possibility for the cancer cells. In other words, immune checkpoint-inhibitors cut the brakes on T lymphocytes. The 2018 Nobel Prize in physiology or medicine was awarded to Dr. J. Allison and Prof. T. Honjo "for their discovery of cancer therapy by inhibition of negative immune regulation" (333). Today, there are inhibitors of both CTLA-4 (e.g. ipilimumab), PD-1 (e.g. nivolumab and pembrolizumab) and PD-L1 (e.g. atezolizumab and durvalumab) in clinical use, both in monotherapy or in combination, and ongoing trials for inhibitors of other immune checkpoint pathways. Immune checkpoint-inhibitors have so far mostly shown effect in patients with cancers caused by external factors such as radiation, carcinogens or microbes and/or tumors with dMMR/MSI-H (334). These kinds of tumors often have a high mutational burden and/or are immunologically “hot” tumors, namely they are T lymphocyte inflamed (Figure 15) (335,336). There is ongoing research to increase immune checkpoint-inhibitors effectiveness in non-responding tumor groups, for example by combining them with other immunotherapies, chemotherapy, radiation or tyrosine kinase inhibitors. It is also evident that the microbiome, especially in the intestines, is important in mediating both response and toxicity to immune checkpoint-inhibitors, and also that immunosuppressants, antibiotics, proton inhibitors (337,338), radiation and chemotherapy may also influence the effect. The main harmful and potentially lethal side-effects are autoimmune attacks of healthy organs. CTLA-4 inhibitors often show more severe autoimmune side effects, as they turn of the breaks on *activation* of all types on T lymphocytes no matter what antigen they may react to, while PD-1/PD-L1 inhibitors mainly *boost an immune reaction peripherally* that is already in place. In this regard, it is worth noticing that anti-PD-1/PD-L1 antibodies have the potential to re-invigorate tumor-reactive T lymphocytes, but do not induce their formation. Immune checkpoint-inhibitors have been revolutionary, as some patients with e.g. metastatic malignant melanoma and non-small cell lung cancer have shown long-term survival, and probably curation (339,340)

Figure 15. High and low mutational burden tumors versus immunologically hot and cold tumors and response to immune checkpoint-inhibitors (335). Reprinted with permission from BMJ Publishing Group Ltd. 2018.



Abbreviations: HNSCC = Head and neck squamous cell carcinoma; HPV = Human Papilloma virus; MCC = Merkel cell carcinoma; MMR CCR; Miss-match repair deficient colorectal cancer; MSS CCR = Micro satellite stabile colorectal cancer; NSCLC = Non-small cell lung cancer; RCC = Renal cell cancer; TMB = Tumor Mutational Burden

Adoptive cell transfer

Adoptive cell transfers/ACT utilize allogenic, or usually autologous, tumor-specific T lymphocytes in cancer treatment. There are multiple different approaches. Tumor-specific TILs can be harvested from the patient's tumor (TIL therapy) or peripheral blood (endogenous T-cell therapy), increased in numbers by cytokine stimulation before transferring them back into the patient. In chimeric antigen receptor CAR T-cell/CAR-T therapy, an artificially made TCR independent of HLA-molecules for activation – called CAR – is added to autologously harvested T lymphocytes and reintroduced to the patient. Because of its independence of HLA-molecule, it can only detect extracellular antigens. CAR-T treatment is mainly relevant for hematological cancers at the moment. Another method is TCR transduced T cells, where harvested T lymphocytes *ex vivo* is genetically modified into expressing certain TCRs (341).

Chemokines

There is ongoing research to block certain chemokine signaling pathways, often in combination with traditional chemotherapy regiments, tyrosine kinase inhibitors or immune checkpoint-inhibitors. The promise is that this might inhibit metastatic mechanisms or modulate the tumor stromal compartment to become less tumor-friendly (312). One example that has shown promise is the CXCR4 inhibitor BL-8040 in combination with PD-1 antagonist pembrolizumab for metastatic pancreatic cancer (342).

Cancer vaccines

There is an important distinction between preventative- and treatment vaccines in cancer. Whereas preventative vaccines are vaccines against oncogenic viruses (for example some strains of HPV), the goal of cancer treatment vaccines is to induce a tumor-specific immune response in an individual with already developed cancer. There are two main approaches: (a) Transfer tumor-antigens or attenuated cancer cells directly or by using microbial vectors, or (b) harvest autologous antigen presenting cells, expose them to tumor antigens *ex vivo*, subsequently re-introducing them back in the blood stream where they can activate an adaptive immune response (343). One example of the latter is Sipuleucel-T used in mCRPC *(see further information in section 1.1.7. Disease management and treatment principals").* Thus far, cancer treatment vaccines have shown promise in initiating an immune response but will probably have to be combined with other immunotherapies for maintenance of immune response.

Oncolytic virus therapy

Oncolytic virus therapy, refers to the use of genetically modified viruses, engineered to only infect cancer cells. The virus is injected directly into the tumor, inducing an anti-cancer immune response by both lytic releasement of tumor antigens, and immunogenic cell death. Immunogenic cell death is a process where cancer cell stress leads to extracellular presentation of damage-associated molecular patterns/DAMPs. DAMPs attract and activates immature DCs, which by phagocytosis and antigen presentation stimulate adaptive immune response (344). The FDA have thus far approved one oncolytic virus therapy called talimogene laherparepvec/T-VEC for in use in malignant melanoma.

Microbes as non-specific immunotherapies

As far back as the 1700s, it has been observed that initiating an immune response towards infectious microbes, can have anti-tumor side-effect (345–347). One well known example still in clinical use today, is Bacillus Calmette–Guérin/BCG-installation in superficial bladder cancer. The injection of attenuated Mycobacterium Bovis into the bladder, creates an immune response against the bacteria, causing an additional anti-cancer immune response in the same area (348,349). However, it is important to be aware of the more common scenario, where infection cause cancer development *(see further information in section "1.2.3. Inflammation and tumorigenesis").*

Other non-specific immuno-modulating drugs

Cytokines such as interferons and interleukins were previously often used in treatment of solid cancers such as metastatic melanoma and renal cancer, causing an unspecific boost of the immune system (350). Thalidomide, lenalidomide, and pomalidomide used in e.g. multiple myeloma, are other examples of non-specific immunomodulating drugs (351). Imiquimod is a drug applied to early-stage non-melanoma skin cancers inducing immune response e.g. by inducing interferon-α and other cytokines (352).

The presence and future of cancer immunotherapy

Even though immunotherapy has been revolutionizing for some patients the last decade, it still has challenges to overcome. Unfortunately, it has not yet generally worked well in common cancer groups such as glioblastoma, prostate-, colorectal-, breast- and pancreatic cancer. However, new immunotherapy approaches aims to (a) make immunologically "cold" tumors, "hot", using e.g. oncolytic virus therapy, and conventional cancer therapies such as chemotherapy and radiation, (b) strengthening adaptive, T lymphocyte immunity by enhancing innate immunity and neutralizing immunosuppressive tumor stromal myeloid cells and Tregs, (c) blocking multiple different immune checkpoints pathways at the same time, and (d) immunotherapy used in combination with targeted treatment. It is now also apparent that the individual's microbiome plays an important role in immunotherapy effectiveness, and more research is needed to possibly manipulate the microbiome to our advantage (353,354).

2. AIM OF THESIS

Our general aim was to contribute to reduce challenges in prostate cancer (PCa) clinical decision making, by improving prognostication, as well as generate hypotheses on PCa biology and possible therapeutic targets. Based in this, we formed the following specific aims for this thesis:

- Establishment of a PCa cohort from the PSA Era, using resected PCa specimens in connection with relevant patient data.
- By immunohistochemistry, investigate the *in situ* prevalence and expression patterns of relevant immune biomarkers in PCa tissue.
- By appropriate statistical analyses, examine the prognostic impact of these immune biomarkers, and if possible, back-up our results by experimental research methods.
- Assess the prognostic impact of the immune biomarkers in relation to other established prognostic factors in PCa.
- If possible, generate hypotheses for possible therapeutic targets.

3. MATERIALS AND METHODS

3.1. PROSTATE CANCER PATIENT COHORT AND PROGNOSTIC BIOMARKERS

To study possible prognostic biomarkers for PCa, we established a contemporary, well-described cohort with relevant outcome data.

3.1.1. Ethics

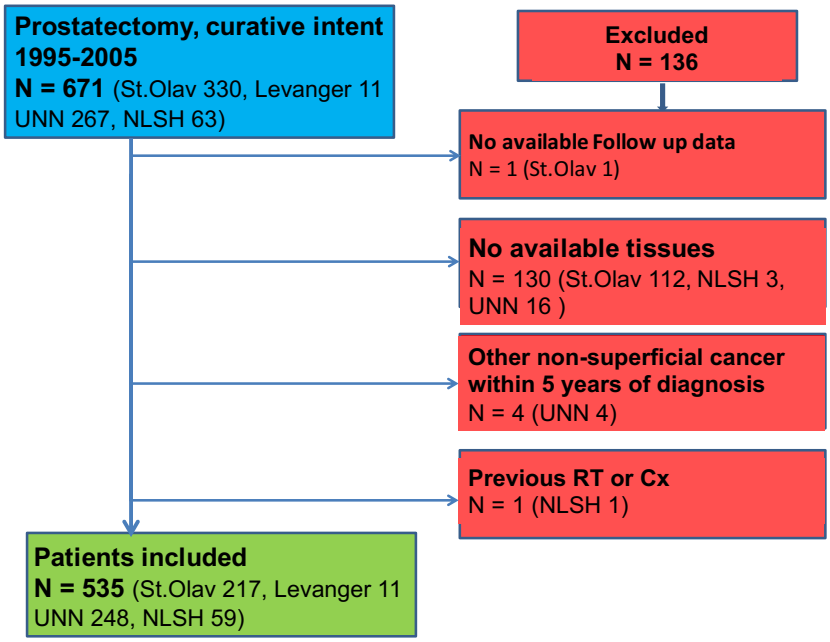
The ethics of this study was approved by The Regional Committees for Medical and Health Research Ethics, REK Nord (Protocol ID: 2009/1393) with extended approval in 2016 and 2019. The Data Protection Official for Research (Norwegian Centre for Research Data, NSD) approved the assembly of the database. The REMARK guidelines (355) were used as reference when reporting material, methods and results.

3.1.2. Prostate cancer patient cohort

Study population

We wanted to establish a cohort of PCa patients representing the PSA era and with a relatively long follow-up enabling survival analyses. It was also vital that each patient had available PCa tissue for biomarker analyses. Hence, the inclusion criteria were men who underwent RP as initial treatment for prostate adenocarcinoma between 01.01.1995 to 31.12.2005. Consequently, we carried out a retrospective search in the databases of the pathological departments of Norway's three most northern hospitals where RPs are performed, serving about 1/5 of Norway's total population for this procedure *(http://www.SSB.no/befolkning).* We identified 671 patients: 267 at the University Hospital of Northern Norway (UNN), 63 at the Nordland Hospital (NLSH), and 341 at the St. Olavs Hospital (of which 11 cases underwent the surgical procedure at the local hospital of Levanger by surgeons from St. Olavs Hospital). 136 patients were excluded (complete information presented in Figure 16) and thus a total of 535 patients with complete medical records and available tumor specimens were included in the cohort.

Figure 16. Patient inclusion and exclusion.



Patient characteristics and clinical data

Complete demographic and clinical data were obtained retrospectively through review of medical records at the operation site and local hospitals by two medical doctors *(Y. Nordby and S. Andersen)* and myself as a trained medical research student. Prostatectomies were retropubic in 435 cases and perineal in 100 cases, and median age at surgery was 62 years (range 45–75 years). Preoperative clinical TNM staging was not routinely stated in the medical files, and data was therefore not obtained. Preoperative PSA measured when admitted to the hospital for surgery was available for 529 of 535 (99%) patients, while the last percent had PCa as an incidental finding at TUR-P before RP was performed. The median preoperative PSA value was 8.8 ng/ml (range 0.7-104.3 ng/ml). For patients with biochemical failure after RP, PSA doubling time (PSA-DT) was calculated using an online calculator (356). The first collection of clinical variables was done in 2011/2012, and a second update was conducted in December of 2015. Clinical variables, patient characteristics and results from univariate survival analyses of these variables are presented in Table 10.

Follow-up and endpoints

Biochemical failure (BF), clinical failure (CF) and PCa-specific death (PCD) were registered as endpoints. We defined BF as a PSA-value ≥ 0.4 ng/ml and rising in a minimum of two different blood samples postoperatively. Biochemical failure-free survival (BFFS) was calculated from the date of surgery to the first measured PSA ≥ 0.4 ng/ml, or the last follow-up date without BF. CF was defined as verified, symptomatic local recurrence in the prostatic bed, or radiologically verified metastasis to bone, visceral organs or lymph nodes after prostatectomy. Clinical failure-free survival (CFFS) was calculated from the date of surgery to the date of CF, or last follow-up date without CF. PCD was defined as death by progressive mCRPC. PCa death-free survival (PCDFS) was calculated from the date of surgery to the date of death, or the last follow-up date without PCD. For Paper I and III, last follow-up was November 2012, and for Paper II, last follow-up was December 2015. Detailed information on follow-up and endpoints are presented in Table 9.

Table 9. Information on follow-up and endpoints

Last follow-up	**November 2012 (Paper I and III)**	**December 2015 (Paper II)**
Median follow-up of survivors in months	89 (range 6-188)	150 (range 17-245)
Postoperative hormonal therapy, n (%)	83 (15.6)	89 (16.6)
Postoperative radiation therapy, n (%)	90 (17.2)	103 (19.2)
Patients with BF, n (%)	170 (31.8)	200 (37.4)
Patients with CF, n (%)	36 (6.7)	56 (10.4)
Patients with PCD, n (%)	15 (2.8)	18 (3.4)
5-year BFFS, %	74	74
10-year BFFS, %	63	62
5-year CFFS, %	96	96
10-year CFFS, %	91	93
10-year PCDFS, %	97	98
Abbreviations: BF = biochemical failure; BFFS = Biochemical free survival; CF = clinical failure; CFFS = clinical failure free survival; PCD = Prostate cancer death; PCDFS = prostate cancer death free survival		

Tumor material and histopathological evaluation

Formalin-fixed and paraffin-embedded (FFPE) prostatectomy specimens were retrieved from the pathology department archives at UNN, NLSH and St. Olavs Hospital, respectively. Representative whole H/E stained sections from each case were evaluated by two experienced pathologists *(E. Richardsen and L.T. Rasmussen Busund).* If needed, new whole sections were cut, mounted and H/E stained. Tumors were histologically classified as adenocarcinomas according to WHO 2004 guidelines (357) and assigned a pathological TNM-stage in agreement with UICC guidelines (358). The Modified Gleason grading system (142,359,360) was used for grading tumors. In 2015, the database was additionally recoded to include the new ISUP Grade Groups (144), and thus both grading systems are presented (Table 10).

Further, the pathologists *(E. Richardsen and L.T. Rasmussen Busund)* registered primary tumor burden as the largest diameter of the index tumor, defined as the largest tumor present (median tumor size was 20 mm, range 2-50 mm). PNI was defined as cancer cells infiltrating the perineural space outside of the prostatic capsular structure, and if cancer cells were observed within a vascular or lymphatic, endothelium-lined space this was defined as LVI. When the tumor extended to the stained surface of the resected specimen, this was registered as PSM. Histopathological variables and results from univariate survival analyses of these variables are presented in Table 10.

All demographic-, clinical- and histopathological data were registered in a SPSS datafile and patients were de-identified. The details of our cohort and clinicopathological variables were published in 2014 (159).

Table 10: Patient characteristics and clinicopathological variables as predictors of biochemical failure, clinical failure and PCa death in PCa patients (n = 535), (univariate analysis; log-rank test) significant p values in bold (threshold ≤ 0.05). Variables from database updated December 2015

Variable	Patients	Patients	BF		CF		PCD	
	(n)	(%)	(200 events)		(56 events)		(18 events)	
			5-year EFS (%)	*p*	*10-year EFS (%)*	*p*	*10-year EFS (%)*	*p*
Age at surgery				0.237		**0.038**		0.404
≤ 65 years	357	67	77		94		98	
> 65 years	178	33	70		91		98	
Surgical proc				0.466		0.308		0.965
Retropubic	435	81	77		92		98	
Perineal	100	19	68		95		99	
Preop PSA				**<0.001**		**0.029**		**0.003**
PSA<10	308	58	80		95		99	
PSA>10	221	41	68		89		97	
Missing	6	1	-		-		-	
Gleason grade / ISUP Grade Group				**<0.001**		**<0.001**		**<0.001**
3+3 / ISUP Grade group 1	183	34	83		98		99	
3+4 / ISUP Grade group 2	219	41	77		94		99	
4+3 / ISUP Grade group 3	81	15	70		90		96	
4+4 / ISUP Grade group 4	17	3	58		87		94	
>8 / ISUP Grade group 5	35	7	37		65		90	
pT-stage				**<0.001**		**<0.001**		**0.001**
pT2	374	70	83		97		99	
pT3a	114	21	60		87		98	
pT3b	47	9	43		74		91	
pN-stage				**<0.001**		**<0.001**		**<0.001**
NX	264	49	79		96		99	
N0	268	50	72		91		97	
N1	3	1	0		33		67	
Tumor Size				**<0.001**		**0.002**		0.085
0-20 mm	250	47	83		96		99	
>20 mm	285	53	68		90		97	
PNI				**<0.001**		**<0.001**		**<0.001**
No	401	75	80		96		99	
Yes	134	25	60		83		95	
PSM				**0.049**		0.198		0.843
No	249	47	69		90		98	
Yes	286	53	81		96		98	
Non-apical PSM				**<0.001**		**<0.001**		**0.022**
No	381	71	82		96		99	
Yes	154	29	57		85		96	
Apical PSM				0.063		0.427		0.128
No	325	61	74		92		98	
Yes	210	39	77		93		99	
LVI				**<0.001**		**<0.001**		**<0.001**
No	492	92	77		95		99	
Yes	43	8	47		69		90	

Abbreviations: BF = biochemical failure; CF = clinical failure; EFS = event free survival in months; ISUP = International Society of Urological Pathology; LVI = lymphovascular infiltration; PCD = Prostate cancer death; PNI = Perineural infiltration; Preop = preoperative; PSA = Prostate specific antigen; PSM = Positive surgical margin; pT-stage = pathological tumor stage; Proc = procedure

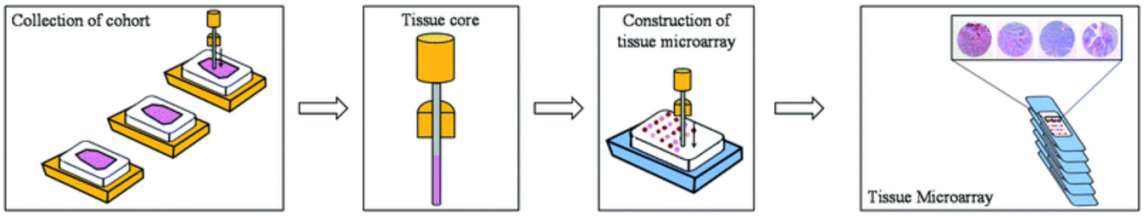
3.1.3. Prognostic biomarkers

A prognostic biomarker is defined to be a molecule or combination of molecules, that separates populations with regard to clinical outcomes. This is different from predictive biomarkers who predicts which patients will have effect of a specific treatment (361). For assessment of possible prognostic biomarkers in the collected tumor specimens, we chose to arrange the tumor tissue in tissue microarrays (TMAs) followed by IHC for detection.

Tissue microarray

The TMA method was first described by Dr. J. Kononen et al. in 1998 (362). TMAs has made it possible to use small amounts of cancer tissue for time- and cost-effective, depersonalized assessment of biomarkers (Figure 17) (363). To select areas for TMA sampling, a uropathologist *(E. Richardsen)* viewed the H&E-stained whole sections from each case using a light microscope, and with different color ink, circled separate areas of the most representative tumor epithelial tissue (meaning the most common Gleason pattern, plus also from areas with higher Gleason grades if present), adjacent tumor stromal tissue, normal epithelial tissue, and normal stromal tissue for each case. An experienced technician *(M. Persson)* used a manual tissue-arraying instrument *(Beecher Instruments Inc, Sun Prairie, WI, US)* with a recommended 0.6 mm diameter needle (364,365) to harvest a total of 6 cores (two tumor epithelial, two tumor stromal, one normal epithelial and one normal stromal) from each case from the corresponding FFPE tissue blocks. Because of natural variation of tumor tissue in the depth of a specimen, the labels had to be re-defined later in the process *(see further information in section on evaluation of IHC staining below).* The harvested cores were inserted into an empty recipient paraffin block, and each cores coordinates were linked to the right case using a digital map. To include all cores, twelve tissue array blocks were constructed. In addition, a TMA multi-block of various solid tumors and normal tissues was constructed and used as controls for the IHC procedure. Before staining with IHC, 4 µm sections were cut with the HM 355S Rotary Microtome *(MICROM International GmbH, Walldorf, Germany)* form each block, dried over night at 60°C to remove endogenous water, affixed to glass slides and sealed with paraffin to minimize the risk of antigen loss. Paraffin coated sections were stored in 4°C and discarded if not used for IHC within 12 months. If the literature for the antibody stated a need for fresh sections for IHC, such was performed.

Figure 17. Illustration of tissue microarray construction (366) Reprinted by permission from Royal Society of Chemistry© 2012.



Immunohistochemistry procedure and antibodies

Since seminal articles by Dr. PK. Nakane (367) and Dr. CR. Taylor and Dr. J. Burns (368) demonstrated the potential of IHC to detect protein biomarkers on archival FFPE material, the method has become fundamental in both clinical practice and research. IHC constitutes of detecting specific antigens through the use of antibodies (immunoglobulins) which can be visualized by chemical reactions yielding a specific color *in situ* where the antigen/antibody-complex is detected. The technique is relatively time- and cost-effective and have the advantages of enabling both quantification and identifying localization of the protein expression in question. Also, through protein profiles it can be used to determine cell type with greater certainty than morphological assessment alone.

The IHC-staining for each marker in this thesis has been thoroughly described in Papers I-III, and the method will thus only be schematically described herein, with detailed information on antibodies and IHC protocols presented in Table 11. All IHC staining was done by experienced technicians *(M. Rakaee, M. Pedersen, M. Nilsen)*. Most of the markers were stained using automated slide-stainers (Table 11). Sections were deparaffinized and rehydrated. Subsequently, heat induced epitope-retrieval/HIER with retrieval solutions was performed to reverse molecular modifications produced by formalin-fixation which potentially mask antigens (369). Further, all antigens were detected through indirect IHC, namely the use of an unlabeled primary antibody to detect the desired antigen (biomarker), and then a secondary labeled antibody that binds to immunoglobulins of the animal species in which the primary antibody was raised. This differs from direct IHC, in which the primary antibody itself is labeled. The label can be an enzyme (for example horseradish peroxidase/HRP) which can react with a substrate (for example 3,3'-Diaminobenzidine/DAB) to yield color, or a linker molecule (for example biotin) which can recruit complexes of enzymes (for example avidin) enabling amplification of a color reaction. Indirect IHC has the

benefits of being more sensitive due to the signal amplification provided by binding of multiple secondary antibodies to each primary antibody (370). To avoid unspecific background staining there are typically two different approaches applied before incubation with antibodies; namely blocking of endogenous peroxidases, and blocking endogenous Fc receptors with e.g. serum from the animal species the antibodies are retrieved from (371). Finally, slides were counterstained to visualize the nuclei and tissue architecture, dehydrated through ethanol series, cleared with Xylene and mounted.

Table 11. Information on primary antibodies used for immunohistochemistry

Target	Clone	Catalog#	Type	Manufacturer	Procedure	Dilution	Incubation	Detection technique	Antigen retrieval
CD3	PS1	790-2921	Mouse mAb	Ventana[1]	Ventana Benchmark XT[1]	Prediluted	24 min 37°C	iView DAB	CC1
CD4	1F6	NCL-CD4-1F6	Mouse mAb	Novocastra[2]	Ventana Benchmark XT[1]	1:5	20 min 37°C	iView DAB	CC1
CD8	1A5	250-2714	Mouse mAb	Ventana[1]	Ventana Benchmark XT[1]	Prediluted	32 min 37°C	iView DAB	CC1
CD20	L26	760-2531	Mouse mAb	Ventana[1]	Ventana Benchmark XT[1]	Prediluted	16 min 37°C	iView DAB	CC1
PD-1	NAT105	ab52587	Mouse mAb	Abcam[3]	Ventana Benchmark XT[1]	1:50	32 min 37°C (ds) 32 min 37°C (ss)	AP-Ultraview Red (ds) HRP-Optiview DAB (ss)	CC1 30 min
PD-L1	E1L3N	13684	Rabbit mAb	Cell Signaling Technology[4]	Ventana Discovery-Ultra[1]	1:25	32 min 37°C	HRP-DAB AMP: 8 min	CC1 64 min
CD8 (ds)	SP57	790-4460	Rabbit mAb	Ventana[1]	Ventana Benchmark XT[1]	Prediluted	12 min RT	HRP-Ultra DAB	CC1 30 min
CXCL16		Ab101404	Rabbit pAb	Abcam[3]	Manually IHC	1:100	Overnight 4°C	Vectastain ABC Vector NovaRed	Citrate buffer pH 6.0 MW 20 min 450W
CXCR6		Ab125115	Goat pAb	Abcam[3]	Manually IHC	1:100	Overnight 4°C	Vectastain ABC Vector NovaRed	Citrate buffer pH 6.0 MW 20 min 450W

Antibody sensitivity and specificity

Both monoclonal and polyclonal antibodies have been used in this thesis (Table 11). Antibodies are made by immunizing animals (e.g. mouse, rabbit, goat etc.) with the antigen in question. Monoclonal antibodies are made from one clone of plasma B cells, immortalized by creating a hybridoma through fusion with a myeloma cell, which produce antibodies recognizing one single epitope of the antigen. Polyclonal antibodies are extracted from purified serum of the infected animal, and made from different clones of plasma B cells, each

producing their own antibody, which together can detect different epitopes on the antigen. Hence, monoclonal antibodies have a higher specificity compared to polyclonal antibodies (lower risk of false positive results), but polyclonal antibodies have a higher probability of detecting the antigen (lower risk of false negative results).

For IHC to be a valid method for biomarker assessment, it is important to solely use antibodies that are highly sensitive (only positive staining if antigen/biomarker is present, avoiding false negative signals) and specific (no positive staining if antigen/biomarker is not present, avoiding false positive signals), and reproducible in the context for which they are used. Thus, before an antibody is selected for further analysis, validation is necessary. Although no uniformly accepted guideline for antibody validation exists, there are several similar recommendations (372–374). At the time of our study, antibodies CD3, CD4, CD8, and CD20 (Paper I and II, Table 11) were already implemented and used in the clinical routine in our laboratory. As these antibodies are considered well-established and trusted for IHC, we performed no further antibody validation (373). Antibodies PD-L1 and PD-1 (Paper II, Table 11), and CXCL16 and CXCR6 (Paper III, Table 11) were chosen after thorough review of current literature. Even though these antibodies had been subjected to in-house validation by their manufacturer, we used multiple steps to assure antibody specificity and sensitivity, including western blot of transfected cell lines, tissue controls and negative staining controls.

Western blots determine antibody specificity against target protein based upon molecular weight and is the standard first step in antibody validation (374). The method has been thoroughly described in Papers II and III and will thus just schematically be described herein. In short, cell lysates with overexpression of the antigen in question were applied onto a gel *(4 to 12 % Bis-Tris gel, Cat# NP0322; Thermo Fisher Scientific, Waltham, MA, US),* and electrophoresis was run to separate proteins by their molecular weight, as smaller proteins migrate faster through the gel. The formed protein bands were then transferred onto a membrane *(Odyssey nitrocellulose membrane, Cat# 926-31092; LI-COR Biosciences, Lincoln, NE, US),* blocked *(Odyssey blocking buffer Cat# 927-40000; LI-COR Biosciences, Lincoln, NE, US),* and incubated with primary and secondary antibodies respectively with washing procedures in-between *(Tris-buffered saline containing 0.05% Tween 20, Cat# T9039, Sigma-Aldrich AS, Oslo, Norway)*. The procedure confirmed antigen-detection in size bands expected in terms of molecular weight of the antigen in question. Rabbit anti-actin

(Cat# A2066, Sigma-Aldrich AS, Oslo, Norway) was used for internal control and all lanes showed 42 KDa molecular weight protein load as expected.

Because validation using western blot only guarantees that the antibody will provide valid results with this exact analysis, the antibody needs to be further validated for IHC on tissues (374). This can be managed through using *positive control tissue,* namely a specimen known to contain the antigen in a given location or structure (372), and *negative control tissue* using a specimen known to not express the antibody (373). With every run of IHC, we included a slide from a TMA multi-block of various solid tumors and normal tissues in which staining was checked to correspond with literature.

Negative staining controls are applied to validate sensitivity of the antibody by examining if positive staining may be due to interactions between the IHC components with endogenous Fc-receptors or other non-specific proteins (372,373). Negative control of non-specific staining was performed through (a) omission of the primary antibody, which controls for nonspecific binding of the secondary antibody, and (b) for monoclonal antibodies, incubation with a subclass isotype-matched control antibody instead of primary antibody.

Evaluation of immunohistochemical staining

All cores were scored by two trained observers (all medical doctors, and always a minimum of one experienced pathologist, Table 12). Before initiating scoring, cores were reviewed to examine IHC quality, agreeing on which tissue compartments to score and deciding on a semiquantitative scoring scale. There are multiple reported scoring systems for quantification of IHC reactivity, such as intensity, density or combined scoring systems like Allred-score, immunoreactive score/IRS and H-score (375). For PD-L1 in clinical use as a predictive marker, many different approaches are used, such as tumor proportion score/TPS (percentage of viable cancer cells with positive membranous staining), combined positive score/CPS (number cancer cells, lymphocytes and histiocytes with positive membranous staining, divided by the total number of viable tumor cells x 100%), and immune cell score/ICS (the percentage of tumor area consisting of immune cells with positive staining) (376).

If staining was homogenous for the tissue compartment in question, a three-level intensity scoring scale was used, and in the case of heterogeneous staining we used a density scale (Table 13). Observers were independent of each other and blinded to clinicopathological data

and patient outcome. In case of major disagreement (scoring difference >1), the core was re-examined until consensus was reached. The intra-class correlation coefficient (reliability coefficient, r) by use of a *two-way random effect model with absolute agreement definition*, was calculated to examine scoring agreement between observers (Table 12). For markers CD3, CD4, CD8 and CD20 (Paper I) as well as PD-1 (Paper II) only positive, lymphocyte-like cells was scored. If possible, positive lymphocytes located intraepithelial and in tumor stromal areas were scored separately. For markers PD-L1 (Paper II) and CXCL16 and CXCR6 (Paper III) expression in tumor epithelial cells and tumor stromal cells was scored separately within the same cores. We defined tumor stroma as stromal cells adjacent to tumor epithelial cells. Stroma was only assessed as a whole, not for subgroups of tumor stromal cells. The scoring value for each core was found by calculating the mean of the two observers scores. The scoring value for each tissue compartment was then found by calculating the mean scoring value of all cores of the same tissue type.

Table 12. Information on observers and reliability

Biomarker	Scorers	Reliability coefficient (r)	Range	p
CD3, CD4, CD8, CD20	Elin Richardsen* Andrej Valkov*	0.95	0.90-0.97	< 0.001
PD-1	Elin Richardsen* Nora Ness	0.96	0.95-0.96	< 0.001
PD-L1	Andrej Valkov * Cecilie V Nordbakken*	0.93	0.92-0.93	< 0.001
CXCL16, CXCR6	Elin Richardsen* Samer Al-Saad*	0.95	0.90-0.97	< 0.001
* Pathologist				

IHC staining can be assessed manually by qualified individuals or with the use of automated systems. To eliminate subjectivity and variability of observers, automated scoring systems are announced as the future. Although the TMA method greatly facilitates automated scoring, at the time we conducted our analyses there were challenges with automated scoring systems regarding ability to recognize artifacts and separate different tissues and cell types. We had the intention of automated scoring, but our in-house ARIOL imaging system *(Applied Imaging Corp., San Jose, CA)* did not meet our expectations. Thus, we chose to score manually in a semiquantitative matter. We did this in two ways – both by digitalizing the slides and viewing the cores on a computer screen (Paper I and III) and by using a manual light microscope (Paper II).

Scoring of digitalized TMA slides

For Paper I and III the ARIOL imaging system was used to scan and digitalize IHC-stained TMA slides. Before the procedure a uropathologist *(E. Richardsen)* reviewed each TMA-core and registered the most prominent tissue in a corresponding slide map. As a result, we ended up with six different tissue types somewhat different than what they initially were chosen as when making the TMAs. Namely, tumor epithelia, tumor stroma, HGPIN, BPH, normal epithelia, and normal stroma. Next, slides were loaded in the SL 50 automated slide loader and scanned at a low resolution (1.25x) and high resolution (20x) using an Olympus BX61 microscope with an automated platform *(Prior Scientific, Cambridge, UK).* Images of the cores were uploaded into the Ariol Software, and the observers scored the samples manually by viewing the cores on a computer screen. A core was scored as missing if the core was physically missing, did not contain the tissue it was labeled as, or considered to be of insufficient quality. For lymphocyte-markers (Paper I), positive lymphocyte-like cells were scored, and location (intraepithelial or in stromal areas) was based on the label of the core. For CXCR6 and CXCL16 (Paper III), all cores were scored in two rounds, one for epithelial cells, and one for stromal cells.

Manual scoring with light microscope

For Paper II, a manual light microscope was used for scoring the IHC-stained TMA slides. A pathologist histologically assured the tissue type for each core and if possible, two cores containing prostate tumor tissue and one core containing normal tissue was scored for each case. When scoring tumor tissues, the investigator ignored any non-cancerous elements in the core and assigned a biomarker score based solely on the present tumor epithelial cells and tumor stromal cells respectively.

Table 13. Information on expression, scoring and cut-offs of biomarkers

Biomarker	Distribution of expression	Localization of expression	Scoring system	Scoring scale	Cut-off
CD3	Focal	Predominantly membranous, some cytoplasmic staining.	Digitalized[1]	Density. Positive cells per 0.6 mm core. 0 = 0-5%; 1 = 6-25%; 2 = 26-75%; 3 = >75%	TEc = 0.50 *(n = 217 low expression, 218 high expression)* TSc = 0.62 (mean) TEc+TSc = 0.50 *(n = 239 low expression, 233 high expression)*
CD4	Focal	Predominantly membranous, some cytoplasmic staining.	Digitalized[1]	Density. Positive cells per 0.6 mm core. 0 = 0-5%; 1 = 6-25%; 2 = 26-75%; 3 = >75%	TEc = 0.44 (mean) TSc = 0.36 (mean) TEc+TSc = 0.50 *(n = 383 low expression, 138 high expression)*
CD8	Focal	Predominantly membranous, some cytoplasmic staining.	Digitalized[1]	Density. Positive cells per 0.6 mm core. 0 = 0-5%; 1 = 6-25%; 2 = 26-75%; 3 = >75%	TEc = 0.42 (mean) *(n = 254 low expression, 182 high expression)* TSc = 0.35 (mean) TEc+TSc = 0.41 (mean) *(n = 279 low expression, 194 high expression)*
CD20	Focal	Predominantly membranous, some cytoplasmic staining.	Digitalized[1]	Density. Positive cells per 0.6 mm core. 0 = 0-5%; 1 = 6-25%; 2 = 26-75%; 3 = >75%	TEc = 0.20 (mean) TSc = 0.25 (mean) TEc+TSc = 0.21 (mean)
PD-1	Focal	Predominantly membranous, some cytoplasmic staining.	Light microscope	Density. Positive cells per 0.6 mm core. 0 = 0-3; 1 = 4-10 cells; 2 = 11-15 cells; 3 = > 15 cells	TS = 1.25
PD-L1	TE: Homogenous TS: Focal	Cytoplasmic and membranous. Intensive intraluminal secretions and intracellular granules were disregarded as artifacts.	Light microscope	TE: Intensity. 0 = Negative; 1 = weak, 2 = intermediate; 3 = strong TS: Density. Positive cells per 0.6 mm core. 0 = 0-3; 1 = 4-10 cells; 2 = 11-15 cells; 3 = > 15 cells	TE = 1.0 TS = 0.54 (mean)
CXCR6	TE: Homogenous TS: Homogenous	Granular and cytoplasmic.	Digitalized[1]	TE: Intensity. 0 = Negative; 1 = weak, 2 = intermediate; 3 = strong TS: Intensity. 0 = Negative; 1 = weak, 2 = intermediate; 3 = strong	TE = 1.91 (mean) *(n = 222 low expression, 244 high expression)* (unpublished results) TE+TS = 1.50 (1-2 vs. 3-4 quartile) *(n = 239 low expression and 237 high expression)*
CXCL16	TE: Homogenous TS: Homogenous	Predominantly cytoplasmic, some membranous staining.	Digitalized[1]	TE: Intensity. 0 = Negative; 1 = weak, 2 = intermediate; 3 = strong TS: Intensity. 0 = Negative; 1 = weak, 2 = intermediate; 3 = strong	TE = 1.12 (1-2 vs. 3-4 quartile) *(n= 240 low expression and 230 high expression)*

[1]ARIOL imaging system (Applied Imaging Corp., San Jose, CA)
Abbreviations: TE = tumor epithelial cells; TEc = positive cells in the tumor epithelial compartment; TS = tumor stromal cells; TSc = positive cells in the tumor stromal compartment

3.1.4. Statistical analysis

All statistical analyses were performed using the statistical package IBM SPSS, version 21 (Paper I), 22 (Paper III) and 23 (Paper II) (SPSS Inc., Chicago, IL). The significance level was set as $p \leq 0.05$ for all analyses. A Wilcoxon signed rank test was used to check for *differences in expression* of the biomarkers between different tissue types (Paper I and III). Spearman's rank-correlation test was used to calculate *correlations* between variables, and moderate or strong correlations ($r > 0.2$) was emphasized. All *survival analyses* were carried out using both BF, CF and PCD as endpoints. Univariate survival curves were drawn by using the Kaplan–Meier method, and the statistical difference between curves was assessed by the log-rank test. The survival curves were terminated at 134 months for Paper I and 192 months for Paper II and III, due to less than 10% of patients at risk after this point. In order to assess the independent prognostic value of the tested biomarkers in regard to endpoints, we used a multivariate backward stepwise Cox regression model with a probability for stepwise entry or removal at both $p = 0.05$ and 0.10. For Paper I and III, significant biomarkers and clinicopathological variables ($p < 0.05$) were entered into the multivariate analysis. For Paper II biomarkers and clinicopathological variables with $p < 0.10$ from the univariate analysis was entered.

3.2. EXPERIMENTAL STUDIES

For Paper III we did experimental *in vitro* studies in addition to using the patient cohort design. Methodological details are published in Paper III and will thus only briefly be stated herein. The goal was to investigate if knockdown of chemokine CXCL16 in PCa cells would affect cancer cell proliferation and -migration.

3.2.1. Cell culture

Two PCa cell lines *(DU145 Cat# HTB81 and PC-3 Cat# CRL-1435 both from ATCC, Manassas, VA, US)* were verified by the Department of Forensic Medicine at UiT The Artic University of Norway, and cultured (details in Paper III). The DU145 cell line was originally derived from a central nervous metastasis and cells are castration resistant, AR-negative, does not express PSA, and has a moderate metastatic potential. The PC3 cell line was originally derived from a metastatic bone lesion and cells are castration resistant, AR-negative, does not express PSA, and have a high metastatic potential. PC3 cells have characteristics of neuroendocrine carcinoma rather than adenocarcinoma (377).

3.2.2. RNA Interference

Cells were transfected with CXCL16 siRNA *(Cat# 4392420, s33809, Silencer Select CXCL16 siRNA, Ambion, Carlsbad, CA, US).* A Cy3-labeled negative control scrambled siRNA *(Cat# AM4621, Silencer Cy3-labeled Negative Control No. 1 siRNA, Ambion, Carlsbad, CA, US)* was used as negative control in all experiments. Transfection efficiency was typically assessed at 85% to 100%.

3.2.3. Proliferation Assay

Trypsinization was used to detach transfected cells, before resuspension in complete growth media. Cells were counted, and with initial titration experiments, optimal cell number per well was determined to be 5000. After baseline measurement, cells were seeded in quadruplicate into the E-plate 16 *(Cat# 05469830001; Roche, Oslo, Norway)* according to the manufacturers protocol. The plate was incubated for 30 minutes at room temperature before positioned in the real-time cell analyzer system xCELLigence RTCA DP instrument *(Real-Time Cell Analyzer Dual-Plate, Cat# 05469759001, Roche, Oslo, Norway)* located in an incubator preserving the same conditions used for routine cultivation of the PCa cell lines. The instrument denoted the cellular growth rate as Cell Index, which is an arbitrary unit reflecting the cell-sensor impedance. The cell index was measured every 15 minutes for the first 24 hours and then every 30 minutes. Growth curves and doubling times were calculated with the RTCA software version 1.2.1 *(Roche, Oslo, Norway).* For each cell line, three independent experiments were performed.

3.2.4. Migration Assay

Ibidi Culture-Inserts *(Inter Instrument AS, Høvik, Norway)* were used to assess migration. The inserts, consisting of two chambers with a 0.5 μm divider, were planted into a 12-well tissue culture dish (one insert per well) using sterile tweezers. 70 μL suspensions containing 4-6 × 10^5 transfected cells/mL were added to each chamber. The cells were left to adhere before the insert was removed and fresh media added. Using a light microscope, images were acquired along the cell-free gap made by the divider at time points 0, 6, and 24 hours. The migration rate into the gap was calculated using the free software TScratch version 1.0 *(Computational Science and Engineering Laboratory, Zurich, Switzerland).* For each cell line, three independent experiments were performed.

4. RESULTS

4.1. PAPER I

The adaptive immune system can potentially have dual roles in cancer development and progression, by either stimulating, or suppressing tumorigenesis. Hence, the aim of this study was to evaluate the prognostic impact of adaptive immune cells residing in different tumor compartments in PCa. For this paper, the density of CD3+, CD4+, CD8+ and CD20+ lymphocytes were analyzed in relation to outcome, as well as their relations to each other and clinicopathological variables.

4.1.1. Expression

Of the total cohort of 535 patients, evaluation of IHC-staining of tumor tissue was possible for 472/535 (88%) cases for CD3, 521/535 (97%) for CD4, 473/535 (88%) for CD8, and 469/535 (87%) for CD20. Lymphocytes were observed microscopically and scored in both intraepithelial and in tumor stromal areas. In statistical analyses we also combined the two locations as an intratumoral score. By quantitative assessment of CD3+ and CD20+ stained lymphocytes – considered to be T lymphocytes and B lymphocytes respectively – intratumoral lymphocytes were found to mainly constitute T lymphocytes (CD3+). There were significantly higher densities of intratumoral CD3+ ($p = < 0.001$), CD4+ ($p = 0.006$) and CD8+ ($p = 0.008$) lymphocytes compared to in non-malignant tissue areas from the cancer patients. There was no such difference observed for CD20+ lymphocytes. The density of intratumoral CD3+ lymphocytes was significantly higher in the intraepithelial compartment compared to tumor stromal areas ($p < 0.001$). For CD4+, CD8+, and CD20+ lymphocytes, no such difference was found.

4.1.2. Correlations

There was no correlation between investigated lymphocyte markers and clinicopathological variables.

4.1.3. Univariate survival analysis

In univariate survival analysis, a high density of intratumoral CD3+, CD4+ and CD8+ lymphocytes were associated with significantly shorter BFFS ($p = 0.046$, $p = 0.026$, $p = 0.003$ respectively). In separate analyses of intraepithelial lymphocytes, the same pattern was

apparent for CD3+ (p = 0.037) and CD8+ lymphocytes (p = 0.010). Even though showing the same negative prognostic tendencies, there were no significant associations between lymphocyte densities and CF or PCD. CD20+ lymphocytes were not associated with outcome. Results presented in Table 14a.

5.1.4. Multivariate survival analysis

In multivariate analysis, a high density of intratumoral CD8+ lymphocytes were an independent, negative predictor of BF (HR = 1.6, CI 95% 1.1-2.2, p = 0.007) together with pT-stage, Gleason score, non-apical PSM and apical PSM. The same association was present when analyzing intraepithelial CD8+ lymphocytes alone (HR = 1.5, CI 95% 1.0-2.0, p = 0.032). Results presented in Table 14a.

4.2. PAPER II

For our next paper we wanted to investigate possible explanations as to why intratumoral CD8+ lymphocytes seemed to be of negative prognostic importance in our cohort. We hypothesized that the lymphocytes we had detected in earlier work (Paper I) indeed were tumor-specific T lymphocytes attracted to especially aggressive tumors, but lacking functionality due to immunosuppression. The PD-1 pathway in intratumoral lymphocytes had recently gained massive attention as an important immunosuppression pathway involved in cancer immune escape, and excitingly, can be targeted by different immune checkpoint-inhibitors. Hence, for this paper we evaluated the expression pattern and prognostic impact of PD-1 and its ligand PD-L1 in our cohort of primary PCa cases.

4.2.1. Expression

Of the total cohort of 535 patients, evaluation of IHC-staining of tumor tissue was possible for 402/535 cases (75%) for PD-L1, and 396/535 (74%) for PD-1.

In general, intratumoral PD-1+ cells were relatively rare (found in 156/396, 39% of patients) and had the morphology of lymphocytes. Additionally, we observed a few intraepithelial PD-1+ cells. Some of these resembled tumor cells, but unfortunately, we were not certain these were tumor cells using morphological assessment alone, and this, in addition to very low numbers, made them impossible to quantify by scoring. Thus, we scored PD-1+ lymphocytes as a whole independently of which tumor department they were located in. 43/396 (11%) cases were categorized as having a high density of PD-1+ lymphocytes. PD-1 and CD8

double staining showed co-expression of CD8 and PD-1, but also lymphocytes with single expression of each marker. However, the brown CD8 staining overpowered the red stain of PD-1, making quantification by scoring very difficult.

PD-L1 expression was both cytoplasmatic and membranous. Intraluminal secretions and some intracellular granules seemed to stain intensively and were disregarded as artifacts. PL-L1 expression by tumor epithelial cells was seen in 92% of cases (371/402), and 236/402 (59%) cases were categorized as having a high PD-L1 expression. In addition, 66% (267/402) of patients had PD-L1+ tumor stromal cells.

4.2.2. Correlation

PD-1 and PD-L1 expression did not correlate to clinicopathological variables, nor to CD3+, CD4+, CD8+ and CD20+ lymphocytes examined in Paper I. The expression of PD-L1+ tumor stromal cells correlated significantly with PD-L1+ tumor epithelial cells ($r = 0.36$, $p = < 0.001$), and had a weak correlation with PD-1+ lymphocytes ($r = 0.21$, $p = < 0.001$).

4.2.3. Univariate survival analysis

In univariate survival analysis, a high density of PD-1+ intratumoral lymphocytes was associated with significantly shorter CFFS in subgroups known to indicate unfavorable PCa prognosis, namely Gleason grade 9 ($p = <0.001$), age at diagnosis < 65 years ($p = 0.025$), preoperative PSA > 10 ($p = 0.039$) and stage pT3 disease ($p = 0.011$). For the whole group the association was limited to a trend only ($p = 0.084$). Expression of PD-L1 by neither tumor epithelial cells nor tumor stromal cells reached statistical significance for predicting BF, CF, or PCD, but there was a trend towards association between a high expression of PD-L1+ tumor epithelial cells and worse outcome, most prominently for shorter BFFS ($p = 0.078$). Results presented in Table 14a and 14b.

4.2.4. Multivariate survival analysis

In multivariate survival analysis, a high density of PD-1+ intratumoral lymphocytes was an independent, negative prognostic factor for CF (HR = 2.5, CI 95% 1.1-5.5, $p = 0.025$) together with Gleason grade and PNI. Results presented in Table 14a and 14b.

4.3. PAPER III

Chemokines and their receptors are important contributors in tumorigenesis as well as in creating anti-cancer immune response. Chemokine CXCL16 and its receptor CXCR6 are important for lymphocyte function and can also affect tumorigenesis through different pathways. Hence, for our next paper we wanted to examine their prognostic significance in our cohort of primary PCa tumors. We analyzed the intensity of expression in both tumor epithelial cells and tumor stromal cells in addition to non-malignant tissue areas.

4.3.1. Expression

CXCR6- and CXCL16 expression was observed on both tumor epithelial and tumor stromal cells microscopically. CXCR6 staining was both granular and cytoplasmic whilst CXCL16 staining was predominantly cytoplasmic, with some membranous staining. There was a significantly higher expression of CXCR6 in tumor tissue compared with non-malignant tissue areas from the cancer patients ($p < 0.001$). CXCR6 was significantly more expressed by tumor epithelial cells compared to tumor stromal cells ($p = 0.008$). There was also significantly higher expression of CXCL16 in tumor tissue compared with non-malignant tissue areas ($p < 0.001$), but no significant difference in expression levels by tumor epithelial cells compared to tumor stromal cells. Co-expression of CXCR6 and CXCL16 was common in tumors ($p < 0.001$) and was significantly higher in tumor tissue compared with non-malignant tissue areas ($p < 0.001$).

4.3.2. Correlation

Co-expression of CXCR6 and CXCL16 in tumor tissue correlated weakly with Gleason score >7 ($p = 0.009$), LVI ($p = 0.020$), and PSM ($p = 0.014$). CXCR6 expression was not correlated to CD3+, CD4+, CD8+ and CD20+ lymphocytes. CXCL16 expression correlated moderately with CXCR6 expression ($p = 0.000$) and there was a weak correlation to CD3+ lymphocytes ($p = 0.034$). R-values below <0,2 (weak) are omitted.

4.3.3. Univariate survival analysis

A high expression of CXCR6 by tumor epithelial cells was associated with significantly shorter BFFS ($p = 0.027$), as well as CFFS ($p = 0.000$) *(unpublished results).* CXCR6 expression by tumor stromal cells alone did not reach statistical significance for any endpoints. A high expression of CXCR6 in tumor tissue (epithelial and tumor stromal cells analyzed as one compartment) was associated with significantly shorter BFFS ($p = 0.003$).

The same trend was apparent for CFFS but did not reach significance (p = 0.063). A high expression of CXCL16 by tumor epithelial cells was associated with significantly shorter CFFS (p = 0.017). CXCL16 expression by tumor stromal cells alone did not reach statistical significance for any endpoints. A high co-expression of CXCL16 and CXCR6 in tumor tissue was associated with significantly shorter BFFS (p = 0.016) and CFFS (p = 0.023). Results presented in 14b.

4.3.4. Multivariate survival analysis

A high expression of CXCR6 by tumor epithelial cells was an independent negative prognostic factor for both BF (HR = 1.5, CI 95% 1.1-2.1, p = 0.010) together with Gleason score, PSM, pT-stage and PNI *(unpublished results)*, and CF (HR = 7.1, CI 95% 2.5-20.4, p = 0.000) together with Gleason score, PSM and PNI *(unpublished results)*. A high expression of CXCR6 in tumor tissue was an independent, negative prognostic factor for both BF (HR = 1.7, CI 95% 1.3-2.4, p = 0.001) together with Gleason grade, PNI, PSM and pT-stage, as well as CF (HR = 2.3, CI 95% 1.1-4.8, p = 0.028) together with Gleason grade, PNI and PSM. A high expression of CXCL16 by tumor epithelial cells was an independent, negative prognostic factor for CF (HR = 2.5, CI 95% 1.2-5.7, p = 0.025) together with Gleason grade, PNI and PSM. A high co-expression of CXCR6 and CXCL16 in tumor tissue was an independent, negative prognostic factor for CF (HR = 5.1, CI 95% 1.6-15.9, p = 0.005) together with Gleason grade, LVI and PSM. Results presented in 14b.

4.3.5. Migration and proliferation assay

PCa cell lines DU145 and PC3 were used for CXCL16 siRNA knockdown analysis. We repeatedly observed that such silencing caused activation of proliferation and an increased migration rate compared to negative controls.

4.4. PROGNOSTIC RESULTS SUMMARIZED

Table 14a. Uni- and multivariate prognostic impact of immunological markers on endpoints. Summary of biomarkers expressed by intratumoral lymphocyte-like cells

Biomarker	Univariate analysis			Multivariate analysis		
	Intraepithelial	**In tumor stromal areas**	**Intratumoral (tumor as one entity)**	**Intraepithelial**	**In tumor stromal areas**	**Intratumoral (tumor as one entity)**
CD3	↑ negative prognostic factor for BF	*NS*	↑ negative prognostic factor for BF	*NS*	*NE*	*NS*
CD4	*NS*	*NS*	↑ negative prognostic factor for BF	*NE*	*NE*	*NS*
CD8	↑ negative prognostic factor for BF	*NS*	↑ negative prognostic factor for BF	↑ negative prognostic factor for BF	NE	↑ negative prognostic factor for BF
CD20	*NS*	*NS*	*NS*	*NE*	*NE*	*NE*
PD-1	*NA*	*NA*	*NS* for the group as a whole, ↑ negative prognostic factor for CF in subgroups with unfavorable prognosis.	*NA*	*NA*	↑ negative prognostic factor for CF

Table 14b. Uni- and multivariate prognostic impact of immunological markers on endpoints. Summary of biomarkers expressed by tumor epithelial cells and tumor stromal cells

Biomarker	Univariate analysis			Multivariate analysis		
	Tumor epithelial cells	**Tumor stromal cells**	**Tumor tissue**	**Tumor epithelial cells**	**Tumor stromal cells**	**Tumor tissue**
PD-L1	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
CXCR6	↑ negative prognostic factor for BF and CF (unpublished results)	*NS*	↑ negative prognostic factor for BF	↑ negative prognostic factor for BF and CF (unpublished results).	*NE*	↑ negative prognostic factor for BF and CF
CXCL16	↑ negative prognostic factor for CF	*NS*	*NE*	↑ negative prognostic factor for CF	*NE*	*NE*
CXCR6/ CXCL16	*NE*	*NE*	↑ negative prognostic factor for BF and CF	*NE*	*NE*	↑ negative prognostic factor for CF
Abbreviations: ↑ = High expression; BF = biochemical failure; CF = clinical failure; NA = not assessed; NE = not entered; NS = non-significant						

5. DISCUSSION

5.1. METHODOLOGICAL CONSIDERATIONS

5.1.1. Ethical considerations

The clinical data and tissues used in our cohort was initially collected and stored in a therapeutic context, and our research has been performed without obtaining informed consent from the patients or their relatives. This presents an important ethical issue. One of the main ethical guidelines for medical research involving human subjects, is the Helsinki Declaration by the Word Medical Association/WMA, which states that physicians performing medical research using identifiable human material or data must seek informed consent for its collection, storage and/or reuse (pt.32, 378). Additionally, Norwegian law binds the researcher to obtain informed, voluntary, explicit and verifiable consent from subjects in medical research (chapter 4, §13, 379). However, both the Declaration of Helsinki (pt.32, 378) and the Norwegian health research law (chapter 6, §28, 379) opens up for exceptions from informed consent if the research is approved by a research ethics committee, which is the case for the herein presented research project. There are multiple arguments as to why informed consent sometimes is not obtained. (a) For big materials it is time consuming, and confiscates time that could otherwise be used for research, (b) cases not consenting could cause a potential bias, (c) majority of cases are dead by the time the research is initiated, (d) it can be considered more compassionate to not contact cases or their relatives to prevent reminding them of a tragic cancer diagnosis, and/or (e) information on the research project may lead to false hope that the research may directly affect the outcome of their cancer disease in a positive way. For medical research to be ethical, the benefit of the study must outweigh the risks. As our data is collected retrospectively, our research has not inflicted the subjects to any physical risk by exposing them to modified or additional procedures (380). Nonetheless, reading, collecting and storing private and sensitive health information poses a potential psychosocial risk if these data were to fall into the hands of outsiders. We minimized the potential risk of this by high standards of data protection, such as complying with the law of confidentiality, including no identity details in publications, and working only with de-identified version of the data and with the original database including the identifiable details locked away with only the research groups leaders *(E. Richardsen and S. Andersen)* having direct access.

5.1.2. Reliability, validity and representativeness of the data material

Reliability refers to the data material being trustworthy, authentic, dependable, accurately recorded and without systemic bias. In our case, the data material benefits from the fact that the same experienced pathologists *(E. Richardsen and L.T. Rasmussen Busund)* registered all histopathological data afresh, not trusting previously stated journals. For clinical data, it is an advantage that an oncologist *(S. Andersen)* was in charge of collection, there was no loss of patients during follow-up, and that data was collected from comprehensive journals as Norway has a strong public health care system with little to no loss of patients to a private system. However, a retrospective study design has some limitations, and will increase the risk of information bias, as verifying information in patient journals is difficult.

When it comes to the reliability issues regarding biomarker analyses, some is described for the pre-analytical handling of tumor specimens in section *5.1.5. Tissue quality and pre-analytical considerations.* Also, a potential problem is the fact that after the IHC procedure, not all cases had viable tumor tissue to assess biomarker expression. This can be due to errors in selection of tissue for the TMA, technical issues during the TMA process, or because of normal, random loss of cores with deeper sectioning of the tissue blocks or during IHC procedures. Another possible negative influencer of reliability is the semiquantitative evaluation of biomarker expression, which, even though optimized, is still a subjective method. Sadly, a potentially considerable reliability issue occurred during collection of the material, when it became apparent that a relatively large proportion of patients from St. Olavs Hospital had to be eliminated because their tumor material was already in use by another research group (Figure 16). Best case scenario, the other research group have chosen their tumors randomly. Worst case, they have chosen the most “biologically interesting” tumors, and/or tumors from patients with hard endpoints, and thus this is a source of uncontrolled selection bias in our material. When comparing clinicopathological variables (Table 15), we found that patients at UNN and NLSH had a higher share of patients with BF compared to St. Olavs Hospital, but there were no significant differences in CF and PCD. In addition, when performing survival analyses for the biomarkers, we sub-stratified for the three hospitals involved as a form of internal validation, and the results as a general rule showed the same tendencies. To validate the analytical and post-analytical condition of our data material, we should ideally have conducted all steps of the analysis first in a training set (hypothesis generating), and then a test set (hypothesis testing).

Validity (also referred to as *internal validity*), encompasses whether the observed results represent the truth in the study population, and thus, are not due to material- or methodological errors. In other words, if we observe a biomarker to be prognostic for an endpoint, is it likely that this is a genuine relationship? To reduce the risk of erroneous influence, we excluded patients where external factors probably could have influenced the TME. In addition, we wanted a very high probability that progression of disease was attributed to primary prostate cancer by avoiding metastases from other malignancies to be wrongly interpreted as prostate cancer CF or PCD. Thus, we excluded patients who had undergone radiotherapy to the pelvic region prior to RP, patients who had received pre-operative hormonal therapy, and patients who had had another malignancy (except basal cell carcinoma, a superficial skin cancer) within 5 years previous to the PCa diagnosis.

Representativeness (also referred to as *generalizability* or *external validation*), of the data material is necessary for the results to be transferable to other groups of interest, e.g. the Norwegian, Nordic or even international population. It is important that the study population is homogenous enough so that other factors than what we are supposed to study do not get to effect results (high internal validity), but at the same time is heterogenous enough to represent a relevant population in "the real world". As most patients get their cancer treatment at public hospitals in their own health region in Norway, the patient cohort presented consist of approximately 1/5 of unselected, Norwegian men undergoing RP as treatment for their localized PCa from 01.01.1995 to 31.12.2005. Additionally, national cancer guidelines in Norway offers a highly standardized, clinical strategy in diagnosis and treatment, which makes it likely that the study population reflect the Norwegian population. For the results to be relevant, it is also important that the study population is representative of the patients we see in the clinic today. Thus, we included patients from the PSA era, which means a relatively large proportion of the patients may have asymptomatic and perhaps indolent cancers detected by unsystematic PSA screening which is a common initiation to getting a PCa diagnosis today.

Table 15. Patient characteristics and clinicopathological variables arranged for the different pathological centers (n = 535), (Pearson's Chi-square test, Mann-Whitney U test) significant p values in bold (threshold ≤ 0.05). Variables from database updated December 2015

Variable	Surgical center			p
	UNN	NLSH	St. Olavs Hospital	
Number of patients, n	248	59	228	
BF, %	48 %	46 %	24 %	**<0.001**
CF, %	12 %	3 %	11 %	0.164
PCD, %	4 %	2 %	3 %	0.635
Total mortality, %	24 %	12 %	15 %	**0.016**
Mean age at surgery, years	62.8	62.6	60.7	**<0.001**
Surgical proc				**<0.001**
Retropubic, %	60 %	100 %	100 %	
Perineal, %	40 %	0 %	0 %	
Mean preop PSA	13.7	7.4	9.3	**<0.001**
Gleason grade / ISUP Grade Group				**<0.001**
3+3 / ISUP Grade group 1, %	29 %	59 %	34 %	
3+4 / ISUP Grade group 2, %	42 %	31 %	43 %	
4+3 / ISUP Grade group 3, %	17 %	7 %	16 %	
4+4 / ISUP Grade group 4, %	4 %	2 %	3 %	
>8 / ISUP Grade group 5, %	9 %	2 %	5 %	
pT-stage				**<0.001**
pT2, %	61 %	97 %	73 %	
pT3a, %	26 %	0 %	22 %	
pT3b, %	13 %	3 %	6 %	
Mean tumor size, mm	15.3	16.8	15.0	**0.050**
PNI, %	21 %	71 %	17 %	**<0.001**
PSM, %	46 %	34 %	67 %	**<0.001**

Abbreviations: BF = biochemical failure; CF = clinical failure; ISUP = International Society of Urological Pathology; NLSH = Nordland Hospital; PCD = Prostate cancer death; PNI = Perineural infiltration; Preop = preoperative; PSA = Prostate specific antigen; PSM = Positive surgical margin; pT-stage = pathological tumor stage; Proc = procedure; UNN = University hospital of Northern Norway

5.1.3. Endpoints issues

We made some perhaps controversial decisions regarding endpoint definitions. Firstly, we defined PSA threshold for BF as ≥ 0.4 ng/ml. Both the AUA (381) and the EAU (119) have recommended defining BF after RP as a serum PSA ≥ 0.2 ng/mL followed by a second confirmatory level. However, at least before PSMA-PET became widely available, others argued that a higher cut-off value had a higher specificity and was better at predicting metastatic progression (382,383). Moreover, we included local recurrence in CF, where most only use metastatic relapse as endpoint (384). Even though this may weaken translation of CF as an endpoint predicting PCD, we wanted to include local recurrence if it had a symptomatic impact on the patient.

Some argue that overall survival is a more meaningful endpoint in cancer research compared to disease-specific survival (385). This is especially relevant when researching treatment regimes, as overall survival also gives information on the potentially lethal side-effects of

cancer therapy. However, in the case of PCa, with such a low mortality rate and a high average patient age which increases risk for fatal comorbidities, disease-specific survival will probably give a more accurate picture when examining prognostic factors. Another problem in retrospective PCa research, is the fact that PCD is in general thought to be overestimated, as many patients with indolent metastatic disease who dies of other, cancer-unrelated causes, are registered as death caused by PCa. To prevent this problem in our cohort, we did not relay on already registered death cause when collecting data. Instead, we thoroughly read the journals and bloodwork of patients to interpret the time leading up to death to conclude if this was directly caused by their PCa.

PCa patients in general have a high disease-specific survival rate, and those who do die of the disease, often do so several years after the initial diagnosis (4). These characteristics prompts the need for a large study population and a long follow-up time to get statistically significant results in prognostic research. As a result, surrogate endpoints such as BF and CF are often used in addition to PCD. This may be problematic as some patients suffering BF after prostatectomy, experience rapid progression and early death, while others have an indolent course and in combination with high age is at risk of dying of other causes (386,387). The use of different endpoints can be confusing and makes it difficult to compare different prognostic biomarker studies, as results for different endpoints are not directly comparable. For this thesis we always performed all survival analyses for all three endpoints, and they as a general rule showed the same tendencies for each variable, even though not always reaching statistical significance.

Additionally, retrospective research does not offer the possibility of standardized protocols for follow-up, thus if some patients are followed more closely than others, e.g. by PSA measurements, this can lead to an earlier detection of BF and/or CF in these patients, affecting survival analyses. Also, differences in survival time after BF or CF can be affected by confounding factors. This is especially relevant if there is made advances in palliative treatment options and/or if new guidelines are implemented during the follow-up, but also because factors such as age and comorbidity, as well as patient wishes, and doctors' preferences will influence if, and when, a palliative treatment is introduced for a patient. Differences in palliative treatment strategies within the study population can impact disease-specific survival time, and thus the reliability of results.

5.1.4. Advantages and disadvantages using tissue microarray

The TMA method has both strength and weaknesses (Table 16) compared to whole sections. One of the biggest concerns when using the technique in prognostic biomarker research, is tumor representativity. Namely, does the expression of the biomarker in the TMA cores reflect the expression in the tumor as a whole (363). However, it is important to remember that even though the gold standard for *in situ* analysis is considered to be whole sections, these also only represent a small portion of a tridimensional tumor. Representativity is an especially relevant concern in a heterogenic, multifocal tumor type such as PCa, but TMA have previously been validated (364) and used (388) in PCa research. The area of which the cores are sampled from, as well as the number of cores sampled is important for representativity. One should also always calculate with a general loss of cores during TMA processing and IHC-staining process of approximately 10-15% (389). A general recommendation is sampling one to four tumor cores per case (390). PCa researchers have recommended that three to four cores from areas of different tumor grades should be sampled. Hence, for each case we sampled a minimum of four cores from representative tumor areas – two from tumor epithelial areas and two from tumor stromal areas. Our intention when making the TMA was to include the most representative tumor tissue for each case, thus we have not specifically targeted areas of tumor inflammation and/or immune cells, such as tertiary lymphoid structures. This can be a potential weakness when analyzing immune markers. However, in general, it is likely that biomarkers of true clinical value will display a relatively uniform staining in tumors.

Table 16. Summary of strengths and weaknesses of tissue microarray

Strengths	Weaknesses
Tissue saving – Consuming only small parts of primary tissue for each analysis.	Possibility of sampling error during core extraction.
Quick evaluation of expression – Close proximity of cores permits more rapid and consistent biomarker evaluation.	Core loss of 10-15% during sectioning, transfer, and staining, leading to lower statistical power.
Cores are non-identifiable, and thus evaluation is blinded from clinical- and outcome data.	Loss in antigenicity with long time between sample preparation and IHC.
All samples on a TMA slide are stained under identical experimental conditions, thereby reducing technical variation.	Small cores may not accurately reflect the whole tumor because of tumor- and biomarker heterogeneity, and the tissue content may change over the length of the core.
Saving scientific resources and costs: Reduced usage of laboratory consumables (IHC) and personnel (evaluation)	Loss of orientation of slide will lead to incorrect connection of biomarker data with clinicopathological data.

5.1.5. Tissue quality and pre-analytical considerations

Multiple factors influence the validity of IHC results, and there is a need for quality in every step when handling of the tumor specimens (Table 17).

Table 17. Analytical variables influencing the reliability of immunohistochemistry

Pre-analytical variables	Surgery time, type and intraoperative conditions Tissue fixation method, time, and volume Tissue processing Storage of blocks Sectioning, processing and storage of slides
Analytical variables	Epitope retrieval Antibody used, antibody titer, incubation time Manual vs. automated staining Detection system Antibody validation and controls
Post-analytical variables	Interpretation of staining (scoring) Choosing cut-off for survival statistics

Biomarker research such as ours, often relies on archival FFPE tissue that initially have been collected and preserved for therapeutic reasons. Thus, pre-analytical aspects may not always be optimal. For our multicenter, retrospective cohort with a ten-year range, it is only natural that all specimens have not had the exact same pre-analytical conditions. There are multiple important elements in the pre-analytical phase. Firstly, the activation of tissue enzymes and autolysis and degradation of biological molecules instantly start when the surgeon cuts the blood supply (391). “Ischemia time” refers to the time between clamping of the arterial blood supply, until fixation of the specimen, and is a major influencer of what we are able to detect by IHC (392). Different markers, patients and tissues have different tolerance level before ischemic degradation begins (393). Another important factor is how fixation is conducted. All specimens in our material have undergone fixation with buffered formalin, which stabilizes proteins and preserves morphology through molecular modulations (369). Understandably, the fixation time in our cohort varied, both between surgical centers, and within each center. This poses a potential issue, as both under- and over-fixation increases the likelihood of false negative IHC. On the one hand, even though tissue penetration of formalin is relatively quick, the chemical reactions responsible for cross-linking and completion of the fixation process is very slow and relies on multiple aspects such as temperature, specimen dimension, and formalin/specimen-volume ratio, which makes under-fixation a common problem (393). On the other hand, prolonged formalin fixation may lead to excessive crosslinking, which makes over-fixation a possible issue. However, epitope retrieval used as a part of IHC, may be able to reverse this, at least for some epitopes (369). To make sure different fixation times between

centers did not corrupt our results, we sub-stratified all biomarker survival analyses for pathological center, and the results as a general rule showed the same tendencies.

Another pre-analytical consideration is how the FFPE prostatectomy specimens, TMA blocks and cut sections are stored pending IHC. Blocks generally has a good durability, but storage of cut sections can induce decline in antigenicity, at least for some epitopes. As stated in section *3.1.3. Prognostic biomarkers,* cut sections were sealed with paraffin, and stored in 4°C, and not used if older than 12 months. There are no general guidelines on how to store sections, but storage in 4 degrees compared to room temperature seems to give a lesser decrease in antigenicity (394–397). When it comes to paraffin coating as protection against oxidation of epitopes, some studies have found it to have the wanted effect (398) while others claim it to not be effective (395), and actually minor adverse effects have been observed (396). Some have also pointed out the importance of long enough processing time to remove endogenous water before storing the tissue (394), and yet others claims that newer epitope retrieval methods have made it possible to detect epitopes on stored slides even if they have not been stored in optimal conditions (396).

5.1.6. Choosing evaluation method of immune cells in tumor tissue

Immune cell content in the TME can be evaluated through IHC, flow cytometry, or gene signatures (399). For flow cytometry, potentially technically challenging tumor tissue disaggregation is necessary. Gene marker signatures can estimate the contribution of different immune cells in the TME based on gene expression (usually RNA). However, many RNAs and proteins are associated with more than one immune cell type, thus each immune cell does not have a general profile signature that all agrees on (400). IHC has multiple limitations (Table 17) but in a prognostic context, mainly because analyses are limited to small areas of tissue, and only a few markers can be examined at a time. One advantage of IHC is the additional information of the *location* of immune cells in the TME. For the immune cell subgroup of TILs is has been suggested clinically relevant if the location is in central parts of the tumor (intraepithelial), in the invasive boarder of the tumor, or in tertiary lymphoid structures close by the tumor (326).

5.1.7. Cut-off determination

In order to perform survival analyses for a variable, and later translate results into a clinical decision, it is necessary to allocate patients into comparable groups of high and low biomarker expression (401). There are different strategies to determine a cut-off point for such dichotomization, each with associated advantages and disadvantages. To avoid cut-off selection bias, it is common to choose an objective approach, such as categorizing cases by mean or median variable expression, or in quartiles. This approach lowers the risk of false positive results (type 1 error) and may be easier for other research groups to reproduce. However, it does not necessarily always reveal a potential biological relationship between biomarker expression and outcome, and thus increases the risk for false negative results (type 2 error). In addressing this, a minimal p-approach, which goal is to find a cut-off to optimize the correlation between biomarker expression and clinical outcome, can be better.

For clinicopathological markers, we mainly based cut-off points on well-known cut-offs from research or clinic. For possible biomarkers, in Paper I we mainly used mean as cut-off, and for Paper II we used a minimal p-approach (optimal cut-off-approach) while also securing statistically sufficient number of cases in each group (Table 13). For Paper III the third quartile or higher was used as cut-off (Table 13). The best possible outcome of biomarker research is to find a general cut-off value for risk. However, the reproducibility of cut-off values is dependent on multiple aspects, particularly how evaluation of IHC is performed. Hence, our exact results are mainly hypothesis generating. Still, even though a specific cut-off value for a biomarker may not be reproducible in different materials, the same prognostic tendency should be.

5.2. DISCUSSION OF RESULTS

5.2.1. Prognostic relevance of adaptive immune cells and immune checkpoint markers – Paper I and II

The general notion is that the adaptive immune system, particularly CD8+ cytotoxic T lymphocytes, are crucial components in cancer immune elimination. Information on how lymphocytes are stimulated and inhibited has further created the foundation for immune checkpoint-inhibitors. So far, there have unfortunately not been convincing evidence that lymphocytes and cancer immune elimination play an important part in PCa biology. Thus, we wanted to explore the prognostic impact of TILs in PCa.

In Paper I, we demonstrated that a high density of lymphocytes positive for the pan T lymphocyte marker CD3, as well as subtypes CD8+ T lymphocytes, and CD4+ T lymphocytes were associated with shorter BFFS in univariate analyses. However, when adjusting for other clinicopathological factors, only high densities of CD8+ T lymphocytes was an independent, negative prognostic factor. This made us conclude that the observed prognostic effect of T lymphocytes was primarily mediated through CD8+ T lymphocytes (Table 14a).

Perhaps surprisingly, our results that intratumoral T lymphocytes, seem to be correlated to poor prognosis in PCa, is actually consistent with most other studies for this cancer form (402–409). However, others have found no association to outcome (410,411), and some even report a high density of intratumoral CD8+ T lymphocytes (412–414) or TILs in general (415) to be associated with a good prognosis. Although some studies report B lymphocytes to be present in higher numbers in malignant, compared to benignant, prostate tumor tissue (416,417), we found no such relationship. Additionally, CD20+ B lymphocytes did not have any prognostic impact on patient outcome, which other reports seems to agree with (403,408,414).

Our results in Paper I, indicates that T lymphocytes, especially of the CD8+ subset, may either act as a direct contributor to prostate tumorigenesis, or is an indirect surrogate marker for a more aggressive PCa phenotype. Either way, this could indicate that initiation of cancer immune elimination, and consequentially cancer immune escape, are important elements of PCa tumorigenesis.

One possible direct mechanism for intratumoral T lymphocytes to contribute to tumorigenesis, is if they act in an immunosuppressive manner. Multiple studies have suggested that CD4+CD25+Foxp3+ Tregs are present and may promote PCa tumor advancement (410,418–421). Additionally, others have concluded that a high tumor density of Foxp3+ cells in PCa patients is associated with worse clinical outcome, without further elucidating which lymphocyte subgroup expressing Foxp3 (411,422–424). Moreover, there are also PCa studies suggesting a rare presence of Foxp3-expressing CD8+ lymphocytes, giving them immunosuppressive abilities contrary to having the cytotoxic characteristics for which they are most known for (280,410,418,424). Henceforth, it could be that at least some of the CD8+ lymphocytes we observe may be characterized as CD8+ Tregs, and that this may partially explain their negative prognostic effect on outcome.

Contrary, if we postulate that the CD8+ T lymphocytes we observe are in fact tumor-specific, cytotoxic lymphocytes, our results could indicate that the most aggressive PCa tumors actually have the ability to initiate an anti-cancer immune response, but that this response for some reason is inadequate for elimination. This could be logical, as it is well known that tumors with a high mutational burden are more likely to cause aggressive disease, as well as triggering an immune response. In fact, PCa tumors with certain mutations linked to aggressive disease such as ERG positivity and PTEN loss are reported to have higher densities of intratumoral T lymphocytes (411). Hence, our results could indicate that CD8+ T lymphocyte density is an indirect marker for tumor aggressiveness and may provide prognostic information in clinical practice.

Furthermore, this interpretation of our results would indicate that CD8+ T lymphocytes are subjected to immunosuppressive mechanisms. PCa research has proposed that tumors actually are infiltrated by tumor-specific, cytotoxic T lymphocytes, but that they are dysfunctional, and that this may partly be caused by PD-1 expression (425,426). The CD8-marker represent a broad population of T lymphocytes with various roles which might weaken its prognostic impact. Lymphocytes exposed to a chronic antigen will stably express PD-1, thus PD-1 is by some proposed as a better marker for tumor-specific, cytotoxic T lymphocytes than CD8 (427). Consequently, for Paper II we investigated the prognostic significance of PD1 as well as its main ligand, PD-L1, in our PCa cohort. Indeed, we found a high density of PD-1+ lymphocytes in PCa tumor tissue to also be an independent, negative prognostic factor, this

time for CF which is an even stronger endpoint than BF. The prognostic impact of PD-1 was stronger than for any of the renowned clinicopathological features, except for Gleason grade. In addition, a high density of PD-1+ lymphocytes were in univariate analyses significantly associated with shorter CFFS in most subgroups related to worse PCa prognosis, such as a low age at the time of diagnosis, high pT-stage, high preoperative PSA, and high Gleason grade (Table 14a).

However, as lymphocyte marker expressions from Paper I did not correlate with PD-1 expression, we could not conclude PD-1 to be a marker for exhausted, tumor-specific CD8+ cytotoxic T lymphocytes. To further explore the relationship between CD8+ and PD-1+ cells, we double-stained for both markers. By microscopic examination we detected co-expression of CD8 and PD-1 on lymphocytes as suspected, but also lymphocytes with one without the other. Another explanation for lack of statistical correlation may be the different scoring methods in Paper I and II. Also limiting the ability for comparison, was that markers expression analysis in Paper II was performed on TMA cores cut from a much deeper tissue level than for Paper I, hampering the ability to correlate these markers in the same tumor areas.

To our knowledge, research on the prognostic effects of PD-1 in PCa, is still scarce. In agreement with our results, is Nardone et al., who described a low density of PD-1+ TILs to be correlated with a longer BFFS in univariate survival analysis (423). Multiple descriptive analyses have reported PD-1+ lymphocytes to be present in prostate tumor regions and/or in adjacent tertiary lymphoid structures (426,428–430). However, Rådestad et al. detected a relatively high proportion of PD-1 positive T lymphocytes in non-malignant prostates, and thus suggested that PD-1 may play a role in controlling the homeostasis of prostate tissue rather than in contributing to cancer associated immune suppression (416).

Nonetheless, for PD-1 to exert a biological effect, it has to be stimulated by one of its ligands; PD-L1 or PD-L2. Of these, PD-L1 is indicated to be the most clinically relevant in solid tumors. Its expression, prognostic and predictive value has been researched in essentially every malignancy, but at the time we conducted our study, only to a small degree in PCa. In our material, PD-L1 expression by tumor epithelial cells was common but produced no statistically significant prognostic results. However, both in univariate and multivariate

analyses there was a consistent tendency that a high expression of PD-L1 by tumor epithelial cells was associated with a worse BFFS (Table 14b).

Since we conducted our study, a meta-analysis (431) with data from five articles (429,432–435) on the prognostic effect of PD-L1 in PCa has been published. It concluded that PD-L1 expression (and PD-L1 promotor methylation) are significant, independent negative prognostic factors in patients with PCa. However, complicating the interpretation of the results, is the fact that the antibody which was used to detect PD-L1 for the largest proportion of the patients in this study (434), EPR1161(2) from Abcam, has since been discontinued by the manufacturer due to a failure to meet their quality criteria (436). Nonetheless, other studies than the ones included in the metanalysis has been performed; Heng Li et al. reported that a high expression of PD-L1 was a significant prognostic factor for shorter BFFS in patients who received adjuvant hormone therapy after RP (437). Petiprez et al. reported that patients with PD-L1 positive tumors in node positive patients had shorter metastasis-free survival (407), and Vicier et al. reported that a high PD-L1 expression had statistically significant association with biochemical- and metastatic relapse and a trend toward poorer overall survival in univariate analysis (413). Contrary, Iocavelli et al. studied tumors from patients with de novo hormone-naïve metastatic PCa and concluded that although 47% of cases were PD-L1 positive, there was no difference in overall survival between patients with PD-L1 positive versus negative tumors (438). Additionally, Zhao et al. used gene expression data from a large number of RP samples, and reported that PD-L1 did not have prognostic significance (408).

In general, studies on PD-L1 expression and its potential role as a prognostic marker, illustrate how challenging it can be to generate general wisdom in translational research questions. This problem is largely caused by variances in study design, making results from different publications hard to compare and combine. Particularly for a heterogenic disease such as PCa different studies often show substantial diversity in design; there are different ways of sampling tumor tissue (RP, TUR-B, biopsy), there is a wide range of disease stages with biological differences (e.g. hormone-naïve versus castration-resistant), and patients may have received treatment prior to baseline which can alter their TME. When it comes to detection assays for PD-L1, which is a relatively new marker in clinical use, different studies often use different antibodies. Even for PD-L1 antibodies regarded as validated, the detection rate for each antibody will differ to some degree, which again can affect the comparability of

results (439). Adding to this, IHC expression can be evaluated in different ways *(see further information in section 3.1.3. Prognostic biomarkers – Evaluation of immunohistochemical staining)*. For instance, a PCa study using the E1L3N antibody, reported PD-L1 expression in tumor associated nerves to be a negative prognostic factor (440), even though this is a known unspecific staining for this antibody (433). Further, dichotomization of marker expression is needed for survival analyses, and such cut-off values commonly varies between different reports. In addition, patients have a relatively long life-expectancy causing use of different, uncomparable surrogate endpoints for outcome analyses.

The lack of common guidelines when it comes to PD-L1 is also evident in the clinical use of PD-L1 as a predictive marker for immune checkpoint-inhibitors. Generally, response to immune checkpoint-inhibitors is observed for several cases with low/negative PD-L1, as well as a lack of response despite PD-L1 tumor expression. Different immune checkpoint-inhibitor drugs and cancer types have a diversity of protocols for PD-L1 analyses, both related to clone of antibody, how the expression is evaluated, and threshold for what is known as PD-L1 positivity *(see further information in section 3.1.3. Prognostic biomarkers – Evaluation of immunohistochemical staining)* (441).

5.2.2. Prognostic relevance of chemokine receptor CXCR6 and its ligand CXCL16 – Paper III

For our final paper, we wanted to examine another possible influencer of lymphocyte function in PCa, by studying the chemokine receptor CXCR6 and its only known ligand, CXCL16. Initially, CXCL16 and CXCR6 were known from inflammatory conditions. CXCR6 is a G-protein-coupled receptor which signals through the AKT/mTOR pathways, and is expressed on several different T lymphocyte subsets, in addition to NKT cells, NK cells, and plasma B cells (Figure 14) (442). CXCL16 exists in a transmembrane-bound form, which can be cleaved of the cell surface by metalloproteases, forming a soluble version (443). The biological effect of CXCL16 can be induced in different ways, namely (a) by binding to CXCR6, activating downstream pathways in the cell expressing the receptor, (b) reverse signaling, where binding of transmembrane CXCL16 to CXCR6 transduce signals exerting biological effect in the *ligand*-expressing cell, enabling a bidirectional signaling between ligand- and receptor-expressing cells, and (c) inverse signaling, where soluble CXCL16 bind to transmembrane CXCL16, transducing signals via its intracellular domain (444).

In many inflammatory diseases, CXCL16 can be upregulated by both parenchymal cells, endothelial cells, and immune cells at the inflamed site (445–447). As a soluble form it causes chemotactic homing of CXCR6-expressing immune cells. As a transmembrane form it can serve as an adhesion molecule for cells expressing CXCR6, as well as a scavenger receptor on phagocytotic cells such as DCs, macrophages/monocytes and B lymphocytes (448).

With their diverse skill sets, chemokines are important in many cancer forms. In PCa, CXCR6 and CXCL16 are expressed on both tumor epithelial and tumor stromal cells (449). This is of interest, as they are related to both inflammatory, and non-inflammatory pro-tumorigenic properties. Upregulated CXCR6 and CXCL16 is proposed as a driving factor of cancer development at sites of inflammation (449,450). CXCR6-CXCL16-interaction can further fuel the pro-inflammatory environment by activating NF-kB and a pro-inflammatory gene expression (451,452). CXCR6-CXCL16-interaction can also serve many other, non-inflammatory, pro-tumorigenic effects, such as shaping the non-inflammatory component of tumor stroma (453), stimulate angiogenesis (454,455), increase proliferation, and induce cellular changes in tumor cells necessary for migration and invasion (450,454,456,457). In addition, it has been proposed that CXCL16-expression by for example bone marrow cells can induce bone metastases of CXCR6-expressing tumor epithelial cells in a seed-and-soil specific manner (456,458,459).

As a deduction, for Paper III we set out to examine the prognostic effect of CXCR6 and CXCL16 in our cohort. We hypothesized that CXCL16 expressed by tumor cells could, as in inflammatory conditions, attract CXCR6-expressing immune cells such as T lymphocytes, which may serve as pro-tumorigenic tumor stromal cells as proposed in Paper I.

We found markers CXCR6 and CXCL16 to be relatively commonly expressed in tumor tissue and more so in tumor tissue compared with non-malignant tissue. This is in agreement with others (450,457,458) and suggest that CXCL16/CXCR6 is increased during prostate tumorigenesis. Cases often had co-expression of markers in their tumor tissue, and a high co-expression was weakly correlated with Gleason score >7, PSM, LVI, and PNI, indicating an association between CXCR6/CXCL16 expression and a more aggressive phenotype of PCa. This is in agreement with other reports (449,458), though some only describe this relationship for CXCR6 and not its ligand (450,454,456).

Further, we found that a high expression of CXCR6 by tumor epithelial cells, and in tumor tissue as a whole, were independent, negative prognostic factors for both BFFS and CFFS. A high expression of CXCL16 by tumor epithelial cells, as well as a high co-expression of CXCL16 and CXCR6 in tumor tissue were independent negative prognostic factors for CFFS (Table 14b).

Despite hypotheses made from tumor biology, CXCR6 was more commonly expressed on tumor epithelial cells than tumor stromal cells, and CXCR6 on stromal cells did not correlate with lymphocyte makers published in Paper I. Thus, the negative prognostic effect we observe is not, at least exclusively, related to TILs. Regrettably, we did not score CXCR6-expression for lymphocyte-like cells only, as this could have made us wiser as to which tumor stromal cells express CXCR6. However, CXCL16 expression was weakly related to intratumoral T lymphocytes (CD3+), indicating tumor expression of CXCL16 may contribute to recruitment of T lymphocytes to the tumor site.

Most other reports have focused on the migration- and invasion properties of CXCR6 when expressed by PCa cell lines (450,454,456,457). Thus, we wanted to test if the negative, prognostic effect of CXCL16 could stem from of stimulation of migration and proliferation of cancer cells. However, contrary to the results from our TMA cohort, our *in vitro* assays showed that silencing of CXCL16 caused increased proliferation and migration in two different PCa cell lines (DU145 and PC3). This can have multiple different explanations, but most likely show the complexity of the TME, and influence of crosstalk between tumor epithelial cells and the stromal compartment. Our *in vitro* assays also eliminate the CXCR6-CXCL16 interaction. Additionally, both cell lines are from metastatic sites, are castration-resistant, and does not express PSA. These are characteristics which tend to be associated with a high degree of independence from origin, whereas tumor cells in primary tumors such as the ones in our TMA, are more dependent on the nutritional and chemical signaling in the milieu of their origin organ (460). Further, neither for our *in vitro* studies, nor in our TMA, did we differentiate between transmembrane and soluble CXCL16. It is proposed that transmembrane and soluble CXCL16 have different biological functions, and that signaling initiated by transmembrane CXCL16 may be anti-oncogenic, whereas soluble CXCL16 may be more pro-oncogenic (461). Even with some shortcomings, we evaluate our results as a relatively strong indicator that CXCR6 and CXCL16 can serve as negative prognostic factors in PCa.

6. CONCLUSIONS

Key discoveries in this thesis

- We were able to establish a largely unselected cohort from the PSA era with a long follow-up.
- Intratumoral lymphocytes was mainly of T lymphocyte subset, and the density of T lymphocytes, as well as subtypes CD4+ and CD8+ T lymphocytes, was more common in tumor tissue than in non-malignant tissue. There was no such difference observed for B lymphocytes (Paper I).
- A high density of intratumoral CD8+ T lymphocytes independently predicted a shorter time to disease relapse in form of biochemical failure (Paper I).
- A high density of intratumoral PD-1+ lymphocytes independently predicted a shorter time to disease relapse in form of clinical failure. In addition, high density of intratumoral PD-1+ lymphocytes were associated with worse prognosis in most subgroups related to poor prognosis, such as low age at the time of diagnosis, high pT-stage, high preoperative PSA, and high Gleason grade (Paper II).
- The very clinically relevant marker PD-L1 was commonly expressed by prostate tumor epithelial cells. Even though a high expression was consistently associated with worse prognosis, these results did not reach statistical significance (Paper II).
- Chemokine receptor CXCR6 and its ligand CXCL16 was more commonly expressed intratumoral compared to in non-malignant tissue (Paper III).
- A high expression of CXCR6 by tumor epithelial cells, and in tumor tissue as a whole, independently predicted a shorter time to disease relapse in form of both biochemical- and clinical failure.
- A high expression of CXCL16 by tumor epithelial cells independently predicted a shorter time to disease relapse in form of clinical failure.
- A high co-expression of CXCL16 and CXCR6 in tumor tissue independently predicted a shorter time to disease relapse in form of clinical failure.
- Silencing of CXCL16 in aggressive, metastatic prostate cancer cell lines increased proliferation and migration in *ex vivo* essays.

The immune system has dual roles in cancer. Immune markers show promise as prognostic and predictive markers in multiple cancer types, and immune checkpoint inhibitors are perhaps the biggest breakthrough in cancer research the last decade. PCa is in need of both

better prognostic tools as well as more personalized treatment options. To achieve this, the biological characteristics that separates indolent from aggressive disease needs to be explored. Thus, our general aim was to investigate immune biomarkers as potential prognostic factors, as well as generate hypotheses on PCa biology and possible therapeutic targets.

The basis for our investigation was our comprehensive, retrospective cohort of 535 patients with primary PCa and their associated primary PCa tumors. We used this material to study the expression of immune markers CD3, CD4, CD8, CD20, PD-1 on intratumoral lymphocytes, as well as PD-L1, CXCR6 and CXCL16 by tumor epithelial cells and tumor stromal cells. We analyzed these markers in relations to patient outcome as well as their association to each other, and well-known clinical- and histopathological parameters for PCa. The main strengths of our study design are a long follow-up, relatively high number of cases, representativeness of cases, multicenter inclusion, and *in situ* evaluation of biomarkers in both tumor epithelial-, tumor stromal and non-malignant compartments. Additionally, we preformed experimental assays examining the role of CXCL16 expression on proliferation and migration in PCa cell lines.

To conclude, the results of our exploratory research suggest that components of the immune system may be important participants in PCa biology. We hope that the results presented herein can contribute to better patient risk stratification, helping doctors and patients regarding treatment strategy, as well as be explored as targets of therapy in future PCa treatment. However, our results need to be investigated in experimental models, and validated in independent patient cohorts, preferably in prospective studies with predefined scoring cut-offs.

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Paper I

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Infiltration of CD8+ Lymphocytes is an Independent Prognostic Factor of Biochemical Failure-Free Survival in Prostate Cancer

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BACKGROUNDS. The adaptive immune system can potentially have dual roles in cancer development and progression by contributing to or suppressing tumor progression and metastasis. The aim of this study was to evaluate the prognostic impact of adaptive immune cells residing in different tumor compartments in prostate cancer.

METHODS. Tissue microarrays from 535 patients were constructed from viable and representative tumor epithelial and stromal areas of primary PC tumors, as well as from normal epithelial and stromal areas. Immunohistochemistry was used to evaluate the density of CD3+, CD4+, CD8+, and CD20+ lymphocytes in both tumor epithelial and tumor stromal areas.

RESULTS. In univariate analysis, a high density of CD3+ ($P = 0.037$) and CD8+ lymphocytes ($P = 0.010$) in tumor epithelial areas was associated with significantly shorter biochemical failure-free survival. When analyzing both tumor epithelial and stromal tissue compartments as one entity, similar relationships were observed for CD3+ ($P = 0.046$), CD4+ ($P = 0.026$), and CD8+ ($P = 0.003$) lymphocytes. In multivariate analysis, high densities of CD8+ lymphocytes limited to tumor epithelial areas (HR = 1.45, $P = 0.032$), as well as in the total tumor tissue (HR = 1.57, $P = 0.007$), were independent negative prognostic factors for biochemical failure-free survival.

CONCLUSIONS. A high density of CD8+ lymphocytes, especially in tumor epithelial areas, is an independent negative prognostic factor for biochemical failure-free survival. *Prostate*

KEY WORDS: **CD8+; T lymphocytes; prostate carcinoma; B lymphocytes; CD3+**

INTRODUCTION

In developed countries, prostate cancer (PC) is the most common male malignancy and the second most common cause of male cancer death [1,2]. There is an increasing use of prostate specific antigen (PSA) testing and, when indicated, follow-up biopsies are performed. The treatment strategy after identifying tumor cells in the biopsy material is still primarily based on risk stratification by use of the preoperative PSA value, Gleason score and cTNM-classification,

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which have not proved adequate for predicting the clinical outcome. A consequence is that a major proportion of patients will receive therapies they in reality do not need [3–5]. To avoid unnecessary costs and side effects, and improve efficacy of treatment, there is a need to develop better prognostic tools as well as more specific treatment methods.

During recent years cancer research has increasingly focused on oncoimmunology [6] and there is compelling evidence of two immunology related areas in this field: (i) the immune system's ability to eliminate premalignant and malignant transformed cells in a process called cancer immunoediting [7], and (ii) the tumor promoting effects of chronic inflammation [8]. Knowledge within these fields has already resulted in practical changes with respect to both prognostic and therapeutic issues. In colorectal cancer, immune scores (IS) [9] appear to be a better prognostic indicator than the TNM system [10], and may be applicable in other cancer types as well. The use of the autologous cellular cancer vaccine Sipuleucel-T has increased survival in metastatic castration-resistant PC [11], suggesting a favorable impact of an anti-tumor immune response towards PC cells.

The prostate is considered an immune-competent organ, meaning it is populated by small numbers of scattered leucocytes, mainly stromal and intraepithelial T and B lymphocytes, macrophages, and mast cells [12–14]. In different conditions such as benign prostatic hyperplasia (BPH), prostatitis, proliferative inflammatory atrophy (PIA), as well as in adenocarcinomas, the immune cell content is often increased and altered compared to normal prostate tissue [12,13]. Comprehending the different immune cells roles and mechanisms in these conditions may lead to valuable knowledge towards better therapeutic management.

The effects of tumor infiltrating lymphocytes (TILs) in cancer development are complex and their prognostic value is determined by several factors including density, subtype, and localization [15,16]. A better understanding of the role of TILs in PC is expected to improve both diagnostic procedures and therapy. Herein, we investigate the prognostic significance of adaptive immune cells in 535 resected primary prostate cancers. We have assessed the prognostic associations of the localization and the density of CD3+, CD4+, CD8+, and CD20+ lymphocytes to biochemical failure (BF)-free survival, as well as their relations to other clinicopathological variables.

MATERIALS AND METHODS

Patients, Clinical and Histopathological Data

Six hundred and seventy one patients who underwent radical prostatectomies as initial treatment for adenocarcinoma from 1995 to 2005 were retrospectively identified from the Departments of Pathology at the University Hospital of Northern Norway (n = 267), Nordland Hospital (n = 63), St. Olavs Hospital (n = 330), and Levanger Hospital (n = 11). Of these, a total of 136 patients were excluded due to: (i) radiotherapy to the pelvic region prior to surgery (n = 1), (ii) other malignancies within 5 years prior to the PC diagnosis (n = 4), (iii) inadequate paraffin-embedded tissue blocks (n = 130), and (iv) lack of follow-up data (n = 1). Thus 535 patients were included in this study. Median follow-up of survivors was 89 (range 6–188) months. Complete demographic and clinical data were obtained from medical records, and histopathological data were registered by two experienced pathologists (ER and LTB) reviewing all cases. The tumors were graded according to the modified Gleason grading system [17,18], and staged according to the WHO guidelines [19]. All demographic-, clinical- and histopathological data (Table I) were recorded in a SPSS data file and patients were de-identified. Last follow-up was 21.11.12.

The Regional Committee for Medical and Health Research Ethics (2009/1393), the Data Protection Official for Research (NSD), and the National Data Inspection Board have approved this study.

Microarray Construction

Tissue microarray (TMA) construction was chosen for high-throughput molecular pathology analysis. For each case, a pathologist (ER) histologically identified and marked two cores with areas of tumor epithelial cells, two cores with tumor-surrounding stromal tissue, one core from areas with normal epithelial cells, and one core with normal stromal tissue. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD). Briefly, we used a 0.6 mm diameter needle to harvest the marked tissue areas from the corresponding paraffin-embedded tissue blocks. The samples were inserted into a recipient paraffin block according to a coordinate pattern. To include all core samples, twelve tissue array blocks were constructed. Multiple 4 μm sections were cut with a Micron microtome (HM355S), affixed to glass slides, and sealed with paraffin. The detailed methodology has been reported previously [20].

Immunohistochemistry (IHC)

The antibodies used in this study were as follows: (i) CD3 (clone PS1), (ii) CD8 (clone 1A5), (iii) CD20 (clone L26) from Ventana Medical (Tucson, AZ), and (iv) CD4 (clone 1F6) from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK). The applied anti-

TABLE I. Patient Characteristics and Clinicopathological Variables as Predictors of Biochemical Failure-Free Survival, Clinical Failure-Free Survival and Disease-Specific Survival (univariate analysis; log-rank test) (N = 535)

Characteristic	Patients (n)	Patients (%)	BF (170 events) 5-year EFS (%)	*P*	CF (36 events) 10-year EFS (%)	*P*	PCD (15 events) 10-year EFS (%)	*P*
Age				0.55		0.085		0.600
≤65 years	357	67	76		92		97	
>65 years	178	33	70		88		96	
pT-stage				<0.001		<0.001		0.027
pT2	374	70	83		96		98	
pT3a	114	21	60		86		98	
pT3b	47	9	43		73		89	
Preop PSA				<0.001		0.085		0.061
PSA<10	308	57	80		93		99	
PSA>10	221	42	67		88		95	
Missing	6	1	–		–		–	
Gleason score				<0.001		<0.001		0.001
3+3	183	34	83		98		99	
3+4	220	41	76		93		98	
4+3	80	15	69		84		95	
4+4	19	4	63		76		94	
>8	33	6	34		67		87	
Tumor size				<0.001		0.019		0.098
0–20 mm	250	47	82		94		99	
>20 mm	285	53	67		88		96	
PNI				<0.001		<0.001		0.002
No	401	75	79		95		98	
Yes	134	25	60		81		93	
PSM				0.04		0.038		0.697
No	249	47	81		94		97	
Yes	286	53	69		89		97	
Circumferential PSM				<0.001		<0.001		0.029
No	381	71	81		95		98	
Yes	154	29	57		81		94	
Apical PSM				0.04		0.484		0.31
No	325	61	73		90		96	
Yes	210	39	77		92		98	
Vasc inf				<0.001		<0.001		0.009
No	492	92	77		93		98	
Yes	43	8	46		71		88	
Surgical proc				0.23		0.41		0.581
Retropubic	435	81	76		90		97	
Perineal	100	19	67		95		98	

BF, biochemical failure; CF, Clinical failure; EFS, event free survival in months; PCD, prostate cancer death; NR, not reached; *P* = *P*-value for log rank statistic for difference in event free survival; PC, Prostate cancer; PNI, Perineural infiltration; Post op RT, postoperative radiotherapy; Preop, preoperative; PSA, Prostate specific antigen; PSA DT, PSA doubling time; PSM, Positive surgical margin; Surgical proc, surgical procedure; Vasc inf, Vascular infiltration.

bodies had been subjected to in-house validation according to the manufacturer's recommendation for immunohistochemistry (IHC) analysis on paraffin-embedded material. Ventana Benchmark XT automated slide stainer (Ventana Medical System, Illkirch, France) was used for IHC. TMA slides were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was done by placing the specimens in 0.01 mol/L citrate buffer (pH 6.0) and exposing them to two repeated microwave heatings of 10 min at 450 W. The DAKO EnVision+ System-Horseradish Peroxidase [diaminobenzidine (DAB)] kit (Dako, Glostrup, Denmark) was used as endogen peroxidase blocking. As negative staining controls, the primary antibodies were replaced with the primary antibody diluents. Primary mouse monoclonal antibodies were

incubated for 24 min (CD3), 20 min (CD4), 32 min (CD8), and 16 min (CD20) at room temperature. The Ventana antibodies were pre-diluted by the manufacturer; CD4 was diluted 1:5 in-house. Biotinylated goat anti-mouse IgG and mouse anti-rabbit IgM, both 200 lg/ml, were used as secondary antibodies. The DAB kit was used to visualize the antigens by application of liquid DAB and substrate substrate-chromogen, yielding a brown reaction product at the site of the target antigen. Finally, slides were counterstained with haematoxylin to visualize the nuclei.

Scoring of IHC

The ARIOL imaging system (Applied Imaging Corp., San Jose, CA) was used to scan and digitalize the IHC stained TMA slides. The slides were loaded in the SL 50 automated slide loader and scanned at a low resolution (1.25x) and high resolution (20x) using an Olympus BX61 microscope with an automated platform (Prior Scientific, Cambridge, UK). Images of the cores were uploaded into the Ariol Software. All samples were de-identified and scored manually by two pathologists (ER and AV) independent of each other. Representative viable tissue sections were scored semi-quantitatively for density as follows: (i) 0 (0–5% cells), (ii) 1 (5–25% cells), (iii) 2 (26–75% cells), or (iv) 3 (75%+ cells) per 0.6 mm tissue core (Fig. 1). In case of major disagreement (scoring difference > 1), the slides were re-examined until consensus was reached. The scoring values for each patient were calculated as the mean of the patients scoring values for each tissue compartment.

To achieve maximal reproducibility in all cases all immune cell scoring-values where dichotomized (low and high density of stained cells). Optimal cut-offs for high and low expression for each marker were chosen at levels securing statistically sufficient numbers in each group. These cut-offs corresponded to mean expression levels for most markers. Hence, the cut-off values varied as follows among the different markers: (i) for CD3+ lymphocytes 0.50 in tumor epithelial areas, 0.62 in tumor stromal areas and 0.50 in tumor tissue as an entity; (ii) for CD4+ lymphocytes 0.44 in tumor epithelial areas, 0.36 in tumor stromal areas and 0.50 in tumor tissue as an entity; (iii) for CD8+ lymphocytes 0.42 in tumor epithelial areas, 0.35 in tumor stromal areas and 0.41 in tumor tissue as an entity; and (iv) for CD20+ lymphocytes 0.20 in tumor epithelial areas, 0.25 in tumor stromal areas and 0.21 in tumor tissue as an entity.

There was good scoring agreement between the two investigating pathologists for all four markers. The intra-class correlation coefficient (reliability coefficient, r) was 0.95, range 0.90–0.97 ($P < 0.001$).

Statistical Methods

All statistical analyses were performed using the statistical package IBM SPSS, version 21 (SPSS Inc.,

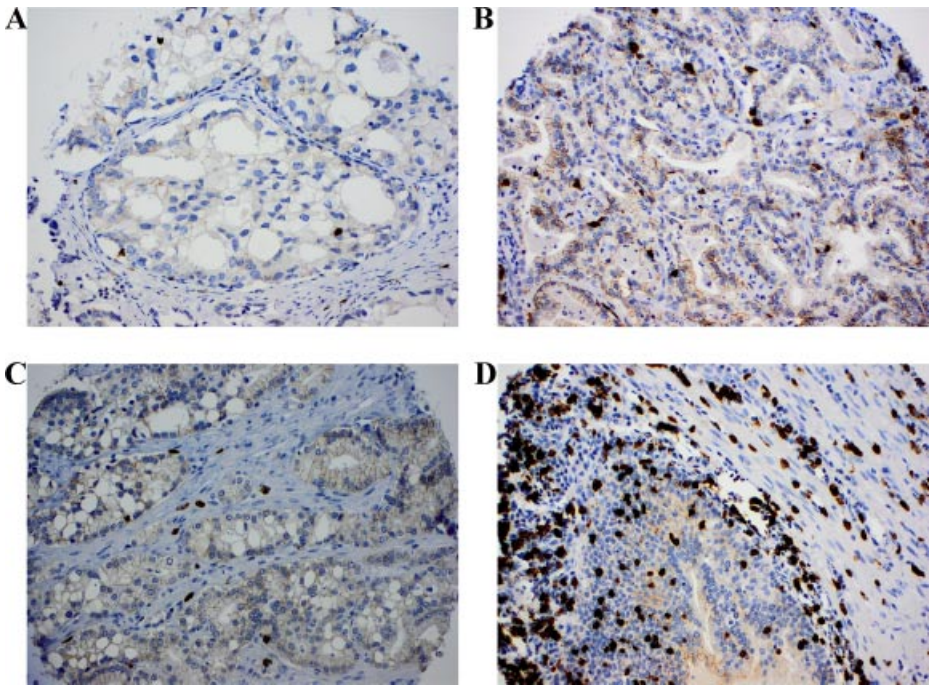

Fig. 1. TMA sections from primary prostate cancer tumors IHC stained for CD8. (**A**) Tumor epithelial area, CD8 low density; (**B**) tumor epithelial area, CD8 high density; (**C**) tumor stromal area, CD8 low density; (**D**) tumor stromal area, CD8 high density. Magnification x400.

Chicago, IL). The IHC scoring values from each pathologist were compared for inter-observer reliability by use of a two-way random effect model with absolute agreement definition. A Wilcoxon signed rank test was used to assess the difference in lymphocyte density in tumor tissue compared to normal prostate tissue in the cancer patients. The $\chi 2$ test was used to examine the associations between molecular marker expressions and the clinicopathological markers. Univariate survival analyses were done by using the Kaplan–Meier method, and the difference between survival curves was assessed by the log-rank test. The survival curves were terminated at 134 months, due to less than 10% of patients at risk after this point. Significant variables from the univariate analyses were further assessed in a multivariate survival analysis using a backward stepwise Cox regression model with a probability for stepwise entry or removal at 0.05 and 0.10, respectively.

The significance level used was $P < 0.05$ for all analyses. All survival analyses were carried out using three different end-points as follows: (i) biochemical failure (BF), (ii) clinical failure (CF), and (iii) PC death (PCD). BF was characterized as a PSA $\geq$ 0.4 ng/ml and rising in a minimum of two different blood samples postoperatively [21]. CF was defined as verified local symptomatic progression and/or radiologically verified metastasis to bone, visceral organs or lymph nodes. PCD was defined as death caused by progressive PC. Survival times were calculated from day of surgery to event.

RESULTS

Clinicopathological Variables and Patient Characteristics

An overview of the demographic, clinical, and histopathological characteristics is presented in Table I. Median age at surgery was 62 (range 45–75). The prostatectomies were retropubic in 435 cases and perineal in 100 cases. At the last follow-up, 170 patients had BF, 36 patients had CF, and 15 patients were dead of PC.

Lymphocyte Infiltration and Correlations

TILs were observed microscopically in both tumor epithelial areas and tumor stromal areas. By quantitative assessment of CD3 and CD20 stained lymphocytes, respectively, tumor lymphocytes were found to be mainly T-lymphocytes (CD3+). There were significantly higher densities of CD3+ ($P < 0.001$), CD4+ ($P = 0.006$), and CD8+ ($P = 0.008$) lymphocytes in tumor tissues compared with non-malignant tissues, whereas no difference was observed for CD20+ lymphocytes. The density of CD3+ lymphocytes was significantly higher in tumor epithelial areas compared to tumor stromal areas ($P < 0.001$). For CD4+, CD8+, and CD20+ lymphocytes, no differences in density levels were found between tumor epithelial and tumor stromal areas.

Expressions of the investigated markers in tumor epithelial or stromal areas did not correlate to pT stage, preoperative PSA (dichotomized at 10 ng/dL), Gleason score, tumor size (dichotomized at 20 mm), perineural infiltration, vascular infiltration or circumferential positive surgical margin (PSM).

Univariate Survival Analysis

The clinicopathological variables pT-stage ($P < 0.001$), preoperative PSA ($P < 0.001$), Gleason score ($P < 0.001$), tumor size ($P < 0.001$), perineural infiltration ($P < 0.001$), PSM ($P = 0.04$), circumferential PSM ($P < 0.001$), apical PSM ($P = 0.04$), and vascular infiltration ($P < 0.001$) were all significantly correlated to BF-free survival in the univariate survival analyses (Table I).

When analyzing both tumor epithelial and tumor stromal areas as one entity, high densities of CD3+, CD4+, and CD8+ ($P = 0.046$, $P = 0.026$, and $P = 0.003$, respectively), but not CD20+ lymphocytes, were associated with significantly shorter BF-free survival (Table II, Fig. 2 panel B and C, Fig. 3 panel B). In separate analyses of tumor epithelial areas, the same pattern was apparent for CD3+ ($P = 0.037$) and CD8+ lymphocytes ($P = 0.010$) (Table II, Fig. 2 panel A, Fig. 3 panel A). In tumor stromal areas, CD3+, CD4+, CD8+, or CD20+ lymphocytes did not predict BF-free survival. There were no significant associations between CF or PCD and the respective immune cell markers.

Multivariate Survival Analysis

Significant clinicopathological variables and immune cell markers from the univariate analyses (Tables I and II) were entered into the multivariate analyses. When analyzing data from tumor epithelial and tumor stromal areas as one entity, high CD8+ density was an independent significant predictor of BF-free survival (HR = 1.565, CI 95% 1.132–2.165, $P = 0.007$), in addition to pT stage, Gleason score, circumferential PSM and apical PSM. (Table III). High CD8+ density also had an independent significant impact when analyzed in the tumor epithelial areas alone (HR = 1.445, CI 95% 1.028–2.032, $P = 0.032$).

TABLE II. Epithelial and Stromal Lymphocyte Subsets and Their Prediction for Biochemical Failure-Free Survival in PC Patients (univariate analysis; log-rank test) (N = 535)

Marker expression	Patients (n)	5-year EFS (%)	10-year EFS (%)	*P*
CD3				
Epithelial compartment				0.037
Low expression	217	80	65	
High expression	218	70	60	
Missing	100			
Stromal compartment				0.65
Low expression	115	70	60	
High expression	69	67	55	
Missing	383			
Both compartments				0.046
Low expression	239	80	65	
High expression	233	70	60	
Missing	63			
CD4				
Epithelial compartment				0.89
Low expression	244	75	62	
High expression	194	74	64	
Missing	97			
Stromal compartment				0.42
Low expression	118	72	61	
High expression	69	67	50	
Missing	348			
Both compartments				0.026
Low expression	383	76	66	
High expression	138	70	55	
Missing	14			
CD8				
Epithelial compartment				0.010
Low expression	254	80	67	
High expression	182	66	57	
Missing	99			
Stromal compartment				0.45
Low expression	127	72	61	
High expression	65	67	54	
Missing	343			
Both compartments				
Low expression	279	78	67	0.003
High expression	194	66	54	
Missing	62			
CD20				
Epithelial compartment				0.14
Low expression	316	76	65	
High expression	115	67	58	
Missing	104			
Stromal compartment				0.86
Low expression	144	70	58	
High expression	44	71	56	
Missing	347			
Both compartments				0.38
Low expression	334	75	71	
High expression	135	64	58	
Missing	66			

EFS, event free survival in months; *P* = *P*-value for log rank statistic for difference in event free survival; Epithelial areas = Numbers based on scoring of TMA cores containing mainly tumour epithelial cells; Stromal areas = Numbers based on scoring of TMA cores containing mainly tumour stromal tissue; Both compartments = Tumour epithelial and tumour stromal tissue cores analysed as an entity.

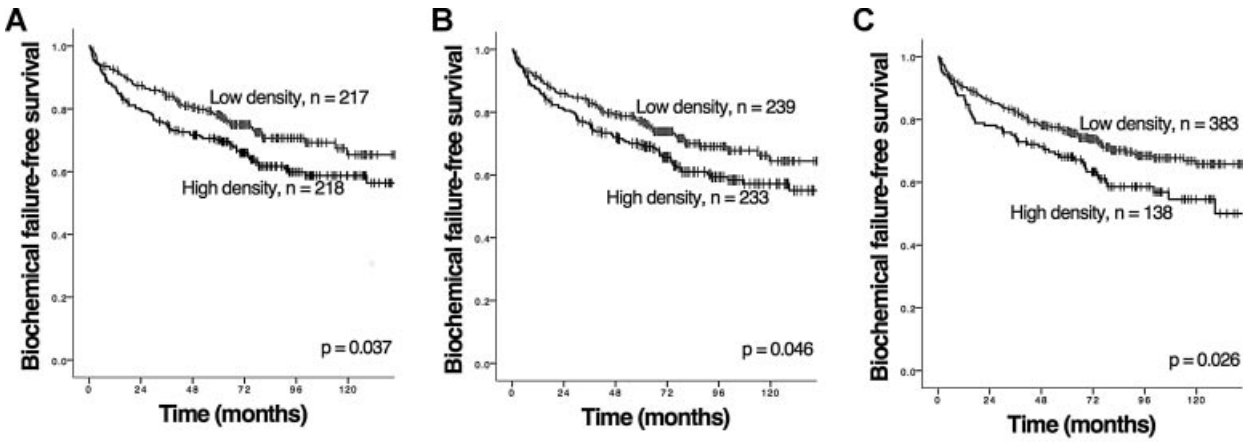

Fig. 2. Biochemical failure-free survival curves for CD3+ lymphocytes in tumor epithelial areas, and CD3+ and CD4+ lymphocytes in tumor epithelial and stromal areas analyzed as one entity. Grey lines indicate low density, whereas black lines indicate high density. (**A**) CD3 epithelial compartment, (**B**) CD3 both tumor tissue compartments, (**C**) CD4 both tumor tissue compartments.

DISCUSSION

In this study, we found that high densities of CD8+ lymphocytes in PC tumor tissue predict BF-free survival after adjustment for clinicopathological prognostic factors. These findings are consistent with results from most previously published studies in the field. Kärjä et al. reported that a high density of TILs was an independent predictor of a shorter BF-free survival [22]. Besides, Richardsen et al. found that a high density of CD3+ lymphocytes in PC primary tumor tissue correlated with metastatic disease [23]. Recently, Flammiger and coworkers published the largest cohort on TILs' prognostic effects in PC [24]. They concluded that patients with either a high or very low number of CD3+ lymphocytes in tumor epithelial areas had a shorter BF-free survival. They did not, however, investigate how the different subsets of T lymphocytes contributed to the clinical outcome. Our findings indicate that the prognostic effect may be mediated primarily through CD8+ lymphocytes rather than the overall density of T lymphocytes as measured by CD3 positivity. In agreement with Flammiger et al. [24], CD20+ lymphocytes did not have any prognostic impact on patient outcome in our cohort.

Conflicting with our findings was a case-control study by Davidsson et al. In this study the researchers evaluated the tumor content of CD4+ and CD8+ lymphocytes, but they did not observe any associations with survival [25]. Instead, they reported that

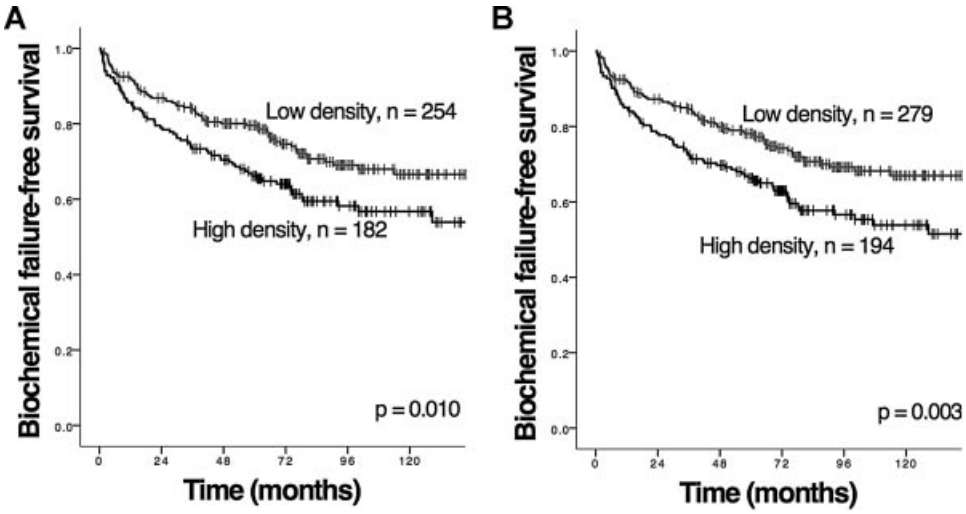

Fig. 3. Biochemical failure-free survival curves for CD8+ lymphocytes in tumor epithelial areas and in tumor epithelial and stromal areas analyzed as one entity. Grey lines indicate low density, whereas black lines indicate high density, (**A**) CD8 epithelial compartment, (**B**) CD8 both tumor tissue compartments.

TABLE III. Cox Regression Analysis Summarizing Prognostic Factors for Biochemical Failure-Free Survival

Factor	HR	95% CI	*P*
pT-stage			<0.001
T2	1.00		
T3a	1.781	1.185–2.676	0.005
T3b	2.649	1.598–4.392	<0.001
Gleason score			0.042
3+3	1.00		
3+4	1.002	0.663–1.512	0.994
4+3	1.540	0.953–2.488	0.078
4+4	1.443	0.660–3.156	0.358
>8	2.323	1.249–4.321	0.008
Circumferential PSM			0.002
No	1.00		
Yes	1.775	1.242–2.535	
Apical PSM			0.008
Yes	1.00		
No	1.637	1.135–2.360	
CD8 both tumor tissue compartments			0.007
Low	1.00		
High	1.57	1.132–2.165	

PSM, Positive surgical margin.

patients with higher numbers of regulatory T cells (CD4+FOXP3+) had an increased risk of dying of PC. The diverging results by Davidsson et al. may be explained by the differences in study design, as their samples were from patients diagnosed with PC before the PSA era, and based on tissue from transurethral resection or adenoma enucleation. This may lead to bias, since the patients were selected based on clinical symptoms indicating more advanced disease than what is the relevant clinical picture of today.

The association between CD8+ lymphocytes and poor prognosis in PC may not necessarily account for causality, but may rather be a covariance due to other mechanisms. One explanation may be that tumor cells with a high degree of malignant mutations are further transformed and thus more immunogenic, while the triggered immune response is inadequate for elimination. The more malignant mutations a tumor cell accumulates, the more possibilities it has to mediate life threatening malignant disease. This could mean that a high density of CD8+ lymphocytes reflects highly transformed cells, and thus indirectly apply as a prognostic factor. If this is the case, T lymphocyte density in PC tumors may be a valid prognostic factor in separating aggressive from indolent tumors in clinical practice. Most PC patients are immune-competent, but it seems that they do not have the ability to generate an effective anti-tumor immune response. The Sipuleucel-T vaccine's effect in patients with metastatic castrate-resistant PC is most likely mediated by triggering a "halted" immune system to exert a more effective anti-tumor immune response. It is still unknown why the immune system is not effective in the first place, and why Sipuleucel-T apparently seems to be effective in patients with metastatic castrate-resistant cancer [11]. Our findings may indicate that the adaptive anti-cancer immune response is halted in some way and thus facilitates tumor escaping immune surveillance. A study by Sfanos et al. in 2009 suggested that CD8+ T lymphocytes in PC tumors undergo a clonal expansion in response to an antigen, but also show a high expression of programmed cell death protein 1 (PD-1) [26], a receptor that upon activation can lead to abnormal activation of the T lymphocytes, as well as apoptosis of CD8+ cytotoxic T lymphocytes [27]. If the CD8+ lymphocytes we detect are in fact impaired CD8+ cytotoxic T lymphocytes, they may be activated with use of immune therapies like Sipuelucel-T, or by attacking specific immunosuppressive mechanisms. To further elucidate these possible immune related mechanisms, experimental studies to investigate lymphocyte-mediated mechanisms in PC are needed.

In contrast, our findings could imply that CD8+ T lymphocytes have immunosuppressive abilities. In an experimental study using TILs from human PC tumors, Kiniwa et al. concluded that both CD4+ and CD8+ T-lymphocyte subpopulations possessed potent immune suppressive activity, and that a subtype of CD25+CD8+ Treg cells expressed FOXP3, and suppressed naïve T-lymphocyte proliferation [28]. A recent study by Flammiger et al. indicates that a high tumor density of FOXP3+ cells in PC patients is associated with a worse clinical outcome, indicating that immunosuppression and subsequent tumor immune escape is an important mechanism for PC development [29]. However, the authors did not use double or triple staining methods to elucidate the cell type harboring the transcription factor. This should be considered to determine which exact cells express FOXP3 in PC tumor tissue. There is also a need to determine the extent to which the different T lymphocyte subpopulations contribute to immunosuppression in different cancer types, and further which ones are to be classified as Tregs.

To our knowledge, this is the first large multicenter cohort assessing the prognostic role of CD4+ and CD8+ lymphocytes in a PC patient material from the PSA era. Furthermore, we investigated the lymphocyte density in tumor epithelial and tumor stromal areas both separately and as one entity. Endpoint issues present one of the main limitations in this study design. Since PC in general have a relatively long expected survival, with disease specific death often

several years after diagnosis, BF is commonly used as endpoint in survival analyses. Although BF is an important prognostic factor, not all patient with BF experience increased morbidity or mortality [30]. In our study, CD8+ density was a strong predictor for BF. When analyzing the association of CD8+ density and CF- and PCD-free survival, the associations showed the same tendency, but did not reach statistical significance in our cohort, probably due to the scarcity of those events during our follow up.

CONCLUSIONS

We report that a high density of T lymphocytes in primary PC tumors, especially of the CD8+ subset, is associated with shorter BF-free survival. Our findings is of clinical significance as a prognostic tool, but also highlights the importance of the adaptive immune system as a target for novel treatment strategies in PC.

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Paper II

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Research Paper

The prognostic role of immune checkpoint markers programmed cell death protein 1 (PD-1) and programmed death ligand 1 (PD-L1) in a large, multicenter prostate cancer cohort

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ABSTRACT

Programmed cell death protein 1 (PD-1) and its ligand Programmed death ligand 1 (PD-L1) have gained massive attention in cancer research due to recent availability and their targeted antitumor effects. Their role in prostate cancer is still undetermined. We constructed tissue microarrays from prostatectomy specimens from 535 prostate cancer patients. Following validation of antibodies, immunohistochemistry was used to evaluate the expression of PD-1 in lymphocytes and PD-L1 in epithelial and stromal cells of primary tumors. PD-L1 expression was commonly seen in tumor epithelial cells (92% of cases). Univariate survival analysis revealed a positive association between a high density of PD-1+ lymphocytes and worse clinical failure-free survival, limited to a trend (p = 0.084). In subgroups known to indicate unfavorable prostate cancer prognosis (Gleason grade 9, age < 65, preoperative PSA > 10, pT3) patients with high density of PD-1+ lymphocytes had a significantly higher risk of clinical failure (p = < 0.001, p = 0.025, p = 0.039 and p = 0.011, respectively). In the multivariate analysis, high density of PD-1+ lymphocytes was a significant negative independent prognostic factor for clinical failure-free survival (HR = 2.48, CI 95% 1.12–5.48, p = 0.025).

INTRODUCTION

Prostate cancer is a major contributor to cancer burden and death among men worldwide [1, 2], and issues multiple challenges regarding diagnostics and disease management. There is a lack of molecular markers suitable for determining the prognosis and thus intensity of treatment, resulting in overtreatment with unnecessary side effects on the one hand and undertreatment and disease progression on the other [3]. Once a patient reaches a state of metastatic castration-resistant disease, no curative treatment options are available. Hence, there is an urgent need for new prognostic markers, as well as better treatment options, for both confined and widespread disease in prostate cancer.

It has become evident that for a cancerous tumor to develop and metastasize it has to escape anti-tumor immune response, especially CD8+ cytotoxic T cell mediated elimination [4]. Multiple mechanisms have been identified, including the exploitation of natural immunosuppressive pathways such as the programmed cell death protein 1 (PD-1) pathway [5, 6]. In healthy individuals, this pathway is important for maintaining self-tolerance, as well as curbing T cells during an immune response, preventing collateral damage to healthy tissues [7]. The pathway consists of the receptor programmed cell death protein 1 (known as PD-1 or CD279) and its ligands programmed death ligand 1 (known as PD-L1, CD274 or B7-H1) and programmed death ligand 2 (known as PD-L2, B7-DC or CD273), where the former is believed to be of

greatest significance. PD-1 can primarily be found on T cells, but also B cells, Natural Killer T (NKT) cells, activated monocytes, and dendritic cells (DCs) [7]. PD-L1 is typically found on antigen-presenting cells such as macrophages, but can be found on a wide range of cells, including human cancer cells [5, 6]. It is proposed that malignant cells express PD-L1 through genetic mutations or epigenetic changes, and as a response to an inflammatory environment [5]. This enables them to directly inactivate tumor infiltrating lymphocytes (TILs), and hence escaping immune destruction. In addition to activating PD-1, PD-L1 also has immunomodulatory effects within the cell on which it is expressed [5].

Knowledge about the PD-1 pathway's immunosuppressive effects lead to the notion that its inhibition could restore T cell mediated immunity towards tumor cells [8]. Currently, drugs that target PD-1 have been approved by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for malignant melanoma and non-small cell lung cancer (NSCLC), and there are currently ongoing trials for drugs targeting PD-L1 [9, 10]. Disappointingly, three recent trials, including a total of 27 patients with metastatic castration-resistant prostate cancer (mCRPC) receiving the PD-1 inhibitor drug nivolumab, demonstrated no clinical benefit [11–13]. In light of the use and development of new PD-1 pathway inhibitors, it is vital to gather information that can shed light on the expression of these immune checkpoint molecules in prostate cancer, and whether their expression is associated with prostate cancer survival. This might aid patient treatment decision-making as well as contributing to future research in PD-1 pathway directed therapies in prostate cancer patients.

Herein, we aimed to examine the potential prognostic significance of PD-1 and/or PD-L1 expression in prostate cancer. Consequently, we investigated 535 primary prostate cancer tumors for expression of PD-L1 in stromal and epithelial cells, as well as the expression of PD-1 and co-expression of PD-1 and CD8 in lymphocytes, and their associations with biochemical and clinical failure-free survival.

RESULTS

Patient characteristics and clinicopathological data

Detailed clinical and histopathological characteristics are presented in Table 1. Median age at surgery was 62 (range 47–75). The prostatectomies were retropubic in 435 cases and perineal in 100 cases. At the last follow-up in December 2015, 200 patients had experienced BF, 56 patients had CF, and 18 patients were dead of prostate cancer. Elaborate information on the cohort has been previously published [14].

Programmed cell death protein 1 and programmed death ligand 1 expression in prostate tumor tissue

Of the total cohort of 535 patients, immunohistochemistry (IHC) tumor scoring was possible for 402 cases for PD-L1, and 396 for PD-1. PD-L1 expression (Figure 1) was both cytoplasmatic and membranous. Intraluminal secretions and some intracellular granules seemed to stain intensively and were disregarded as artifacts. PD-L1 staining in tumor epithelial (TE) cells was positive in 371/402 (92%) cases, and 236/402 (59%) cases had a high PD-L1 intensity score. In addition, 267/402 (66%) of patients had PD-L1+ stromal cells. In general, PD-1+ cells were sparse (Figure 1) and fit the morphology of lymphocytes. In total, 156/396 (39%) cases had such intratumoral PD-1+ lymphocytes, and 43/396 (11%) cases had a high density. In addition, we observed few intraepithelial PD-1+ cells. Some of these resembled tumor cells, as recently described for malignant melanoma [15]. Unfortunately, we were not certain these were tumor cells using only morphological assessment, and this, in addition to low numbers, made them impossible to quantify by scoring. CD8 and PD-1 double staining showed co-expression of CD8 and PD-1, but also lymphocytes with single expression of one marker (Figure 1). However, the brown CD8 staining overpowered the red stain of PD-1, making quantification by scoring difficult.

Correlations between programmed cell death protein 1, programmed death ligand 1, lymphocyte markers and clinicopathological variables

The expression of PD-L1+ tumor stromal (TS) cells correlated significantly with PD-L1+ TE cells (r = 0.36, $p = < 0.001$), and had a weak correlation with intratumoral PD-1+ lymphocytes ($r = 0.21$, $p = < 0.001$). The expression of PD-L1 in TE cells and TS cells, in addition to intratumoral PD-1+ lymphocytes did not correlate to previously published [16] tumor tissue expression of lymphocyte markers CD3, CD4, CD8 and CD20. The expression of PD-1+ lymphocytes and PD-L1 in TS and TE was not correlated to clinicopathological variables (age, pT stage, preoperative PSA, Gleason grade, tumor size, perineural infiltration, lymphovascular infiltration and non-apical positive surgical margin).

Univariate survival analysis

The results of the univariate survival analyses are presented in Table 1 and Figures 2 and 3. Neither PD-L1+ TE cells nor PD-L1+ TS cells reached statistical significance for predicting biochemical failure (BF) or clinical failure (CF), but there was a trend towards a negative association between PD-L1 expression in TE cells and outcome, most prominently for biochemical

failure-free survival (BFFS) (HR: 1.34 (CI95% 0.97–1.85) $p = 0.078$, Table 1, Figure 2). With regard to PD-1+ lymphocytes, there was a trend for worse clinical failure-free survival (CFFS) in the entire patient material (HR: 1.96 (CI95% 0.90–4.25), $p = 0.084$, Table 1, Figure 3), but subgroups known to indicate unfavorable prostate cancer prognosis had a significantly higher risk for CF if they had a high density of intratumoral PD-1+ lymphocytes: age < 65 ($p = 0.025$), pT3 stage ($p = 0.011$), preoperative PSA > 10 ($p = 0.039$), and Gleason grade 9 ($p = < 0.001$) (Figure 3).

Multivariate survival analysis

Clinicopathological variables and PD-1 and PD-L1 variables with $p < 0.10$ from the univariate analyses (Table 1) were entered into three different multivariate models and results are presented in Table 2. High expression of intratumoral PD-1+ lymphocytes was a significant negative independent prognostic factor for CFFS (HR = 2.48, CI95% 1.12–5.48), $p = 0.025$) together with Gleason grade and perineural infiltration.

DISCUSSION

In our large, multicenter cohort of 535 prostate cancer cases, we observed a high density of PD-1+ lymphocytes in prostate cancer tumor tissue to independently predict shorter CFFS. The prognostic impact of PD-1 was stronger than for any of the renowned clinicopathological features, except for Gleason grade. In addition, a high density of PD-1+ lymphocytes was significantly associated with shorter CFFS in most subgroups related to worse prostate cancer prognosis, such as low age, high pT-stage, high preoperative PSA, and high Gleason grade. Furthermore, 92% of the cases had some level of PD-L1 expression in TE cells, but no significant association between the marker and outcome was observed.

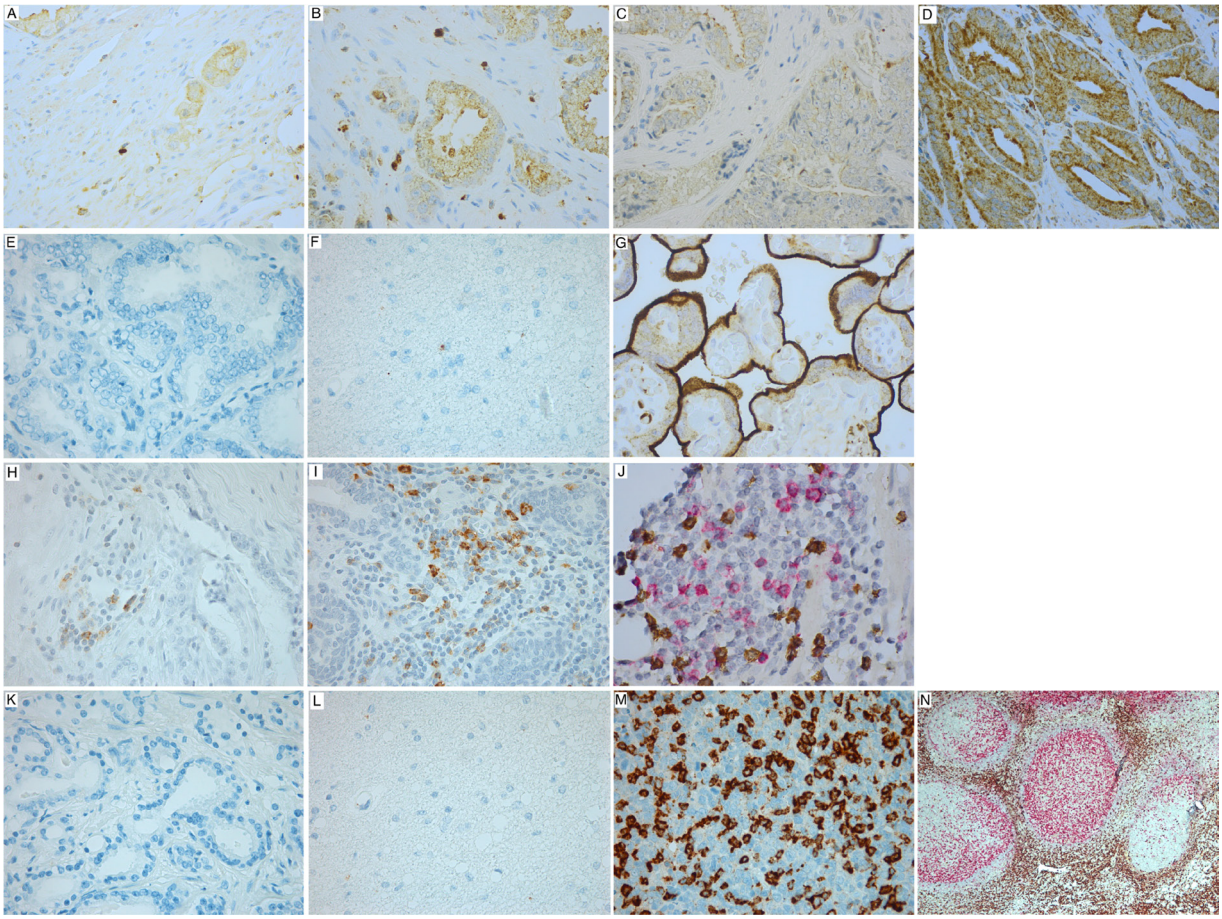

Figure 1: Immunohistochemical analysis. (**A**) Low density PD-L1+ stromal cells, (**B**) High density PD-L1+ stromal cells, (**C**) Low intensity PD-L1+ tumor epithelial cells, (**D**) High intensity PD-L1+ tumor epithelial cells, (**E**) Negative isotype control antibody for PD-L1 (prostate TMA), (**F**) Negative control for PD-L1 (brain), (**G**) Positive control for PD-L1 (placenta), (**H**) Low density of intratumoral PD-1+ lymphocytes, (**I**) High density of intratumoral PD-1+ lymphocytes, (**J**) PD-1 and CD8 double stain with pink showing PD-1 positivity, and brown showing CD8 positivity, (**K**) Negative isotype control antibody for PD-1, (**L**) Negative control for PD-1 (brain), (**M**) Positive control for PD-1 (tonsil), (**N**) Positive control for PD-1 and CD8 double stain (tonsil). Magnification ×400 for all, except (**N**) which shows ×50 magnification.

Table 1: Patient characteristics, clinicopathological variables, and molecular markers as predictors of biochemical- and clinical failure in prostate cancer patients (*n* = 535), (univariate analysis; log-rank test) significant *P* values in bold (threshold ≤ 0.05)

Variable	Patients	BF		CF	
	(*n*)	(200 events)		(56 events)	
		5-year EFS (%)	*p*	10-year EFS (%)	*p*
Age			0.237		**0.038**
≤ 65 years	357	77		94	
> 65 years	178	70		91	
pT-stage			**< 0.001**		**< 0.001**
pT2	374	83		97	
pT3a	114	61		87	
pT3b	47	43		74	
Preop PSA			**< 0.001**		**0.029**
PSA<10	308	81		95	
PSA>10	221	68		89	
Missing	6	-		-	
Gleason grade			**< 0.001**		**< 0.001**
3+3 / Grade group 1	183	83		98	
3+4 / Grade group 2	219	77		94	
4+3 / Grade group 3	81	70		90	
4+4 / Grade group 4	17	58		86	
>8 / Grade group 5	35	37		65	
Tumor Size			**< 0.001**		**0.002**
0-20 mm	250	83		96	
>20 mm	285	68		90	
PNI			**< 0.001**		**< 0.001**
No	401	80		96	
Yes	134	60		83	
PSM			**0.049**		0.198
No	249	69		90	
Yes	286	81		96	
Non-apical PSM			**< 0.001**		**< 0.001**
No	381	82		96	
Yes	154	57		85	
Apical PSM			0.063		0.427
No	325	74		92	
Yes	210	77		93	
LVI			**< 0.001**		**< 0.001**
No	492	77		95	
Yes	43	47		69	
Surgical proc			0.466		0.308
Retropubic	435	77		92	
Perineal	100	68		95	

PD-1+ lymphocytes in TS			0.489		0.084
Low	353	74		94	
High	43	69		87	
Missing	139				
PD-L1+ TS cells			0.899		0.680
Low	245	28		92	
High	157	74		91	
Missing	133				
PD-L1+ TE cells			0.078		0.603
Low	166	77		92	
High	236	71		92	
Missing	133				

Abbreviations: BF = biochemical failure; CF = clinical failure; EFS = event free survival in months; LVI = lymphovascular infiltration; p = *p* value for difference in event free survival with log rank analysis; PD-1 = programmed cell death protein 1; PD-L1 = programmed death-ligand 1; PNI = Perineural infiltration; Preop = preoperative; PSA = Prostate specific antigen; PSM = Positive surgical margin; pT-stage = pathological tumor stage; Proc = procedure; TE = tumor epithelial cells; TS = tumor stromal cells

To our knowledge, this is the first study to examine the prognostic impact of both PD-1 and its ligand PD-L1 in the same prostate cancer cohort, and the first to explore prognostic effects of PD-1+ lymphocytes in prostate cancer altogether. In addition to novelty, two major strengths in our study are the large, unselected patient population and the long follow-up time enabling us to calculate prognoses with regard to relevant clinical endpoints. Since no antibody for quantifying PD-1, and especially PD-L1, in formalin-fixed paraffin embedded (FFPE) tissue is uniformly accepted as standard, the antibodies used herein underwent stringent confirmatory validation in our laboratory, in addition to the manufacturers in-house validation.

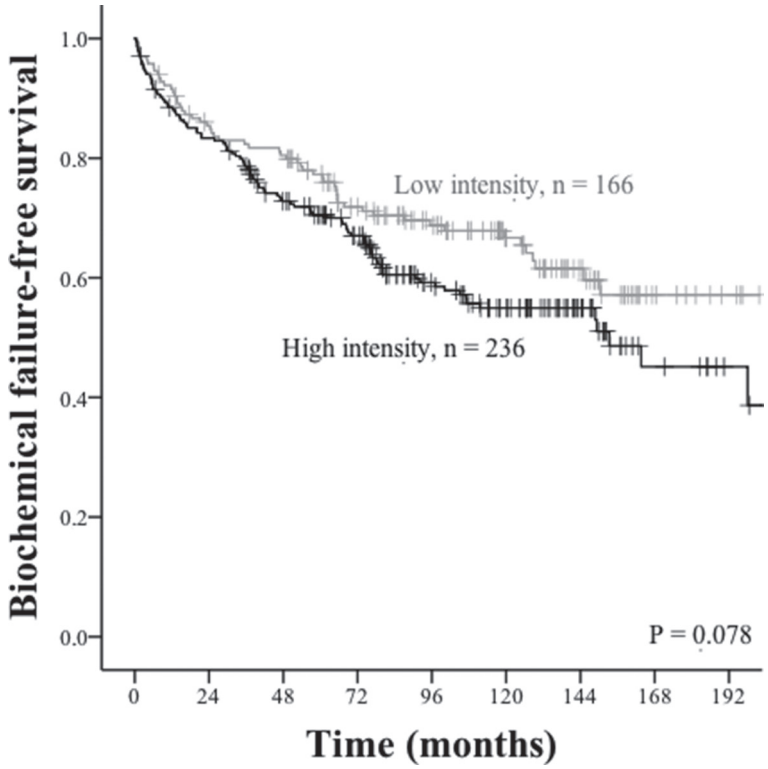

Figure 2: Biochemical failure-free survival curves for PD-L1 intensity in tumor epithelial cells. Grey lines indicate low intensity, whereas black lines indicate high intensity.

Table 2: Independent predictors for biochemical- and clinical failure in prostate cancer patients (*n* = 535), (cox regression analysis, backward conditional model)

Variable	Model 1 (clinicopathological)				Model 2 (PD-L1+ TE)		Model 3 (PD-1+ lymphocytes in TS)	
	BF		CF		BF		CF	
	HR (95% CI)	*p*	HR (95% CI)	*p*	HR (95% CI)	p	HR (95% CI)	*p*
Age	NE		NS		NE		NS	
≤ 65 years								
> 65 years								
pT-stage		***0.001***	NS			***0.003***	NS	
pT2	1.00				1.00			
pT3a	1.48 (1.02–2.14)	***0.040***			1.50 (1.00–2.27)	***0.050***		
pT3b	2.34 (1.47–3.74)	***< 0.001***			2.41 (1.45–4.00)	***0.001***		
Preop PSA		***0.033***	NS		NS		NS	
PSA < 10	1.00							
PSA > 10	1.37 (1.03–1.84)							
Gleason grade		***0.040***		***< 0.001***		***0.011***		***< 0.001***
3 + 3/Grade group 1	1.00		1.00		1.00		1.00	
3 + 4/Grade group 2	1.24 (0.86–1.78)	0.249	3.74 (1.40–9.98)	***0.009***	1.02 (0.67–1.56)	***0.920***	4,70 (1,31–16,81)	***0.017***
4 + 3/Grade group 3	1.73 (1.12–2.68)	***0.013***	5.08 (1.73–14.88)	***0.003***	1.98 (1.21–3.25)	***0.007***	6,26 (1,66–23,63)	***0.007***
4 + 4/Grade group 4	2.13 (1.06–4.31)	***0.035***	5.95 (1.41–25.14)	***0.015***	2.05 (0.96–4.37)	0.063	10,10 (2,04–50,17)	***0.005***
> 8/Grade group 5	1.92 (1.09–3.39)	***0.025***	13.09 (4.46–38.40)	***0.000***	1.83 (1.00–3.36)	***0.050***	20,34 (5,71–72,48)	***< 0.001***
Tumor size	NS		NS		NS		NS	
0–20 mm								
> 20 mm								
PNI		***0.045***	NS			***0.017***		***0,012***
No	1.00				1.00		1.00	
Yes	1.40 (1.01–1.94)				1.56 (1.08–2.25)		2,32 (1,21–4,47)	
Non-apical PSM		***0.001***	NS			***0.026***	NS	
No	1.00				1.00			
Yes	1.73 (1.25–2.38)				1.50 (1.05–2.14)			
Apical PSM		***0.026***	NE		NS		NE	
No	1.00							
Yes	0.71 (0.52–0.96)							
LVI	NS		NS		NS		NS	
No								
Yes								
PD-L1 + TE cells	NE		NE		NS		NE	
Low								
High								
PD-1 + lymphocytes in TS	NE		NE		NE			***0.025***
Low							1.00	
High							2.48 (1.12–5.48)	

Abbreviations: BF = biochemical failure; CF = clinical failure; CI = confidence interval; HR = hazard ratio; LVI = lymphovascular infiltration; NE = not entered in analysis; NS = not significant; p = *p* value for difference in survival with Cox regression analysis; PD-1 = programmed cell death protein 1; PD-L1 = programmed death-ligand 1; PNI = Perineural infiltration; Preop = preoperative; PSA = Prostate specific antigen; PSM = Positive surgical margin; pT-stage = pathological tumor stage; TE = tumor epithelial cells; TS = tumor stromal cells

This study was conducted as a further elaboration of our previous observation that CD8+ lymphocytes are independent negative prognostic markers in prostate cancer [16]. Based on this discovery, we proposed that the detected CD8+ lymphocytes were indeed tumor-specific CD8+ T cells summoned to particularly aggressive tumors, but lacking functionality due to immunosuppression, for example due to activation of the PD-1 pathway. The CD8-marker represents a broad population of T cells with various roles, which might weaken its prognostic impact. A recent study in melanoma patients concluded that PD-1 expression on CD8+ T cells identifies the subpopulation of tumor-specific effector cells [17] and hence, PD-1 may be a more specific prognostic marker than CD8. Surprisingly, our previously published lymphocyte marker expressions (CD3, CD4, CD8 and CD20) [16] did not correlate with PD-1 expression. To further explore the relationship between CD8+ and PD-1+ cells, we double-stained for both markers. By microscopic examination we detected co-expression of CD8 and PD-1 on lymphocytes as suspected, but also cells with single PD-1 or CD8 marker expression. Another explanation for lack of statistical correlations may be the different scoring methods of lymphocyte markers and PD-1 [16]. Also limiting the ability for comparison, the current study was performed on TMA cores cut from a much deeper tissue level than the lymphocyte study [16] hampering the ability to correlate these markers in the same tissue areas.

So far, translational studies regarding the prognostic impact of PD-1 and PD-L1 in human prostate cancer are sparse. For PD-1, only descriptive analyses have been published, all reporting PD-1+ lymphocytes to be present in prostate cancer carcinoma regions and/or in adjacent TLS [18–20]. With regard to PD-L1 positive tumor epithelial cells, descriptive analyses have been conflicting: Some research groups have reported lack of tumor epithelial positivity [12, 13, 20], while others have observed sparse expression [21] or cases with high expressions [18]. In a recent study, Gevensleben et al. found a high PD-L1 expression in TE cells to be an independent negative prognostic factor of BFFS in a cohort of 902 men with prostate cancer [22]. We could not fully reproduce this result, but in both univariate and multivariate analyses there was a consistent tendency of high expression of PD-L1 in TE cells in patients with a worse BFFS. In a larger study population, this association may have reached statistical significance.

There may be multiple possible biological explanations as to why we and others [18, 22] observe such high expression levels of PD-L1 in prostate tumor

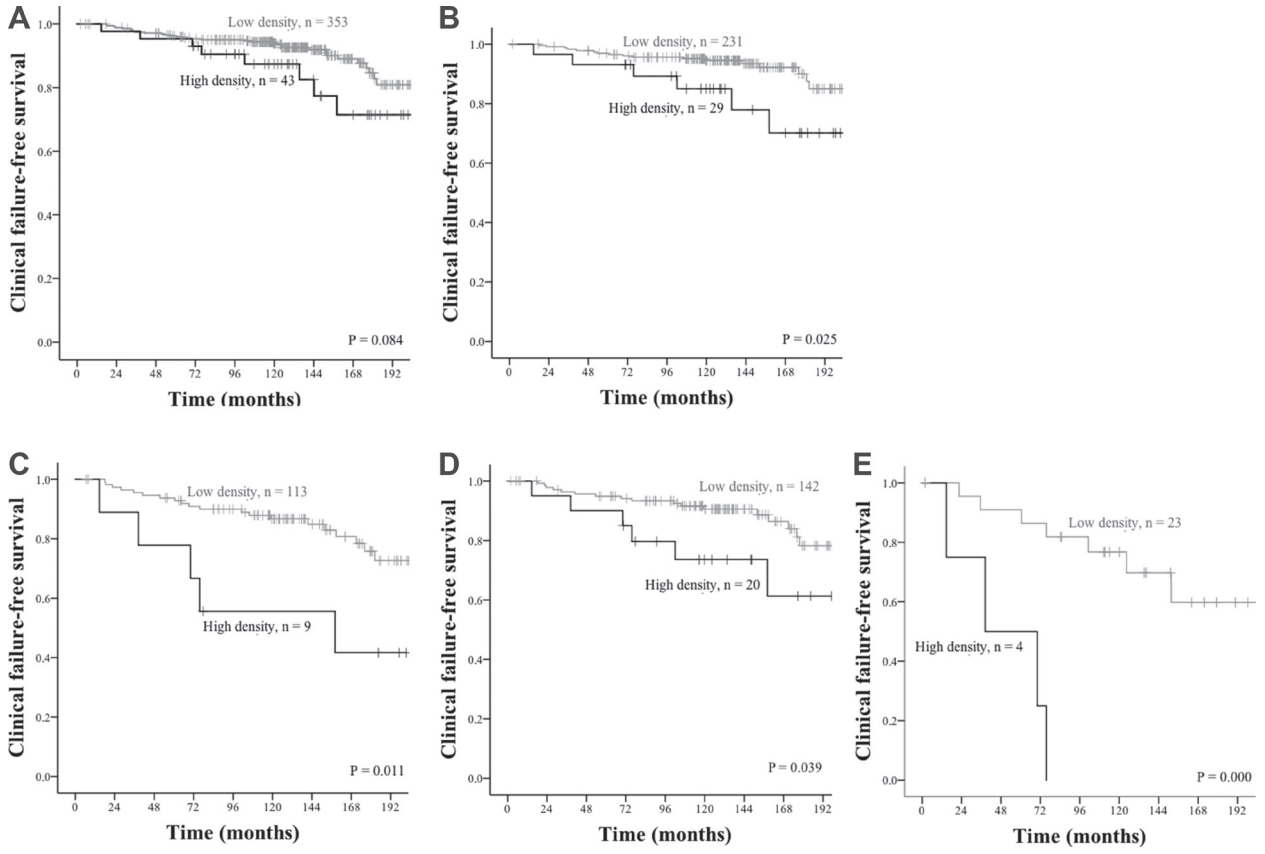

Figure 3: Clinical failure-free survival curves for PD-1+ lymphocytes in tumor stromal areas. Grey lines indicate low density, whereas black lines indicate high density. (**A**) All patients, (**B**) Patients with age < 65, (**C**) Patients with pTstage = 3, (**D**) Patients with preoperative PSA > 10, (**E**) Patients with Gleason grade = 9.

epithelial cells. The most well-known mechanism of PD-L1 induction on tumor epithelial cells is cytokines such as IFNγ produced by adaptive immune cells in the tumor microenvironment ('adaptive immune resistance') [21, 23, 24]. However, we find that PD-L1 expression do not correlate to adaptive immune cell markers, which may suggest there is another mechanism at play. Several studies in different cancers have demonstrated that intrinsic oncogenic pathways may induce PD-L1 expression ('innate immune resistance'). Some examples include EGFR mutations [25, 26] and loss of phosphatase and tensin homolog (PTEN) [27, 28]. To our knowledge, no such relationships have been found between intrinsic pathways and PD-L1 expression in human prostate cancer [21].

Our finding that an augmentation of the PD-1 pathway leads to a worse prostate cancer prognosis may indicate that tumor immune escape, and thus tumor immune elimination, are important mechanisms in prostate cancer. CD8+ cytotoxic T cells are proposed to be one of the most important protagonists in tumor immune elimination, and the mechanisms by which tumor cells avoid attack by tumor-specific CD8+ T cells are crucial parts of the immune escape process [4]. Different escape routes have been proposed. FOXP3+CD25+CD4+ Tregs are known suppressors of CD8+ cytotoxic T cells, and are observed up-regulated in multiple cancer types, including prostate cancer [29–31]. In addition, the process of antigen presentation is often impaired in tumors, leading to inadequate activation and boosting of T cells [32]. Moreover, tumor-specific CD8+ T cells have been found to express exhaustion markers such as PD-1 and T-cell immunoglobulin and mucin-domain containing-3 (Tim-3) indicating that their presence not necessarily implies an effective ongoing immune elimination process [33–35]. Contributing to this, different tumor cells have been found to express molecules such as indoleamine-2,3-dioxygenase (IDO) [36] and PD-L1, known to impair function of CD8+ cytotoxic T cells [5].

There have been recent breakthroughs in PD-1 pathway inhibition in other cancer diseases [9]. Our study has found the pathway molecules to be present in prostate cancer, and their presence to be associated to poor prognosis and as such, proposing them as attractive targets for inhibition. However, results from prostate cancer studies have so far proven mostly disappointing. At the time we conducted this study there had been published results from three different clinical trials with a total of 27 prostate cancer patients treated with the PD-1 inhibitor nivolumab [11–13]. Unfortunately, no clinical benefits were observed for these cases, which may have several possible explanations. For an immune checkpoint inhibitor to be effective in cancer treatment, the cancer in question must be able to evoke an immune response. Thus, one possible reason why PD-1 blockage does not appear to work in prostate cancer, may be that it is not an immunogenic cancer type. However, there are several aspects contradicting this proposition. Firstly, prostate cancer can express multiple tumor-associated antigens necessary in triggering anti-tumor immune response [such as prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), prostate-specific membrane antigen (PSMA), and prostate stem cell antigen (PSCA)] [37, 38]. Secondly, the FDA approved autologous dendritic cell-based vaccine Sipuleucel-T extends survival in patients with mCRPC. Though its exact mechanisms are not known, the most likely explanation is that it generates a tumor-specific T cell mediated immune response [31]. Furthermore, the mechanisms by which anti-androgen treatment increases survival in prostate cancer are believed to be partly explained by its ability to boost a tumor specific immune response [38, 40–41].

A likely reason why nivolumab-trials have failed to show effect in prostate cancer patients may be differences in patient and tumor characteristics. Patients in both our and Gevensleben et al. [22] cohorts were hormone naïve while the mentioned nivolumab trials [11–13] only included patients with mCRPC. Hence, immunosuppression through the PD-1 pathway may be a less efficient mechanism in late stage, widespread cancer disease, and/or there may be a more direct relationship between androgens and the PD-1 pathway. In addition, all prostate cancer patients included in the nivolumab-studies was reported to be negative for tumor expression of PD-L1 (< 5% PD-L1 positive cells) [12, 13]. As no prostate cancer patients with a high degree of PD-L1 positive tumor cells have received nivolumab, the trials give no genuine data on the efficacy of PD-1 pathway inhibitor treatment. However, we have, corroborating others [22], demonstrated that such expression is common in primary prostate cancer tumors from patients with localized disease. Though there is an ongoing debate regarding whether PD-L1 tumor expression can predict treatment effect, there are multiple indications that PD-L1 positivity enrich for response to PD-1 pathway inhibitors [43]. Hence, PD-1 pathway inhibitors should not be completely disregarded as ineffective in prostate cancer treatment, and in a recent trial with pembrolizumab 3/10 patients had an almost complete PSA regression [44].

To conclude, we find PD-1+ lymphocytes in prostate cancer tumors to be an independent negative prognostic marker in post-prostatectomy hormone naïve patients. In addition, our observations imply that PD-1 pathway inhibitors may yield therapeutic benefit in selected groups of prostate cancer patients.

MATERIALS AND METHODS

Patient characteristics and clinicopathological data

Six hundred and seventy-one patients who underwent radical prostatectomy as initial treatment for prostate adenocarcinoma from 1995 to 2005 were

retrospectively identified from the Departments of Pathology at the University Hospital of Northern Norway (n = 267), Nordland Hospital (n = 63), St. Olavs Hospital (n = 330), and Levanger Hospital (n = 11). One hundred and thirty-six patients did not meet the inclusion criteria due to: (i) radiotherapy to the pelvic region prior to surgery (n = 1), (ii) other malignancies within 5 years prior to the prostate cancer diagnosis (n = 4), (iii) inadequate paraffin-embedded tissue blocks (n = 130), and (iv) lack of follow-up data (n = 1). Thus, a total of 535 patients were included in this study. Complete demographic and clinical data were obtained from medical records. Two experienced pathologists (ER and LTB) reviewed all cases and registered histopathological data. Tumors were histologically classified according to WHO guidelines [45], graded in accordance with both the modified Gleason grading system [46, 47] and the new contemporary Gleason grading system [48], and staged in agreement with International Union Against Cancer (UICC) guidelines [49]. All demographic-, clinical- and histopathological data (Table 1) were registered in a SPSS data file and patients were de-identified. This report includes follow-up data as of December 2015. Median follow-up of survivors was 150 (range 17–245) months. For extensive information regarding our cohort, see our previous report [14]. The ethics of this study has been approved by The Regional Committees for Medical and Health Research Ethics (Protocol ID: 2009/1393, extended approval 2015), The Norwegian Data Protection Authority, and The Data Protection Official for Research (The Norwegian Social Science Data Service). Informed consent was not obtained, but the data was analyzed de-identified and this report contains no identifiable details.

Tissue microarray construction

For each case, a pathologist (ER) histologically identified and marked separate areas of the most representative TE tissue, adjacent TS tissue, normal epithelial (NE) tissue, and normal stromal (NS) tissue. In brief, a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD) with a 0.6 mm diameter needle was used to harvest a total of 6 cores from each case from the corresponding paraffin-embedded tissue blocks. The samples were inserted into a recipient paraffin block, and from each block 4 µm sections were cut with a Micron microtome (HM355S), affixed to glass slides, and sealed with paraffin.

Validation of antibody specificity

The primary antibodies used in this study were as follows: (i) PD-L1 rabbit monoclonal antibody (Cat#13684, clone: E1L3N, Cell Signaling Technology, Danvers, MA, USA), (ii) PD-1 mouse monoclonal antibody (Cat#ab52587, clone: NAT105, Abcam, Cambridge, UK) and (iii) CD8 rabbit monoclonal antibody (clone SP57; Ventana; Cat#790-4460). All applied antibodies had been subjected to in-house validation by their manufacturer. In addition, we performed confirmatory validation for PD-L1 and PD-1 to further accredit antibody specificity. Overexpressed human HEK293T cell lysates were utilized from OriGene for PD-L1 (#LY415473), PD-1 (#LY401555) and HEK293 as empty vector (#LY500001/negative control). Cells were incubated with 2xSDS sample buffer (OriGene) for 10 minutes at 100°C. Equal amounts of protein lysates were resolved on to a 4 to 12% Bis-Tris gel (Cat#NP0322; Life Technologies), and transferred onto an Odyssey nitrocellulose membrane (Cat#926-31092, LI-COR). The membrane was subsequently blocked for 1 hour at room temperature using Odyssey blocking buffer (Cat#927-40000, LI-COR). For PD-L1 1/1000, and for PD-1 1/50 dilution of primary antibody was applied and the membrane incubated overnight at 4˚C. PD-L1 (Cat#926-32213, LI-COR), and PD-1 (Cat#926-32212, LI-COR) RDye 800CW secondary antibodies with 1:1000 dilution was then applied, and the membrane incubated 1 hour at room temperature. Between antibody incubations, the membrane was washed 3 times for 5 minutes, each time in tris-buffered saline containing 0.05% Tween 20 (Cat#T9039, Sigma-Aldrich). Molecular weight markers used were the MagicMark XP Western Protein Standard (Cat#LC5603, Invitrogen) and SeeBlue Plus2 Pre-stained Standard (Cat#LC5925, Invitrogen). The most prominent bands (Supplementary Figure 1) represent the observed molecular weight of the detected protein, which correspond intimately with the predicted weight. Rabbit anti-actin, diluted 1:1000 (Cat#A2066, Sigma-Aldrich) was used as internal control and all lanes showed 42 KDa molecular weight protein load as predicted (Supplementary Figure 1).

Immunohistochemistry

Prior to IHC analysis, all slides were heated at 60°C for tissue fixation. PD-L1 IHC was performed on a Discovery-Ultra immunostainer (Ventana Medical Systems, Tucson, AZ). Slides were deparaffinized on-board in three 8-minute cycles. Antigen retrieval was done by using the EDTA-based solution (pH 8.0–8.5) CC1 reagent (Cat#950-124) at 95°C and incubating for 64 minutes. Endogenous peroxidase was blocked by incubating with Discovery inhibitor (Cat#760-4840) for 8 minutes. Primary antibody PD-L1 with 1/25 dilution was added and slides were incubated for 32 minutes at 37°C. Secondary antibody used was UltraMap anti-rabbit HRP (Cat#760-4315) incubating for 20 minutes, followed by 8 minutes HRP amplification. Finally, ChromoMap DAB (Cat#760-159) was used to visualize the antigens.

PD1/CD8 dual and PD1 IHC were performed using the Ventana Benchmark XT automated immunostainer (Ventana Medical Systems, Tucson, AZ). Antigen retrieval

was done for 30 minutes at approximately 100°C with CC1 reagent (Cat#950-124). Primary CD8 prediluted antibody was incubated for 12 minutes and visualized using the polymer-based Ventana ultraView DAB detection kit (Cat#760-500). The protocol followed by an ultraWash step to wash off excess antibody. Antibody denaturation for 4 minutes at 90°C was performed to ensure that the first primary antibody was completely inactivated before applying the second primary antibody. The PD-1 primary antibody in a 1/50 dilution was incubated for 32 minutes. The primary antibody was visualized using the Ventana ultraView Universal Alkaline Phosphatase Red Detection Kit (Cat#760-501) for double stain. The single staining of PD-1 was performed with same antigen retrieval procedure and was visualized with ultraView DAB detection kit.

To visualize the nuclei, all slides were counterstained with Ventana Hematoxylin II reagent (Cat# 790-2208) for 32 minutes, followed by a Bluing reagent (Cat# 760-2037) for 8 minutes, and then dehydrated, cleared and mounted on glass slides. All double stained sections were compared with the corresponding single stained slide. Two different controls were applied. First, control staining with an isotype-matched control antibody without the primary antibody, under the same staining protocol as for the primary antibody. Rabbit and mouse isotype-matched negative control antibodies were obtained from Abcam (PD-L1, Cat#ab27478; PD-1, Cat#ab18443). Second, multiple organ TMA as positive and negative tissue controls were used to verify the specificity of the staining in every staining procedure. The positive tissue controls comprised placenta for PD-L1 and tonsil for PD-1 (Figure 1). Negative tissue controls were samples of normal brain and ventricle for both PD1 and PD-L1.

To confirm staining homogeneity of PD-L1 throughout the tumor epithelium, we selected six patients from TMA slides with different tumoral expression (low, moderate, high). Whole tissue sections of these patients were further stained with PD-L1 (Cat#13684, clone: E1L3N), and analyzed by an experienced pathologist who approved staining homogeneity (Supplementary Figure 2).

Scoring of immunohistochemistry

All tissue samples were scored semi-quantitatively by two investigators independent of each other, and blinded to clinicopathological data and patient outcome. PD-L1 was scored by two experienced pathologists (AV, CN) and PD-1 was scored by one experienced pathologist (ER) and one trained MD (NN). For each tissue core the most experienced pathologist histologically assured the tissue type, and if possible 2 cores of TE, 2 of TS, 1 of NE and 1 of NS was scored for each case. Because PD-L1 was uniformly homogenously expressed in epithelial cells, an intensity scoring scale was chosen, and were as follows: no staining = 0, weak staining = 1, moderate staining = 2, and strong staining = 3. PD-L1+ stromal cells and PD-1+ lymphocytes were scored as number of positive stained cells per 0.6 mm diameter core as follows: 0 = 0–3, 1 = 4–10, 2 = 11–15, and 3 = > 15. In case of major disagreement (scoring difference > 1), the core was re-examined and consensus was reached. For each case, the mean score was calculated for each tissue compartment, and further dichotomized into low and high expression. Cut-off values for dichotomization were chosen according to a minimal *P*-value approach (optimal cut-off) while also securing statistically sufficient numbers in each group, and high scores were defined as follows: (i) ≥ 0.54 (mean) for PD-L1+ TS cells (ii) ≥ 1.0 for PD-L1 TE cells, and (iii) ≥1.25 for intratumoral PD-1+ lymphocytes. Scoring agreement between investigators was excellent for both markers. The intra-class correlation coefficient (reliability coefficient, r) was 0.93 (CI95% 0.92–0.93, $p < 0.001$) for PD-L1 and 0.96 for PD-1 (CI95% 0.57–0.96, $p < 0.001$). Slides with CD8 and PD-1-double staining were examined, but not quantified by scoring.

Statistical analysis

All statistical analyses were performed using the statistical package IBM SPSS, version 23 (SPSS Inc., Chicago, IL). The IHC scoring values from each observer were compared for inter-observer reliability by use of a two-way random effect model with absolute agreement definition. Spearman's rank-correlation was used to examine the associations between PD-L1 and PD-1 expressions, previously published lymphocyte markers [15] and clinicopathological markers. Presented r-values are the Spearman's rank correlation coefficient. Univariate Cox regression analysis was used to generate HR for each individual variable. Univariate survival analyses were done by using the Kaplan–Meier method, and the difference between survival curves was assessed by the log-rank test. The survival curves were terminated at 192 months, as less than 10% of patients were at risk after this point. All significant variables from the univariate analyses were assessed in multivariate survival models using a backward stepwise Cox regression model with a probability for stepwise entry or removal at $p = 0.05$ and 0.10, respectively. The significance level was $p < 0.05$ for all analyses. All survival analyses were carried out using BF and CF as endpoints. BF was characterized as a PSA ≥ 0.4 ng/ml, and rising in a minimum of two different blood samples postoperatively. BFFS was calculated as time from surgery to last follow-up date or the date PSA was first measured above threshold. CF was defined as verified local, symptomatic cancer recurrence and/or radiological verified metastasis to bone, visceral organs or lymph nodes after prostatectomy. CFFS was calculated from date of surgery to last follow-up date without CF or to date of CF.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Paper III

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BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

The Prognostic Significance of CXCL16 and Its Receptor C-X-C Chemokine Receptor 6 in Prostate Cancer

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The chemokine CXCL16 and its receptor, C-X-C chemokine receptor (CXCR6), affect tumor progression through different pathways, including leukocyte recruitment and function, cellular senescence, tumor cell proliferation, survival, invasion, and metastasis. We examined how the expression of CXCL16/CXCR6 in prostate cancer (PC) was related to clinicopathological features and activation of inflammatory cells. Tissue microarrays from 535 patients were constructed from tumor epithelial and tumor stromal areas of primary PC. Immunohistochemistry was used to evaluate the expression of CXCL16/CXCR6, $CD3^+$ T cells ($CD4^+$, $CD8^+$), and $CD20^+$ B cells. Survival analyses were used to evaluate their prognostic impact. Expression of CXCL16 in PC cell lines (DU145 and PC3) and the effect on proliferation and migration were examined. High expression levels of CXCL16 [hazard ratio (HR), 2.52; 95% CI, 1.12—5.68; $P = 0.026$] and CXCR6 (HR, 2.29; 95% CI, 1.10—4.82; $P = 0.028$) were each independent predictors for clinical failure. High co-expression of CXCL16 and CXCR6 (HR, 5.1; 95% CI, 1—15.9; $P = 0.05$) was associated with negative prognostic factors, such as Gleason grade 4 + 3, Gleason score ≥7, vascular infiltration, and positive surgical margins. As a conclusion, high protein expression of CXCL16 and high protein co-expression of CXCL16/CXCR6 in PC were independent predictors for a worse clinical outcome. *(Am J Pathol 2015, 185: 2722—2730; http://dx.doi.org/10.1016/j.ajpath.2015.06.013)*

Despite recent advances in treatment and clinical management of prostate cancer (PC), it is still a leading cause of cancer death. To improve the diagnostics and the therapy of both localized and metastatic PC, identification of molecules involved in development and progression of this heterogeneous disease is vital.

Chemokines belong to a superfamily of chemotactic cytokines. In both physiological and pathological conditions, activation of the chemokine or chemokine receptor leads to a coordinated recruitment of leukocytes. This also includes regulation of cell differentiation, proliferation, survival, and senescence.[1,2] CXCL16 and its receptor, C-X-C chemokine receptor 6 (CXCR6), belong to the CXC chemokine family, and CXCL16 exists both in a transmembrane and a soluble form.[3—5] The chemokine receptor, CXCR6 (alias STRL33, Bonzo, or TYMSTR), is expressed on the surface of $CD4^+$ and $CD8^+$ T cells,[5] natural killer cells,[6] plasma cells,[7] fibroblasts, keratinocytes, and cancer cells of different origins.[8—11] Interaction between the receptor, CXCR6, and its ligand, CXCL16 (alias SR-PSOX), is involved in a diversity of biological processes, including selective regulation of lymphocyte subsets, chronic inflammation, cell adhesion and survival, tumor development and progression, and antitumor immunity.[4,12—16] Chemokine receptor engagement is known to enhance tumor cell proliferation and survival through activation of the mitogen-activated protein/extracellular signal—regulated kinase pathway. This is also the case for soluble

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CXCL16 (S-CXCL16), which up-regulates its own receptor, CXCR6, resulting in increased cell migration.[13] Furthermore, CXCL16 and CXCR6 are reported to be markers and promotors of inflammation-associated cancer.[17–19]

The biology of human PC is believed to arise from precancerous processes associated with inflammation, such as proliferative inflammatory atrophy.[20] Proliferative inflammatory atrophy is proposed to lead to prostatic intraepithelial neoplasia and cancer.[21] In PC cell line studies, CXCL16 and CXCR6 are up-regulated in proliferative inflammatory atrophy, prostatic intraepithelial neoplasia, and tumor tissue compared with normal tissue, and correlate with cancer stage and grade.[17] CXCL16 and CXCR6 are up-regulated in aggressive PC and bone cancer metastasis[22] and are associated with poor treatment outcome.[23–25]

In 535 prostatectomy specimens, we explored the prognostic impact of CXCL16 and CXCR6 expression in epithelial tumor cells and tumor stromal areas and their prognostic impact in PC; investigated the impact of CXCL16 on proliferation and migration in PC cell lines DU145 and PC3; and correlated the CXCL16/CXCR6 expression with the presence of T lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$ cells) and B lymphocytes ($CD20^+$).

Materials and Methods

Patients and Tissue Samples

Primary tumor tissue from 535 radical prostatectomy patients diagnosed at the University Hospital of Northern Norway (Tromsø), the St. Olav's University Hospital (Trondheim, Norway), and Nordland Hospital (Bodø, Norway), from 1995 through 2005, were used in this study.

Adequate paraffin-embedded tissue blocks and complete demographic and clinicopathological data were obtained for all patients. The tumors were graded according to the new modified Gleason grading system and staged according to the World Health Organization guidelines.[26,27] All primary tumors were histologically reviewed by two pathologists (E.R. and L.-T.B.). Follow-up time was assigned from the date of first examination until the date of last follow-up, which was November 31, 2012, date of biochemical failure (BF), or date of clinical failure (CF). BF was characterized as a prostate-specific antigen (PSA) $\geq$0.4 ng/mL and increasing in a minimum of two different blood samples postoperatively. CF was defined as progression of local symptoms or metastasis to bone, visceral organs, or lymph nodes, verified by computed tomography, magnetic resonance imaging, bone scan, ultrasonography, or death due to PC. Data were censored for death from causes other than PC. The median follow-up of survivors was 89 months (range, 6.3 to 188.3 months). The cohort and the tissue microarrays (TMAs) are thoroughly described and used in a previously published article.[28] The study has been approved by The Regional Committee for Medical and Health Research Ethics (2009/1393), the Data Protection Official for Research (Norwegian Social Science Data Services), and the National Data Inspection Board.

Microarray Construction

TMA construction was chosen for high-throughput molecular pathology analysis. For each case, a pathologist (E.R.) histologically identified and marked two cores with areas of tumor cells (epithelial tumor cells), two cores with tumor stromal tissue, one core from areas with normal epithelial cells, and one core with normal stromal tissue. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD). Briefly, we used a 0.6-mm-diameter needle to harvest the marked tissue areas from the corresponding formalin-fixed, paraffin-embedded tissue blocks. The samples were inserted into an empty recipient paraffin block, according to a coordinate pattern. To include all core samples, 12 tissue array blocks were constructed. Multiple sections (4 μm thick) were cut with a Micron microtome (model HM355S; Thermo Scientific, Oslo, Norway), affixed to glass slides, and sealed with paraffin. The detailed method has been reported previously.[29]

Immunohistochemistry

CXCL16 (rabbit polyclonal, ab101404, 1:100) and CXCR6 (goat polyclonal, ab125115, 1:100) antibodies from Abcam (Cambridge, UK) were used in the study. The antibodies were validated by the manufacturer for immunohistochemistry on paraffin-embedded material. In addition, in-house validation by Western blot analysis was performed. Cut sections were deparaffinized with xylene and rehydrated with ethanol. Antigen retrieval was performed by placing the sections in 0.01 mol/L citrate buffer, pH 6.0, before microwave heating for 20 minutes at 450 W. Endogenous peroxidase was blocked by incubation in 3% H_20_2 for 10 minutes. Sections were blocked in 5% goat or rabbit serum for 1 hour before overnight incubation with the primary antibodies at 4°C. The primary antibodies were visualized by adding a secondary biotin-conjugated antibody, followed by an Avidin/Biodin/Peroxidase complex (Vectastain ABC Elite-kit; Vector Laboratories, Burlingame, CA) and substrate (Vector NovaRed; Vector Laboratories). As negative staining controls, the primary antibodies were replaced with the primary antibody diluent. All slides were counterstained with hematoxylin to visualize the nuclei.

The antibodies were as follows: CD3 (clone PS1), CD8 (clone 1A5), and CD20 (clone L26) from Ventana Medical (Tucson, AZ), and CD4 (clone 1F6) from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK). The applied antibodies had been subjected to in-house validation, according to the manufacturer's recommendation for immunohistochemical analysis on paraffin-embedded material. The detailed method has been reported previously.[30]

Scoring of Immunohistochemistry

The ARIOL imaging system (Applied Imaging Corp, San Jose, CA) was used to scan and digitalize the immunohistochemical-stained TMA slides. The slides were loaded in the SL 50 automated slide loader (Vision BioSystems, Buffalo Grove, IL) and scanned at a low resolution (×1.25) and high resolution (×20) using an Olympus BX61 microscope with an automated platform (Prior Scientific, Cambridge, UK). Images of the cores were uploaded into the Ariol Software version 3.1.2. Two pathologists (E.R. and S.A.-S.) scored independently and semiquantitatively viable parts of each anonymized core by light microscopy. The dominant staining intensity of epithelial tumor cells (TEs) and tumor stromal cells (TSs) was scored as follows: 0, negative; 1, weak; 2, intermediate; and 3, strong. A core was scored as missing if the core was missing or considered to be of insufficient quality to score by both observers. Consequently, all reported marker expressions are based on the evaluation of two separate tissue cores. High expression of CXCL16 and CXCR6 was defined as expression at the third quartile or higher ($\geq$1.12 and $\geq$1.5, respectively) and was measured in the following manner: 0, no positive cells; 1, <25% positivity; 2, 25% to 50% positivity; and 3, 51% to 100% positivity. The scoring method for T and B cells has been reported previously.[30]

Western Blot Analysis

Cells were washed in ice-cold phosphate-buffered saline, and lysate was added directly in NuPAGE LDS Sample Buffer (NP0007; Life Technologies-Fisher Scientific, Oslo, Norway) with dithiothreitol. Equal amounts of protein lysates were resolved onto a 4% to 12% Bis-Tris gel (NP0322; Life Technologies). The resolved proteins were transferred onto an Odyssey nitrocellulose membrane (catalog number 926-31092; LI-COR Biosciences, Lincoln, NE), and the membrane was subsequently blocked for 1 hour at room temperature using the Odyssey blocking buffer (catalog number 927-40000; LI-COR Biosciences). Primary and secondary antibodies were diluted in the Odyssey blocking buffer, and the membrane was incubated with antibody dilution at room temperature. Antibodies used were rabbit polyclonal anti-CXCL16 antibody, 1:1000 (ab101404; Abcam), rabbit anti-actin, 1:3000 (A2066; Sigma-Aldrich AS, Oslo, Norway), and IRDye 800CW donkey anti-rabbit, 1:10,000 (number 926-32213; LI-COR Biosciences). Between antibody incubations, the membrane was washed three times for 5 minutes each time in tris-buffered saline containing 0.05% Tween 20 (Sigma-Aldrich). Molecular weight markers used were the MagicMark XP Western Protein Standard (catalog number LC5603; Invitrogen-Fisher Scientific, Olso, Norway) and SeeBlue Plus2 Pre-stained Standard (catalog number LC5925; Invitrogen-Fisher Scientific).

Cell Culture

The PC cell lines DU145 (HTB-81) and PC3 (CRL-1435), both from ATCC (Manassas, VA), were cultured in RPMI 1640 media (catalog number R8758; Sigma-Aldrich) supplemented with 10% fetal bovine serum (catalog number S0415; Biochrom, Berlin, Germany) and 1× penicillin-streptomycin antibiotic mixture (catalog number P0781; Sigma-Aldrich). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. The cell line authenticity was verified by the Department of Forensic Medicine at UiT The Arctic University of Norway (Tromsø).

RNA Interference

Cells were transfected with CXCL16 siRNA (catalog number 4392420, s33809, Silencer Select CXCL16 siRNA; Ambion) using the transfection reagent Lipofectamine 2000 (catalog number 11668-019; Invitrogen-Fischer Scientific). A Cy3-labeled negative control scrambled siRNA (catalog number AM4621, Silencer Cy3-labeled Negative Control No. 1 siRNA) was included in all experiments. Transfection efficiency was typically assessed to 85% to 100%.

Proliferation Assay

The proliferation potential of the cells was measured using the real-time cell analyzer system xCELLigence, RTCA DP (catalog number 05469759001; Roche) fitted with the E-plate 16 (catalog number 05469830001; Roche, Oslo, Norway). Cells were trypsinized until detached, resuspended in complete growth media, and counted. By initial titration experiments, optimal cell number per well was determined to be 5000. According to the manufacturer's protocol, cells were seeded in quadruplicate into the E-pale after baseline measurement. The plate was incubated for 30 minutes at room temperature before positioned in the RTCA DP (Real-Time Cell Analyzer Dual-Plate; Roche) instrument. The RTCA DP instrument was located in an incubator preserving the same conditions used for routine cultivation of the PC cell lines. The instrument denotes the cellular growth rate as Cell Index, which is an arbitrary unit reflecting the cell-sensor impedance. The cell index was measured every 15 minutes for the first 24 hours (for better resolution at attachment and spreading phase), and then every 30 minutes. Growth curves and doubling time were calculated with the RTCA software version 1.2.1 (Roche). For each cell line, three independent experiments were performed.

Migration Assay

To assess migration, the ibidi culture insert (Inter Instrument AS, Høvik, Norway) was used. The insert consists of two 0.22-cm^2 chambers separated by a 0.5-mm divider. The inserts were planted into a 12-well tissue culture dish (one insert per well) using sterile tweezers. To each chamber, 70 μL pre-transfected cell suspensions containing 4 to 6 × 10^5 cells/mL were added. The cells were left to adhere before the insert was removed and fresh media added. Images were acquired along the cell-free zone at time points 0, 6, and 24 hours.

Table 1 Patient Characteristics, Clinicopathological Variables, and Their Prognostic Variables for BF, CF, and PCD

			BF (170 events)		CF (36 events)		PCD (15 events)	
Characteristic	No. of patients	% of Patients	5-Year EFS (%)	*P* value	10-Year EFS (%)	*P* value	10-Year EFS (%)	*P* value
Age, years								
≤65	357	67	76		92		97	
>65	178	33	70		88		96	
pT stage				<0.001		<0.001		<0.05
2	374	70	83		96		98	
3a	114	21	60		86		98	
3b	47	9	43		73		89	
Preoperative PSA				<0.001				
<10	308	57	80		93		99	
>10	221	42	67		88		95	
Missing	6	1						
Gleason score				<0.001		<0.001		<0.05
3 + 3	183	34	83		98		99	
3 + 4	220	41	76		93		98	
4 + 3	80	15	69		84		95	
4 + 4	19	4	63		76		94	
>8	33	6	34		67		87	
Tumor size, mm				<0.001		<0.05		
0–20	250	47	82		94		99	
>20	285	53	67		88		96	
PNI				<0.001		<0.001		<0.05
No	401	75	79		95		98	
Yes	134	25	60		81		93	
PSM				<0.05		<0.05		
No	249	47	81		94		97	
Yes	286	53	69		89		97	
Circumferential PSM				<0.001		<0.001		<0.05
No	381	71	81		95		98	
Yes	154	29	57		81		94	
Apical PSM				<0.05				
No	325	61	73		90		96	
Yes	210	39	77		92		98	
Vasc inf			<0.001	<0.001		<0.001		<0.05
No	492	92	77		93		98	
Yes	43	8	46		71		88	
Surgical procedure								
Retropubic	435	81	76		90		97	
Perineal	100	19	67		95		98	

Univariate analysis; log-rank test. $N = 535$.

BF, biochemical failure; CF, clinical failure; EFS, event-free survival; PCD, prostate cancer death; PNI, perineural infiltration; PSA, prostate-specific antigen; PSM, positive surgical margin; Vasc inf, vascular infiltration.

The migration rate into the 0.5-mm gap was calculated using the free software TScratch version 1.0 (Computational Science and Engineering Laboratory, Zurich, Switzerland). For each cell line, three independent experiments were performed.

Statistical Analysis

All statistical analyses were performed using the statistical package IBM SPSS version 22 (SPSS Inc., Chicago, IL). Scoring values from each pathologist (E.R. and S.A.-S.) were compared for interobserver reliability by use of a two-way random-effect model with absolute agreement definition. The intraclass correlation coefficient (reliability coefficient, r) was 0.95 (range, 0.90 to 0.97; $P < 0.001$). A Wilcoxon signed rank test was used to check for differences in expression of CXCL16 and CXCR6 between normal and cancer tissues. A Spearman correlation test was performed to examine associations between CXCL16, CXCR6, clinicopathological markers, and the immune cells ($CD3^+$, $CD4^+$, $CD8^+$ T cells, and $CD20^+$ B cells). All survival analyses were performed using two different end points: biochemical failure-free survival (BFFS) and clinical failure-free survival (CFFS). BF was characterized as a PSA ≥0.4 ng/mL and increasing in

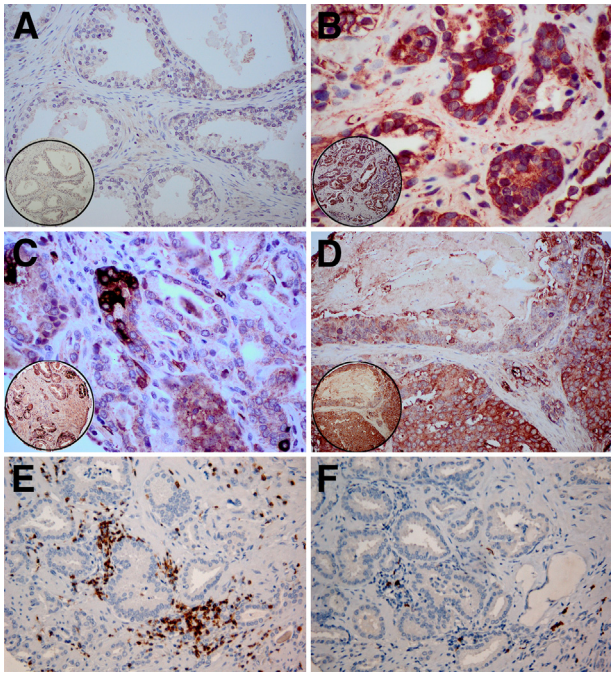

Figure 1 Immunohistochemistry microscopic images of tissue microarray representing expression of CXCL16 in normal prostatic tissue with no expression (**A**) and high expression (**B**) in tumor epithelial cells. C-X-C chemokine receptor 6 is highly expressed in single tumor epithelial cells (**C**) and in larger areas of tumor tissue (**D**). Amounts of CD3+ (**E**) and CD20+ (**F**) T cells in one core from the same area. Original magnification: ×100; ×400 (**insets**).

minimum of two different blood samples postoperatively. CF was defined as verified local symptomatic progression and/or verified metastasis to bone, visceral organs, or lymph nodes on computed tomography, magnetic resonance imaging, bone scan, or ultrasonography. The Kaplan-Meier method was used to make plots of BFFS and CFFS in high versus low expression of CXCL16 and CXCR6. Statistical differences were tested with log-rank. Significant variables from the univariate analyses were included in the multivariate survival analysis using a backward stepwise Cox regression model with a probability for stepwise entry removal at 0.05 and 0.10, respectively. The significance level used was $P < 0.05$ for all analyses.

Results

Clinicopathological Variables and Patient Characteristics

The patients' demographic, clinical, and histopathological characteristics are presented in Table 1. Their median age at surgery was 62 (range, 47 to 75) years. The prostatectomies were retropubic in 435 cases and perineal in 100 cases. The patients did not receive preoperative hormonal therapy. At last follow-up, 170 (32%) of the patients had BFFS, 36 (7%) of the patients had CFFS, and 15 (3%) of the patients were dead of PC. The median PSA was 8.8 (range, 0.7 to 104) ng/mL. The median tumor size was 20 (range, 2.0 to 50) mm.

Expression of CXCL16 and CXCR6 and Correlations

The staining of CXCL16 was predominantly cytoplasmic, with some membranous staining. CXCR6 staining was both granular and cytoplasmic (Figure 1). There was frequent co-expression between CXCL16 and CXCR6 ($P < 0.001$). Overall, there was a high co-expression of CXCL16/CXCR6 in tumor tissue compared with normal prostatic tissue and benign hyperplasia (both $P < 0.001$). When analyzing the chemokines separately, we found a high expression of

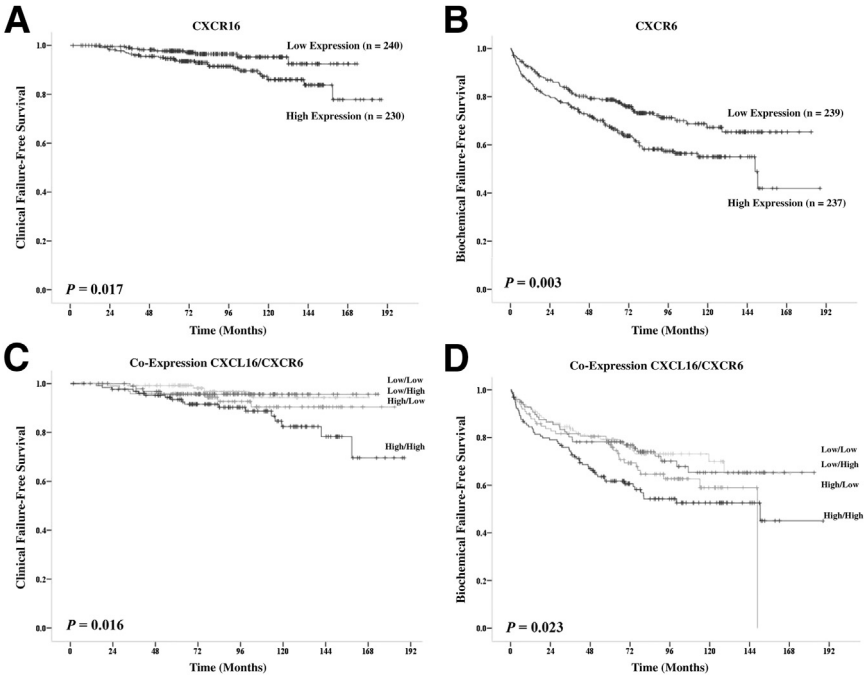

Figure 2 Disease-specific survival according to biochemical failure (biochemical failure-free survival) and clinical failure (clinical failure-free survival) in the total cohort: expression of CXCL16 (**A**) and C-X-C chemokine receptor 6 (CXCR6; **B**). **C** and **D:** Co-expression of CXCL16/CXCR6.

Table 2 Multivariate Models Including Significant Univariate Analyses for Clinical Failure in All Patients

Factor	HR	95% CI	*P* value
Model 1	CF (n = 36)		
Gleason score			
3 + 3	1		<0.05
3 + 4	2.30	0.72–7.39	
4 + 3	2.78	0.80–9.74	
>7	7.04	2.19–22.61	<0.05
Perineural infiltration	2.17	1.04–4.54	<0.05
Positive surgical margin	2.92	1.40–6.08	<0.05
CXCL16	2.52	1.12–5.68	<0.05
Model 2			
Gleason score			
3 + 3	1		<0.05
3 + 4	2.53	0.80–7.98	
4 + 3	3.87	1.12–13.33	<0.05
>7	7.20	2.25–22.99	<0.001
Perineural infiltration	2.39	1.15–4.96	<0.05
Positive surgical margin	2.90	1.39–6.05	<0.05
CXCR6	2.29	1.10–4.82	<0.05
Model 3			
Gleason score			
3 + 3	1		<0.05
3 + 4	2.17	0.68–6.96	
4 + 3	3.52	1.00–12.34	<0.05
>7	6.46	1.96–21.32	<0.05
Vascular infiltration	2.92	1.24–6.89	<0.05
Positive surgical margin	3.36	1.62–6.95	<0.001
CXCL16/CXCR6 (low/low)	1		<0.05
CXCL16/CXCR6 (high/low)	2.23	0.64–7.77	
CXCL16/CXCR6 (low/high)	2.50	0.58–10.82	
CXCL16/CXCR6 (high/high)	5.11	1.65–15.87	

Cox regression, backward conditional; n = 535.

CF, clinical failure; CXCR, C-X-C chemokine receptor; HR, hazard ratio.

CXCL16 in tumor tissue compared with normal tissue ($P < 0.001$), but there were no differences when comparing TE with TS ($P = 0.446$). High expression of CXCR6 was found in tumor tissue compared with normal tissue ($P < 0.001$). CXCR6 was significantly higher expressed in TE compared with TS ($P = 0.008$).

Co-expression of CXCL16/CXCR6 was correlated with Gleason score >7 ($P = 0.009$), vascular infiltration ($P = 0.020$), and positive circumferential margins ($P = 0.014$).

We also found correlations between high expression of CXCL16 in tumor compartment (TE + TS) and $CD3^+$ T cells ($P = 0.034$). A trend in the same direction was seen for $CD4^+$ T cells, but did not reach statistical significance ($P = 0.079$). CXCL16 and CXCR6 were not correlated to $CD3^+$ T cells, $CD8^+$ T cells, or $CD20^+$ B cells.

Univariate Analysis

Clinicopathological variables significant for BFFS were as follows: pT stage ($P < 0.001$), preoperative PSA ($P < 0.001$), Gleason score ($P < 0.001$), tumor size ($P < 0.001$), perineural infiltration (PNI; $P < 0.001$), positive surgical margin (PSM; $P = 0.04$), circumferential PSM ($P < 0.001$), apical PSM ($P = 0.04$), and vascular infiltration ($P < 0.001$). Clinicopathological variables significant for CFFS were as follows: pT stage ($P < 0.001$), pN stage ($P < 0.001$), Gleason grade ($P < 0.001$), tumor size ($P = 0.019$), PNI ($P = 0.001$), PSM ($P = 0.038$), nonapical surgical margin ($P < 0.001$), and vascular infiltration ($P < 0.001$) (Table 1).

When analyzing both tumor epithelial cells and tumor stromal areas as one compartment, high expression of CXCL16 was significantly associated with a reduction in CFFS ($P = 0.017$) (Figure 2A). High expression of CXCR6 was associated with a reduction in BFFS ($P = 0.003$) (Figure 2B), and a trend was found for CFFS ($P = 0.063$). High co-expression of CXCL16/CXCR6 was significantly associated with a reduction in CFFS ($P = 0.023$) (Figure 2C) and BFFS ($P = 0.016$) (Figure 2D).

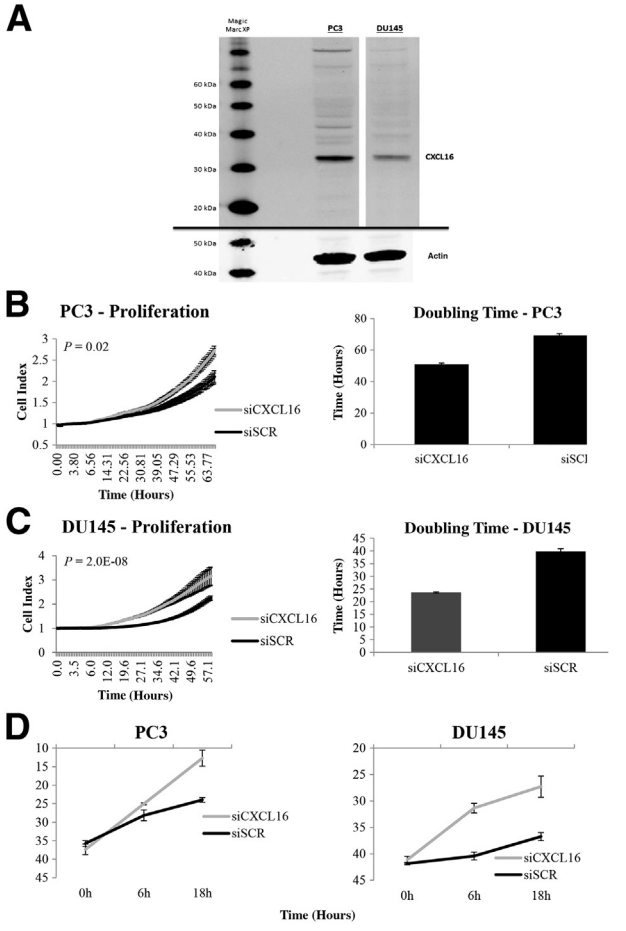

Figure 3 **A:** The specificity of the CXCL16 antibody was determined by Western blot analysis on cell lysate from the prostate cancer cell lines PC3 and DU45. Actin was used as a loading control. In both cell lines, CXCL16 is detected at the predicted molecular weight, with relatively small amounts of unspecific binding. **B** and **C:** The silencing of CXCL16 resulted in increased proliferation for both PC3 and DU145 cells. **D:** The rate of migration into the open area, after removing the ibidi insert, increases when CXCL16 is silenced. siSCR, small interfering scrambled.

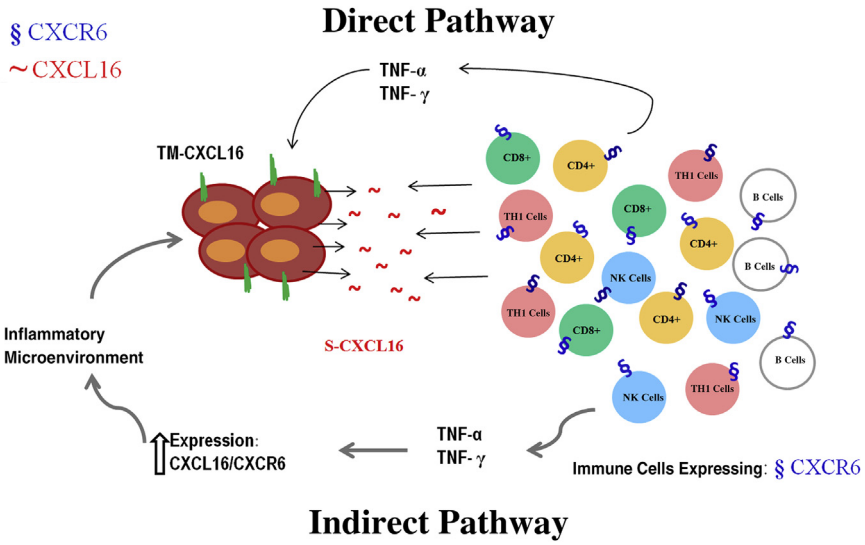

Figure 4 Enhanced tumorigenesis directly or indirectly through positive feedback loops between tumor cells, chemokines, and leukocytes. Soluble (S)-CXCL16 secreted from cancer cells leads to a positive feedback loop: directly through cross talk with tumor cells, or indirectly by generating inflammatory microenvironment facilitating tumor cell growth. CXCR, C-X-C chemokine receptor; NK, natural killer; TH1, type 1 helper T cell; TM, transmembrane; TNF, tumor necrosis factor.

When stratifying the analyses for the different surgical centers (University Hospital of Northern Norway, St. Olav Hospital, and Nordland Hospital), the results were the same.

Multivariate Analysis

High expression of CXCL16 (hazard ratio, 2.5; 95% CI, 1.2−5.7; $P = 0.025$) was an independent predictor for CFFS in addition to Gleason score >7, PNI, and PSM. High CXCR6 expression (hazard ratio, 2.3; 95% CI, 1.1−4.8; $P = 0.028$) was also an independent prognostic factor for CFFS as well as Gleason grade 4 + 3, Gleason score >7, PNI, and PSM. High co-expression of CXCL16 and CXCR6 (hazard ratio, 5.1; 95% CI, 1.6−15.9; $P = 0.005$), Gleason grade 4 + 3, Gleason score >7, vascular infiltration, and PSM were independent prognostic factors for CFFS (Table 2).

Western Blot Analysis

Western blot analysis was used to verify the specificity of the CXCL16 antibody (Figure 3A). The molecular weight of the detected protein (strongest bands) corresponded well with the predicted weight, as with the data provided by the manufacturers. The weaker extra bands may represent chemically modified degraded proteins or products of alternative splicing. siRNA targeted against CXCL16 caused a marked decrease in the intensity of the bands, compared with scrambled control siRNA. This confirms the specificity of the antibodies.

Migration and Proliferation Assays

To uncover possible biological effects of CXCL16 in PC cell lines, we evaluated effects of transient knockdown of CXCL16 on cell proliferation and migration. To study proliferation, we used the xCELLigence platform (Roche). This is a microelectric assay based on changing impedance of bottom electrodes in the presence of the cells. We repeatedly observed that knockdown of CXCL16 with siRNA did cause activation of proliferation compared with the negative scrambled control. This was evident from both the growth curves and the doubling time calculations (Figure 3B). To study migration, we used the ibidi culture insert. The insert consists of two chambers separated by a divider, and cells are loaded into the chamber. After cell adhesion, the insert was removed and the migration rate into the gap was calculated. Both DU145 and PC3 cells exhibited increased migration ability after transient knockdown of CXCL16 (Figure 3, C and D). The experiment was repeated three times.

Discussion

In this large multicenter-cohort, we found that CXCL16 and CXCR6, separately or co-expressed, were independent predictors for CF in PC. Furthermore, CXCL16 and CXCR6, independently or co-expressed, correlated with Gleason score >7, positive circumferential margins, vascular infiltration, and PNI, indicating a more aggressive PC phenotype. CXCL16 and CXCR6 were both highly expressed in PC tissue compared with normal epithelial cells and normal stromal areas. Although positive expression of CXCL16 was found in both tumor epithelial cells and tumor stromal areas, CXCR6 was largely restricted to tumor stromal areas.

The major strength of our study is the large multicenter cohort and the long follow-up. Our results were internally validated in three different cohorts. Few previous studies have reported on the association between CXCL16, CXCR6, and clinicopathological features in radical prostatectomy specimens.

Consistent with others, we found a correlation between high expression of CXCL16 and PC,[19,22,25] and more

aggressive PC.[4–6] There have been conflicting reports regarding the role of CXCR6 in PC,[5,31] and whether CXCR6 is linked to PC prognosis. We found that high expression of CXCR6 in tumor stromal areas directly correlated to an increased risk of having BF, and a more aggressive cancer. Moreover, we found a high correlation between CXCL16 and $CD3^+$ T cells.

CXCL16 and CXCR6 are expressed in inflammation-associated cancers.[17,18] The mechanisms are thought to be a positive feedback loop between CXCL16, CXCR6, and the tumor microenvironment.[17–19] Precancerous or cancer cells secrete CXCL16, which contributes to CXCR6-mediated recruitment of leukocytes.[31,32] Darash-Yahana et al[17] found that CXCL16 and CXCR6 were expressed on $CD3^+$ T cells in the tumor microenvironment. Others have reported that CXCR6 is expressed on $CD4^+$ T cells, $CD8^+$ T cells, natural killer cells, and plasma cells,[4–7,11] whereas CXCL16 is expressed on tumor-associated macrophages, dendritic cells, and B cells.[8–10] We found a positive correlation between CXCL16 and $CD3^+$ T cells, but not B cells. We found no correlation between CXCR6 and $CD3^+$ T cells ($CD4^+$, $CD8^+$), or $CD20^+$ B cells, which is consistent with some previous studies,[17] but in contrast to others.[3,10] We have previously shown that infiltration of $CD8^+$ T cells in tumor stromal areas predicts an increased rate of BF in PC.[30] However, in this study, no correlation between $CD8^+$ T cells and CXCL16/CXCR6 was observed. Herein, we demonstrated a correlation between CXCL16 and $CD3^+$ T cells, suggesting that this chemokine may serve as a marker for a relationship between inflammation and PC.

Experimental studies have shown that CXCL16/CXCR6 induces cell proliferation and migration,[13,16,18,33] and that the ligand and its receptor are expressed in PC cell lines.[23,25] Herein, we evaluated the effects of transient knockdown of CXCL16 using siRNA on cell proliferation and migration in two PC cell lines (DU145 and PC3). By siRNA-mediated knockdown, nevertheless, we observed increased cell proliferation and migration. This may be explained in two ways: CXCL16 exists in a transmembrane form (TM-CXCL16) and S-CXCL16,[8,10] and tumor microenvironment plays an important role in the cross talk between tumor epithelial cells and tumor stromal cells (Figure 4).[17,31,32] S-CXCL16 induces immunocyte chemotaxis, whereas TM-CXCL16 mediates cell-cell adhesion by binding to CXCR6.[18,34] Loss of cell adhesion may, therefore, explain why CXCL16 knockdown leads to increased cell proliferation through the loss of contact inhibition. When decreased cell adhesion takes place, it might promote detachment of single cancer cells to migrate and establish elsewhere as metastatic foci and contribute to worse prognosis. Our immunohistochemistry findings showed that CXCL16 was located in both cell membrane and cytoplasm, indicating that CXCL16 was expressed in both the transmembrane and soluble form. Transmembrane CXCL16 acts like a cell adhesion molecule for cells expressing CXCR6, whereas S-CXCL16 induces the migration of CXCR6-expressing cancer cells and enhances the proliferation of these cells.[3,8,10,17] In the functional studies, we did not distinguish between TM-CXCL16 or S-CXCL16, and this may have influenced our findings. We can only speculate whether our results were mediated by TM-CXCL16, S-CXCL16, or both.

More important, the PC cell lines we studied are not in contact with any tumor microenvironment. In the absence of stroma, the cross talk between tumor cells and the microenvironment cannot take place. A study by Hojo et al[34] on colorectal cancer observed higher levels of $CD4^+$ and $CD8^+$ T cells in cancer tissue, with high TM-CXCL16 expression. TM-CXCL16 predicted good prognosis, whereas high serum-soluble CXCL16 was a signal of poor prognosis.[34] However, it still remains to uncover which forms of the CXCL16 are involved in PC aggressiveness, and how CXCL16 and CXCR6 contribute in prostate carcinogenesis.

Conclusion

CXCL16 and CXCR6 are highly expressed in aggressive prostatic tumors and are independent negative predictors for clinical failure-free survival. Our findings underline the influence of these chemokines in PC and strengthen the argument for immunological approaches in its treatment.

Acknowledgments

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