UiT
THE ARCTIC
UNIVERSITY
OF NORWAY

Faculty of Health Sciences
Department of Clinical Medicine

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

—

Yngve Nordby
A dissertation for the degree of Philosophiae Doctor – May 2018

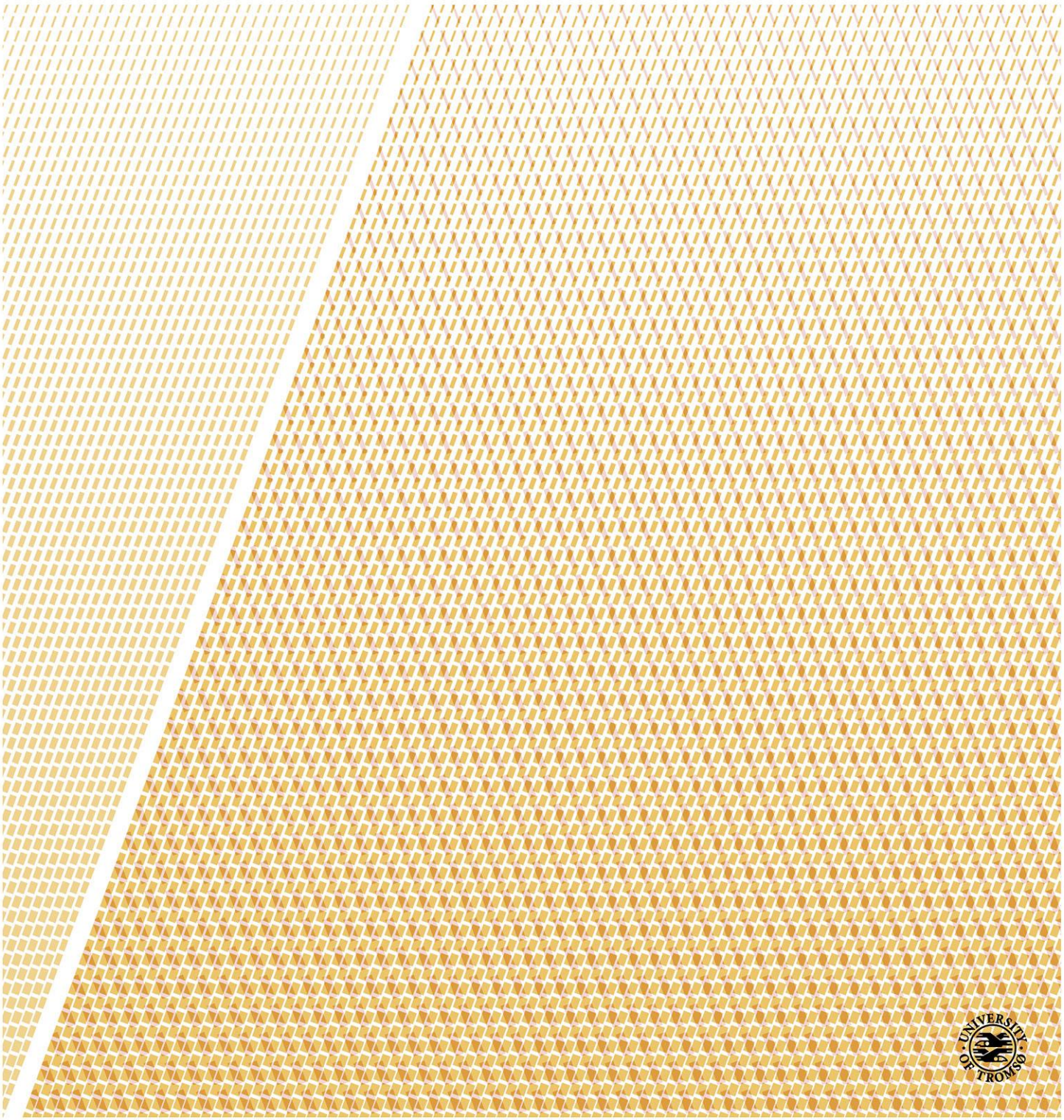


Table of Contents

List of Figures

List of Tables

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 *in-situ* hybridization
CT	Computer tomography
DRE	Digital rectal exam
EAU	European Association of Urology
EMT	Epithelial to mesenchymal transition
IHC	Immunohistochemistry
ISH	*In-situ* 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.

It has been a long and winding road from Dr. Stig Müller presented me to Dr. Sigve Andersen and their then newly started prostate project, to the completion of this thesis. The combination of working as a surgeon in training and as a Ph.D student has not always been without challenges, but it has been a privilege to work with the experienced and accomplished researchers in the Translational Cancer Reasearch Group in such a friendly and inspiring environment.

First I would like thank my main supervisor Sigve Andersen for his endless patience, exceptional positive attitude and rock solid supervision. I thank my co-supervisors dr. Elin Richardsen and professors Roy Bremnes, Lill-Tove Busund and Hiten Patel for their invaluable supervision. Thanks to my other colleagues in the Translational Cancer Research Group. A special thanks to the former head of the Urological Department, Tore Knutsen, for facilitating time to research between surgical shifts and for helping me to keep the goal in sight when despair has seemed imminent.

My warmest thanks goes to my family. To Ann-Margaret, for her warm heart and infinite care for our family. For her infinite patience when I have worked countless nights. To our daughters, Maja and Martine, for their laughs and smiles <3

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 prostatectomy[1]

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 recurrence[2]

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]

Scientific reports **7**, 16308, doi:10.1038/s41598-017-16556-2 (2017)

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 varibles 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 worldwide[4]. 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 overtreatment[7-9]. 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-effects[10].

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 validation[11]. 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 males[12]. It secrets 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

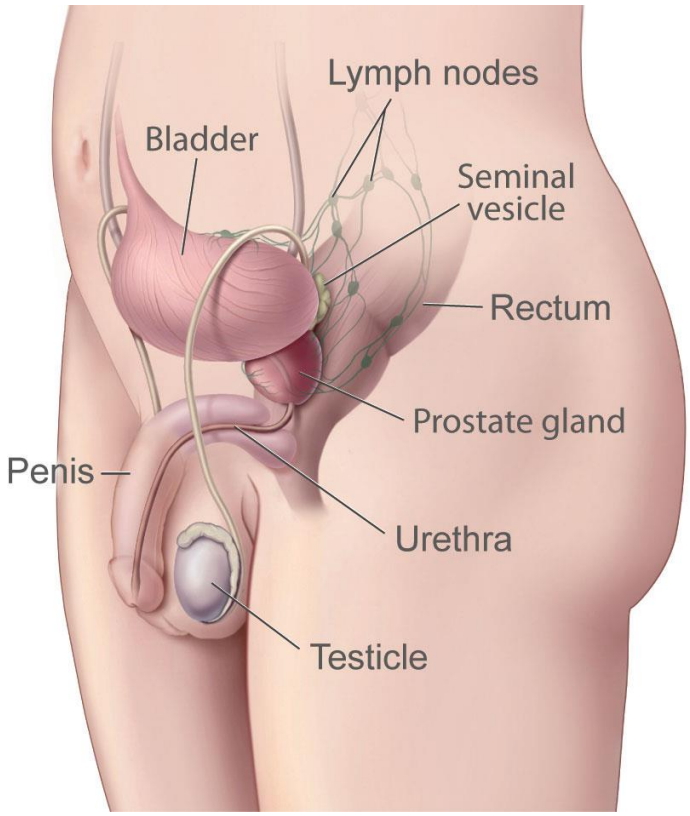


Figure 1. Illustration of the prostate location and anatomy. *The prostate can be palpated in a digital rectal exam. Reprinted with permission from www.cancer.gov.*

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 index[13].

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 hyperplasic (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 α1-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 65[16]. 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 men[17]. 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 prevented[22]. 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 5α-reductase inhibitors finasteride and dutasteride, more commonly used for treatment of BPH. 5α-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 PC[25-27]. Some research suggests that aspirin daily might lower the risk of PC[28]. 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 PC[29].

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 men[6]. 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, considerable 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 uncertain[30-32]. 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 2015[6].

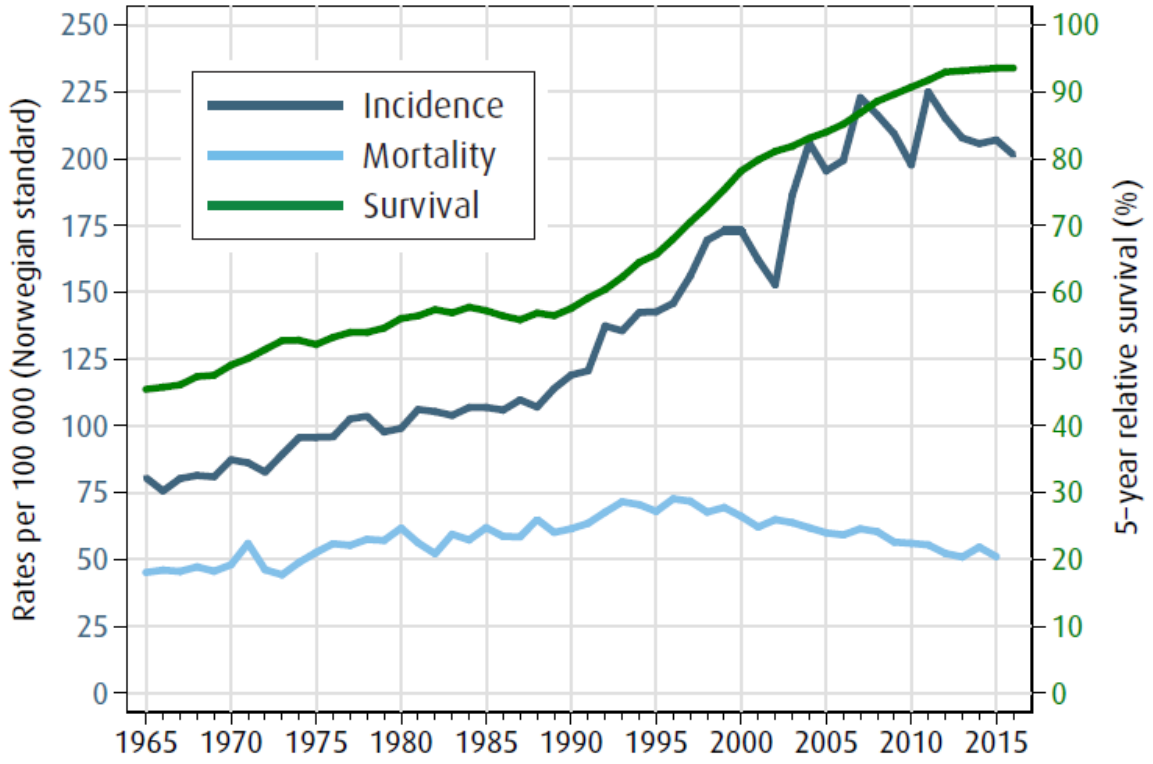


Figure 2. Trends in incidence and mortality rates and 5-year relative survival proportions. *Although incidence and survival has increased rapidly from the 1990, mortality has declined. However, 5 year survival is a poor measurement of quality of PC treatment, as PC often has a long preclinical fase. 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 gland[33]. BPH usually develops in the transition zone, whereas 75% of PC develops in the peripheral zone[34]. 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 Worth 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 help[35]. Two independent studies has 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 Norway[38].

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 PCs[39]. Although not as common, around 7 % of PC patients in Norway are initially diagnosed with metastatic PC, where bone pain may be the presenting symptom[6].

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 outmost importance for patients considered for curative treatment, where the most accurate method for determination of N-stage is an operative extended lymphadenectomi. 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 stage[40]. 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 PC[41]. 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 information[46]. In conjuction 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 mortality[47]. 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 conjuction 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 considered[48]. 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 patients[49].

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) probe[48]. 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 system[50]. 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 pattern[51]. 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

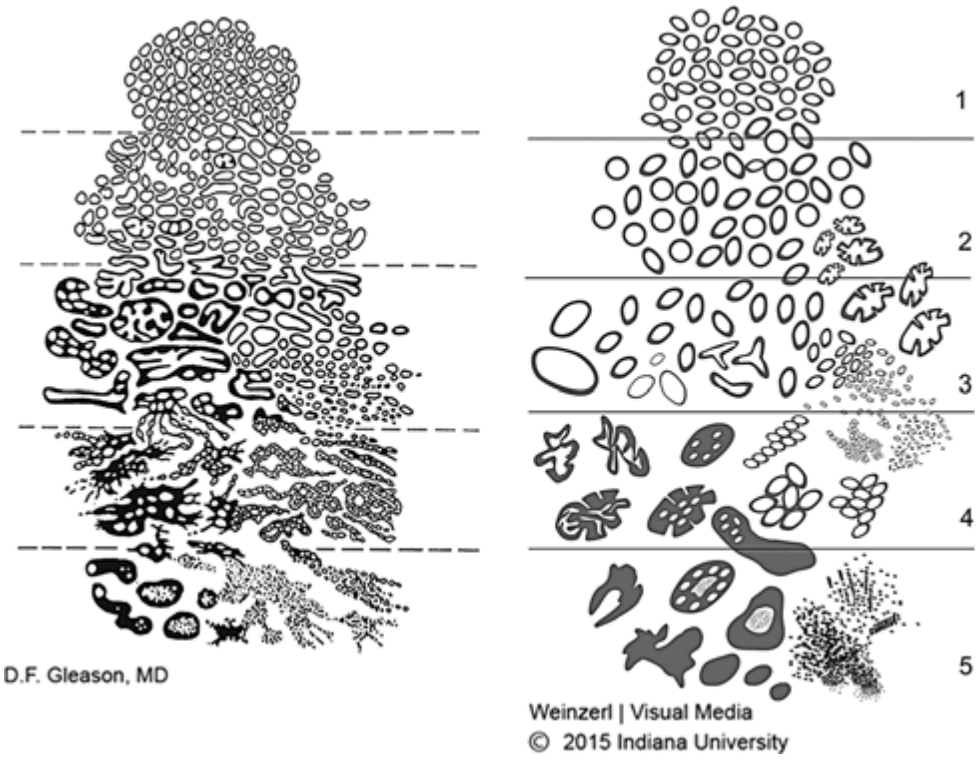


Figure 3. Prostatic adenocarcinoma histologic patterns. *Original (left) and 2015 Modified ISUP Gleason schematic diagrams. Reprinted with permission from Wolters Kluwer Health, Inc.*

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 stratification[52-54]. 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 PC[48]. 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 2016[55]

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 Urologcal 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 age[56]. The post-surgical score (CAPRA-S) is a tool for prediction of outcomes after radical prostatectomy, and it's points are assigned according to Table 5[57].

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 treatment[58]. 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 progression[59]. This may also be discussed for subgroups of patients with intermediate risk PC[60]. 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 treatment[59]. 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 1949[61-63]. 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 plummeted[64].

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 used[65]. 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-Axelson 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 betters 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 recurrence[71]

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 lymphedema[72].

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 patients[73].

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 monitoring[74].

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 0.4[75]. 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 tumors[78]. 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 (doxetaxel) 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 treatment[79]. It is not recommended to treat patients for non-metastatic CRPC outside of a clinical trial[48].

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 environment[82]. 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 recurrence[82]. 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 system[83]. 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 TME[84].

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 cancer[85]. 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 added[86].

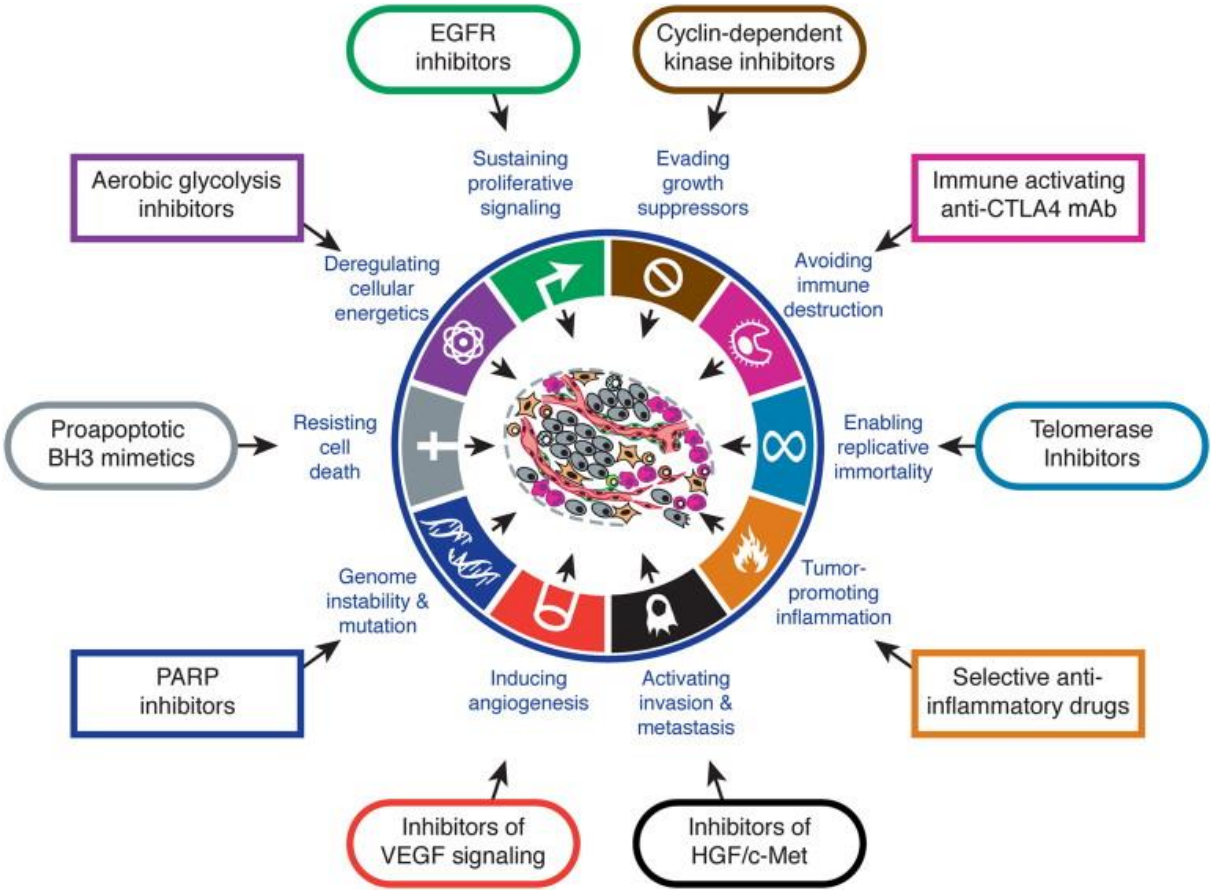


Figure 4. The hallmarks of cancer. *Biological capabilities acquired during the multistep development of human tumors and potential drugs for targeted therapies. Reprinted with permission from Elsevier*[86].

The hypothesis that tumor growth is angiogenesis dependent was first stated by Folkman in the early seventies[87]. Today, much evidence underlines tumor dependence on angiogenesis in order to progress[88]. 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 dies[91].

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 growths[92].

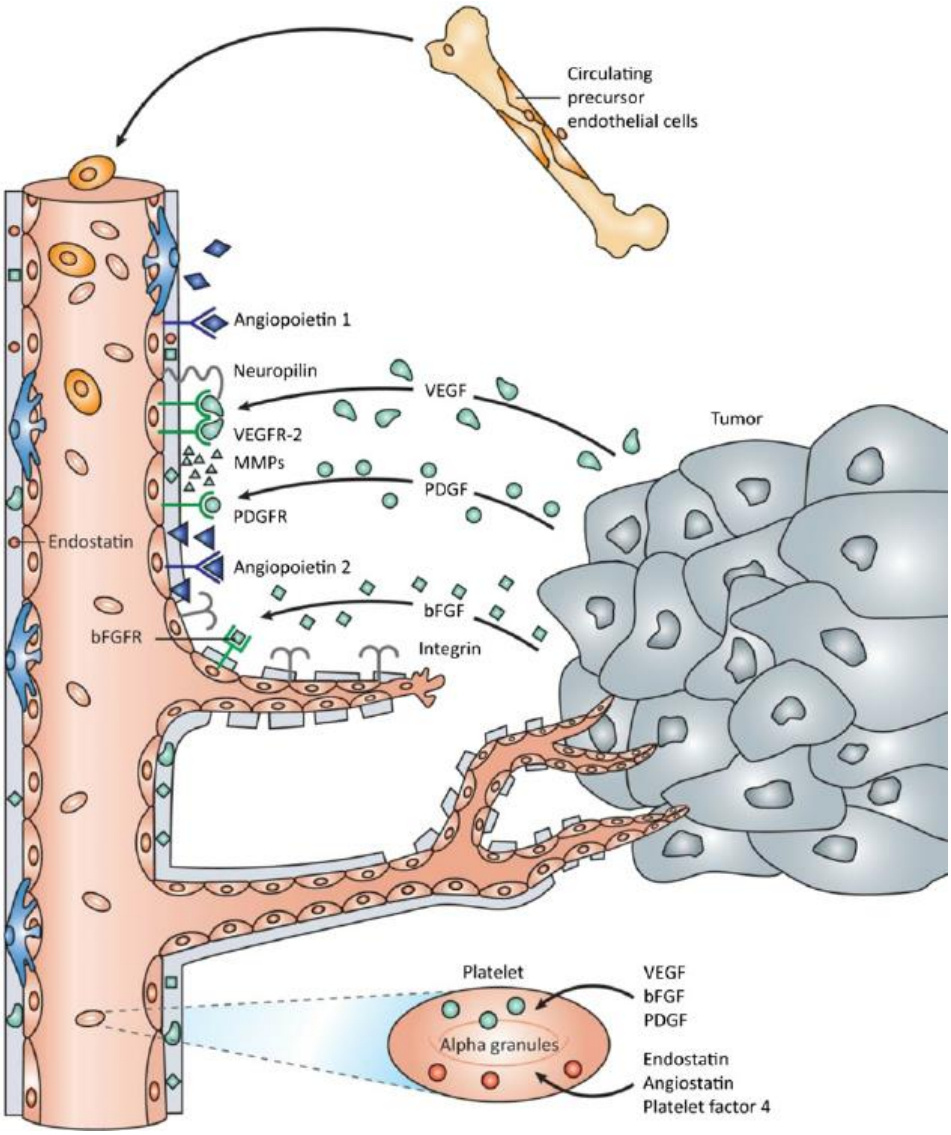


Figure 5. Tumor angiogenesis mechanisms. *Soluble angiogenic factors (e.g., VEGF, PDGF, FGF) are secreted from the tumor and surrounding cells to induce and regulate key steps in angiogenesis. Reprinted with permission from Nature Reviews*[93].

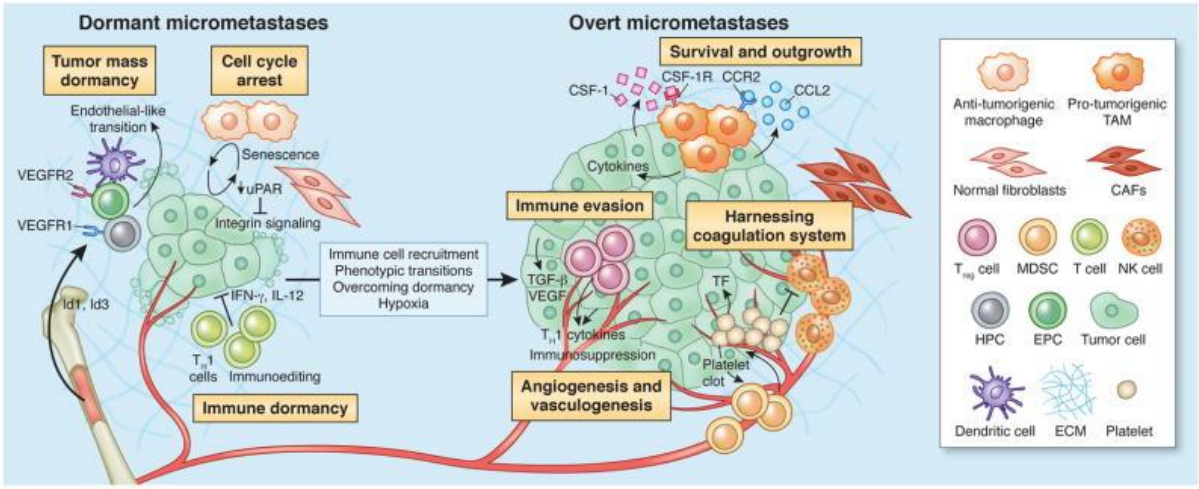


Figure 6. Overcoming tumor dormancy, and initiation of secondary outgrowth in metastatic niches. *Dormant micrometastases are held in check by several mechanisms. Tumor mass dormancy is when proliferation is balanced by apoptosis, owing to a lack of vasculature and limited supply of nutrients and oxygen. Multiple cell types contribute to the re-establishment of vascularity 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 Nature*[82].

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 metastasis[95]. PC has the ability to produce MMPs, VEGF, TGFβ, 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 architecture[96].

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 outcome[97]. Taking into account the essential role of angiogenesis in PC development, angiogenesis is suggested to lead to further improvements in PC diagnosis and staging[98].

Meta-analyses have shown that high VEGF levels in PC cells are associated with poor prognosis[99]. 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 controversial[98]. 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 carcinoma[105]. 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.

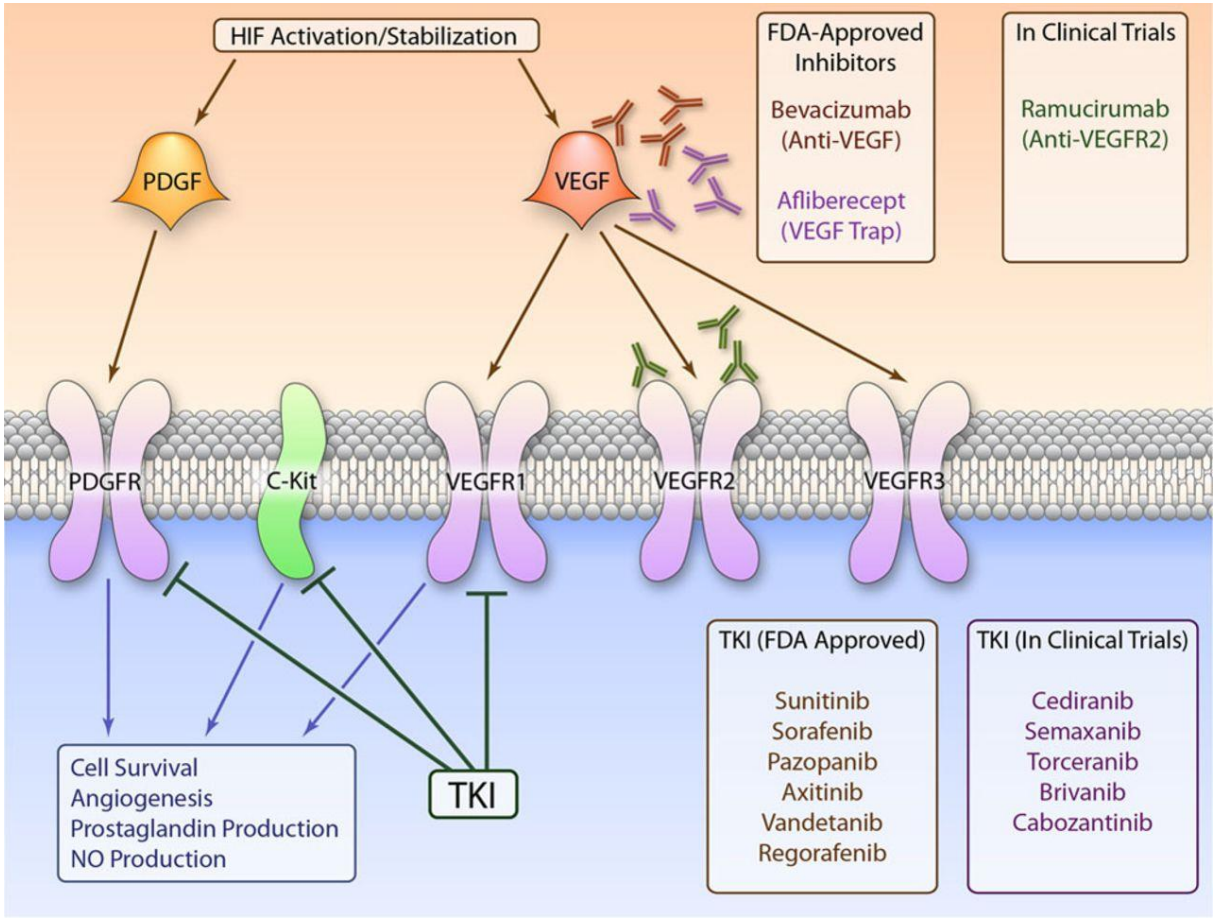


Figure 7. Angiogenesis inhibitors (VEGF signaling pathway (VSP) inhibitors) being tested in human cancer trials. *Although these agents are being referred to as VSP inhibitors, drugs such as sunitinib inhibit many other receptor tyrosine kinases, allowing them to be approved for the treatment of other 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, Inc*[108].

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 PC[109]. 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 mCRPC[110]. 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 aflibercepte[111]. 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 metastases[112]. Although encouraging symptomatic relief, results from the phase 3 trial COMET-1 did not show improvement in overall survival[113]. 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 is several cancers[115-118]. 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 cancer[119]. For PC, the few previous clinicopathological studies regarding the VEGFs have not yielded consistent results, and their stromal expressions had hardly been previously assessed[120-127]. 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 (PDGSs)

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 microenvironment[128]. 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 cancer[129-135]. 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 action[138]. 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 protuberans[119]. 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 metastasis[141]. High levels of PDGFR-β in PC tumor stroma and non-malignant prostate tissue have been associated with shorter cancer specific survival for PC patients[142]. 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 disease[143-146]. 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 stage[147]. 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 trials[150].

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 PC[151-158]. 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 study[159], 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 angiogenetic 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 nomenclature[51]. 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 analyses[162]. 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 microtone (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 methods[163]. 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 pattern[164].

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 assessments[165].

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 suggested[168]. 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 decisions[169].

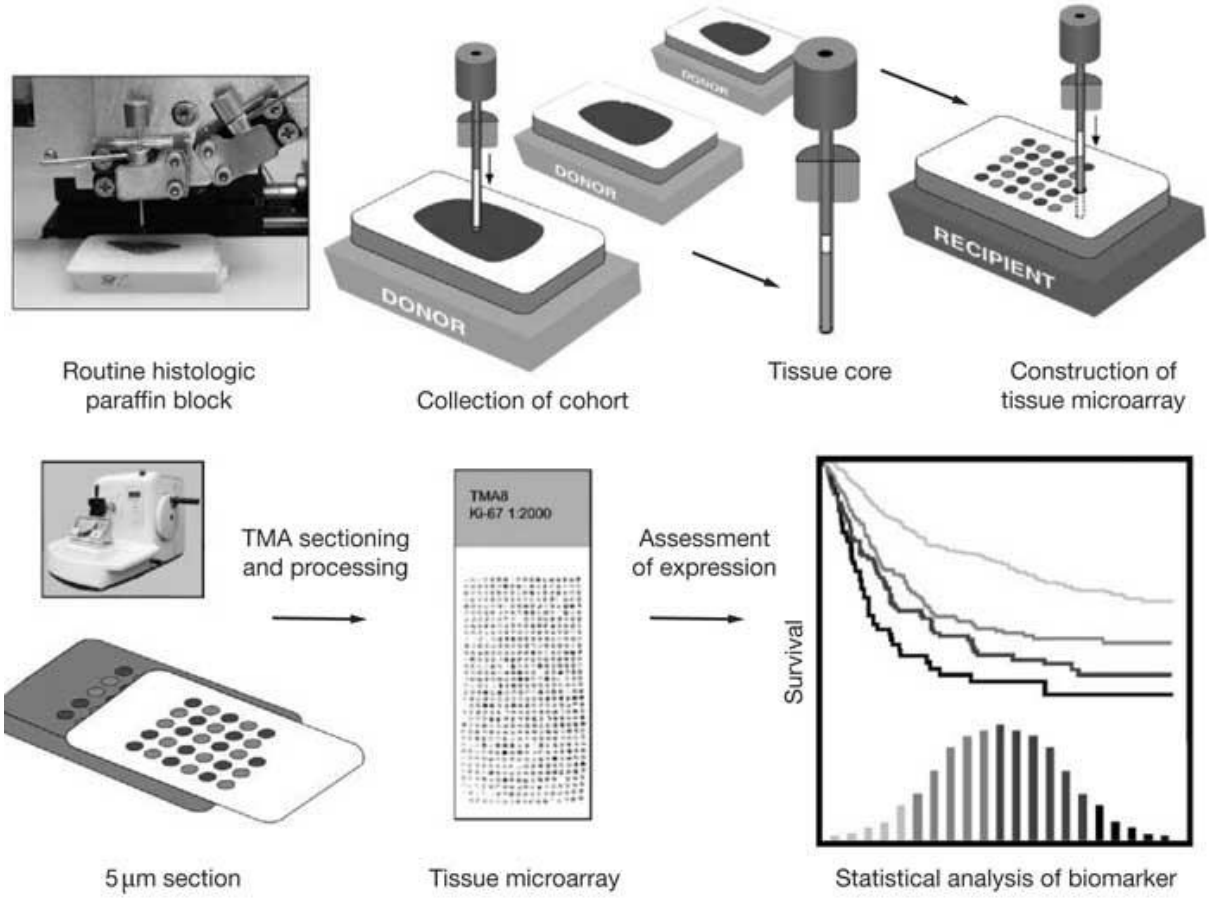


Figure 8. Construction and use of tissue microarrays for biomarker identification. *Paraffin-embedded, formalin-fixed tissues are collected. Representative areas from each donor tumor block are punched into cores of 0.6 mm in diameter and arrayed into a recipient TMA block. Sections of the resultant 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, ©2004*[170].

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 methods[171]. 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 analysis[172]. 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 results[172].

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 literature[173]. 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 manufacurers' 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

Simular 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 immunohistochemistry[174]. 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.

Simular 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 ≥ 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 reapprovement 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

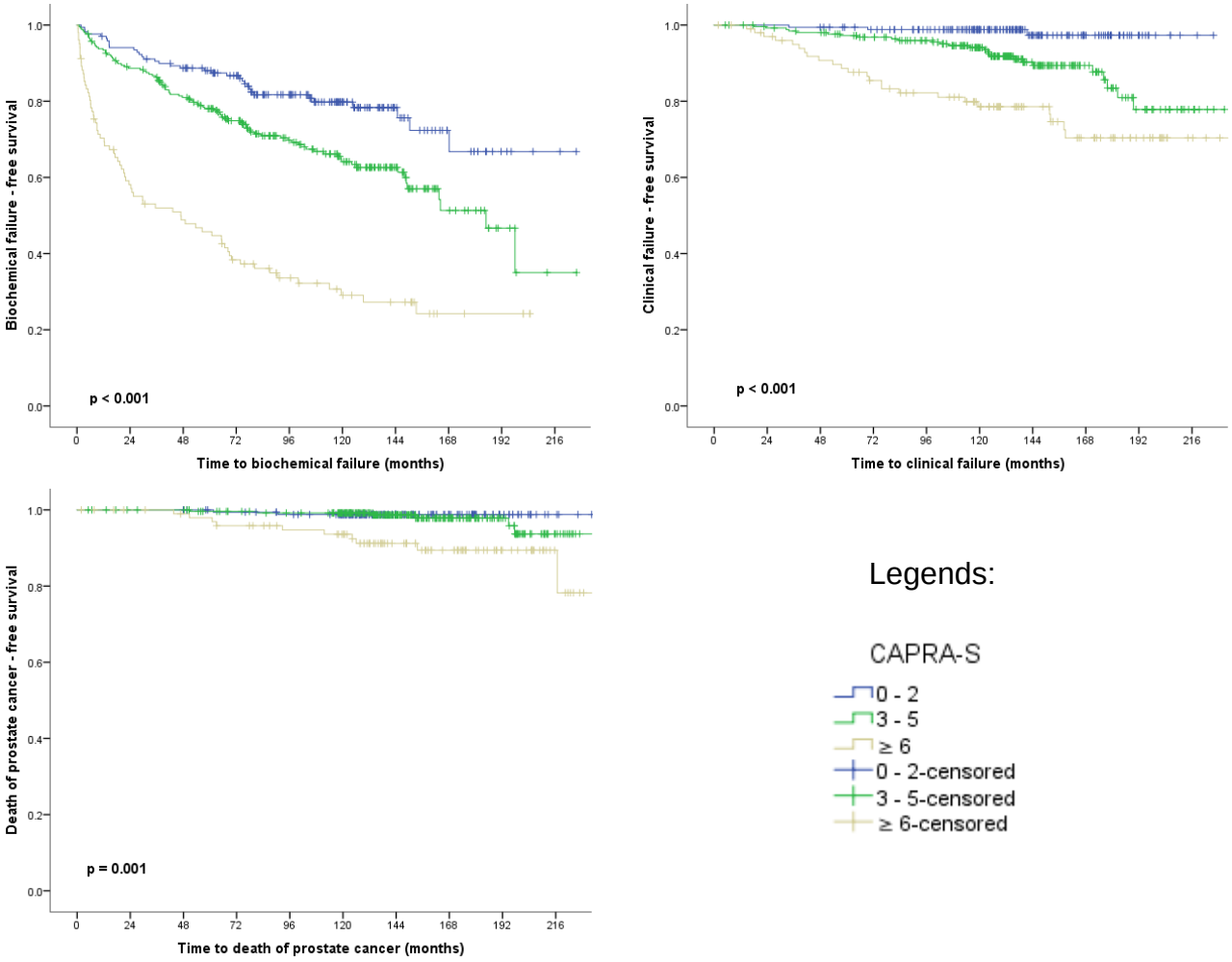


Figure 9. Event-free survival on all endpoints stratified upon CAPRA-S Score.

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			
	UNN	Bodø	St.Olav	p
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

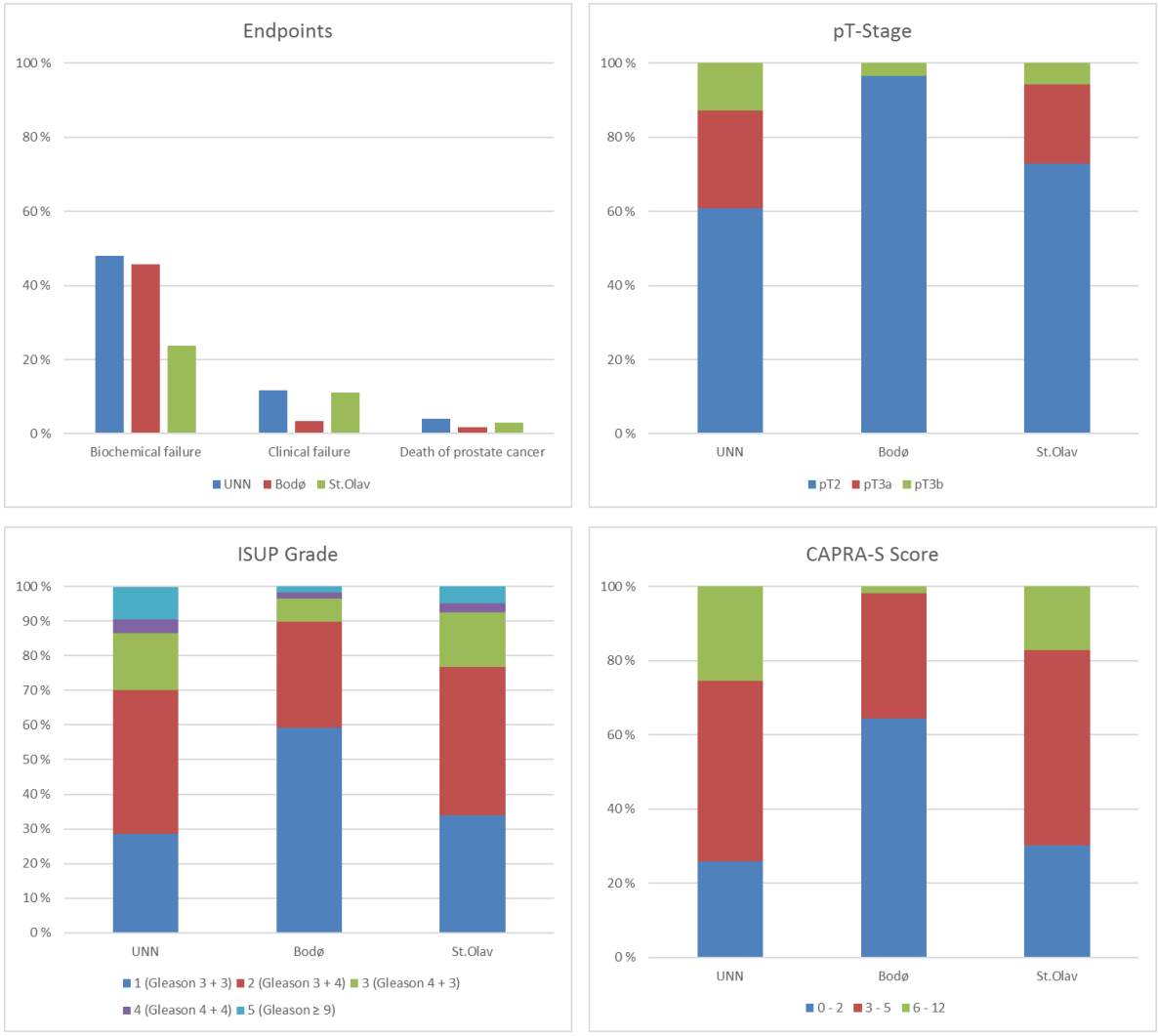


Figure 10. Comparison of patients' endpoints and histopathological parameters between the different surgical centers.

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			
	BF		CF		BF		CF	
	HR	P	HR	P	HR	P	HR	P
VEGF-A epithelium		NS		NS		NE		NE
VEGF-A stroma, high expr	1.49	0.013		NS	1.51	0.016		NE
VEGFR-2 epithelium		NS		NS		NE		NE
VEGFR-2 stroma expr	1.42	0.032	2.28	0.031		NS		NS
VEGF-A and VEGFR-2 in stroma		0.003		NS		0.011		NE
Both low expr	1				1			
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		0.029		0.095		0.038
Both stroma and epithelium low expr	1		1		1		1	
Either stroma, epithelium or both high expr	1.52		4.33		1.45		4.56	
VEGF-C epithelium		NS		NS		NE		NE
VEGF-C stroma		BS		NS		NE		NE
VEGFR-3 epithelium		NS		NS		NE		NE
VEGFR-3 stroma		NS		NS		NE		NE
PDGF-B epithelium		NS		NS		NS		NS
PDGF-D epithelium		NS		NS		NS		NS
PDGFR-β stroma, high expr	1.73	< 0.001	2.63	0.001	1.58	0.002	2.17	0.01
miR-205 in epithelium, high expr	1.61	0.003		NS	1.70	0.001		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 VEFGR-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 homogenously 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 assembeled 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 anylses	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 epithelium[127].

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 our without neoadjuvant axitinib prior to prostatectomy for patients with known or suspected lymph node metastasis is currently recruiting[176]. 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 therapy[177]. 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 time[142]. 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 system[83].

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 disease[180]. 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 samples[181]. 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. If 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 BF[159].

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 cells[182-184]. 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.

Futher 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 patients[155]. Further, Gandellini et al. found miR-205 to prevent malignant interplay between PC cells and associated fibroblasts[151].

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 fundamented 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.

7 References

1 Nordby, Y. *et al.* Stromal expression of VEGF-A and VEGFR-2 in prostate tissue is associated with biochemical and clinical recurrence after radical prostatectomy. *Prostate* **75**, 1682-1693, doi:10.1002/pros.23048 [doi] (2015).

2 Nordby, Y. *et al.* High expression of PDGFR-beta in prostate cancer stroma is independently associated with clinical and biochemical prostate cancer recurrence. *Scientific reports* **7**, 43378, doi:10.1038/srep43378 (2017).

3 Nordby, Y. *et al.* High miR-205 expression in normal epithelium is associated with biochemical failure - an argument for epithelial crosstalk in prostate cancer? *Scientific reports* **7**, 16308, doi:10.1038/s41598-017-16556-2 (2017).

4 Ferlay, J. *et al.* Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer* **136**, E359-386, doi:10.1002/ijc.29210 (2015).

5 Ferlay, J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **127**, 2893-2917, doi:10.1002/ijc.25516 [doi] (2010).

6 Kreftregisteret - Cancer Registery of Norway. Cancer in Norway 2016 - Cancer incidence, mortality, survival and prevalence in Norway. (2017).

7 Bill-Axelson, A. *et al.* Long-term distress after radical prostatectomy versus watchful waiting in prostate cancer: a longitudinal study from the Scandinavian Prostate Cancer Group-4 randomized clinical trial. *Eur. Urol* **64**, 920-928, doi:10.1016/j.eururo.2013.02.025 (2013).

8 Etzioni, R. *et al.* Increasing use of radical prostatectomy for nonlethal prostate cancer in Sweden. *Clin. Cancer Res* **18**, 6742-6747, doi:10.1158/1078-0432.CCR-12-1537 (2012).

9 Fossa, S. D. *et al.* Treatment and 5-year survival in patients with nonmetastatic prostate cancer: the Norwegian experience. *Urology* **83**, 146-152, doi:10.1016/j.urology.2013.08.081 (2014).

10 Bratt, O., Hugosson, J., Klotz, L. & Gnanapragasam, V. The drama of prostate cancer diagnostics. *Lancet Oncol* **18**, e132, doi:10.1016/s1470-2045(17)30121-3 (2017).

11 Martin, N. E. New developments in prostate cancer biomarkers. *Current opinion in oncology* **28**, 248-252, doi:10.1097/cco.0000000000000279 (2016).

12 Harper, D. *Online Etymology Dictionary*, <http://www.etymonline.com/index.php?term=prostate> (2017).

13 Fowke, J. H. *et al.* The association between body size, prostate volume and prostate-specific antigen. *Prostate cancer and prostatic diseases* **10**, 137-142, doi:10.1038/sj.pcan.4500924 (2007).

14 Chang, R. T., Kirby, R. & Challacombe, B. J. Is there a link between BPH and prostate cancer? *The Practitioner* **256**, 13-16, 12 (2012).

15 Orsted, D. D., Bojesen, S. E., Nielsen, S. F. & Nordestgaard, B. G. Association of clinical benign prostate hyperplasia with prostate cancer incidence and mortality revisited: a nationwide cohort study of 3,009,258 men. *European urology* **60**, 691-698, doi:10.1016/j.eururo.2011.06.016 (2011).

16 American Cancer Society. *Prostate Cancer*, <https://www.cancer.org/cancer/prostate-cancer.html> (2017).

17 Tan, D. S., Mok, T. S. & Rebbeck, T. R. Cancer Genomics: Diversity and Disparity Across Ethnicity and Geography. *J Clin Oncol* **34**, 91-101, doi:10.1200/jco.2015.62.0096 (2016).

18 Hemminki, K. Familial risk and familial survival in prostate cancer. *World journal of urology* **30**, 143-148, doi:10.1007/s00345-011-0801-1 (2012).

19 Jansson, K. F. *et al.* Concordance of tumor differentiation among brothers with prostate cancer. *European urology* **62**, 656-661, doi:10.1016/j.eururo.2012.02.032 (2012).

20 Ewing, C. M. *et al.* Germline mutations in HOXB13 and prostate-cancer risk. *The New England journal of medicine* **366**, 141-149, doi:10.1056/NEJMoa1110000 (2012).

21 Lynch, H. T. *et al.* Screening for familial and hereditary prostate cancer. *International journal of cancer* **138**, 2579-2591, doi:10.1002/ijc.29949 (2016).

22 Leitzmann, M. F. & Rohrmann, S. Risk factors for the onset of prostatic cancer: age, location, and behavioral correlates. *Clinical epidemiology* **4**, 1-11, doi:10.2147/clep.S16747 (2012).

23 Blanc-Lapierre, A. *et al.* Metabolic syndrome and prostate cancer risk in a population-based case-control study in Montreal, Canada. *BMC public health* **15**, 913, doi:10.1186/s12889-015-2260-x (2015).

24 Esposito, K. *et al.* Effect of metabolic syndrome and its components on prostate cancer risk: meta-analysis. *Journal of endocrinological investigation* **36**, 132-139, doi:10.1007/bf03346748 (2013).

25 Andriole, G. L. *et al.* Effect of dutasteride on the risk of prostate cancer. *The New England journal of medicine* **362**, 1192-1202, doi:10.1056/NEJMoa0908127 (2010).

26 Andriole, G. L. *et al.* Mortality results from a randomized prostate-cancer screening trial. *The New England journal of medicine* **360**, 1310-1319, doi:10.1056/NEJMoa0810696 (2009).

27 Kramer, B. S. *et al.* Use of 5-alpha-reductase inhibitors for prostate cancer chemoprevention: American Society of Clinical Oncology/American Urological Association 2008 Clinical Practice Guideline. *J Clin Oncol* **27**, 1502-1516, doi:10.1200/jco.2008.16.9599 (2009).

28 Bilani, N., Bahmad, H. & Abou-Kheir, W. Prostate Cancer and Aspirin Use: Synopsis of the Proposed Molecular Mechanisms. *Frontiers in pharmacology* **8**, 145, doi:10.3389/fphar.2017.00145 (2017).

29 Johannessen, K., Berge. *Nasjonalt handlingsprogram med retningslinjer for diagnostikk, behandling og oppfølging av prostatakreft*, <http://www.helsebiblioteket.no/retningslinjer/prostatakreft/forord> (2015).

30 Bray, F., Lortet-Tieulent, J., Ferlay, J., Forman, D. & Auvinen, A. Prostate cancer incidence and mortality trends in 37 European countries: an overview. *European journal of cancer (Oxford, England : 1990)* **46**, 3040-3052, doi:10.1016/j.ejca.2010.09.013 (2010).

31 Kvale, R. *et al.* Interpreting trends in prostate cancer incidence and mortality in the five Nordic countries. *Journal of the National Cancer Institute* **99**, 1881-1887, doi:10.1093/jnci/djm249 (2007).

32 Kvale, R. *et al.* Prostate and breast cancer in four Nordic countries: A comparison of incidence and mortality trends across countries and age groups 1975-2013. *International journal of cancer* **141**, 2228-2242, doi:10.1002/ijc.30924 (2017).

33 McNeal, J. E. The zonal anatomy of the prostate. *Prostate* **2**, 35-49 (1981).

34 Applewhite, J. C., Matlaga, B. R., McCullough, D. L. & Hall, M. C. Transrectal ultrasound and biopsy in the early diagnosis of prostate cancer. *Cancer control : journal of the Moffitt Cancer Center* **8**, 141-150 (2001).

35 Engstrom, G., Walker-Engstrom, M. L., Loof, L. & Leppert, J. Prevalence of three lower urinary tract symptoms in men-a population-based study. *Family practice* **20**, 7-10 (2003).

36 Gjengsto, P., Eide, J., Frugard, J., Bakke, A. & Hoisaeter, P. A. The potentially curable prostate cancer patient and the pathways leading to diagnosis and treatment. *Scandinavian journal of urology and nephrology* **38**, 15-18, doi:10.1080/00365590310019990 (2004).

37 Hughes, A. M., Sladden, M. J., Hirst, G. H. & Ward, J. E. Community study of uncomplicated lower urinary tract symptoms among male Italien immigrants in Sydney, Australia. *European urology* **37**, 191-198, doi:20117 (2000).

38 Norway, K.-C. R. o. Årsrapport 2016 - Annual report of 2016. (2016).

39 Siegel, R., Naishadham, D. & Jemal, A. Cancer statistics, 2012. *CA: a cancer journal for clinicians* **62**, 10-29, doi:10.3322/caac.20138 (2012).

40 Polascik, T. J., Oesterling, J. E. & Partin, A. W. Prostate specific antigen: a decade of discovery--what we have learned and where we are going. *The Journal of urology* **162**, 293-306 (1999).

41 Rana, A., Karamanis, K., Lucas, M. G. & Chisholm, G. D. Identification of metastatic disease by T category, gleason score and serum PSA level in patients with carcinoma of the prostate. *British journal of urology* **69**, 277-281 (1992).

42 Schroder, F. H. *et al.* Screening and prostate-cancer mortality in a randomized European study. *The New England journal of medicine* **360**, 1320-1328, doi:10.1056/NEJMoa0810084 (2009).

43 Schroder, F. H. *et al.* Prostate-cancer mortality at 11 years of follow-up. *The New England journal of medicine* **366**, 981-990, doi:10.1056/NEJMoa1113135 (2012).

44 Draisma, G. *et al.* Lead time and overdiagnosis in prostate-specific antigen screening: importance of methods and context. *Journal of the National Cancer Institute* **101**, 374-383, doi:10.1093/jnci/djp001 (2009).

45 Loeb, S. *et al.* Overdiagnosis and Overtreatment of Prostate Cancer. *European urology* **65**, 1046-1055, doi:10.1016/j.eururo.2013.12.062.

46 Frosch, D. L., Kaplan, R. M. & Felitti, V. The evaluation of two methods to facilitate shared decision making for men considering the prostate-specific antigen test. *Journal of general internal medicine* **16**, 391-398 (2001).

47 Ilic, D., Neuberger, M. M., Djulbegovic, M. & Dahm, P. Screening for prostate cancer. *The Cochrane database of systematic reviews*, Cd004720, doi:10.1002/14651858.CD004720.pub3 (2013).

48 European Association of Urology. (2018).

49 Thompson, I. M. *et al.* Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *The New England journal of medicine* **350**, 2239-2246, doi:10.1056/NEJMoa031918 (2004).

50 Epstein, J. I. An update of the Gleason grading system. *J. Urol* **183**, 433-440, doi:10.1016/j.juro.2009.10.046 (2010).

51 Epstein, J. I. *et al.* The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. *The American journal of surgical pathology* **40**, 244-252, doi:10.1097/pas.0000000000000530 (2016).

52 Graefen, M. *et al.* International validation of a preoperative nomogram for prostate cancer recurrence after radical prostatectomy. *J Clin Oncol* **20**, 3206-3212, doi:10.1200/jco.2002.12.019 (2002).

53 Kattan, M. W., Eastham, J. A., Stapleton, A. M., Wheeler, T. M. & Scardino, P. T. A preoperative nomogram for disease recurrence following radical prostatectomy for prostate cancer. *Journal of the National Cancer Institute* **90**, 766-771 (1998).

54 Zelefsky, M. J. *et al.* Pretreatment nomogram predicting ten-year biochemical outcome of three-dimensional conformal radiotherapy and intensity-modulated radiotherapy for prostate cancer. *Urology* **70**, 283-287, doi:10.1016/j.urology.2007.03.060 (2007).

55 Brierley, A., et al.,. (2016).

56 Cooperberg, M. R. *et al.* The University of California, San Francisco Cancer of the Prostate Risk Assessment score: a straightforward and reliable preoperative predictor

of disease recurrence after radical prostatectomy. *The Journal of urology* **173**, 1938-1942, doi:10.1097/01.ju.0000158155.33890.e7 (2005).

57 Cooperberg, M. R., Hilton, J. F. & Carroll, P. R. The CAPRA-S score: A straightforward tool for improved prediction of outcomes after radical prostatectomy. *Cancer* **117**, 5039-5046, doi:10.1002/cncr.26169 (2011).

58 Albertsen, P. C., Hanley, J. A., Gleason, D. F. & Barry, M. J. Competing risk analysis of men aged 55 to 74 years at diagnosis managed conservatively for clinically localized prostate cancer. *Jama* **280**, 975-980 (1998).

59 Albertsen, P. C. Observational studies and the natural history of screen-detected prostate cancer. *Current opinion in urology* **25**, 232-237, doi:10.1097/mou.0000000000000157 (2015).

60 Klotz, L. *et al.* Clinical results of long-term follow-up of a large, active surveillance cohort with localized prostate cancer. *J Clin Oncol* **28**, 126-131, doi:10.1200/jco.2009.24.2180 (2010).

61 Lim, S. K., Kim, K. H., Shin, T. Y. & Rha, K. H. Current status of robot-assisted laparoscopic radical prostatectomy: how does it compare with other surgical approaches? *International journal of urology : official journal of the Japanese Urological Association* **20**, 271-284, doi:10.1111/j.1442-2042.2012.03193.x (2013).

62 Memmelaar, J. Total prostatovesiculectomy; retropubic approach. *The Journal of urology* **62**, 340-348 (1949).

63 Millin, T. Retropubic prostatectomy: a new extravesical technique report on 20 cases. 1945. *The Journal of urology* **167**, 976-979; discussion 980 (2002).

64 Walsh, P. C., Lepor, H. & Eggleston, J. C. Radical prostatectomy with preservation of sexual function: anatomical and pathological considerations. *Prostate* **4**, 473-485 (1983).

65 Muller, S., Gronning, L. E., Nilsen, F. S., Mygland, V. & Patel, H. R. Robotic and minimal access surgery: technology and surgical outcomes of radical prostatectomy for prostate cancer. *Expert review of anticancer therapy* **14**, 1317-1321, doi:10.1586/14737140.2014.965689 (2014).

66 Heidenreich A, B. M., Joniau S, an der Kwast TH, atveev V, Mason MD. *Guidelines on prostate cancer*, <http://www.uroweb.org/fileadmin/tx_eauguidelines/2009/Full/Prostate_Cancer.pdf> (2009).

67 Bill-Axelson, A. *et al.* Radical prostatectomy or watchful waiting in early prostate cancer. *The New England journal of medicine* **370**, 932-942, doi:10.1056/NEJMoa1311593 (2014).

68 Bill-Axelson, A. *et al.* Radical prostatectomy versus watchful waiting in early prostate cancer. *The New England journal of medicine* **364**, 1708-1717, doi:10.1056/NEJMoa1011967 (2011).

69 Wilt, T. J. *et al.* Radical prostatectomy versus observation for localized prostate cancer. *The New England journal of medicine* **367**, 203-213, doi:10.1056/NEJMoa1113162 (2012).

70 Wilt, T. J. *et al.* Follow-up of Prostatectomy versus Observation for Early Prostate Cancer. *The New England journal of medicine* **377**, 132-142, doi:10.1056/NEJMoa1615869 (2017).

71 Stephenson, A. J. *et al.* Predicting the outcome of salvage radiation therapy for recurrent prostate cancer after radical prostatectomy. *J Clin Oncol* **25**, 2035-2041, doi:10.1200/jco.2006.08.9607 (2007).

72 American Cancer Society. *Radiation Therapy for Prostate Cancer*, <https://www.cancer.org/cancer/prostate-cancer/treating/radiation-therapy.html> (2018).

73 Kalbasi, A. *et al.* Dose-Escalated Irradiation and Overall Survival in Men With Nonmetastatic Prostate Cancer. *JAMA oncology* **1**, 897-906, doi:10.1001/jamaoncol.2015.2316 (2015).

74 Hamdy, F. C. *et al.* 10-Year Outcomes after Monitoring, Surgery, or Radiotherapy for Localized Prostate Cancer. *The New England journal of medicine* **375**, 1415-1424, doi:10.1056/NEJMoa1606220 (2016).

75 Toussi, A. *et al.* Standardizing the Definition of Biochemical Recurrence after Radical Prostatectomy-What Prostate Specific Antigen Cut Point Best Predicts a Durable Increase and Subsequent Systemic Progression? *The Journal of urology* **195**, 1754-1759, doi:10.1016/j.juro.2015.12.075 (2016).

76 Gallee, M. P. *et al.* Variation of prostate-specific antigen expression in different tumour growth patterns present in prostatectomy specimens. *Urological research* **18**, 181-187 (1990).

77 Yamamoto, S. *et al.* M1 prostate cancer with a serum level of prostate-specific antigen less than 10 ng/mL. *International journal of urology : official journal of the Japanese Urological Association* **8**, 374-379 (2001).

78 Pagliarulo, V. *et al.* Contemporary role of androgen deprivation therapy for prostate cancer. *European urology* **61**, 11-25, doi:10.1016/j.eururo.2011.08.026 (2012).

79 Smith, M. R. *et al.* Natural history of rising serum prostate-specific antigen in men with castrate nonmetastatic prostate cancer. *J Clin Oncol* **23**, 2918-2925, doi:10.1200/jco.2005.01.529 (2005).

80 Joyce, J. A. & Fearon, D. T. T cell exclusion, immune privilege, and the tumor microenvironment. *Science (New York, N.Y.)* **348**, 74-80, doi:10.1126/science.aaa6204 (2015).

81 Turley, S. J., Cremasco, V. & Astarita, J. L. Immunological hallmarks of stromal cells in the tumour microenvironment. *Nature reviews. Immunology* **15**, 669-682, doi:10.1038/nri3902 (2015).

82 Quail, D. F. & Joyce, J. A. Microenvironmental regulation of tumor progression and metastasis. *Nature medicine* **19**, 1423-1437, doi:10.1038/nm.3394 (2013).

83 Bremnes, R. M. *et al.* The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. *J. Thorac. Oncol* **6**, 209-217, doi:10.1097/JTO.0b013e3181f8a1bd [doi] (2011).

84 Liotta, L. A. & Kohn, E. C. The microenvironment of the tumour-host interface. *Nature* **411**, 375-379, doi:10.1038/35077241 (2001).

85 Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).

86 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:S0092-8674(11)00127-9 [pii];10.1016/j.cell.2011.02.013 [doi] (2011).

87 Folkman, J. Tumor angiogenesis: therapeutic implications. *The New England journal of medicine* **285**, 1182-1186, doi:10.1056/nejm197111182852108 (1971).

88 Folkman, J. What is the evidence that tumors are angiogenesis dependent? *Journal of the National Cancer Institute* **82**, 4-6 (1990).

89 Carmeliet, P. & Jain, R. K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249-257, doi:10.1038/35025220 (2000).

90 Holmgren, L., O'Reilly, M. S. & Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nature medicine* **1**, 149-153 (1995).

91 Taverna, G., Grizzi, F., Colombo, P. & Graziotti, P. Is angiogenesis a hallmark of prostate cancer? *Frontiers in oncology* **3**, 15, doi:10.3389/fonc.2013.00015 (2013).

92 Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-364 (1996).

93 Folkman, J. Angiogenesis: an organizing principle for drug discovery? *Nature reviews. Drug discovery* **6**, 273-286, doi:10.1038/nrd2115 (2007).

94 Donnem, T. *et al.* Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment? *Cancer medicine* **2**, 427-436, doi:10.1002/cam4.105 (2013).

95 Li, Y. & Cozzi, P. J. Angiogenesis as a strategic target for prostate cancer therapy. *Medicinal research reviews* **30**, 23-66, doi:10.1002/med.20161 (2010).

96 Chung, L. W., Baseman, A., Assikis, V. & Zhau, H. E. Molecular insights into prostate cancer progression: the missing link of tumor microenvironment. *The Journal of urology* **173**, 10-20, doi:10.1097/01.ju.0000141582.15218.10 (2005).

97 Humphrey, P. A. Gleason grading and prognostic factors in carcinoma of the prostate. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* **17**, 292-306, doi:10.1038/modpathol.3800054 (2004).

98 Russo, G., Mischi, M., Scheepens, W., De la Rosette, J. J. & Wijkstra, H. Angiogenesis in prostate cancer: onset, progression and imaging. *BJU international* **110**, E794-808, doi:10.1111/j.1464-410X.2012.11444.x (2012).

99 Zhan, P., Ji, Y. N. & Yu, L. K. VEGF is associated with the poor survival of patients with prostate cancer: a meta-analysis. *Translational andrology and urology* **2**, 99-105, doi:10.3978/j.issn.2223-4683.2013.06.03 (2013).

100 Bok, R. A. *et al.* Vascular endothelial growth factor and basic fibroblast growth factor urine levels as predictors of outcome in hormone-refractory prostate cancer patients: a cancer and leukemia group B study. *Cancer Res* **61**, 2533-2536 (2001).

101 George, D. J. *et al.* The prognostic significance of plasma interleukin-6 levels in patients with metastatic hormone-refractory prostate cancer: results from cancer and leukemia group B 9480. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 1815-1820, doi:10.1158/1078-0432.ccr-04-1560 (2005).

102 Kerbel, R. S. in *ASCO Annual Meeting* (2016).

103 Ferrara, N. & Adamis, A. P. Ten years of anti-vascular endothelial growth factor therapy. *Nature reviews. Drug discovery* **15**, 385-403, doi:10.1038/nrd.2015.17 (2016).

104 Kerbel, R. S. Tumor angiogenesis. *The New England journal of medicine* **358**, 2039-2049, doi:10.1056/NEJMra0706596 (2008).

105 Lainakis, G. & Bamias, A. Targeting angiogenesis in renal cell carcinoma. *Current cancer drug targets* **8**, 349-358 (2008).

106 Gettman, M. T. *et al.* Role of microvessel density in predicting recurrence in pathologic Stage T3 prostatic adenocarcinoma. *Urology* **54**, 479-485, doi:S0090-4295(99)00202-2 [pii] (1999).

107 Weidner, N., Carroll, P. R., Flax, J., Blumenfeld, W. & Folkman, J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am. J. Pathol* **143**, 401-409 (1993).

108 Ky, B., Vejpongsa, P., Yeh, E. T., Force, T. & Moslehi, J. J. Emerging paradigms in cardiomyopathies associated with cancer therapies. *Circulation research* **113**, 754-764, doi:10.1161/circresaha.113.300218 (2013).

109 McKay, R. R. *et al.* A Randomized Phase II Trial of Short-Course Androgen Deprivation Therapy With or Without Bevacizumab for Patients With Recurrent Prostate Cancer After Definitive Local Therapy. *J Clin Oncol* **34**, 1913-1920, doi:10.1200/jco.2015.65.3154 (2016).

110 Kelly, W. K. *et al.* Randomized, double-blind, placebo-controlled phase III trial comparing docetaxel and prednisone with or without bevacizumab in men with metastatic castration-resistant prostate cancer: CALGB 90401. *J. Clin. Oncol* **30**, 1534-1540, doi:JCO.2011.39.4767 [pii];10.1200/JCO.2011.39.4767 [doi] (2012).

111 Tannock, I. F. *et al.* Aflibercept versus placebo in combination with docetaxel and prednisone for treatment of men with metastatic castration-resistant prostate cancer

(VENICE): a phase 3, double-blind randomised trial. *Lancet Oncol* **14**, 760-768, doi:10.1016/s1470-2045(13)70184-0 (2013).

112 Smith, M. R. *et al.* Cabozantinib in Chemotherapy-Pretreated Metastatic Castration-Resistant Prostate Cancer: Results of a Phase II Nonrandomized Expansion Study. *J. Clin. Oncol* **32**, 3391-3399, doi:JCO.2013.54.5954 [pii];10.1200/JCO.2013.54.5954 [doi] (2014).

113 Smith, M. *et al.* Phase III Study of Cabozantinib in Previously Treated Metastatic Castration-Resistant Prostate Cancer: COMET-1. *J Clin Oncol* **34**, 3005-3013, doi:10.1200/jco.2015.65.5597 (2016).

114 Ferrara, N., Hillan, K. J., Gerber, H. P. & Novotny, W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat. Rev. Drug Discov* **3**, 391-400, doi:10.1038/nrd1381 [doi];nrd1381 [pii] (2004).

115 Eisma, R. J., Spiro, J. D. & Kreutzer, D. L. Vascular endothelial growth factor expression in head and neck squamous cell carcinoma. *Am. J. Surg* **174**, 513-517, doi:S0002961097001669 [pii] (1997).

116 Fontanini, G. *et al.* Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: a prospective study. *J. Natl. Cancer Inst* **89**, 881-886 (1997).

117 Manders, P. *et al.* The prognostic value of vascular endothelial growth factor in 574 node-negative breast cancer patients who did not receive adjuvant systemic therapy. *Br. J. Cancer* **87**, 772-778, doi:10.1038/sj.bjc.6600555 [doi] (2002).

118 Takahashi, Y., Kitadai, Y., Bucana, C. D., Cleary, K. R. & Ellis, L. M. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* **55**, 3964-3968 (1995).

119 Felleskatalogen. (2017).

120 Acuna, P. *et al.* [Vascular endothelial growth factor in malignant and non malignant prostatic tissue: Association with tumor recurrence at one year after prostatectomy]. *Rev. Med. Chil* **141**, 153-159, doi:S0034-98872013000200002 [pii];10.4067/S0034-98872013000200002 [doi] (2013).

121 Gravdal, K., Halvorsen, O. J., Haukaas, S. A. & Akslen, L. A. Proliferation of immature tumor vessels is a novel marker of clinical progression in prostate cancer. *Cancer Res* **69**, 4708-4715, doi:69/11/4708 [pii];10.1158/0008-5472.CAN-08-4417 [doi] (2009).

122 Peyromaure, M., Camparo, P., Badoual, C., Descazeaud, A. & Dinh-Xuan, A. T. The expression of vascular endothelial growth factor is associated with the risk of cancer progression after radical prostatectomy. *BJU. Int* **99**, 1150-1153, doi:BJU6734 [pii];10.1111/j.1464-410X.2007.06734.x [doi] (2007).

123 Talagas, M. *et al.* VEGFR1 and NRP1 endothelial expressions predict distant relapse after radical prostatectomy in clinically localized prostate cancer. *Anticancer Res* **33**, 2065-2075, doi:33/5/2065 [pii] (2013).

124 Teishima, J. *et al.* Accumulation of FGF9 in prostate cancer correlates with epithelial-to-mesenchymal transition and induction of VEGF-A expression. *Anticancer Res* **34**, 695-700, doi:34/2/695 [pii] (2014).

125 Vergis, R. *et al.* Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. *Lancet Oncol* **9**, 342-351, doi:S1470-2045(08)70076-7 [pii];10.1016/S1470-2045(08)70076-7 [doi] (2008).

126 Wang, Q., Diao, X., Sun, J. & Chen, Z. Stromal cell-derived factor-1 and vascular endothelial growth factor as biomarkers for lymph node metastasis and poor cancer-specific survival in prostate cancer patients after radical prostatectomy. *Urol. Oncol* **31**, 312-317, doi:S1078-1439(11)00007-X [pii];10.1016/j.urolonc.2011.01.006 [doi] (2013).

127 Wu, T. T. *et al.* Expression of vascular endothelial growth factor in Taiwanese benign and malignant prostate tissues. *J. Chin Med. Assoc* **70**, 380-384, doi:S1726-4901(08)70024-8 [pii];10.1016/S1726-4901(08)70024-8 [doi] (2007).

128 Appiah-Kubi, K. *et al.* Platelet-derived growth factor receptor/platelet-derived growth factor (PDGFR/PDGF) system is a prognostic and treatment response biomarker with multifarious therapeutic targets in cancers. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **37**, 10053-10066, doi:10.1007/s13277-016-5069-z (2016).

129 Andrae, J., Gallini, R. & Betsholtz, C. Role of platelet-derived growth factors in physiology and medicine. *Genes & development* **22**, 1276-1312, doi:10.1101/gad.1653708 (2008).

130 Chen, P. H., Chen, X. & He, X. Platelet-derived growth factors and their receptors: structural and functional perspectives. *Biochimica et biophysica acta* **1834**, 2176-2186, doi:10.1016/j.bbapap.2012.10.015 (2013).

131 Chu, J. S. *et al.* Expression and prognostic value of VEGFR-2, PDGFR-beta, and c-Met in advanced hepatocellular carcinoma. *Journal of experimental & clinical cancer research : CR* **32**, 16, doi:10.1186/1756-9966-32-16 (2013).

132 Cimpean, A. M. *et al.* Platelet-derived growth factor and platelet-derived growth factor receptor-alpha expression in the normal human thymus and thymoma. *International journal of experimental pathology* **92**, 340-344, doi:10.1111/j.1365-2613.2011.00777.x (2011).

133 Jechlinger, M. *et al.* Autocrine PDGFR signaling promotes mammary cancer metastasis. *The Journal of clinical investigation* **116**, 1561-1570, doi:10.1172/jci24652 (2006).

134 Kubo, T. *et al.* Platelet-derived growth factor receptor as a prognostic marker and a therapeutic target for imatinib mesylate therapy in osteosarcoma. *Cancer* **112**, 2119-2129, doi:10.1002/cncr.23437 (2008).

135 Matei, D. *et al.* PDGF BB induces VEGF secretion in ovarian cancer. *Cancer biology & therapy* **6**, 1951-1959 (2007).

136 Abramsson, A., Lindblom, P. & Betsholtz, C. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. *The Journal of clinical investigation* **112**, 1142-1151, doi:10.1172/jci18549 (2003).

137 Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A. & Betsholtz, C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development (Cambridge, England)* **126**, 3047-3055 (1999).

138 Roskoski Jr., R. The role of small molecule platelet-derived growth factor receptor (PDGFR) inhibitors in the treatment of neoplastic disorders. *Pharmacological Research*, doi:https://doi.org/10.1016/j.phrs.2018.01.021 (2018).

139 Mathew, P. *et al.* Accelerated disease progression in prostate cancer and bone metastases with platelet-derived growth factor receptor inhibition: observations with tandutinib. *Cancer chemotherapy and pharmacology* **68**, 889-896, doi:10.1007/s00280-011-1567-2 (2011).

140 Rosenberg, A. & Mathew, P. Imatinib and prostate cancer: lessons learned from targeting the platelet-derived growth factor receptor. *Expert. Opin. Investig. Drugs* **22**, 787-794, doi:10.1517/13543784.2013.787409 [doi] (2013).

141 Huang, W. *et al.* A novel function for platelet-derived growth factor D: induction of osteoclastic differentiation for intraosseous tumor growth. *Oncogene* **31**, 4527-4535, doi:onc2011573 [pii];10.1038/onc.2011.573 [doi] (2012).

142 Hagglof, C. *et al.* Stromal PDGFRbeta expression in prostate tumors and non-malignant prostate tissue predicts prostate cancer survival. *PLoS. One* **5**, e10747, doi:10.1371/journal.pone.0010747 [doi] (2010).

143 Hammond, S. M. RNAi, microRNAs, and human disease. *Cancer chemotherapy and pharmacology* **58 Suppl 1**, s63-68, doi:10.1007/s00280-006-0318-2 (2006).

144 Hammond, S. M. An overview of microRNAs. *Advanced drug delivery reviews* **87**, 3-14, doi:10.1016/j.addr.2015.05.001 (2015).

145 Pichler, M. & Calin, G. A. MicroRNAs in cancer: from developmental genes in worms to their clinical application in patients. *British journal of cancer* **113**, 569-573, doi:10.1038/bjc.2015.253 (2015).

146 Zhang, W., Liu, J. & Wang, G. The role of microRNAs in human breast cancer progression. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* **35**, 6235-6244, doi:10.1007/s13277-014-2202-8 (2014).

147 Orang, A. V., Safaralizadeh, R. & Hosseinpour Feizi, M. A. Insights into the diverse roles of miR-205 in human cancers. *Asian Pacific journal of cancer prevention : APJCP* **15**, 577-583 (2014).

148 Salajegheh, A. *et al.* Modulatory role of miR-205 in angiogenesis and progression of thyroid cancer. *Journal of molecular endocrinology* **55**, 183-196, doi:10.1530/jme-15-0182 (2015).

149 Hu, Y. *et al.* miRNA-205 targets VEGFA and FGF2 and regulates resistance to chemotherapeutics in breast cancer. *Cell death & disease* **7**, e2291, doi:10.1038/cddis.2016.194 (2016).

150 Christopher, A. F. *et al.* MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *Perspectives in clinical research* **7**, 68-74, doi:10.4103/2229-3485.179431 (2016).

151 Gandellini, P. *et al.* miR-205 hinders the malignant interplay between prostate cancer cells and associated fibroblasts. *Antioxidants & redox signaling* **20**, 1045-1059, doi:10.1089/ars.2013.5292 (2014).

152 Gandellini, P. *et al.* miR-205 regulates basement membrane deposition in human prostate: implications for cancer development. *Cell death and differentiation* **19**, 1750-1760, doi:10.1038/cdd.2012.56 (2012).

153 Hagman, Z. *et al.* miR-205 negatively regulates the androgen receptor and is associated with adverse outcome of prostate cancer patients. *British journal of cancer* **108**, 1668-1676, doi:10.1038/bjc.2013.131 (2013).

154 Hulf, T. *et al.* Epigenetic-induced repression of microRNA-205 is associated with MED1 activation and a poorer prognosis in localized prostate cancer. *Oncogene* **32**, 2891-2899, doi:10.1038/onc.2012.300 (2013).

155 Kalogirou, C. *et al.* MiR-205 is progressively down-regulated in lymph node metastasis but fails as a prognostic biomarker in high-risk prostate cancer. *International journal of molecular sciences* **14**, 21414-21434, doi:10.3390/ijms141121414 (2013).

156 Larne, O. *et al.* miQ--a novel microRNA based diagnostic and prognostic tool for prostate cancer. *International journal of cancer* **132**, 2867-2875, doi:10.1002/ijc.27973 (2013).

157 Martens-Uzunova, E. S. *et al.* Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. *Oncogene* **31**, 978-991, doi:10.1038/onc.2011.304 (2012).

158 Tucci, P. *et al.* Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 15312-15317, doi:10.1073/pnas.1110977109 (2012).

159 Melbo-Jorgensen, C. *et al.* Stromal expression of MiR-21 predicts biochemical failure in prostate cancer patients with Gleason score 6. *PloS one* **9**, e113039, doi:10.1371/journal.pone.0113039 (2014).

160 Stephenson, A. J. *et al.* Defining biochemical recurrence of prostate cancer after radical prostatectomy: a proposal for a standardized definition. *J Clin Oncol* **24**, 3973-3978, doi:10.1200/jco.2005.04.0756 (2006).

161 Helpap, B. & Egevad, L. Modified Gleason grading. An updated review. *Histol. Histopathol* **24**, 661-666 (2009).

162 Bremnes, R. M. *et al.* High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. *J. Clin. Oncol* **20**, 2417-2428 (2002).

163 Simon, R. Applications of tissue microarray technology. *Methods in molecular biology (Clifton, N.J.)* **664**, 1-16, doi:10.1007/978-1-60761-806-5_1 (2010).

164 Bubendorf, L., Nocito, A., Moch, H. & Sauter, G. Tissue microarray (TMA) technology: miniaturized pathology archives for high-throughput in situ studies. *The Journal of pathology* **195**, 72-79, doi:10.1002/path.893 (2001).

165 Torhorst, J. *et al.* Tissue microarrays for rapid linking of molecular changes to clinical endpoints. *The American journal of pathology* **159**, 2249-2256, doi:10.1016/s0002-9440(10)63075-1 (2001).

166 Howat, W. J. & Wilson, B. A. Tissue fixation and the effect of molecular fixatives on downstream staining procedures. *Methods (San Diego, Calif.)* **70**, 12-19, doi:10.1016/j.ymeth.2014.01.022 (2014).

167 Schlomm, T. *et al.* Marked gene transcript level alterations occur early during radical prostatectomy. *European urology* **53**, 333-344, doi:10.1016/j.eururo.2007.03.075 (2008).

168 Sauter, G. Representativity of TMA studies. *Methods in molecular biology (Clifton, N.J.)* **664**, 27-35, doi:10.1007/978-1-60761-806-5_3 (2010).

169 Kallioniemi, O. P., Wagner, U., Kononen, J. & Sauter, G. Tissue microarray technology for high-throughput molecular profiling of cancer. *Human molecular genetics* **10**, 657-662 (2001).

170 Giltnane, J. M. & Rimm, D. L. Technology insight: Identification of biomarkers with tissue microarray technology. *Nature clinical practice. Oncology* **1**, 104-111, doi:10.1038/ncponc0046 (2004).

171 Brennan, D. J., O'Connor, D. P., Rexhepaj, E., Ponten, F. & Gallagher, W. M. Antibody-based proteomics: fast-tracking molecular diagnostics in oncology. *Nature reviews. Cancer* **10**, 605-617, doi:10.1038/nrc2902 (2010).

172 Bordeaux, J. *et al.* Antibody validation. *BioTechniques* **48**, 197-209, doi:10.2144/000113382 (2010).

173 Goldsmith, J. D., Fitzgibbons, P. L. & Swanson, P. E. Principles of Analytic Validation of Clinical Immunohistochemistry Assays. *Advances in anatomic pathology* **22**, 384-387, doi:10.1097/pap.0000000000000094 (2015).

174 Jensen, E. Technical review: In situ hybridization. *Anatomical record (Hoboken, N.J. : 2007)* **297**, 1349-1353, doi:10.1002/ar.22944 (2014).

175 S, P. *Axitinib Before Surgery in Treating Patients With High-Risk Prostate Cancer*, <https://clinicaltrials.gov/ct2/show/NCT01385059> (2014).

176 ClinicalTrials.gov. *Neoadjuvant Axitinib in Prostate Cancer*, <https://clinicaltrials.gov/ct2/show/NCT01409200?term=axitinib+prostate&rank=1> (2017).

177 McKay, R. R. *et al.* A Randomized Phase II Trial of Short-Course Androgen Deprivation Therapy With or Without Bevacizumab for Patients With Recurrent Prostate Cancer After Definitive Local Therapy. *J Clin Oncol*, doi:10.1200/jco.2015.65.3154 (2016).

178 Sethi, S., Macoska, J., Chen, W. & Sarkar, F. H. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *American journal of translational research* **3**, 90-99 (2010).

179 Ustach, C. V. *et al.* A novel signaling axis of matriptase/PDGF-D/ss-PDGFR in human prostate cancer. *Cancer Res* **70**, 9631-9640, doi:10.1158/0008-5472.can-10-0511 (2010).

180 Najy, A. J. *et al.* Cediranib inhibits both the intraosseous growth of PDGF D-positive prostate cancer cells and the associated bone reaction. *Prostate* **72**, 1328-1338, doi:10.1002/pros.22481 (2012).

181 Conley-LaComb, M. K. *et al.* PTEN regulates PDGF ligand switch for beta-PDGFR signaling in prostate cancer. *The American journal of pathology* **180**, 1017-1027, doi:10.1016/j.ajpath.2011.11.021 (2012).

182 Correction: A Network Biology Approach Identifies Molecular Cross-Talk between Normal Prostate Epithelial and Prostate Carcinoma Cells. *PLoS computational biology* **12**, e1005172, doi:10.1371/journal.pcbi.1005172 (2016).

183 Macara, I. G., Guyer, R., Richardson, G., Huo, Y. & Ahmed, S. M. Epithelial homeostasis. *Current biology : CB* **24**, R815-825, doi:10.1016/j.cub.2014.06.068 (2014).

184 Trevino, V. *et al.* A Network Biology Approach Identifies Molecular Cross-Talk between Normal Prostate Epithelial and Prostate Carcinoma Cells. *PLoS computational biology* **12**, e1004884, doi:10.1371/journal.pcbi.1004884 (2016).

Paper 1

Stromal Expression of VEGF-A and VEGFR-2 in Prostate Tissue Is Associated With Biochemical and Clinical Recurrence After Radical Prostatectomy

Yngve Nordby,[1,2]* Sigve Andersen,[1,3] Elin Richardsen,[4,5] Nora Ness,[5] Samer Al-Saad,[4,5] Christian Melbø-Jørgensen,[5] Hiten RH Patel,[1,2] Tom Dønnem,[1,3] Lill-Tove Busund,[4,5] and Roy M Bremnes[1,3]

[1]*Department Clinical Medicine, The Arctic University of Norway, Tromso, Norway*
[2]*Department Urology, University Hospital of North Norway, Tromso, Norway*
[3]*Department Oncology, University Hospital of North Norway, Tromso, Norway*
[4]*Department Clinical Pathology, University Hospital of North Norway, Tromso, Norway*
[5]*Department Medical Biology, The Arctic University of Norway, Tromso, Norway*

BACKGROUND. There is probably significant overtreatment of patients with prostate cancer due to a lack of sufficient diagnostic tools to predict aggressive disease. Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are potent mediators of angiogenesis and tumor proliferation, but have been examined to a limited extent in large prostate cancer studies. Meanwhile, recent promising results on VEGFR-2 inhibition have highlighted their importance, leading to the need for further investigations regarding their expression and prognostic impact.

DESIGN. Using tissue microarray and immunohistochemistry, the expression of VEGFs (VEGF-A and VEGF-C) and their receptors (VEGFR-2 and VEGFR-3) were measured in neoplastic tissue and corresponding stroma from radical prostatectomy specimens in 535 Norwegian patients. Their expression was evaluated semiquantatively and associations with event-free survival were calculated.

RESULTS. High expression of 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 VEGF-A, VEGFR-2 or both in stroma was independently associated with a higher incidence of biochemical failure (HR = 1.77, $P = 0.011$).

CONCLUSIONS. This large study highlights the prognostic importance of VEGF-A and VEGFR-2 stromal expression. Analyses of these biomarkers may help distinguish which patients will benefit from radical treatment. Together with previous studies showing efficiency of targeting VEGFR-2 in prostate cancer, this study highlights its potential as a target for therapy, and may aid in future selection of prostate cancer patients for novel anti-angiogenic treatment. *Prostate 75:1682–1693, 2015.* © 2015 Wiley Periodicals, Inc.

KEY WORDS: **prostate cancer; veg; angiogenesis; tissue microarray; immunohistochemistry**

INTRODUCTION

Prostate cancer (PC) is the most frequent cancer in men, and the second most common cause of male cancer death in developed countries [1]. However, once diagnosed with PC, the mortality of PC is estimated to be only 2–3%. The challenge is to

*Correspondence to: Yngve Nordby, MD MSc, Dept Urology, University Hospital of North Norway, P.O.B. 93, N-9038 Tromso, Norway.
E-mail: yngve.nordby@unn.no
Received 23 January 2015; Accepted 5 June 2015
DOI 10.1002/pros.23048
Published online 13 August 2015 in Wiley Online Library (wileyonlinelibrary.com).

distinguish between patients with an aggressive and potentially deadly form of PC, versus patients with more indolent disease.

Clinical prognostic risk stratification using preoperative PSA value, cTNM and Gleason score are well-established, but imprecise. This results in a significant overtreatment (radical therapy), but possibly also undertreatment of some patients [2–4]. There is a need for better prognostic tools to aid in the prediction of which patients will benefit from curative treatment.

Angiogenesis is a well-studied hallmark of cancer [5]. Without sufficient blood flow, the malignant tumor cannot grow to a self-sustaining tumor of significant size. The vascular endothelial growth factor-A (VEGF-A) is a central regulator of tumor--induced angiogenesis and is critical for tumor growth and metastasis [6]. The vascular endothelial growth factor receptor-2 (VEGFR-2) plays an important role in angiogenesis, endothelial cell proliferation, migration, and survival.

VEGF-A overexpression has been associated with tumor progression and poor prognosis in colorectal carcinoma [7], breast cancer [8], lung cancer, [9] and in squamous cell carcinoma of the head and neck [10]. For prostate cancer, the few previous clinicopathological studies of VEGF expression have not yielded consistent results. Few previous studies have evaluated the expression of VEGF-A in epithelium and its association to relapse from PC [11–15]. Stromal expression of VEGF-A in PC has hardly been studied. Wu et al. observed that high Gleason grade tumors and advanced disease had significantly higher frequency of VEGF expression in stroma but not in glandular epithelium [16]. However, two recent studies found no association between VEGF-A expression and PC relapse [17,18]. VEGFR-2 is known to be expressed in vascular endothelium, particularly enriched for neoangiogenesis with cancer [19].

In a randomized phase 2 study, the MET/VEGFR-2 inhibitor cabozantinib led to reduced pain in 57% of patients with metastatic castration-resistant prostate cancer (mCRPC) [20], but preliminary results failed to show improvement in overall survival in the phase 3 study COMET-1 [21]. In addition, the anti-angiogenic drug tasquinimod has also showed encouraging results in a phase 2 study [22]. Also, tasquinimod reduced the risk of radiographic cancer progression and death compared to placebo in men with mCRPC. However, the drug did not extend overall survival [23]. The VEGFR-2 inhibitor ramucirumab inhibited cell proliferation in vitro, as well as tumor progression in mouse xenograft models of human cancer. A phase 2 study in prostate cancer found ramucirumab to have encouraging results, but to our knowledge the results have so far only been published as an abstract [24]. Ramucirumab was recently approved by the FDA as treatment for advanced non-small cell lung cancer.

As previous studies have shown conflicting results, we systematically investigated both tumor and stromal expression of the anti-angiogenic ligands VEGF-A and VEGF-C, and their respective receptors VEGFR-2 and VEGFR-3 as biomarkers in a large cohort of 535 prostatectomized patients. Herein, we explored the associations with clinical outcome in terms of biochemical recurrence, clinical recurrence, and death from PC.

MATERIALS AND METHODS

Patients

671 patients who underwent radical prostatectomy with curative intent for adenocarcinoma in the prostate from 1995 to 2005 were retrospectively identified from the Departments of Pathology at the University Hospital of Northern Norway (n = 267), Nordland Hospital (n = 63), St. Olavs Hospital (n = 330) and Levanger Hospital (n = 11). Of these, 136 patients were excluded due to (i) previous non-superficial cancer within 5 years of PC diagnosis (n = 4), (ii) radiotherapy to the pelvis prior to surgery (n = 1), (iii) inadequate paraffin-embedded tissue blocks (n = 130), and (iv) lack of follow-up data (n = 1), leaving a total of 535 patients included in the study. None of the patients had received pre-operative hormonal therapy. The cohort is thoroughly described in a previous paper [25].

We collected relevant data from medical journals: Demographical data, age at surgery, previous medical history, retropubic, or perineal surgery, and preoperative PSA measured immediately before surgery. Further, we collected data until the last follow-up date (31.12.12) or until patients' death. The patients' clinical outcome was recorded for a median follow-up of 7.4 years (range 0.5–16 years). These data were: Postoperative PSA values, as well as postoperative therapy (radio-, hormonal, and/or chemotherapy). The following endpoints were used: Biochemical failure (BF) defined as postoperative PSA $\geq$0.4 or intervention with adjuvant therapy; Clinical failure (CF) defined as clinically palpable tumor 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.

Tissues and Tissue Microarray Construction

Tumor tissues, consisting of formalin-fixed paraffin-embedded blocks of prostate tissue from the

patients' prostatectomies, were collected from the archives of the pathological departments. One experienced pathologist (E.R.) reevaluated the prostate samples and classified them according to the updated WHO guidelines [26,27]. Two pathologists (E.R. and L.T.B.) identified the most representative areas of cancer epithelium cells and tumor-near stroma. Each area was biopsied with at least two 0.6 mm cores. In addition, two biopsies from normal tissue of each patient were also sampled. The cores were arranged in tissue microarray (TMA) blocks for large-scale analysis. To include all core samples, TMA blocks were constructed. Multiple 4 μm sections were cut with a Micron microtone (HM355S), affixed to glass slides and stained by specific antibodies for immunohistochemical analysis (IHC). The detailed methodology has been reported previously [28].

Immunohistochemistry

The antibodies used were VEGF-A rabbit polyclonal (Thermo–Fisher; cat.no AB-9031; 1:50 dilution), VEGF-C rabbit polyclonal (Invitrogen; cat.no 18-2255; 1:25 dilution), VEGFR-2 rabbit monoclonal (Cell Signaling Technology; clone 55B11; cat.no #2479; 1:100 dilution) and VEGFR-3 mouse monoclonal (Merck Millipore; clone 9D9F9; cat.no MAB-3757; 1:100 dilution). 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% H_2O_2 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 450 W. 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 BenchMark 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.

For validation, two different controls for our staining method were applied. First, control staining of the sections with an isotype-matched control antibody without the primary antibody. Secondly, multiple organ tissue microarray as positive and negative tissue controls were used to verify the specificity. The positive tissue controls comprised of human angiosarcoma for VEGF-A and VEGFR-2, colon carcinoma for VEGF-C and lymph node for VEGFR-3.

Scoring of Immunohistochemistry

The IHC stained TMA slides were scanned and digitalized using the ARIOL imaging system (Applied Imaging Corp., San Jose, CA), and uploaded into the ARIOL software. Two pathologists (E.R, S.A-S.) independently and semiquantatively scored viable parts of each anonymized core by light microscopy. The pathologists 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. The core was scored as "missing" if the core was missing or considered of insufficient quality to score by both observers. A final score for marker expression in both tumor epithelium (tumor) and tumor-near stroma (stroma) for each patient was calculated using the mean values of the observers' scoring of the patients cores. Scoring of IHC cores were dichotomized to low and high expressions. Cut-off values were chosen in order to secure statistically sufficient numbers in each group. In general, there was a low expression of VEGF-A in the tumor stromal areas (cut-off 0.63). For VEGFR-2, there was a high expression in tumor stromal areas (cut-off 2.17), and a low expression in tumor epithelial areas (cut-off 0.7).

Statistical Methods

SPSS 21.0.0 (Chicago, IL) was used for all statistical analyses. Correlations were analyzed using Spearman's rank correlation coefficient. Univariate survival analyses were done by the Kaplan–Meier method, and the statistical significant difference between survival curves was assessed by the log-rank test. Presentation of the survival curves were terminated at 134 months, due to less than 10% of patients at risk after this point. The significance level (P-value) was not corrected for multiply hypotheses testing, due to a relatively large number of patients and few hypotheses giving little chance for Type I errors. 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.

Ethics

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

RESULTS

Clinicopathological Variables and Patient Characteristics

The patients' clinicopathological data are presented in the first part of Table I. Median age at surgery was 62 (47–75). The prostatectomies were retropubic in 81% of cases, and perineal in 19% of cases. At the last follow-up, 32% of the patients had BF, 6.7% of the patients had CF, and 2.8% of the patients had PCD. Median PSA was 8.8 (range 0.7–104) and the median tumor size was 20 mm (2.0–50).

Expressions and Correlations

The staining of VEGF-A was both nuclear and cytoplasmic. There was generally a low expression of VEGF-A in tumor stromal areas compared with VEGFR-2, which was strongly expressed. The staining intensity of VEGF-C was restricted to granular cytoplasmic staining in a few endothelial cells. For VEGFR-3 there was a strong nuclear staining intensity and a weaker cytoplasmic expression. Representative light microscopic examples of normal tissue as well as weak and strong expression of VEGF-A and VEGFR-2 in epithelium and stroma are shown in Figure 1. None of the biomarkers or their combinations had any direct correlation to any of the clinicopathological variables.

In the control cores, there was in general no expression of VEGF-A and VEGFR-2 in normal epithelium or stroma (Fig. 1). VEGFR-2 was expressed in vascular endothelium in both normal and cancerous prostate specimen, as expected from previous studies [29].

In 45% of the cases where VEGF-A was highly expressed in stroma, epithelium was also highly expressed, leaving 55% of the cases where high expression occurred in the stroma alone. Besides, there was no significant correlation between positive VEGF-A staining in the epithelium versus stroma ($P = 0.074$).

For VEGFR-2, high epithelial expression was observed along with high stromal expression in 55% of the cases, leaving 45% of the cases with high expression in stroma alone. There was no significant correlation between positive VEGFR-2 staining in epithelium versus stroma ($P = 0.184$).

Based on the staining distribution and the absence of correlation between epithelial and stromal staining, the IHC staining was considered to be specific. Besides, there was no expression of VEGF-A and VEGFR-2 in control cores of normal prostate tissue.

Univariate Analyses

Results for the clinicopathological variables are presented in Table I. For BF, significant prognostic factors were pT-stage ($P < 0.001$), pN-stage ($P < 0.001$), preoperative PSA ($P < 0.001$), Gleason score ($P < 0.001$), tumor size ($P < 0.001$), perineural infiltration ($P < 0.001$), positive surgical margin [($P = 0.040$); subclasses: apical ($P = 0.042$) and non-apical margins ($P < 0.001$)] and vascular infiltration ($P < 0.001$). For CF, significant prognostic factors were pT-stage ($P < 0.001$), pN-stage ($P < 0.001$), Gleason score ($P < 0.001$), tumor size ($P < 0.013$), perineural infiltration ($P < 0.001$), positive surgical margin [($P = 0.031$); with subclass non-apical margin ($P < 0.001$)] and vascular infiltration ($P < 0.001$). The significant prognostic factors for PCD were pT-stage ($P = 0.027$), pN-stage ($P < 0.001$), Gleason score ($P < 0.001$), perineural infiltration ($P = 0.002$), non-apical positive surgical margin ($P = 0.029$) and vascular infiltration ($P = 0.009$).

Results from the univariate analyses of molecular markers according to BF, CF and PCD endpoints are presented in Table I and Figures 2, 3 and 4. Patients with high expression of VEGF-A in stroma ($P = 0.013$), high expression of VEGFR-2 in stroma ($P = 0.032$) and a combination of high expression of either VEGF-A or VEFGR-2 in stroma ($P = 0.003$) had significantly worse outcome regarding BF. For CF, patients with high expression of VEGFR-2 in stroma ($P = 0.031$) and high expression of VEGFR-2 in either stroma, epithelium or both ($P = 0.029$) had a significantly worse outcome. None of the markers were significantly associated with worse outcome regarding PCD, though VEGFR-2 tended towards significance ($P = 0.076$).

Univariate analyses of VEGF-C and VEGFR-3 expressions showed no significant differences in BF, CF and PCD.

Multivariate Analyses

Results from two of three multivariate models regarding clinicopathological variables and biomarkers are shown in Table II. Three models were calculated as it is prohibited to analyze combinations of the same marker in one Cox regression model. Model 1 shows that besides clinicopathological variables [pT-status ($P < 0.001$), Gleason ($P = 0.010$), positive non-apical margin ($P = 0.003$) and positive apical

TABLE I. Patient Characteristics, Clinicopathological Variables and Expressions of VEGF-A and VEGFR-2 in 535 Prostate Cancer Patients (univariate analyses; log-rank test)

			BF (n = 170)			CF (n = 36)			PCD (n = 15)		
	Patients		Mean	5 year		Mean	5 year		Mean	5 year	
Characteristics	(n)	(%)	EFS	EFS	*P*	EFS	EFS	*P*	EFS	EFS	*P*
Age					0.555			0.056			0.600
<65 years	357	67%	128	77%		179	97%		183	99%	
≥65 years	178	33%	122	70%		159	95%		169	100%	
pT-stage					<0.001			<0.001			0.027
pT2	374	70%	145	83%		183	98%		184	99%	
pT3a	114	21%	96	60%		165	94%		181	100%	
pT3b	47	9%	60	43%		144	86%		163	95%	
pN-stage					<0.001			<0.001			<0.001
NX	264	50%	131	79%		182	98%		185	100%	
N0	268	50%	118	71%		171	95%		180	99%	
N1	3	1%	23	0%		56	33%		97	100%	
Preoperative PSA					<0.001			0.063			0.061
<10	308	58%	138	80%		179	98%		184	99%	
>10	221	41%	110	67%		171	94%		178	99%	
Missing	6	1%	—			—					
Gleason					<0.001			<0.001			0.001
3 + 3	183	34%	127	83%		169	99%		173	100%	
3 + 4	220	41%	135	76%		172	96%		178	100%	
4 + 3	80	15%	108	69%		171	94%		175	99%	
4 + 4	19	4%	87	63%		156	95%		167	94%	
>8	33	6%	53	34%		134	87%		155	97%	
Tumor size					<0.001			0.013			0.098
≤20 mm	250	47%	138	82%		180	98%		183	99%	
>20 mm	285	53%	118	67%		170	94%		180	99%	
Perineural infiltration					<0.001			<0.001			0.002
No	401	75%	130	79%		175	98%		180	99%	
Yes	134	25%	101	60%		161	91%		175	99%	
Positive surgical margin					0.040			0.031			0.697
No	249	47%	136	81%		180	98%		183	99%	
Yes	286	53%	113	69%		171	95%		180	99%	
Non-apical positive surgical margin					<0.001			<0.001			0.029
No	381	71%	140	81%		182	98%		185	99%	
Yes	154	29%	92	57%		160	92%		176	99%	
Apical positive surgical margin					0.042			0.593			0.313
No	325	61%	124	73%		174	96%		180	99%	
Yes	210	39%	126	77%		176	96%		183	99%	
Vascular infiltration					<0.001			<0.001			0.009
No	492	92%	131	77%		178	97%		183	99%	
Yes	43	8%	79	46%		139	85%		160	97%	
Surgical procedure					0.232			0.383			0.581
Retropubic	435	81%	130	76%		175	96%		181	99%	
Perineal	100	19%	118	67%		173	98%		179	100%	
VEGF-A in stroma					0.013			0.890			0.357
Low	331	62%	134	76%		175	96%		180	99%	
High	148	28%	112	67%		169	96%		180	99%	
Missing	56	10%	—			—			—		
VEGFR-2 in stroma					0.032			0.031			0.076
Low	231	43%	132	77%		175	99%		179	100%	
High	248	46%	121	71%		173	94%		179	99%	
Missing	56	10%	—			—			—		

(*Continued*)

TABLE I. (*Continued*)

			BF (n = 170)			CF (n = 36)			PCD (n = 15)		
	Patients		Mean	5 year		Mean	5 year		Mean	5 year	
Characteristics	(n)	(%)	EFS	EFS	*P*	EFS	EFS	*P*	EFS	EFS	*P*
VEGF-A and VEGFR-2 in stroma					0.003			0.345			0.757
Both low	149	28%	138	81%		167	99%		171	100%	
Either VEGF-A or VEGFR-2 high	257	48%	123	70%		175	96%		182	99%	
Both high	68	13%	102	67%		167	93%		176	98%	
Missing	61	11%	—			—			—		
VEGFR-2 in stroma and epithelium					0.053			0.029			0.230
Both stroma and epithelium low	113	21%	125	83%		159	100%		161	100%	
Either stroma, epithelium or both high	344	64%	127	73%		174	95%		181	99%	
Missing	78	15%	—			—			—		

BF, biochemical failure; CF, clinical failure; PCD, prostate cancer death; EFS, event free survival in months

margin ($P = 0.003$)], a high VEGF-A expression in stroma correlates with increased BF (HR = 1.51, $P = 0.016$). In model 2 we computed a co-expression variable of VEGF-A and VEGFR-2. We found 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 ($P = 0.011$). Besides, the same clinicopathological variables that were significant in model 1 also came out significant in model 2. In addition, a third model was analyzed (not presented), in which the results revealed that a VEGFR-2 expression in either stroma, epithelium or both was associated with worse CF-free survival (HR = 4.56, $P = 0.038$).

DISCUSSION

The current results demonstrate that overexpression of VEGF-A and VEGFR-2 in prostate adenocarcinoma is independently and significantly associated with biochemical and clinical recurrence in PC patients treated by radical prostatectomy. In our cohort, the risk of biochemical failure is nearly doubled (HR 1.77) provided high expression of VEGF-A or VEGFR-2 in stroma, while the risk of clinical failure is quadrupled (HR 4.56) if VEGFR-2 is overexpressed in either tumor epithelium, stroma or both.

VEGFR-2 has so far been scarcely studied in clinicopathological studies, as the major focus has been on VEGF-A. Marker studies involving both tumor epithelium and tumor stroma are even more rare. Our data indicate that VEGFR-2 is a stronger prognosticator than VEGF-A, and particularly that overexpression in the tumor-near stroma is of great significance.

The strength of our study is the large number of patients, the long clinical follow-up and that both tumor epithelium and stroma have been examined, as opposed to previous studies. In contrast to RT-PCR techniques, IHC markers allow us to visualize and assess expressions of antibodies in both the epithelial and stromal compartments.

Despite the long clinical follow-up, a weakness of this study is the low numbers of clinical recurrence and prostate cancer specific deaths (36 and 15 events, respectively). This shows that larger studies and longer follow-up are needed to properly evaluate the significant endpoints.

Our data demonstrating that VEGF-A is a poor prognostic factor in prostate cancer is consistent with the majority of previous studies in this disease [11–15]. Interestingly, our results emphasize that it is the VEGF-A overexpression in the tumor--near stroma rather than the tumor epithelium that is of greatest importance. Corroborating our findings, Wu et al. investigated 51 radical prostatectomy specimens and observed that high Gleason grade tumors and advanced disease had a significantly higher frequency of VEGF-A expression in tumor--near stroma, than the tumor epithelium [16]. Importantly, Vergis and coworkers, studying prostate cancer tissues from 308 prostatectomized patients and 289 patients undergoing prostate biopsies prior to radiotherapy, reported that increased VEGF-A expression was significantly and independently associated with a reduced time to biochemical failure [14]. In a smaller cohort (n = 40), Peyromaure et al. found that VEGF-A expression was the most significant predictive factor of cancer progression after radical prostatectomy [15]. In a more recent

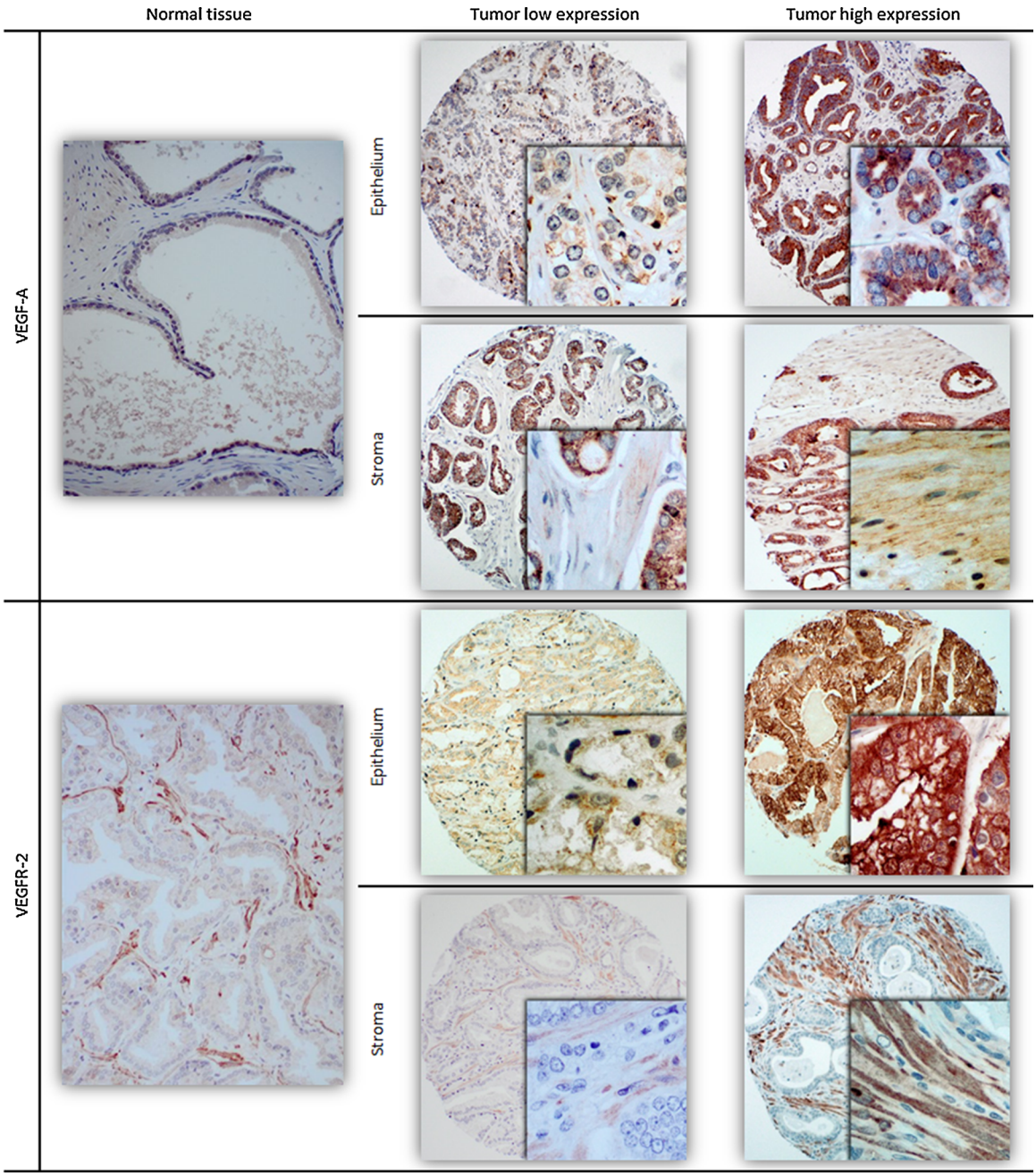


Fig. 1. Examples of low and high expressions of VEGF-A and VEGFR-2 immunohistochemical staining in tissue microarray cores of prostate cancer epithelium and stroma. 100x (main) and 400× (embedded) magnification.

investigation of 148 prostate cancer patient undergoing radical prostatectomy for clinically localized disease, Wang et al. found that high VEGF-A expression was more correlated to N+ prostate carcinoma and strongly predicted biochemical progression after prostatectomy [12]. In addition, Gravdal et al. reported that high vascular proliferation was significantly related to adverse clinicopathological features and was a strong and independent predictor for biochemical failure when investigating prostate cancer specimens from 104 cancer patients with localized disease [13]. However, stromal expression has not been specifically addressed in any of these studies.

Two recently published studies reported no association between VEGF-A expression and recurrence [17,18]. These studies were, however, of limited size, with shorter follow-up and without stromal assessments, emphasizing in particular the need for larger studies.

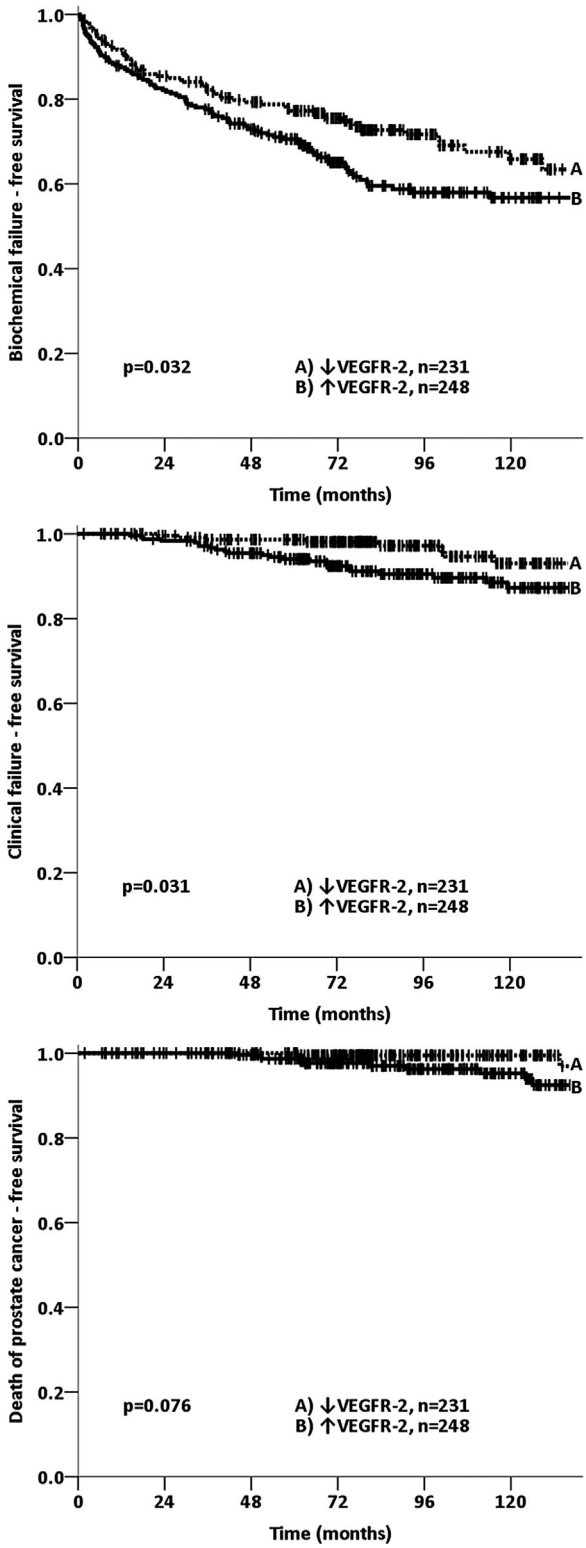


Fig. 2. Kaplan-Meier curves of low or high expression of VEGFR-2 in stroma for (top) biochemical failure, (middle) clinical failure and (bottom) death of prostate cancer.

The importance of our stromal findings appears biological plausible: 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, mediated through direct cell-cell contacts or by secreted molecules. Thus, minor changes in one compartment may cause dramatic alterations in the whole system [30].

The inhibition of angiogenic pathways is an established treatment for several common solid tumors. But its role in the management of prostate cancer is, however, still unclear. Several phase III studies of antiangiogenic agents in metastatic PC have yielded disappointing results: Adding the VEGF-A inhibitor bevacizumab to docetaxel chemotherapy in CRPC patients showed no significant improvement in overall survival, but led to increased toxicity and treatment related deaths [31]. Studies on sunitinib, the tyrosine kinase inhibitor (TKI) against VEGFR-2/platelet-derived growth factor receptor, in patients with advanced CRCP were discontinued due to ineffectiveness [32]. In a large randomized phase III study comparing docetaxel plus lenalidomide (an anti-angiogenic/immunomodulatory agent) versus docetaxel plus placebo, there was no improvement in overall survival in the experimental arm [33]. A recent phase II study of the VEGFR-targeting TKI pazopanib administered to 23 patients with CRPC failed to show sufficient activity in general to warrant further evaluation. Importantly, four patients had a long-term benefit, suggesting that targeting the VEGFR pathway may be highly relevant in selected patients, emphasizing the need for better predictive markers in these patients [34].

The rationale for further studies on antiangiogenic therapy remains strong as novel agents in this field have shown promising results. The dual VEGFR-2/MET targeting TKI cabozantinib has been shown to suppress angiogenesis, metastasis, and tumor growth in preclinical models, and led to significant survival benefits in a medullary thyroid cancer phase III study [35,36]. In a phase II non-randomized discontinuation trial for patients with mCRPC, cabozantinib yielded impressive palliation of bone pain and verified reduced bone metastases [20]. Although data showed encouraging symptomatic relief, preliminary results from the phase 3 trial COMET-1 did not show improvement in overall survival. Tasquinimod has been shown to decrease blood vessel density, though the exact mechanism of action is still unclear. In a randomized placebo-controlled phase II study in males with minimally symptomatic mCRPC, tasquinimod led to improved progression-free survival, and the treatment was well tolerated [22]. The phase III

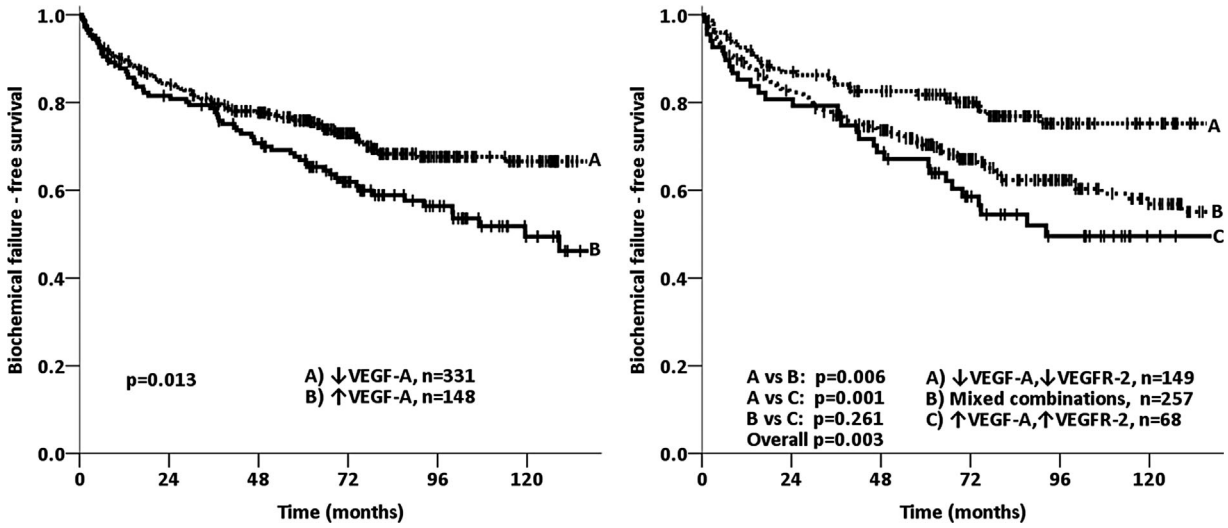


Fig. 3. Kaplan-Meier curves of (left) low or high expression of VEGF-A in stroma for biochemical failure, and (right) combinations of low and high expressions of VEGF-A and VEGFR-2 in stroma for biochemical failure.

trial failed, however, to improve in overall survival [23]. Preliminary results of a phase II study of the VEGFR-2 inhibitor ramucirumab plus mitoxantrone and prednisone in patients with mCRPC led to encouraging progression-free and overall survival [24]. PC is clinically and molecularly a heterogeneous disease and the lack of available predictive biomarkers for patient selection is apparently one of the key reasons why several large trials have produced disappointing results. Specific biomarkers associated with response to therapy are urgently needed to guide treatment selection among prostate cancer patients.

To our knowledge, 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 prostate cancer [37]. Hence, the therapeutic combined inhibition of the VEGF-A/VEGFR-2 signaling may in the future be added to radical treatment of prostate cancer. Although first it will be necessary to further clarify the role of VEGF-A and VEGFR-2 in prostate cancer progression and relapse.

In conclusion, our results indicate that VEGF-A and VEGFR-2, primarily in stroma, are strong independent predictors of prostate cancer recurrence. With further validation of these results, VEGF-A and VEGFR-2 appear be important prognosticators and may in the future aid in treatment allocation of PC patients. As novel therapeutic agents such as

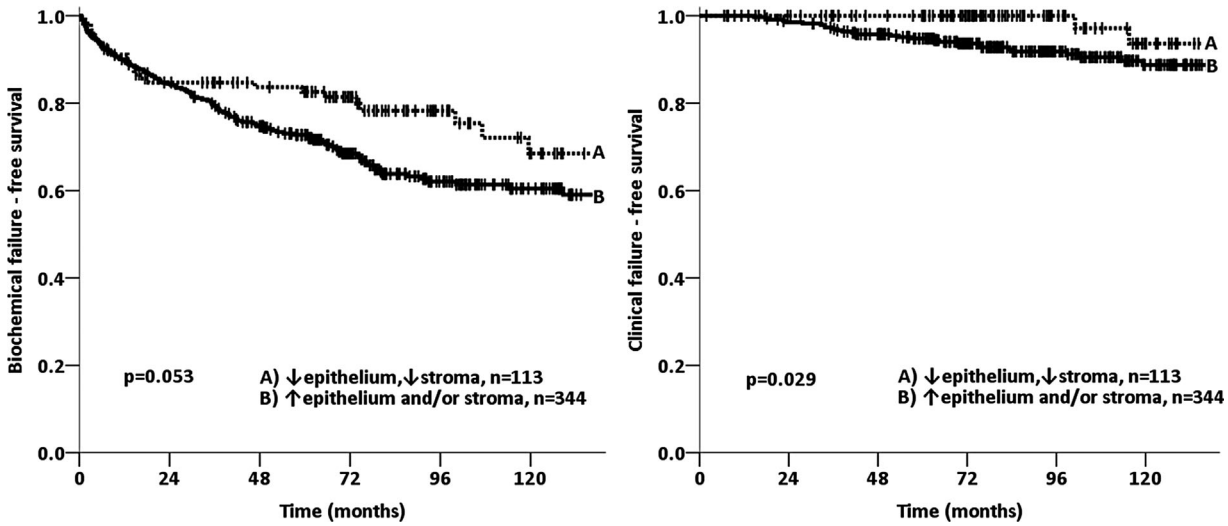


Fig. 4. Kaplan-Meier curves for low expressions of VEGFR-2 in stroma and epithelium versus high expression of VEGFR-2 in either stroma or epithelium or both for (left) biochemical failure and (right) clinical failure.

TABLE II. Expression of VEGF-A and VEGFR-2 in Prostate Tissue as Prognostic Factors in 535 Prostate Cancer Patients (multivariate analyses; Cox regression with backward conditional model)

Model 1	BF (n = 170)			CF (n = 36)		
Factor	HR	95 %CI	*P*	HR	95 %CI	*P*
pT status			<0.001			NE
pT2	1					
pT3a	1.87	1.27–2.76	0.002			
pT3b	2.59	1.58–4.24	<0.001			
Preoperative PSA > 10			NS			NE
Gleason			0.010			0.019
3+3	1			1		
3+4	1.09	0.72–1.65	0.684	2.68	0.84 - 8.61	0.097
4+3	1.65	1.03–2.64	0.036	3.80	1.10 - 13.1	0.034
4+4	1.95	0.92–4.13	0.081	3.52	0.64 - 19.5	0.149
> 8	2.55	1.41–4.61	0.002	7.79	2.33 - 26.0	0.001
Perineural infiltration			NS	2.29	1.09 - 4.85	0.030
Positive non-apical margin	1.70	1.20–2.42	0.003	0.40	0.19 - 0.84	0.016
Positive apical margin	0.59	0.41–0.83	0.003			NE
High expression of VEGF-A in stroma	1.51	1.08–2.10	0.016			NE
High expression of VEGFR-2 in stroma	1.32	0.95–1.84	0.094	1.98	0.90 - 4.36	0.088
Model 2*	BF (n = 170)			CF (n = 36)		
Factor	HR	95%CI	*P*	HR	95%CI	*P*
pT status			0.003			NE
pT2	1					
pT3a	1.69	1.13–2.53	0.011			
pT3b	2.26	1.35–3.77	0.002			
Preoperative PSA > 10	1.33	0.95–1.86	0.096			NE
Gleason			0.013			0.019
3+3	1			1		
3+4	1.07	0.71–1.62	0.751	2.45	0.87–6.90	0.090
4+3	1.63	1.08–2.62	0.042	2.87	0.91–9.10	0.073
4+4	1.92	0.91–4.05	0.086	2.73	0.52–14.2	0.223
> 8	2.57	1.39–4.73	0.003	6.74	2.21–20.6	0.001
Perineural infiltration			NS	2.48	1.23–5.04	0.012
Positive non-apical margin	1.74	1.22–2.48	0.002	3.22	1.56–6.64	0.002
Positive apical margin	0.58	0.41–0.83	0.003			NE
VEGF-A and VEGFR-2 in stroma			0.011			NE
Low expression of both	1					
High for either VEGF-A or VEGFR-2	1.77	1.14–2.58	0.009			
High expression of both	2.02	1.22–3.34	0.006			

BF, biochemical failure; CF, clinical failure; NE, not entered into Cox regression due to not significant in univariate analyses; NS, not significant and removed by backward model before last step of analyses.
*Two models are needed as it it prohibited to analyse combinations of the same marker in one analysis.

cabozantinib recently showed promising results in patients with CRPC, the VEGFR-2 axis appears to be of clinical importance from a therapeutic perspective.

ACKNOWLEDGMENT

We are endebted to our lab engineer Marit Nilsen for high quality IHC staining work on TMAs.

REFERENCES

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010;127:2893–2917.
2. Bill-Axelson A, Garmo H, Holmberg L, Johansson JE, Adami HO, Steineck G, Johansson E, Rider JR. Long-term distress after radical prostatectomy versus watchful waiting in prostate cancer: A longitudinal study from the Scandinavian Prostate

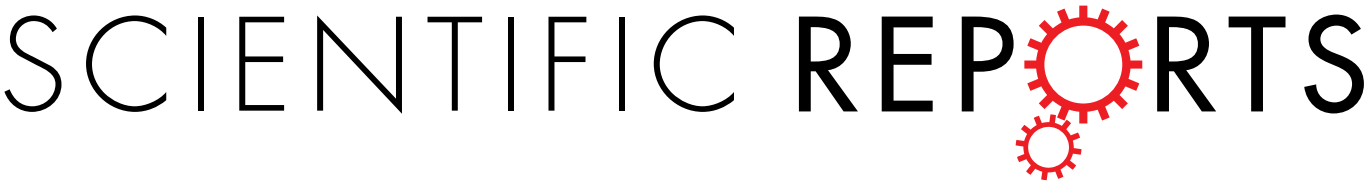
Cancer Group-4 randomized clinical trial. Eur Urol 2013;64: 920–928.

3. Etzioni R, Mucci L, Chen S, Johansson JE, Fall K, Adami HO. Increasing use of radical prostatectomy for nonlethal prostate cancer in Sweden. Clin Cancer Res 2012;18:6742–6747.
4. Fossa SD, Nilssen Y, Kvale R, Hernes E, Axcrona K, Moller B. Treatment and 5-year survival in patients with nonmetastatic prostate cancer: The Norwegian experience. Urology 2014;83: 146–152.
5. Hanahan D, Weinberg RA. Hallmarks of cancer: The next generation. Cell 2011;144:646–674.
6. Ferrara N, Hillan KJ, Gerber HP, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat Rev Drug Discov 2004;3:391–400.
7. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer Res 1995;55: 3964–3968.
8. Manders P, Beex LV, Tjan-Heijnen VC, Geurts-Moespot J, Van Tienoven TH, Foekens JA, Sweep CG. The prognostic value of vascular endothelial growth factor in 574 node-negative breast cancer patients who did not receive adjuvant systemic therapy. Br J Cancer 2002;87:772–778.
9. Fontanini G, Lucchi M, Vignati S, Mussi A, Ciardiello F, De LM, De PS, Basolo F, Angeletti CA, Bevilacqua G. Angiogenesis as a prognostic indicator of survival in non-small-cell lung carcinoma: A prospective study. J Natl Cancer Inst 1997;89:881–886.
10. Eisma RJ, Spiro JD, Kreutzer DL. Vascular endothelial growth factor expression in head and neck squamous cell carcinoma. Am J Surg 1997;174:513–517.
11. Teishima J, Yano S, Shoji K, Hayashi T, Goto K, Kitano H, Oka K, Nagamatsu H, Matsubara A. Accumulation of FGF9 in prostate cancer correlates with epithelial-to-mesenchymal transition and induction of VEGF-A expression. Anticancer Res 2014;34:695–700.
12. Wang Q, Diao X, Sun J, Chen Z. Stromal cell-derived factor-1 and vascular endothelial growth factor as biomarkers for lymph node metastasis and poor cancer-specific survival in prostate cancer patients after radical prostatectomy. Urol Oncol 2013;31:312–317.
13. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. Proliferation of immature tumor vessels is a novel marker of clinical progression in prostate cancer. Cancer Res 2009;69:4708–4715.
14. Vergis R, Corbishley CM, Norman AR, Bartlett J, Jhavar S, Borre M, Heeboll S, Horwich A, Huddart R, Khoo V, Eeles R, Cooper C, Sydes M, Dearnaley D, Parker C. Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: A retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. Lancet Oncol 2008;9:342–351.
15. Peyromaure M, Camparo P, Badoual C, Descazeaud A, Dinh--Xuan AT. The expression of vascular endothelial growth factor is associated with the risk of cancer progression after radical prostatectomy. BJU Int 2007;99:1150–1153.
16. Wu TT, Wang JS, Jiann BP, Yu CC, Tsai JY, Lin JT, Huang JK. Expression of vascular endothelial growth factor in Taiwanese benign and malignant prostate tissues. J Chin Med Assoc 2007;70:380–384.
17. Acuna P, Ellwanger A, Ramirez A, Cardemil F, Vega J, Casalino R, Madrid E. [Vascular endothelial growth factor in malignant and non malignant prostatic tissue: Association with tumor recurrence at one year after prostatectomy]. Rev Med Chil 2013;141:153–159.
18. Talagas M, Uguen A, Garlantezec R, Fournier G, Doucet L, Gobin E, Marcorelles P, Volant A. DE BM. VEGFR1 and NRP1 endothelial expressions predict distant relapse after radical prostatectomy in clinically localized prostate cancer. Anticancer Res 2013;33:2065–2075.
19. Tardy I, Pochon S, Theraulaz M, Emmel P, Passantino L, Tranquart F, Schneider M. Ultrasound molecular imaging of VEGFR2 in a rat prostate tumor model using BR55. Invest Radiol 2010;45:573–578.
20. Smith MR, Sweeney CJ, Corn PG, Rathkopf DE, Smith DC, Hussain M, George DJ, Higano CS, Harzstark AL, Sartor AO, Vogelzang NJ, Gordon MS, de Bono JS, Haas NB, Logothetis CJ, Elfiky A, Scheffold C, Laird AD, Schimmoller F, Basch EM, Scher HI. Cabozantinib in Chemotherapy-Pretreated Metastatic Castration-Resistant Prostate Cancer: Results of a Phase II Nonrandomized Expansion Study. J Clin Oncol 2014;32:3391–3399.
21. Exelixis. Exelixis Announces Results from the COMET-1 Phase 3 Pivotal Trial of Cabozantinib in Men with Metastatic Castration-Resistant Prostate Cancer. 2014.
22. Armstrong AJ, Haggman M, Stadler WM, Gingrich JR, Assikis V, Polikoff J, Damber JE, Belkoff L, Nordle O, Forsberg G, Carducci MA, Pili R. Long-term survival and biomarker correlates of tasquinimod efficacy in a multicenter randomized study of men with minimally symptomatic metastatic castration-resistant prostate cancer. Clin Cancer Res 2013;19:6891–6901.
23. Active Biotech. Active Biotech and Ipsen announce their decision to discontinue the development of tasquinimod in prostate cancer. 2015.
24. M Hussain, E. Dana Rathkopf, Glenn Liu, J. Andrew Armstrong, William Kevin Kelly, C. Anna Ferrari, D. John Hainsworth, Ling Yang, D. Jonathan Schwartz, S. Celestia , Higano. A phase II randomized study of cixutumumab (IMC-A12: CIX) or ramucirumab (IMC-1121B RAM) plus mitoxantrone (M) and prednisone (P) in patients (pts) with metastatic castrate-resistant prostate cancer (mCRPC) following disease progression (PD) on docetaxel (DCT) therapy 2012.
25. Andersen S, Richardsen E, Nordby Y, Ness N, Storkersen O, Al-Shibli K, Donnem T, Bertilsson H, Busund LT, Angelsen A, Bremnes RM. Disease-specific outcomes of radical prostatectomies in Northern Norway; a case for the impact of perineural infiltration and postoperative PSA-doubling time. BMC Urol 2014;14:49.
26. Epstein JI An update of the Gleason grading system. J Urol 2010;183:433–440.
27. Helpap B, Egevad L. Modified Gleason grading. An updated review. Histol Histopathol 2009;24:661–666.
28. Bremnes RM, Veve R, Gabrielson E, Hirsch FR, Baron A, Bemis L, Gemmill RM, Drabkin HA, Franklin WA. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non--small-cell lung cancer. J Clin Oncol 2002;20:2417–2428.
29. Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K, Koji T. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. Br J Cancer 1999;80:309–313.
30. Bremnes RM, Donnem T, Al-Saad S, Al-Shibli K, Andersen S, Sirera R, Camps C, Marinez I, Busund LT. The role of tumor

stroma in cancer progression and prognosis: Emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. J Thorac Oncol 2011;6:209–217.

31. Kelly WK, Halabi S, Carducci M, George D, Mahoney JF, Stadler WM, Morris M, Kantoff P, Monk JP, Kaplan E, Vogelzang NJ, Small EJ. Randomized, double-blind, placebo-controlled phase III trial comparing docetaxel and prednisone with or without bevacizumab in men with metastatic castration-resistant prostate cancer: CALGB 90401. J Clin Oncol 2012;30:1534–1540.
32. Michaelson MD, Oudard S, Ou YC, Sengelov L, Saad F, Houede N, Ostler P, Stenzl A, Daugaard G, Jones R, Laestadius F, Ullen A, Bahl A, Castellano D, Gschwend J, Maurina T, Chow ME, Wang SL, Lechuga MJ, Paolini J, Chen I. Randomized, placebo--controlled, phase III trial of sunitinib plus prednisone versus prednisone alone in progressive, metastatic, castration-resistant prostate cancer. J Clin Oncol 2014;32:76–82.
33. Celgene. Celgene Will Discontinue Phase III MAINSAIL® Trial in Castrate-Resistant Prostate Cancer. 2011.
34. Sridhar SS, Joshua AM, Gregg R, Booth CM, Murray N, Golubovic J, Wang L, Harris P, Chi KN. A Phase II Study of GW 786034 (Pazopanib) with or without Bicalutamide in Patients With Castration-Resistant Prostate Cancer. Clin Genitourin Cancer 2015;13:124–129.
35. Elisei R, Schlumberger MJ, Muller SP, Schoffski P, Brose MS, Shah MH, Licitra L, Jarzab B, Medvedev V, Kreissl MC, Niederle B, Cohen EE, Wirth LJ, Ali H, Hessel C, Yaron Y, Ball D, Nelkin B, Sherman SI. Cabozantinib in progressive medullary thyroid cancer. J Clin Oncol 2013;31: 3639–3646.
36. Yakes FM, Chen J, Tan J, Yamaguchi K, Shi Y, Yu P, Qian F, Chu F, Bentzien F, Cancilla B, Orf J, You A, Laird AD, Engst S, Lee L, Lesch J, Chou YC, Joly AH. Cabozantinib (XL184), a novel MET and VEGFR2 inhibitor, simultaneously suppresses metastasis, angiogenesis, and tumor growth. Mol Cancer Ther 2011; 10:2298–2308.
37. Pal S. Axitinib Before Surgery in Treating Patients With High-Risk Prostate Cancer. ClinicalTrials.gov; 2014.

Paper 2



OPEN

Received: 26 October 2016
Accepted: 23 January 2017
Published: 24 February 2017

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

Yngve Nordby[1,2], Elin Richardsen[3,4], Mehrdad Rakaee[4], Nora Ness[4], Tom Donnem[1,5], Hiten R. H. Patel[1,2], Lill-Tove Busund[3,4], Roy M. Bremnes[1,5] & Sigve Andersen[1,5]

Due to a lack of sufficient diagnostic tools to predict aggressive disease, there is a significant overtreatment of patients with prostate cancer. Platelet derived growth factors (PDGFs) and their receptors (PDGFRs) are key regulators of mesenchymal cells in the tumor microenvironment, and has been associated with unfavorable outcome in several other cancers. Herein, we aimed to investigate the prognostic impact of PDGFR-β and its ligands (PDGF-B and PDGF-D) in a multicenter prostatectomy cohort of 535 Norwegian patients. Using tissue microarrays and immunohistochemistry, the expression of ligands PDGF-B and PDGF-D and their corresponding receptor, PDGFR-β, was assessed in neoplastic tissue and tumor-associated stroma. PDGFR-β was expressed in benign and tumor associated stroma, but not in epithelium. High stromal expression of PDGFR-β was independently associated with clinical relapse (HR = 2.17, p = 0.010) and biochemical failure (HR = 1.58, p = 0.002). This large study highlights the prognostic importance of PDGFR-β expression, implicating its involvement in prostate cancer progression even in early stage disease. Hence, analyses of PDGFR-β may help distinguish which patients will benefit from radical treatment, and since PDGFR-β is associated with relapse and shorter survival, it mandates a focus as a therapeutic target.

Prostate cancer (PC) is the most frequent malignancy in men[1]. Despite a relatively low mortality rate, the sheer PC incidence rate makes it the second most common cause of male cancer death in developed countries. Differentiation between patients with an aggressive and potentially deadly form of PC versus patients with indolent disease remains a challenge. Contemporary risk stratification leads to a significant overtreatment (radical therapy), but possibly also an undertreatment of some patients[2–4]. There is a definite need for better prognostic tools to aid in the prediction of which patients will benefit from curative treatment.

The platelet derived growth factor ligands (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 microenvironment[5]. 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[6]. 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 cancer.

There are five different known isoforms of PDGF ligands: PDGF-AA (PDGF-A), PDGF-BB (PDGF-B), PDGF-CC (PDGF-C), PDGF-DD (PDGF-D) and AB heterodimer (PDGF-AB)[7]. These interact in a specific manner with tyrosine kinase receptors of three different isoforms: PDGFR-αα (PDGFR-α), PDGFR-ββ (PDGFR-β)

[1]Dept Clinical Medicine, The Arctic University of Norway, Tromso, Norway. [2]Dept Urology, University Hospital of North Norway, Tromso, Norway. [3]Dept Clinical Pathology, University Hospital of North Norway, Tromso, Norway. [4]Dept Medical Biology, The Arctic University of Norway, Tromso, Norway. [5]Dept Oncology, University Hospital of North Norway, Tromso, Norway. Correspondence and requests for materials should be addressed to Y.N. (email: yngve.nordby@uit.no)

and αβ heterodimer (PDGFR- αβ). The different ligand isoforms have variable affinities for the receptor isoforms causing cross reactivity. PDGFR-β is activated by PDGF-B or PDGF-D.

Although several PDGFR inhibitors are approved for clinical use in other cancer types, attempts at PDGFR inhibition in PC patients have so far been unsuccessful with no improvement in disease specific survival, despite robust pre-clinical results[8–10].

Alternations of PDGFRs have been detected in several cancers including pancreatic, ovarian, breast, gastric, thymoma, gastrointestinal stromal tumor, osteosarcoma, hepatocellular and hematologic cancers among others[11–15]. In PC, PDGF-D seems to be involved in osteoclastic differentiation and establishment of bone metastasis[16]. High levels of PDGFR-β in PC tumor stroma and non-malignant prostate tissue have been associated with shorter cancer specific survival for PC patients[17]. However, PDGFR-β expression for PC patients with a localized disease and its prognostic value post radical treatment has, to our knowledge, not been previously examined.

In our pursuit of new prognostic biomarkers and potential targets for novel therapeutic strategies, we systematically assessed both PC tumor and stromal expression of PDGFR-β and its ligands PDGF-B and PDGF-D, as well as associations with clinical outcome in a large multicenter cohort of 535 prostatectomy patients.

Materials and Methods

Patients. 671 patients who underwent radical prostatectomy with curative intent for adenocarcinoma in the prostate from 1995 to 2005, were retrospectively identified from the Departments of Pathology at the University Hospital of Northern Norway (n = 267), Nordland Hospital (n = 63), St. Olavs Hospital (n = 330) and Levanger Hospital (n = 11). Of these, 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), and (iv) lack of follow-up data (n = 1), leaving a total of 535 eligible patients for the cohort. None of the patients had received pre-operative hormonal therapy. The cohort is thoroughly described in a previous paper[18].

We collected relevant data from medical journals involving: Demographical data, age at surgery, previous medical history, retropubic or perineal surgery, and preoperative serum PSA level measured immediately before surgery. Further, we collected outcome data until the last follow-up date (December 01, 2015) or until patients' death. The surviving patients' disease-specific outcomes were recorded for a median follow-up of 12.4 years (range 1.5–20 years). These data included postoperative PSA values and postoperative therapy (radio-, hormonal- and/or chemotherapy). The following endpoints were used: Biochemical failure (BF) defined as postoperative PSA $\geq$ 0.4 or intervention with salvage therapy; Clinical failure (CF) defined as clinically palpable tumor 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.

Tissues and tissue microarray construction. Tumor tissues, consisting of formalin-fixed paraffin-embedded blocks of prostate tissue from the patients' prostatectomies, were collected from the archives of the pathological departments. One experienced pathologist (E.R.) reevaluated the prostate samples and classified them according to the updated WHO guidelines[19,20]. Two pathologists (E.R. and L.T.B.) identified the most representative areas of cancer epithelium cells and adjacent stroma. Each area was biopsied with at least two 0.6 mm cores. The cores were arranged in tissue microarray (TMA) blocks for large-scale analysis. Multiple 4 μm TMA sections were cut with a Micron microtone (HM355S) and stained by specific antibodies for immunohistochemical analysis (IHC). The detailed methodology has been reported previously[21].

Immunohistochemistry. Immunohistochemical analysis 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 following primary antibodies were loaded: PDGF-D (R&D system, #AF1159, goat, polyclonal, 1/40 dilution), PDGF-B (Sigma, #A81363, rabbit, polyclonal, 1/25 dilution) and PDGFR-β (Cell Signaling, #3169, rabbit, monoclonal, 1/25 dilution). 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.

Two different controls for our staining method were applied. Firstly, control staining of the sections with an isotype-matched control antibody without the primary antibody. Secondly, multiple human organ TMA as positive and negative tissue controls were used to verify the specificity of the staining in every staining procedure. Positive tissue controls comprised of colon carcinoma and placenta for PDGFs, while negative tissue controls comprised of normal tonsil and brain.

Scoring of immunohistochemistry. One experienced pathologist (E.R) and one experienced oncologist trained in assessing histopathological slides (S.A) independently and semiquantatively scored viable parts of each anonymized core 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. In addition, each core was also scored by density according to the fraction of marker positive cells in stroma: 0 = 0% positive cells; 1 = 1–50% positive cells; 2 = 50–75% positive cells; 3 $\geq$ 75% positive cells. 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. A final score for both intensity

and density marker expression in both epithelium and stroma for each patient was calculated using the mean values of the observers' scoring of the patients cores. Scoring of IHC cores were dichotomized into low and high expressions. Cut-off values was set at median to secure reproducibility and statistically sufficient numbers in each group. High or low expression of PDGF-B or PDGF-D were not significantly associated with endpoints for any cut-off. For PDGFR-β, there was no expression of the marker in epithelium. In stroma though, there was a heterogeneous distribution of density (cut-off 1.50), while there was a relatively high expression of intensity (cut-off 2.25).

Statistical methods. SPSS 23.0.0.0 (Chicago, IL) was used for all statistical analyses. Correlations were analyzed using Spearman's rank correlation coefficient. Comparing means of expressions between different tissues were analyzed using the non-parametric Wilcoxon signed rank test. 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. 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.

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

Results

Clinicopathological variables and patient characteristics. The patients' clinicopathological data are presented in the first part of Table 1. Median age at surgery was 62 (47–75) years. At the last follow-up, 37% of the patients had BF, 11% had CF and 3.4% were dead of PC. Median preoperative serum PSA was 8.8 (range 0.7–104) and the median tumor size was 20 mm (2.0–50).

Expressions. For PDGF-D, intensity was scored in both tumor and normal epithelium. Stroma was not scored due to weak staining of fibromuscular stroma, and the positive staining in stroma was mainly in lymphoid cells. Density of PDGF-D was not scored due to homogenous distribution. While macrophages and lymphoid cells were positive stained, fibroblasts did not express PDGF-D. The staining was cytoplasmic and granular. PDGF-D was expressed at a higher level in tumor epithelium (mean = 2.13) compared to normal epithelium (mean = 1.85, $p < 0.001$).

For PDGF-B, only intensity was scored as density was homogenously distributed. Stroma could not be scored due to an overall strong staining of fibromuscular stroma. Intensity of both tumor epithelium and normal epithelium was scored separately in two groups. PDGF-B expression was overall cytoplasmic in the luminal and basal cells of the epithelium. There was no significant difference in PDGF-B expression in tumor epithelium (mean = 1.48) versus normal epithelium (mean = 1.52, $p = 0.194$).

Both intensity and density were scored for PDGFR-β. But since PDGFR-β was not expressed in epithelium, only stroma was scored. Both tumor stroma and normal stroma were scored into two separate groups. The staining was cytoplasmic and granular, and no membrane staining was seen. Intensity of PDGFR-β was higher in tumor stroma (mean = 2.35) compared to normal stroma (mean = 1.85, $p < 0.001$), and staining density was also higher in tumor stroma (mean = 1.85) compared to normal stroma (mean 1.28, $p < 0.001$).

Representative light microscopic examples of PDGFR-β high and low intensity and density are shown in Fig. 1.

Correlations. There was a high intraclass correlation between the two scorers, with a correlation coefficient of 0.95 (CI = 0.94–0.95, $p < 0.001$). None of the biomarkers correlated to any of the clinicopathological variables except a weak correlation between mean density of PDGFR-β in stroma and perineural infiltration ($r = 0.25$, $p < 0.001$). For the cases where there were two valid scores of stroma or epithelium, the intra-case heterogeneity was calculated using the intraclass correlation procedure. For intensity of PDGFR-β stroma scores, there was a correlation coefficient of 0.78 (CI = 0.37–0.89, $p < 0.001$) of absolute agreement. For density of PDGFR-β stroma scores, the correlation coefficient was 0.79 (CI = 0.65–0.86, $p < 0.001$).

Univariate analyses. Results from the univariate analyses of the clinicopathological variables are presented in Table 1. For BF, significant prognostic clinicopathological factors were pT-stage ($p < 0.001$), preoperative PSA ($p < 0.001$), Gleason score ($p < 0.001$), tumor size ($p < 0.001$), perineural infiltration ($p < 0.001$), lymphovascular infiltration ($p < 0.001$) and positive surgical margin ($p = 0.049$) with its subclass non-apical margin ($p < 0.001$). For CF, significant prognostic factors were age ($p = 0.038$), pT-stage ($p < 0.001$), preoperative PSA ($p = 0.029$), Gleason score ($p < 0.001$), tumor size ($p < 0.002$), perineural infiltration ($p < 0.001$), vascular infiltration ($p < 0.001$) and positive non-apical margin ($p < 0.001$). The significant prognostic factors for PCD (not presented in tables) were pT-stage ($p < 0.001$), preoperative PSA ($p = 0.003$), Gleason score ($p < 0.001$), perineural infiltration ($p < 0.001$), lymphovascular infiltration ($p < 0.001$) and positive non-apical surgical margin ($p = 0.022$).

Statistical analyses found no difference in endpoints with respect to expressions in tumor stroma respective normal stroma. Hence, all stromal scorings were pooled. Intensity and density of PDGFR-β in stroma versus endpoints in univariate analyses were examined. Results showed that both intensity and density of PDGFR-β were correlated to BF and CF, but density yielded stronger results in means of higher hazard ratio (HR) and significance than intensity. In addition, a backward Cox regression analysis, comparing intensity and density versus endpoints, was performed, and intensity was removed before the last step in the analysis. Hence, we chose to focus on expression as density of PDGFR-β in all stromal scorings.

Characteristics	Patients		BF (200 events)		CF (56 events)	
	(n)	(%)	5 year EFS (%)	*p*	10 year EFS (%)	*p*
Age				0.237		**0.038**
≤65 years	357	67	77		94	
>65 years	178	33	70		91	
pT-stage				**<0.001**		**<0.001**
pT2	374	70	83		97	
pT3a	114	21	61		87	
pT3b	47	9	43		74	
Preop PSA				**<0.001**		**0.029**
PSA < 10	308	57	81		95	
PSA > 10	221	42	68		89	
Missing	6	1	—		—	
Gleason				**<0.001**		**<0.001**
3 + 3	183	34	83		98	
3 + 4	219	41	77		94	
4 + 3	81	15	70		90	
4 + 4	17	4	58		86	
≥9	35	6	37		65	
Tumor Size				**<0.001**		**0.002**
0–20 mm	250	47	83		96	
>20 mm	285	53	68		90	
Perineural infiltration				**<0.001**		**<0.001**
No	401	75	80		96	
Yes	134	25	60		83	
Lymphovascular infiltration				**<0.001**		**<0.001**
No	492	92	77		95	
Yes	43	8	47		69	
Positive surgical margin				**0.049**		0.198
No	249	47	81		96	
Yes	286	53	69		90	
Apical positive surgical margin				0.063		0.427
No	325	61	74		92	
Yes	210	39	77		93	
Non-apical positive surgical margin				**<0.001**		**<0.001**
No	381	71	82		96	
Yes	154	29	57		85	
Surgical procedure				0.466		0.308
Retropubic	435	81	77		92	
Perineal	100	19	68		95	
PDGFR-β in stroma				**<0.001**		**0.001**
Low expression	267	50	80		94	
High expression	262	49	70		91	
Missing	6	1				

Table 1. Patient characteristics, clinicopathological variables and expressions of PDGFR-β in 535 prostate cancer patients (univariate analyses; log-rank test). Abbreviations: BF = biochemical failure; CF = clinical failure; EFS = event free survival in months.

Results from the univariate analyses of the molecular markers according to BF and CF endpoints are presented in Table 1 and Fig. 2. Patients with a high expression of PDGFR-β in stroma had significantly worse outcome regarding BF ($p < 0.001$) and CF ($p = 0.001$) 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.

Expression levels of PDGFR-β versus BF and CF stratified according to AJCC (American Joint Committee on Cancer) PC stage are presented in Table 2. For BF, high expression of PDGFR-β is associated with a worse outcome in stage IIB ($p = 0.007$) and III ($p = 0.029$). For CF, high expression of PDGFR-β is associated with a worse outcome in stage IIA ($p = 0.011$) and IIB ($p = 0.027$).

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

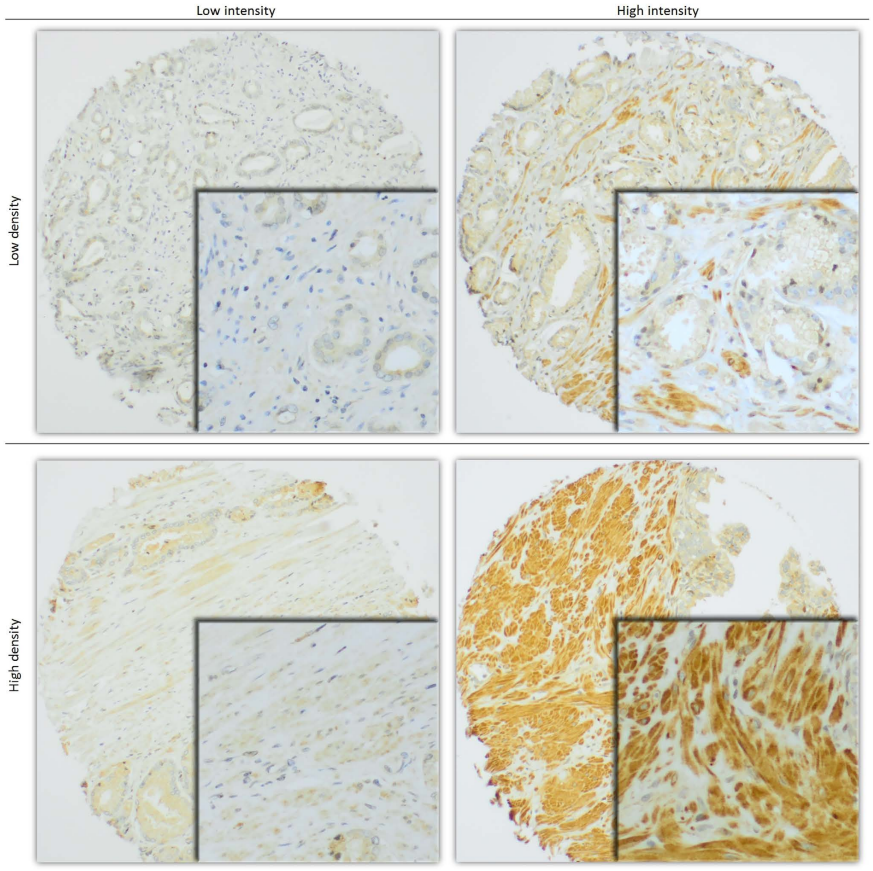


Figure 1. Examples of high and low intensity and density of PDGFR-β immunohistochemical staining in tissue microarray cores of prostate cancer stroma. 100× (main) and 400× (embedded) magnification.

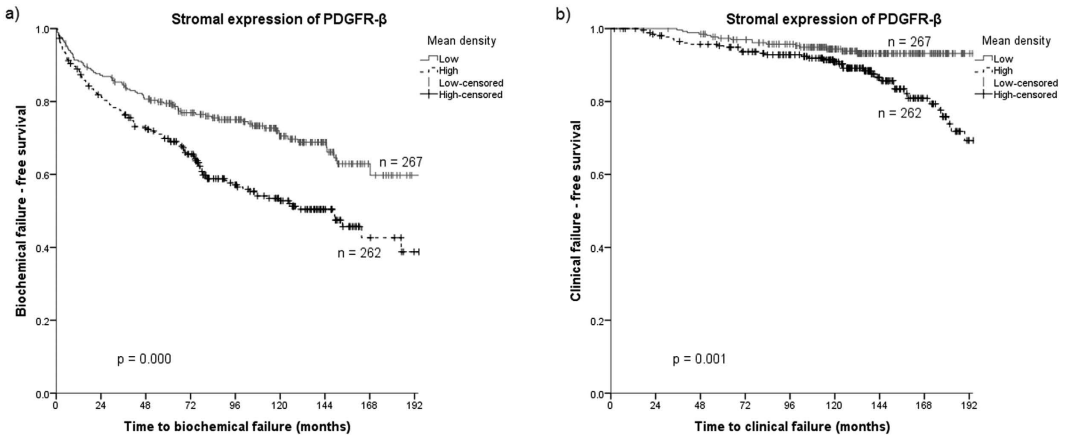


Figure 2. Kaplan-Meier curves of low and high expression of PDGFR-β in prostate cancer stroma for (**a**) biochemical failure and (**b**) clinical failure.

Multivariate analyses. Results from a multivariate model of clinicopathological variables and biomarkers are shown in Table 3. We observed that in addition to clinicopathological variables [pT-stage ($p < 0.001$), pre-operative PSA ($p = 0.014$), Gleason $4 + 3$ ($p = 0.039$), Gleason ≥ 9 ($p = 0.018$) and positive non-apical margin ($p = 0.003$)], a high expression of PDGFR-β in stroma correlates to a worse BF (HR = 1.58, $p = 0.002$). For CF, the only factors that correlate to a significantly worse outcome are Gleason score ($p < 0.001$) and high expression of PDGFR-β in stroma (HR 2.17, $p = 0.010$).

Group		10 year EFS (%)					
		Biochemical failure			Clinical failure		
		Low expr	High expr	p	Low expr	High expr	p
I	(n = 43)			NS			NS
IIA	(n = 111)	76	64	0.082	100	92	0.007
IIB	(n = 219)	82	64	0.007	98	96	0.026
III	(n = 159)	48	29	0.029			NS
IV	(n = 3)			NS			NS

Table 2. Ten year EFS for patients with low or high levels of PDGFR-β stromal expression in relation to prognostic groups of prostate cancer. The stratification of our cohort into prognostic groups are constructed according to the American Joint Committee on Cancer (AJCC) TNM system. Abbreviations: EFS = Event free survival; NE = No events; NS = Not significant ($p > 0.10$); expr = expression of PDGFR-β.

Characteristics	BF (200 events)			CF (56 events)		
	HR	CI 95%	*p*	HR	CI 95%	*p*
Age	NE			NS		
pT-stage			**<0.001**	NS		
pT2	1					
pT3a	1.56	1.07–2.25	**0.019**			
pT3b	2.46	1.55–3.90	**<0.001**			
Preop PSA			**0.014**	NS		
PSA < 10	1					
PSA > 10	1.45	1.08–1.95				
Gleason			0.064			**<0.001**
3 + 3	1			1		
3 + 4	1.19	0.82–1.70	0.360	3.37	1.36–8.37	**0.009**
4 + 3	1.59	1.02–2.47	**0.039**	4.45	1.61–12.3	**0.004**
4 + 4	1.98	0.98–4.00	0.058	5.40	1.35–21.7	**0.017**
≥9	1.95	1.12–3.38	**0.018**	15.1	5.83–39.2	**<0.001**
Tumor Size	NS			NS		
0–20 mm						
>20 mm						
Perineural infiltration	NS			NS		
No						
Yes						
Lymphovascular infiltration	NS			NS		
No						
Yes						
Non-apical positive surgical margin			**0.005**	NS		
No	1					
Yes	1.57	1.15–2.15				
PDGFR-β in stroma			**0.002**			**0.010**
Low expression	1			1		
High expression	1.58	1.18–2.13		2.17	1.20–3.90	

Table 3. Expression of PDGFR-β in prostate tissue as a prognostic factor in 535 prostate cancer patients (multivariate analyses; Cox regression with backward conditional model). Abbreviations: BF = biochemical failure; CF = clinical failure; NE = not entered into Cox regression due to not significant in univariate analyses; NS = not significant and removed by backward model before last step of analyses.

Discussion

We demonstrate a high expression of PDGFR-β in prostate cancer stroma to be independently and significantly associated with biochemical and clinical recurrence in PC patients treated by radical prostatectomy. We found the mean expression of PDGFR-β to be higher in tumor stroma compared to normal stroma. In our cohort, PDGFR-β outperforms well-established prognostic factors like pT-stage, preoperative PSA, tumor size, PNI, lymphovascular infiltration and positive surgical margin as a prognostic tool. There was no significant difference in clinical outcome according to PDGF-B or PDGF-D expression.

PDGF pathway studies are scarce in PC and the majority has been performed *in vitro*. The absence of marker studies involving normal and malignant tissues in both epithelial and stromal compartments further underpins the need for further investigation in this field. The strengths of our study are the size of our multicenter cohort, the long clinical follow-up, and the examination of both tumor epithelium and stroma. In contrast to RT-PCR techniques, IHC markers allow us to visualize and assess the expression of antibodies *in situ*. Despite the long clinical follow-up (mean 12.4 years), the relatively low incidence of clinical recurrence and prostate cancer-specific death leaves a relatively low numbers of events. This demonstrates the need for even larger PC studies to properly evaluate these endpoints. This study is biased towards the selected group of patients that are considered healthy enough to undergo prostatectomy and towards stages of PC that are perceived as surgically curable.

In a phase II study of the PDGFR-inhibitor SU101 for patients with hormone-refractory PC, PDGFR-β was shown by IHC analysis to be upregulated in most primary and metastatic PC cells[22]. Corroborating our findings, Singh *et al.* revealed, by using a gene microarray on 235 tumor samples, that PDGFR-β is one of at least five genes that predict PC recurrence after prostatectomy[23].

Hagglof *et al.* found that a high expression of PDGFR-β in both normal and tumor stroma was associated with poor survival and advanced disease in a natural course of the disease, without radical intervention[17]. Their study was based on PC tissue specimens collected from approximately 300 patients subjected to transurethral resection of the prostate (TURP) during 1975–1991. As radical treatment had not been implemented as medical practice at the time, their sampled TURP material differs from the intervention prostatectomies of our study. Our results show that high expression of PDGFR-β is a prognostic factor after prostatectomy intervention with curative intent, and as such unveils the importance of PDGFR-β expression at a more relevant clinical setting. While Hagglof *et al.* did not observe significant prognosticators in the multivariate analysis, our multivariate results showed that Gleason score and expression of PDGFR-β were independent significant prognostic factors for clinical failure. Although the clinical setting is different from the study by Hagglof *et al.*, our results build on their results and demonstrate that PDGFR-β in either benign or malignant stroma of PC tissue is a prognostic biomarker both in the natural history of PC and after prostatectomy.

When investigating the ligands PDGF-B and PDGF-D, PDGF-D was expressed at a higher level in tumor epithelium compared to normal epithelium. Other studies have suggested that PDGF-D seems to be involved in development of bone metastasis, and is associated with increased Gleason and tumor stage[24,25]. However, we found no associations between expression of PDGF-D and clinical outcome. A reason for this may be that our sample selection consists of patients with localized disease, whereas earlier studies of PDGF-D have been studies implicating a more advanced disease[26]. Our results indicate that PDGF-D is not significantly associated with cancer relapse in earlier stages of the disease.

We found no difference in levels of PDGF-B expression in normal versus tumor epithelium, nor was there any associations between expressions and prognosis. These findings are supported by previous clinical studies demonstrating that both PDGFR-β and PDGF-D are up-regulated in primary prostate cancers and bone metastases, whereas PDGF-B is not frequently detected in clinical samples[27]. Hence, it is the upregulation of the receptor PDGFR-β that seems to be of clinical significance for patients considered for radical treatment.

In our cohort, the risk of BF increased 58% (HR 1.58) as a result of high PDGFR-β expression in stroma. Even more importantly, the only two factors that predict clinical failure in our cohort are Gleason score and high expression of PDGFR-β. If fact, high PDGFR-β expression more than doubles the risk of clinical failure. Expressions of PDGFR-β have a significant impact on BF and CF for the intermediate risk groups IIA, IIB and III. This is of particular interest as we are in desperate need for better prognostic tools in this patient group.

Our results show that both normal and malignant stroma are of clinical importance. 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 system[28].

Treatment with inhibitors of the PDGF pathways has been established for several cancer types. The tyrosine kinase inhibitor (TKI) imatinib is a potent inhibitor of the PDGFR, and is used to treat gastrointestinal stromal tumors, some forms of leukemia, and myeloproliferative diseases among others. Despite robust pre-clinical data, imatinib has proven ineffective in Phase I and II clinical trials for patients with metastatic castration-resistant PC (mCRPC)[10]. A Phase II trial even showed that PDGFR inhibition with tandutinib was associated with accelerated disease progression, hypothesizing that PDGF contributes to the homeostasis of bone metastases from PC[8]. Other attempts of PDGF inhibition in PC has been no more successful[29,30].

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[31,32]. 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.

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. PDGFR upregulation has been suggested as a mechanism of evading different targeted drug therapies in some preclinical studies, and further exploration in a clinical relevant setting is warranted[33]. Specific prognostic biomarkers, associated with response to therapy, are also warranted in order to guide treatment stratification. There are still several unresolved aspects regarding PDGFR inhibition as PC treatment. Hitherto, no studies involving PDGFR-inhibition has been carried out in early stage prostate cancer. According to translational research data, it can be speculated that such therapy may prove effective in the primary setting.

In conclusion, our results indicate PDGFR-β in either benign or tumor associated stroma to be a strong, independent predictor of prostate cancer recurrence. Although PDGF inhibition so far has been disappointing, its implication in PC relapse warrants further exploration in an optimal setting. As a prognosticator, PDGFR-β in PC stroma consistently appears to be associated with poor prognosis, particularly in the important intermediate risk subgroups. Prospective validation should be considered for future studies.

References

1. Ferlay, J. *et al.* Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **127,** 2893–2917, doi: 10.1002/ijc.25516 (2010).
2. Bill-Axelson, A. *et al.* Long-term distress after radical prostatectomy versus watchful waiting in prostate cancer: a longitudinal study from the Scandinavian Prostate Cancer Group-4 randomized clinical trial. *Eur. Urol* **64,** 920–928, doi: S0302-2838(13)00137-1 [pii]; 10.1016/j.eururo.2013.02.025 (2013).
3. Etzioni, R. *et al.* Increasing use of radical prostatectomy for nonlethal prostate cancer in Sweden. *Clin. Cancer Res* **18,** 6742–6747, doi: 1078-0432.CCR-12-1537 [pii]; 10.1158/1078-0432.CCR-12-1537 (2012).
4. Fossa, S. D. *et al.* Treatment and 5-year survival in patients with nonmetastatic prostate cancer: the Norwegian experience. *Urology* **83,** 146–152, doi: S0090-4295(13)01251-X [pii]; 10.1016/j.urology.2013.08.081 (2014).
5. Appiah-Kubi, K. *et al.* Platelet-derived growth factor receptor/platelet-derived growth factor (PDGFR/PDGF) system is a prognostic and treatment response biomarker with multifarious therapeutic targets in cancers. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine* **37,** 10053–10066, doi: 10.1007/s13277-016-5069-z (2016).
6. Andrae, J., Gallini, R. & Betsholtz, C. Role of platelet-derived growth factors in physiology and medicine. *Genes & development* **22,** 1276–1312, doi: 10.1101/gad.1653708 (2008).
7. Chen, P. H., Chen, X. & He, X. Platelet-derived growth factors and their receptors: structural and functional perspectives. *Biochimica et biophysica acta* **1834,** 2176–2186, doi: 10.1016/j.bbapap.2012.10.015 (2013).
8. Mathew, P. *et al.* Accelerated disease progression in prostate cancer and bone metastases with platelet-derived growth factor receptor inhibition: observations with tandutinib. *Cancer chemotherapy and pharmacology* **68,** 889–896, doi: 10.1007/s00280-011-1567-2 (2011).
9. Mathew, P. *et al.* Platelet-derived growth factor receptor inhibition and chemotherapy for castration-resistant prostate cancer with bone metastases. *Clinical cancer research: an official journal of the American Association for Cancer Research* **13,** 5816–5824, doi: 10.1158/1078-0432.ccr-07-1269 (2007).
10. Rosenberg, A. & Mathew, P. Imatinib and prostate cancer: lessons learned from targeting the platelet-derived growth factor receptor. *Expert. Opin. Investig. Drugs* **22,** 787–794, doi: 10.1517/13543784.2013.787409 (2013).
11. Chu, J. S. *et al.* Expression and prognostic value of VEGFR-2, PDGFR-beta, and c-Met in advanced hepatocellular carcinoma. *Journal of experimental & clinical cancer research: CR* **32,** 16, doi: 10.1186/1756-9966-32-16 (2013).
12. Cimpean, A. M. *et al.* Platelet-derived growth factor and platelet-derived growth factor receptor-alpha expression in the normal human thymus and thymoma. *International journal of experimental pathology* **92,** 340–344, doi: 10.1111/j.1365-2613.2011.00777.x (2011).
13. Jechlinger, M. *et al.* Autocrine PDGFR signaling promotes mammary cancer metastasis. *The Journal of clinical investigation* **116,** 1561–1570, doi: 10.1172/jci24652 (2006).
14. Kubo, T. *et al.* Platelet-derived growth factor receptor as a prognostic marker and a therapeutic target for imatinib mesylate therapy in osteosarcoma. *Cancer* **112,** 2119–2129, doi: 10.1002/cncr.23437 (2008).
15. Matei, D. *et al.* PDGF BB induces VEGF secretion in ovarian cancer. *Cancer biology & therapy* **6,** 1951–1959 (2007).
16. Huang, W. *et al.* A novel function for platelet-derived growth factor D: induction of osteoclastic differentiation for intraosseous tumor growth. *Oncogene* **31,** 4527–4535, doi: onc2011573 [pii]; 10.1038/onc.2011.573 (2012).
17. Hagglof, C. *et al.* Stromal PDGFRbeta expression in prostate tumors and non-malignant prostate tissue predicts prostate cancer survival. *PLoS. One* **5,** e10747, doi: 10.1371/journal.pone.0010747 (2010).
18. Andersen, S. *et al.* Disease-specific outcomes of radical prostatectomies in Northern Norway; a case for the impact of perineural infiltration and postoperative PSA-doubling time. *BMC. Urol* **14,** 49, doi: 1471-2490-14-49 [pii];10.1186/1471-2490-14-49 (2014).
19. Epstein, J. I. An update of the Gleason grading system. *J. Urol* **183,** 433–440, doi: S0022-5347(09)02704-9 [pii]; 10.1016/j.juro.2009.10.046 (2010).
20. Helpap, B. & Egevad, L. Modified Gleason grading. An updated review. *Histol. Histopathol* **24,** 661–666 (2009).
21. Bremnes, R. M. *et al.* High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. *J. Clin. Oncol* **20,** 2417–2428 (2002).
22. Ko, Y. J. *et al.* A multi-institutional phase ii study of SU101, a platelet-derived growth factor receptor inhibitor, for patients with hormone-refractory prostate cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research* **7,** 800–805 (2001).
23. Singh, D. *et al.* Gene expression correlates of clinical prostate cancer behavior. *Cancer cell* **1,** 203–209 (2002).
24. Sethi, S., Macoska, J., Chen, W. & Sarkar, F. H. Molecular signature of epithelial-mesenchymal transition (EMT) in human prostate cancer bone metastasis. *American journal of translational research* **3,** 90–99 (2010).
25. Ustach, C. V. *et al.* A novel signaling axis of matriptase/PDGF-D/ss-PDGFR in human prostate cancer. *Cancer Res* **70,** 9631–9640, doi: 10.1158/0008-5472.can-10-0511 (2010).
26. Najy, A. J. *et al.* Cediranib inhibits both the intraosseous growth of PDGF D-positive prostate cancer cells and the associated bone reaction. *Prostate* **72,** 1328–1338, doi: 10.1002/pros.22481 (2012).
27. Conley-LaComb, M. K. *et al.* PTEN regulates PDGF ligand switch for beta-PDGFR signaling in prostate cancer. *The American journal of pathology* **180,** 1017–1027, doi: 10.1016/j.ajpath.2011.11.021 (2012).
28. Bremnes, R. M. *et al.* The role of tumor stroma in cancer progression and prognosis: emphasis on carcinoma-associated fibroblasts and non-small cell lung cancer. *J. Thorac. Oncol* **6,** 209–217, doi: 10.1097/JTO.0b013e3181f8a1bd (2011).
29. Armstrong, A. J. *et al.* A phase 2 multimodality trial of docetaxel/prednisone with sunitinib followed by salvage radiation therapy in men with PSA recurrent prostate cancer after radical prostatectomy. *Prostate cancer and prostatic diseases* **19,** 100–106, doi: 10.1038/pcan.2015.59 (2016).
30. Dahut, W. L. *et al.* Phase II clinical trial of cediranib in patients with metastatic castration-resistant prostate cancer. *BJU international* **111,** 1269–1280, doi: 10.1111/j.1464-410X.2012.11667.x (2013).
31. Donnem, T. *et al.* Vessel co-option in primary human tumors and metastases: an obstacle to effective anti-angiogenic treatment? *Cancer medicine* **2,** 427–436, doi: 10.1002/cam4.105 (2013).
32. Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144,** 646–674, doi: S0092-8674(11)00127-9 [pii];10.1016/j.cell.2011.02.013 (2011).
33. Paulsson, J., Ehnman, M. & Ostman, A. PDGF receptors in tumor biology: prognostic and predictive potential. *Future oncology (London, England)* **10,** 1695–1708, doi: 10.2217/fon.14.83 (2014).

Author Contributions

Wrote the main manuscript: Y.N. Figure preparation: Y.N. Participated in IHC optimization: E.R., M.R. Participated in scoring expressions: Y.N., S.A. and E.R. Establishing database and retrieving clinical information: Y.N., S.A., E.R., L.T.B. Reviewed the manuscript: All authors. Have agreed on all content: All authors. Performing and controlling statistics: Y.N. and S.A. Conceived the research idea: S.A., Y.N., L.T.B., R.B., T.D., E.R.

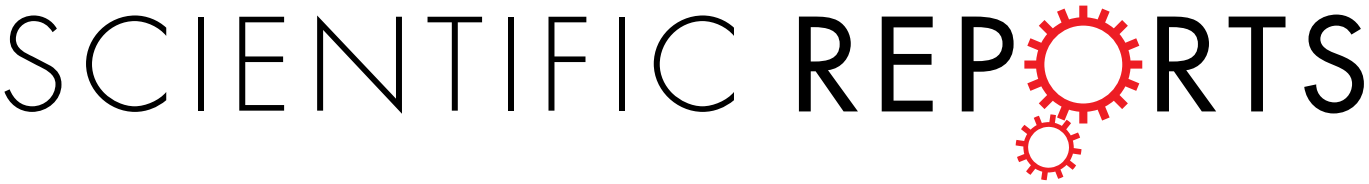
Additional Information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Nordby, Y. *et al.* High expression of PDGFR-β in prostate cancer stroma is independently associated with clinical and biochemical prostate cancer recurrence. *Sci. Rep.* **7**, 43378; doi: 10.1038/srep43378 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Paper 3



OPEN

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

Received: 24 May 2017
Accepted: 14 November 2017
Published online: 24 November 2017

Yngve Nordby[1,2], Elin Richardsen[4,5], Nora Ness[5], Tom Donnem[1,3], Hiten R. H. Patel[1,2], Lill-Tove Busund[4,5], Roy M. Bremnes[1,3] & Sigve Andersen[1,3]

Due to insufficient prognostic tools, failure to predict aggressive prostate cancer (PC) has left patient selection for radical treatment an unsolved challenge. This has resulted in overtreatment with radical therapy. Better prognostic tools are urgently warranted. MicroRNAs (miRs) have emerged as important regulators of cellular pathways, resulting in altered gene expressions. miR-205 has previously been observed downregulated in PC, acting as tumor suppressor. Herein, the expression of miR-205 in prostate tissue was examined in a large, well-described cohort of 535 Norwegian prostatectomy patients. Using *in situ* hybridization, miR-205 expression was semiquantatively measured in normal and tumor tissues from radical prostatectomy specimens. Associations with clinicopathological data and PC relapse were calculated. Expression of miR-205 was lower in tumor epithelium compared to normal epithelium. No association was observed between miR-205 expression in primary tumor epithelium and cancer relapse. In contrast, high expression of miR-205 in normal epithelium was independently associated with biochemical relapse (HR = 1.64, p = 0.003). A prognostic importance of miR-205 expression was only found in the normal epithelium, raising the hypothesis of epithelial crosstalk between normal and tumor epithelium in PC. This finding supports the proposed novel hypothesis of an anti-cancerogenous function of normal epithelium in tumor tissue.

Prostate cancer (PC) is the most common malignancy in men[1]. The majority of prostate tumors is detected at early stages with uncertain prognosis. Prognostic factors like prostate specific antigen (PSA) and histologic scores are well established. These are, however, imprecise and fail to accurately predict PC outcome. This has led to a significant overtreatment with radical therapy (prostatectomy or radiation), while most patients probably would have managed better without treatment[2–5]. Side effects and lack of benefit for costly treatment is discrediting aggressive treatment. However, high incidence and uncertain prognostication makes PC the second most common cause of cancer death in men[6]. Thus, there is an urgent need for better prognostic tools to aid treatment selection, in the interest of both patients and the public.

The micro-RNAs (miRs) are small noncoding RNAs regulating protein expression and numerous cellular processes[7]. These are involved in the normal functioning of cells, while dysregulations of miRs are associated with disease. Various dysregulations of certain miRs (oncomirs) associated with specific cancers have been identified, and they may have either tumor suppressor or oncogenic functions able to modulate nearly all stages of cancer progression including proliferation, apoptosis, cell migration, angiogenesis and stem cell maintenance[8–10]. miR-205 acts either as an oncogene or as a tumor suppressor by facilitating or repressing tumor initiation and

[1]Department of Clinical Medicine, The Arctic University of Norway, Tromso, Norway. [2]Department of Urology, University Hospital of North Norway, Tromso, Norway. [3]Department of Oncology, University Hospital of North Norway, Tromso, Norway. [4]Department of Clinical Pathology, University Hospital of North Norway, Tromso, Norway. [5]Department of Medical Biology, The Arctic University of Norway, Tromso, Norway. Correspondence and requests for materials should be addressed to Y.N. (email: yngve.nordby@unn.no)

proliferation depending on type of cancer and stage[11]. Recently, there has been a major effort to target these non-coding RNAs therapeutically, and a few miRs have entered preclinical and clinical trials[12].

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 PC[13,14]. miR-205 is found to be down-regulated in PC tissue compared to benign tissues, and loss of miR-205 seems to be associated with an invasive phenotype and poor clinical outcome[15]. miR-205 has a tumor suppressive function by inhibiting the transition from epithelial to mesenchymal tissue, cell migration and invasion in the prostate[16]. In a recent study carried out in PC clinical samples, miR-205 was demonstrated to act against tumor initiation and progression by basement membrane maintenance or repressing the mitogen-activated protein kinase and androgen receptor-signaling pathway[17]. However, high miR-205 expression has also been associated with adverse outcome in PC patients[14].

Since miR-205 was consistently downregulated for a selected group of 14 PC patients with rapid biochemical failure in our previous screening array of 1435 miRs in tumor tissue[18], we set out to investigate the prognostic role of miR-205 in our large and well-described cohort of 535 Norwegian prostatectomy patients with extensive follow-up.

Materials and Methods

Patients. 671 patients who underwent radical prostatectomy with curative intent for prostatic adenocarcinoma from 1995 to 2005, were retrospectively identified from the respective Departments of Pathology associated with the University Hospital of Northern Norway (n = 267), Nordland Hospital (n = 63) and St. Olavs Hospital (n = 330) and Levanger Hospital (n = 11). 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), and (iv) lack of follow-up data (n = 1), leaving a total of 535 eligible patients in the cohort. None of the patients had received pre-operative hormonal therapy. The cohort is thoroughly described in a previous paper[19].

We collected relevant data from medical journals involving: Demographical data, age at surgery, previous medical history, retropubic or perineal surgery, and preoperative serum PSA level measured immediately before surgery. Further, we collected outcome data until the last follow-up date (December, 2015) or until patients' death. The surviving patients' disease-specific outcomes were recorded for a median follow-up of 12.4 years (range 1.5–20 years). These data included postoperative PSA values and postoperative therapy (radio-, hormonal- and/or chemotherapy). The following endpoints were used: Biochemical failure (BF) defined as postoperative PSA ≥ 0.4 or intervention with salvage therapy; Clinical failure (CF) defined as clinically palpable tumor recurrence in the prostate bed or metastasis verified by radiology; and Prostate cancer specific death (PCD), defined as death caused by PC stated in the patients' journal.

Tissues and tissue microarray construction. Tumor tissues, consisting of formalin-fixed paraffin-embedded (FFPE) blocks of prostate tissue from the patients' prostatectomies, were collected from the archives of the pathological departments. An experienced pathologist (E.R.) re-evaluated the prostate samples and classified them according to the updated WHO guidelines[20,21]. Two pathologists (E.R. and L.T.B.) identified the most representative areas of cancer epithelium cells and adjacent stroma. Each area was sampled with at least two 0.6 mm cores. The cores were arranged in tissue microarray (TMA) blocks for large-scale analysis. Multiple 4 µm TMA sections were cut with a Micron microtone (HM355S). The detailed methodology has been reported previously[22].

***In situ* hybridization (ISH).** Chromogen *in situ* hybridization (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, (hsa-miR-205-5p, No. 18099-15), positive control (U6 hsa/mmu/rno, No.99002-15) and negative control (scrambled-miRNA, No. 99004-15) were used. Positive and negative tissue controls for miR-205 comprised of a stained TMA multi-organ block. The controls comprised 12 different organs with both normal and tumor tissues. Hybridization, stringent wash temperatures and concentrations were optimized for each probe. Elix RNAse-free water was used during the process to minimize the risk of RNA degradation.

4 µm FFPE TMA slides were dried overnight at 59 °C to attach cores to Super Frost Plus slides. To ensure good distribution of reagents and to protect sections from drying, LCS (Liquid Coverslip oil, Roche 650–010) was added to all incubations in Discovery. Sections were deparaffinized in EZ Prep (Roche 950–100) at 68 °C (3×12 min). Heat mediated pretreatment was done at 95 °C with CC1 (Roche 950–500), 40 min for hsa-miR-205 and 24 min for scrambled miRNA. A combination of heat mediated and enzymatic pretreatment was done for U6, CC1 for 8 min at 95 °C and Protease III (Roche 760–2020) for 16 min at 37 °C. Probe concentrations were 25 nM for miR-205, 10 nM for scrambled miRNA, and 0.5 nM for U6. Denaturation was set to 8 min at 90 °C for all sections. Hybridization was performed for 60 min at 50 °C for miR-205, 57 °C for scramble miRNA and 55 °C for U6. Stringent washes were done 2×8 min with 2.0X RiboWash. Sections were blocked with alkaline phosphatase (AP) anti-DIG (Roche 760–4825) and were incubated for 20 min at 37 °C for immunologic detection. The enzymatic reactions was carried out with NBT/BCIP (CromoMap Blue kit, Roche (760–161) for 20 min at 37 °C. Finally, sections were counterstained and mounted.

Scoring of *in situ* hybridization and cutoff. An experienced pathologist (E.R) and one Ph.D.-student/surgeon-in-training (Y.N) independently and semiquantatively scored viable parts of each anonymized core by light microscopy. The scorers were blinded for each other's score. Intraclass correlation was calculated to assess agreement between the two observers. miR-205 expression was assessed and scored in tumor epithelium, normal epithelium and stroma. Each core was scored by the dominant intensity of staining: 0 = no staining; 1 = weak staining; 2 = moderate staining; 3 = strong staining, and classified as either normal or tumor epithelium. The core

was scored as "missing" if the core was missing or the tissue was considered of insufficient quality to score. A final score from tumor epithelium and normal epithelium for each patient was calculated using the mean values of the observers' scoring of the patients cores. Scoring of IHC cores were dichotomized into low and high expressions. Cut-off values were set at median to secure reproducibility and statistically sufficient numbers in each group. There was no significant difference in outcome regarding the choice of mean or median as cut-off value, hence median was preferred to avoid the influence of extreme values.

Statistical methods. SPSS 23.0.0.0 (Chicago, IL) was used for all statistical analyses. Correlations were analyzed using Spearman's rank correlation coefficient. Mean ranks of expressions between different tissues were compared by using the non-parametric Wilcoxon signed rank test. Univariate survival curves were drawn by the Kaplan-Meier method, and the statistical significance between survival curves was assessed by the log-rank test. 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.

Ethics. The reporting of clinicopathological variables, survival data and biomarker expressions was conducted in accordance with the REMARK guidelines. This study has been approved by The Regional Committee for Medical and Health Research Ethics, REK Nord, project application 2009/1393, including a mandatory reapprovement 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.

Results

Clinicopathological variables and patient characteristics. The patients' clinicopathological data are presented in the first part of Table 1. Gleason score was converted to the standards of the new International Society of Urological Pathology 2014 Grades (ISUP Grade) terminology[23]. The validated score for prediction of outcomes after radical prostatectomy, Cancer of the Prostate Risk Assessment Postsurgical Score (CAPRA-S Score), was calculated based on PSA, Gleason, surgical margin, extracapsular extension, seminal vesicle invasion and lymph node invasion[24,25]. Median age at surgery was 62 (47–75) years. At the last follow-up, 37% of the patients had BF, 11% had CF and 3.4% were dead of PC. 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 was 12.4 years.

Expressions. Figure 1 shows examples of high and low expression of miR-205. miR-205 was expressed in both normal and tumor epithelium, where expression in tumor epithelium (mean score = 1.79) was lower when compared to normal epithelium (mean score = 1.85, $p = 0.008$). There was no expression of miR-205 in stroma. There was a significantly 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. For validation, miR-205 staining of the multi control TMA block was compared to previous known expression profiles of different tissues[26–29]. miR-205 expression in the TMA multi control tissues was expressed negative or positive according to previous known miR-205 expression profiles.

Correlations. The intraclass correlation coefficient between the two scorers was 0.86 (CI = 0.82–0.89, $p < 0.001$). 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 correlated significantly to expression in normal epithelium ($r = 0.27$, $p < 0.001$).

Correlations between miR-205 expression and expressions of previously analyzed angiogenic markers [(VEGF-A, VEGF-C, VEGFR-2 and VEGFR-3) and (PDGF-B, PDGF-D and PDGFR-β)] were calculated[30,31]. miR-205 in tumor epithelium correlated to PDGF-D in tumor epithelium ($r = 0.41$, $p < 0.001$), PDGF-B in tumor epithelium ($r = 0.22$, $p < 0.001$), PDGFR-β in stroma ($r = 0.21$, $p < 0.001$), VEGF-A in epithelium ($r = 0.18$, $p < 0.001$), VEGF-C in epithelium ($r = 0.25$, $p < 0.001$) and VEGFR-2 in epithelium ($r = 0.23$, $p < 0.001$). miR-205 in normal epithelium correlated to PDGF-D in normal epithelium ($r = 0.29$, $p < 0.001$) and PDGF-B in normal epithelium ($r = 0.24$, $p < 0.001$).

Univariate analyses. Results from the univariate analyses of the clinicopathological variables are presented in Table 1. The significant prognostic clinicopathological factors for BF were pT-stage ($p < 0.001$), preoperative PSA ($p < 0.001$), ISUP Grade ($p < 0.001$), positive surgical margin ($p = 0.049$) with its subclass non-apical margin ($p < 0.001$), CAPRA-S Score ($p < 0.001$), tumor size ($p < 0.001$), perineural infiltration ($p < 0.001$) and lymphovascular infiltration ($p < 0.001$). Significant prognostic factors for CF and PCD were previously reported[30,31]. Regarding the miR-205 biomarker, we found no association between expression in tumor epithelium and endpoints for any cut-offs (for mean cut-off and BF: $p = 0.864$). In contrast, high expression of miR-205 in normal epithelium was associated with BF ($p = 0.003$). There was a trend towards 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. A Kaplan-Meier survival curve of miR-205 expression versus BF for all patients is presented in Fig. 2.

Survival analyses for BF stratified according to clinicopathological factors were calculated to explore if 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), $p = 0.001$]. But no significant association was observed between the biomarker and BF in patients with ISUP Grade 3 (Gleason 4 + 3) or higher [n = 114, HR = 1.12 (95% CI = 0.66–1.88), $p = 0.676$]. A Kaplan-Meier survival plot of miR-205 and BF stratified on ISUP

Characteristics	Patients		Biochemical failure		
	(n)	(%)	5 year EFS (%)	HR (95% CI)	p
Age					**0.237**
≤65 years	357	67	77	1	
>65 years	178	33	70	1.19 (0.89–1.59)	
pT-stage					**<0.001**
pT2	374	70	83	1	
pT3a	114	21	61	2.30 (1.67–3.15)	
pT3b	47	9	43	4.41 (3.01–6.47)	
Preop PSA					**<0.001**
PSA < 10	308	57	81	1	
PSA > 10	221	42	68	1.65 (1.24–2.18)	
Missing	6	1	—		
ISUP Grade					**<0.001**
1 (Gleason 3 + 3)	183	34	83	1	
2 (Gleason 3 + 4)	219	41	77	1.35 (0.95–1.92)	
3 (Gleason 4 + 3)	81	15	70	2.14 (1.41–3.26)	
4 (Gleason 4 + 4)	17	4	58	3.14 (1.59–6.19)	
5 (Gleason ≥9)	35	6	37	4.30 (2.63–7.03)	
Positive surgical margin					**0.049**
No	249	47	81	1	
Yes	286	53	69	1.33 (1.00–1.76)	
Apical positive surgical margin					**0.063**
No	325	61	74	1	
Yes	210	39	77	0.76 (0.56–1.02)	
Non-apical positive surgical margin					**<0.001**
No	381	71	82	1	
Yes	154	29	57	2.25 (1.69–2.97)	
CAPRA-S Score					**<0.001**
0–2	169	32	88	1	
3–5	258	48	78	1.85 (1.25–2.73)	
6–12	102	19	46	5.28 (3.51–7.93)	
NC due to missing PSA	6	1	—		
Tumor size					**<0.001**
0–20 mm	250	47	83	1	
>20 mm	285	53	68	1.79 (1.34–2.39)	
Perineural infiltration					**<0.001**
No	401	75	80	1	
Yes	134	25	60	2.16 (1.63–2.88)	
Lymphovascular infiltration					**<0.001**
No	492	92	77	1	
Yes	43	8	47	2.26 (1.29–3.41)	
Surgical procedure					**0.466**
Retropubic	435	81	77	1	
Perineal	100	19	68	1.14 (0.81–1.60)	
miR-205 in epithelium					**0.003**
Low expression	220	41	81	1	
High expression	245	46	78	1.61 (1.18–2.21)	
Missing	70	13	—		

Table 1. Patient characteristics, clinicopathological variables and expressions of miR-205 and their associations with biochemical failure in 535 prostate cancer patients (univariate analyses; log-rank test, unadjusted Cox proportional hazard ratios). Abbreviations: EFS = event free survival in months; HR = hazard ratio; NC = not computable.

Grade is shown in Fig. 2. 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].

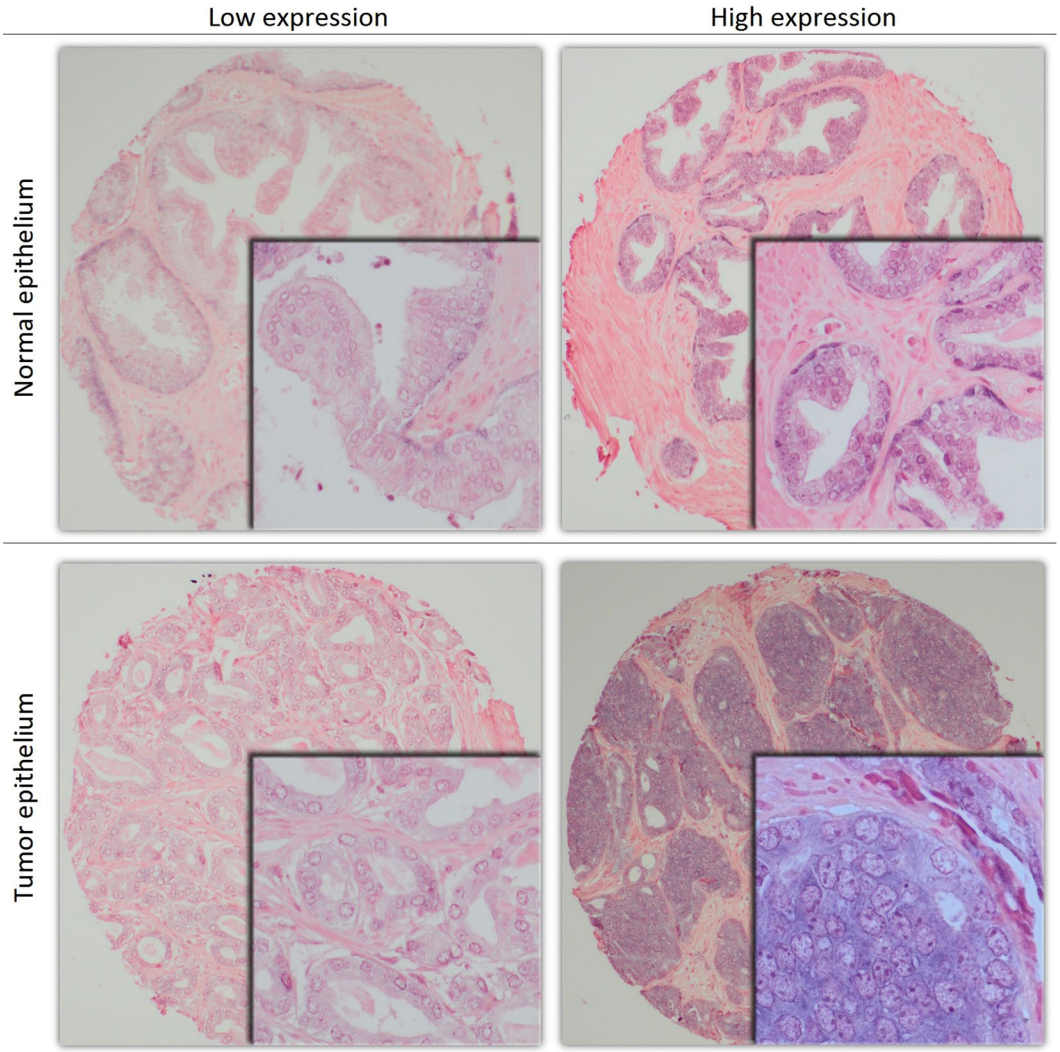


Figure 1. Examples of high and low expression of miR-205 in tissue microarray cores of prostate cancer tissue. 100x (main) and 400x (embedded) magnification.

Multivariate analyses. Results from a multivariate model of clinicopathological variables and miR-205 vs BF for all patients are presented in Table 2. In addition to the clinicopathological factors CAPRA-S Score ($p < 0.001$) and perineural infiltration ($p = 0.001$), a high expression of miR-205 correlates to a worse BF (HR = 1.70, $p = 0.001$). Clinicopathological factors associated to CF and PCD in our cohort are previously reported[19]. When further exploring which clinicopathological subgroups the miR-205 expression had prognostic value, a multivariate model stratified on ISUP Grade was calculated and is presented in Table 3. 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 ($p < 0.001$).

Discussion

We found no association between PC relapse and miR-205 expression assessed in tumor epithelium of PC patients treated by radical prostatectomy. However, we demonstrate that high expression of miR-205 in normal prostate epithelium is independently and significantly associated with biochemical recurrence. Interestingly, our findings raise the hypothesis of the potential impact of normal epithelium in prostate tumors. There was a significantly higher mean expression of miR-205 in normal epithelium compared to tumor epithelium, confirming results from previous studies. We found no association between miR-205 expression and CF or PCD, possibly due to a low number of events in these subgroups.

To our knowledge, this is, hitherto, the largest study of miR-205 expression vs clinical outcome in PC patients. The strengths of our study are the size of the multicenter cohort, the long clinical follow-up, and the *in-situ* examination in both normal and tumor epithelium and stroma. Although the ISH technique is labor-intensive, its strength compared to the widely used real-time polymerase chain reaction (RT-PCR) technique, is the ability to assess marker expressions in the different tissue compartments and cell types. In PC this is highly attractive due

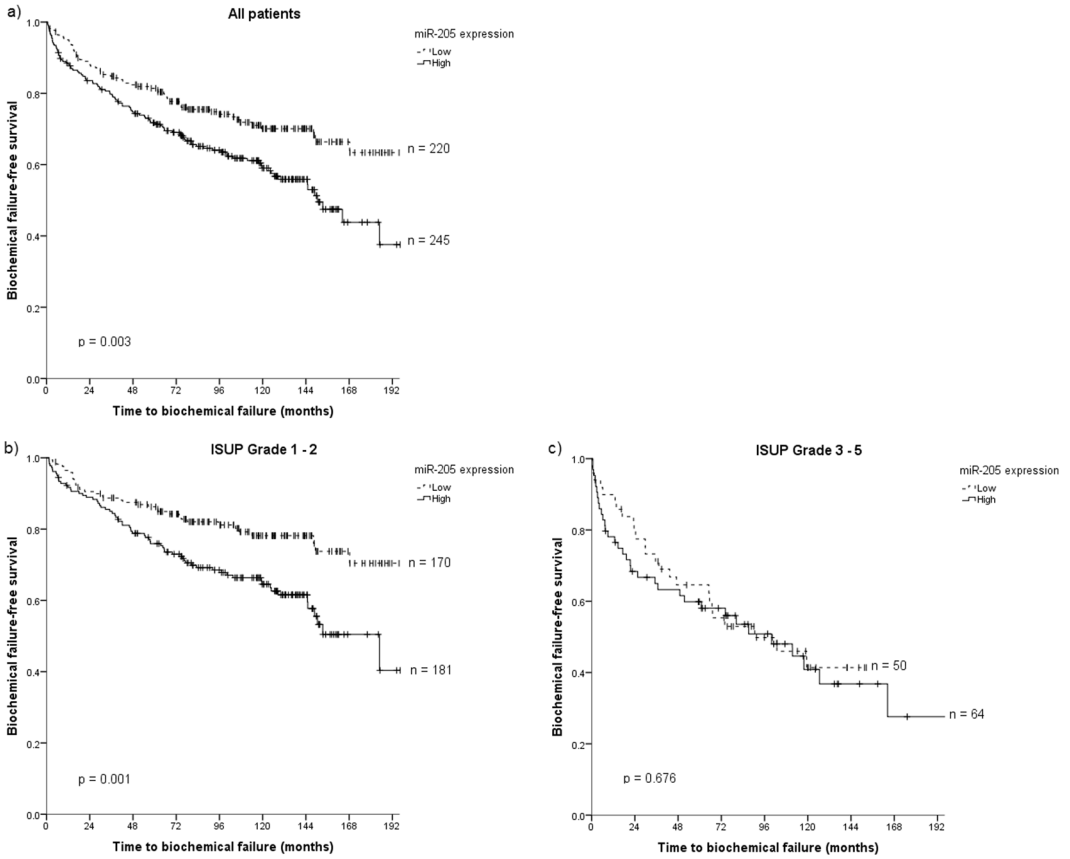


Figure 2. Kaplan-Meier curves of high and low miR-205 expression in normal epithelium in tissue microarray cores of prostate cancer tissue for (**a**) all patients, (**b**) patients with ISUP Grade 1 or 2 and (**c**) patients with ISUP Grade 3–5.

to the multifocal nature of the tumor tissue. Despite the long clinical follow-up (mean 12.4 years), the relatively low incidence of clinical recurrence and prostate cancer-specific death leaves a relatively low number of events. This demonstrates the need for even larger PC studies with longer follow-up to properly evaluate these endpoints. Other weaknesses are the retrospective design and that this study is inherently biased towards the selected group of patients that are considered healthy enough to undergo prostatectomy and towards stages of PC which are perceived surgically curable.

Several previous studies have consistently characterized miR-205 as a tumor suppressor generally downregulated in PC[13–15,32]. However, these studies were based on patients with higher histological grades of cancer in contrast to our patients' localized disease. Hulf *et al.*, however, showed that epigenetic-induced repression of miR-205 is associated with worse prognosis, validated by a cohort with localized PC cases consisting of 149 patients who had had a radical prostatectomy performed[33]. They found overexpression of miR-205 in PC cells to negatively affect cell viability, consistent with a tumor suppressor function.

We found that miR-205 was less expressed in tumor epithelium compared to normal epithelium, consistent with previous studies[13–15,17,32–35]. In line with this, we did not find any association between miR-205 expression in tumor epithelium and clinical outcome. Surprisingly, the prognostic impact of miR-205 was exclusively related to the normal prostate epithelium in PC patients. Initially, our data appeared contradicting as the active tissues in the carcinogenic processes traditionally has been considered to be tumor epithelium and stromal cells. Studies of the interplay between normal morphological and neoplastic epithelial cells have been limited. Hence, little is known about the function of normal epithelium in tumorigenesis. However, a few recent studies have revealed that normal epithelial cells, in addition to normal cells surrounding the tumor, can exert an anti-tumor activity on prostate carcinoma cells. Trevino *et al.* proposed that normal epithelial cells have the potential to revert some of the traits of tumor cells, effectively normalizing the phenotypic characteristics of the tumor cells[34,36]. The integrity and homeostasis of the epithelium are of vital importance to survival. These processes are maintained during growth and in response to damage by the evolvement of defensive mechanisms[37]. This suggests that normal epithelium may have a more important role in controlling tumor expansion than previously acknowledged, although crosstalk between normal and neoplastic epithelial cells is not fully understood.

Based on the studies cited above and our presented results, we hypothesize that the morphologically normal epithelial cells in PC specimens are potentially active functional cellular constituents counteracting the carcinogenic processes of tumor cells, in which one of the counteracting mechanisms might be expression of the tumor suppressor miR-205 in low and intermediate grade tumors. In our cohort, the prognostic importance of miR-205

Characteristics	Patients		Biochemical failure	
	(n)	(%)	HR (95% CI)	p
CAPRA-S Score				**<0.001**
0–2	169	32	1	
3–5	258	48	1.83 (1.19–2.81)	
6–12	102	19	4.55 (2.88–7.20)	
Missing	6	1	—	
Tumor size				**NS**
0–20 mm	250	47		
>20 mm	285	53		
Perineural infiltration				**0.001**
No	250	47	1	
Yes	285	53	1.78 (1.27–6.93)	
Lymphovascular infiltration				**NS**
No	492	92		
Yes	43	8		
miR-205 in epithelium				**0.001**
Low expression	220	41	1	
High expression	245	46	1.70 (1.23–2.35)	
Missing	70	13	—	

Table 2. Prognostic factors and their independent associations with biochemical failure in prostate tissue in 535 prostate cancer patients (multivariate analyses; Cox regression with backward conditional model). Abbreviations: HR = hazard ratio; NS = not significant and removed by backward model before last step of analyses.

expression was primarily found in low-risk cancers such as ISUP Grade Group 1–2 (Gleason 3 + 3 and 3 + 4) and CAPRA-S Score < 6. A possible mechanism why low-risk cancers with high expression of miR-205 in the normal epithelium are more prone to BF, might be that these tumors inhabit properties to recur, while tumors lacking this ability does not induce protective upregulation of miR-205 in normal epithelium to prevent development of tumor aggressiveness. For the more advanced tumors (ISUP Grade ≥3, CAPRA-S Score ≥6), one may postulate that the tumor cells have overcome the influence of the normal epithelium and have inhibited or bypassed their tumor suppressive properties. Thereby, high miR-205 expression in the normal epithelium may be a marker of the normal epithelium's efforts to prevent more aggressive tumors to develop. It has been suggested that normal epithelial cells, secreting tumor suppressive factors such as Il-6[38], TNFα[39,40] and TGFβ1[39], play an important role in influencing the molecular and physiological state of tumor cells[36,41]. In support of our hypothesis, recent studies suggest that, at the initial phase of tumor expansion, normal epithelium may provide a tumor suppressive environment which cancer cells need to overcome to cause tumor progression[34,36,41].

Our findings are also supported by Kalogirou *et al*. They found a consistent tendency for miR-205 upregulation to correlate with an adverse outcome of PC patients, supporting our findings of an association between high expression of miR-205 and BF[14]. They suggested that miR-205 expression might be tightly controlled at different tumor stages, affecting the expression of either tumor suppressors or oncogenes. However, their RT-PCR study could not differentiate between expressions in normal or tumor epithelium, nor epithelium or stroma. Gandellini *et al*. found pathological loss of miR-205 in PC to favor tumorigenesis by creating discontinuities in the basal membrane, and demonstrated that therapeutic replacement of miR-205 can restore basal membrane deposition, thus hampering cancer progression[17]. In a later study, they found miR-205 to prevent the malignant interplay between prostate cancer cells and associated fibroblasts[35], supporting our hypothesis.

We also observed a positive correlation between miR-205 and VEGF-A/VEGFR-2. It has previously been shown that high expression of VEGF-A and VEGFR-2 is correlated to BF and CF in prostatectomy patients[30]. miR-205 directly targets VEGF-A, functioning as a tumor suppressor in breast cancer[42]. No studies have, to our knowledge, assessed associations between PDGFs and miR-205. However, we found a strong correlation between PDGF-D and miR-205, whereas PDGF-B and PDGFR-β correlated to miR-205 to a less extent as well.

In conclusion, our results add support to the potential prognostic role of normal epithelium in PC and its potential crosstalk to surrounding tissues.

As high miR-205 expression in normal epithelium was an overall predictor for BF for patients with localized disease, we propose normal epithelium acts 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. Although speculative, this intriguing postulation mandates further research to clarify the mechanisms of crosstalk between tissues. Considerable resources are currently being invested in the development of miR anti-cancer therapy. However, the success of such specific therapeutic targeting will rely on a deeper understanding of the biological mechanics at play.

Characteristics	Patients		Biochemical failure			
			ISUP Grade 1–2		ISUP Grade 3–5	
	(n)	(%)	HR (95% CI)	p	HR (95% CI)	p
pT-stage				NS		**<0.001**
pT2	374	70			1	
pT3a	114	21			2.78 (1.47–5.25)	
pT3b	47	9			4.99 (2.66–9.35)	
Preop PSA				NS		NS
PSA < 10	308	57				
PSA > 10	221	42				
Missing	6	1				
Positive surgical margin				NS		NS
No	249	47				
Yes	286	53				
Tumor size				NS		NS
0–20 mm	250	47				
>20 mm	285	53				
Perineural infiltration				**0.003**		NS
No	250	47	1			
Yes	285	53	1.93 (1.25–2.97)			
Lymphovascular infiltration				NS		NS
No	492	92				
Yes	43	8				
miR-205 in epithelium				**0.001**		NS
Low expression	220	41	1			
High expression	245	46	2.07 (1.37–3.15)			
Missing	70	13	—			

Table 3. Prognostic factors and their independent associations with biochemical failure in prostate tissue in 535 prostate cancer patients (multivariate analyses; stratified Cox regression with backward conditional model). Abbreviations: HR = hazard ratio; NS = not significant and removed by backward model before last step of analyses.

Equipment and settings. Figure 1 is edited in Adobe Photoshop for cropping, while frames and shadows are made in Microsoft Excel. No adjustments to color, brightness or contrast were done. The subpanels of Fig. 2 are exported from SPSS and joined in Microsoft Paint. All figures and tables are made by the first author, Y.N.

Availability of materials and data. The datasets generated during and analysed during the current study are not publicly available in respect to patients' privacy.

References

1. Ferlay, J. *et al*. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int. J. Cancer* **127**, 2893–2917, https://doi.org/10.1002/ijc.25516 (2010).
2. Bill-Axelson, A. *et al*. Long-term distress after radical prostatectomy versus watchful waiting in prostate cancer: a longitudinal study from the Scandinavian Prostate Cancer Group-4 randomized clinical trial. *Eur. Urol* **64**, 920–928, https://doi.org/10.1016/j.eururo.2013.02.025 (2013).
3. Etzioni, R. *et al*. Increasing use of radical prostatectomy for nonlethal prostate cancer in Sweden. *Clin. Cancer Res* **18**, 6742–6747, https://doi.org/10.1158/1078-0432.CCR-12-1537 (2012).
4. Fossa, S. D. *et al*. Treatment and 5-year survival in patients with nonmetastatic prostate cancer: the Norwegian experience. *Urology* **83**, 146–152, https://doi.org/10.1016/j.urology.2013.08.081 (2014).
5. Van der Kwast, T. H. & Roobol, M. J. Defining the threshold for significant versus insignificant prostate cancer. *Nature reviews. Urology* **10**, 473–482, https://doi.org/10.1038/nrurol.2013.112 (2013).
6. Siegel, R., Ma, J., Zou, Z. & Jemal, A. Cancer statistics, 2014. *CA: a cancer journal for clinicians* **64**, 9–29, https://doi.org/10.3322/caac.21208 (2014).
7. Pichler, M. & Calin, G. A. MicroRNAs in cancer: from developmental genes in worms to their clinical application in patients. *British journal of cancer* **113**, 569–573, https://doi.org/10.1038/bjc.2015.253 (2015).
8. Hammond, S. M. RNAi, microRNAs, and human disease. *Cancer chemotherapy and pharmacology* **58**(Suppl 1), s63–68, https://doi.org/10.1007/s00280-006-0318-2 (2006).
9. Hammond, S. M. An overview of microRNAs. *Advanced drug delivery reviews* **87**, 3–14, https://doi.org/10.1016/j.addr.2015.05.001 (2015).
10. Zhang, W., Liu, J. & Wang, G. The role of microRNAs in human breast cancer progression. *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine* **35**, 6235–6244, https://doi.org/10.1007/s13277-014-2202-8 (2014).
11. Orang, A. V., Safaralizadeh, R. & Hosseinpour Feizi, M. A. Insights into the diverse roles of miR-205 in human cancers. *Asian Pacific journal of cancer prevention: APJCP* **15**, 577–583 (2014).
12. Christopher, A. F. *et al*. MicroRNA therapeutics: Discovering novel targets and developing specific therapy. *Perspectives in clinical research* **7**, 68–74, https://doi.org/10.4103/2229-3485.179431 (2016).

13. Hagman, Z. *et al*. miR-205 negatively regulates the androgen receptor and is associated with adverse outcome of prostate cancer patients. *British journal of cancer* **108**, 1668–1676, https://doi.org/10.1038/bjc.2013.131 (2013).
14. Kalogirou, C. *et al*. MiR-205 is progressively down-regulated in lymph node metastasis but fails as a prognostic biomarker in high-risk prostate cancer. *International journal of molecular sciences* **14**, 21414–21434, https://doi.org/10.3390/ijms141121414 (2013).
15. Tucci, P. *et al*. Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 15312–15317, https://doi.org/10.1073/pnas.1110977109 (2012).
16. Martens-Uzunova, E. S. *et al*. Diagnostic and prognostic signatures from the small non-coding RNA transcriptome in prostate cancer. *Oncogene* **31**, 978–991, https://doi.org/10.1038/onc.2011.304 (2012).
17. Gandellini, P. *et al*. miR-205 regulates basement membrane deposition in human prostate: implications for cancer development. *Cell death and differentiation* **19**, 1750–1760, https://doi.org/10.1038/cdd.2012.56 (2012).
18. Melbo-Jorgensen, C. *et al*. Stromal expression of MiR-21 predicts biochemical failure in prostate cancer patients with Gleason score 6. *PloS one* **9**, e113039, https://doi.org/10.1371/journal.pone.0113039 (2014).
19. Andersen, S. *et al*. Disease-specific outcomes of radical prostatectomies in Northern Norway; a case for the impact of perineural infiltration and postoperative PSA-doubling time. *BMC. Urol* **14**, 49, https://doi.org/10.1186/1471-2490-14-49 (2014).
20. Epstein, J. I. An update of the Gleason grading system. *J. Urol* **183**, 433–440, https://doi.org/10.1016/j.juro.2009.10.046 (2010).
21. Helpap, B. & Egevad, L. Modified Gleason grading. An updated review. *Histol. Histopathol* **24**, 661–666 (2009).
22. Bremnes, R. M. *et al*. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. *J. Clin. Oncol* **20**, 2417–2428 (2002).
23. Epstein, J. I. *et al*. The2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. *The American journal of surgical pathology* **40**, 244–252, https://doi.org/10.1097/pas.0000000000000530 (2016).
24. Cooperberg, M. R., Hilton, J. F. & Carroll, P. R. The CAPRA-S score: A straightforward tool for improved prediction of outcomes after radical prostatectomy. *Cancer* **117**, 5039–5046, https://doi.org/10.1002/cncr.26169 (2011).
25. Cooperberg, M. R. *et al*. TheUniversity of California, San Francisco Cancer of the Prostate Risk Assessment score: a straightforward and reliable preoperative predictor of disease recurrence after radical prostatectomy. *The Journal of urology* **173**, 1938–1942, https://doi.org/10.1097/01.ju.0000158155.33890.e7 (2005).
26. Childs, G. *et al*. Low-level expression of microRNAs let-7d and miR-205 are prognostic markers of head and neck squamous cell carcinoma. *The American journal of pathology* **174**, 736–745, https://doi.org/10.2353/ajpath.2009.080731 (2009).
27. Bishop, J. A. *et al*. Accurate classification of non-small cell lung carcinoma using a novel microRNA-based approach. *Clinical cancer research: an official journal of the American Association for Cancer Research* **16**, 610–619, https://doi.org/10.1158/1078-0432.ccr-09-2638 (2010).
28. Bera, A., VenkataSubbaRao, K., Manoharan, M. S., Hill, P. & Freeman, J. W. A miRNA signature of chemoresistant mesenchymal phenotype identifies novel molecular targets associated with advanced pancreatic cancer. *PloS one* **9**, e106343, https://doi.org/10.1371/journal.pone.0106343 (2014).
29. Duan, B. *et al*. miR-205 as a biological marker in non-small cell lung cancer. *Biomedicine & pharmacotherapy=Biomedecine & pharmacotherapie* **91**, 823–830, https://doi.org/10.1016/j.biopha.2017.04.086 (2017).
30. Nordby, Y. *et al*. Stromal expression of VEGF-A and VEGFR-2 in prostate tissue is associated with biochemical and clinical recurrence after radical prostatectomy. *Prostate* **75**, 1682–1693, https://doi.org/10.1002/pros.23048 (2015).
31. Nordby, Y. *et al*. High expression of PDGFR-beta in prostate cancer stroma is independently associated with clinical and biochemical prostate cancer recurrence. *Scientific reports* **7**, 43378, https://doi.org/10.1038/srep43378 (2017).
32. Larne, O. *et al*. miQ–a novel microRNA based diagnostic and prognostic tool for prostate cancer. *International journal of cancer* **132**, 2867–2875, https://doi.org/10.1002/ijc.27973 (2013).
33. Hulf, T. *et al*. Epigenetic-induced repression of microRNA-205 is associated with MED1 activation and a poorer prognosis in localized prostate cancer. *Oncogene* **32**, 2891–2899, https://doi.org/10.1038/onc.2012.300 (2013).
34. Correction: A Network Biology Approach Identifies Molecular Cross-Talk between Normal Prostate Epithelial and Prostate Carcinoma Cells. *PLoS computational biology* **12**, e1005172, https://doi.org/10.1371/journal.pcbi.1005172 (2016).
35. Gandellini, P. *et al*. miR-205 hinders the malignant interplay between prostate cancer cells and associated fibroblasts. *Antioxidants & redox signaling* **20**, 1045–1059, https://doi.org/10.1089/ars.2013.5292 (2014).
36. Trevino, V. *et al*. A Network Biology Approach Identifies Molecular Cross-Talk between Normal Prostate Epithelial and Prostate Carcinoma Cells. *PLoS computational biology* **12**, e1004884, https://doi.org/10.1371/journal.pcbi.1004884 (2016).
37. Macara, I. G., Guyer, R., Richardson, G., Huo, Y. & Ahmed, S. M. Epithelial homeostasis. *Current biology: CB* **24**, R815–825, https://doi.org/10.1016/j.cub.2014.06.068 (2014).
38. Giri, D., Ozen, M. & Ittmann, M. Interleukin-6 is an autocrine growth factor in human prostate cancer. *The American journal of pathology* **159**, 2159–2165, https://doi.org/10.1016/s0002-9440(10)63067-2 (2001).
39. Campbell, C. L., Savarese, D. M., Quesenberry, P. J. & Savarese, T. M. Expression of multiple angiogenic cytokines in cultured normal human prostate epithelial cells: predominance of vascular endothelial growth factor. *International journal of cancer* **80**, 868–874 (1999).
40. Mechergui, Y. B. *et al*. The profile of prostate epithelial cytokines and its impact on sera prostate specific antigen levels. *Inflammation* **32**, 202–210, https://doi.org/10.1007/s10753-009-9121-7 (2009).
41. Hogan, C., Kajita, M., Lawrenson, K. & Fujita, Y. Interactions between normal and transformed epithelial cells: Their contributions to tumourigenesis. *The international journal of biochemistry & cell biology* **43**, 496–503, https://doi.org/10.1016/j.biocel.2010.12.019 (2011).
42. Wu, H., Zhu, S. & Mo, Y. Y. Suppression of cell growth and invasion by miR-205 in breast cancer. *Cell research* **19**, 439–448, https://doi.org/10.1038/cr.2009.18 (2009).

Author Contributions

Wrote the main manuscript: Y.N. Figure preparation: Y.N. Participated in *in-situ* hybridization: E.R. Participated in scoring expressions: Y.N., S.A. and E.R. Establishing database and retrieving clinical information: Y.N., S.A., E.R., N.N., L.T.B. Reviewed the manuscript: All authors. Have agreed on all content: All authors. Performing and controlling statistics: Y.N. and S.A. Conceived the research idea: S.A., Y.N., L.T.B., R.B., T.D., E.R.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.