

Angiogenic biomarkers in prostate cancer

A study into the prognostic significance of angiogenesis related growth factor ligands and receptors and miR-205 in a cohort of Norwegian prostatectomy patients

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Abbreviations

AJCC	American Joint Committee on Cancer
BF	Biochemical failure
BFFS	Biochemical failure-free survival
BPH	Benign prostate hyperplasia
BRCA2	Breast cancer gene 2
CAPRA	Cancer of the Prostate Risk Assessment
CAPRA-S	Post-surgical CAPRA
CF	Clinical failure
CFFS	Clinical failure-free survival
CISH	Chromogenic <i>in-situ</i> hybridization
CT	Computer tomography
DRE	Digital rectal exam
EAU	European Association of Urology
EMT	Epithelial to mesenchymal transition
IHC	Immunohistochemistry
ISH	<i>In-situ</i> hybridization
ISUP	International Society of Urological Pathology
miR	micro-RNA
MRI	Magnetic resonance imaging
PC	Prostate cancer
PCD	Death of prostate cancer
PCDFS	Death of prostate cancer free survival
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PSA	Prostate specific antigen
RALP	Robot-assisted laparoscopic prostatectomy
TKI	Tyrosine kinase inhibitors
TMA	Tissue microarray
TNM	Tumor Node Metastasis - Classification of malignant tumors
TRUS	Transrectal ultrasound
TUR-P	Transurethral resection of the prostate
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization
WTS	Whole tissue sections

Acknowledgements

For nearly six years, from August 2011 to June 2017, I was employed as a resident doctor at the Department of Urology at the University Hospital of Northern Norway. The work was basically divided into 50 % research in form of a Ph.D project and 50 % clinical work as a doctor specializing in general surgery, starting on a path to training as an academic urologist. Presented in this thesis is a summary of the results from my work as a Ph.D. student from that period.

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List of papers

PAPER I (published)

Nordby Y, Andersen S, Richardsen E, Ness N, Al-Saad S, Melbø-Jørgensen C, Patel HRH, Dønnem T, Busund L-T, Bremnes RM

Stromal expression of VEGF-A and VEGFR-2 in prostate tissue is associated with biochemical and clinical recurrence after radical prostatectomy¹

Prostate **75**, 1682-1693, doi:10.1002/pros.23048 [doi] (2015)

PAPER II (published)

Nordby Y, Richardsen E, Rakaee M, Ness N, Dønnem T, Patel HRH, Busund L-T, Bremnes RM, Andersen S

High expression of PDGFR- β in prostate cancer stroma is independently associated with clinical and biochemical prostate cancer recurrence²

Scientific reports **7**, 43378, doi:10.1038/srep43378 (2017)

PAPER III (published)

Nordby Y, Richardsen E, Ness N, Dønnem T, Patel HRH, Busund L-T, Bremnes RM, Andersen S

High miR-205 expression in normal epithelium is associated with biochemical failure - an argument for epithelial crosstalk in prostate cancer?³

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Abstract

Background: Prostate cancer is a heterogeneous disease, ranging from indolent and slow growing, to aggressive and lethal. Due to insufficient prognostic tools, there is a significant overtreatment of patients with harmless disease. Differentiating which patients benefit from radical treatments remains a huge challenge, and there is an urgent need to find new and better prognostic tools that may aid in treatment allocation. Angiogenesis is a well-studied hallmark of cancer. Without sufficient blood flow, the malignant tumor cannot grow to a self-sustaining tumor of significant size. The prognostic impacts of selected angiogenic biomarkers in our cohort were explored, with the aim to uncover novel biomarkers to contribute to the knowledge of prostate cancer aggressiveness for improved risk stratification. In addition, a deeper understanding of the molecular characteristics and functional pathways for different stages in prostate cancer is essential in order to succeed in development of novel therapeutic agents for targeted therapy.

Methods: Patient data and prostatectomy specimens from 535 Norwegian patients treated for prostate cancer with curative intent were collected. Using tissue microarrays with several cores from predefined areas of the specimens, staining with immunohistochemistry and *in-situ* hybridization were performed for renowned angiogenic biomarkers. Correlations between expression levels of biomarkers and clinicopathological variables were explored, event-free survival times were calculated according to expression levels, and to assess their independent prognostic impact, the markers were entered into multivariate analyses.

Main results: High expression of vascular endothelial growth factor receptor 2 (VEGFR-2) in either stroma or epithelium was independently associated with a higher incidence of prostate cancer relapse (HR = 4.56, p = 0.038). A high combined expression of either VEGFR-2, vascular endothelial growth factor A (VEGF-A) or both in stroma was independently associated with a higher incidence of biochemical failure (HR = 1.77, p = 0.011). High stromal expression of platelet derived growth factor receptor β (PDGFR- β) was independently associated with clinical relapse (HR = 2.17, p = 0.010) and biochemical failure (HR = 1.58, p = 0.002). High expression of microRNA (miR)-205 in normal epithelium was independently associated with biochemical relapse (HR = 1.64, p = 0.003). When assessing expression of miR-205, we found novel indications of a crosstalk between normal epithelium and tumor epithelium, suggesting an anti-cancerogenous function of normal epithelium.

Conclusions: We found positive associations between prostate cancer relapse and several biomarkers associated with angiogenesis. Especially PDGFR- β seems promising as a new biomarker as it outperforms traditional established prognosticators. A common finding for all three papers was that the prognostic impact of angiogenic markers was mostly found in tissue *outside* the actual tumor epithelium, highlighting the complex interplay in prostate cancer tumors. This may have implications for tissue sampling for research and in a therapeutic perspective, these pathways may also be attractive targets for targeted therapy.

1 Introduction

Next to lung cancer, prostate cancer (PC) is the second most commonly diagnosed cancer worldwide⁴. It is the fifth leading cause of death from cancer in men, with an estimated 307,000 deaths worldwide representing 6.6% of the total male cancer mortality. However, in developed countries, PC is the most common malignancy in men, constituting 29 % of all new cancers diagnosed in Norwegian men in 2015, as well as being the second most common cause of cancer death^{5,6}. While most PCs are indolent and non-aggressive, some develop into a metastasized and deadly form of PC. Most PCs are diagnosed at an early stage, and due to insufficient prognostic tools, failure to predict which cases lead to an advanced form has led to a significant overtreatment⁷⁻⁹. After availability of radical treatments, treatment allocation has been to the concept of “better safe than sorry” as many patients and clinicians usually prefer to err on the safe side not to miss the window of cure for a cancer that could later be lethal. Most men with localized PC are hence treated and left with permanent post-therapeutic sequelae and side-effects¹⁰.

There is an urgent need for better prognostic tools to aid decisions in which patients to offer curative treatment. The use of a wide variety of biomarkers are utilized for a variety of different cancers with PC being a major exception due to lack of prospective validation¹¹. Biomarkers may function as predictors of disease outcome (prognostic markers) and/or to aid selection of patients for different therapies (predictive markers).

1.1 Prostate cancer

1.1.1 The prostate – functions and anatomy

The prostate (from Ancient Greek: “protector”, “guardian”, “one who stands before”) is an exocrine gland found only in males¹². It secretes the milky white fluid that constitutes about 30 % of semen. Most of the fluid is produced by the seminal vesicles located just behind the prostate, and the rest of the semen consists of spermatozoa. To prolong the lifespan of sperm, the alkalinity of the prostate ejaculate helps neutralize the acidity of the vaginal tract. The prostate is located below the bladder and in front of the rectum, and its posterior regions are palpable in a digital rectal exam. The gland increases in size during puberty, and attains its

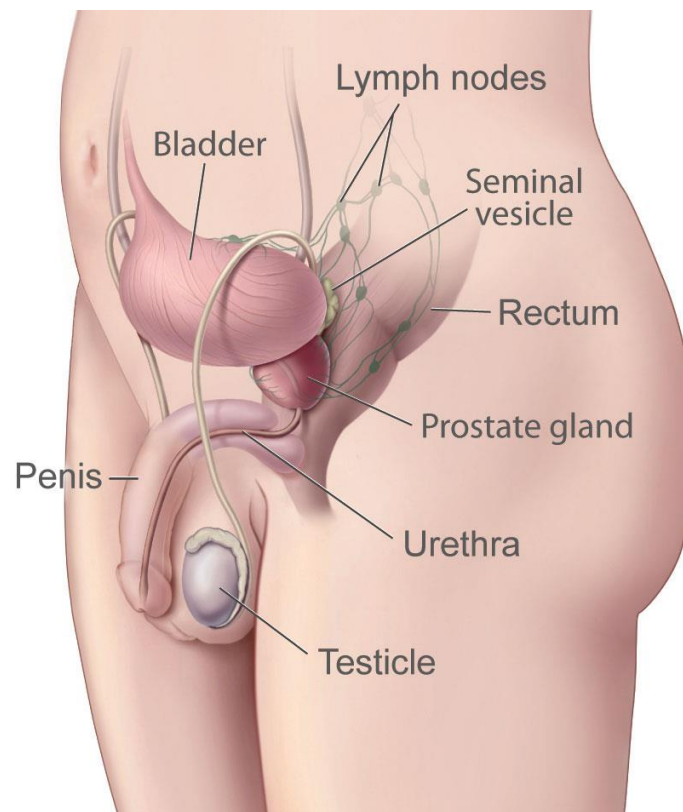


Figure 1. Illustration of the prostate location and anatomy. *The prostate can be palpated in a digital rectal exam. Reprinted with permission from www.cancer.gov.*

full size of a walnut during the early twenties and remains stable thereafter. Sometimes after the age of 40 the cells in the prostate gland undergo multiplication and cause the gland to further enlarge. For adult males, the mean weight of a normal prostate range from 7 – 16 grams, and is related to body mass index¹³.

The prostate is dependent of male hormones (androgens) to function properly, where the testosterone metabolite dihydrotestosterone (DHT) predominantly regulates the prostate.

The prostate may, like all other organs, be subject to different diseases. Inflammation of the prostate gland, prostatitis, may be caused by bacterial infections or by other non-bacterial inflammations like male chronic pelvic pain syndrome. Benign prostatic hyperplastic (BPH) is common among older men, and many of its symptoms are shared with those of PC, including increased urination hesitancy or frequency of urination due to enlargement of the prostate. A growing prostate can cause obstruction of the prostatic urethra, leading to difficulties in urination and may result in urine retention. Medical treatment of BPH consists mainly of $\alpha 1$ -receptor blockers that relaxes the muscle fibers in the prostate and urethra, and 5α -reductase

inhibitors (antiandrogen) that shrinks the prostate and hence reduces pressure on the urethra, allowing for easier passage of urine. The most common surgical treatment for BPH is a transurethral resection of the prostate (TUR-P), where obstructive prostatic tissue is resected to allow better flow of urine. In extreme cases, a surgical removal of the prostate (ex. Millins open prostatectomy in form of enucleation of adenoma) is needed. An estimated 50% of men have histologic evidence of BPH by the age of 50. Although prostate specific antigen (PSA) levels may be elevated in men affected by BPH because of increased organ volume and inflammation due to urinary tract infections, BPH does not lead to cancer or increase the risk of cancer^{14,15}.

As BPH and PC share many symptoms, there is a need to differentiate benign from malign disease for men with symptoms of BPH or PC.

1.1.2 Risk factors and causes

The chance of having PC rises rapidly after the age of 50, where 6 in 10 cases of PC are found in men older than 65¹⁶. Race/ethnicity is also a risk factor, where African-American men are more than twice as likely to die of PC as white men and generally have a more lethal course of disease, while PC occurs less often in Asian and Latino men compared to white men¹⁷. While PC is less common in Asia, Africa, Central and South America, it is more common in North America, Northwestern Europe, Australia and on the Caribbean Islands. Family history is a risk factor, where having a father or brother with PC more than doubles the risk for developing PC^{18,19}. The risk is much higher for men with several affected relatives, particularly for relatives with PC in young age. Some studies have found that inflammation in the prostate may contribute to PC. Smoking and obesity, however, has not been shown to increase the risk of PC.

Exact etiology of PC are unknown, but on a basic level, PC is caused by DNA changes in normal PC tissue. 5 to 10 % of PCs are hereditary cancers, where some inherited mutated genes linked to hereditary PC includes mutations of MSH2 and MLH1 (Lynch syndrome / hereditary non-polyposis colorectal cancer) or mutations of BRCA2 (more commonly known for breast cancer in women) amongst others. However, most gene mutations related to PC seem to be acquired mutations (somatic) developed during a man's life rather than being inherited (germline), and does not pass on to offspring^{20,21}.

Regarding prevention of PC, risk factors such as age, race and family history cannot be prevented²². Although the effects of body weight, physical activity and diet on PC risk are not clear, a healthy diet, being physically active and staying at a healthy weight might lower the risk^{23,24}. Some drugs might help reduce the risk of PC, including the 5 α -reductase inhibitors finasteride and dutasteride, more commonly used for treatment of BPH. 5 α -reductase inhibitors might have the potential for preventing or delaying the development of PC (for Gleason 6 cancers only), but has the potential small increased risk of high-grade PC²⁵⁻²⁷. Some research suggests that aspirin daily might lower the risk of PC²⁸. However, it is not clear whether the benefits of these drugs outweighs the risks for most men, and more studies are needed. According to the Norwegian national guidelines for diagnosis, treatment and follow-up of PC, there is currently no basis for general recommendations on chemoprophylaxis to prevent PC, whereas the EAU guidelines state that no definitive recommendation can be provided for specific preventive or dietary measures to reduce the risk of developing PC²⁹.

1.1.3 Epidemiology

5118 new cases of PC were diagnosed in Norway in 2016, which accounted for almost one third of all cancer cases in men⁶. Based on today's cancer incidence in Norway, approximately every eighth man (13.6 % in 2011-2014) will be diagnosed with PC before the age of 75 in Norway (lifetime risk in the absence of competing causes of death). However, considerably fewer men die of PC every year. 1045 men died of PC in 2015 in Norway, accounting for about 19 % of all cancer deaths in men. The lifetime risk before death of PC before the age of 75 is approx. 1.4% (about one in 70 men).

A decrease in mortality of PC in Norway (Figure 2) and in many other countries from the beginning of the 1990s and beyond has been observed, although the cause of decline is uncertain³⁰⁻³². New cases of PC increase in all age groups, but PC is primarily the old men's disease. Almost half of all cases occur among men over 74 years, and the proportion of the population of this age group is increasing. As a result of higher overall life expectancy, the incidence of PC has more than quadrupled in 2015 compared to the 1950s. As a result of a marked increase in the use of PSA as a diagnostic tool combined with the fact that more men are diagnosed with PC each year than the number of people who die from the disease, the

number of men living with PC and who need some sort of follow-up has doubled from approximately 22 000 to 44 000 in a ten year span from 2005 to 2015⁶.

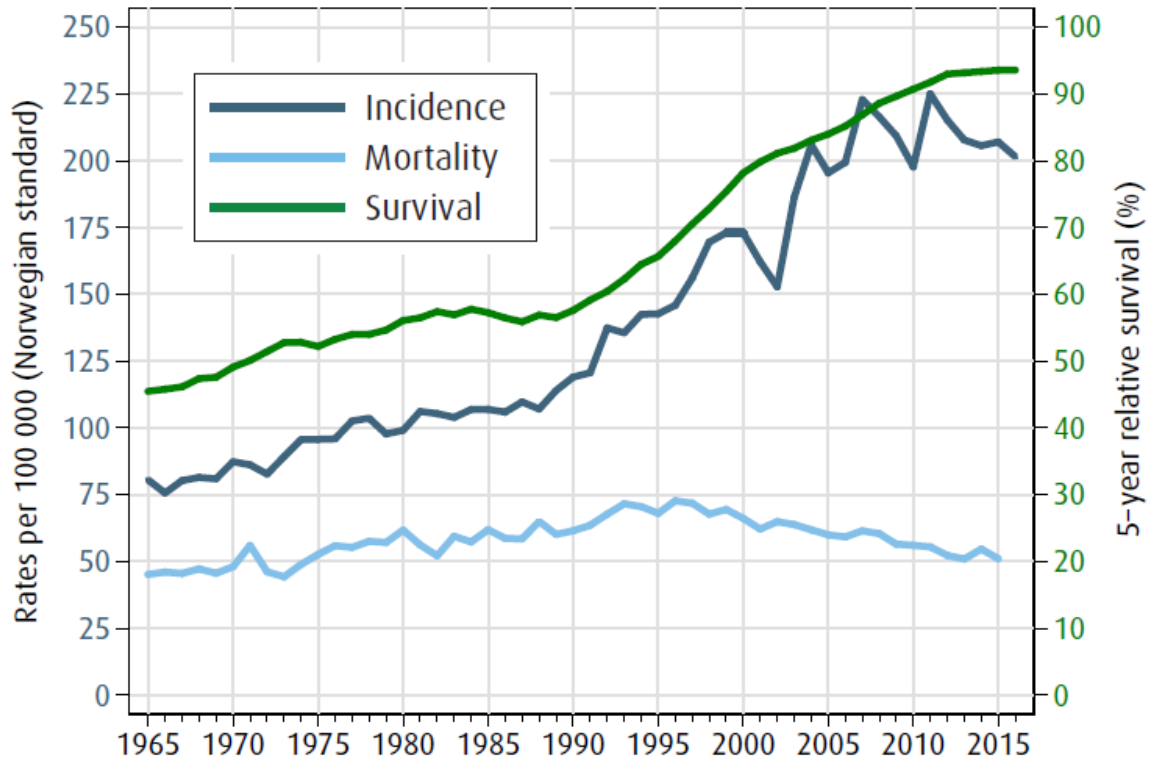


Figure 2. Trends in incidence and mortality rates and 5-year relative survival proportions. *Although incidence and survival has increased rapidly from the 1990, mortality has declined. However, 5 year survival is a poor measurement of quality of PC treatment, as PC often has a long preclinical fase. Mortality is, on the contrary, not affected by this type of bias. Reprinted with permission the Cancer Registry of Norway.*

1.1.4 Histopathology

The prostate is divided into four histological regions: The peripheral zone, central zone, transition zone and anterior fibromuscular stroma, where the peripheral zone comprises approximately 70 % of the gland³³. BPH usually develops in the transition zone, whereas 75% of PC develops in the peripheral zone³⁴. The prostate gland is surrounded by the prostatic “capsule” where the neurovascular bundles outside of the capsule are responsible for erectile function. Given its proximity to the distal rectum, the posterior aspect of the prostate is most prominent on digital rectal exam (DRE).

PC is classified according to the World Health Organization classification of tumors. More than 95 % of the PC are adenocarcinomas, arising from the prostate epithelial cells. Less than 5% of prostate carcinomas are variants of adenocarcinoma which often have very poor prognosis (ductal carcinoma, mucosal carcinoma, signet cell carcinoma and small cell carcinoma).

1.1.5 Clinical presentation of PC

Most patients with PC are asymptomatic, particularly in the early stages of disease. Only a minor part of men with urinary disorders seek medical help³⁵. Two independent studies have found that concern for PC, rather than the degree of urinary disorders, determines whether one is seeking a doctor^{36,37}. As such, many patients are still asymptomatic at the time of diagnosis, as patient requested screenings by PSA measurements with the following biopsies are commonly performed. Detection of elevated PSA in general health controls in healthy men has been an increasing cause of referral to an urologist, and as of 2016, elevated PSA was the main reason for a diagnosis of PC in Norway³⁸.

Local progression may result in lower urinary tract obstruction associated with BPH, and symptoms such as weak stream, hesitancy, urgency, frequency, nocturia, straining, intermittency, incomplete emptying, and various degrees of incontinence may occur. PC tumors may bleed, presenting hematuria. Approximately 90 % of all new incidents in the United States have been reported as localized or regional PCs³⁹. Although not as common, around 7 % of PC patients in Norway are initially diagnosed with metastatic PC, where bone pain may be the presenting symptom⁶.

1.1.6 Diagnosis, staging and prognosis

The primary assessment of PC stage is usually done with DRE, measurement of PSA, and for men with higher risk disease skeletal scintigraphy, optionally supplemented by computer tomography (CT) or MRI. Local T-staging is based on the findings on DRE and optionally MRI. N-stage is of utmost importance for patients considered for curative treatment, where the most accurate method for determination of N-stage is an operative extended lymphadenectomy. M-stage is best assessed with MRI or skeletal scintigraphy due to its predominant metastatic spread to skeletal tissue. The TNM classification for adenocarcinomas of the prostate is presented in Table 3.

1.1.6.1 PSA discussion

The measurements of PSA levels revolutionized the ability to diagnose PC at an early stage⁴⁰. In addition, a serum PSA level before treatment of more than 100 ng/ml has been found to be strongest indicator of metastatic disease with a positive predictive value of 100 % in a prospective study of 60 patients with newly diagnosed PC⁴¹. However, mass screening of the asymptomatic patient with PSA measurements remains a controversial subject, and argumentations are complex. Briefly summarized, PSA screening for PC has not shown a gain in overall survival although the European Randomized Study of Screening for Prostate Cancer (ERSPC) study has shown that PSA screening reduced the risk of death from PC^{42,43}. The benefit of reduced mortality of PC must be weighed against potential adverse effects of overdiagnosis and complications of treatment such as urinary leakage, erection failure and dysfunction of the intestine. It is estimated that 23 - 42 % of PCs detected as a result of PSA screening has been overdiagnosed^{44,45}. This is based on estimated expected life of the diagnosis and estimated chance that the disease will produce clinical symptoms from PC without PSA screening. In conclusion, PSA testing of potentially healthy men for PC will probably lead to reduced mortality, but at the cost of over diagnosis and overtreatment of tumors that may not give symptoms throughout the man's life. An American study found that the proportion of men who wanted to undergo PSA testing was halved after being given extensive information⁴⁶. In conjunction with the recommendations of the European Association of Urology (EAU) and US Preventive Services Task Force, population-based screening is not recommended and this has been implemented in the Norwegian national guidelines for diagnosis, treatment and follow-up of PC. There is still no level 1 evidence that PSA mass screening is cost-effective in reducing PC mortality⁴⁷. Exceptions should be made for middle-aged men with family disposition for PC or other high risk groups such as patients with known mutations in BRCA2. PSA tests can be offered to the patient on an individual basis, but should not be taken without the patient being fully informed of the pros and cons.

The PC diagnosis is most often determined by the appearance of cancer tissue in biopsies from the prostate or from TUR-P tissue, while some patients are primarily diagnosed with metastasis and highly elevated PSA. The general practitioners tools for detection of PC are PSA and DRE. In conjunction with the patient, the practitioner decides whether to refer the patient to a specialist for biopsy following a thorough examination, evaluation of current and prior serum PSA values and DRE findings. The need for prostate biopsy is based on PSA

level and/or suspicious DRE, while age, potential comorbidity, and therapeutic consequences should also be considered⁴⁸. Limited PSA elevation alone should not prompt immediate biopsy. PSA level should be verified after a few weeks using the same assay under standardized conditions. However, DRE is limited because it only allows the posterior surface of the gland to be digitally examined, and the examination is highly subjective with poor inter-examination reliability. On the other hand, some types of PC only mildly increase PSA levels, justifying the DRE as an important examination. In asymptomatic men with moderately elevated PSA and with life expectancy below 10 years and negative DRE, one can be reluctant regarding biopsies.

Table 1. Frequency of PC according to low PSA levels in 2950 patients⁴⁹.

PSA level (ng/ml)	Risk of PC
0.0 – 0.5	6.6 %
0.6 – 1.0	10.1 %
1.1 – 2.0	17.0 %
2.1 – 3.0	23.0 %
3.1 – 4.0	26.9 %

The tissue sampling is usually done under local anesthetics guided by a transrectal ultrasound (TRUS) probe⁴⁸. The majority of tissue sampling is in the peripheral zone, with the number of biopsies ranging from eight to 16. In the case of repeated benign biopsies and persistent elevated PSA levels, a multiparametric magnetic resonance imaging (MRI) of the prostate and targeted biopsies can be considered. While CT and TRUL are not recommended for local staging for any risk group, for intermediate-risk patients ISUP Grade 3 or high-risk localized for locally advanced PC patients, MRI is recommended.

1.1.6.2 Tissue aggressiveness

Grading refers to the microscopic description of cancer aggressiveness. The biopsies are graded according to the Gleason Scoring system⁵⁰. The Gleason grading system consists of histopathological patterns graded from well-differentiated grade 1 to poorly-differentiated grade 5, where grade 1 and 2 are not considered to be cancer and are rarely used. The two most dominant Gleason grades are summed to obtain a Gleason Score. More than 40 years after Gleason's grading score was invented by Douglas Gleason, this is still one of the most important prognostic factors in PC.

Recent years, the International Society of Urological Pathology (ISUP) have recommended using their new grading system based on a consensus conference held in 2014, where morphological criteria were clarified including updated definitions of Gleason pattern⁵¹. The ISUP grading system is based upon the Gleason's grading system, and has the benefit of facilitating patient communication. ISUP grades and the corresponding Gleason grades are presented in Table 2. The corresponding histologic patterns for prostatic adenocarcinoma are presented in Figure 3.

Table 2. ISUP grades and the corresponding Gleason grades

ISUP grade	Gleason grade
Grade 1	≤ 6
Grade 2	3 + 4
Grade 3	4 + 3
Grade 4	8
Grade 5	9 – 10

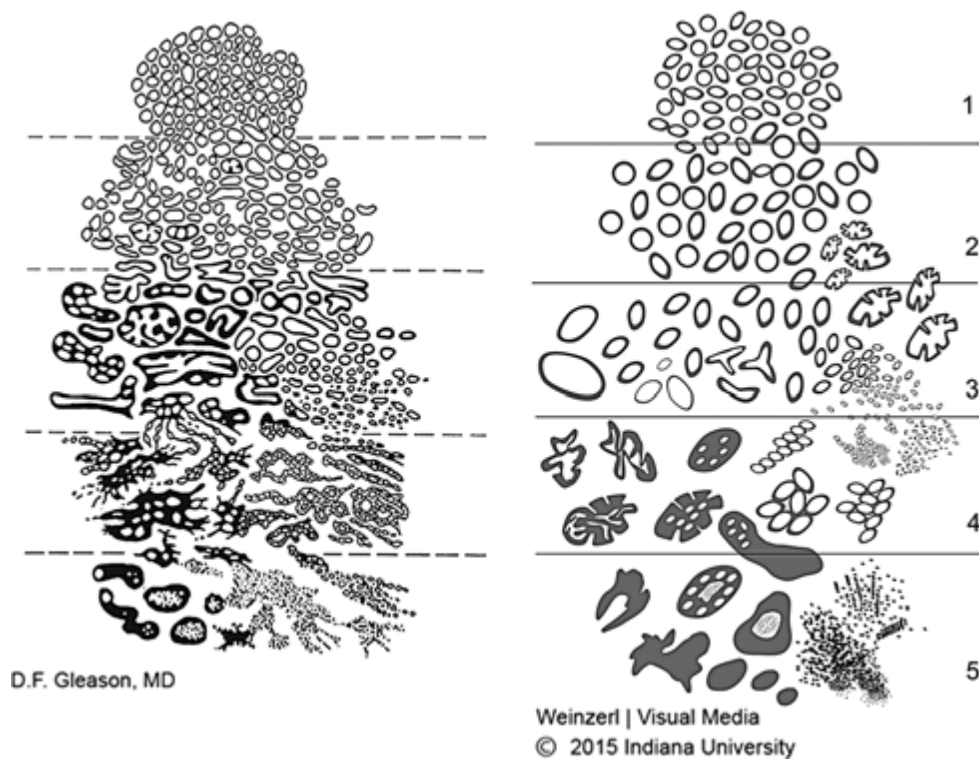


Figure 3. Prostatic adenocarcinoma histologic patterns. *Original (left) and 2015 Modified ISUP Gleason schematic diagrams. Reprinted with permission from Wolters Kluwer Health, Inc.*

1.1.6.3 TNM and risk groups

Risk stratification to separate PC patients with a potential curative disease and patients in a palliative setting is imperative regarding choice of therapy. The division of these groups is not clear, but several risk stratification tools mostly based on PSA, Gleason Score and T stage are used to help risk stratification⁵²⁻⁵⁴. The EAU Guidelines of 2017 uses the 2017 TNM classification of PC and the EAU risk group classification, which is essentially based on D'Amico's classification system for PC⁴⁸. The EAU risk group for biochemical recurrence of localized and locally advanced PC is presented in Table 4.

Table 3. Tumor Node Metastasis (TNM) classification of prostate cancer adenocarcinomas of 2016⁵⁵

Primary Tumor (T)		
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 (for example, because of elevated PSA)
T2	Tumor that is palpable and confined within the prostate	
	T2a	Tumor involves one-half of one lobe or less
	T2b	Tumor involves more than one-half of one lobe but not both lobes
	T2c	Tumor involves both lobes
T3	Tumor extends through the prostatic capsule	
	T3a	Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement
	T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall	
Regional Lymph Nodes (N)		
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastasis	
N1	Regional lymph node metastasis	
Distant Metastasis (M)		
M0	No distant metastasis	
M1	Distant metastasis	
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s)

Table 4. EAU risk groups for biochemical recurrence of localized and locally advanced prostate cancer. *GS=Gleason score; ISUP=International Society for Urological Pathology; PSA=prostate-specific antigen.*

Low-risk	Intermediate-risk	High-risk	
PSA < 10 ng/mL and GS < 7 (ISUP grade 1) and cT1-2a	PSA 10-20 ng/mL or GS 7 (ISUP grade 2/3) or cT2b	PSA > 20 ng/mL or GS > 7 (ISUP grade 4/5) or cT2c	any PSA any GS (Any ISUP) cT3-4 or cN+
Localized			Locally advanced

1.1.6.4 The CAPRA-S score

Cancer of the Prostate Risk Assessment (CAPRA) score is a validated score developed to predict PC recurrence based on the pretreatment data preoperative PSA, Gleason score, clinical T stage, biopsy results and age⁵⁶. The post-surgical score (CAPRA-S) is a tool for prediction of outcomes after radical prostatectomy, and its points are assigned according to Table 5⁵⁷.

Table 5. The CAPRA-S score. Points are assigned for each variable: Up to 3 for prostate specific antigen (PSA) level in ng/ml, up to 3 for pathologic Gleason score, 2 each for positive surgical margin (SM) and seminal vesicle invasion (SVI), and 1 each for extracapsular extension (ECE) and lymph node invasion (LNI). Points are summed to yield the CAPRA-S score.

Variable	Level	Points
PSA	0 – 6	0
	6.01 – 10	1
	10.01 – 20	2
	>20	3
Surgical margin	Negative	0
	Positive	2
Seminal vesicle invasion	No	0
	Yes	2
Gleason	2 – 6	0
	3 + 4	1
	4 + 3	2
	8 – 10	3
Extracapsular extension	No	0
	Yes	1
Lymph node invasion	No	0
	Yes	1

1.1.7 Management of curative prostate cancer

PC is a heterogeneous disease, where some patients may have a dramatic and aggressive development while other patients will stay asymptomatic without treatment⁵⁸. The choices of treatment include active surveillance, watchful waiting, and radical treatment with prostatectomy or radiation. Brachytherapy or combinations of radical treatments, with or without the supplement of hormone treatment, may also be options, but is less used.

1.1.7.1 Active surveillance

Active surveillance aims to avoid unnecessary treatment in curable men with low risk PC by treating only those showing signs of progression⁵⁹. This may also be discussed for subgroups of patients with intermediate risk PC⁶⁰. These must be followed with frequent PSA controls and also rebiopsy after one year or at PSA rise. If the PC shows signs of progression, radical treatment may be offered if the patients are healthy enough to undergo treatment.

1.1.7.2 Watchful waiting

Watchful waiting is a deferred or symptom-guided treatment⁵⁹. It refers to conservative management, until the development of local or systemic progression with (imminent) disease-related complaints. Patients are then treated according to their symptoms, in order to maintain quality of life. In contrast to active surveillance, which aims for a curative intent, watchful waiting is intended as a palliative strategy.

Table 6. Definitions of active surveillance and watchful waiting

	Active surveillance	Watchful waiting
Treatment intent	Curative	Palliative
Follow-up	Predefined schedule	Patient-specific
Assessment/markers used	DRE, PSA, re-biopsy, mpMRI	Not predefined
Life expectancy	> 10 years	< 10 years
Aim	Minimize treatment-related toxicity without compromising survival	Minimize treatment-related toxicity
Comments	Low-risk patients	Can apply to patients at all stages

1.1.7.3 Radical prostatectomy

Open radical prostatectomy, with surgical removal of the prostate gland and usually the seminal vesicles, is usually performed with a retropubic access through a midline incision, although a perineal access is an option. In 1947, Millin carried out retropubic prostatectomy, followed by Memmelaar with the first radical retropubic prostatectomy in 1949⁶¹⁻⁶³. However, it was not until the 70s and 80s when Walsh reported his techniques of anatomical and physiological radical retropubic prostatectomy (RRP), that complication rates plummeted⁶⁴.

In recent years, the minimal invasive techniques of laparoscopy and robot-assisted laparoscopic prostatectomy (RALP) has gained popularity with robot-assisted techniques being the most frequently used⁶⁵. The development of these techniques has resulted in shorter hospitalization and faster rehabilitation compared to open prostatectomy, but it is unclear whether the minimal invasive techniques result in better oncological long-term results and less late complications than open surgical techniques.

Regarding complications of radical prostatectomy, perioperative mortality is very low (0-1.5%)⁶⁶. Major perioperative complications are also rare, but the most common include urinary fistulas, damage to the rectum, major bleeding, deep venous thrombosis and pulmonary embolism. The main problem of surgery are the long-term side effects in form of persistent severe stress incontinence (0-15%) and erectile dysfunction (29-100%).

Evidence supporting radical prostatectomy as treatment for early PC is based on the well-documented Swedish study by Bill-Axelsson et al., where 695 men with early PC were randomly assigned to watchful waiting or radical prostatectomy from 1989 to 1999^{7,67,68}. Radical prostatectomy was associated with a reduction in the rate of PC deaths. However, results from recent studies such as the PIVOT trial found no significant differences in mortality between men undergoing surgery for localized PC versus those treated with observation only^{69,70}. Persisting uncertainty regarding non-fatal health outcomes and long-term mortality underpins the need for better prognostic markers.

Radical prostatectomy is a well-established and recommended treatment for patients with cT1-cT2 stage, yielding life expectancy of more than 10 years. For cT3 cancers, radical prostatectomy may be performed in selected cases with supplementary regional lymph node

dissection. Supplement of adjuvant or salvage radiation and/or hormonal therapy may be needed.

In patients with pT3 tumors and/or positive surgical margin after prostatectomy, adjuvant radiation therapy reduces the risk of distant metastasis and leads to better overall survival. An alternative strategy is to provide salvage radiation therapy in case of biochemical or local recurrence. Observational studies have shown that up to 50% of these patients achieve disease control if salvage radiation therapy is initiated in early biochemical recurrence⁷¹

1.1.7.4 External beam radiotherapy

External beam radiotherapy (EBRT) is another option of curative treatment, and functions by damaging the DNA of malignant cells leading to cell death. Shaped radiation beams are aimed from several angles of exposure to intersect at the tumor, focusing a much larger radiation dose at the malignant target rather than in the surrounding healthy tissue. Intensity-modulated radiotherapy (IMRT), with or without image-guided radiotherapy (IGRT), is considered the best standard for external beam radiotherapy (EBRT)⁴⁸. Some of the side effects (temporary or chronic) from EBRT of the prostate with margins includes radiation proctitis, radiation cystitis, urine incontinence, urethral stricture, erectile dysfunction, impotence, fatigue and lymphedema⁷².

Several RCTs have shown that dose escalation (range 74-80 Gy) has a significant positive impact on relapse-free five-year survival. The best evidence of an OS benefit for patients with intermediate-risk or high-risk PC, but not with low-risk PC, comes from a retrospective analysis of the U.S. National Cancer Database covering a total of 42 481 patients⁷³.

The PROTECT study compared active monitoring, radical prostatectomy and external-beam radiotherapy for treatment of clinically localized PC following a PSA testing. At a median of 10 years, PC-specific mortality showed no significant difference among treatments. Surgery and radiotherapy were associated with a lower incidence of disease progression and metastases than was active monitoring⁷⁴.

1.1.8 After radical treatment

Postoperative disease activity can largely be monitored using PSA measurements. The PSA level is expected to be unmeasurable within six weeks after radical prostatectomy. Increasing PSA indicates disease progression in most cases, where 61 % progress after a rise to 0.2 and

74 % rise further after a measured value of 0.4⁷⁵. It should be noted that PSA production of the most undifferentiated tumors may be low^{76,77}. Rapid PSA doubling time may indicate remote metastasis, while a slow-rising PSA concentration with longer doubling time often indicates local recurrence or residual disease.

By evaluation of the post-operative histology, consideration should be given to the need for adjuvant radiation therapy. A PSA increase or new symptoms, which give suspicion of recurrence, should lead to further investigation. The general consensus of biochemical recurrence, called biochemical failure (BF), after radical prostatectomy is defined as two PSA values ≥ 0.2 ng/ml for 2 different measurements at least one week apart. 27-53 % of patients treated in curative intent will experience a rise in PSA within 10 years of ended treatment.

Patients with indications of local PC recurrence, called clinical failure (CF), following radical prostatectomy and a life expectancy of at least 10 years, should be offered salvage radiation therapy to the prostatic bed. Adjuvant hormone therapy for salvage radiotherapy is still controversial as addition of hormone therapy has only reduced biochemical relapse and clinical progression and not surely reduced mortality. For patients with a histological verified local recurrence after radical radiation treatment and a life expectancy of at least 10 years may be referred to one of the few highly specialized centers where salvage prostatectomy may be performed. However, the procedure is considered technically challenging and there is a considerable risk of urine incontinence, although salvage prostatectomy may yield cancer control.

1.1.8.1 Metastasized prostate cancer

For over 50 years, primary androgen deprivation therapy (ADT) has been the standard care of metastatic PC, and represents one of the most effective systemic palliative treatments known for solid tumors⁷⁸. There is no evidence for, or against, a specific type of ADT, whether bilateral orchiectomy (surgical castration), an LHRH analogue or antagonist. The exception is for patients with impending spinal cord compression for whom either a surgical castration or an LHRH antagonist are the preferred options. For patients whose first presentation is M1 disease, castration combined with chemotherapy (doxorubicin) is offered for patients who are fit enough for chemotherapy.

Patients with castrate serum testosterone < 50 mg/dL and either PSA progression or radiological progression are defined with a castration-resistant PC (CRPC)⁴⁸. For patients with non-metastatic CRPC, frequent post-treatment PSA surveillance has resulted in earlier detection of progression. One-third of men with a rising PSA will develop bone metastases within two years, but there are no available studies suggesting a benefit for immediate treatment⁷⁹. It is not recommended to treat patients for non-metastatic CRPC outside of a clinical trial⁴⁸.

First-line treatment of patients with metastatic CRPC (mCRPC) comprises of continuing ADT in conjunction with different agents such as abiraterone (androgen receptor antagonist), enzalutamide (androgen receptor antagonist) and docetaxel (chemotherapy) + prednisone as life prolonging agents. A symptomatic approach such as treatment for painful bone metastases are treated early on with palliative measures such as RT and adequate use of analgesics.

1.2 Tumor microenvironment

The tumor microenvironment (TME) is a complex of extracellular matrix (ECM) and a number of cell types such as fibroblasts, vascular cells, immune cells and soluble factors such as cytokines and chemokines^{80,81}. By secreting signal molecules such as growth factors or by cell-to-cell interaction, tumor cells can modulate their stromal environment⁸². A dynamic and mutualistic interaction between tumor cells and the surrounding stroma may promote the initiation, progression, metastasis and chemoresistance of solid tumors. Unlike tumor cells, stromal cell types within the TME are genetically stable and thus represent an attractive therapeutic target with reduced risk of resistance and tumor recurrence⁸². The stromal microenvironment is an active and important biological component, as there is continuous and bilateral molecular crosstalk between normal cells and tumor cells of the stromal compartment. Thus, minor changes in one compartment may cause dramatic alterations in the whole system⁸³. The TME exerts an important role in tumor progression by modulating the metabolism and fostering tumor growth, progression, and metastasis to distant sites. Pro- and anti-angiogenic factors are not exclusively produced by tumor cells, but also by stromal cells of the TME⁸⁴.

1.3 Angiogenesis in prostate cancer

1.3.1 Hallmarks of cancer and angiogenesis

As proposed by Hanahan and Weinberg in their acknowledged publication from 2000, the hallmarks of cancer comprise six biological properties a tumor must acquire in order to develop into cancer⁸⁵. These include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. In their updated review from 2011, more emerging hallmarks were added⁸⁶.

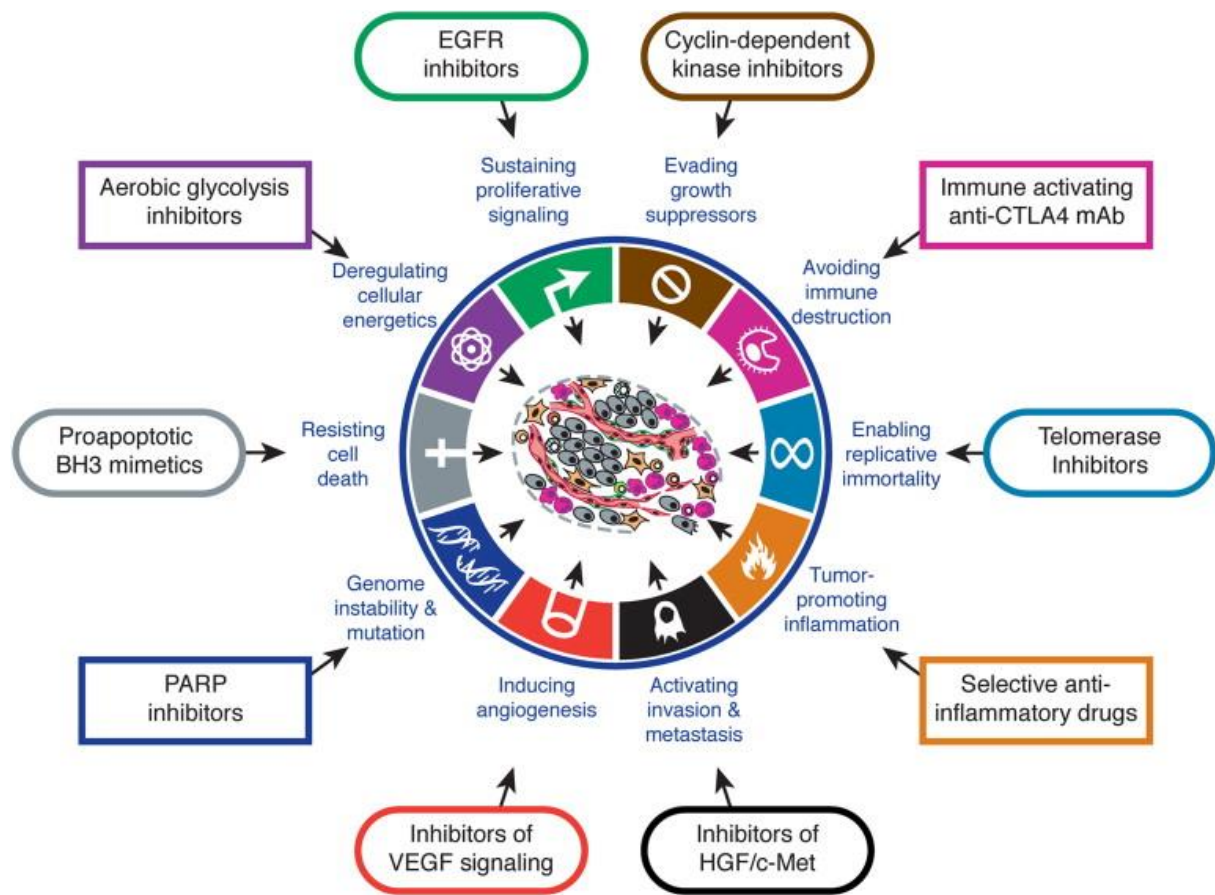


Figure 4. The hallmarks of cancer. *Biological capabilities acquired during the multistep development of human tumors and potential drugs for targeted therapies. Reprinted with permission from Elsevier⁸⁶.*

The hypothesis that tumor growth is angiogenesis dependent was first stated by Folkman in the early seventies⁸⁷. Today, much evidence underlines tumor dependence on angiogenesis in order to progress⁸⁸. The stroma is a hostile metabolic microenvironment characterized by hypoxia and acidosis. Tumor outgrowth is usually restricted to no more than 1–2 mm in diameter during the avascular phase of tumor development. In this phase, the tumor is nourished by diffusion of oxygen and nutrients provided by nearby blood vessels^{89,90}. Avascular tumors can reach a dormant steady state, where tumor cell proliferation and death are in balance and where a net increase in tumor volume does not occur. In some non-malignant diseases, such as lobular capillary hemangioma or keloid formation, angiogenesis is self-limited. In the case of tumor angiogenesis, once begun, it continues indefinitely until the entire tumor is eradicated or the host dies⁹¹.

Tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by the process of angiogenesis, addresses these needs. During tumor progression, an angiogenic switch is activated and remains on, causing normally quiescent vasculature to continually sprout new vessels that help sustain expanding neoplastic growths⁹².

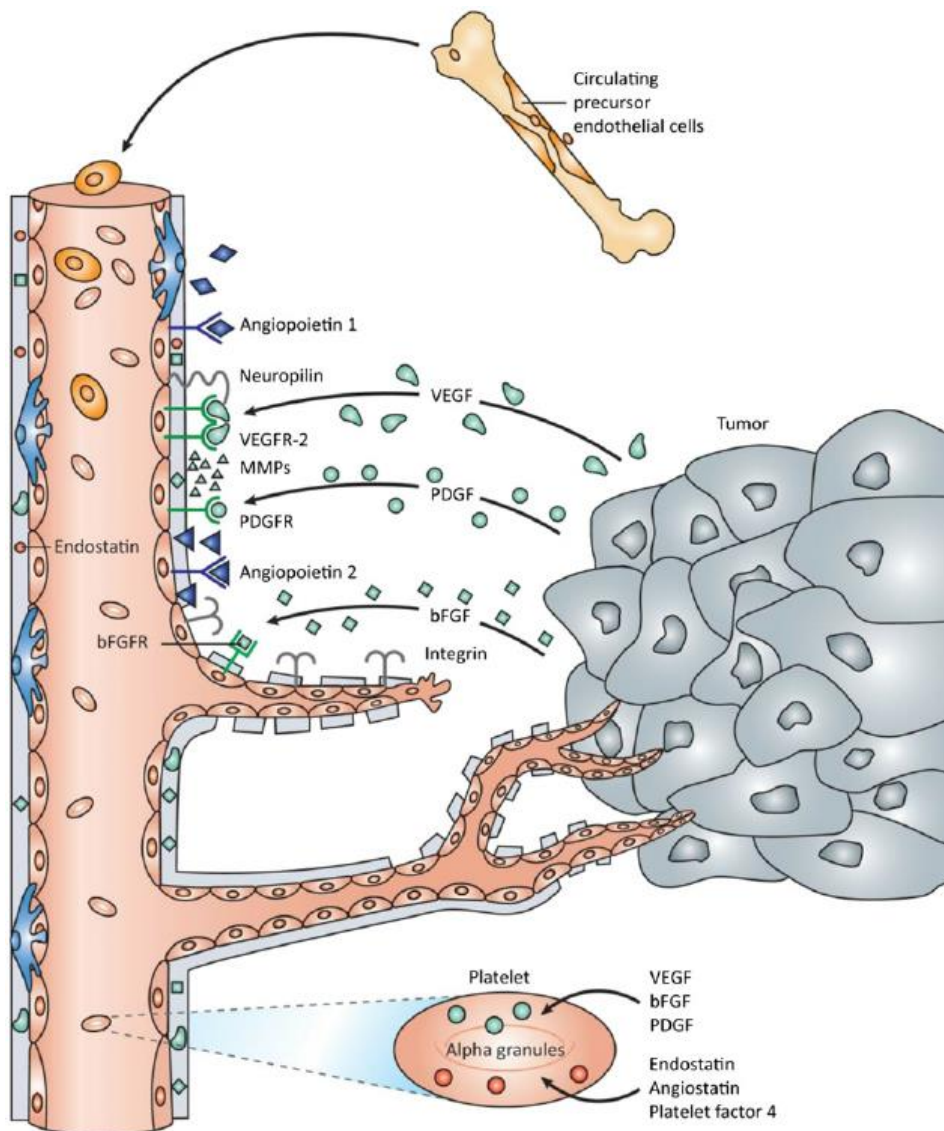


Figure 5. Tumor angiogenesis mechanisms. Soluble angiogenic factors (e.g., VEGF, PDGF, FGF) are secreted from the tumor and surrounding cells to induce and regulate key steps in angiogenesis. Reprinted with permission from Nature Reviews⁹³.

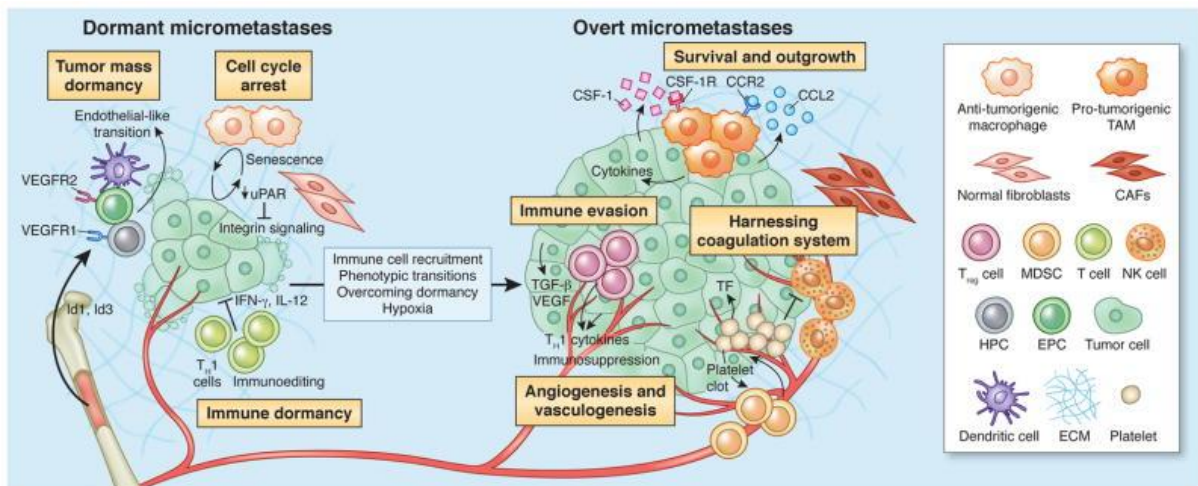


Figure 6. Overcoming tumor dormancy, and initiation of secondary outgrowth in metastatic niches. *Dormant micrometastases are held in check by several mechanisms. Tumor mass dormancy is when proliferation is balanced by apoptosis, owing to a lack of vasculature and limited supply of nutrients and oxygen. Multiple cell types contribute to the re-establishment of vasculature at the secondary site, including hematopoietic and endothelial progenitor cells (HPCs; EPCs) expressing VEGF receptors, and dendritic cell precursors which can differentiate into an endothelial-like state. Tumor cells can also exist in a state of cellular dormancy, whereby proliferation is arrested in G0. Last, tumor cells can enter immune-induced dormancy whereby immunogenic cells are cleared, and cells that are able to survive enter a state of equilibrium. Immune suppressor cells are recruited to tumors in response to this process, and contribute to the establishment of an immunosuppressive state within secondary tissues. Once micrometastases overcome dormancy, they become receptive to signals and cell types within their microenvironment to further support their expansion. Platelets, and components of the coagulation system, such as tissue factor (TF), are also important mediators of metastatic outgrowth, as they interfere with the ability of NK cells to destroy micrometastases, and support clot formation, which in turn causes the recruitment of MDSCs. Reprinted with permission from Springer Nature⁸².*

Although angiogenesis as endothelial sprouting is regarded as a hallmark of cancer development, several studies have shown primary tumors and metastases to be able to progress without angiogenesis^{86,94}. The concept of vascular co-option implies that tumors can obtain blood supply by overtaking the native vasculature and let tumor cells migrate along the

vessels of the host organ. Intussusception (or splitting angiogenesis) implies the mechanism where preexisting vessels split into daughter vessels. These relatively new considerations suggest that the vasculature of human tumors is more comprehensive than previously regarded, and have been introduced as a potential explanation of antiangiogenic drug resistance.

Angiogenesis is also an important process in the needed development of tumor vasculature for PC progression, being critical to tumorigenicity and metastasis⁹⁵. PC has the ability to produce MMPs, VEGF, TGF β , and cyclooxygenase 2 (COX-2), as well as several endogenous inhibitors of angiogenesis such as angiostatin, endostatin, PSA, TSP1, interleukin 8, and interferons. Bidirectional cellular interactions between neoplastic PC cells and stromal cells are mandatory for local tumor progression and metastasis, and influence the tumor microvascular architecture⁹⁶.

At present, PC grade is evaluated by histological Gleason or ISUP score, as a measure of cell differentiation, widely accepted as a pathological indicator correlating with stage and metastatic potential. However, its grading based on prostate biopsies remains a poor predictor of pathological outcome⁹⁷. Taking into account the essential role of angiogenesis in PC development, angiogenesis is suggested to lead to further improvements in PC diagnosis and staging⁹⁸.

Meta-analyses have shown that high VEGF levels in PC cells are associated with poor prognosis⁹⁹. Moreover, VEGF levels in plasma and urine of patients with mCRPC are independent predictors of overall survival^{100,101}.

However, the significance of angiogenesis in PC still remains controversial⁹⁸. While there are currently no markers for net angiogenic activity of PC, which may help investigators to design specific anti-angiogenic treatment strategies, it is reasonable to assume that the quantification of various aspects of tumor vasculature may provide an indication of angiogenic activity.

The research interest in angiogenesis and PC has declined recent years, probably due to the setback of many of the angiogenesis inhibitors. A Pubmed search (angiogenesis and prostate cancer) reveals that the peak interest was around 2013 with a subsequent sharp decline.

1.4 Anti-angiogenic therapy

Tumor angiogenesis factors are secreted by tumor cells, and stimulate the formation of new blood vessels in and around tumors. Essential among these are the vascular endothelial growth factors (VEGF) and their receptors (VEGFRs)¹⁰². Ligand binding to VEGFR-2 sets in motion a number of intracellular signalling pathways that lead to multiple mechanisms inducing sprouting neoangiogenesis, including cell division, migration, vascular permeability, and promotion of cell survival^{103,104}.

The four types of approved VEGF pathway–targeting drugs in oncology are:

- I. Monoclonal neutralizing antibodies to the circulating VEGF ligand
- II. Monoclonal VEGFR-2 blocking antibodies
- III. Oral small-molecule TKIs (tyrosine kinase inhibitors) that primarily act intracellularly to block the catalytic signaling function of VEGFR-2
- IV. Antibody-like decoy trap agent that binds strongly to VEGF and placental growth factor.

Inhibition of angiogenic pathways has proven an effective strategy for the treatment of several common solid tumors like renal cell carcinoma¹⁰⁵. However, a role in the management of PC is yet to be defined. As a histological measure of tumor angiogenesis, microvessel density (MVD) has been shown to correlate with Gleason score and predict cancer progression^{106,107}. Whether neovascularization is a primary pathogenic event or a response to the hypoxic microenvironment of a growing tumor, this observation provides a rationale for investigating anti-angiogenic therapy as a treatment strategy for PC.

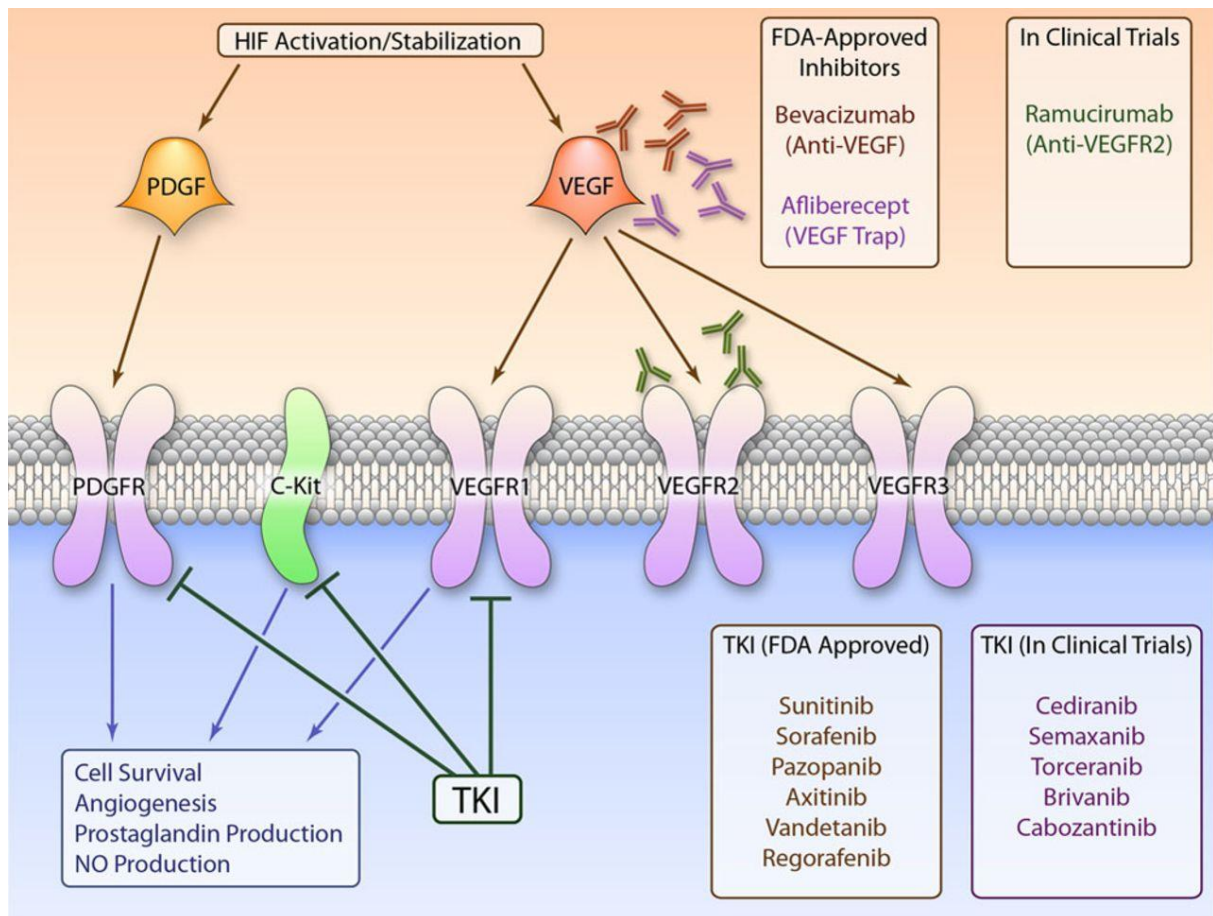


Figure 7. Angiogenesis inhibitors (VEGF signaling pathway (VSP) inhibitors) being tested in human cancer trials. Although these agents are being referred to as VSP inhibitors, drugs such as sunitinib inhibit many other receptor tyrosine kinases, allowing them to be approved for the treatment of other cancers while, at the same time, creating the possibility for a wide range of off-target toxicities. Abbreviations: FDA = Food and Drug Administration; HIF = hypoxia-inducible factor. Reprinted with permission from Wolters Kluwer Health, Inc¹⁰⁸.

Examples of trials with angiogenesis inhibitors are many with some of the largest/recent presented here:

- A recent phase 2 trial employed the VEGF-A inhibitor bevacizumab in combination with short-term androgen deprivation therapy (ADT) in patients with hormone-sensitive recurrent PC¹⁰⁹. Results showed that patients treated with bevacizumab in addition to ADT had a significant improvement in relapse-free survival.

- A phase 3 trial investigated a potential clinical benefit in addition of bevacizumab to standard docetaxel and prednisone therapy in patients with mCRPC¹¹⁰. An improvement in progression-free survival for patients treated in the docetaxel + prednisone/bevacizumab arm was demonstrated. However, combined treatment was associated with more common grade 3 or greater treatment-related toxicity compared to the control group. Furthermore, the incidence of treatment-related deaths in the docetaxel + prednisone/bevacizumab arm was greater. In addition, this trial also failed to show an improvement of overall survival for patients treated additionally with bevacizumab compared to docetaxel + prednisolone monotherapy.
- A phase 3 study investigated the impact of the VEGF-R inhibitor aflibercept¹¹¹. Aflibercept in combination with docetaxel and prednisone given as first-line chemotherapy for men with metastatic castrate-resistant PC resulted in no improvement in overall survival and added toxicity compared with placebo. Docetaxel plus prednisone remains the standard treatment for such men who need first-line chemotherapy.
- In a phase II non-randomized discontinuation trial for patients with mCRPC, the dual VEGFR-2/MET targeting TKI cabozantinib yielded impressive palliation of bone pain and verified reduced bone metastases¹¹². Although encouraging symptomatic relief, results from the phase 3 trial COMET-1 did not show improvement in overall survival¹¹³. However, cabozantinib had some activity in improving bone scan response, radiographic progression-free survival, symptomatic skeletal events circulation tumor cells conversions and bone biomarkers, but not PSA outcomes.

There are still a few antiangiogenesis studies in progress, identified through Clinicaltrials.gov:

- Tivozanib (oral VEGF-R1/R2/R3 TKI) + enzalutamide in advanced PC
- Cabozantinib (VEGFR-2/MET targeting TKI) + docetaxel and prednisone for advanced PC
- Trebananib (Ang1 and Ang2 inhibitor) and abiraterone for advanced PC
- Docetaxel, Thalidomide (antiangiogenic activity by unknown mechanism), prednisone and bevacizumab to treat metastatic PC

1.5 Angiogenic markers covered in this thesis

1.5.1 Paper I - Vascular endothelial growth factors (VEGFs)

VEGF-A is a central regulator of tumor induced angiogenesis and is critical for tumor growth and metastasis^{103,114}. Overexpression of VEGF-A has been associated with tumor progression and poor prognosis in several cancers¹¹⁵⁻¹¹⁸. The vascular endothelial growth factor receptor-2 (VEGFR-2) plays an important role in angiogenesis, endothelial cell proliferation, migration, and survival. Anti-VEGF therapy is approved for clinical use. For example, bevacizumab is a recombinant humanized monoclonal antibody that blocks angiogenesis by inhibiting VEGF-A, and is approved in Norway for treatment of metastatic colorectal cancer, metastatic breast cancer, non-small celled lung cancer, advanced or metastatic kidney cancer, epithelial ovarian cancer or primary peritoneal cancer, and cervix cancer¹¹⁹. For PC, the few previous clinicopathological studies regarding the VEGFs have not yielded consistent results, and their stromal expressions had hardly been previously assessed¹²⁰⁻¹²⁷. Due to the lack of stromal assessment and conflicting results, we systematically investigated both tumor and stromal expressions and associations with clinical outcome for VEGF-A, VEGF-C and their respective receptors VEGFR-2 and VEGFR-3.

1.5.2 Paper II - Platelet derived growth factors (PDGFs)

PDGFs and their receptors (PDGFRs) have emerged as key regulators of cell growth and division, and mediate significant impact on malignant cells and the tumor microenvironment¹²⁸. As potent mitogens for cells of mesenchymal origin, the PDGFs are important regulatory proteins for fibroblasts, smooth muscle cells and glial cells. They are involved in embryonic development, cell proliferation, cell migration and stimulate wound healing in the adult. In particular, these factors play a significant role in angiogenesis in which mutational activation or upregulation of the PDGFs or PDGFRs may lead to uncontrolled blood vessel formation and cancer¹²⁹⁻¹³⁵. Their specific role has been implied in stabilizing recently formed vasculature through pericyte recruiting and lining of pericytes around blood vessels^{136,137}.

From a therapeutic perspective, important drugs are inhibiting PDGF action¹³⁸. As an example, imatinib (PDGFR TKI) is approved in Norway for treatment of some forms of chronic myelogenous leukemia, acute lymphoblastic leukemia and eosinophil leukemia,

metastatic malignant gastrointestinal tumors and dermatofibrosarcoma protuberans¹¹⁹. However, inhibition of PDGFs in PC has so far been unsuccessful^{139,140}.

In PC, PDGF-D seems to be involved in osteoclastic differentiation and establishment of bone metastasis¹⁴¹. High levels of PDGFR- β in PC tumor stroma and non-malignant prostate tissue have been associated with shorter cancer specific survival for PC patients¹⁴². However, PDGFR- β and both ligands' expressions for PC patients with a localized disease and its prognostic value post radical treatment have not been examined previously. Thus, we systematically investigated both tumor and stromal expressions and associations with clinical outcome for PDGF-B, PDGF-D and their corresponding receptor PDGFR- β .

1.5.3 Paper III - Micro-RNA 205

The micro-RNAs (miRs) are small noncoding RNA molecules that function as regulators of protein expressions and are involved in numerous cellular processes, from normal functioning of cells to dysregulations associated with disease¹⁴³⁻¹⁴⁶. miR-205 acts either as an oncogene or as a tumor suppressor by facilitating or repressing tumor initiation and proliferation depending on type of cancer and stage¹⁴⁷. miR-205 plays a crucial role in angiogenesis and targets VEGF-A and fibroblast growth factor-2 (FGF2), leading to decreased activity of PI3K/AKT signaling pathway^{148,149}.

There has been a major effort to target these noncoding RNAs therapeutically the last years, and a few miRs have entered the preclinical and clinical trials¹⁵⁰.

While studies have demonstrated that miR-205 in general is involved in both normal development and cancer, the prognostic role of miR-205 in PC is not unambiguously clarified in PC¹⁵¹⁻¹⁵⁸. miR-205 is found to be downregulated in PC tissue compared to benign tissues, and loss of miR-205 seems to be associated with invasive phenotype and poor clinical outcome. miR-205 has a tumor suppressive function by inhibiting the transition from epithelial to mesenchymal tissue (EMT), cell migration and invasion in the prostate. However, high miR-205 expression has also been shown to correlate to adverse outcome in PC patients. As miR-205 was consistently downregulated for a selected group of 14 patients with rapid biochemical failure in a screening array of 1435 miRs in presumed tumor tissue in our 2014 study¹⁵⁹, we set to investigate the prognostic role of miR-205 in our cohort using *in situ* hybridization on tissue microarray blocks.

2 Aim of thesis

The aim of the work included in this thesis was to investigate associations of important angiogenic biomarkers with patient outcome after curative treatment with radical prostatectomy.

More specifically, the aims of this thesis are:

- Establishment of a prostatectomy cohort and collecting relevant patient data for the database.
- By immunohistochemistry (IHC) or *in-situ* hybridization (ISH), investigate the *in-situ* expressions of important angiogenic biomarkers in both normal and tumor epithelium and surrounding stroma.
- Examine the prognostic impact by estimating correlations between biomarker expression and patient outcome.
- Assess the prognostic impact of the biomarkers in question in relation to other established prognostic factors.

3 Materials and methods

3.1 Patient cohort

All patients (n = 671) treated with radical prostatectomy with curative intent for adenocarcinoma in the prostate from 1995 up to 2006 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). The patients' formalin-fixed paraffin-embedded prostatectomy specimens were collected from the respective hospitals Pathology Departments and their biobanks. Of these, 136 patients were excluded due to

- (i) previous non-superficial cancer within five years of PC diagnosis (n = 4)
- (ii) radiotherapy to the pelvis prior to surgery (n = 1)
- (iii) inadequate paraffin-embedded tissue blocks (n = 130)
- (iv) lack of follow-up data (n = 1)

None of the patients had received pre-operative hormonal therapy, leaving a total of 535 eligible patients.

During 2011-2012, the patient database was formed by collecting relevant data from the patients' medical journals. To gain access to the local hospitals electronic patient journals, Yngve Nordby, Sigve Andersen and Nora Ness did travels to the hospitals of Trondheim, Levanger and Bodø. To ensure even longer follow-up, Nordby contacted the patients' local hospitals and follow-up centers to retrieve additional data after the patients no longer were followed by their operating centre. We used SPSS to record patient data, and the database was de-identified after all relevant data was retrieved to protect the patients' privacy. The identified database was stored on a secure server at the University Hospital of North Norway, only accessible to a few key persons, and all analyses was performed using the de-identified version of the database. Andersen further updated the database with renewed follow-up data in December 2015.

We collected relevant patient data from medical journals involving:

- (i) demographical data
- (ii) age at surgery

- (iii) center of surgery
- (iv) previous medical history
- (v) retropubic or perineal surgery
- (vi) preoperative serum PSA level measured immediately before surgery.
- (vii) postoperative serum PSA levels
- (viii) postoperative therapy
 - a. Radiotherapy
 - b. Hormonal therapy
 - c. Chemotherapy

We collected outcome data until the last follow-up date (December 01, 2015) or until patients' death.

3.1.1 Endpoints and patient cohort discussion

The following endpoints were defined and recorded in the database:

Biochemical failure (BF) – defined as postoperative raise in PSA levels ≥ 0.4 ng/ml in at least two consecutive postoperative blood samples according to Stephenson et al.¹⁶⁰, or intervention with salvage therapy due to rising PSA.

Clinical failure (CF) – defined as local symptomatic recurrence in the prostate bed or metastasis verified by radiology.

Prostate cancer specific death (PCD) – defined as death caused by PC stated in the patients' journal.

Although international consensus define biochemical failure as two postoperative consecutive PSA rises > 0.2 ng/mL, others have argued for a higher cut-off of 0.4 ng/mL for patients at high risk of clinical progression. Hence, we chose to set cutoff at 0.4 ng/mL to ensure a more clinically relevant cutoff. By using 0.4 ng/mL, the endpoint becomes more specific for patients at high risk of clinical progression, and hence increases PSAs usage as a surrogate marker for clinical useful endpoints.

To avoid bias in patient selection, patients with previous non-superficial cancer within five years of PC diagnosis (n = 4) or radiotherapy to the pelvis prior to surgery (n = 1) were excluded due to risk of bias of other cancer relapse or plausible introductions of changes in

tumor microenvironment not caused by PC. Skin cancers were not regarded to influence cancer specific mortality.

The advantages of the retrospective cohort study design compared to the prospective are many: The studies may be conducted on a smaller scale; require less time to complete; diseased people have already been identified so retrospective studies are helpful in addressing diseases of low incidence; generally less expensive than prospective studies partly due to already occurred exposure and outcome. Among the disadvantages of the retrospective cohort study design are the introduction of significant biases that may affect the selection of controls and in the recall of past exposure to risk factors. For example, cause of death can be biased by subjective interpretation when collecting medical information. Hence, in our analyses of PCD, the patients included have stated death by PC in their medical journal and was reviewed by us.

Variations over time between the surgical centers regarding patient selection for treatment and changes in histological grading protocol represents an important confounder. To avoid this, analyses of patients outcomes stratified upon clinicopathological variables were calculated, while all tissues were reevaluated for an updated histologic assessment.

3.2 Tissues and histopathological evaluations

All prostatectomy samples were reevaluated by two experienced pathologists, Elin Richardsen and Lill-Tove Busund, and classified according to the updated WHO (World Health Organization) guidelines^{50,161}. Gleason score was converted to Grade Group according to the consensus of the International Society of Urological Pathology (ISUP) for an updated nomenclature⁵¹. The following histological properties of the samples were evaluated and recorded in the database:

- (i) Gleason score / Grade Group
- (ii) TNM classification
- (iii) Tumor size
- (iv) Perineural infiltration
- (v) Lymphovascular infiltration
- (vi) Surgical margin
 - a. Positive apical margin

b. Positive non-apical margin

3.2.1 Tissue microarray

Tissue microarray (TMAs) technology were used in order to obtain high-throughput histological analyses¹⁶². An experienced pathologists, Elin Richardsen, identified the most representative areas of cancer epithelial cells and adjacent stroma in the patients' prostatectomy specimens. Each area was biopsied with at least two 0.6 mm cores and arranged in TMAs for large-scale analysis. Multiple 4 µm TMA sections were cut with a Micron microtome (HM355S) and stained by specific antibodies for immunohistochemical analysis (IHC) or *in situ* hybridization.

TMA has become a standard tool for tissue-based research. Most histological and molecular techniques available for whole tissue section (WTS) can be applied to TMA sections, including IHC, ISH and immunofluorescence methods¹⁶³. Its advantages comprise a high throughput volume, saving valuable tissue, time and reagents. A large number of specimen might be rapidly analyzed, and reliable allocation of clinical data to the tissue specimen is permitted by the uniform shape and highly organized array pattern¹⁶⁴.

The standardization of tissue staining ensures elimination of staining variations between all cases and control tissues as these are stained under identical experimental conditions. Compared to WTS, the observers may directly compare staining intensities between multiple tumors on each TMA slide, improving the semiquantitative assessments¹⁶⁵.

The TMA technique is not without challenges. Preanalytic factors such as ischemic time, fixation type and fixation time may vary, and analytical factors such as intra- and interobserver differences during scoring may also affect the performance characteristics of the TMA analyses^{166,167}.

A major concern about TMA has been the issue of tumor heterogeneity. It may not be clear whether the small cores are representative for donor tissue or not, and what size and number of cores are optimal. It is important to sample the most representative areas of each tumor. Using larger tissue cores, or multiple cores, from the same donor tissue to enhance the representativity has been suggested¹⁶⁸. Studies have validated the reliability of the TMA method by applying TMA technique to reproduce previously well-established associations between molecular alterations and clinical outcome^{165,166}.

Finally, the TMA technology is used as a population-level research tool as it is not intended for making individual case decisions¹⁶⁹.

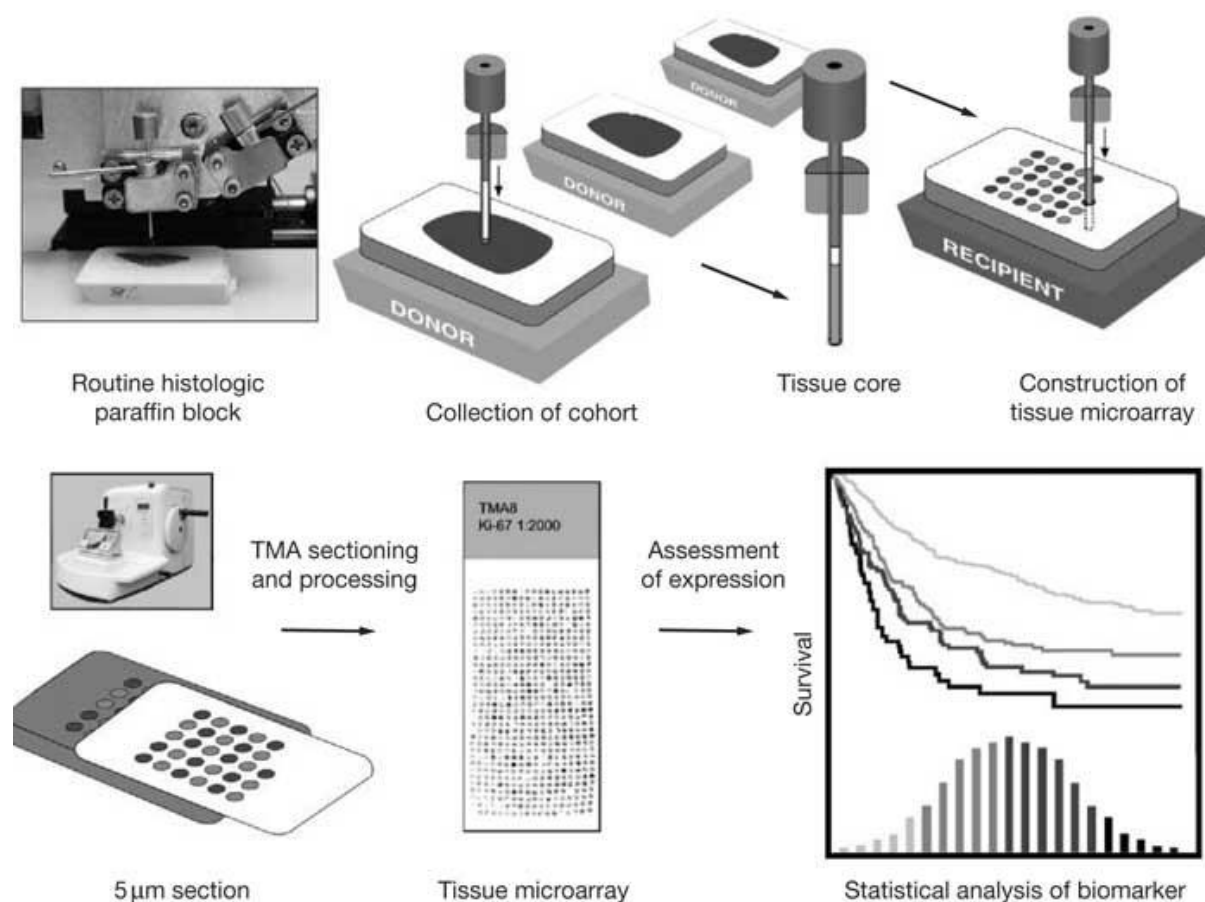


Figure 8. Construction and use of tissue microarrays for biomarker identification.

Paraffin-embedded, formalin-fixed tissues are collected. Representative areas from each donor tumor block are punched into cores of 0.6 mm in diameter and arrayed into a recipient TMA block. Sections of the resultant tissue microarray are cut and transferred to glass slides for processing of biomarker status by immunohistochemistry or in situ hybridization techniques. Biomarker expressions are assessed and the data linked to clinical information. The graph shows a histogram and Kaplan–Meier survival plot from expression analysis of quartiles. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Clinical Oncology, ©2004¹⁷⁰.

3.3 Immunohistochemistry

Regarding the VEGFs and PDGFs, detecting selective proteins (antigens) in the TMAs was performed by using immunohistochemical analyses (IHC). IHC is widely used in basic research to image the distribution and localization of biomarkers, as well as in routine diagnostics of abnormal cells such as those found in cancerous tumors. The general steps in an indirect IHC preparation are

- (i) Application of a specific primary antibody: Binds to the antigen of interest (the biomarker to be detected).
- (ii) Application of a secondary antibody: Binds to the primary antibody.
- (iii) Application of a chromogen: Visualizes the antibody-antigen complex.

The direct method is a one-step staining method where a labeled antibody reacts with the antigen of interest. This method is simple and rapid, but sensitivity is lower due to little signal amplification and is hence used less frequently. In our material, the indirect method was used as it is more sensitive than direct detection strategies because of signal amplification due to the binding of several secondary antibodies to each primary antibody if the secondary antibody is conjugated to the fluorescent or enzyme reporter. The antibodies used in this thesis are summarized in Table 7.

3.3.1 Advantages and challenges of IHC

One of the main advantage of using IHC is that it allows the *in-situ* assessment of the distribution and localization of specific cellular components in different compartments of tissues. It is a relatively inexpensive method, and is established in most laboratories. In addition, it can be performed on archived tissue, and is stained manually or in a high-throughput automated process.

Factors that may affect tissue antigenicity, such as variability in tissue collection, fixation variability, tissue processing and antigen retrieval method may be challenges to the IHC method. Detection of antigens may vary according to choice of antibody (clone, type), variability in staining, application of secondary antibody and antigen detection methods¹⁷¹. A thorough optimization of all steps of the IHC process are mandatory to achieve reproducible and reliable IHC results.

3.3.2 Antibodies

The selection of antibodies is a critical step in performing a reliable IHC analysis¹⁷². By immunizing animals with antigen, polyclonal antibodies are produced by different B-cell clones. Polyclonal antibodies bind to various epitopes on an antigen, and have slightly different specificities and affinities. In contrast, monoclonal antibodies are generated by a single B-cell clone from a single animal, resulting in a homogenously directed antibody against a single epitope.

As polyclonal antibodies can recognize multiple epitopes on the target molecule, they are more robust reagents in terms of less influence of the results caused by variations in the pre-analytic processing of specimens. They have a higher probability for detection, and false negative IHC results are less common. However, there is an increased risk of cross-reactivity with other proteins, producing false positive results.

Due to the lack of variability of polyclonal antibodies, monoclonal antibodies have high lot-to-lot consistency and are more specific. However, they are more likely to work in only one set of conditions, and due to weaker signals more prone to false negative IHC results¹⁷².

The main challenges for IHC antibody selection lies in avoiding issues such as non-specific antibodies, strong background staining and weak target antigen staining. The antigen must be identifiable in tissues with both low and high expression. The fact that it is impossible to show that the antibody staining corresponds to the protein of interest, makes reliable IHC results dependent on methods controls and an acceptance of what is considered appropriate staining according to medical literature¹⁷³. To evaluate antibody specificity, the use of positive and negative control tissues is essential.

Regarding antibodies chosen in this thesis, we antibodies that had been successfully used by others by reviewing previously published studies including the antibody of interest. Further, the manufacturers' information and online databases were consulted. To verify specificity of the antibodies, multiple different tumors and normal tissues was stained as control tissues according to Table 7.

3.3.3 IHC procedures in this thesis

VEGF-A, VEGF-C and VEGFR-2 were stained manually with the Dako EnVision detection kit (Dako, Glostrup, Denmark). In brief, after drying overnight, the slides were deparaffinized in xylene and dehydrated with alcohols. Endogenous peroxidase activity was inhibited by incubating the sections in 1.5% H₂O₂ for 10 min, and antigen retrieval for primary antibodies 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 450W. Nonspecific binding sites were blocked by 10% normal goat serum for 30 min. The sections were incubated with primary antibodies overnight, and then incubated with the secondary antibody (Dako Real Envision/HRP, K5007) for 30 min. Sections were counterstained with hematoxylin and mounted for examination with light microscope.

VEGFR-3 was stained using the automated Bench-Mark XT stainer (Ventana Medical Systems, Inc., Tucson, AZ). Epitope retrieval was accomplished on the automated stainer with CC1 solution (Ventana Medical Systems, Inc., Tucson, AZ). The VEGFR-3 antibody was incubated for 32 min and was detected by using the iVIEW DAB Detection Kit (Ventana Medical Systems, Inc., Tucson, AZ). Finally, to visualize the nuclei, the slides were counterstained with Ventana Hematoxylin II reagent for 8 min, followed by a Bluing reagent for 4 min.

IHC analysis for the PDGFs and their receptor was performed on Discovery-Ultra immunostainer (Ventana Medical Systems, Tucson, AZ). Slides were deparaffinized in three 8-minute cycles. On-board CC1 antigen retrieval incubated for PDGF-D, PDGF-B and PDGFR- β , 32, 24 and 48 minutes respectively. Discovery inhibitor (Cat #760–4840) blocked endogenous peroxidase for 8 minutes. The primary antibodies were loaded and the slides were incubated for 32 minutes at 37 °C. Antibody dilution buffer (Ventana, #ADB250) were used for all antibodies except for PDGF-D where Discovery antibody diluent (Ventana, #760–108) was utilized. Slides were developed using corresponding secondary antibody for 20 minutes, followed by 12 minutes HRP amplification for PDGFR- β and were detected using ChromoMap DAB (Cat #760–159). Finally, the slides were counterstained to detect the nuclei with Ventana Hematoxylin II reagent for 32 minutes, followed by a Bluing reagent for 8 minutes and dehydrated, cleared and mounted as in our routine processing.

3.4 *In-situ* hybridization

Similar to IHC for detection of proteins, *in-situ* hybridization (ISH) can be used for detection of the presence of specific micro-RNAs (miRs). A labeled complementary RNA strand (probe) localizes a specific RNA sequence in a portion or section of tissue (*in-situ*).

Around year 2000, chromogenic *in-situ* hybridization (CISH) was developed and combines the chromogenic signal detection of IHC with ISH. CISH and IHC are different as IHC measures protein expression whereas CISH measures RNA amplification.

The advantage of ISH is that it enables determination of how the distribution of specific nucleic acids is related to protein products of the target gene and their relation with cellular structures using immunohistochemistry¹⁷⁴. CISH enables examination of gene amplification, gene deletion, chromosomal translocations, and chromosomal number. The major advantages of CISH includes that signals are stable over time, low cost, assessed using a light microscope, and permanent staining.

Similar to the IHC method, reliable ISH results requires precise optimization, for each tissue examined and each probe used. A disadvantage of applying ISH techniques is the difficulty in identifying targets with low DNA and RNA copies.

3.4.1 ISH procedure in this thesis

The complete procedure is presented in Paper III. In brief, CISH was performed on Ventana Discovery Ultra instrument. Buffers and detection reagents were purchased from Roche and Labeled locked nucleic acid (LNA) modified probes from Exiqon. Positive and negative controls were used. Positive and negative tissue controls for miR-205 was a stained TMA multi-organ block comprised of 12 different organs with both normal and tumor tissues.

Table 7. Antibodies and IHC procedure.

Antibody	Vendor	Catalog number	Clone	Host species and clonality	Primary antibody titer	Primary antibody time/temp	Secondary antibody	Positive tissue control	Negative tissue control
VEGF-A	Thermo-Fisher	AB-9031		Rabbit polyclonal	1:50	Overnight / 5°C	Dako Real Envision/HRP, K5007	Angiosarcoma	
VEGF-C	Invitrogen	18-2255		Rabbit polyclonal	1:25	Overnight / 5°C	Dako Real Envision/HRP, K5007	Colon carcinoma	Normal brain
VEGFR-2	Cell Signaling	2479	55B11	Rabbit monoclonal	1:100	Overnight / 5°C	Dako Real Envision/HRP, K5007	Angiosarcoma	
VEGFR-3	Merck Millipore	MAB-3757	9D9F9	Mouse monoclonal	1:100	32 min / 37°C	iVIEW DAB Detection Kit (Ventana)	Lymph node	
PDGF-B	Sigma	A81363		Rabbit polyclonal	1:25	32 minutes / 37°C	UltraMap anti-Rb HRP (Ventana)	Colon carcinoma and placenta	Normal tonsil and brain
PDGF-D	R&D System	AF1159		Goat polyclonal	1:40	32 minutes / 37°C	UltraMap anti-Gt HRP (Ventana)	Colon carcinoma and placenta	Normal tonsil and brain
PDGFR-β	Cell Signaling	3169	28E1	Rabbit monoclonal	1:25	32 minutes / 37°C	UltraMap anti-Rb HRP (Ventana)	Colon carcinoma and placenta	Normal tonsil and brain

3.5 Scoring of expressions

Expressions of proteins or miRs was semiquantatively scored by two persons. The mean of the observers' scores were used and the observers' scores were assessed for agreement in terms of intraclass correlation. For the VEGFs (Paper I), the IHC stained TMA slides were scanned and digitalized using the ARIOL imaging system and uploaded into the ARIOL software. Two pathologists, Elin Richardsen and Samer Al-Saad, independently scored viable parts of each anonymized core by light microscopy. They recorded their respective scoring values into the ARIOL software, and the scores were then exported to the SPSS database for statistics by Nordby. For the PDGFs (Paper II) and miR-205 (Paper III), two persons independently scored each core while their scores were consecutively recorded manually into an Excel sheet by a third person. The PDGFs were scored by Richardsen and Andersen, and recorded by Nordby. miR-205 was scored by Richardsen and Nordby, and recorded by Andersen. All scores were exported into the SPSS database and prepared for statistics by Nordby.

Every core was independently and semiquantatively scored by light microscopy. The scorers were blinded for each other's score. Each core was scored by the dominant intensity of staining: 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining. For heterogeneous distributions of stromal staining, each core was also scored by density according to the estimated fraction of marker positive cells: 0 = 0 % positive cells; 1 = 1 – 50 % positive cells; 2 = 50 – 75 % positive cells; 3 \geq 75 % positive cells. Normal and tumor stroma and epithelium were scored independently if the marker was expressed in these compartments. The core was scored as "missing" if the core was missing or considered of insufficient quality to score by both observers.

Table 8. Overview of expression assessments for each biomarker.*Abbreviations: NS = Not scored; NE = Not expressed*

Marker	Tumor epithelium	Normal epithelium	Tumor stroma	Normal stroma
VEGF-A	Intensity	NS	Intensity	NS
VEGF-C	Intensity	NS	Intensity	NS
VEGFR-2	Intensity	NS	Intensity	NS
VEGFR-3	Intensity	NS	Intensity	NS
PDGF-B	Intensity	Intensity	NS	NS
PDGF-D	Intensity	Intensity	NS	NS
PDGFR-β	NS	NS	Intensity and density	Intensity and density
miR-205	Intensity	Intensity	NE	NE

3.6 Cut-off values

Scoring of IHC cores were dichotomized into low and high expressions. Statistical analyses regarding associations between biomarkers and endpoints were calculated for every cut-off, but eventually cut-off values were set at median to secure reproducibility and statistically sufficient numbers in each group. The exception was for stromal VEGF-A, where cut-off was set a bit higher than median (median = 0.5; cut-off 0.63) as the median would not give groups of approximately equal size (there was a high frequency with score = 0.5).

A cut-off near mean or median values lowers the probability of type 1 errors (false positive), but may not necessarily be the biological correct threshold, resulting in increased type 2 errors (false negatives). Optimal cut-off, in terms of searching for the cut-off that yields the most significant statistical differences, will, on the other hand increase the chance of type 1 errors (false positives).

3.7 Statistical analyses

SPSS 23.0.0.0 (Chicago, IL) and SPSS 24.0.0.0 was used for all statistical analyses. For cross-tabs, difference between groups were estimated using Pearson X^2 test or Fisher's exact test. Correlations were analyzed using Spearman's rank correlation coefficient. Comparing

mean ranks of expressions between different tissues were analyzed using the non-parametric Wilcoxon signed rank test. Comparing means between more than two groups (age, tumor mm) were analyzed by the non-parametrical Kruskal-Wallis H test due to non-normal distribution. Univariate survival curves were drawn by the Kaplan-Meier method, and the statistical significant difference between survival curves was assessed by the log-rank test. Calculations of unadjusted hazard ratios for univariate associations between variables and endpoints were analyzed using Cox regression. Presentations of the survival curves were terminated at 194 months due to less than 10 % of patients at risk after this point. For multivariate analyses, the backward conditional Cox-regression analysis was used with a probability for stepwise entry at 0.05 and stepwise removal of 0.10. A $p < 0.05$ was considered statistically significant for all analyses.

3.8 Ethics

The reporting of clinicopathological variables, survival data and biomarker expressions was conducted in accordance with the REMARK guidelines. These studies have been approved by The Regional Committee for Medical and Health Research Ethics, REK Nord, project application 2009/1393, including a mandatory reapproval January 22, 2016. REK Nord waived the need for patient consent for this retrospective study. The Data Protection Official for Research (NSD) approved the establishment of the database.

4 Results

4.1 Patient characteristics

Demographic, clinical and histopathological variables for all included patients and their associations with endpoints are presented in Table 9. Median age at surgery was 62 (47-75) years. At the last follow-up in December 2015, 37 % of the patients had BF, 11 % had CF and 3.4 % were dead of PC. Total mortality was 19.1 %.

Median preoperative serum PSA was 8.8 (range 0.7 - 104) and the median tumor size was 20 mm (2.0 - 50). Mean follow-up time of survivors was 12.4 years.

A total of 19.3 % (n = 103 patients) received salvage radiotherapy to the prostatic bed after prostatectomy due to

- Rising PSA, 14.6 % (n = 78)
- Persisting PSA, 0.9 % (n = 5)
- Not free surgical margins, 3.6 % (n = 19)

16.6 % (n = 89) received endocrine treatment after prostatectomy, while 3.6 % (n = 19) received palliative chemotherapy within the follow-up period.

Figure 9 shows event-free survival of BF, CF and PCD according to CAPRA-S score 0-2, 3-5 and 6-12.

The patients' surgical centers and differences in histopathological data are presented in Table 10 and Figure 10.

Uni- and multivariate prognostic impacts of the angiogenetic markers assessed in this thesis are summarized in Table 11.

Table 9 shows clinicopathological variables such as T-stage, PSA and Gleason are associated with increased BF, CF and PCD. Figure 9 shows that increased CAPRA-S score is correlated to increased events of BF, CF and PCD.

When comparing differences between operating centers, the patients operated at Levanger Hospital were added to the St. Olavs Hospitals' group due to the low number of patients

operated at Levanger (n = 10), same demographic belonging and that the patients were operated by the same surgeons as the patients operated at St. Olavs.

While there were no significant differences in CF and PCD, the patients at UNN had higher BF and overall mortality. However, there was a significant difference in the patients' baseline between the surgical centers: The patients at UNN were older, had higher pT-stage, higher ISUP Histologic Grade and higher preoperative PSA, as presented in Table 10.

Table 9. Patient characteristics, clinicopathological variables, and their associations with endpoints for 535 prostate cancer patients.*(univariate analyses; log-rank test, unadjusted Cox proportional hazard ratios)*

Characteristics	Patients		BF (200 events = 37.4%)			CF (56 events = 10.5%)			PCD (18 events = 3.4%)		
	(n)	(%)	5 year EFS (%)	HR (95% CI)	p	10 year EFS (%)	HR (95% CI)	p	10 year EFS (%)	HR (95% CI)	p
Age					0.237			0.038			0.404
≤ 65 years	357	67	77	1		94	1		98	1	
> 65 years	178	33	70	1.19 (0.89-1.59)		91	1.75 (1.02-2.98)		98	1.50 (0.58-3.90)	
pT-stage					<0.001			<0.001			0.001
pT2	374	70	83	1		97	1		99	1	
pT3a	114	21	61	2.30 (1.67-3.15)		87	2.93 (1.61-5.34)		99	1.96 (0.62-6.25)	
pT3b	47	9	43	4.41 (3.01-6.47)		74	4.54 (2.24-9.21)		98	6.60 (2.20-19.8)	
Preop PSA					<0.001			0.029			0.003
PSA < 10	308	57	81	1		95	1		99	1	
PSA > 10	221	42	68	1.65 (1.24-2.18)		89	1.82 (1.06-3.14)		97	4.62 (1.52-14.1)	
Missing	6	1	-			-					
ISUP Grade					<0.001			<0.001			<0.001
1 (Gleason 3 + 3)	183	34	83	1		98	1		99	1	
2 (Gleason 3 + 4)	219	41	77	1.35 (0.95-1.92)		94	3.52 (1.42-8.73)		99	1.99 (0.26-10.8)	
3 (Gleason 4 + 3)	81	15	70	2.14 (1.41-3.26)		90	4.70 (1.71-13.0)		96	8.18 (1.65-40.7)	
4 (Gleason 4 + 4)	17	4	58	3.14 (1.59-6.19)		86	6.22 (1.55-24.9)		94	6.85 (0.72-76.0)	
5 (Gleason ≥ 9)	35	6	37	4.30 (2.63-7.03)		65	18.0 (7.00-46.6)		91	15.8 (3.06-81.8)	
Positive surgical margin					0.049			0.198			0.843
No	249	47	81	1		96	1		98	1	
Yes	286	53	69	1.33 (1.00-1.76)		90	1.44 (0.82-2.52)		98	1.10 (0.42-2.87)	
Apical positive surgical margin					0.063			0.427			0.128
No	325	61	74	1		92	1		98	1	
Yes	210	39	77	0.76 (0.56-1.02)		93	0.80 (0.46-1.39)		98	0.45 (0.16-1.29)	
Non-apical positive surgical margin					<0.001			<0.001			0.022
No	381	71	82	1		96	1		99	1	
Yes	154	29	57	2.25 (1.69-2.97)		85	2.63 (1.55-4.47)		96	2.84 (1.12-7.24)	
CAPRA-S Score					<0.001			<0.001			0.001
0 - 2	169	32	88	1		99	1		99	1	
3 - 5	258	48	78	1.85 (1.25-2.73)		94	5.73 (1.73-18.9)		99	1.76 (0.35-8.73)	
6 - 12	102	19	46	5.28 (3.51-7.93)		79	13.6 (4.08-45.2)		94	7.28 (1.58-33.5)	
NC due to missing PSA	6	1									
Tumor size					<0.001			0.002			0.085
0 - 20 mm	250	47	83	1		96	1		99	1	
> 20 mm	285	53	68	1.79 (1.34-2.39)		90	2.39 (1.34-4.28)		97	2.41 (0.86-6.76)	
Perineural infiltration					<0.001			<0.001			<0.001
No	250	47	80	1		96	1		99	1	
Yes	285	53	60	2.16 (1.63-2.88)		83	2.70 (1.59-4.59)		95	5.75 (2.15-15.4)	
Lymphovascular infiltration					<0.001			<0.001			<0.001
No	492	92	77	1		95	1		99	1	
Yes	43	8	47	2.26 (1.29-3.41)		69	4.23 (2.32-7.70)		90	6.50 (2.49-17.0)	
Surgical procedure					0.466			0.308			0.965
Retropubic	435	81	77	1		92	1		98	1	
Perineal	100	19	68	1.14 (0.81-1.60)		95	0.66 (0.30-1.47)		99	0.97 (0.28-3.37)	

Abbreviations: BF = biochemical failure; CF = clinical failure; PCD = prostate cancer death; EFS = event free survival in months; NC = not computable

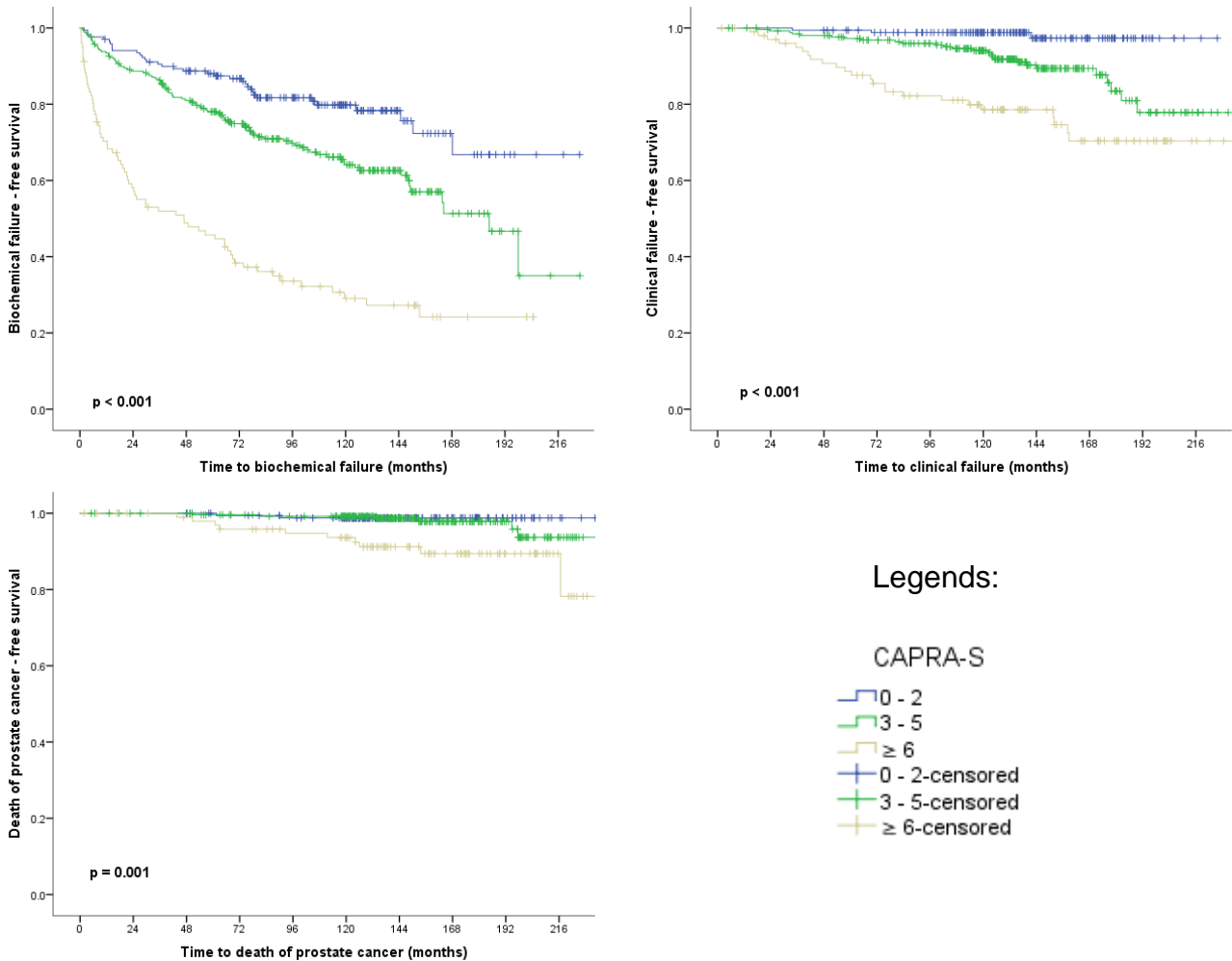


Figure 9. Event-free survival on all endpoints stratified upon CAPRA-S Score.

The CAPRA-S score is a combined prognostic postoperative score comprising of PSA, Gleason score, T-stage and surgical margin. Increased CAPRA-S Score is associated with increased BF, CF and PCD.

Table 10. Endpoints and histopathological parameters for patients operated at the different surgical centers.*(Pearsons Chi-square test, Mann-Whitney U test)*

Characteristics	Surgical center			p
	UNN	Bodø	St.Olav	
Number of patients (n)	248	59	228	
Mean CAPRA-S Score	4.2	2.2	3.8	<0.001
CAPRA-S Score				<0.001
0 - 2	26 %	64 %	30 %	
3 - 5	49 %	34 %	53 %	
6 - 12	25 %	2 %	17 %	
NC due to missing PSA (n)	0	0	6	
Biochemical failure	48 %	46 %	24 %	<0.001
Clinical failure	12 %	3 %	11 %	0.164
Death of prostate cancer	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
Preop PSA	13.7	7.4	9.3	<0.001
pT-stage				<0.001
pT2	61 %	97 %	73 %	
pT3a	26 %	0 %	22 %	
pT3b	13 %	3 %	6 %	
ISUP Grade				0.001
1 (Gleason 3 + 3)	29 %	59 %	34 %	
2 (Gleason 3 + 4)	42 %	31 %	43 %	
3 (Gleason 4 + 3)	17 %	7 %	16 %	
4 (Gleason 4 + 4)	4 %	2 %	3 %	
5 (Gleason ≥ 9)	9 %	2 %	5 %	
Mean tumor size (mm)	15.3	16.8	15.0	0.050
Perineural infiltration	21 %	71 %	17 %	<0.001
Lymphovascular infiltration	9 %	7 %	7 %	0.619
Positive surgical margin	46 %	34 %	67 %	<0.001
Surgical procedure				<0.001
Retropubic	60 %	100 %	100 %	
Perineal	40 %	0 %	0 %	

Abbreviations: BF = biochemical failure; CF = clinical failure; EFS = event free survival in months; NC = not calculable

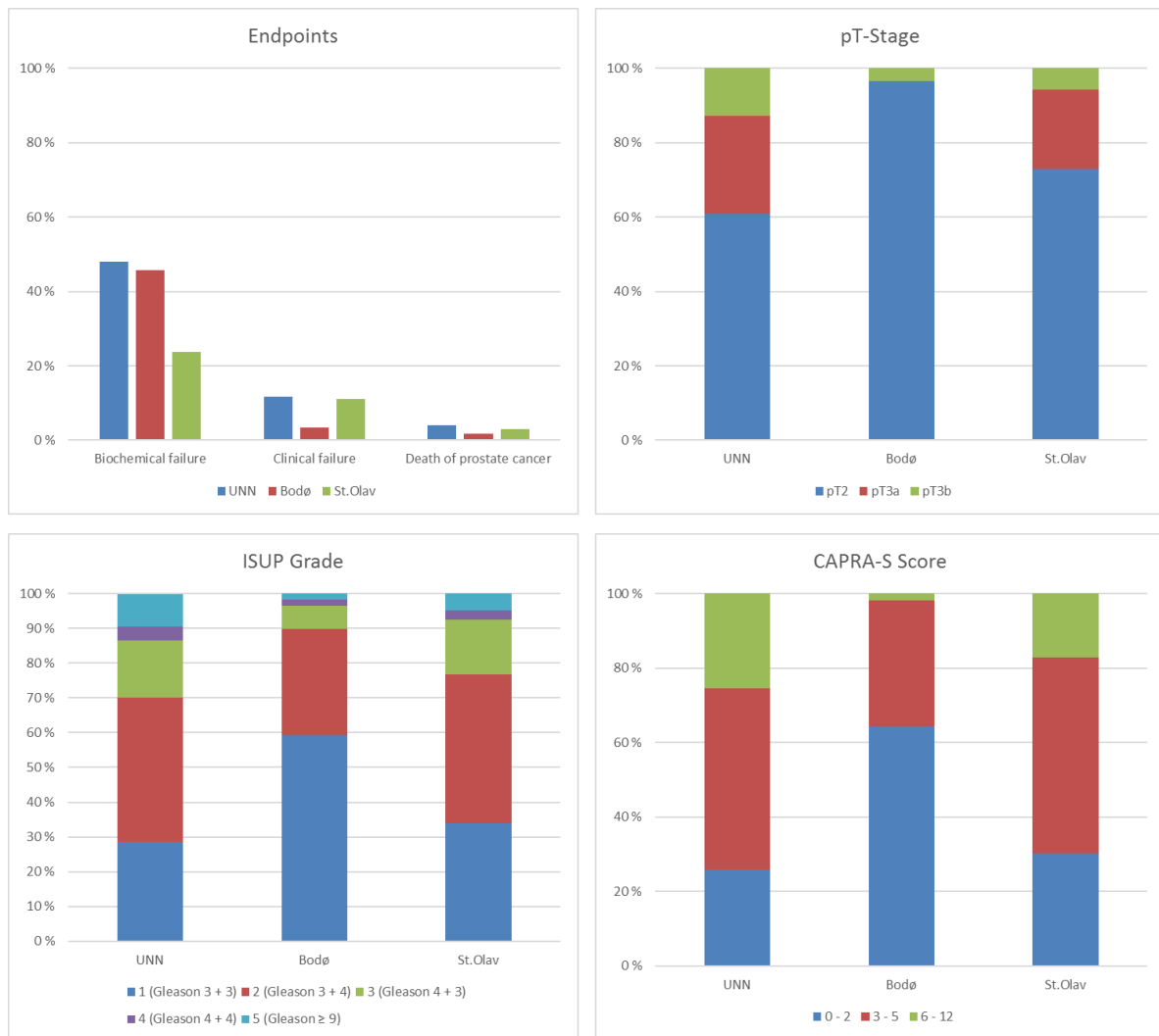


Figure 10. Comparison of patients' endpoints and histopathological parameters between the different surgical centers.

Table 11. Univariate and multivariate analyses of all biomarkers assessed in this thesis.

(Univariate analyses; log rank test, unadjusted Cox proportional hazard ratios. Multivariate analyses; Cox regression with backward conditional model)

Marker	Univariate analysis			Multivariate analysis		
	HR	BF	P	HR	BF	P
VEGF-A epithelium			NS			NE
VEGF-A stroma, high expr	1.49		0.013	1.51		0.016
VEGFR-2 epithelium			NS			NE
VEGFR-2 stroma expr	1.42		0.032	2.28		0.031
VEGF-A and VEGFR-2 in stroma			0.003			NS
Both low expr	1			1		0.011
Either VEGF-A or VEGFR-2 high expr	1.75			1.77		
Both high expr	2.21			2.02		
VEGFR-2 in stroma and epithelium			0.053			0.095
Both stroma and epithelium low expr	1			1		1
Either stroma, epithelium or both high expr	1.52			1.45		4.56
VEGF-C epithelium			NS			NE
VEGF-C stroma			BS			NE
VEGFR-3 epithelium			NS			NE
VEGFR-3 stroma			NS			NE
PDGF-B epithelium			NS			NS
PDGF-D epithelium			NS			NS
PDGFR-β stroma, high expr	1.73		<0.001	2.63		0.001
miR-205 in epithelium, high expr	1.61		0.003	1.70		0.001
			NS			NS

Abbreviations: BF = biochemical failure; CF = clinical failure; HR = hazard ratio; NS = Not significant; NE = Not entered due to NS in univariate analysis; expr = expression

4.2 Paper I – VEGFs

4.2.1 Expressions and correlations

Both VEGF-A and VEGFR-2 were expressed in both epithelium and stroma. There was no expression of the biomarkers in the control cores, except VEGFR-2 expression in vascular endothelium as expected. There was no correlation between epithelial and stromal expression. Neither of the VEGFs had any direct correlation to any of the clinicopathological variables.

4.2.2 Univariate analyses

Published univariate results for the clinicopathological variables are presented in Paper 1, while the updated results after last database update are presented in Table 9. As presented in Paper 1 and Table 11, patients with high expression of VEGF-A in stroma (HR 1.49, $p = 0.013$), high expression of VEGFR-2 in stroma (HR 1.43, $p = 0.032$) and a combination of high expression of either VEGF-A or VEGFR-2 in stroma ($p = 0.003$) had significantly worse outcome regarding BF. For CF, patients with high expression of VEGFR-2 in stroma (HR 2.28, $p = 0.031$) and high expression of VEGFR-2 in either stroma, epithelium or both (HR 4.33, $p = 0.029$) had a significantly worse outcome. None of the markers were significantly associated with worse outcome regarding PCD, though VEGFR-2 tended towards significance (HR 3.00, $p = 0.076$). Univariate analyses of VEGF-C and VEGFR-3 expressions showed no significant differences in BF, CF and PCD.

4.2.3 Multivariate analyses

Published multivariate results for the clinicopathological variables are presented in Paper 1, while the updated results after last database update are presented in Table 9. As presented in Paper 1 and Table 11, high VEGF-A expression in stroma correlated to increased BF (HR 1.51, $p = 0.016$). High expression of either VEGF-A or VEGFR-2 in stroma (HR = 1.77) or both (HR 2.02) were significantly associated with increased BF ($p = 0.011$). Although not significant for BF ($p = 0.095$), a high VEGFR-2 expression in either stroma, epithelium or both was significant and independently associated with worse CF-free survival (HR = 4.56, $p = 0.038$).

4.3 Paper II – PDGFs

4.3.1 Expressions and correlations

For PDGF-D, intensity was scored in both tumor and normal epithelium. Stroma was not scored due to weak staining, and density was not scored due to homogenous distribution. PDGF-D was expressed at a higher level in tumor epithelium compared to normal epithelium (mean 2.13 vs 1.85, $p < 0.001$). For PDGF-B, only intensity was scored as density was homogeneously distributed. Stroma was not scored due to overall strong fibromuscular staining. There was no significant difference in PDGF-B expression in tumor epithelium compared to normal epithelium (mean 1.48 vs 1.52, $p = 0.194$). PDGFR- β was not expressed in epithelium, hence only stroma was scored. Both intensity and density was scored, but statistics found density to yield stronger results in means of higher hazard ratio and significance than intensity. Hence, all published evaluations were based on PDGFR- β density scoring.

Neither of the PDGFs correlated to the clinicopathological variables except a weak correlation between mean density of PDGFR- β in stroma and perineural infiltration ($r = 0.25$, $p < 0.001$).

4.3.2 Univariate analysis

Results for the univariate analyses of clinicopathological variables are presented in Paper 2 and Table 9. Results from the univariate analyses of PDGFs are presented in Paper 2 and summarized in Table 11.

Univariate analyses of PDGF-B and PDGF-D expressions showed no significant associations with BF, CF and PCD.

For PDGFR- β , statistical analyses found no difference in endpoints with respect to expressions in tumor stroma respective normal stroma. Hence, all stromal scorings were pooled. Assessing stromal density of high expression yielded stronger results in means of higher hazard ratio (HR) and significance than intensity, thus results from analyses of density scores were published. Patients with a high expression of PDGFR- β in stroma had significantly worse outcome regarding BF (HR = 1.73, $p < 0.001$) and CF (HR = 2.63, $p = 0.001$) compared to patients with low expression of PDGFR- β . For PCD (3.4% of cases), no

significant outcome difference was observed regarding high or low PDGFR- β expression subgroups.

4.3.3 Multivariate analysis

Results from a multivariate model of clinicopathological variables and biomarkers are presented in Paper 2 and summarized in Table 11.

A high expression of PDGFR- β in stroma correlated to a worse BF (HR = 1.58, $p = 0.002$). For CF, the only factors that correlated to a significantly worse outcome in our model was Gleason score ($p < 0.001$) and high expression of PDGFR- β in stroma (HR 2.17, $p = 0.010$).

4.4 Paper III – miR-205

4.4.1 Expressions and correlations

miR-205 was expressed in both normal and tumor epithelium, where expression in tumor epithelium (mean score = 1.79) was reduced compared to normal epithelium (mean score = 1.85, $p = 0.008$). There was no expression of miR-205 in stroma. There was a significant higher expression of miR-205 in normal epithelium for patients that suffered BF (mean score = 1.99) compared to patients without BF (mean score = 1.77, $p = 0.001$). No difference in miR-205 expression in tumor epithelium was observed comparing patients with or without BF.

None of the clinicopathological variables correlated to ($r < 0.2$) expression of miR-205 in tumor or normal epithelium. miR-205 expression in tumor epithelium was correlated to expression in normal epithelium ($r = 0.27$, $p < 0.001$).

As presented in Paper 3, miR-205 correlates to various VEGFs and PDGFs.

4.4.2 Univariate analysis

Results for the clinicopathological variables are presented in Paper 3 and Table 9. Results from the univariate analysis of miR-205 are presented in Paper 3 and Table 11.

We found no associations between miR-205 expression in tumor epithelium and endpoints for any cut-offs (for mean cut-off and BF: $p = 0.864$). However, high expression of miR-205 in normal epithelium was associated with BF ($p = 0.003$). There was a trend of association between high miR-205 and CF, but the association was not significant ($p > 0.100$). For PCD, no significant outcome difference was observed regarding high or low miR-205 expression subgroups for any cut-off.

We further assessed whether there were possible subgroups where expression of miR-205 had a particular significant impact on prognosis. For patients with ISUP Grade 1 or 2 (Gleason 3+3 or 3+4), there was a significant association between BF and high miR-205 expression [$n = 351$, HR 1.94 (95% CI = 1.30-2.91), $p = 0.001$]. No significant association between miR-

205 and BF was observed for patients with ISUP Grade 3 (Gleason 4+3) or higher [n = 114, HR = 1.12 (95% CI = 0.66-1.88), p = 0.676].

Regarding the post-prostatectomy outcome predictor CAPRA-S Score, there was a significant association between high miR-205 expression and BF for patients with CAPRA-S Score 0-5 [n = 374, HR = 1.75 (95% CI = 1.18-2.62), p = 0.005] , while there was no significant association for patients with CAPRA-S Score 6-12 [n = 86, HR = 1.38 (95% CI = 0.81-2.355), p = 0.235].

4.4.3 Multivariate analysis

Results from a multivariate model of clinicopathological variables and miR-205 are presented in Paper 3 and summarized in Table 11.

In addition to the clinicopathological factors CAPRA-S Score (p < 0.001) and perineural infiltration (p = 0.001), a high expression of miR-205 was significantly and independently associated with a worse BF (HR = 1.70, p = 0.001) in our model. In the same model for ISUP Grade 1-2, the only significant prognostic factors associated with increased BF were perineural infiltration (HR = 1.93, p = 0.003) and high miR-205 expression (HR 2.07, p = 0.001). Regarding ISUP Grade 3-5, the only factor associated with increased BF was pT-stage (p < 0.001).

5 Discussion

5.1 Study design

Weaknesses and strengths of the study design are discussed in more detail in Chapter 3. A summary is listed in Table 12.

Table 12. Summary of weaknesses and strengths.

	Strengths	Weaknesses
Study design and database	Large cohort Extensive follow-up Minimal selection bias due to inclusion of consecutive patients and centralized treatment Data collected by clinicians optimizes quality of database All tissues reexamined and staged according to most recent classification by experienced pathologists	No validation of results in external patient cohorts Long inclusion period may result in differences in patient selection due to different treatment trends over time and different practices between surgical centers. Information bias due to retrospective collection of data Low percentage of PCD results in limited number of events (n = 18), increasing the risk of type 2 errors (false negative results)
Tissue microarray, IHC and ISH	Well-validated and high throughput method saves time, tissue, reagents and money Assessments of both epithelium and stroma, normal and tumor tissues possible Standardization of analysis Validated antibodies The use of mean cut-offs reduces the chance of type 1 errors	Time-consuming and requires technical skill when TMA is first assembled Variability introduced by preanalytic factors (e.g. fixation), experimental conditions and antigen quality. Intraobserver variability Monoclonal antibodies are more prone to false negative results (type 2 errors)
Scoring and data analyses	Semi-quantitative scoring is low-cost, quick, transferable into clinical practice Scores from two independent scorers	Manual scoring is difficult to reproduce and compare between studies Continuous variables has more information than ordinal variables

5.2 Paper I – VEGFs

Prior to our study, a few clinicopathological studies had reported conflicting results on VEGF-A expression in PC and their prognostic value, while stromal expression had hardly been studied. Due to the uncertainty, we systematically investigated both tumor and stromal expressions.

We demonstrated that overexpression of VEGF-A and VEGFR-2 is independently and significantly associated with BF and CF in PC patients treated with prostatectomy. The risk of BF is nearly doubled provided high stromal expression of VEGF-A or VEGFR-2, while the risk of CF is quadrupled if VEGFR-2 is overexpressed in either tumor epithelium, tumor-adjacent stroma or both. Our data demonstrating VEGF-A as an prognostic factor in PC is consistent with the majority of previous studies^{121,122,124-126}. However, we found VEGFR-2 to be a stronger prognosticator than its more commonly studied ligand VEGF-A. While most previous studies have not evaluated stromal expression, our results regarding VEGF-A emphasize that it is the overexpression in tumor-adjacent stroma rather than the tumor epithelium that is of greatest importance. This is supported by a smaller study of 51 radical prostatectomy specimens where high Gleason grade tumors and advanced disease had a significantly higher frequency of VEGF-A expression in tumor-near stroma rather than tumor epithelium¹²⁷.

An explanation of why previous studies have yielded conflicting results lays in their lack of differentiation or evaluation of tumor- and stromal expression, due to either not analyzed or due to the limitations of RT-PCR technique where tissues are pooled. Another explanation may lay in the choice of antibodies, where the use of thoroughly validated high-quality antibodies are essential to produce reliable results.

In conclusion, our results supports most previous studies, but in addition clarifies stromal expression of VEGF-A and VEGFR-2 as strong independent predictors of PC recurrence. VEGFR-2 has previously been scarcely studied in clinicopathological studies, and our results demonstrate VEGFR-2 to outperform VEGF-A as a prognostic factor for PC relapse.

Anti-VEGF treatments are established for various cancers, but attempts at anti-VEGF treatment in PC has so far been unsuccessful. Targeting the VEGF-A/VEGFR-2 pathway is

not previously studied in patients with localized PC. At the present, however, a randomized phase II trial of the VEGFR-1, -2 and -3 inhibitor axitinib, administered prior to surgery, is ongoing in high-risk PC (started 2011, ending in 2018)¹⁷⁵. A Phase II trial of androgen deprivation therapy with or without neoadjuvant axitinib prior to prostatectomy for patients with known or suspected lymph node metastasis is currently recruiting¹⁷⁶. Androgen deprivation therapy combined with bevacizumab resulted in an improved PSA relapse-free survival for patients with hormone-sensitive PC in a randomized phase II trial for patients with recurrent PC after definitive local therapy¹⁷⁷. In this trial, long-term follow-up is needed, but the study provides rationale for combining vascular endothelial growth factor-targeting therapy with ADT in hormone-sensitive PC.

Hence, the therapeutic combined inhibition of the VEGF-A/VEGFR-2 signaling may in the future be added to radical treatment of PC. However, a thorough understanding of the active pathways in order to succeed in targeted therapy is crucial.

5.3 Paper II – PDGFs

Prior to our study, PDGF pathways studies were scarce in PC and the majority had been performed *in vitro*. Thus, the lack of clinicopathological studies of the PDGFs and the absence of biomarker studies in PC involving both normal and malignant tissues in epithelial and stromal compartments, mandated the need for further investigation.

We found a high expression of PDGFR- β in stroma to be independently and significantly associated to BF (HR = 1.58, p = 0.002) and CF (HR = 2.17, p = 0.010) in PC patients treated with radical prostatectomy. In our cohort, PDGFR- β outperforms well-established prognostic factors like pT-stage, preoperative PSA, tumor size, PNI, lymphovascular infiltration and a positive surgical margin as a prognostic factor.

Stromal overexpression of PDGFR- β had previously been found to be associated with poor survival and advanced disease in a natural course of the disease, prior to the implementation of radical prostatectomy as medical practice at the time¹⁴². However, PDGFR- β as a prognostic factor for cancer recurrence post prostatectomy had previously not been examined for patients with a perceived curable localized disease.

As for the main results for the VEGFs, the prognostic impact was in stromal expression. Our results show that both normal and malignant stroma are of clinical importance regarding PDGFR- β . The stromal microenvironment is an active and important biological compartment. Mediated through direct cell-cell contacts or by secreted molecules, there is a continuous and bilateral molecular crosstalk between both normal cells and tumor cells of the stromal compartment. Accordingly, minor changes in one compartment may cause dramatic alterations in the whole system⁸³.

We found no associations between PDGF-D expression and clinical outcome, although other studies have suggested that PDGF-D seems to be involved in development of bone metastasis and is associated with increased Gleason grade and tumor stage^{178,179}. A reason for this may be that our sample selection consists of patients with localized disease, whereas previous studies of PDGF-D have been implicating a more advanced disease¹⁸⁰. There was no associations between PDGF-B expression and prognosis, supported by previous clinical studies demonstrating that both PDGFR- β and PDGF-D are upregulated in primary PC and

bone metastases, whereas PDGF-B is not frequently detected in clinical samples¹⁸¹. Our results indicate that neither PDGF-B nor PDGF-D is associated with cancer relapse in earlier stages of the disease. Hence, it is the upregulation of the receptor PDGFR- β that seems to be of clinical significance for patients considered for radical treatment.

An important result is that the only two factors that predict CF in our cohort are Gleason score and high expression of PDGFR- β . In fact, high PDGFR- β expression more than doubles the risk of clinical failure, and has a significant impact on BF and CF for the intermediate American Joint Committee of Cancer (AJCC) risk groups IIA, IIB and III. This is of particular interest as we are in desperate need for better prognostic tools in intermediate risk patients.

5.4 Paper III – miR-205

Previous studies have characterized miR-205 as a tumor suppressor, downregulated in prostate tumor tissue^{151-153,158}. As a prognostic marker, conflicting results have been published. In our screening array of 1435 miRs in tumor tissue from our 2014 study (not included in dissertation), miR-205 was consistently downregulated for the 14 PC patients with rapid BF¹⁵⁹.

We found miR-205 to be downregulated in tumor epithelium compared to normal epithelium, corroborating previous studies^{151-153,155,156,158}. However, expression of miR-205 in tumor epithelium was not associated with PC relapse in our cohort. Paradoxically, the prognostic impact of miR-205 was exclusively related to the normal prostate epithelium, as high expression of miR-205 in normal epithelium was independently and significantly associated with BF. Traditionally, the active tissues in the carcinogenic processes has been considered to be tumor epithelium and stroma. Studies of interplay between normal morphological and neoplastic epithelial cells has been limited, and little is known about the function of morphological normal epithelium in tumorigenesis. However, a few recent studies have revealed that perceived normal epithelial cells, in addition to normal cells surrounding the tumor, can exert an anti-tumor activity on prostate carcinoma cells¹⁸²⁻¹⁸⁴. This suggests that normal epithelium may have a more important role in controlling tumor expansion than previously acknowledged, though the crosstalk between normal and neoplastic epithelial cells is not understood.

Further analyses revealed that the prognostic importance of miR-205 was primarily found in low-risk cancers such as ISUP Grade Group 1-2 and CAPRA-S Score < 6. Based on our presented results and the few studies suggesting normal epithelium might exert anti-tumor activity, we hypothesize that the normal epithelial cells in PC specimens are potential functionally active cellular constituents counteracting the carcinogenic processes of tumor cells. One of the counteracting mechanisms might be overexpression of the tumor suppressor miR-205 in low and intermediate grade tumors. Thereby, the miR-205 overexpression in normal epithelium could be a marker of the normal epitheliums efforts to hinder the more aggressive tumor to develop.

Our results are supported by a study by Kalogirou et al., as they found a consistent tendency for miR-205 to correlate with an adverse outcome for PC patients¹⁵⁵. Further, Gandellini et al. found miR-205 to prevent malignant interplay between PC cells and associated fibroblasts¹⁵¹.

In conclusion, our results add support of the potential role of normal epithelium and its potential crosstalk to surrounding tissues in PC. We propose normal epithelium to hinder further aggressiveness in the more aggressive low-grade tumors. This can be by exerting tumor suppressor effects of miR-205 in low- and intermediate grade PC tumors. However, our results warrants validation both in functional experimental studies and in clinical validation cohorts. There is always the risk of this being a type I error, a false positive.

Considerable resources are currently being put in the development of miR anti-cancer therapy, and the success of specific targeting in a therapeutic perspective rely on a deeper understanding of the biological mechanics at play.

6 Conclusions

In this thesis, results from an established comprehensive prostatectomy cohort and three published papers are presented. We have examined expressions of important angiogenic biomarkers and their associations with patient outcome as well as histopathological parameters in prostatectomy tissues.

Amongst the strengths of the prostatectomy cohort is the extensive follow-up time. While most PC clinicopathological studies have shorter follow-up time and fewer patients, the median follow-up time of more than 12 years and 535 included patients are one of the major strengths. This greatly reduces the chance of false negative errors. Despite this, the low incidence of CF and PCD results in a relatively low number of these events and BF is a controversial endpoint. As PC is, in most cases, a slowly developing disease, the need for long follow-up and large cohorts cannot be underestimated.

Another strength is the use of IHC and ISH to assess protein expressions in specific compartments. In contrast to the RT-PCR studies that are widely common amongst the comparable studies, the use of IHC and ISH allows assessment of both tumor and stromal compartments, as well as assessment of expressions in normal and tumor tissues within the same patient. Interestingly, the main findings of the VEGFs and PDGFs was found in the stromal compartments, as opposed to epithelial expressions. Currently, one has become more aware of the importance of the stromal microenvironment and the crosstalk between epithelium and the surrounding stroma, in contrast to earlier perceptions of epithelium as the major active component in tumorigenesis. These results clearly demonstrate the superiority of ISH and ICH methods compared to the more widely used RT-PCR, and results, in our opinion, in more robust and nuanced results. To further support this statement, the prognostic importance of miR-205 was found in normal epithelium in contrast to tumor epithelium, raising the hypothesis of a cross-talk between normal and tumor epithelium in tumorigenesis. Little is known about epithelial cross-talk and the potential mechanisms of a tumor suppressor function by normal epithelium. These results propose novel and interesting biological mechanisms not previously described in detail and mandates further studies. Part of the contradicting results from previous studies may be explained by the use of RT-PCR techniques and antibodies of uncertain quality in those studies.

Regarding the VEGFs, the aim was to clarify their prognostic value in PC patients with a localized disease as previous results have been contradicting and not unambiguously clarified. Our results of VEGF-A and VEGFR-2 as predictors for PC recurrence are solid fundamental in the superiority of the IHC assessment and thoroughly validated antibodies. Anti-VEGF therapy have so far failed in PC patients, but recent ongoing trials have been promising, awaiting results. Our results suggest that the VEGFR-2 axis is of clinical importance in PC. In addition of presenting VEGFR-2 as an independent prognostic biomarker for PC recurrence, the VEGFR-2 axis appears to be of clinical importance from a therapeutic perspective. As a clinically and molecularly heterogeneous disease, the lack of available prognostic biomarkers for PC patient stratification regarding therapy is one of the key reasons why several trials have produced disappointing results. Specific prognostic biomarkers, associated with response to therapy, are also warranted in order to guide treatment stratification.

As a biomarker, PDGFR- β expression has not previously been assessed for patients with a localized disease. Our results indicate PDGFR- β in either benign or tumor associated stroma to be a strong, independent predictor of PC recurrence. Although PDGF inhibition so far has been disappointing, its implication in PC relapse warrants further exploration to identify the optimal setting in which to exploit its impact. Hitherto, no studies involving PDGFR-inhibition has been carried out in early stage PC. According to translational research data, it can be speculated that such therapy may prove effective in the primary setting. Prospective validation should be considered for future studies.

A major implication of this study is the need to pay particular attention to stringent tissue sampling and evaluation in PC studies. Our finding that almost all significant prognostic results were outside of the neoplastic cells themselves, could have been masked or could have been falsely interpreted as been associated to tumor cells by a non *in-situ* approach.

Some future perspectives for the studies needed in this area of angiogenesis markers in PC should be mentioned; they should focus on interplay between compartments and cells, they should be confirmed by experimental models and clinical validations in different cohorts should be included before prospective studies. The road to clinical useful prognostic biomarkers in PC is indeed long and winding.

In conclusion, this thesis present promising new biomarkers that may aid in future treatment selection of PC patients. Our studies will hopefully provide stepping stones for future contributions regarding prognostic markers, eventually improving treatment strategies for the most common cancer in men.

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Paper 1

Paper 2

Paper 3