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Faculty of Health Sciences
Institute of Medical Biology

The role of steroid hormone receptors in prostate cancer

A study of estrogen- and progesterone receptors in adenocarcinoma of the prostate

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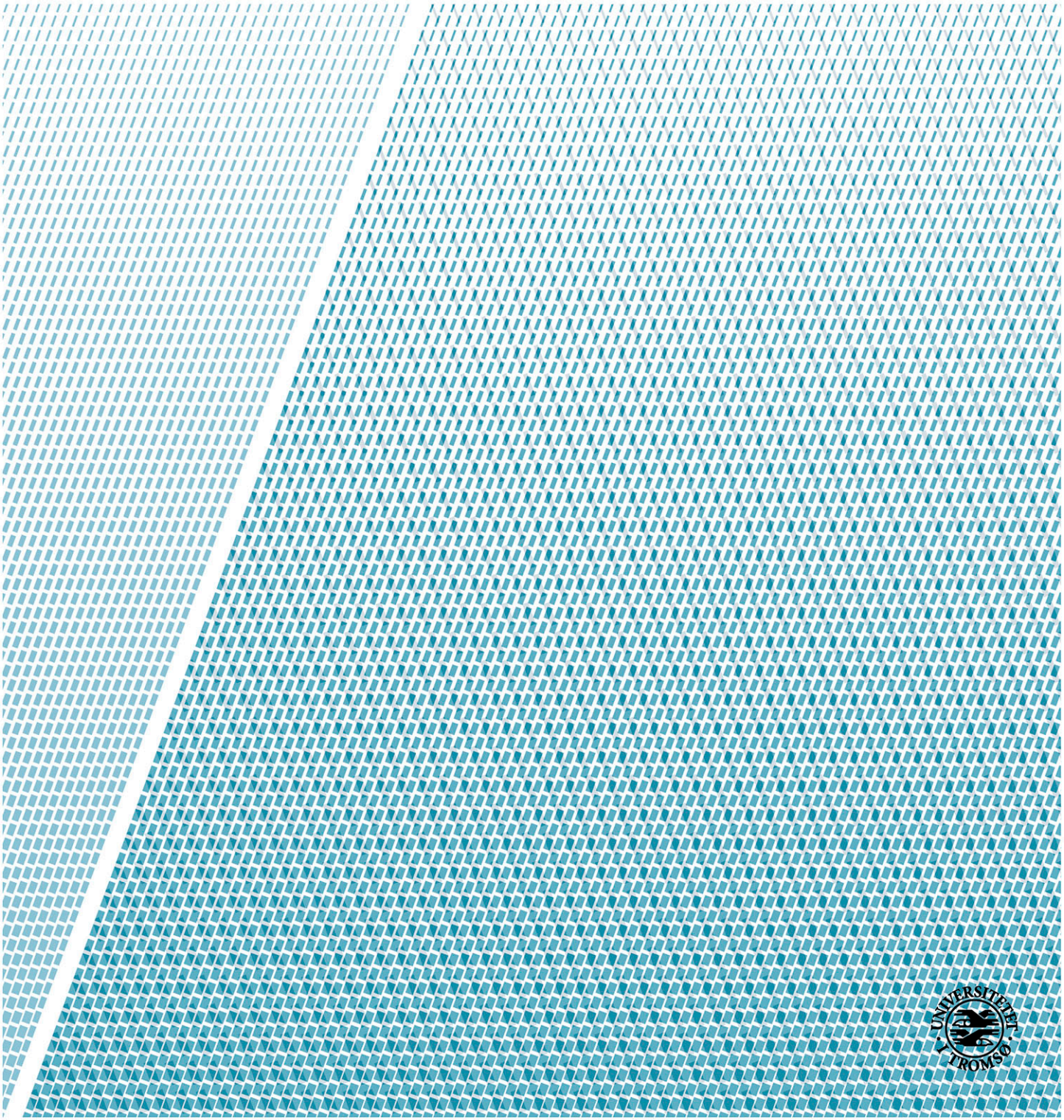
A dissertation for the degree of Philosophiae Doctor – June 2018



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SUMMARY

For decades, prostate cancer (PC) has been ranked amongst the most frequent occurring cancers affecting men, especially in the western world[1]. Despites 5-year survival rates exceeding 90 % and climbing, it remains one of the most mortal cancers[2,3]. The PC has a heterogeneous nature which is exceedingly hard to predict. At one end, PCs can present as small, well-differentiated tumors which will remain indolent throughout life. One the other side are the progressing, aggressive cancers resulting in metastatic disease and death. Great efforts have been made throughout the years to develop additional prognostic markers that can aid decisions of treatment strategies and thus reduce unnecessary invasive procedures. Yet, the challenge of separating indolent from aggressive disease withstands and overtreatment remains a challenge[4]. That PC depends on androgens to develop and progress has been acknowledged for over 60 years[5,6]. Androgens are, in addition to estrogen and progesterone, sex steroid hormones, belonging to a large family of steroid hormones. These hormones exert their effects by binding and stimulating their cognate steroid hormone receptor (SHR)[7]. The previous paradigm of androgens being the "male" hormone and estrogen and progesterone a "female" hormone has shifted due to advances in several research fields. It is now appreciated that sex steroid hormones are vital for numerous physiological functions in both sexes and that their receptors are expressed in various tissues throughout the human body[8]. We sought to examine the tissue distribution of a selection of steroid hormone related biomarkers and their association with the clinical endpoints: biochemical failure (BF), clinical failure (CF) and PC death (PCD). The included biomarkers where the progesterone receptor (PGR) (**Paper I**) and its isoforms (PGRA, PGRB) (**Paper III**), in addition to the estrogen receptor (ER) α, ERβ and aromatase (**Paper II**), the enzyme converting androgens to estrogen. These markers were investigated in both tumor cells and the tumor microenvironment (TME) of prostate adenocarcinomas. This was achieved by constructing tissue microarrays from 535 prostatectomy specimens. The material was retrospectively collected from patients initially treated with radical prostatectomy for their cancer, and who were naïve to hormonal and radiation therapy. Patient follow up time was initially 7.4 years (**Paper I**), and after a patient update it was extended to 12.4 years (**Paper II and II**). A significant and independent prognostic value was observed for all investigated markers. ERα (**Paper II**) and PGRA (**Paper III**) expression was mainly restricted to tumor associated stroma (TS), while the remaining markers where expressed in both TS and tumor epithelial (TE) tissue compartments. In TS, ERα was a positive prognostic factor regarding CF and

PCD and aromatase with regards to BF, while ERβ was a negative prognosticator for BF (**Paper II**). In TE, aromatase (**Paper II**) and pan-PGR (**Paper I**) expression were associated with CF. Aromatase as a positive prognosticator and pan-PGR as a negative. When investigating the PGR isoforms separately (**Paper III**), PGRB in TE remained a negative prognosticator for CF, while PGRA expression in TE was absent. Indicating that the initial negative effect observed for pan-PGR was effectuated by the PGRB isoform. Based on these observations, we suggest a role of these sex-SHRs in the pathogenesis of PC and propose a prognostic and possibly therapeutic potential.

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Paper I

High Progesterone Receptor Expression in Prostate Cancer Is Associated with Clinical Failure Thea Grindstad, Sigve Andersen, Samer Al-Saad, Tom Donnem, Yury Kiselev, Christian Nordahl Melbø-Jørgensen, Kaja Skjefstad, Lill-Tove Busund, Roy M. Bremnes, Elin Richardsen. PLoS One. 2015; 10(2): e0116691. Published online 2015 Feb 27. doi:10.1371/journal.pone.0116691

Paper II

Estrogen receptors α and β and aromatase as independent predictors for prostate cancer outcome

Thea Grindstad, Kaja Skjefstad, Sigve Andersen, Nora Ness, Yngve Nordby, Samer Al-Saad, Silje Fismen, Tom Donnem, Mehrdad Rakaee Khanehkenari, Lill-Tove Busund, Roy M. Bremnes & Elin Richardsen. Scientific Reports volume 6, Article number: 33114 (2016), published online: 09 September 2016, doi:10.1038/srep33114

Paper III

Progesterone receptors in Prostate Cancer: Progesterone receptor B is the isoform associated with disease progression

Thea Grindstad, Elin Richardsen, Sigve Andersen, Kaja Skjefstad, Mehrdad Rakaee khanehkenari, Tom Donnem, Nora Ness, Yngve Nordby, Roy M. Bremnes, Samer Al-Saad & Lill-Tove Busund. (Submitted)

ABBREVIATIONS

AAH	Adenomatous hyperplasia
AF1/ AF2	Activation function domain ½
APAAP	Alkaline phosphatase-antialkaline phosphatase
AR	Androgen receptor
ASAP	Atypical small acinar proliferation
BF	Biochemical failure
BPH	Benign Prostate Hyperplasia
BRCA	BReast CAncer gene
CDK	Cyclin dependent kinases
CF	Clinical failure
CT	Computed tomography
DAB	Diaminobenzidine
DBD	DNA binding domain
DNA	Deoxyribonucleotideacid
DRE	Digital Rectal Examination
E2	17β-estradiol
ECM	Extracellular matrix
EPE	Extraprostatic extension
ER	Estrogen receptor
FFPE	Formalin Fixed Paraffin Embedded
ISH	In situ hybridization
FSH	Follicle stimulating hormone
GnRH (/LHRH)	Gonadotropin releasing hormone
GR	Glucocorticoid receptor
H&E	Hematoxylin and Eosin
HOXB13	Homeobox gene 13
IDCP	Intraductal carcinoma of the prostate
IHC	Immunohistochemistry
LBD	Ligand binding domain
LH	Luteinizing hormone
LUTS	Lower urinary tract symptoms
LVI	Lymphovascular infiltration
MRI	Magnetic Resonance Imaging
MAPK	Mitogen-activated protein kinase
PAP	Peroxidase-antiperoxidase
PC	Prostate cancer
PCA3	Prostate cancer antigen 3
PCD	PC death
PCR	Polymerase chain reaction
PGR	Progesterone receptor
PI3K	Phosphoinositide-3- kinase
PIN	Prostatic intraepithelial neoplasia
PNI	Perineural infiltration
PET	Positron emission tomography
PSA	Prostate specific antigen
PSM	Positive surgical margin
RNA	Ribonucleic acid
SHBG	Sex-hormone binding globulin
SHR	Steroid hormone receptors
SNP	Single nucleotide polymorphism
SPCG 4	Prostate cancer Group Study Number 4
SUMO	Small ubiquitin-like modifier
TE	Tumor epithelial cells
TMA	Tissue microarray
TME	Tumor microenvironment
TMPRSS2	Transmembrane protease serine 2
TNM	Tumor, Node, Metastasis
TS	Tumor-associated stromal cells

1 INTRODUCTION

1.1 Prostate cancer

1.1.1 Epidemiology

Globally, PC accounts for an estimated 1.600 000 new cases and 366 000 deaths annually. This made PC the most frequently occurring cancer in men worldwide in 2015[1]. A regional difference in PC incidence is observed, and the highest rates occur in the developed countries of the western world. The odds of developing PC by the age of 79 can be as high as 1/6 in highly developed countries, like the U.S. and the Nordic countries, and as low as 1/47 in developing countries[1]. In the U.S., 161 360 new cases of PC are expected in 2017, accounting for 19 % of new cancer cases in males[3]. In Norway, PC was the most common cancer in 2016 with 5118 new cases (Figure 1). This accounts for 29 % of all new cancer cases in males in 2016 and places Norway amongst the European countries with highest incidence rates[2]. PC is more prevalent in older men, and the diagnosis is most frequent in the age group 65-74. In Norway, the cumulative risk of developing PC by the age of 75 is 13,4 %[2].

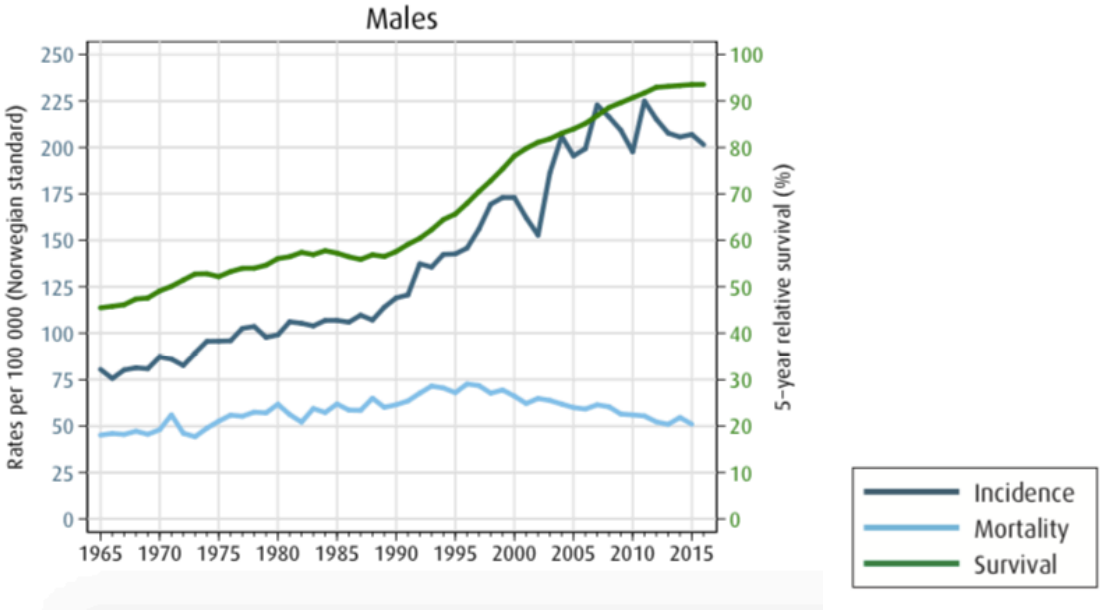

Figure 1 – PC incidence, mortality and survival rates (national standard) in Norway. Cancer Registry of Norway, Cancer in Norway 2016©[2]

1.1.2 Trends in incidence-, survival- and mortality rates

A drastic rise in PC incidence was observed in the beginning of the 1990´s, especially amongst younger men (< 70 years) and in highly developed countries[2,3,9]. Additionally, a

stage migration towards a greater extent of less aggressive PCs at initial diagnosis was observed[10]. In Norway, this increase has persisted since then, but seems to have stabilized in recent years with an age-standardized, world standard population, incidence rate of 107 per 100.000 person years in the time period 2012 – 16 (Figure 1)[9]. In the U.S., a gradual decline following the major peak in the 1990´s has been observed with a reduction in PC incidence of approximately 10% annually in the period 2010 to 2013. Besides increased life expectancy, these changes conceivably reflect the prostate specific antigen (PSA)-test application patterns for the detection of asymptomatic PC[3].

An increase in survival rates has been observed parallel with the increased incidence rates in the 1990´s (Figure 1)[2,9]. The 5-year relative survival rate of PC was 93.6 % in Norway in the time period 2012 – 16, compared 91.7% in 2007 – 11 and 85.1 % in 2002 - 06[2]. Despite this, PC remains one of the cancers taking most lives annually. Today, it ranges as the 3rd leading cause of cancer related death amongst men in both the U.S. and Norway. In the U.S. nearly 27.000 cases of death due to PC is expected in 2017 and the median age of death is around 80 years[3]. In Norway 1045 men died of PC in 2015[2]. It is important to remember that PC development is variable, and it can take decades from cancer development to manifestation of clinical cancer. This is demonstrated by the high incidence rates combined with the high 5-years survival rates. Fortunately, survival data indicates that the mortality rates are declining[2,3]. After a steady increase in PC mortality rates towards the early-mid 1990´s, a continued decrease in mortality rates has been observed since, especially amongst younger men (< 70 years)[2,3,9] (Figure 1). In Norway, a steady decline in mortality rates with annual declines ranging from 1.9 – 2.7 % has been observed from the mid- to late-1990s[9]. In 2015, the age-standardized mortality in Norway was 51.1 per 100 000 person-years, compared to 61.9 per 100.000 between 1980 – 1984[2,9]. This is comparable to the U.S. where mortality rates have decreased by approximately 3 % annually since 1999[3]. However, this change is small compared to the aforementioned increase observed within incidence- and survival rates. Additionally, for the past decade, survival of patients with distant metastatic disease has remained unchanged at approximately at 36.5 % in Norway[2]. The increase in incidence rates and the subsequent decline in mortality rates can indicate improved PC management, including earlier diagnosis, new treatment options and enhanced awareness amongst patients. However, it also raises the question of overdiagnosing patients and even worse, over-treating.

1.1.3 Etiology and risk factors

Although PC accounts for one of the leading causes of cancer and cancer related death, the cause of PC is not as evident as for other major cancer types, such as lung cancer. An unbalanced distribution globally with much higher incidence rates in the western world[1] could indicate environmental- or life style factors as a major contributor to cancer development. However, it is important to consider the PSA-test as a probable confounder to this difference. Since the use of PSA-test is applied more extensively in the western world, it could in turn increase incidence rates. Nevertheless, there are several known factors associated with PC risk. These are age, ethnicity, genetics and possibly diet and life-style factors.

1.1.3.1 Age

There is a strong correlation between age and development of PC. The majority of PCs are diagnosed in the older population. Only 1 % of PCs are clinically detected in men < 50 years of age and the majority of patients have reached 60 years before the diagnosis, whit a peak in the age group 65 – 69[1]. In Norway, the median age at diagnosis is 69 years old, a few years younger than in the previous decades, and the diagnosis is rarely given before the age of 50 (Figure 2). Similarly, in the U.S. the risk of developing PC evolves from 1.9 % (1/52) in the age group 50 – 59 to 9.1 % (1/11) for those > 70[3]. A plausible explanation for an observed decline in incidence after the age of 70 (Figure 2) could be that fewer men are being examined for possible PC in this age group. Several autopsy studies support this age correlation by confirming an increasing frequency of latent PC with age demonstrating occult cancer in as much as 40 – 73 % of the patients in the age group 81 – 90 years[11].

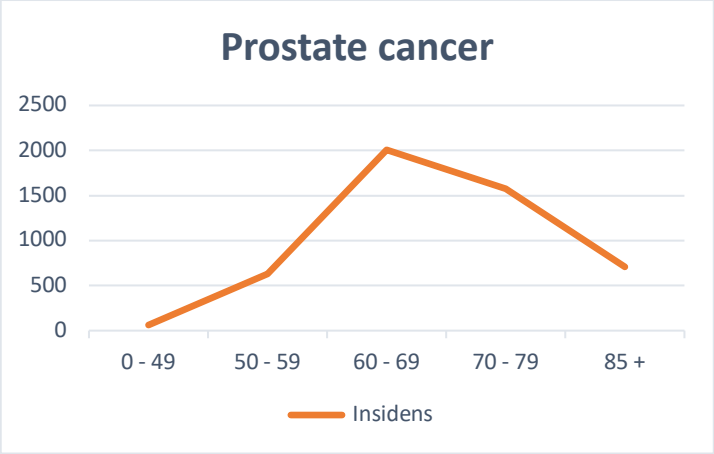

Figure 2 - Depicting the number of prostate cancers (n) diagnosed by age-group in Norway 2016. The table is based on numbers from The Norwegian Cancer Association´s annual cancer statistics. Figure: Thea Grindstad

1.1.3.2 Ethnicity

The risk of PC and PC mortality rates are markedly elevated in black males of West-African ancestry[12]. In the U.S. the risk of developing PC is 74 % higher in African-American males compared to Caucasians and Hispanics[3]. Additionally, African-American males appear to have an earlier disease onset[13] and more aggressive disease[14]. Such differences are also evident in African males from Sub-Sahara, the Caribbean and United Kingdom[12]. The reason for these differences is not fully understood but is likely complex and multifactorial. Confounding factors such as differences in received health care or disease literacy have been implicated as the major reason for the observed difference[15,16]. However, emerging evidence also indicates genetic variations as the underlying cause[17,18].

1.1.3.3 Inheritance

The risk of PC is increased 2.5-times when a single first-degree relative is affected, and with two or more affected first-degree relatives the risk is increased 5-fold[19]. Presumably, the majority of PCs are a result of spontaneous acquired (somatic) mutations, this is, however, strongly indicative of inheritable risk factors for PC as well. Presently, several inherited (germline) genetic factors associated with PC have been identified. This includes rare, but high-risk germline mutations, in addition to more frequent, low-risk genetic factors identified through genome-wide association studies (GWAS)[20].

One acknowledged rare, but high-risk germline mutation in PC is *BRCA2*[a] mutation. *BRCA2*[a] is a DNA repair gene with an acknowledged association with familiar PC, in addition to the more familiar association with breast- and ovarian cancer risk. This mutation can be inherited from both parents, and PC on the father's side is not necessary for the risk to be increased in the mutation carrier. The relative risk of developing PC by the age of 65 is estimated to be 2 – 7 times higher for *BRCA2*[a] mutation carriers compared to those without the mutation[21]. *BRCA2*[a] mutation is also associated with earlier disease onset and a more aggressive phenotype[22,23]. Another predisposition gene identified is the homeobox gene (*HOXB13*)[b]. In a large scale study on populations with mostly European descent, men with *HOXB13*[b] mutations (G84E variant) has significantly higher odds for developing PC and the mutation was associated familial PC and earlier disease onset[24]. Lynch syndrome[c] is the most frequent cause of hereditary colorectal cancer and is associated with malignancies in several other organs in both genders. An increased predisposition for PC has been proposed due to observed increase in life time risk amongst men with Lynch syndrome compared to the general population[25]. However, a benefit of increased screening in this patient group is not confirmed. Other extensively studied candidate susceptibility genes in hereditary PC are the inflammatory and infection response genes *RNASEL*, *ELAC2* and *MSR1,* but their impact on prostate carcinogenesis remains uncertain[20]. Several GWAS studies have identified a great number of single nucleotide polymorphisms[d] (SNP´s) associated with familial PC. Due to substantial heterogeneity throughout the population, so far, no single gene variant has been associated with a larger proportion of familial PC. Nor can a single gene variant distinguish between indolent and more aggressive phenotypes[20]. There is, however, emerging evidence indicating that accumulation of specific SNP´s can increase PC risk exponentially[26].

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[a] **BRACA1/ 2:** Tumor suppressor genes located on human chromosome 13 at locus 13q12.3. Encodes protein essential for DNA repair pathways, suppressing formation of chromosomal rearrangements. Mutations are associated with several cancers and is inherited in an autosomal dominant pattern

[b] **HOXB13:** Transcription factor encoding gene that belongs to the homeobox gene superfamily. Regulates various gene transcripts essential for embryonic development and tissue differentiation, including the prostate.

[c] **Lynch syndrome:** An autosomal dominant disorder that is caused by a germline mutation in one of several DNA mismatch repair (MMR) genes (*MLH1, MSH2, MSH6, and PMS2*)

[d] **SNP:** Single nucleotide polymorphism - a variation in a single nucleotide at a single position in a DNA stretch between members of a species or paired chromosomes in an individual, creating different alleles

1.1.3.4 Other risk factors

The effect of obesity on PC incidence is not fully determined. There is, however, evidence indicating that obese men are more likely to develop an aggressive PC compared to those with normal body mass index[27]. This observation that is supported in a recent "umbrella" review of risk factors and PC[28]. Cellular damage and a persistent inflammatory milieu are associated with cancer development[29]. Numerous environmental carcinogens that could cause damage and inflammation to prostatic cells have been investigated and several potential candidates have emerged, e.g. red meat, dairy products and diets high in calcium. Several protective candidates are proposed, e.g. omega-3-rich foods and certain vegetables, including tomato. In the aforementioned review, the majority of dietary factors investigated yielded only moderate to weak evidence of an association with PC[28]. So far, there is not sufficient evidence available to advocate specific nutritional supplements to prevent PC. Other environmental carcinogens, such as tobacco use, is associated with a minor increased risk of PC death amongst those with established disease[30].

1.2 Anatomy and histopathology of the prostate

1.2.1 Normal prostate

The prostate is an exocrine gland unique to males. This firm, elastic structure, approximately the size of a walnut after puberty[31]. However, in the aging male the prostate enlarges[32]. The prostate is located at the base of the urinary bladder, anterior to the rectum (Figure 3). A section of the urethra courses through the prostate and merges with the ejaculatory duct where secretions from the prostatic glands, *vas deferens* and seminal vesicles empty together, contributing to the composition of semen[31].

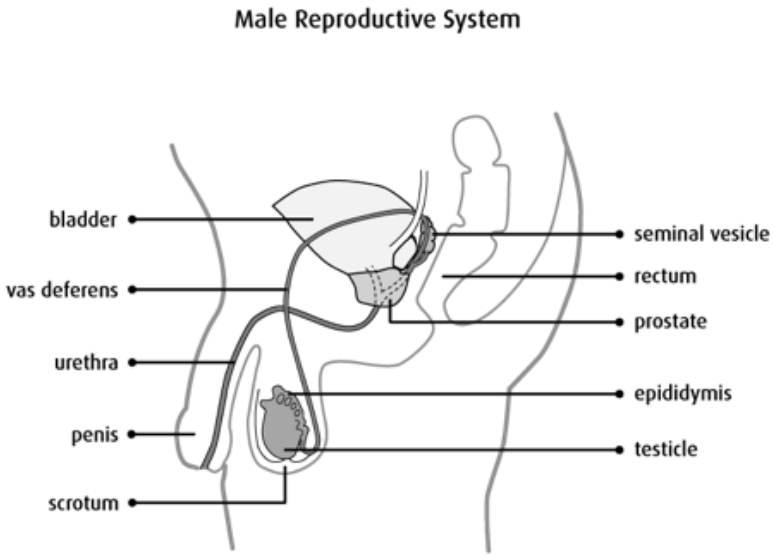

Figure 3 – Anatomical illustration of the male reproductive system. Reprinted with permission from the Canadian Cancer Society © (http://www.cancer.ca/en/cancer-information/cancer-type/prostate/prostate-cancer/the-prostate/?region=on)

The prostate gland is divided into four general zones. These zones differ in their histological composition and are predilection sites for specific prostatic diseases are discussed in the sections below[33] (Figure 4)

1) **Peripheral zone** – Constitutes the majority of the gland (approximately 70 %)
    a. Forms the mid and the apex of the prostate
    b. The easiest accessible area when performing digital rectal examination (DRE)
2) **Central zone** – The area surrounding the ejaculatory ducts (20 %)
3) **Transition zone** – Makes up approximately 5 % of the gland and is the area around the proximal prostatic urethra
4) **The anterior fibromuscular stroma**
    a. Composed of collagen and smooth muscle cells

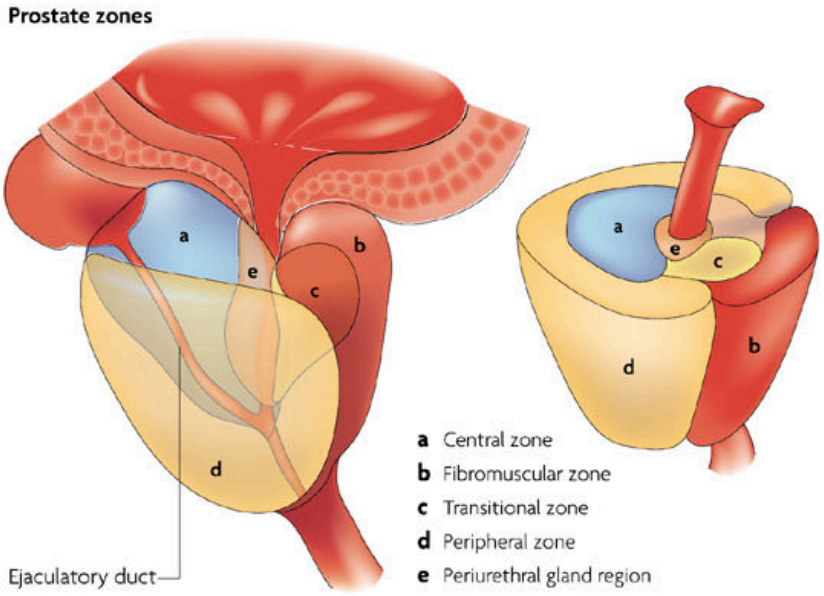

Figure 4 – The zonal anatomy of the prostate

The anatomical zones of the prostate. Reprinted with permission from Nature© Nature Reviews Cancer[34], 2007. The drawing is adapted from an image on Understanding PC website (http://studentweb.usq.edu.au/home/q9210374/site/index.html).

The prostate is made up of branched tubular-acinar glands (30 – 50) forming a convoluted pattern which is surrounded by stroma (Figure 5). These glands drain directly into the urethra through several ducts[35]. The architecture of the glands is simpler in the transition zone and peripheral zone compared to the central zone, which contains large, irregular acini[33]. The stroma consists mainly of collagenous fibrous tissue and smooth muscle fibers and extracellular matrix (ECM). The transition and central and zones have more compacted

stroma and denser muscle bundles. Fibrous septa separate the gland into lobules. Columnar secretory cells, typically with a prominent, round basal nucleus and pale cytoplasm, constitute the main epithelial cell type in the glands. In addition, small, flat basal cells are located at the base of the gland and are in contact with the basement membrane. The basal cells harbor the function as stem cells and can become distinct in cases of benign prostatic hyperplasia (BPH). A capsule comprised of condensed fibromuscular layer of the stroma encloses the posterior and lateral parts of the prostate, while the anterior and apical are restricted by the anterior fibromuscular stroma which solely consists of muscle fibers and collagenous stroma[35]. Outside the prostatic capsule and fibromuscular layer lies neurovascular bundles necessary for the penile erectile function[31].

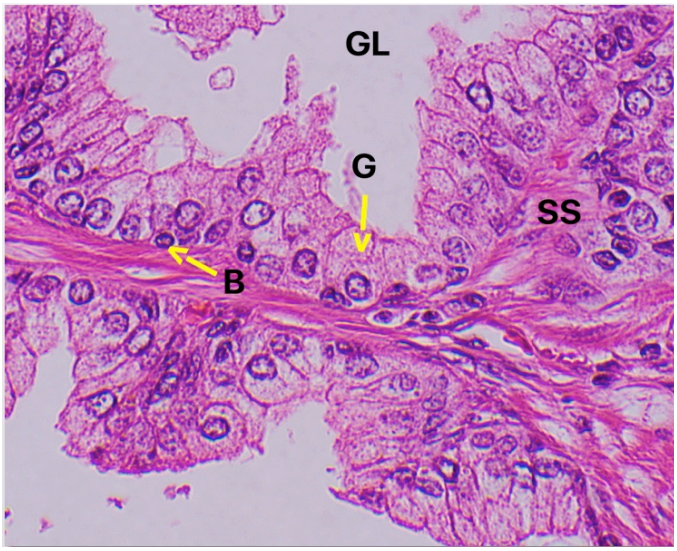

Figure 5 – Normal prostate histology

Detailed picture of prostate gland histology marking off basal cells (B), supporting stroma (SS), glandular epithelial cells (G) and lumen of a prostatic gland (GL). Figure: Thea Grindstad

1.2.2 Benign Prostate hyperplasia (BPH)

BPH is a benign prostatic enlargement and not considered a risk factor for PC[36]. Although extensively investigated, the pathogenesis of remains not fully comprehended. The incidence of BPH increases with age[32], and symptoms of BPH include those referred to as lower urinary

tract symptoms[e] (LUTS). BPH is primarily a disease of the stroma and develops predominantly in the central- and transitional zone of the prostate (Figure 5)[37]. This is in contrast to PC which originates from epithelial cells and has the peripheral zone as a predilection site[38]. The typical histological traits of BPH are hyperplastic nodules with an increased stroma to epithelium ratio and an intact, continuous basal cell layer. The glands can be cystic and dilated, crowded and small, or a combination. Additionally, the glandular architecture can appear more complex with luminal foldings and papillary branches[37]. Typical traits of BPH are depicted in Figure 6 below.

1.2.3 Precancerous lesions

There are four main entities associated with precancerous lesions in the prostate. The major being prostatic intraepithelial neoplasia (PIN). The remaining are atypical small acinar proliferation (ASAP)[39], adenomatous hyperplasia (AAH) (also referred to as adenosis)[40] and proliferative atrophic lesions[41]. The pathological term ASAP is applied when one identifies a lesion suspicious of, but not diagnostic of carcinoma. The cancerous potential in AAH and atrophy, on the other, hand is undetermined. PIN is discussed in detail below. In addition, a fifth lesion referred to as intraductal carcinoma of the prostate (IDCP) has recently been defined[42]. Detailed morphological description and classification for precursor and invasive lesions was recently published in an updated version by the World health organization (WHO)[43].

1.2.3.1 Prostate intraepithelial neoplasia

PIN is a histologic diagnosis that can only be made by microscopic examination of prostatic tissue. The epithelial cells in PIN contain morphological changes and characteristics similar with that of malignant lesions and inhabit many similarities regarding distribution and localization in the prostatic tissue (Figure 6)[44]. However, in PIN the neoplastic epithelial cells are confined to the prostatic ducts and do not form a tumor mass. Additionally, tissue architecture remains more or less intact[45]. PIN is commonly divided into low-grade (LGPIN) and high-grade lesions (HGPIN). PIN, especially HGPIN, has the potential of malignant transformation. Over time, progression to invasive cancer can occur, but there is no guarantee

[e] **LUTS:** Includes symptoms related to the enlarge prostate and the potential obstruction it can cause on the urethra. E.g. problems with emptying the bladder, frequency, nocturia, hesitant urination and decreases force in urine flow

of this transformation. The discovery of HGPIN also is prevalent in proximity to cancerous lesions[45]. Because of this, active treatment of PIN is not considered beneficial, but re-biopsies or close monitoring is necessary when HGPIN is discovered. Repeat biopsies reveal cancer after initial isolated HGPIN in as many as 25 % - 39 % of investigated cases[46,47].

1.2.3.2 Intraductal carcinoma of the prostate

This lesion is defined as large acinar ducts filled with malignant epithelial cells and can be difficult to distinguish from high grade PIN[42,43]. In contrast to PIN, IDCP is in the majority of cases associated with invasive adenocarcinoma. When IDCP is detected on prostate biopsies it warrants active treatment.

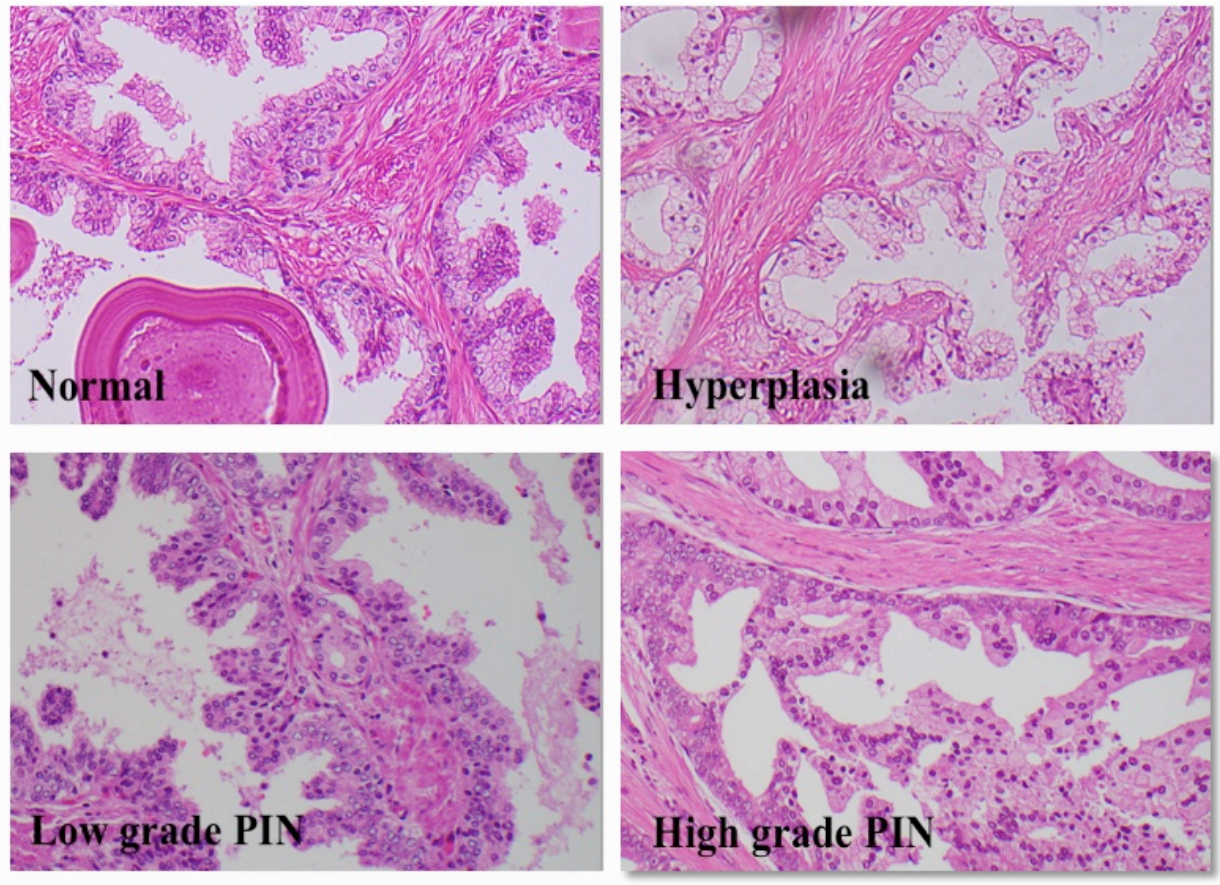

Figure 6 – Histopathology

High resolution histopathological pictures (20x) providing examples of normal, benign and pre-malignant lesions of the prostate. Figure: Thea Grindstad

1.2.4 Malignant tumors

1.2.4.1 Multifocality

A majority of PC tumors (50 - 80 %) develops in a multifocal manner[48–52]. The term multifocal implies tumor development in individual, separate lesions in the prostate, with normal tissue in between (Figure 7). The multifocal lesions of PC frequently display heterogeneity in their prognostic features such as Gleason score, tumor volume and extraprostatic extension[48,50,51,53]. Further, lesions containing BPH, normal stromal tissue or PIN can usually be detected in the prostate simultaneously. When including the prostate stromal tissue, this creates a cancer consisting of a complex mixture of different tissue types, e.g. hyperplasia, LG- and HGPIN, normal tissue and tumor tissue (Figure 7). Given that different tumor lesions within one gland can have different metastatic potential, this heterogeneity further complicates the nature of the PC[48,54,55].

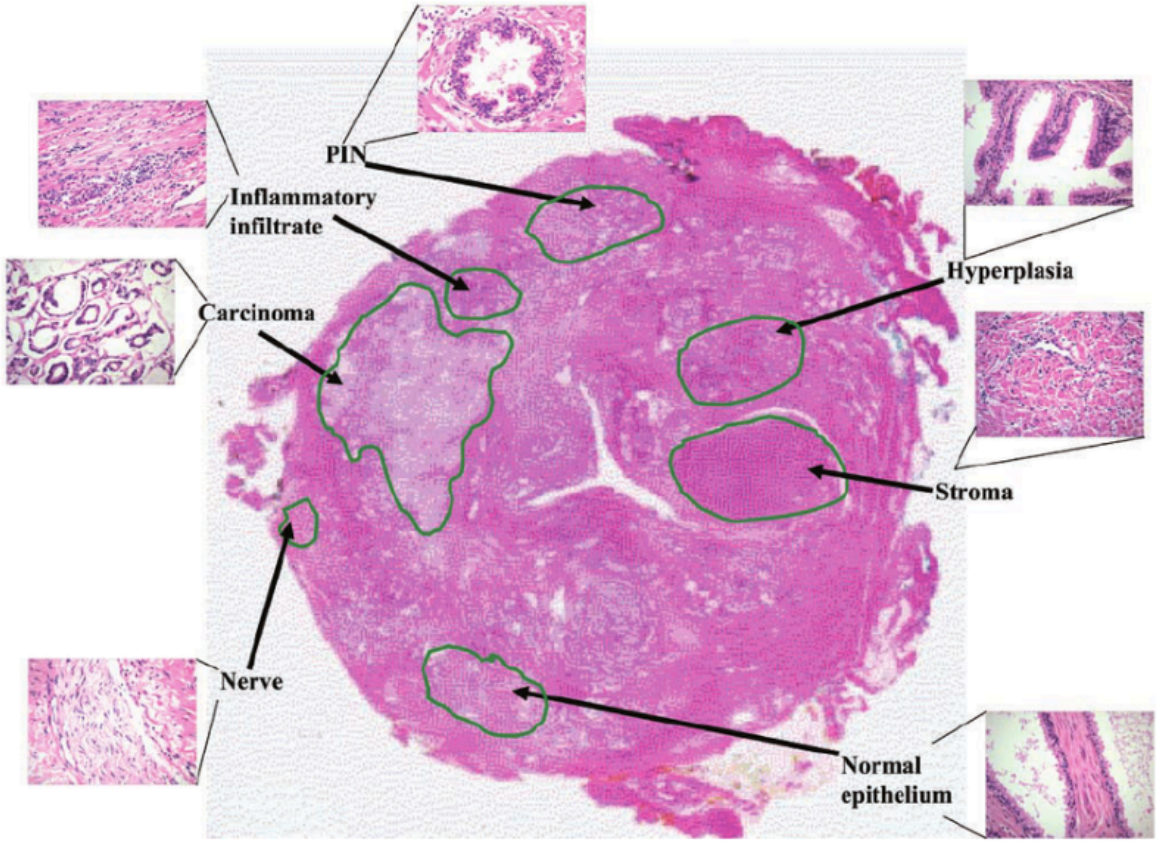

Figure 7 - Illustration of PC multifocality with different diagnostic histopathological areas within one gland. Reprinted with permission from Sage Journals ©, 2005[56]. **Abbreviations:** PNI = Perineural infiltration

1.2.4.2 Adenocarcinoma

Adenocarcinoma accounts for the majority (> 90 %) of PCs and is an invasive carcinoma originating from the glandular epithelium in the prostate. The epithelial cells have a secretory differentiation and are arranged in a variety of morphological patterns, conventional acinar adenocarcinoma accounting for the vast majority (Figure 9)[43]. A limited number of adenocarcinomas of the prostate (5 - 10 %) will have rare histological features like ductal carcinoma, mucinous (colloid) carcinoma, and signet ring cell carcinoma[43]. These subgroups can be diagnostic challenging but are important to identify due to prognostic differences, with the majority having a worse prognosis[57]. Typically, these histological variants are seen in association with conventional acinar adenocarcinoma. The broad histologic spectrum of adenocarcinomas of the prostate, in addition to the numerous pre-malignant lesions which can occur in the prostate and resemble invasive cancer, frequently challenges the accuracy in the diagnosing of PC. Other, less prevalent cancers that can affect the prostate (< 5 %) are e.g. neuroendocrine tumors, carcinosarcomas, transitional cell carcinomas, basal cell carcinomas, stromal sarcomas and lymphomas[43].

According to McNeal´s model, approximately 70 % of the prostate carcinomas are situated in the prostate´s peripheral zone and the majority in the posterior/ posterolateral peripheral part (Figure 5). Just below 10 % are solely located in the transition zone, and the remaining in both zones or with an intermediary location. The cancer can exist both uni- or bilaterally. Tumors arise rarely in the central zone are, they are rather evolvement of tumors from the aforementioned locations[38].

Disruption of the basal cell layer is an early event in PC pathogenesis, this was confirmed by detecting a loss of "basal cell specific antibodies" in PC[58]. Other typical traits of prostatic adenocarcinoma are architectural and cellular atypia (Figure 7). This includes infiltrating glands of smaller character than those from benign tissue with irregular, enlarged, hyperchromatic nuclei and prominent nucleoli. The lumen is often rounder, and more oval compared to the benign glands which are defined by branched lumen with papillary folding inwards. Further, the less differentiated the tumors are, the more disorganized and asymmetrical the architecture of glands appear, until they are more or less lacking. Additionally, when visualized using hematoxylin and eosin (H&E) staining, the cytoplasm of adenocarcinomas often appears darker than the corresponding benign epithelium. Less

specific, but also common traits of PC are intraluminal crystalloids. These are dense, eosinophilic crystal-like structures in various geometrical shapes. Intraluminal bluely colored mucin is also an indicator, but not specific to PC. The same accounts for pink amorphous luminal secretions[59,60]. Three histopathological features are considered pathognomonic of PC (Figure 8)[43]: Mucinous fibroplasia (collagenous micronodules), glandular glomerulations and perineural infiltration (PNI). Mucinous fibroplasia is loose fibrous tissue with fibroblast ingrowth. Glomerulations are tissue areas with architecture resembling a nephron's glomeruli due to the cribriform formations attached to the gland. PNI is defined as tumor cells tracing or encircling a nerve[61].

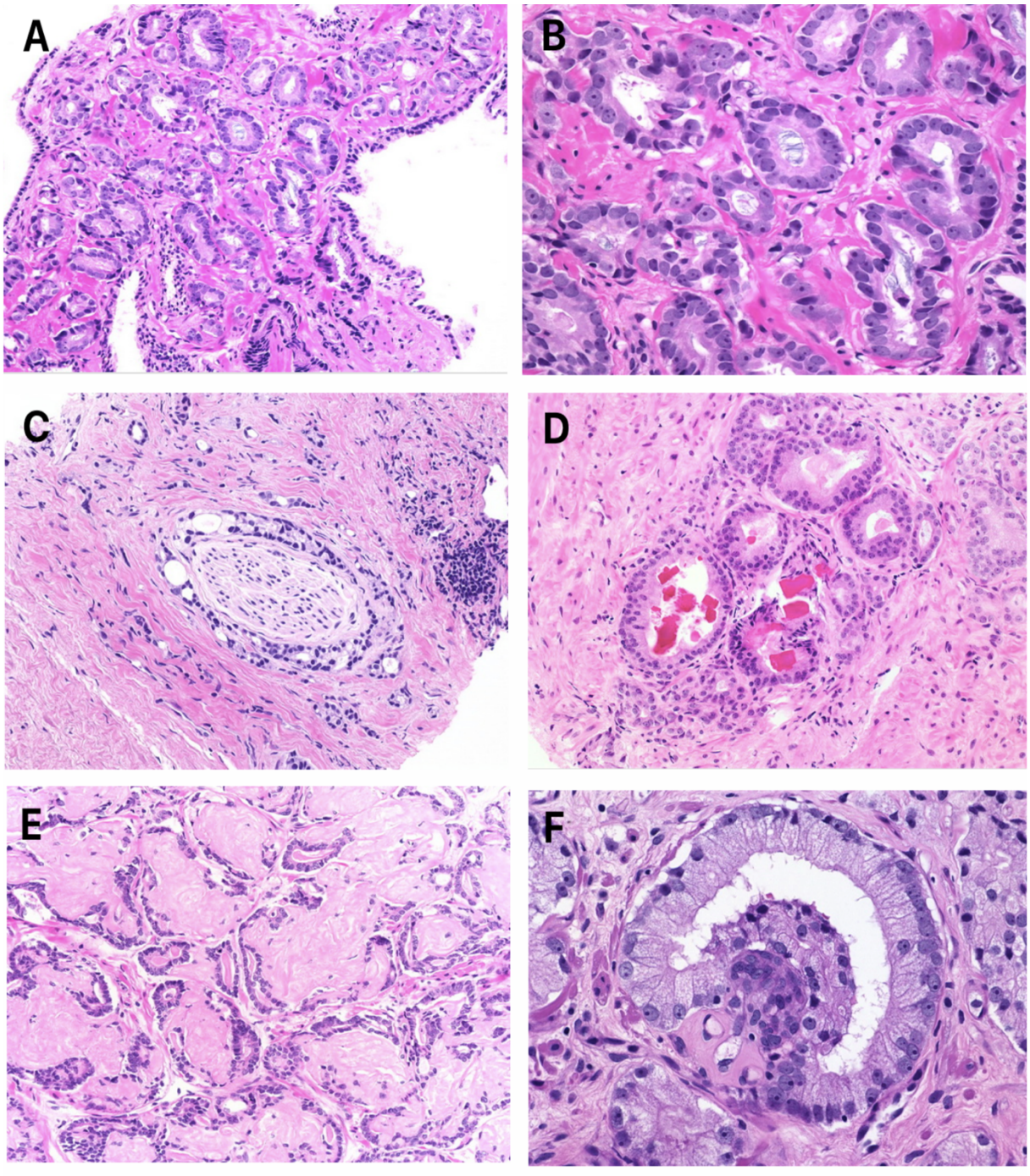

Figure 8 - Picture displaying different histological characteristics typical of adenocarcinoma of the prostate

A) Small atypical glands, with round or oval lumens, infiltrating between larger, benign glands. Basal cells are also lacking **B)** PC with small glands, round or oval lumen, lack of basal cell layer, nuclear enlargement, hyperchromasia, prominent nucleoli and intraluminal blue mucin. **C)** Perineural infiltration **D)** Atypical glands with intraluminal eosinophilic crystalloids **E)** Numerous collagenous micronodules in a focus of PC **F)** Foci of PC with glomerulations, including loss of basal cells. Reprinted with permission from WebPathology.com©, pictures by Dr. Dharam Ramnani.

Acinar adenocarcinoma of the prostate has various growth patterns leading to different architectural arrangements of the tissue. These patterns are associated with the cancer´s aggressiveness and form the basis for the Gleason grading system and the new Gleason grade groups[62,63]. Such growth patterns can include:

- Fused glands: Groups of glands no longer entirely separated by stroma
- Cribriform glands: A proliferation of glands with characteristic lumina
- Poorly defined glands: A cluster of glands with absent or deformed lumina
- Glomeruloid glands: Dilated glands with a cribriform proliferation attached to one side of the gland, resembling a glomerulus as described above.

1.3 Metastatic prostate cancer

As for many other cancer types, PC initially develops and grows locally in the gland. The first invasive step is usually the disruption of the capsule, followed by growth into surrounding fat tissue and nearby structures. This usually includes the vesiculae seminales and also the urethra and bladder. Distant metastatic spread of the cancerous cells can occur both lymphatically, this entails cancerous cells infiltrating lymphatic vessels which allows spread first to regional and further to distant lymph nodes, and hematogenously, which is when the cancerous cells are transported through blood vessels[64]. The regional lymph nodes are nodules of true pelvis below the bifurcation of common iliac arteries and the distant lymph nodules lie outside the true pelvis. Red hematopoietic bone marrow (spine, humerus, femur, pelvis, ribs, sternum) is a predilection site for distant metastasis. Other metastatic sites includes internal organs, commonly the lungs and liver[64]. Replacement of hematopoietic bone marrow with cancerous cells causes anemia and renders the patient at increased risk of infection. Further, an increased osteoblastic activity[f] in the bone metastasis creates painful osteosclerotic lesions[g]. These lesions change the bone architecture[65] and increase the risk of fracture, hypercalcemia and spinal cord compression[66,67]. Death from PC is frequently secondary to such complications following bone metastasis[66,68].

[f] **Osteoblastic activity**: Bone forming activity

[g] **Osteosclerotic lesions**: Bone metastases characterized by increased osteoblastic activity

1.4 Diagnosis

In general practice, the presence of risk factors, positive family history or symptoms suspicious of PC usually leads to a DRE and PSA testing. Suspicious findings further initiate referral to secondary care for transrectal ultrasound (TRUS) and biopsy of the prostate[69].

1.4.1 Symptoms

Early stage PC gives few, or no symptoms. As a consequence, many cancers are detected due to opportunistic PSA-testing or an abnormal DRE[70]. If tumors grow to exert pressure on the urethra, LUTS develops as described for BPH. However, these symptoms are not PC specific and are often a result of concomitant BPH. Impotence is another unspecific PC symptom. If the cancer becomes locally invasive, symptoms like pain, hematuria and hydroureter/ hydronephrosis can occur due to bladder- neck or base infiltration. These symptoms are, however, rare. Hematospermia is another rare symptom[69]. Metastatic disease to the bone is painful and can lead to neurologic symptoms due to spinal cord compression[66,67]. Weight loss is an additional symptom of advanced disease[69].

1.4.2 Digital rectal examination

On DRE, nodules, indurations, and asymmetry are suggestive of PC. DRE can detect tumors in the posterior and lateral parts (peripheral zone) of the prostate and an abnormal DRE, even with PSA levels below cut-off, has a strong association with PC[71,72]. TNM-Stage T1 cancers and the cancers situated in other parts of the prostate (25 – 35 %) are not distinguishable using DRE[73].

1.4.3 Prostate specific antigen

PSA, also known as kallikrein-3 (KLK3), is a serine protease and a member of the kallikrein-related peptidase family/ human tissue kallikrein gene family. It is produced by the epithelial cells of the prostate gland and secreted into the lumen where it merges with the seminal fluid, and its enzymatic activity contributes to semen lubrication[74,75]. Upon discovery, PSA was considered highly specific of the prostate, but later studies have revealed extra-prostatic production of the protease in both genders, however, without influencing serum PSA levels[76,77]. In healthy men, marginal levels of PSA enter the bloodstream. However, malignant epithelial cells also produce PSA and destruction of the basement membrane of prostate epithelial cells and capillaries can result in excessive leakage of PSA into the circulation.

Consequently, correlation has been demonstrated between increasing serum PSA-levels and the risk of PC[78,79]. However, very importantly, serum – PSA levels are not PC specific. Other benign conditions, such as BPH, infections in the prostate (prostatitis), can elevate the PSA level[80]. The PSA-levels will also rise with age[81], and biological variations in reference levels between individuals are prevalent[82]. So are fluctuation in a healthy individual's PSA-levels[83]. PSA reference level is currently set to ≤ 4.0 ng/mL. The major disadvantage of the PSA test is its lack of specificity and the low positive predictive value. In this case it means the lack of ability to distinguish an innocent condition from an aggressive, potential lethal disease, especially amongst men with PSA levels in the low-intermediate range[70,84–86]. Increasing the reference level to ≤ 10.0 ng/mL, improves specificity markedly, but the risk of not detecting clinically significant cancers increases concurrently. It must also be noted that PC can occur despite PSA-levels below reference level (≤ 4.0 ng/mL), also reducing the test's sensitivity. In the PC prevention trial, of the men with PSA below 4 ng/mL, PC was detected in 15 %, and out of these 15 % had high-grade disease[87]. However, an elevated PSA level is usually the first sign of a disease relapse and the test serves a purpose as a predictor for the recurrence of PC after initial treatment attempt[88,89].

1.4.4 The question of prostate specific antigen screening

After the development of the PSA test, it was quickly advocated as a cancer screening tool by several expert associations in the early 1990s[90]. As described in the "Epidemiology" section, this led to a vast increase in PC incidence, especially of clinically localized cancers, and subsequently an increase in the application of aggressive treatment strategies[10]. However, a subsequent fall in mortality rates was not observed. Since then, PC screening and PSA testing have been a topic of controversy.

Several comprehensive studies have attempted to clarify the effect of PSA-screening on PC mortality. The large European Randomized Study of Screening for PC ERSPC[91] produced evidence of a reduction in PC mortality in the screening group, however with a modest effect. The absolute risk reduction of death from PC at 13 years follow-up was 0.11 per 1000 person-years, equivalent to one PC death averted per 27 additional PCs detected. In the United States Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial[86], with a median follow-up time of 14.8 years, no mortality reduction was observed between the screened group and the control group. In 2011, a Cochrane meta-analysis including five randomized

controlled trials (RCTs), with a total of 341,351 participants, investigated screening vs. non-screening for PC. Herein, no significant difference in PC mortality was observed between the groups, but PC was diagnosed significantly more often in the screened group[85]. The ERSPC and PLCO trials are large, comprehensive trials, however, several aspects of the trials have been widely debated. The major concern remains that if screening does reduce mortality, benefits of screening are outweighed by the harms of overdiagnosing and overtreatment. Thus, opportunistic screening or nationwide screening programs are not implemented today. This is in conjunction with the recommendations by European Association of Urology (EAU) and US Preventive Services Task Force[92]. Notably, the current trends are pointing towards a decrease in opportunistic PSA-testing and incidence of early stage PCs[93].

1.4.5 Biopsy

Through prostate biopsies, prostate tissue is collected for pathological examination. This allows for an assessment of the Gleason grade and other histopathological traits suspicious of cancer. Biopsies of the prostate can be performed both transperineal and transrectal. Most commonly today is transrectal biopsies with TRUS assistance[94]. The main concern with this approach is bacterial contamination and septicemia. Today, a 12-needle biopsy strategy is recommended to secure tissue which adequately represents the prostate. The samples should be taken bilaterally and evenly distributed, with emphasis on the lateral aspects[95]. Magnetic resonance imaging (MRI) are also available to aid targeted biopsies e.g. by making lesions registered on MRI available for TRUS-guided biopsies. This is becoming increasingly accessible, although not applied routinely outside Norway[96]. A prostate biopsy is indicated in men with a DRE that is suspicious for cancer, regardless of the serum PSA. An isolated PSA elevation, however, is not necessarily sufficient for a referral to biopsy given the physiological fluctuations in PSA levels[83]. This decision is based the initial PSA level and/or re-evaluation of PSA levels with a few weeks interval. Additionally, potential DRE findings, symptoms and the patients age and general health are considered[97].

1.4.6 Immunohistochemistry

Immunohistochemistry (IHC) can be a useful diagnostic tool in selected tissue samples where the diagnosis of invasive cancer is uncertain. The absence of expression of the basal cell markers, such as the high molecular weight cytokeratin 34BE12 and the nuclear protein p63 which is present in basal cells of the prostate, is indicative of invasive cancer[98]. The

expression of the enzyme alpha-methylacyl-CoA racemase (AMACR/ P504S) on both mRNA and protein level is another marker for prostatic adenocarcinoma[99].

1.4.7 Radiologic investigations

Patients at high risk or with symptoms suspicious of aggressive disease are evaluated radiologically. Today, this is a constantly evolving field in medicine. Currently, the clinical utility of several improved imaging modalities is awaiting validation. When evaluating bone metastasis, technetium-99m bone scintigraphy has been a frequently applied radiological imaging technique. This can be supplemented with computed tomography (CT) or MRI of the bone if necessary[97]. Positron emission tomography (PET)-CT has also emerged as a useful method for detecting bone metastasis when conventional bone scans are insufficient, and this technique continues to evolve and improve[100]. In addition to MRI guided biopsies, the application of MRI in the detection, evaluation and staging of PC, is also a progressing research field[101].

1.5 Staging, classification, and prognostication

1.5.1 Prostate cancer progression

The risk evaluation and choice of therapeutic strategies for PC are currently largely based on clinical and pathological observations, including TNM-stage, Gleason score, and serum PSA-levels, in addition to patient characteristics such as comorbidity and life expectancy[73]. The application of PSA led to stage migration and shift in diagnostics, with more PCs being diagnosed at lower stages[10]. Today, > 80 % of PC patients are diagnosed with a localized disease for which the 5-year relative survival rate is close to 100%[3], however, there are evidence of an ongoing trend-shift with reductions in opportunistic PSA screening and detection of early stage cancers[93]. When distant metastasis develops, however, the survival rate is drastically reduced to approximately 36 % or less[2,3].

The predicament with PC is the heterogeneity in progression patterns, which makes the clinical outcome challenging to predict (Figure 9). PC is a slow growing disease, and decades can pass by form beginning of cancer development to manifestation of clinical cancer[102,103]. For a large portion of patients, the cancer will remain indolent throughout life, not affecting the patient's life quality or survival. However, some tumors are aggressive and will progress

quickly to metastatic disease and result in significant morbidity and cancer related death. The challenge lies in predicting the nature of the particular cancer in question[4].

In case of relapse after initial curative treatment, the progression pattern also varies. The first sign of cancer relapse is usually an asymptomatic rise in PSA levels, referred to as biochemical failure (BF). The most appropriate definition of BF after radical prostatectomy is a debated topic[104,105]. Currently, the consensus is two consecutive PSA values > 0.2 ng/mL and rising[106]. Regarding radiation therapy, a PSA level 2 ng/mL above the post-radiation nadir is considered evidence of BF[107]. Compared to radical prostatectomy, where PSA reaches undetectable levels weeks after surgery, it take years before PSA levels reaches nadir after radiation therapy[107]. BF can be followed by a clinical manifestation of the disease, referred to as clinical failure (CF). The proportion of patients who experience BF within 10 years after radical prostatectomy or radiation therapy with curative intent ranges from 27 – 53 %[97], and varies to a great extent in intermediate and high-risk patients[108]. Additionally, after BF, only a fraction (6 – 40 %) of patients will progress to CF and PCD, and the time to progression is usually protracted and variable[89,109–111]. This time span can range from 15 years in patients with low risk cancer to only one year in the highest risk groups, with a median time span of 8 – 10 years[89,109–111]. The risk of cancer progression and time to BF and CF can to some extent be estimated based on the aforementioned clinical and pathological parameters, however evidence from the SPCG-4 trial indicates that the prognostic value of the clinical parameters alone is not adequate[112].

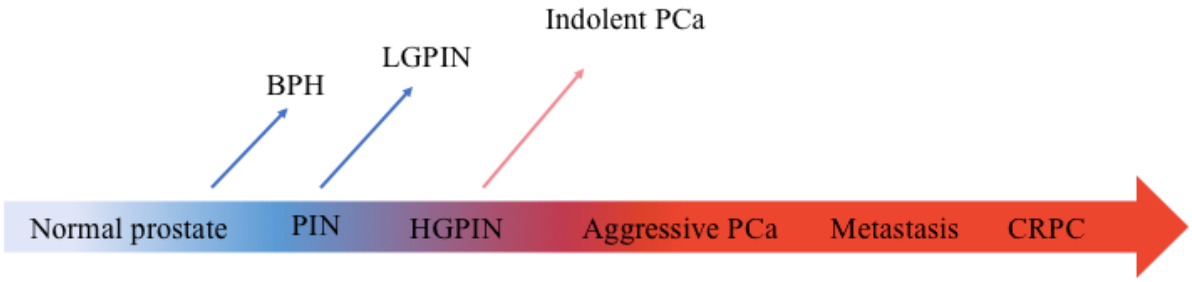

Figure 9 - The multistage process of PC development and tumor progression.

Abbreviations: PCa = PC; BPH = benign prostate hyperplasia; PIN = Prostate intraepithelial neoplasia; LG = Low grade; HG = High grade: PCa = PC; CRPC = Castrate resistant PC. Figure: Thea Grindstad

1.5.2 The Gleason grading system and Gleason Grade Groups

The Gleason grading system was first described in 1966 by Dr. Donald Gleason and colleagues[113] and was initially based on a study of 270 patients from the Minneapolis Veterans Administration Hospital. Their investigations demonstrated a progressive increase in cancer specific mortality with an increase in their scoring system[113]. It has since been modified several times, but the basic grading categories have remained unchanged. For the past four decades, it has been the most commonly accepted PC grading system and it remains the best available predictor for the pathological and clinical outcome of PC. Currently, the applied Gleason scoring system is according to the 2014 international Society of Urological Pathology Consensus Conference on Gleason Grading of Prostatic Carcinoma[62].

A great advantage with the Gleason scoring system is that it takes into calculation the heterogenic growth pattern of PC. This is achieved by basing the scoring system on the primary and secondary architectural growth pattern of the PC tumors, which is related to the aggressiveness of the cancer (Figure 10). The two most dominant tumor patterns receive a score, originally ranging from 1 to 5, reflecting the degree of differentiation[114]. In contemporary practice, only scores 3 to 5 are applied[62]. Finally, the scores are added (e.g. 3 + 3 / 3 + 4 / 4 + 3 etc.), with the first number in the calculation represent the most prevalent pattern of the two, thus creating a combined score ranging from 6 (3 + 3) to 10 (5 + 5). If there is only one grade present, that grade will be doubled[114].

In recent years, the International Society for Urologic Pathology (ISUP) and the World Health Organization (WHO) has developed a revised Gleason grading system with updated histological criteria and included grade groups numbered 1 to 5 (Table 1). These grade groups are comparable to the different Gleason scores: Grade group 1 to Gleason score 6 (≤3 + 3), grade group 2 can be compared to Gleason score 7a (3 + 4) tumor, grade group 3 to Gleason score 7b (4 + 3) tumors, grade group 4 to Gleason score 8 tumors, and grade group 5 to Gleason score 9 and 10 tumors[62].

Grade Group	Gleason score	Gleason pattern	Histological definitions
1	≤6	≤3+3	Solely separate, discrete, well-formed glands
2	7	3+4	Mainly well-formed glands and minor components of less-developed / fused cribriform glands
3	7	4+3	Mainly less developed/fused/cribriform glands with minor components of well-defined glands
4	8	4+4, 3+5, 5+3	Solely less developed/fused/cribriform glands OR mainly well-defined glands and smaller components lacking glands OR predominantly lack of glands and few components of well-formed glands
5	9 or 10	4+5, 5+4, or 5+5	Lacks gland formation, or including necrosis. With or without less developed/fused/cribriform glands

Table 1- Gleason Grade Groups

The newly defined Gleason Grade Groups, association with Gleason score and pattern in addition to brief histological definitions. Adapted from: 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[62]

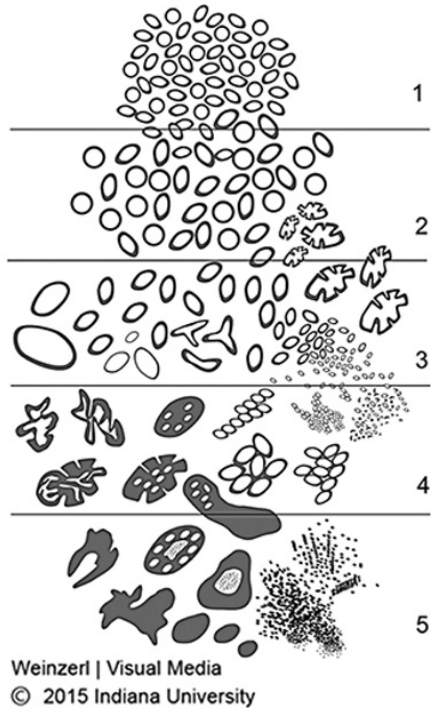

Figure 10 - Revised and modified schematic Gleason diagram, created for the 2016 WHO Classification of Tumors of the Urinary System and Male Genital Organs with the assistance of David Grignon. Reprinted with permission from Indiana University School of Medicine ©.

This new system is beneficial in the way that it can be more comprehensible for the patients and aid the decision making of treatments strategies in a more constructive manner. For instance, previously Gleason score 6 (3 + 3) was the lowest reported score, however, this could be misleading given that the original Gleason sum score ranged from 2 to 10. Now, the scoring system begins with grade group 1, which more accurately reflects the least aggressive cancers and lowest applicable Gleason score. Further, by placing Gleason score sum 7 in two separate groups, grade group 2 (3 + 4) and grade group 3 (4 + 3), it more correctly demonstrates the prognostic differences of these two scoring groups. Finally, Gleason score 8 has demonstrated different prognostic values compared to Gleason score 9 and 10, thus by dividing these into grade group 4 and 5, this difference is also better represented[63,115,116].

1.5.3 Tumor, node, metastasis (TNM) classification

When PC is diagnosed, the patient is assigned a stage which describes the extent of the disease and aids prognostication and treatment strategies. The standard system for staging newly diagnosed PC is the TNM-system developed jointly by the American Joint Committee on Caner (AJCC) and the International Union Against Cancer (UICC). This system is based on the anatomic extent of the disease, including the primary tumor size and confinement **(T)**, the absence or presence and extent of regional lymph node metastasis **(N)** and the presence or absence of distant metastasis **(M)** (Table 2). The current edition, 8th, was published in December 2016[73] and implemented January 1st 2018, staging of the material in this thesis is based on the previous edition from 2010[117,118].

There are two types of T staging, clinical (cT) and pathological (pT). Assessment of cT is accomplished through DRE and evaluation of transurethral resection of the prostate (TUR-P) specimens or biopsy material. There are no palpable findings in cT1, only evidence of malignancy in resected material, when cT2 is applied the tumor is palpable and presumably confined within the prostate. cT3/T4 implies that the tumor extends beyond the capsule[73]. Assessment of pT is done on radical prostatectomy specimens. The latter enables more accurate prognostication given that more information can be obtained from the resected prostate specimens[119,120].

Evaluation of regional nodal involvement (N) can be achieved through the classic technique of pelvic lymph node dissection, which is the current most reliable method. The procedure is

however invasive, comprehensive and expensive. Non-invasive approaches such as radiological investigations with either CT or MRI can also be applied. However, this approach has low sensitivity for detecting malignant nodules of smaller size[121]. However, much more sensitive techniques like PSMA-PET shows great promise to increase sensitivity markedly[100]. Currently, the recommended strategy to asses risk of lymph node involvement and the need for further diagnostic measures are nomograms[h] based on e.g. PSA and Gleason score, such as Partin tables[i,122]. Finally, The M-stage is determined radiologically as described in the "diagnosis" section.

[h] **Nomograms**: A diagram representing the relations between three or more variable quantities by means of a number of scales

[i] **Partin table**: The Partin tables asses' clinical features of PC including Gleason score, serum PSA and clinical stage to make predications of whether the tumor will be confined to the prostate

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

Table 2 – The Tumor Node Metastasis (TNM) classification system of malignant tumors.

Developed jointly by the American Joint Committee on Caner (AJCC) and the International Union Against Cancer (UICC). Adapted from: AJCC Cancer Staging Manual, Eighth Edition (2017), Springer International Publishing ©[73].

1.5.4 Risk grouping

Finally, the PC staging can further be stratified into pre-treatment risk-groups based on their estimated risk of recurrence. This improves prognostics further and aids treatment decisions. Multiple risk stratification systems based on TNM-stage, pre-treatment PSA and Gleason scores have been developed. Widely applied, and listed in the WHO's tumor classification manual[43], is the AJCC prognostic stage groups. This was first implemented in the 7th edition of AJCC Cancer staging manual[118]. Further, the EAU Guidelines of 2017 applies the EAU risk groups, which is an adaption of D'Amico's classification system[97]. The EAU grade groups are also applied in Norway. These risk-groups are created by incorporating TNM-stage with pre-treatment PSA and Gleason grade groups. Such risk groups range from low (I) to very high (IV), with some stages divided into subgroups (A, B etc.) (Table 3). As a rule of thumb, the lower the number and letter, the less the cancer has progressed. The risk groups predict the risk of BF after therapy with curative intent, and appropriate treatment can thus be decided by which category the patient belongs to[73,123].

The main changes to the PC TNM staging in recent AJCC/ UICC update (8th edition) is the elimination of pathologically organ confined cancer (pT2) sub-staging. Further, now both Gleason score and the newly defined Gleason Grade Group[62] should be used to more accurately reflect tumor grade. Regarding AJCC prognostic stage groups, stage II is now subdivided by Gleason Grade Group and stage III includes selected organ confined tumors based on PSA level, which is unusual compared to other cancers where AJCC stage group III invariably is associated with non-organ confined disease. A final change is the implementation of statistical prediction models validated by the Precision Medicine Core, so far two models have met the strict criteria and are implemented in the 8th edition of AJCC Staging Manual[73,123].

When T is...	And N is...	And M is...	And PSA is...	And Grade Group is...	Then the stage group is...
cT1a-c, cT2a	N0	M0	<10	1	I
pT2	N0	M0	<10	1	I
cT1a-c, cT2a, pT2	N0	M0	≥10 <20	1	IIA
cT2b-c	N0	M0	<20	1	IIA
T1-2	N0	M0	<20	2	IIB
T1-2	N0	M0	<20	3	IIC
T1-2	N0	M0	<20	4	IIC
T1-2	N0	M0	≥20		IIIA
T3-4	N0	M0	Any	1- 4	IIIB
Any T	N0	M0	Any	1- 4	IIIC
Any T	N1	M0	Any	Any	IVA
Any T	Any N	M1	Any	Any	IVB
NOTE: When either PSA or Grade Group is not available, grouping should be determined by T category and/or either PSA or Grade Group as available.					

Table 3 - A reproduction of the prostate cancer risk grouping constructed by AJCC. Adapted from: AJCC Cancer Staging Manual, Eighth Edition (2017), Springer International Publishing ©[73]

1.5.5 Histopathological prognosticators

Several features of prostatic adenocarcinoma have been connected to disease outcome and progression. Some are well validated, e.g. positive surgical margin (PSM). The results regarding other features, e.g. PNI, lymphovascular infiltration (LVI) and maximum tumor diameter are however ambiguous and the prognostic value is uncertain.

1.5.5.1 Perineural infiltration

As described in the "malignant tumor" section, PNI is one of the three histopathological features considered pathognomonic of PC. It is frequently discovered in prostatectomy specimens, its prognostic value, however, is undetermined. PNI in a prostate biopsy has been reported a strong indicator of extra-prostatic extension (EPE) of the cancer, which is a feature of a TNM stage pT3a cancer and represents a locally advanced disease[124,125]. The presence of PNI in biopsies can thus influence treatment decisions and choice of surgical techniques. Several studies have further detected an independent negative prognostic impact of PNI[124,126], while others fail to detect association with clinical endpoints[125,127]. The definite prognostic

value of PNI has thus not been concluded, but it is recommended to include in the pathology report[97].

1.5.5.2 Lymphovascular infiltration

Lymphovascular infiltration (LVI) can be defined as the presence of tumor cells in structures lined with endothelium, e.g. blood- and lymphatic vessels. It can usually be detected in a small subgroup (approximately 10 %) of radical prostatectomy specimens from patients with localized PC[128]. As with PNI, although extensively investigated, it´s prognostic value is not concluded due to varying reports[129]. There is, however, vast amount of evidence describing an association between LVI and early BF and cancer aggressiveness[128]. Further, independent associations with cancer specific mortality has also been reported[130]. LVI is also recommended included in pathology reports[97].

1.5.5.3 Positive surgical margin

Positive surgical margin (PSM) is a pathological assessment defined as tumor cells reaching the inked surface of the resected prostate specimen after radical prostatectomy (Figure 11). This is an acknowledged post-operative prognostic parameter for disease progression of PC[97]. Investigations have revealed that the probability of being progression free five years after radical prostatectomy ranges from 58 – 64 % for patients with positive surgical margins and 81 – 83 % for patients with negative margins[131,132]. In these studies, progression was defined as either BF or evidence of local recurrence or distant metastasis.

The location and number of PSMs and its impact on prognosis has, however, been a debated topic. This has been investigated in numerous reports, but a consensus has not been reached. Several reports find an independent association between PSM at the base or non-apical locations, but question the influence of a positive apical margin[133,134]. Others could not find the PSM location to independently predict disease, but rather that the multitude of positive margins was significant in regards to disease progression[135].

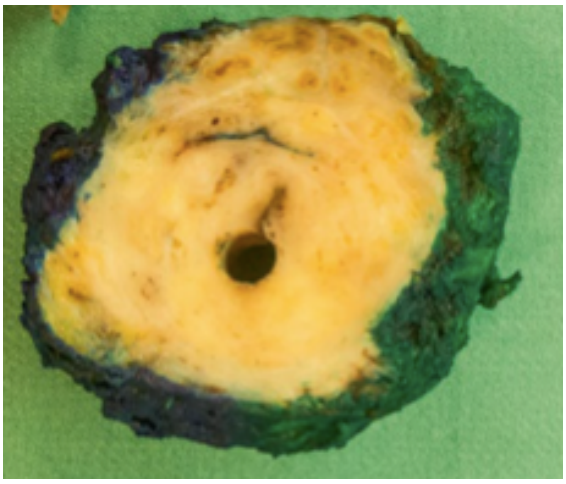

Figure 11 - Whole-mount tissue section of resected prostate. For orientation and with regard to surgical reception margins, the prostate is inked blue to the right, green to the left and black at the posterior surgical margin. Reprinted with permission from Elin Richardsen ©

1.5.5.4 Tumor size - Maximum tumor diameter

Amongst other factors associated with prognosis after radical prostatectomy are maximum tumor diameter and tumor volume. Like other histopathological factors, the independent prognostic value is not fully determined. Additionally, due to the multi-focal growth pattern of PC, selecting an index tumor and measuring tumor diameter is more complicated in other solid cancers. However, there are several studies reporting tumor size as an independent prognostic predictor of cancer recurrence, and a tumor diameter > 20 mm has been associated with increased risk of BF[136,137]. In the pathology report, it is recommended to include the diameter and/ or volume in addition to an estimate of total tumor tissue percentage[97].

1.5.6 The search for novel prognostic biomarkers

A biomarker can be described as an objectively measurable variable that can serve as an indicator of biological or pathological processes, or pharmacological response to therapautic inerventions. It can be a single variable (such as PSA), or a variable composed of several measurements. In theory, a biomarker can be detected in a broad range of human material such as tissue samples, urine and serum. Further, the biomarkers can have many forms, including patterns of gene expression, a particulare gene variant or levels of RNA- and protein expression. Biomarkers are placed in two categories: 1) Prognostic, which indicated the natural disease progression and likely outcome, and can thus indicate who would benefit from treatment. 2) Predictive, which indicates the response to a specific therapy[138]. In PC research, great efforts have been made to reveal new biomarkers that will aid the therapeutic

decision making and reduce overtreatment. Numerous biomarkers with proposed significance in prognostic models have emerged throughout the recent years. So far no ideal individual biomarker with sufficient sensitivity and specificity has emerged[139]. Thus, the search for biomarkers in PC continues as an important step to offer improved, personalized treatment strategies adapted to the cancers aggressiveness. A selection of emerging biomarkers with promise are discussed below.

Several derrivatives of PSA have been investigated to strengthen the test specifisity and its pre-biopsy predictive value. The aim to improve discrimination between indolent and aggressive disease, specially in men with a PSA value ranging between 4 – 10 ng/mL. This include: PSA-velocity and doubling time[j,140], PSA density[k,141] and age- and race specific reference ranges[81,142]. There are evidence of moderate prognostic improvement using these strategies. However, presently there is no consensus on using any of the aforementioned PSA derivatives in the clinic. This is largely due to the persisting risk of under-detection of clinically significant PC and challenges with implementing these strategies in clinical routine. In serum, PSA can exist in two forms. The majority of the PSA is in a complexed form, bound to protease inhibitors, and the remaining in an unbound, free form (5 – 35 %). A lower ratio of free-to-total PSA is suspicious of PC, and the percentage of free-PSA in the blood has demonstrated improved diagnostic specificity over total-PSA alone[143]. Various isoforms of free-PSA have been identified, including benign-PSA, inactive/ intact-PSA and pro-PSA (with several subgroups)[144]. Recently, several of these variables have been integrated in new diagnostic models, including the prostate health index[l] (PHI)[145] and the Four kallikrein assays[m] (4K-index)[146]. Both have demonstrated promise in the detection of clinically significant PC.

Other advancements in early detection biomarkers are PC antigen 3 (PCA3) and TMPRSS2:ERG gene fusions. Both biomarkers can be detected in biopsy specimens and

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[j] **PSA velocity:** The change in PSA over time

[k] **PSA density:** PSA value divided by the prostate volume measured by TRUS

[l] **PHI:** A blood test detecting free PSA, total PSA, and the [-2] proPSA isoform of free PSA and combining the results in to a mathematical model calculating one common score.

[m] **4K-index:** Serum levels total PSA, free PSA, intact PSA and Human Kallikrein Antigen 2 is measured. Additionally, DRE results and any prior biopsies results are included in the model. In brief, results are compared to a database and a percentage risk of "significant" PC is calculated.

quantified in urine after prostatic massage. PCA3 is a non-coding messenger RNA (mRNA) which can be measured in urine samples using real-time polymerase chain reaction (PCR). PCA3 levels are higher in PC compared to benign conditions. It has further demonstrated greater specificity than total-PSA and percentage of free-PSA in detecting PC[147]. Currently, it is approved in the U.S. for use in decision making regarding the need for repeat biopsies in men with previously negative biopsies, but persistently elevated PSA levels[148]. Its value as a urine marker in directing the need for initial biopsy is inconclusive, in part due to difficulties with cut-off levels[147]. Sporadic PC is, like all cancers, associated with a variety of somatic mutations and chromosomal abnormalities. Chromosomal rearrangements, resulting in fusion of members of the ETS family of oncogenic transcription factors is a prevalent event in PC. The most frequent is the fusion with the androgen regulated TMPRSS2 gene, resulting in TMPRSS2-ERG fusions gene. This fusion has been estimated to occur in > 50 % of PCs[149]. The detection of the TMPRSS2-ERG fusions gene could provide additional value in PC diagnostics, and help distinguish between aggressive and indolent disease at initial diagnosis[150]. Its presence in radical prostatectomy specimens has also been associated with increased risk of cancer recurrence after surgery for localized cancer[151]. Additional prognostic value in the detection of PC with high-grade risk has also been observed when combining serum-PSA score with PCA3 and TMPRSS2-ERG gene assays in urine[152].

Other tissue-based prognostic markers based on specific gene expressions are also emerging. The Cell Cycle Progression (CCP) score is based on the mRNA expression of several cell cycle associated genes[153]. Another is the Genomic Prostate Score (GPS), based on the mRNA expression of 17 genes associated with PC progression[154]. Both have presented promising results in regards to aiding stratification of indolent and aggressive disease in men with newly diagnosed PC. However, further validation is deemed necessary before implementation into routine practice.

1.6 Steroid hormones

1.6.1 The endocrine system

In an organism, the endocrine system constitutes an important communication system. The system consists of endocrine glands producing hormones that work as chemical signal molecules. Upon production, the hormones are secreted into the bloodstream and can exert their function distant from the production site, by binding to and stimulating their cognate

receptors. Hormones can be divided into subgroups based on their chemical composition and solubility. One major entity is the large group of hydrophobic hormones, the steroid hormones, which are all derived from cholesterol (Figure 12). The main constituents of this group are the corticosteroids, vitamin D, and the sex steroid hormones: androgens, estrogens and progesterone[8]. Steroid hormones are involved in numerous physiological processes in the human body, including metabolism, cell differentiation and proliferation and cell cycle regulation, and are essential for normal development, growth and reproduction[8]. Due to their hydrophobic nature, the hormones depend on proteins for transportation through the bloodstream to reach their destination. Such proteins include sex-hormone binding globulin (SHGB) as well as albumin, which functions as an unspecific transporter. Only a small, unbound fraction of the hormones in the blood is in an active form that will bind and stimulate the receptor. An equilibrium will constantly exists between the bound and unbound form, making the protein binding a means of storage[8].

1.6.2 Steroid hormone production

Steroid hormones are primarily synthesized in the adrenal glands and gonads of men and women through the same synthetic pathway (Figure 12). The majority of sex-steroid hormone production occurs in the gonads. The adrenal glands synthesize glucocorticoids and small amounts of androgens, but larger amounts of androgen precursors. The synthesis occurs in different zones of the adrenal gland according to different hormone products[8]. Due to enzymatic activity in peripheral tissue, hormonal precursors produced in the adrenal glands can be converted to sex steroid hormones in other tissues than the gonads[155,156].

The production of steroid hormones from cholesterol is a complex interplay between the cells' mitochondria and the endoplasmic reticulum. Different members of the cytochrome P450 enzyme – family (CYP-450) are important regulators in the conversion of cholesterol to the different steroid hormones[157]. The steroid hormone production and secretion is regulated by the hypothalamic - pituitary axis (H-P axis). Both the hypothalamus and the pituitary gland secrete hormones which exert an executive function with regards to the peripheral steroid hormone production. Corticotropin-releasing hormone (CRH) stimulates secretion of adrenocorticotropin (ACTH) from the pituitary gland which further stimulates synthesis of cortisol and androgen precursors in the adrenal cortex. Gonadotropin releasing hormone (GnRH) regulates the secretion of two gonadotropic hormones from the anterior pituitary

gland, the luteinizing hormone (LH) and the follicle stimulating hormones (FSH). These hormones further stimulate the production of the sex-steroid hormones in the gonads (ovaries and testes) predominantly synthesizing estrogens and androgens, respectively. The H-P axis is controlled by a negative feedback mechanism which entails that increased hormone levels in the peripheral system will result in decreased secretion of regulatory hormones from the hypothalamus and the pituitary gland[8].

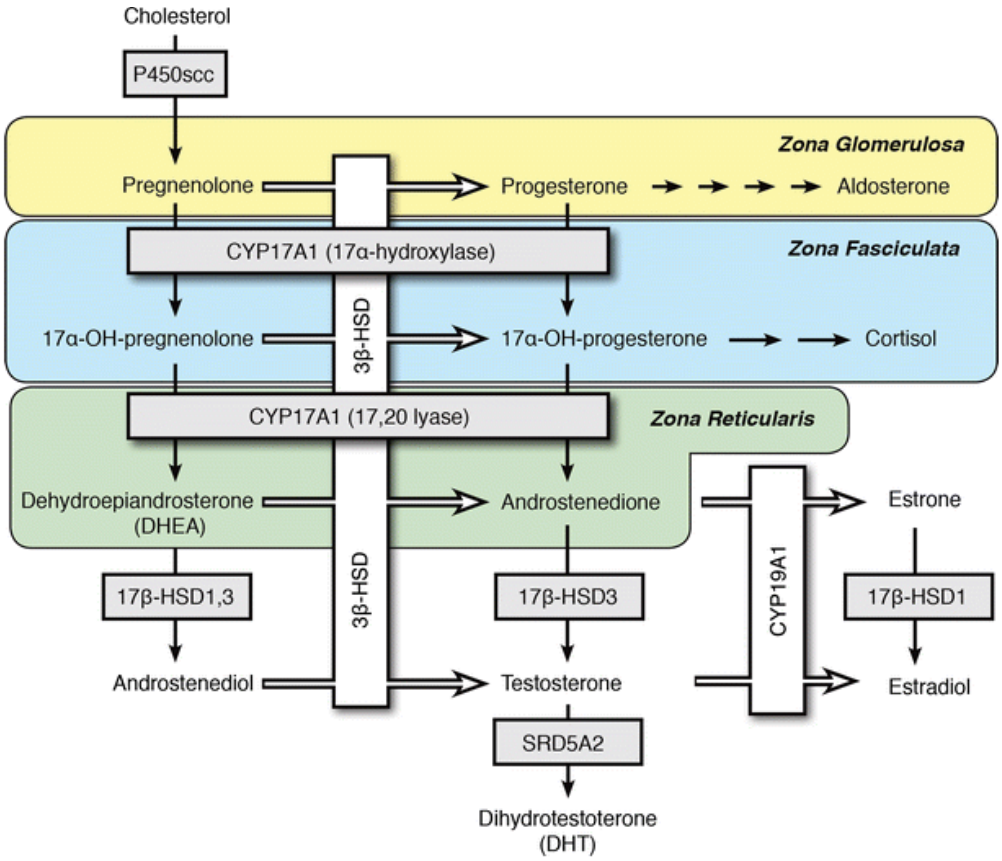

Figure 12 - Adrenal steroid hormone synthesis pathway. Reprinted with permission from Springer U.S. ©, 2016[158]

1.6.3 The physiological role of steroid hormones - estrogen and progesterone

Estrogens include the ligands that bind and stimulate the ERs: 17β-estradiol (E2), estrone, and estriol (from most to least potent), in addition to synthetic estrogenic ligands[157]. All estrogens are synthesized from androgens through the enzymatic activity of the CYP-450 enzyme aromatase (CYP19A1) (Figure 12). Aromatase activity and estrogen receptors (ER) expression have been demonstrated in various tissues of both men and women, including adipose tissue, brain, bone, prostate and testicle[155]. The important role of adipose tissue in aromatization of androgens is demonstrated through the correlation between body mass index

and plasma levels of estrogens[159]. Progestogen is a common denominator of any substance capable of stimulating the PGRs, reflecting its role in in promoting and supporting pregnancy. Progesterone (P4) is the only naturally occurring progestogen in humans. Synthetic progesterones constitute a diverse group of compounds referred to as progestins[160].

Traditionally, estrogens and progesterone were considered female reproductive hormones. In collaboration, estrogens and progesterone orchestrates numerous cellular processes in female reproductive organs, including uterus, ovaries and breast. Thereby exerting a vital role in pregnancy and fetal development[8]. Today, the understanding of their physiological roles has broadened. Estrogens and progesterones, in addition to the other steroid hormones, are now considered vital in numerous non-reproductive physiological functions in both genders[161]. For instance, progesterone is essential in male reproductive physiology, e.g. by facilitating the acrosome reaction of sperm cells[162]. Progesterone has also been established as an important neurosteroid due to its developmental and protective roles in the central and peripheral nervous system[163]. Estrogens are vital in bone and muscle homeostasis as well as prevention of osteoporosis, in addition to regulation of metabolism and the cardiovascular system[161,164].

1.6.4 Steroid hormone receptors

Given the hydrophilic nature of the steroid hormones, their cognate receptors are located intracellularly, thus steroid hormones bind after diffusing through the cell membrane. The connection between hormone and receptor occurs in the cytoplasm with subsequent import to the nucleus, or the receptor can be located in the nucleus. When connected to their respective receptors, they can function as transcription factors that modulate the expression of target genes. Nuclear SHRs have a considerable homologous structure. Through comparison of the amino acid sequences of the SHRs, a high level of conservation in the DNA binding domains (DBDs) has been revealed. This has led to the definition of the SHR superfamily, a part of the nuclear receptor family, classified as a type 1 nuclear receptor[7].

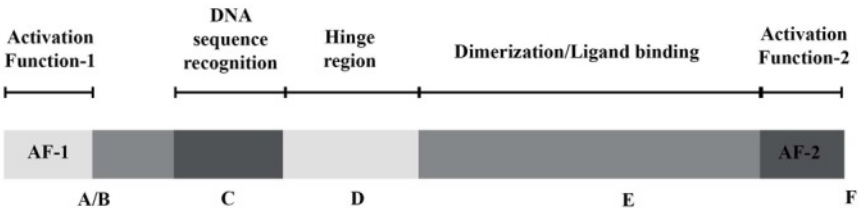

Figure 13 - General structure of the nuclear receptor, reprinted with permission from Bentham Science Publishers Ltd ©, 2001[165]

In general, nuclear receptors consists of a DBD, a ligand binding domain (LBD) that include an activation function 2 domain (AF-2), and an additional activation function domain (AF-1)[165]. The A/B domain at the N-terminal encodes the AF-1, an essential domain for interaction with co-regulators. The C domain encodes the centrally located DBD, essential for sequence-specific binging of SHRs to DNA. The D domain is a hinge region with amino acid sequences that facilitate post-translational modification of the receptor and stimulate nuclear localization signaling. The E/F domain, located in the C-terminal region, contains an LBD. This serves as a receptor specific ligand binding site, and coactivator interaction site. The AF-2 domain, which similar to the AF-1, is involved in transcriptional regulatory activity. All SHRs have several phosphorylation sites that can be subjected to kinase activity, the majority of those discovered are located in the N-terminal domain[165].

1.6.4.1 Mechanism of action

When a receptor in the cytoplasm is not bound by its ligand, it exists in an inactive form regulated by chaperone protein complexes. In the classical model (also referred to as genomic pathway) (Figure 13), a conformational change occurs in the receptor upon ligand binding. The chaperone complexes dissociate, the receptor dimerizes, and nuclear translocation is facilitated. Inside the nucleus, the complex interacts with specific DNA-sequences, classified as hormones response elements (HREs), frequently as homodimers. This leads to the assembly of co-regulatory molecules which influence transcription of target genes[166] (Figure 13). Other models of non-classical genomic steroid hormone action also exist. For instance, ligand bound SHRs can interact with other DNA-bound transcription factors to modulate their activity. Further, given the numerous phosphorylation sites of the SHRs, their function can be altered as a result of phosphorylation by various intracellular kinases, both in the absence and presence of ligand[167]. The importance of other rapid, non-genomic mechanisms of action for the activated SHRs have also been revealed. Steroid hormones can bind membrane bound

receptors, or receptors in the cytoplasm, and initiate various rapid intracellular signaling cascades. The activation of these signaling cascades results in changes of intracellular ion concentrations and activate second messenger systems[168–170] (Figure 14). Adding to the complexity, is the bidirectional collaboration between the genomic and non-genomic pathways working together to exert the steroid hormones mechanism of action[171]. Finally, she steroid hormones receptors are expressed in various tissue types throughout the human body, and in different cellular compartments within tissues. Often, multiple SHR subgroups are expressed simultaneously either in the same cell types or in different cells within the same tissue.

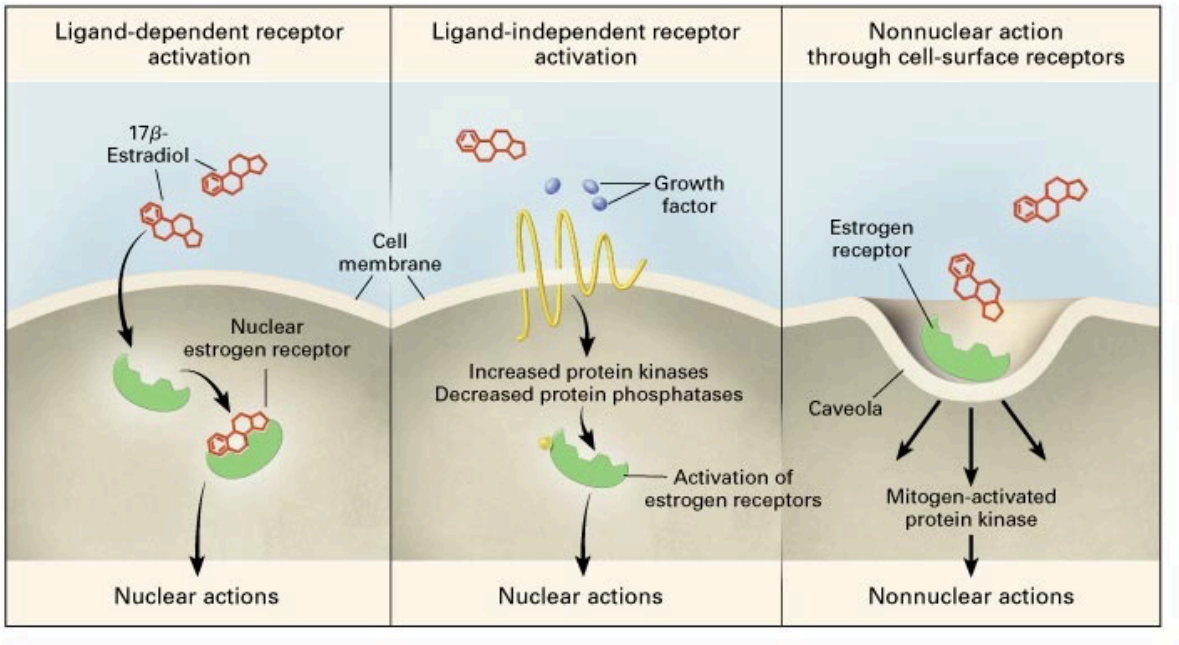

Figure 14 – Steroid hormone receptor signaling

Examples of SHRs different mechanisms of actions, demonstrated through estrogen. Form left – right: 1) Classical genomic pathway, 2) Ligand independent receptor activation e.g. through growth factors 3) Non-genomic signaling linked to the mitogen activated protein kinase (MAPK) pathway. Reprinted with permission from Massachusetts Medical Society ©, 2002[172]

1.6.4.2 Detailed description of steroid hormone receptors investigated in this thesis

Estrogen receptors

The effects of estrogens are mediated through two different nuclear estrogen receptors, ERα and ERβ. ERα was discovered as early as 1966[173]. Until 1995, when ERβ was cloned from rat prostate[174], ERα was believed to be the only ER. The newly discovered ERβ subtype

displayed high affinity to estrogens similar to ERα. The ER genes are located on different chromosomes, ERα on chromosome 6 and ERβ on chromosome 14. ERβ is smaller than ERα, but the receptors have a considerable degree of sequence homology in the DBD (96 %), making the receptors able to recognize the same HRE on DNA. In the LBD, the sequence homology is only 58 %, and it is even less in the AF-1(N-terminal domain)[174]. This divergence in the LBD allows the ERs to bind ligands with different affinities. As an example, several synthetic ligands have demonstrated ERα agonistic activity and at the same time total ERβ antagonistic activity[175]. Today, several ER selective ligands have been discovered and continues to develop[164].

Although the receptors are similar in many respects, different tissue distribution and biological effects of the two receptors have been observed and is an evolving research field. Technically, the ERs can regulate expression of target genes through both homo- and hetero-dimerization. Though homo-dimers are considered most frequent[176]. Genomic analyzes have uncovered that the gene sets regulated by the two receptors differ to a great extent (> 70 %)[177]. Further, in some tissues, both ERs are expressed at similar levels, while in others one ER predominates. Additionally, both receptors can be present simultaneously, but in different cell types[178]. Hence, the effect of estrogens in different tissues is seemingly dependent on the relative levels of ERs.

Several ERα and ERβ isoforms have been identified. In addition to the wild-type ERα (ERα66), a broad range of truncated ERα variants have been described. Of example are the splice variants ERα36, which is lacking both AF-1 and AF-2[179], and the ERα46[180]. In addition to the wild type, ERβ1, four C-terminally truncated ERβ splice variants have been isolated in humans, ERβ2 to 5. The only functional isoform is ERβ1. ERβ2/βcx, ERβ4 and ERβ5 can heterodimerize with ERβ1, working in a regulatory fashion[181].

Progesterone Receptor

Progesterone binds and stimulates the progesterone receptor (PGR) which was discovered in the late 1960s[182]. The PGR exists in two isoforms, PGRA and PGRB. Both receptors are transcribed from one single gene. They are separated only by additional 164 amino acids found in the upstream N-terminal region of PGRB, also termed B-upstream segment. Despite these small differences, this specific region renders the PGRB with an extra transactivating

function (AF-3)[183]. The expression of the PGRs is controlled by ligand bound ERs, making PGRs downstream effectors of ERs. The PGRA and PGRB transcription is controlled by different ER-regulated promoters[184].

Both PGR isoforms are functionally distinct. Each have their own response genes, mediating the wide specter of physiological effects of progesterone with little overlap[185,186]. PGRB is in general considered more active compared to PGRA, and it seems that the majority of progesterone targeted genes are regulated through PGRB[186–188]. Notably, reports have been made of PGRB, but nor PGRA, being able to induce rapid intracellular signaling[168]. Previous investigations has resulted in the assumption that PGRA and PGRB are expressed at homogenous levels in normal human tissue, and that a disruption of this 1:1 ratio can be a step in disease development[189]. Because of the two isoforms, there is potential for the dimerized ligand bound PGR complex to exists as homodimers (A:A or B:B) or heterodimers (A:B). These dimerization variants determines target gene specificity and further contributes to the complexity of progesterone signaling[186,188]. Although a 1:1 ratio of PGRA and PGRB expression is considered standard, certain physiological alterations in receptor expression ratio is expected, e.g. in the endometrium during the normal menstrual cycle[190]. A third, less recognized PGR isoform, PGRC, is smaller than the others with a truncated N-terminal. Hitherto, PGRA and PGRB are considered the dominantly functional isoforms[191]

1.7 Tumor biology

When cancer cells develop, several biological changes occur in basic cellular processes, both within the malignantly transformed cells, but also in involved tissue environments. Such changes were eloquently summarized by in the paper ”Hallmarks of cancer” (2000)[192], and in the revised 2011 version[29]. The hallmarks include adverse changes to key processes in the cell cycle and its environment, e.g. apoptosis, proliferative signaling, cell cycle control, metabolism and interactions with surrounding tissue. Importantly, the nature of these changes will differ between cancer types, and individuals. This makes heterogeneity an important aspect of cancer development. The detailed mechanisms underlying the SHR involvement in malignant transformation is an intricate and complex research field, constantly evolving, and extending beyond the scope of this thesis. There is, however, a vast amount of evidence linking the SHR to various malignancies and their action to several of the “cancer hallmarks”. This will be outlined the sections below.

1.7.1 Steroid hormone-related cancers

One excellent example of hormone related malignancies is PC. In individuals with complete androgen insensitivity, prostate gland development is absent, confirming that the AR is essential for prostate growth and development[193]. The AR, however, also contributes to initiate and maintain carcinogenesis in the prostate. This was demonstrated already in the 1940's through the beneficial effect suppressing testicular androgen synthesis had on metastatic disease[5,6]. This strategy of androgen deprivation therapy (ADT), in addition to anti-androgen treatment, continues to be an acknowledged treatment strategy of advanced PC today, and will be discussed in detail in the "treatment section". Further underscoring the hormone dependency of the PC is the effectiveness of new treatment strategies targeting enzymatic steps in the steroid hormone synthesis pathways[194,195]. However, the treatment effect of this type of hormone manipulation is only temporary, and disease progression to castrate resistant PC (CRPC) is inevitable, despite castration levels of serum androgens. How the cancer develops compensatory strategies to continue proliferation and growth is not fully understood. Interestingly, evidence indicates that even in a castrate resistant state, the PC continues to be dependent on androgens for further growth[196].

Similarly, in other hormone responsive organs, such as the breast, sex-SHR are directly associated with cancer development. In normal breast epithelium, ERα is expressed in approximately 10 %. However, 50 – 80 % of malignant tumors express ERα. In contrast, ERβ is expressed in approximately 80 % of normal breast epithelial cells[197]. Blocking of estrogen actions in ERα positive breast cancer can reduce disease recurrence and prolong disease specific survival. So far, this is achieved through two pharmacological strategies: Selectively modulating the binding of estrogen to the ERα, and by blocking the production of estrogen through inhibition of aromatase[198]. Further, the lack of effect of receptor blockage in ERα negative breast cancer, supports the notion of hormonal influence on cancer progression. Consequently, ERα and PGR, given its role as an ER response gene, are established as biomarkers in routine breast cancer diagnostics, to predict prognosis and evaluate initiation to hormonal therapy[199]. Unfortunately, as for PC, many breast cancers also develop resistance to hormone therapy by a mechanism which are not fully defined.

Steroid hormones also play a vital role in the tumorigenesis of endometrial cancer where the exposure to estrogens unopposed by progestins can result in cancer development[200]. This context became evident in the 1970s when women using sequential contraceptive pills developed an increased risk of endometrial cancer[201]. In contrast, combined oral contraceptives, which includes a combination of estrogen and progestin, is associated with a reduction in cancer risk. Consequently, hormone therapy is widely applied in ER^+/PGR^+ endometrial cancers, and progestins are most frequently used. Other alternatives are equal to those applied in ER^+/PGR^+ breast cancer, with the goal of inhibiting the estrogenic growth stimulation[202]. ERα and PGR status are also considered positive prognosticators for disease specific survival[200].

In addition to the positive prognostic and therapeutic role of the PGRs in endometrial cancer[200], protective roles of SHRs in other cancers are evident. For decades, synthetic glucocorticoids have been applied in the treatment of hematopoietic malignancies derived from the lymphoid cell lineage[203]. Stimulation of the glucocorticoid receptor (GR) in this setting induces apoptosis in the malignant cells, but the mechanism is not fully understood[204]. In ovarian cancer, high levels of both PGR and ERβ have been suggested a positive predictor of survival[205]. Together, such results support the notion that steroid hormone-induced biological outcomes occur in a context-dependent manner in multiple malignancies.

1.7.2 Steroid hormones and cancer hallmarks

Considering the steroid hormones extensive involvement in human physiology, a solely tumor promoting or tumor suppressive role of steroid hormones is highly unlikely. Certainly, the SHR action will depend on context and vary according to cross reactivity with other SHRs, the influence of co-regulatory molecules on the receptors transcriptional activity and non-genomic actions of the receptors.

A tightly controlled cell cycle, and a correct cellular response to DNA damage, are crucial to prevent a malignant transformation of a cell. In cancer development, the balance between cell proliferation and apoptosis is lost, and unopposed cell proliferation reigns. The progression through the cell cycle is regulated, in part, by specific proteins called cyclins and the cyclin dependent kinases (CDK). In cancer development, mutations and overexpression of cyclins and other cell regulatory molecules are frequent events[29]. Recently, the close connections

between SHR and such components of the cell cycle machinery were outlined in a comprehensive review by Zheng & Murphy[206]. In PC, the AR's ability to regulate progression through the initial phases of the cell cycle by controlling CDK activity has been uncovered in several experimental studies[207]. There are further extensive connections between cellular machineries that handle DNA damage and the SHRs. A bidirectional regulatory relationship between the SHR and the DNA repair mediators is indicated, meaning that SHR can regulate DNA repair mediators and that DNA repair mediators can influence the SHRs function[208]. Consequently, dysregulated SHR activity has the potential to support a malignant transformation and contribute to cancer progression in several ways.

Examples of the relationship between SHR and the DNA repair machinery can be drawn from ADT's ability to improve radiation therapy-response in PC. The underlying mechanism for this effect is not fully understood. It is, however, hypothesized that the AR can positively regulate DNA repair mediators in a manner which promotes resistance to radiation therapy-induced DNA damage[209,210]. Hence, suggesting that inhibition of specific SHRs will sensitize the cells to cancer treatment that is based on inducing DNA damage. Further, functional studies have demonstrated that SHRs can promote malignant transformation by inducing DNA damage and subsequent oncogenic genomic rearrangements[211–213]. This is however complicated by evidence of SHRs with DNA stabilizing abilities[214].

SHRs act as signal transductors that decipher and transfer hormonal signals in both a paracrine and an autocrine manner. Several of these signals have oncogenic functions if transmitted aberrantly, e.g. through transcription of proliferative target genes and through direct initiation of proliferative signaling cascades[168–170]. The Src tyrosine kinase is a recognized proto-oncogene involved in regulation of proliferation, adhesion and invasion. Sex-SHRs' interaction with Src can trigger Src activation and subsequent progression through the cell cycles due to upregulation of necessary target genes[168,169,215]. In breast and PC cell lines, sex steroid hormones can initiate mitogenic pathways such as the PI3K/Akt and the Src/Ras/ Erk[n,168,170]. Such pathways transmit proliferative signals to the cell nuclei, and dysregulation are frequent events in cancer development[170]. There are also examples of SHRs being regulated directly through phosphorylation of mitogenic protein kinases, including the

[n] **Erk**: Also known as mitogen-activated protein kinase (MAPK)

cell cycle regulators, CDKs and mitogen activated protein kinase (MAPK), which activation in turn can be elicited by growth factors[167].

Finally, alterations to SHR's structure and function as the cancer progresses must also be considered. Regarding the AR, somatic mutations (e.g. splice variants, amplifications and point mutations) are considered later event in the prostate carcinogenesis and associated with development of CRPC. Such AR alterations can lead to increased AR activity, render the receptors constitutively active or result in SHRs receptive to stimulation by hormones other than their cognate hormones[196]. Recently, the development of an mutant AR responding to progesterone stimulation has been connected to progression of advanced disease[216]. SHR mutations could further allow interaction with different co-regulators rendering alternate transcriptional activity in response to ligand binding.

1.7.3 Regulators of steroid hormone receptor transcriptional activity and implications in hormone dependent cancer

The SHRs' initiation of target gene transcription is a complex, multilayered process regulated at several levels in the cell. This includes phosphorylation, interaction with co-regulators and other modifying proteins such as ubiquitin and small ubiquitin-like modifier (SUMO) proteins[217]. Co-regulators are essential for SHRs' transcriptional activity and their physiological functions. These regulatory proteins are recruited to the target gene promoters by ligand bound SHRs. Here they assemble in large complexes, eventually enhancing or repressing transcriptional activity. Co-regulators exert their regulatory functions through several mechanisms. This include recruitment of transcriptional machinery, chromatin remodeling, modification of enzymes and histone proteins and actions as chaperone proteins[218]. An immense amount (> 400) of SHR co-regulators have been identified since the first discoveries in the mid 90's[219]. Further, aberrant co-regulatory activity is implicated in the development of hormone-related cancers[220]. The most extensively characterized family of co-regulators, and also the first to be described, where the steroid receptor co-activators (SRC: SRC-1, SRC-2, and SRC-3) belonging to the p160 family of co-activators[219]. These, and several other co-regulators, has demonstrated interaction with different members of the SHR family, including ARs, ERs and PGRs [220]. There are also numerous examples of their oncogenic abilities in hormone dependent cancers[220]. Of example are the observed roles in PC where SRC-1 expression is correlated with tumor aggressiveness and increased AR

activity[221], SRC-2 has demonstrated a role in metabolic reprogramming in development of metastatic disease[222], and SRC-3 is overexpressed and correlated with cancer cell proliferation[223]. This knowledge opens the door to the concept that the functional outcome of SHR signaling depends to a large extent on the interacting co-regulatory molecules.

1.7.4 Steroid hormone receptor crosstalk

Outside the laboratory, tissues rarely express one or another SHR, but rather various receptors combined in the same or different cell types. This co-existence lays the foundation for receptor "cross talk" in this homogenous receptor family, and furthermore, the ligand cross reactivity[7]. A large amount of evidence of steroid hormone interactions in cancer is emerging. Some examples are the interactions which have been revealed between the AR and ERα through the ability of the AR to bind and regulate estrogen response elements in breast cancer[224]. Other interactions with steroid hormones in breast cancer are demonstrated between PGRs and ERα[225–227]. In ER+/PR+ breast cancer, an ERα, PGR-B and Proline-, glutamic acid- and leucine-rich protein 1 (PELP-1) complex has demonstrated proliferative abilities in the presence of estrogen[225]. It is further indicated that the regulation of the PGR by ERα involves a complex crosstalk and physical interaction between these two receptors[226,227].
In advanced PC, overlapping functions between the GR and AR has been suggested a mechanism of cancer progression in ADT resistant PC[228,229]. The development of an mutant AR responding to progesterone stimulation has also been connected to progression of advanced disease[216]. Besides, a dysregulated interplay between the different receptors can result in malignant transformation, as is suggested for an imbalance of the PGRA:PGRB ratio in breast cancer[189].

1.7.5 The tumor microenvironment and tumor-associated stroma

As outlined in the updated version of "Hallmarks of cancer", the cells and tissue surrounding a malignant tumor is no longer considered passive bystanders in tumorigenesis, but rather vital contributors to the development of certain "cancer hallmarks"[29]. The microenvironment surrounding a PC includes stromal cells, such as fibroblasts, endothelial cells and pericytes, in addition to nerves, ECM and immune cells, including the signaling molecules they secrete (Figure 15)[230]. As previously depicted, the non-malignant prostate stroma consists mainly of collagenous fibrous tissue and smooth muscle fibers. In a malignant environment stimulated by various signaling molecules and growth factors, the stroma surrounding the cancer changes

and acquires different abilities. Such changes include stromal tissue populated with cancer associated fibroblasts (CAFs), and the stroma transforms into a "reactive stroma". This reactive stroma is actively involved in the acquisition of malignant traits, such as growth and metastasis, and gradually provides a favorable environment for cancer development[231]. One example is CAFs contributing with increased ECM and production of growth factors and factors inducing increased angiogenesis and vascular permeability, thereby facilitating metastasis[232]. Cancer development is dependent on reciprocal interactions with tumor cells and the tumor associated stroma[29]. It is becoming evident that SHRs are not only expressed in tissue parenchyma, but also in stromal cells[233]. This is addressed in detail in the "discussion of main results" section. Further, SHRs are connected to several malignant traits in cancer development (as discussed in the previous sections). This indicates that SHRs most likely influence tumor progression from two angles, through their actions in both tumor associated stromal cells and tumor epithelial cells. Additionally, given the stromal-epithelial interplay, pro-tumorigenic factors produced in the TME will also influence the SHRs and their actions in the malignant transformation[234].

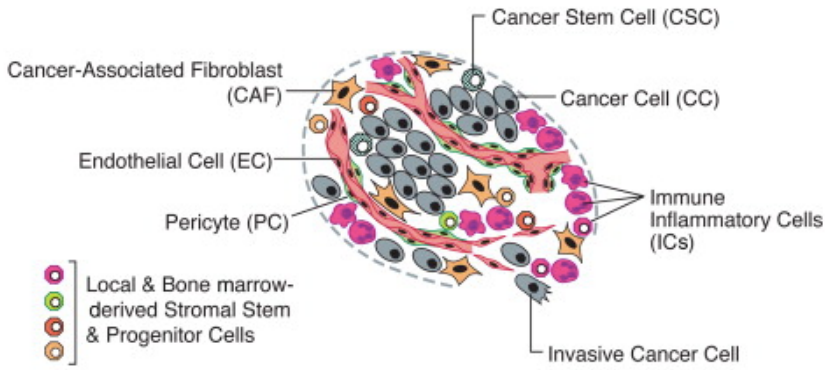

Figure 15 – The tumor cells and the tumor microenvironment. Reprinted with permission from Elsevier ©, 2011[29]

1.8 Prostate cancer treatment

1.8.1 Risk stratification and treatment choice

Today, a broad range of treatment options exists for PC patients depending on the risk category the cancer is placed in (Table 3) in addition to the patient's comorbidities, performance status, life expectancy and preferences[235,236]. Although, perioperative mortality is low, all of the presently available invasive PC treatments have several adverse effects strongly

associated with reduced quality of life[237]. The objective is thus to offer treatment customized to the patient's risk and avoid overtreatment. Patients with localized PCs categorized as clinically low-risk are often recommended a strategy of active surveillance[4]. For patients with localized cancer in the low-intermediate risk category, treatment with curative intent diverge from experimental focal therapy[238], radiation therapy to radical prostatectomy[239]. Patients with locally or regionally localized, high-risk PC typically receives treatment with radiation therapy or radical prostatectomy[239], often with adjuvant androgen deprivation therapy (ADP) including pelvic radiation therapy or pelvic lymph node dissection when indicated[240]. Patients with disease progression despite surgery or radiation therapy, or presenting with disseminated disease, are recommended ADP to suppress serum testosterone levels[241]. Chemotherapy can also be offered when cancer recurrence occurs, or the cancer has progressed to castration resistance[242]. Additionally, second generation hormonal therapies and systemic radionuclides have increased survival in patients who has acquired resistance to ADP[243,194,195,244]. Recently, immunotherapies have also been implemented in the treatment of CRPC[235,245].

1.8.2 Active surveillance and watchful waiting

Active surveillance involves regular testing and assessment of signs of cancer progression. This includes repeated measurements of serum PSA, DRE and prostate biopsies, MRIs can also be applied. The purpose of active surveillance is to reduce overtreatment and avoid treatment related complications in patients with cancers where progression is highly unlikely even if left untreated. With signs of progression, the curative treatment can be initiated[4]. The drawback is the risk of seemingly low-risk cancers to develop into lethal cancers or risk of existing undiscovered high-grade carcinomas. Recent investigations with 15 years follow-up have however revealed very low PC metastasis and mortality rates (< 3 %) for the active surveillance groups[246].

Active surveillance must not be confused with watchful waiting. Whereas active surveillance maintains the possibility of curative treatment, watchful waiting is a conservative strategy including only symptomatic treatment. In some circumstances it is considered that the patient will not benefit from curative treatment attempts, this often entails elderly patients with comorbidities and short life expectancy. In these cases, a watchful waiting strategy can be considered. This includes deferral from curative treatment attempts, but rather treating PC

related symptoms when necessary. Also in this strategy, the purpose is to reduce overtreatment and treatment related side-effects[237,247].

1.8.3 Radical prostatectomy

Radical prostatectomy is the surgical removal of the entire prostate, including the prostatic urethra and both vesiculae seminales. This is a treatment option for patients with localized cancer. A high risk of extracapsular spread of the cancer is usually a contraindication to surgery[236]. The goal is total removal of the entire PC and also minimize adverse effects such as urinary incontinence and erectile dysfunction[237]. The surgery can be performed as open surgery, laparoscopic and with robot assisted laparoscopy. No recognized differences exist in oncological results or post-operative adverse effects, although a lower a perioperative morbidity has been associated with robot assisted surgery[248]. Nerve sparing surgery, to avoid erectile dysfunction, is an alternative for some patients with localized disease. Adjuvant radiation therapy to the prostate region is considered in patients with unfavorable histopathological reports[236]. Due to the high frequency of adverse effects that follows radical prostatectomies, large-scale randomized clinical trials (RCTs), with approximately 20 years of follow up, have been constructed to consider the effect on overall survival when selecting radical prostatectomy over watchful waiting in patients with localized disease. The Scandinavian PC Group (SPCG)-4 trial[102] provide evidence that radical prostatectomy improves overall survival or delays metastatic development compared to patients assigned to watchful waiting. However, these patients were diagnosed before the PSA-era which could inflict a stage migration in the cohort, with fewer low grade cancers than we see today. In contrast, The PC Intervention or Observation Trial (PIVOT) study[103] was initiated in the early PSA-era. Herein, no significant benefit in overall survival or reduction in PC specific mortality was discovered for the radical prostatectomy group. Invasive treatment did however reduce disease progression and subsequent treatment, but was associated with increased adverse effects. The critique against the PIVOT trial has been lack of study power. It must also be noted that neither of these trials assesses the effect of an active surveillance strategy. The ProtecT trial is another RCT from the PSA-era currently comparing the outcome in low risk PC patients receiving either active monitoring (similar to active surveillance), radical prostatectomy and radiation therapy. Published results after 10 years of follow up did not detect any difference in prostate-cancer-specific mortality between the groups, however mortality was overall low after only 10 years, and more years of follow up are needed[249].

1.8.4 Radiation therapy

Radiation therapy is considered equivalent to radical prostatectomy as a curative option for patients with clinically localized PC[239]. Radiation therapy can also be used as first line treatment for locally advanced PC, with or without nodal involvement, in combination with hormonal therapy[240] and as an adjuvant treatment to radical prostatectomy when positive resection margins are discovered. In case of rising PSA-values after surgery, early salvation radiation provides a possibility to cure patients[250]. Radiation therapy, which in PC typically is delivered by photons or protons, causes DNA damage and subsequent cellular death to a greater extent in malignant tissue compared to normal tissue[251]. In PC, radiation therapy is most often administered using external beam radiation therapy (EBRT), but brachytherapy or a combination of the two are also possible. Dose-escalated intensity-modulated radiation therapy (IMRT) is the gold standard for EBRT[236]. The radiation doses are administered by small daily fractions over weeks. Due to evidence of significant improvements in biochemical failure free survival (BFFS) when applying larger cumulative doses, doses in the range 74 – 80 Gy is now recommended[252]. In brachytherapy, a radiation source is implanted into or next to the PC using image guiding. The most common brachytherapy approach in PC for curative intent is low-dose rate brachytherapy, an option for patients with low risk disease or a subgroup of patients with intermediate risk disease. The advantage is the ability to deliver high doses of radiation directly to the tumor, saving normal tissue[253]. The most common side effect of radiation therapy is bowl dysfunction due to irritation of the mucosal area affected by radiation. Bladder irritation due to radiation also occurs, resulting in incontinence and dysuria. This is, however, more common after radical prostatectomy. Erectile dysfunction is also more prevalent after radical prostatectomy but can develop slowly over time after radiation therapy. Further, both radiation therapy and radical prostatectomy are associated with reduced quality of life compared to patients under active surveillance[237].

1.8.5 Focal therapy

Besides radical prostatectomy and radiation therapy, other experimental focal therapeutic strategies for patients with low-intermediate risk, clinically localized PC have emerged with the aim to preserve healthy tissue and save nerves and adjacent structures, thereby reducing adverse outcomes. Various ablative strategies are used in focal treatment. Cryoablation therapy involves the induction of extremely low temperatures and subsequent thawing, High-

Intensity Focused Ultrasound (HIFU), where ultrasonic waves administered transrectal through an ultrasound probe, are used to initiate cellular damage. These agents are administered intravenously, reaches the malignant prostatic lesions and is subsequently exposed to light by laser via the perineum[254]. Such treatment options in low-intermediate risk localized PC have been compared to radical prostatectomy and radiation therapy in a systematic review[238], however due to lack of follow-up time and the high risk of bias across the included studies there was insufficient evidence to make recommendations of focal therapy over radical prostatectomy or radiation therapy. The recommendation is currently that focal treatment strategies should be considered experimental[236].

1.8.6 Androgen deprivation therapy

As described in the "PC progression and prognostication" section, a varying degree of patients will experience BF following initial curative treatment[108], and only a subgroup off patients will progress to CF and PCD[89,109–111]. Additionally, the progression time to CF is highly variable amongst patients. This challenge the decision of which treatment to initiate and when. Following BF, the treatment options include observation, salvage radiation therapy of the prostate bed or ADT delivered either continuously, alternating or anticipating.

1.8.6.1 Castration naïve disease

A castration naïve disease indicates a PC that is still dependent on androgens for growth and progression. In ADT, ablation of testicular androgen synthesis is achieved. This can be accomplished through both medical and surgical castration, both methods are considered equal[255]. Surgical castrations include an orchiectomy, which very efficiently reduces serum androgen levels. It is debated whether ADT for patients with more advanced disease should be given at an early stage or not until the patients has clinical symptoms and/or metastatic disease. Presently, ADT is offered instantly to patients who present with symptomatic metastatic disease. In asymptomatic patients with locally advanced disease, or BF after initial curative treatment, routine ADT use is not recommended. In this situation, ADT should only be considered if PSA is >50 ng/mL and PSA DT <12 months[256]. A consensus has not been reached regarding use of ADT in asymptomatic metastatic patients. In patients with localized, non-metastasized cancer, who are not suitable for curative treatment, it is recommended to offer ADT only when palliation is required[235]. Increased survival in patients with hormone-naïve metastatic PC receiving docetaxel (chemotherapy) in addition to ADT has recently been

demonstrated, and is considered in patients presenting with high-burden metastatic disease who are fit enough to handle chemotherapy[257,258].

With medical castration, analogs to GnRH or GnRH antagonists are most commonly utilized as first-line treatment of metastatic disease[235]. Their effect is exerted by stimulating the GnRH receptors and activating the negative feedback of the HP-axis or by direct blockage of the GnRH receptors through competitive binding, respectively (Figure 16)[241]. However, his treatment strategy does not block synthesis of hormone precursors in the adrenal gland. First generation AR antagonists, such as bicalutamide and flutamide, bind to AR and competitively block the binding of the androgens testosterone and dihydrotestosterone (DHT). These treatments do not block the HP-axis and subsequently does not reduce testosterone levels (Figure 16). Further, because of dihydrotestosterone's (DHT) high affinity to the AR, these agents were not potent enough to sufficiently block all AR activity. Thus, they are not suitable for monotherapy, but are often used in combination with first-line LHRH analogues to optimize the ADT, or as secondary endocrine therapy in castrate-resistant PC (CRPC)[235]. When testicular androgen production is inhibited, and serum androgens reach castration levels (testosterone level < 50 mg/dl), the cancer and metastases will go into remission. This remission last for 2 - 3 years on average. When the cancer inevitably progresses, despite continued treatment and serum castrations levels, it is now castrate resistant[259]. Although ADT is initially considered very effective it is associated with several adverse effects including hot-flashes, sexual dysfunction, osteoporosis, increased cardiovascular risk and gynecomastia[235].

1.8.6.2 Castration resistant disease

CRPC is defined as biochemical (PSA > 2 ng/mL and consecutive rise in PSA with one-week interval) or radiological (≥ 2 new bone lesions or one new soft tissue lesion) disease progression despite castration levels of testosterone. Castration treatment is continued also after the cancer becomes castration resistant. In addition, both cytostatic (e.g. docetaxel, cabazitaxel) and new hormonal treatment strategies with enzalutamide and abiraterone can be added as second-line treatment[235]. Enzalutamide is an AR antagonist which inhibits AR more potently than first generation anti-androgens (Figure 11)[243,260]. Upon castration therapy, extra-gonadal androgen synthesis is unaffected. Abiraterone, however, is a cytochrome P-450 17A1 inhibitor (CYP-17A1) which inhibits the synthesis of androgens in extra-gonadal sites, e.g.

the adrenal glands, by blocking an enzymatic step in the synthesis pathway (Figure 16). It is given in combination with prednisolone since abiraterone also inhibits cortisol synthesis, this is to replace deficient cortisol and suppress a subsequent rise in ACTH levels due to the negative feedback in the HP-axis[194,195]. Both have demonstrated increased survival in CRPC patients[243,194,195]. There is also recent evidence of benefits of adding abiraterone to standard ADP as initial treatment of metastatic disease[261].

1.8.7 Other

The bone-targeted drug radium[223], an alpha emitter delivering bone metastasis targeted internal radiation, is associated with a survival benefit in metastatic CRPC[244]. Both zoledronic acid and denosumab[o], have demonstrated efficacy in delaying or preventing skeletal related events in CRPC patients, but no effect on disease specific survival has been observed[262]. Additionally, chemotherapy with cabazitaxel is approved as a second-line treatment following treatment with traditional chemotherapy in CRPC[235,263]. Immunotherapy is another new concept in PC treatment, and the PC vaccine Sipuleucel-T has demonstrated overall survival benefits in metastatic PC patients[245]. It is currently approved in the U.S. as the first therapeutic cancer vaccine for asymptomatic or minimally symptomatic patients with metastatic CRPC, but is not available in Europe[235]. Despite these current advances in treatment strategies, CRPC inevitably progresses, and PC death usually occurs within 2 – 4 years[264].

[o] **Denosumab**: Antibody directed against RANKL (receptor activator of nuclear factor κB ligand), a key mediator of osteoclast formation, function, and survival

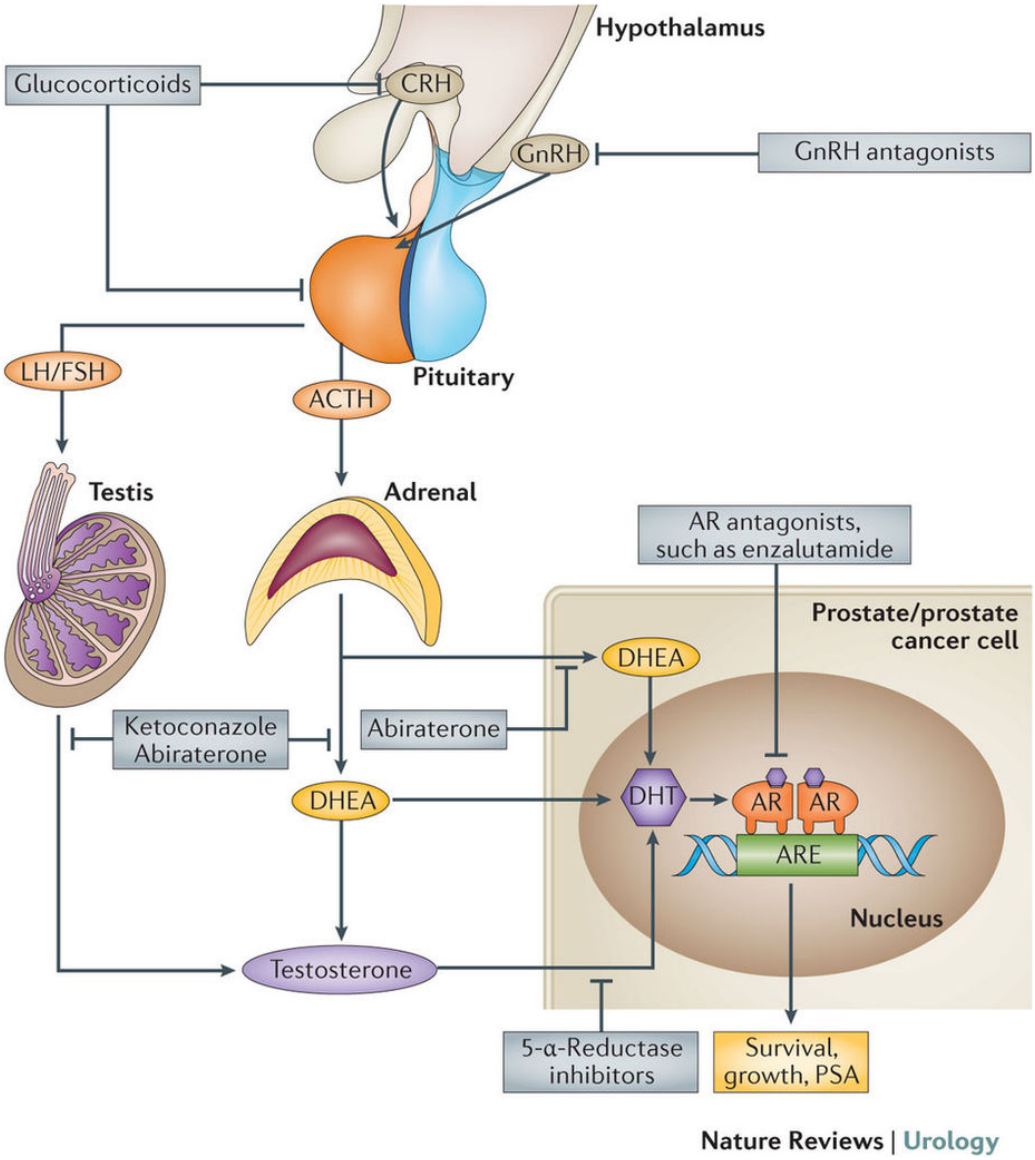

Figure 16 - Schematic illustration of pharmaceutical agents that reduce androgen synthesis and signaling. These drugs include gonadotropin-releasing hormone/ luteinizing-hormone releasing hormone (GnRH/LHRH) inhibitors that inhibit LH action, glucocorticoids that inhibit corticotropin-releasing hormone (CRH) release from the hypothalamus and ACTH from the pituitary gland, and abiraterone that inhibit CYP-17A1activity and thereby inhibiting adrenal and testicular androgen production, AR antagonists, including enzalutamide, that interfere with androgen binding to ARs and thereby inhibit AR signaling. Importantly, abiraterone is also able to block intratumoral synthesis of androgens in PC cells. Reprinted with permission from Springer Nature©, Nature reviews Urology, 2016[265]. **Abbreviations**: AR = Androgen receptor; DHT = Dihydrotestosterone; DHEA = dehydroepiandrosterone; FSH = follicle-stimulating hormone

2 AIMS OF THE THESIS

The executive aim of this thesis was to explore the expression of the "feminine" sex SHRs in prostate adenocarcinoma and their association with disease progression. Thereby assessing the markers tissue distribution and prognostic value in PC. In detail, we aimed to:

- To examine the *in situ* tissue distribution of the sex-SHRs: ERα, ERβ, pan-PGR, PGRA, PGRB in addition to the aromatase enzyme in the different tissue compartments of prostate adenocarcinoma using tissue microarray (TMA) and IHC
- To retrospectively evaluate the prognostic impact of marker expression on the clinical outcomes: BF, CF and PCD using survival analyzes
- To evaluate the correlation of these markers with other prognostic markers in prostate cancer

3 MATERIALS AND METHODS

3.1 Patient cohort

3.1.1 Data acquisition

Radical prostatectomy specimens and complete follow up data from 671 patients were retrospectively identified from the time period 01.01.1995 through 31.12.2005. Primary tumor blocks were collected from the archives of the Departments of Pathology at St. Olav Hospital/ Trondheim University Hospital (n = 341), Nordland hospital Bodo (NLSH) (n = 63) and the University Hospital of Northern Norway (UNN) (n = 267) (Figure 17).

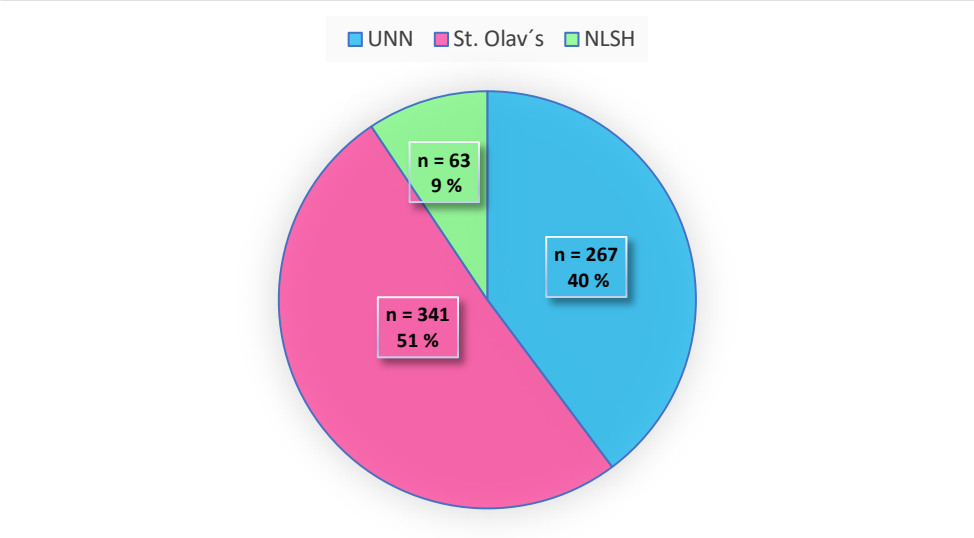

Figure 17 - Patient distribution between the contributing hospitals' departments of pathology

Of the initial 671, a total of 136 patients were excluded from the study (Figure 18). Reasons for exclusion were: I) radiotherapy to the pelvic region prior to surgery (NLSH n = 1), II) other malignancies (other than superficial skin cancers) within 5 years prior to the PC diagnosis (UNN n = 4), III) inadequate paraffin-embedded tissue blocks (St. Olav n = 112, NLSH n = 3, UNN n = 15), IV) lack of clinical follow-up data (St. Olav n = 1). None of the included patients had received hormonal therapy prior to or at the time of the prostatectomy. Thus, 535 patients with adequate tissue blocks for re-evaluation and complete follow-up data were included.

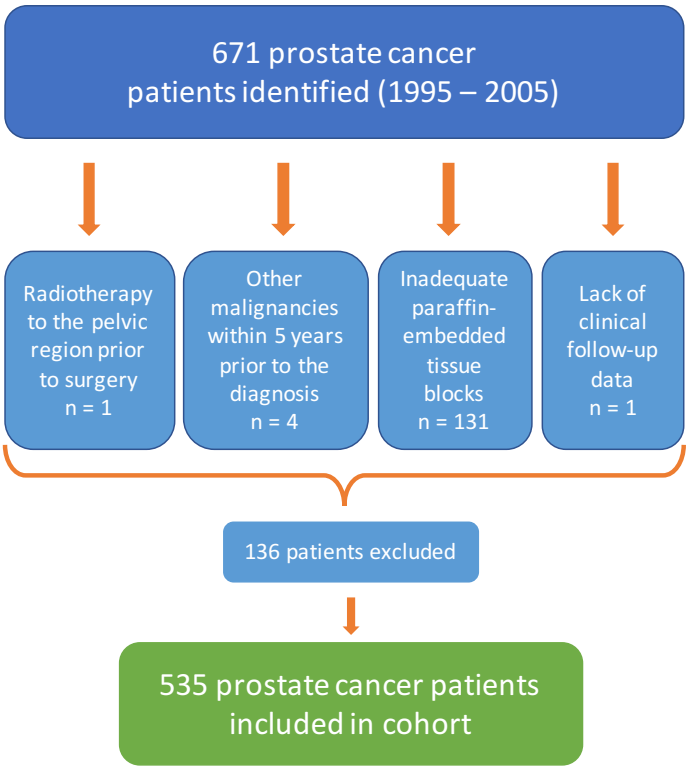

Figure 18 - The prostate cancer cohort and inclusion criteria

3.1.2 Definition of end-points and clinical variables

Three clinical endpoints were defined and evaluated in this cohort **(Paper I, III and III):** Biochemical failure (BF), Clinical failure (CF) and PC death (PCD). BF was determined as PSA recurrence ≥ 0.4 ng/mL in a minimum of two different blood samples postoperatively, as has been previously discussed[105]. BFFS was calculated from the date of surgery to the last follow-up date for BF, which was the last date of a measured PSA. CF was defined as verified local symptomatic progression beyond cure or by findings of metastases to bone, visceral organs or lymph nodes by CT, MR, bone scan or ultrasonography. Clinical failure free survival (CFFS) was calculated from the date of surgery to the last follow-up date for CF, which was the last date without symptoms or any evidence of metastasis. PC death (PCD) was defined as death caused by progressive and disseminated CRPC. PC death free survival (PCDFS) was calculated from the date of surgery to the date of death by PC.

PSA values preoperatively were measured just before surgery. The exception was a minor group of patients who underwent transurethral resection of the prostate (TUR-P) for other reasons than PC, prior to surgery. For these patients, PC was an incidental finding.

Postoperatively, for all patients, up to four PSA measurements taken at least with six weeks interval, were included. PSA doubling time (PSA-DT) was calculated using an online calculator (http://nomograms.mskcc.org/Prostate/PsaDoublingTime.aspx) based on a previously defined algorithm[89]. PSA-DT was further stratified into groups, < 3 months, 3 – 9 months, 9 – 15 months and >15 months, constructed based on significant differences in prognostication for CF and PCD, as previously reported[109].

Demographic and patient clinicopathological data (Table 4) were acquired from medical records, compiled into a database and de-identified. The patients were retrospectively included from 01.01.1995 and clinical data was last updated in December 2015. At the last follow-up median follow-up time was 12.5 years (range 1.5 – 20.4) and 200 patients (37 %) had experienced BF, 56 (11 %) CF, whereas 18 (3 %) had died due to PC. Patients that did not experience the specific endpoint, e.g. were alive or without relapse at the last follow-up date or could for some reason not be followed, were censored. Patient data update was performed by studying the patients' medical records at the operating centers and at local hospital. In **Paper I and II** the thesis was generated with data from the previous patient update in November 2012, with a median patient follow-up time of 7.4 years (range ½ – 15.6). The final paper (**Paper III**) included data from the most recent update in 2015. A detailed description of the patient cohort has been previously described[266]. The database has also been basis for previous publications[267–273].

Characteristics	Patients		BF (n = 200, 37%)			CF (n = 56, 11%)		PCD (n = 18, 3%)	
	n	%	5 -year EFS (%)	10-year EFS (%)	p	10-year EFS (%)	p	10-year EFS (%)	p
Age					0.24		**0.038**		0.40
≤ 65	357	67	77	64		94		98	
> 65	178	33	70	59		91		98	
pT-stage					**< 0.001**		**< 0.001**		**0.001**
pT2	374	70	83	73		97		99	
pT3a	114	21	61	45		87		98	
pT3b	47	9	43	22		74		90	
pN-stage					**<0.001**		**< 0.001**		**< 0.001**
NX	264	49	79	68		96		99	
N0	268	50	72	58		91		97	
N1	3	1	0	0		33		67	
Preoperative PSA					**< 0.001**		**0.029**		**0.003**
PSA ≤ 10	308	57	81	68		95		99	
PSA >10	221	42	68	54		89		97	
Missing	6	1							
Gleason grade group					**< 0.001**		**< 0.001**		**< 0.001**
1 (3+3)	183	34	83	70		98		99	
2 (3+4)	219	41	77	68		94		99	
3 (4+3)	81	15	70	47		90		96	
4 (4+4)	17	3	58	28		86		94	
5 (≥ 9)	35	7	37	29		65		91	
Tumor size					**< 0.001**		**0.002**		0.09
≤ 20 mm	250	47	83	70		96		99	
> 20 mm	285	53	68	55		90		97	
PNI					**<0.001**		**< 0.001**		**< 0.001**
No	401	75	80	70		96		99	
Yes	134	25	60	41		83		95	
PSM					**0.049**		0.20		0.84
No	249	47	80	66		96		98	
Yes	286	53	70	59		90		98	
Circumferential PSM					**< 0.001**		**< 0.001**		**0.022**
No	381	71	82	70		96		99	
Yes	154	29	57	44		85		96	
Apical PSM					0.063		0.43		0.13
No	325	61	74	58		92		98	
Yes	210	39	77	68		93		99	
LVI					**< 0.001**		**< 0.001**		**< 0.001**
No	492	92	77	64		95		99	
Yes	43	8	47	39		70		90	
Surgical procedure					0.47		0.31		0.96
Retropubic	435	81	77	63		92		98	
Perineal	100	19	68	58		95		99	

Table 4 - Patient characteristics and clinicopathological variables in 535 PC patients (univariate analyzes; log-rank test). Significant p-values in bold (threshold p ≤ 0.05). Abbreviations: EFS = Event free survival; BF = Biochemical failure; CF = Clinical failure; PCD = PC death; PSM = Positive surgical margin; LVI = Lymphovascular infiltration

3.1.3 Characteristics of study population

Details regarding the cohort's demographic and clinicopathological variables are presented in Table 4. The hospitals contributing to our database are a part of the mid- and northern health regions in Norway and are located in Central- and North Norway, excluding Finnmark. Together they constitute two of the six university hospitals in Norway. These hospitals are the major health centers, which serve about 1/5 of the total Norwegian population (www.SSB.no).

3.1.4 Ethics

This project was approved by the Regional Committee for Medical and Health Research Ethics, REK Nord, project application 2009/1393. A mandatory re-approval was conducted in January 2016. As this was a retrospective study where the majority of the material was more than ten years old, and where most of the patients were deceased, REK Nord considered a written patient consent as not necessary. All patients were anonymized and given a trial number. The Data Protection Official for Research (NSD) approved the assembly of the database. The reporting of clinicopathological variables, survival data and biomarker expressions was conducted in accordance with the REMARK guidelines[274].

3.2 Tissue preparation

3.2.1 Tissue re-evaluation

Prior to inclusion, all prostate specimens were histologically re-evaluated and re-staged by an experienced pathologist (ER) according to the 2010 revision (7th edition) of the TNM classification system[117,118]. Further, the tumors were initially graded according to the 2005 International Society of Urological Pathology Modified Gleason System[275]. Concurrent with the last patient update in December 2015, the Gleason grading was revised according to the most recent version of the modified Gleason grading system[62,63].

3.2.2 Tumor specifications

The applied tumor size is the largest measured diameter of the index tumor. A positive surgical margin (PSM) was defined as tumor extending to the stained surface of the resected specimen. Observed tumor cells within lymphatic- or blood vessels that were in contact with endothelial cells or filling the luminal space was considered as lymphovascular infiltration

(LVI). PNI was defined as tumor cells infiltrating the perineural space outside the prostatic capsule.

3.2.3 Tissue fixation and processing

Optimal preservation of tissue is critical for maintaining good tissue quality for further histological examination. This was achieved through three main steps (Figure 22): **I)** Tissue fixation **II)** Tissue processing **III)** Tissue slide mounting and drying. The applied fixation procedure for the cohort **(Paper I, II and III),** and the most extensively utilized fixation procedure, is formalin fixation in 10 % neutral buffered formalin, which consists of a 4 % formaldehyde solution buffered to a neutral pH[276]. Formalin inhibits cellular processes and tissue degradation. Additionally, it removes pathogens and conserves tissue architecture. The tissue fixation occurs by the formation of cross-links between proteins, or proteins and nucleic acids, and the formation of hydroxymethyl bridges[277]. Tissue processing is the conversion of tissue fixed in a liquid solution, such as formalin, to embedding in paraffin. The result is formalin fixed paraffin-embedded tissue (FFPE). The processing includes tissue dehydration, -washing and the final incubation in a warm embedding solution, preferably paraffin[278]. Finally, The FFPE tissue blocks are stored at room temperature in a dark storage room.

3.2.4 Tissue microarray

TMA consists of paraffin blocks where numerous individual tissue cores ($n \leq 1000$) have been inserted in a predefined coordinate pattern. It was developed by Kononen and colleagues in 1998 as a high throughput technology, which very efficiently would facilitate the analysis of molecular markers in numerous tissue specimens[279]. The TMAs are constructed by acquiring cylindrical tissue cores, usually from whole section FFPE tissue specimens, and arraying them into a recipient TMA block (Figure 19)[280]. The size of the TMA cores ranges from the standard size of 0,6 mm. and up to 2.0 mm. in diameter[280]. The number and the size of the TMA cores needed depends on the target tissue. The TMAs are available for detection of a broad specter of molecular targets, including DNA, RNA and protein level, and all available techniques for examination of histological sections can be applied, e.g. immunohistochemistry (IHC) and DNA in situ hybridization (ISH)[279,280]. Number of TMA-sections that can be cut from the recipient TMA paraffin block depends on the thickness of the donor tissue block, but with sufficient tissue depth in the donor block, hundreds of TMAs

can be constructed. A standard FFPE donor tissue block should be preferably 3- 4 mm thick and no less than 1 mm thick[280].

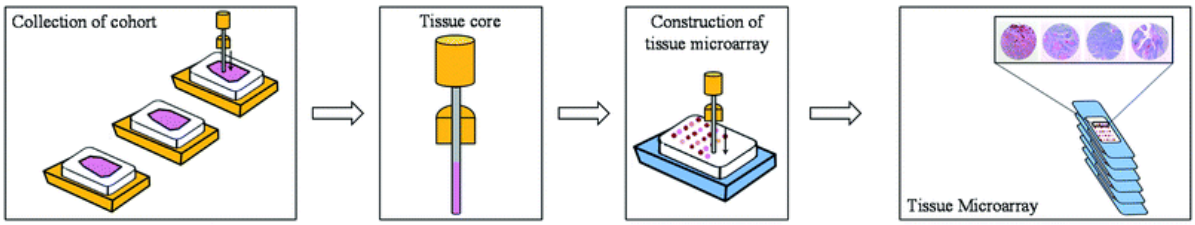

Figure 19 – Tissue microarray construction. Adapted in part from Pallua, J. D. et al. Fourier transform infrared imaging analysis in discrimination studies of squamous cell carcinoma[281]. Reprinted with permission from Royal Society of Chemistry© 2012

3.2.5 Tissue microarray construction in our prostate cancer cohort

Tissue microarrays (TMAs) was the chosen method for analysis of the PC tissue samples in our cohort **(Paper I, II and III)**. FFPE tissue blocks were collected from the included patients. All blocks were sliced and stained with hematoxylin and eosin (H&E). For each case, a pathologist (ER) identified and marked representative areas of the prostate specimens. This included areas with tumor epithelial cells (TE), tumor associated stromal cells (TS), normal epithelial cells (NE), normal stromal cells (NS) in addition to areas with benign prostate hyperplasia (H) and prostate intraepithelial neoplasia (PIN). From each of these areas, central cores were sampled from the donor block to construct TMA blocks. To include all core samples, twelve tissue array blocks were constructed. This included a total of 2236 cores from the selected tissue areas of the prostates, the majority containing both epithelial and stromal cells (Figure 20): 1070 cores from tumor tissue, 535 cores from stromal areas, 456 cores from normal tissue areas, 89 cores from areas with PIN and 86 cores with hyperplasia.

A tissue-arraying instrument (Beecher Instruments, Silver Springs, MD, USA) was used to harvest the cores from the marked tissue areas in the donor blocks, using a standard 0.6 mm diameter needle. The samples were subsequently inserted into an empty recipient paraffin block according to a predefined coordinate pattern. Afterwards, multiple four µm sections were cut from the paraffin blocks with a Micron microtome (HM355S), dried over night at 60 °C and affixed to glass slides. The process is illustrated in Figure 19. If the TMAs were

stored before IHC procedure, the slides were sealed with paraffin and stored in a refrigerator at approximately + 4°C for no more than a year.

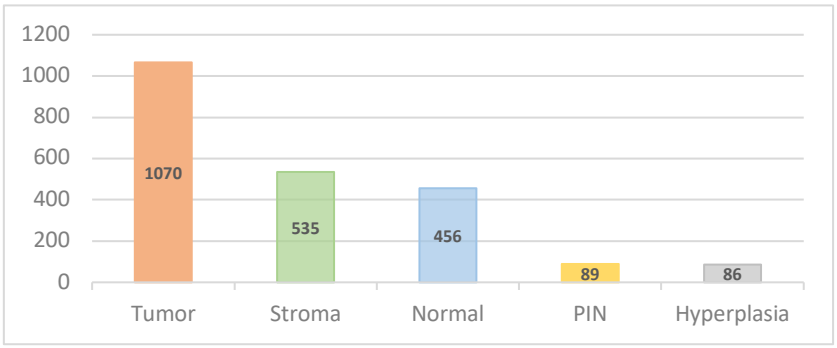

Figure 20 - Distribution of tissue microarray cores (n) from prostate cancer tissue areas

3.3 Immunohistochemistry

The principle of immunohistochemistry (IHC) can be traced back to 1934, as professor Marrack used staining reagents in order to detect microorganisms[282]. Since then, this technique has expanded and has been optimized. Today, IHC is an important part of diagnostic pathology to acquire an accurate diagnosis[98,99,199], and is extensively applied in research for prognostic and predictive molecular markers[276]. IHC is an umbrella term referring to numerous methods for recognition of specific cellular or extracellular components (antigens) within tissue sections. The antigen detection is accomplished by binding of antibodies (immunoglobulins) specific to the antigen of interest. Using antibodies conjugated to enzyme labels, with the ability to utilize coloring substrates (chromogens), the antigen-antibody complex can be visualized (Figure 21)[283]. The color of the reaction depends on the chosen chromogen, usually DAB, which gives off a brown color[284].

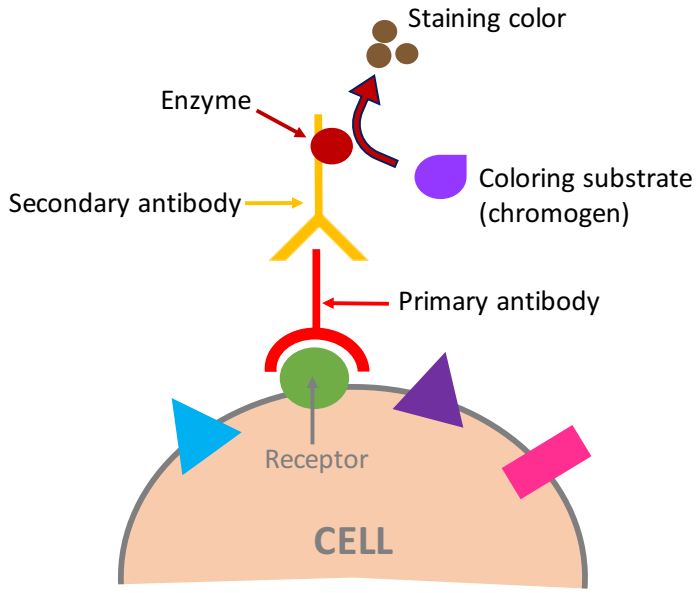

Figure 21 - Illustration of indirect immunohistochemistry method in detection of specific receptor using primary and secondary antibodies. Figure by Thea Grindstad

Different techniques and reagents can be utilized in the IHC processes. However, the objective, to detect and quantify a specific tissue component is the universal. In brief, the IHC process includes three phases: **I)** Pre-analytic phase **II)** Analytic phase **III)** Post – analytic phase[285] (Figure 22). The antigen retrieval step in the analytic phase is an important step, given that it increases accessibility to tissue antigens in formalin fixed material[286].

IHC can also be divided into an indirect and direct method. In the direct IHC, a primary antibody is directly conjugated to an enzyme label. Indirect IHC refers to the utilization of detection systems. In this method, a labeled secondary antibody with the ability to bind the unlabeled primary antibody, achieves visualization of the antigen[283] (Figure 21). Several approaches for indirect IHC exists, some frequently applied detection methods are the unlabeled antibody enzyme complex techniques: peroxidase-antiperoxidase (PAP)[287] and alkaline phosphatase-antialkaline phosphatase (APAAP)[288], the avidin – biotin complex (ABC) technique[289], the labeled streptavidin-biotin complex (LSAB)[290] and the more recent polymer-based detection system and tyramine amplification system[276]

In general, indirect IHC is considered more complex and time consuming compared to direct IHC. It is, however, also considered more sensitive[283]. The decision of which method to apply

requires expertise and experience, and is an individual consideration based on chosen antibodies and target tissue.

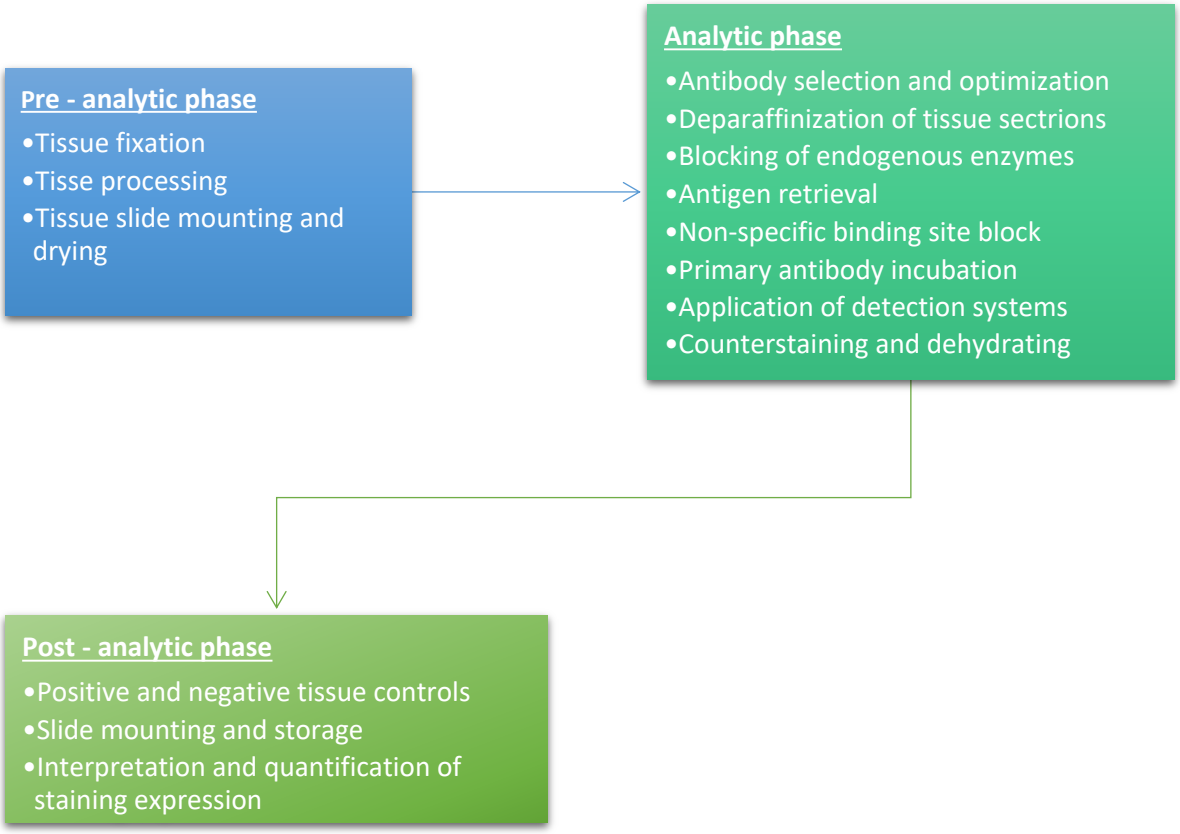

Figure 22 – Steps of the immunohistochemistry process. Figure by Thea Grindstad

3.3.1 Antibodies

Antibodies are glycoproteins secreted by specialized B lymphocytes (plasma cells). They represent one of the principal effectors of the adaptive immune system and inhabit the ability to effectively and accurately bind specific antigens. This ability has led to their ubiquitous use within different scientific procedures. The majority of antigens are very complex and present many epitopes that can be recognized by a large number of lymphocytes. A polyclonal antibody response occurs as the B lymphocytes differentiate into antibody-producing plasma cells directed against the different epitopes of the specific antigen. The counterpart is monoclonal antibodies which are identical antibodies. They are produced by one specialized B lymphocyte and directed against one single epitope on the antigen[291]. In research settings,

both monoclonal and polyclonal antibodies are applied and both types were utilized in this thesis. In brief, the monoclonal antibodies are constructed in immunized animals by injecting a purified antigen. An immortal hybrid cell is then created by fusing isolated B cells from the animal (mostly mice or rabbit) with myeloma cells. This hybrid produces antibodies specific for a single antigen epitope[292]. Polyclonal antibodies are obtained from immunization of a broad range of animals (e.g. rabbit, goat, monkey, mouse etc.) with specific molecules containing the antigen of interest. The immune response against the antigen will result in the production of numerous of plasma cell clones producing different antibodies[293].

In IHC- based research, choosing a satisfactory antibody for the procedure is a vital step. When selecting the antibodies for this thesis, extensive literature review and careful considerations were made by experienced technicians (Table 5). The online datasheets from provided by the manufacturer were consulted and evidence of the chosen antibodies being successfully applied by others on previous occasions was obtained. As is recommended, positive and negative tissue controls were utilized when assessing all applied antibodies[276,285]. Tissue controls were fixed and stained in the same manner as the investigated tissue. Tissue known to express the target antigen is used as positive tissue control[285]. If the antibody was not validated by the manufacturer, or if it was explicitly requested by reviewers, in-house validation of primary antibodies was conducted using Western blot, cell lines and transfected cell lysates to verify antibody specificity. This was the case for pan-PGR, ERα, ERβ and aromatase, detailed validation protocol description is accompanied in supplementary data for **Paper I and II**.

3.3.2 In-house immunohistochemistry procedure

In this thesis **(Paper I, II and III)**, all IHC-staining was performed with both manual (ERα, ERβ, Aromatase) and automated (pan-PGR, PGRA, PGRB) protocols. The antibodies and details regarding IHC procedures are presented in Table 5. In the first steps, the TMA slides were deparaffinized with either xylene (manual protocol) or EZ Prep buffer (automated protocol). Antigen retrieval was performed by using manufacturers retrieval solutions and microwave heating. Subsequently, sections were incubated to block endogenous peroxidase activity. The sections were then incubated with primary antibodies, and after washing, incubated with the corresponding secondary antibodies. The immune complexes were visualized with the detection kits applied by the manufacturer. As negative staining controls,

primary antibodies were replaced with the antibody diluent. To visualize the nucleus and tissue architecture, the slides were counterstained with hematoxylin and bluing reagent. Finally, the sections were dehydrated through an ethanol series, cleared in xylene and slide-mounted.

Antibody	Vendor	Catalogue number	Clone number	Host species and clonality	Antigen retrieval	Primary antibody titer	Primary antibody time/ temperature	Secondary antibody	Detection system	Tissue controls	Validation
Pan-PGR	Ventana	790–4296	1E2	Rabbit Monoclonal	CC1 (60 min)	Pre-diluted	36°C, 24 min	Integrated with detection kit	UltraView Universal DAB	Breast	HEK 293 transfectant
PGRA	Novocastra	NCL-L-PGR-312	16	Mouse Monoclonal	CC1 (64 min)	1\25	36°C, 60 min	Integrated with detection kit	Optiview DAB	Endometrium	-
PGRB	Thermo-Fisher	MA5-12642	hPRa2	Mouse Monoclonal	CC1 (48 min)	1/50	36°C, 1 Hour	Integrated with detection kit	Optiview DAB	Endometrium	-
ERα	Santa Cruz	SC-543		Rabbit polyclonal	Citrate buffer (20 min)	1/100	4°C, over night	Goat anti-rabbit IgG	Vectastain ABC-HRP Kit	Ovary	A549, NCI-H460, DU145, PC3, MCF7, MDA-MB-231 cell lines;
ERβ	AbD Serotec	MCA1974s	PPG5/10	Mouse monoclonal	Citrate buffer (20 min)	1/10	4°C, over night	Horse anti-mouse IgG	Vectastain ABC-HRP Kit	Ovary	A549, NCI-H460, DU145, PC3, MCF7, MDA-MB-231 cell
Aromatase (CYP-19)	Santa Cruz	SC-14245		Goat polyclonal	Citrate buffer (20 min)	1/100	4°C, over night	Rabbit anti-goat IgG	Vectastain ABC-HRP Kit	Placenta	A549, NCI-H460, DU145, PC3, MCF7, MDA-MB-231 cell

Table 5 - Overview of applied antibodies and immunohistochemical procedures

Abbreviations: PGR = Progesterone receptor; PGRA = Progesterone receptor isoform A; PGRB = Progesterone receptor isoform B; ER = Estrogen receptor; CC1= cell conditioning 1, DAB= 3,3′-Diaminobenzidine; HRP= horseradish peroxidase

3.3.3 Microscopic evaluation of immunohistochemistry staining and scoring

An overview of published markers and their scoring systems are presented in Table 6. The IHC marker expression of the applied antibodies **(Paper I, II and III)** were all semi-quantitatively evaluated. This was conducted by manually quantifying an estimation of the biomarker distribution and color variation. Two independent scoring systems were developed based on the markers expression profile, as is frequently done in IHC based research[294]. One system was based on the percentage of positive cells, density, and subdivision into percentage ranges. This included assessing the percentage of stained cells in relative to the total number of target cells. The other system was the division into groups based on the scorers' subjective opinion of variations in staining intensity. When possible, we also created a combined intensity and density score by calculating the mean value of the density and intensity scores. For all markers but aromatase **(Paper II)** and PGRA and PGRB **(Paper III)**, both intensity and density were considered. Due to lack of variation in density, aromatase **(Paper II)** was the only IHC marker solely given an intensity score. In **Paper I**, only density of pan-PGR yielded significant results. Due to this, only density of PGRA and PGRB was scored in **Paper III**. However, for the majority of markers, it was the variation in density levels that was significantly associated with disease progression **(Paper I, II and III)**.

The presence of brown staining color in one or several cellular components, including cellular membrane, cytoplasm or nucleus of target cells was considered as a positive staining. The percentage-groups (density) reflecting positive cells was converted into a score ranging from the lowest value 0 to the highest value 3, according to a predefined model by the investigators. The intensity was also given a score of 0 – 3, where 0 was absent and 3 very strong expression of the same antigen. A core was scored as "missing" either if it was missing or considered of insufficient quality to score. The IHC marker expression in stromal and epithelial cells was investigated individually and separate scores were given. This rendered a series of categorical data for further analyzes.

The IHC marker expression was always scored by two experienced investigators independent of each other and blinded to any pathological- or clinical information, **Paper I**: ER and SAS, **Paper II**: ER, SFI and ER, TG, **Paper III**: MRK and ER. The scoring was conducted manually using paired light microscopes (**Paper III)**, or through the ARIOL imaging system (Applied Imaging Corp., San Jose, CA, USA) **(Paper I and II)**. When using paired light

microscopes **(Paper III)**, a third party (TG) recorded the scoring values that was wordlessly signaled by the independent investigators. In case of discrepancy (score difference > 1), the slides were re-examined, and a consensus reached. Finally, the scoring data was transferred to a SPSS database. The mean score value, based on the scores from the two investigators, was calculated and then connected to the patient's clinical and histopathological information. The AIROL scans and digitalizes IHC stained TMA slides by loading the slides in the SL 50 automated slide loader and then scanning the slides at low resolution (1.25x) and high resolution (20x), using an Olympus BX61 microscope with an automated platform (Prior Scientific, Cambridge, UK). This enables scoring of tissue samples on a computer screen and relives the investigators from scoring simultaneously. Digital images of IHC stained slides were also obtained and saved.

3.4 Cut-off levels

To apply our ordinal scoring data in a productive manner in statistical survival analysis, it was necessary to stratify patients into distinct groups based on scoring values. This approach makes it easier to compare a variable with an outcome, and is conducted by dividing the variable at a cut-off point[295]. Thus, a cut off value was chosen and the scoring values were dichotomized into low and high expression. To secure reproducibility and sufficient patient number in each group only standardized cut-off values[p] were considered and applied in all articles (Table 6). Finally, based on a minimum p-value approach[295] in the univariate survival analyzes, the cut off value best differentiating the groups according to event-free survival was chosen.

[p] **Standardized cut off values**: zero, quartiles, mean, median

Paper	Published marker	Tissue compartment	Scoring system	Mean scoring value	Median scoring value	Chosen Cut-off value
I	**PGR (both isoforms)**	TE	Density 0 = 0 % 1 = >0 - ≤ 5 % 2 = > 5 - 50 % 3 = > 50 %.	0,52	0,38	0,75 (4th q.)
		TS		1,43	1,50	1,75 (4th q.)
II	**ERα**	TS	Density 0 = 0 % 1 = > 0 - ≤ 5 % 2 = > 5 - 50 % 3 = > 50 %	1,19	1,25	0,75 (1st q.)
	ERβ	TS		1,27	1,50	1,50 (median)
	Aromatase (CYP-19)	TE	Intensity 0 = negative 1 = weak 2 = moderate 3 = strong	1,10	1,00	1,00 (median)
		TS		1,10	1,00	0,63 (1st q.)
III	**PGRA**	TS	Density 0 = 0 % 1 = > 0 - ≤ 25 % 2 = >25 - 50 % 3 = > 50 %	1,34	1,35	1,34 (mean)
	PGRB	TE		1,34	1,25	1,34 (mean)
		TS		0,89	0,50	0,89 (mean)

Table 6 - Overview - published markers

Density: The percentage of positive cells compared to the total number of nucleated cells in the compartment
Intensity: The relative color intensity of stained nucleated cells in the compartment
Abbreviations: PGR = Progesterone receptor; PGRA = Progesterone receptor isoform A; PGRB = Progesterone receptor isoform B; ER = Estrogen receptor; TE = Tumor epithelial cells; TS = Tumor associated stromal cells; q. = Quartile

3.5 Statistics

All statistical analyzes were performed using SPSS version 21.0.0.0 – 24.0.0.0 (SPSS Inc., Chicago, IL, USA). The IHC scoring values from each pathologist were compared for inter-observer reliability by two-way random effect model with absolute agreement definition. Correlation analyzes were conducted using Spearman´s rank correlation coefficient to assess the correlation between IHC marker expression, the clinicopathological variables, and other previously published markers. A correlation coefficient (r) of 0.3 – 0.49 was considered a moderate to weak correlation, r of 0.5 – 0.69 moderate to strong and finally $r \geq 0,7$ as strong. In our material, only $r > 0.3$ was taken into consideration. The Wilcoxon signed ranks test was used to compare marker expression within the different PC tissue areas. Univariate survival analysis was conducted using the Kaplan-Meier method to draw survival curves for each variable group. The log-rank test was used to assess the statistical significance between the survival curves of the model. The following end-points were considered in all survival analyzes: BF, CF, PCD. All significant variables from the univariate analysis were entered in the multivariate analysis using a backward stepwise Cox regression model with a probability for stepwise entry removal at 0.05 and 0.1, respectively. Proportional hazards assumption (log-minus-log plot) was tested for each variable to ascertain its capability to be included in the multivariate model. We considered a p-value < 0.05 as statistically significant for all analyzes. Presentations of the survival curves were terminated at 192 months in paper III due to < 10 % of patients were at risk after this point, in paper I and II, < 10 % were at risk after 134 months.

4 MAIN RESULTS

Overview of main results from univariate and multivariate analyzes are presented in Table 8 and outlined in the next chapters. **Paper I – III**, chapters on results, are referred to for an in-depth description of the published results. Univariate results for the cohort's clinicopathological variables, after the last patient update, are presented in Table 4. Detailed description of the previous cohort can be found in Table I, **Paper I and II**. Table 7 provides an overview of the clinicopathological characteristics, and patient outcome data of the patient cohort examined in this thesis, including detailed description of the two most recent patient updates. Analyzes investigating correlations between the investigated biomarkers in this thesis, other emerging biomarkers investigated in this group and clinicopathological variables were conducted for all biomarkers. However, only weak ($r < 0.3$), or non-significant ($p > 0.05$), results were detected. Because of this, these results will not be discussed further herein.

4.1 Patient characteristics

	Paper I and II	Paper III
Hospital	St. Olav's, NLSH, UNN	
Number of patients	535	
Time of inclusion	01.01.1995 - 31.12.2005	
Median age at surgery	62 years (range 47 – 76)	
Median PSA	8.8 (range 0.7 – 104)	
Median tumor size (index tumor)	20 mm (2.0 – 50)	
Last follow-up	**Nov. 2012**	**Dec. 2015**
Median follow-up time of survivors	89 months (range 6 – 188)	150 months (range 18 – 245)
Postop. hormonal therapy, n (%)	83 (15.6 %)	89 (16.6 %)
Postop. radiation therapy, n (%)	90 (17.2 %)	103 (19.2 %)
Patients with BF (%)	170 (31.8 %)	200 (37.4 %)
Patients with CF (%)	36 (6.7 %)	56 (10.4 %)
Patients with PCD (%)	15 (2.8 %)	18 (3.4 %)
Median BFFS (months)	70.3	86.8
Median CFFS (months)	90.2	133.7
Median PCDFS (months)	93.5	146.3
Median survival free time: BF – CF (months)	61.2	29.0
Median survival free time: BF – PCD (months)	68.4	51.0
5-year BFFS	74 %	74 %
10-year BFFS	63 %	62 %
5-year CFFS	96 %	96 %
10-year CFFS	91 %	93 %
10-year PCDFS	97 %	98 %

Table 7 – Overview over clinicopathological characteristics and patient outcome date from the cohort examined in Paper I - III

4.2 Paper I

The PGR is an established prognostic marker in breast cancer, and it has been a topic of interest in PC research for decades. Throughout this time, a general agreement of its presence in stromal cells of the PCs has evolved. Epithelial tissue distribution and the receptors role in prostate carcinogenesis, however, remains undefined. In our first paper, we thus sought to investigate the pan-PGR distribution in our cohort of 535 primary PC specimens, and the association with the clinical endpoints, BF, CF, and PCD. In our analyzes, we separated between stromal and epithelial receptor expression and evaluated the marker expression in the different diagnostic areas the heterogeneous PC is composed of.

4.2.1 Receptor expression

PGR was expressed in both stromal and epithelial cells of normal and malignant tissue. A significantly higher density of PGR was detected in TS compared to TE ($p < 0.001$).

4.2.2 Univariate analyzes

In both TE and TS, PGR was associated with a worse prognosis. Patients with a high density of PGR expression experienced reduced CFFS compared to those with low PGR expression levels, TE ($p = 0.006$) and TS ($p = 0.045$) respectively. No significant association with BF nor PCD was detected, although high PGR levels in TE displayed a similar tendency for increased BF. This was, however, not statistically significant ($p = 0.144$). Finally, when merging the PGR density levels in TE and TS, patients with high (high/high) levels had significantly increased risk of CF ($p = 0.019$) compared to the other groups (low/low, high/low, low/ high).

4.2.3 Multivariate analyzes

All significant variables from the univariate analyzes were tested in the multivariate model. PGR in TE emerged as an independent predictor of CF (HR: 2.51, 95% CI: 1.23–5.17, $p = 0.012$) alongside Gleason grade ($p = 0.001$) and non-apical surgical margin ($p = 0.006$). Patients with a high density of PGR expression had 2.5 times greater risk of experiencing CF compared to those with low expression.

4.3 Paper II

ERα is another established prognostic marker and also a therapeutic target in breast cancer. After the discovery of ERβ, a new paradigm of a protective role of ERβ in cancer

development has emerged, but it remains to be confirmed. Both ER isoforms have been detected in PC. The prognostic value of the different receptors and their role in prostate carcinogenesis is still debated. Using the same cohort as in Paper I, we sought to determine the prognostic significance of both ERs, in addition to aromatase, the enzyme synthesizing these hormones from androgens. The receptors and enzyme were investigated in the same manner as our previous marker, separating between stromal and epithelial cells and different tissue compartments.

4.3.1 Receptor expression

Epithelial staining of ERα was predominantly negative in both malignant and normal epithelial cells. ERβ staining was overall positive in stromal and epithelial cells of both benign and malignant prostate tissue. There was, however, a variance in the percentage of positive cells (density) and the density of ERβ was significantly higher in TE compared to TS ($p < 0.001$). Aromatase staining was cytoplasmic, with an overall positive staining in both stromal and epithelial cells. There was, however, variance in the staining intensity. There was a stronger expression of aromatase detected in NS compared to TS ($p < 0.001$). Finally, a positive correlation was detected between ERα and ERβ in TS ($r = 0.5$, $p < 0.001$) and between the ERs and aromatase in their respective tissue compartments; ERα and aromatase in TS ($r = 0.36$ $p < 0.001$), ERβ and aromatase in TS ($r = 0.53$, $p < 0.001$), and ERβ and aromatase in TE ($r = 0.43$, $p < 0.001$).

4.3.2 Univariate analyzes

We found both ERs and aromatase to be significantly associated with PC outcome. In TS, ERα was beneficial, and a high density of ERα expression was associated with increased time to CFFS ($p = 0.042$) and PCDFS ($p = 0.019$). This trend was, however, not displayed with regards to BF. For the small selection of patients with a positive epithelial ERα expression, no significant difference in BFFS, CFFS or PCDFS was found. A high density of ERβ expression in TS, on the other hand, was associated with reduced BFFS ($p = 0.002$), but not with other endpoints. Finally, a high intensity level of aromatase in TS was favorable with respect to BF ($p = 0.016$), but displayed no association with CF nor PCD. High intensity level of aromatase in TE was also beneficial. This was associated with an increased CFFS ($p = 0.036$) and displayed a similar tendency with PCD without reaching statistical significance ($p = 0.061$).

4.3.3 Multivariate analyzes

In the multivariate model, both ERβ (HR: 1.70, 95% CI: 1.19–2.42, p = 0.004) and aromatase (HR: 0.55, 95% CI: 0.38–0.80, p = 0.002) in TS were independent prognostic factors for BF. Patients with a high ERβ level displayed a 1.7 times increased risk of BF compared to those with low levels. Patients with high levels of aromatase had a 45 % lower risk of developing BF compared to those with low levels. ERα in TS was an independent positive prognosticator for CF (HR: 0.43, 95% CI: 0.22–0.87, p = 0.018) and PCD (HR: 0.28, 95% CI: 0.10–0.78, p = 0.015). Patients with high ERα in TS had a 57 % and 72 % risk reduction for CF and PCD, respectively, compared to those with low levels. Aromatase in TE also emerged as an independent positive prognostic marker for CF (HR: 0.43, 95% CI: 0.21–0.90, p = 0.024), with a 57 % reduction in risk of progression to CF for patients with high levels compared to those with low levels. Other independent prognosticators, alongside the biomarkers in the multivariate models were PSM (non-apical: p = 0.002, apical: p = 0.038), Gleason score ≥ 9 (p = 0.007) and pT-stage (p < 0.001) for BF, non-apical PSM (p = 0.002), PNI (p = 0.043) and Gleason score ≥ 9 (p = 0.001) for CF, regarding PCD is was PNI (p = 0.034) and Gleason score ≥ 9 (p = 0.015).

4.4 Paper III

Considering our findings of a negative effect of a high PGR level on PC outcome in the first paper, we sought to further elucidate the significance of PGR in PC. This time we systematically assessed the two receptor isoforms, PGRA and PGRB, their stromal and epithelial distribution and association with clinical outcome. In addition, the clinicopathological data in our cohort had recently been updated, rendering longer follow-up time and more events. The receptors were otherwise investigated in the same manner as our previous markers.

4.4.1 Receptor expression

PGRA expression was detected exclusively in stromal cells in both normal and malignant tissue. Expression of PGRB was both stromal and epithelial, and PGRB was located in all tissue compartments. The expression of PGRA in stromal cells was significantly higher compared to PGRB in both NS (p < 0.001) and TS (p < 0.001). Regarding PGRB, the epithelial expression was overall higher than the expression in the surrounding stroma (p <

0.001). Finally, a strong and significant correlation between PGRB expression in TE and TS was detected ($r = 0.82$, $p < 0.001$).

4.4.2 Univariate analyzes

Patients with a high density of PGRB had a significant decrease in both BFFS and CFFS. This applied to both TE expression (BFFS: $p < 0.001$, CFFS: $p = 0.006$) and TS expression (BFFS: $p = 0.034$, CFFS: $p = 0.034$). No additional prognostic value was evident when merging PGRB expression in TE and TS. No association with clinical endpoints was discovered for PGRA expression.

4.4.3 Multivariate analyzes

A high density of PGRB expression in TE remained an independent prognostic marker for both BF (HR: 2.0, 95% CI: 1.45 – 2.76, $p < 0.001$) and CF (HR: 2.5, 95% CI: 1.29 – 4.85, $p = 0.006$). Non-apical PSM ($p = 0.016$), Gleason grade group 3 and 4 ($p = 0.032$ and $p = 0.008$), PNI ($p = 0.002$), preoperative PSA ($p = 0.021$) and pT-stage 3b ($p = 0.001$) were additional independent prognosticators for BF. Regarding CF, age ≥ 60 ($p = 0.026$), LVI ($p = 0.028$) and Gleason grade group 1 through 5 ($p = 0.013$) were additional independent prognosticators. Patients with a high PGRB level in TE had twice the risk of experiencing BF and 2.5 times the risk of CF compared to patients with low levels. PGRB in TS did not reach statistical significance in multivariate analyzes.

4.5 Overview of the main results

Paper	Bio-marker	End-point	Results					
			Univariate			Multivariate		
			TE	TS	TE + TS	TE	TS	TE + TS
Paper I	**PGR**	BF	ns	ns	ns			
		CF	**p = 0.006**	**p = 0.045**	**p = 0.019**	**HR 2.51 (1.23–5.17) p = 0.012**	ns	ns
		PCD	ns	ns	ns			
Paper II	**ERα**	BF		ns				
		CF		**p = 0.042**			**HR 0.43 (0.22–0.87) p = 0.018**	
		PCD		**p = 0.019**			**HR 0.28 (0.10–0.78) p = 0.015**	
	ERβ	BF	ns	**p = 0.002**	ns		**HR 1.70 (1.19–2.42) p = 0.004**	
		CF	ns	ns	ns			
		PCD	ns	ns	ns			
	Aromatase	BF	ns	**p = 0.016**	ns		**HR 0.55 (0.38–0.80) p = 0.002**	
		CF	**p = 0.036**	ns	ns	**HR 0.43 (0.21–0.90) p = 0.024**		
		PCD	ns	ns	ns			
Paper III	**PGRA**	BF		ns				
		CF		ns				
		PCD		ns				
	PGRB	BF	**p < 0.001**	**p = 0.034**	ns	**HR 2.00 (1.45 - 2.76) p < 0.001**	ns	
		CF	**p = 0.006**	**p = 0.034**	ns	**HR 2.50 (1.29 - 4.85) p = 0.006**	ns	
		PCD	ns	ns	ns			

Table 8 - Overview of main results from uni- and multivariate analysis in paper I – III. (Univariate analyzes: log rank test, multivariate analyzes: Cox regression analyzes, backwards stepwise model) **Abbreviations:** TE = Tumor epithelial cells; TS = Tumor associated stromal cells; PGR = Progesterone receptor; ER = Estrogen receptor; BF = Biochemical failure; CF = Clinical failure; PCD = PC death; HR = Hazard ratio; ns = Not significant; gray shaded square = not entered in analysis

5 DISCUSSION

5.1 Patient cohort

A strength of this thesis is the unselected study population from Central and Northern Norway. Although this is a relatively small populace, several aspects make this a heterogeneously composed cohort. The median life expectancy for men in Norway was 80.6 years in 2015. In the study population **(Paper I, II and III)** it ranges from one of the lowest estimates of 79.0 years in Nordland to one of the highest with 80.6 years in More and Romsdal. Life quality surveys have also revealed a broad specter the regarding percentage of population classifying as obese (BMI ≥ 27), ranging from 22 % to 35 % with some of the counties well above the national average. Further, the part of the population who never exercise varies from 12 % – 17 % depending on the county, and from 10 % to 16 % of the men are daily or "some-times" smokers (www.SSB.no, numbers from 2015 – 2016). These factors are all variables associated with PC, however the influence on cancer progression cannot fully be determined[28].

A reasonable estimate, according to the head of the department of Urology, UNN Tromso, T. Knudsen, is that ≥ 95 % of men diagnosed with PC in these regions during the inclusion period were operated at the hospitals participating in this study. This thesis is based on a large PC cohort of 535 patients. We did, however, encounter a potential selection bias when collecting PC tissue for the cohort from St. Olav´s hospital. A great number of PC specimens from this hospital were appropriated by another research group (n = 100) and not available at the time of tissue collection. Thus, a part of the St. Olav´s cohort could not be included in our material, reducing the total cohort size and representability.

A retrospective study is less expensive and more time efficient compared to e.g. prospective studies. The retrospective design has its natural limits in the access to additional information regarding e.g. life style and comorbidities. It also removes the option of collecting additional material that could be of interest, e.g. blood samples. Additionally, it always renders the possibility of information bias as we have limited ways of verify the information retrospectively collected from patient journals. The retrospective design also excludes the possibility to standardize follow-up procedures. For example; if one center uniformly measured PSA levels every 3 months they could have more BF events and shorter BFFS. Another bias occurs in studies that includes a mixture of PC material collected before and

after the introduction of the PSA-test. Introduction of the PSA-test has resulted in increased detection of PC with indolent disease[10]. Our material is collected between 1995 – 2005, and thus placed in the PSA-area. This renders the assumption of a more homogenous and comparable material.

When tumor material has been collected over a long period of time, a challenge resides in alterations to the diagnostic guidelines and procedures. This could affect the tumor types included in the material and results in variations in cancer staging. This potential bias is evaded in our material by the re-classification of tumors according to updated diagnostic systems. This material was further collected prior to the introduction of image guided biopsies[296]. It is thus reasonable to assume that the diagnostic procedures for detecting PCs were uniform with the standard DRE, PSA testing, and TRUS with needle biopsies[69].

This thesis benefits from a material with a long follow-up time. Due to the nature of PC, the number of PC specific deaths remians low despite decades of follow up. By evaluating other endpoints associated with disease progression (BF, CF)[89,109], in addition to PCD, more robust statistical results could be produced due to a greater number of events. It must, however, be noted that, although BF is a early sign of disease activity, the time to progression to CF varies to a great extent. Further, not every patient with BF will experience CF and PCD within their lifetime[89,109,111]. The international definition of BF is currently two consecutive postoperative rises in PSA level > 0.2 ng/mL[106]. This is, however, a topic of controversy. There are several arguing that that a higher PSA level cut-off of ≥ 0.4 ng/mL is stronger associated with continued systemic progression, and consequently makes a more clinically relevant cut-off[104,105]. Based on this assumption, we chose to establish cut-off for BF at 0.4 ng/mL to ascertain that the patients identified where those at high risk of clinical progression. Whether to evaluate disease specific survival or overall survival is another consideration. PC affects older men and, for majority of cases, the cancer progression is slow[4]. Comorbidity and death from other causes than PC is prevalent; disease specific survival is therefore the chosen parameter herein. Disease specific survival is further dependent on accurate entries regarding the cause of death, this can be biased by subjective interpretation.

A confounder when performing survival analyzes on material with a long follow-up time is changes in post-operative treatment regimes. Benefitting our material, the standardized

treatment strategies and the equality in health care distribution in Norway enables a relatively homogenous study population. However, with the introduction of new treatment strategies, such as new generation hormonal therapies[243,194] and improved bone targeted terapy[244], great advances have been made in treatment of castrate resistant- and metastatic PC. This has led to an increase in survival rates over the past years[2] and can consequently affect the results regarding impact of different molecular markers on disease specific survival. On the other hand, these new treatment strategies have little impact on other clinical endpoints such as BF and CF.

In this material, the exclusion criteria I and II were included to minimize bias introduced by mechanisms that may alter the TME in a manner not related to PC biology. Radiation therapy might induce necrosis or alter the protein structure in the tumor tissue. Previous malignancies can change the host's biological response to the current malignancy, its treatment could have affected the PC tissue and metastatic disease could be misinterpreted to represent the wrong primary cancer. Criteria III represents the greatest number of excluded patient and is important to minimize bias related to tissue processing and analyzes.

5.2 Methodological considerations

5.2.1 Tissue fixation and processing

A great benefit of FFPE tissue is that it eliminates the need for fresh or fresh – frozen tissue, it conserves tissue morphology, and it can be stored for many years and still exhibit stabile immunostaining for most antigens[297,298]. Our material was collected over a ten – year period and thus using FFPE was the most applicable preservation method. Cutting sections from TMA can be technically more challenging than cutting whole tissue sections, making TMA more prone to certain artifacts and tissue loss. FFPE preserved over many years can lose its elasticity, which would lead to challenges in "punching out" i.e. obtaining cylindrical cores for constructing TMAs[299]. This resulted in a number of "missing" cores in our cohort, which was somehow higher than expected.

In theory, every step in the pre-analytic phase (Figure 22) has several variables that challenge standardization, which has been thoroughly reviewed by Engel & Moore[278]. A major challenge with formalin fixation has been alterations of the tissue proteins three-dimensional structure (e.g. cross-linking of proteins and DNA), thereby masking or damaging epitopes[277].

Today, this has been improved by antigen retrieval methods. This technique increases the accessibility to tissue antigens, and was considered a breakthrough for IHC based research when developed in 1991[286]. As reviewed by Engel & Moore, formalin fixation is both a time-dependent and time-consuming method. If the tissue has been placed in formalin for a long time (> 24 – 48 hours), it can result in "over-fixation" which can weaken or destroy the tissue antigenicity and result in a false negative staining[278]. The development of antigen retrieval methods has improved the epitope detection following prolonged tissue fixation[286], but standardizations in tissue fixation times are lacking. Formalin fixation has an average tissue penetration time of 1 mm per hour[277]. Thus, the optimal fixation time can vary depending on multiple factors, including the size and the consistency of the specimen. Consequently, longer fixation is needed for larger tissue samples to prevent autolysis and deterioration of tissue antigenicity[300,301]. There are also several aspects of tissue processing techniques that can compromise antigenicity. For instance, non-specific staining can occur from inadequate tissue dehydration protocols and by the use of dehydration reagents of insufficient quality prior to paraffin embedding. Additionally, differences in temperatures during paraffin embedding can also affect IHC quality[278]. Given that our cohort was collected retrospectively from several pathology institutions over a time period of 10 years, variations in tissue fixation was expected and must be taken into account. Additional quality assessments were made by stratifying the significant results based on the different donor institutions and 5-year time intervals. In our studies, the same trends in results were observed throughout time and pathological centers **(Paper I, II and III)**, although not always with significant results in each subgroup. This could probably be due to the reduced number of patients and the reduced number of events in each subgroup.

5.2.2 Tissue microarray procedure

A major benefit with TMAs is the tissue utilization, in addition to the time and cost-effectiveness. A single TMA experiment can provide data on the molecular characteristics of as many as 1000 specimens at once[279]. This also minimizes experimental variability since the same experimental conditions are applied to all tissue specimens on one master-slide simultaneously, increasing the reproducibility of the staining reactions. In contrast, the conventional whole section analyzes will contain only one tissue sample. As a result, up to a thousand separate analyzes, with a new experimental procedure for each slide, is required to obtain the same results as for a single TMA slide. Given the frequent limitation in tissue

availability for research purposes, TMAs can multiply the number of studies one tissue sample can supply. TMAs also provides the possibility of assembling a TMA panel with acquired healthy tissue samples, which is a great benefit. By doing this, controls tissues will be readily available when needed for different laboratory procedures, e.g. antibody validation.

Having said this, we are aware of that malignant tumors can be heterogeneous in general, and that prostate cancer is in particular known to show a varying grade of heterogeneity[48–52]. Typically, the tumor cores in PC are sampled from the most dominant tumor (index tumor). However, observations have been made of metastatic tissue not sharing genetic changes with the index tumor and of non-dominant tumor foci giving rise to metastasis[54,55]. This can present an even bigger challenge when using TMA as a method, and there has been concerns regarding the representatively of this method compared to the conventional whole tissue sections[302,303]. However, several studies have demonstrated a good reproducibility of biomarker expression in TMAs compared with whole section tissue analyzes[304,305] and clinical endpoints[306], including PC[307]. An overview of advantages and weaknesses with TMA is presented in Figure 23.

Several of the initial TMA studies were conducted on FFPE PCs. As a result, throughout the years, knowledge on how to apply TMA based PC research has been optimized. It continues to day to serve as an acknowledged method for gene and protein detection in PC[308]. In several tissues, two or more cores have been found to supply satisfactory information regarding antigen prevalence adequately to a whole tissue sections[304,309]. However, three to four cores are needed to optimally investigate biomarkers expression in PCs due to the aforementioned tumor heterogeneity[310]. Importantly, using larger cohorts, like ours, one would additionally benefit from dilution of potential sampling errors[302]. In our cohort, an average of four cores per patient were collected, with an average of two of four being cores from index tumor. Additionally, the cores were selected by an experienced pathologist, which is essential to ascertain tissue representativeness. Our TMA sections were cut 4µm thick, as is recommended, given that thicker sections can result in increased background staining[276]. Inevitably, tissue morphology will change through the length of the tissue core as more TMA slides are cut. To prevent incorrect classification of tumor areas, repeated H&E re-staining and re-evaluations of the sections (every 50th section or so) is recommended. The TMA slides applied in this thesis were cut early in the process and were thus affected to a minor degree by

this bias. Finally, when FFPE material is cut and affixed to glass slides the antigen epitopes are exposed and vulnerable, thus if the IHC process is delayed the quality could deteriorate[299]. Being conscious of this, either IHC was preformed within a day, or the slides were prepared for storage by sealing them with paraffin and then kept in refrigerator storage at approximately + 4°C for no more than one year.

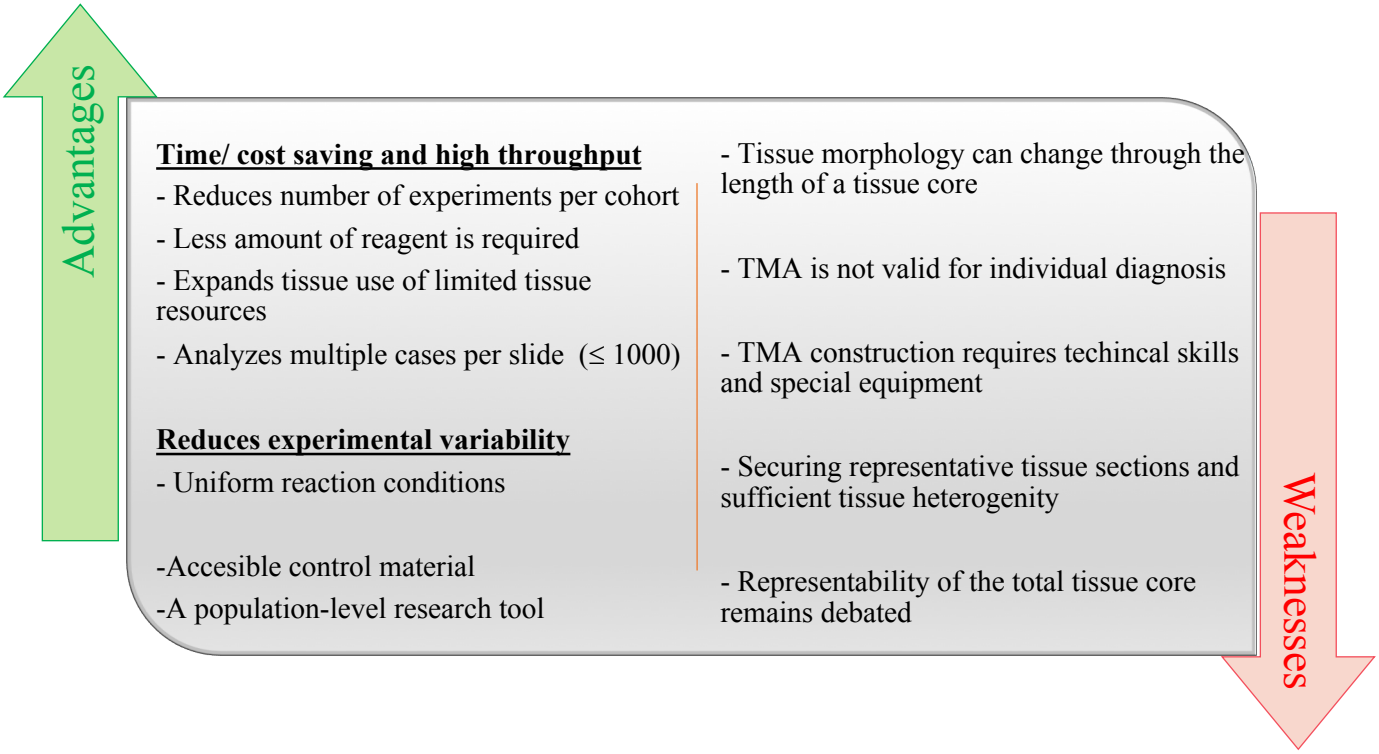

Figure 23 – Advantages and weaknesses of tissue microarray. Figure by Thea Grindstad

5.2.3 Antibodies

Regarding antibodies, the objective is to achieve a strong, specific antigen-antibody signal with minimal background staining. Although the use of IHCs is extensive, a generalization of the selection and validation process is lacking[311]. Today, antibodies are commercially available from numerous manufacturers, automatically introducing challenges as price and quality. Additionally, different antibodies are suited for different tissues and procedures, representing a selection bias. Insufficient antibody selection and validation has always been, and continues to be, a common concern[312,313]. Binding of an antibody to an epitope is a reversible process and depends on precise antibody – antigen interaction and their binding affinity[291]. Changes in antigen conformation can affect the strength of this interaction, e.g. the challenge with formalin tissue fixation[278]. Antibodies cross-reacting with similar epitopes on different antigens and background staining, due to hydrophobic and ionic interactions and

endogenous enzyme activity represents additional challenges[283,291]. We met these challenges by carefully selecting antibodies based on experience, availability and expertise, and by always applying recommended positive and negative controls and non-IHC validation methods when deemed necessary. The applied pan-PGR and PGRA antibodies have been extensively validated and applied in routine breast cancer diagnostics[199,314]. Pan-PGR, ERα, ERβ, and aromatase were subjected to further in-house validation. Additionally, all applied antibodies were validated by the manufacturer. Despite these precautions, the validity of our ERβ antibody (clone PPG5/10) has been questioned in recent publications[312,313]. This antibody is directed against the ERβ1 isoform, the wild type ERβ, and is one of the most widely applied ERβ antibodies today.

In this thesis, both monoclonal and polyclonal antibodies were applied: Monoclonal: ERβ **(Paper II),** PGRA and PGRB **(Paper III)**. Polyclonal: pan-PGR **(Paper I)**, ERα and aromatase (**Paper II).** Each have their own benefits and disadvantages (Figure 24). Polyclonal antibodies are technically easier, less expensive and less time consuming to produce compared to monoclonal antibodies[291]. They also have higher detection sensitivity and are stable in a broader range of conditions compared to monoclonal antibodies. They are, however, also at greater risk of creating false positive results due to cross-reactivity with other epitopes[291]. Monoclonal antibodies are highly specific by binding to one single epitope on an antigen, thus in less risk of cross-reactivity[292]. They are however less stable and threatened by conformational changes to a larger extent than polyclonal antibodies. This is given that the detection depends on only one epitope which increases the risk of false negative results. Monoclonal antibodies are also a constant renewable source when generated, whilst production of polyclonal antibodies is limited to the applied animal and its lifespan[291].

Monoclonal	Polyclonal
•Expensive	•Less expensive
•Time consuming	•Less time consuming
•Technically demanding	•Easier technically
•Homogeneous: recognizes only one epitope	•Heterogeneous: recognizes several epitopes
•Higher detection specificity – less false positives	•Higher detection sensitivity – less false negatives
•Reduced risk of background staining	•Increased risk of background staining - increased risk of cross reactivity and false positives
•Influenced by analytic and conformational changes - Increased risk of false negatives	•Limited resource
•Constant renewable source	

Figure 24 – The main differences of monoclonal and polyclonal antibodies summarized. Figure by Thea Grindstad

5.2.4 Immunohistochemistry procedure

IHC is a well-established method for *in situ* evaluation of the prevalence of various antigens, as well as their localization and distribution in different tissue compartments. It can be performed on both fresh tissue samples and on fixed tissue, including both small (e.g. TMA, biopsies) and whole mount sections. Herein **(Paper I, II and III)**, an indirect IHC technique was applied which is a more sensitive method of antigen detection compared to the direct IHC. This is due to the signal amplification provided by the secondary labeled antibody, which makes it well suited for detection of infrequent and heterogeneously expressed antibodies. It does, however, require several additional stages of incubation[283]. Direct IHC is more applicable for the detection of highly expressed antigens, and represents a less demanding methodology[283]. Indirect IHC as a procedure harbor several steps (Figure 21) which can bias valid antigenicity. These steps include blocking of endogenous enzymes, antigen retrieval methods, antibody dilutions, incubation, temperature, pH, buffers, choice of detection systems. To minimize the variability in antigen detection, all IHC protocols were

performed by experienced laboratory technicians, as is recommended. Further, a protocol was constructed to maintain the expected standard. The data sheets with manufacturers methodological recommendations where carefully consulted in advance of the protocol construction and rigorously established laboratory procedures were employed and followed. However, the variation in applied IHC processes challenges comparison of IHC based research and collaboration between different health centers. Another benefit of IHC staining is that tissue slides maintain staining quality at room-temperature for several years after the IHC procedures which allow for a re-evaluations of tissue sections if necessary[315].

5.2.5 Scoring of immunohistochemical marker expression and selection of cut-off levels

5.2.5.1 Scoring

In IHC based search for prognostic molecular markers, a challenge resides in the quantification of the markers prevalence. The reported expression level of the marker is the result of a subjectively measured visual scores. These scores are converted into quantitative data, rendering a semi-quantitative scoring system. There is a lack of standardization also at this post-analytic stage of IHC. This introduces inter- and intra-scorer reproducibility bias[294]. Several semi-quantitative scoring systems, combining intensity and density into one score, have been developed. This includes H-score[316], immunoreactive score (IRS)[317], Allred score[318] and the "quick-scoring system"[319], all striving to minimize individual variations in scoring results and to increase the reproducibility. However, these are all time-consuming systems which also suffer from variations in intra- and inter-observer variability[320]. No system has at this point emerged as superior.

SHRs can be localized in the cell membrane, cytoplasm and nucleus whereas the aromatase enzyme is localized in the cytoplasm[165]. Staining patterns of the investigated markers were evaluated in connection to their anticipated biological behavior. Accordingly, we consider membranous, cytoplasm and nuclear expression of pan-PGR, PGRA, PGRB, ERα and ERβ, as a positive expression. Aromatase positivity was, as expected, only appreciated in the cytoplasm. We were also conscious of the false positive staining that can occur in leucocytes, e.g. granulocytes, due to endogenous peroxidase activity, hence these cells were not considered positive when displaying a brown color.

All markers **(Paper I, II and III)** were scored in a semi-quantitative manner, as described in the methods section (chapter 3.3.3). We attempted to secure reproducibility and consistency by ensuring that the scoring of marker expressions was always performed by two observers. Both observers were blinded to each other's results and to clinicopathological data. The scoring systems were established prior to the scoring procedure. Additionally, the scoring agreement between investigators was evaluated using a correlation coefficient (reliability coefficient, r) decided by two-way random effect model with absolute agreement definition. For all investigated markers, there was a good scoring agreement between the investigators (r - range: 0.78 – 0.93, $p < 0.001$).

Our applied system of dividing scoring assessments into percentage ranges is a common approach[276]. A challenge, however, resides in different research groups having different percentage ranges for the same antigen expression, which makes comparison difficult. Our method is also less specific than an accurate count of positive cells would be. However, the aims of the three papers are explorative and hypothesis generating, where the main focus is unveiling trends in patient outcome based on marker expression level. We thus deemed our methods of scoring time-efficient and pragmatic for the investigated markers and our research aims.

5.2.5.2 Determining cut-off levels

Analyzing a continuous variable is very time demanding and not as compatible with survival analyzes, and transferable to clinical practice, as dichotomized variables. By making a variable categorical one enables the stratification of patients into risk groups, treatment strategies and clinical trials[295]. So far, in the search for clinical relevant biomarker, the investigators are free to choose cut-off levels due to lack of standardized systems[276]. This arbitrary selection of cut-off levels makes one great source of discrepancy in prognostic tumor biomarker research[321]. When deciding cut-off levels by "fishing" for optimal results, the risk of type 1 errors[q] increases, thereby increasing the risk of false positive findings[295,322]. To avoid this, a standardized approach was chosen for all the investigated markers, strictly adhering to pre-defined standard cut off values (zero, mean, median, quartiles) and then choosing the one best differentiating the patient groups. Conversely, this increases the risk of

[q] **Type 1 error:** The risk of incorrectly discarding the H_0 hypothesis

type 2 errors[r], and thereby the risks of not detecting the impact of the biological markers on patient outcome. This approach facilitated further comparison of the biomarkers within this thesis.

5.3 Discussion of main results

5.3.1 Paper I and III: The progesterone receptor and its isoforms in prostate cancer

In our first work, we observed a significant and independent negative prognostic effect of pan-PGR expression in TE. A high pan-PGR level in TE was associated with reduced CFFS. The prognostic effect was strong (HR 2.5) and, in our model, predicted outcome alongside Gleason grade and non-apical PSM. Continued investigations of the PGR isoforms unveiled a solely stromal expression of PGRA, whereas PGRB expression was observed in both stromal and epithelial tissues. PGRB in TE continued to be a strong, independent, negative prognosticator for CF, and also BF, with as much as 2.5 times increase in risk of CF. No such associations were observed for the PGRA. On the basis of these results, the assumption can be drawn that our previously observed impact of PGR expression in TE was indeed effectuated the PGRB isoform.

Throughout the decades, there has been a lack of agreement regarding the cellular distribution of the pan-PGR in PC. Whilst the majority of investigations detect a stromal presence of the pan-PGR[323–331], the epithelial distribution is disputed[323,326,329–331], this discrepancy also applies to cell line studies[329,332–334]. There are, however, in line with our results, several reports of PGR expression in TE[324,327,328,332]. Of note, the majority of these results are derived from investigations of a smaller selection tissue samples. A negative regulatory role of PGR in TE was suggested by Bonkhoff et al. after they detected an increase in the PGR expression level from low to high grade tumors. The most extensive increase, however, in PGR expression in their material was detected in castrate resistant and metastatic lesions[324]. This is however disputed by others, Hobisch et al. was not able to detect the pan-PGR by IHC in metastatic PC tissue[333]. Hiramatsu et al., on the other hand, detected PGR expression to various extents in both TE and TS, but observed that patients with advanced surgical stages had significantly lower PGR levels in both tumor and stromal cells[328].

[r] **Type 2 error:** The risk of incorrectly accepting the H_0 hypothesis

Fewer investigations have been made of the PGR isoforms distribution, and it is only recently that antibodies directed against the PGRA became commercially available. In more recent analyzes, Yu et al. observed PGRA and PGRB only in a subset of stromal cells, and detected no epithelial expression. In their cohort (n = 27), PGRB expression was downregulated in TS compared to adjacent NS, but not associated with either Gleason score or serum PSA concentrations. Based on results from cell line studies, they suggested a favorable role of both PGR isoforms in regulating the stromal environment as an underlying mechanism for their observations[323,334]. In a larger cohort (n = 194), using a pan-PGR antibody, they described a great distribution of stromal PGR, but no associations with stromal pan-PGR expression and Gleason score or clinical endpoints was detected[335]. Thus, an agreement regarding the distribution and role of PGR and its isoforms in PC has not been reached.

The PGR and its isoforms are to a greater extent investigated in female reproductive tissue and malignancies. As mentioned in the "steroid hormone dependent malignancies" section, PGR is an acknowledge prognostic marker in breast- and endometrial cancer, as a surrogate marker for ERα expression[199]. Further, altered functions of PGR isoforms are assumed to be involved in the pathogenesis of tumors that arise hormone dependent tissues. The results regarding the PGRs role in breast cancer pathogenesis is however ambiguous. A benefit of stimulating PGR in breast cancer has been presented[336], but these results are obstructed by the association between certain progestins in menopausal hormone therapy and an increased risk of breast cancer[337]. However, new evidence on this topic continues to emerge[338]. It is debated that these discrepancies depend on the choice of ligand (progestogen) and administered dose[160]. Through immunohistochemical studies of healthy human breast tissue in pre-menopausal women, Mote et al. observed PGRA and PGRB expression confined to epithelial cells and expressed in a 1:1 ratio[189]. Also in healthy endometrium, PGRA and PGRB were a expressed in a 1:1 ratio through most stages of the menstrual cycle[190]. Reports of stromal PGRA or PGRB expression in human breast tissue are lacking, but in endometrial stroma cells PGRA has been described as the dominant isoform[190].

A disruption of the expression ratio of 1:1 in epithelial cells has been demonstrated in hormone responsive cancers. In breast carcinogenesis, a predominance of one isoform has been reported as an early event[189,339]. Studies on breast tissue in BRCA1 or BRCA2 mutation carriers have revealed altered PGR isoform expression, with a PGRA predominance, in

mutations carriers compared to those without, but no difference in ERα expression[340]. Thus, BRCA mutations could potentially exploit a proliferative effect of PGRs in cancer further increasing the cancer risk, a theory that could be transferred to PC pathogenesis. In the endometrium, however, progesterone inhibits estrogen-driven growth. In endometrial cancer, the loss of equilibrium in PGRA/PGRB ratio in epithelial cells, and the subsequent predominance of either of the isoforms has been observed as an early event in tumorigenesis. Further, expression of only one PGR isoform was associated with a higher clinical grade[341]. Although the PGRs have been detected in, and associated with, several hormone dependent malignancies, it is also important to consider that the receptors very likely function in a manner that is tissue specific. Thus, challenges lie in deciphering the context dependent and tissue specific actions of the PGR isoforms and their interplay. The aforementioned evidence indicates a disrupted PGRA:PGRB balance in hormone dependent cancers. Although the distribution of PGR isoforms in normal prostate has not been established, there is evidence of a PGR disequilibrium in our cohort given the differences in tissue distribution. It is possible that a disruption in receptor distribution can result in altered cellular responses to the hormone stimuli, eventually resulting in malignant transformation.

As a target for cancer therapy, there is limited research considering the PGRs in PC. Currently, one ongoing phase I/II clinical trial is investigating the effect of the type-I PGR modulator (PGR antagonist), onapristone, in patients with confirmed PGR expression. This is, however, a study including patients with advanced disease who were progressing on enzalutamide or abiraterone treatment[342].

5.3.2 Paper II - The estrogen receptor α, estrogen receptor β and aromatase in prostate cancer

Herein, we observed a mainly stromal ERα expression, and in TS a high expression of ERα was associated with increased CFFS and PCDFS. The reduction in risk compared to patients with high levels was very strong (HR 0.43 and 0.28, respectively) and predicted outcome alongside PNI and Gleason grade ≥ 9. ERβ, on the other hand, was expressed to a great extent in both epithelial and stroma cells, and a high level of ERβ in TS was associated with reduced BFFS, but not CFFS nor PCDFS. Finally, a high aromatase level in both TS and TE was favorable with respect to BFFS and CFFS, respectively. This gave a 45 % and 57 % relative

risk reduction, respectively, compared to patients with low levels. Aromatase in TE predicted outcome alongside non-apical PSM, Gleason score and PNI.

Estrogens involvement in prostate carcinogenesis has been acknowledged for decades[5]. Previously, estrogens were used as the main treatment of PC due to their ability to suppress serum testosterone levels via negative feedback on luteinizing hormone (LH) production[5,343]. However, due to association with serious cardiovascular side effects, other treatment modalities were developed[343]. Knowledge of direct estrogenic action on the prostate gland through the ERs, however, is more recent. Although development and growth of the normal and cancerous prostate is mainly accredited to AR mediated effects, there is compelling evidence of a proliferative role of estrogen in the prostate, as it is in breast and uterine cancer[344]. However, this is complicated by the different ERs, and their distinct effects in different hormone responsive tissues. As previously described, ERα has a well-established tumor promoting action in breast cancer. The role of ERβ however, remains controversial. A vast amount of evidence has emerged, claiming an anti-proliferative, pro-apoptotic and tumor-suppressive role of ERβ. However, it appears that the black and white image of an oncogenic ERα and a tumor suppressive of ERβ, at least in breast cancer, is too simple. Further, that a more complex, two-sided role of ERβ and should be considered[345]. This is transferable to PC, where highly diverging results regarding the role of ERs in carcinogenesis have been emerging for decades. Also in PC, the evidence points towards a less simplistic model than a tumor promoting ERα and a tumor suppressive ERβ[344].

Reports from normal prostate tissue describes ERα expression to be restricted mainly to stromal cells[346–349], with some reporting expression to a lesser extent in the basal cell layer[346]. This is in agreement with our observation of a predominant stromal ERα expression. We further detected ERβ in both epithelial and stromal cells, but to a greater extent in epithelial cells. This is in agreement with current literature[347,349–351], although some report ERβ to be predominantly localized in the basal cell epithelial cells[348].

Alas, reports of ERs expression and distribution in PC are highly diverging, including their association with cancer progression. Our observation of a positive prognostic effect of ERα in TS is supported by many, in both cell line[352,353] and IHC studies[350,354]. In agreement with our investigations, Slavin et al. investigated hormone naïve, primary PC specimens and detected

an increased risk of BF in patients lacking stromal ERα expression[352]. Through further *in vitro* and *in vivo* studies they detected a protective effect of ERα expressing CAF in the later stages of PC progression[352,353]. On the basis of their discovery they proposed utilizing ERα levels in CAF as prognostic markers in PC. Celhay et al. investigated ERα expression in patients initially treated with, and responding to, ADT and found a dominant stromal ERα expression[354]. No significant difference in ERα expression levels were observed between hormone sensitive and hormone refractory cancers, however a significant reduction in BFFS was detected in patients with low stromal ERα expression compared to those with high levels[354]. Improved clinical outcome for patients with stromal ERα expression and CRPC has also been detected[350].

We detected ERα epithelial positivity in only a small subgroup of patients (NE and TE negativity in 70 % and 64 %, respectively), which is in agreement with several others[328,349,350,355]. In our material, no significant difference in expression level was detected between normal and cancerous epithelial areas, nor was there an association with any clinical outcomes. However, there exists contradicting reports supporting a tumor promoting role of ERα, similar to that in breast cancer. Such reports describe an upregulation of the ERα in epithelial cells of the progressing PC. Bonkhoff et al. reported an upregulation of ERα in TE as the cancer progressed, describing ERα as a late event in prostate carcinogenesis. The highest levels were detected in CRPC and metastatic disease[346]. This observation has been supported by others, however in studies of smaller sample size (n = 36)[356]. No stromal ERα expression was detected by Bonkhoff et al[346], while Royuela et al. observed only stromal ERα in normal tissue, but intense epithelial immunostaining in TE[348]. A correlation between ERα in TS and Gleason grade has also been described, despite downregulation of ERα compared to corresponding normal stromal areas[351].

Similar to breast cancer, ERβ has been implicated as an anti-proliferative effector and tumor suppressor, which is partly lost during prostatic carcinogenesis[355,357,358]. In our large cohort, ERβ was expressed in a majority of both stromal and epithelial cells. There was a significantly higher density of ERβ TE compared to TS. However, no significant difference in receptor level when comparing tumor tissue with adjacent normal tissue, or correlation with tumor stage or Gleason grade was detected. In contrast, a significant reduction of ERβ in TS compared to adjacent NS has been reported by Daniels et. al, however, without any

correlation between the expression level of ERβ in TS and clinicopathological variables[351]. Grover et al. presented immunohistochemical detection of lower ERβ levels in cancerous tissue compared to normal prostatic tissue[357]. Further, a poorer cancer specific survival for patients with lower ERβ has been reported by Fujimura et al[355]. However, both observations were made in a smaller cohort and with short follow-up time. By Fixemer et al., a partial loss of ERβ was first described after exposure to ADP, and a with a substantial loss primarily occurring in CRPC[359]. In contrast there are many reports, including ours, suggest a negative role of ERβ expression on PCa prognosis[349,350,360]. Of note, the majority observes a negative effect of an epithelial expression or does not separate between tissue compartments. Recently, in a large cohort of primary prostatectomy specimens (n = 566), Schade et al. observed a negative association between high intensity of ERβ cytosol and BFFS and PCDFS. They also detected a negative association between the nuclear expression of the ERβ splice variant ERβ2 and PC outcome[360]. This latter isoform was, however, not examined in our material. In the study by Zellweger et al., ERβ expression in TE of patients naïve to hormonal treatment was significantly associated with a poor overall survival[350]. They also detected an increase in ERβ level after ADT and progression to CRPC[350]. Horvath et al. on the other hand registered ERβ-positive in the majority of normal prostates and a progressive loss of ERβ as the cancer progressed. However, the small group of patients whose cancer was ERβ positive (n = 18) had a higher rate of relapse and decreased BFFS compared with those where ERβ expression had been lost[349]. Additionally, in the study by Royuela et al. an increased intensity of ERβ immunostaining in TE compared to NE and BPH was observed[348].

As outlined above, there have been several descriptions of a dynamic evolvement of ERβ expression in PC progression. This is also evident in the studies by Leav et al., where ERβ expression at the protein and transcript levels was lost in high-grade dysplasia, but reappearance in grade 3 cancers and was diminished in grade 4/5 cancers. Further, they observed a re-establishment of ERβ expression in the majority of bone and lymph node metastases[347]. Other observations have also have been made of a regained ERβ expression in metastatic tissue in CRPC tissue[361], which is in direct contrast to the aforementioned observations by Fixemer et al. Later, the group of Leav et al. proposed that reversible epigenetic regulations by methylation of ERβ could be the underlying mechanism for the ERβs distinct and different roles at various stages in the evolution and progression of PC[362].

Despite diverging results, there are strong indications of an involvement of estrogens in PC. Due to this, selective ER modulators[s] (SERM) and ER antagonists as potential PC treatment approaches have received attention and continues to be an evolving research field. So far, the majority of studies involve patients with castrate resistant disease, and SERMs have to this point not been investigated for use in patients with treatment-naïve PC. Raloxifen and fulvestrant are some of the most extensively evaluated therapeutical agents, and although it is well tolerated in most patients, only limited clinical responses have been observed so far[363].

A lack of correlation between circulating sex steroid hormone levels and PC has been demonstrated in epidemiological studies[364,365]. Further observations have been made of diverging levels between circulating sex steroid hormone and intra-tissue levels of sex steroid hormone in PC[366]. This indicates a role of the local production of active sex steroid hormones from their precursors in PC. In our cohort we observed aromatase expression in a majority of the PC specimens. Notably, there was a stronger expression in NS compared to TS. This can be interpreted as a confirmation of a local production of estrogens in the prostate whit decreased activity in cancerous tissue. This substantiates the notion of estrogens ability to directly act upon the prostate gland, not only thorough negative feedback on the HP-axis.

We detected aromatase expression in both epithelial and stromal cells of the PCs in our cohort. Although, several have previously described aromatase expression in PC, an agreement regarding its cellular expression is currently lacking[354,367–369]. In contrast to the ERs, there are a limited number of recent studies investigating the prognostic significance of aromatase in various prostatic tissue compartments like us. In one recent study by Miftakhova et al., aromatase expression was detected at significantly higher levels in primary PC compared to BPH using IHC. There was not observed any difference between expression level in metastatic tissue compared to primary tumors. They reported predominantly cytoplasmic aromatase expression, similar to us, but did not separate TS and TE expression. Further reports were made of a subset of patients with upregulated aromatase mRNA

[s] **SERM**: A common denominator of compounds that interacts with the ERs as either agonists or antagonists in a tissue specific manner.

expression being associated with reduced BFFS, although this finding was limited to a small portion of patients the included patients (n = 4/135)[370].

In the aforementioned study by Celhay et al., the immunohistochemical detection of aromatase was mainly epithelial. Further, a high expression of aromatase on biopsy was associated with a shorter time to hormonal relapse in patients initially traded with ADP for their PC[354]. Given that the patients in our cohort were hormone naïve, this result cannot be directly compared with our results. Ellem et al. observed a solely stromal expression of aromatase in normal prostates, but reported that aromatase was abnormally expressed in PC cell lines and in TE of samples from malignant tissue. They thus hypothesized an induction of aromatase gene expression with the onset of malignancy[369]. This is contradicting our findings of a beneficial prognostic role of aromatase in both TE and TS, however, the methodology differs from greatly from ours and Ellem et. al does not investigate aromatase' prognostic value. Supporting the observations of Celhay et al. one study found a 30-fold upregulation of the gene encoding aromatase in metastatic CRPC[371]. Similar to the observations from the ERs, this could indicate a variation in expression over the course of prostate carcinogenesis, and also depending on the treatment situation. So far, a few attempts have been made to investigate the effect of aromatase inhibitors on CRPC in small clinical trials. A beneficial effect has, however, not been observed[372,373].

As for PC, there are few reports of the prognostic value of aromatase expression in other hormone dependent malignancies. In breast cancer, aromatase expression is not established as a prognostic marker although the tumor cells have demonstrated the ability to produce a large amount of estrogens due to elevated levels of aromatase[374]. There has, however, been reports of aromatase expression in TS or TE of $ER\alpha^{+}/PGR^{+}$ breast cancer being positively associated with long-term outcomes[375]. This included relapse free- and cancer specific survival following neoadjuvant endocrine treatment.

5.4 Conclusion

Key discoveries in this thesis

- Patients with high levels of pan-PGR expression in TE had significantly reduced time to CF compared to patients with low levels **(Paper I).**
- High levels of PGRB in TE was an independent negative prognosticator for BF and CF in the same manner as pan-PGR. Indicating that PGRB isoform represented the negative prognostic value initially observed for the pan-PGR **(Paper III).**
- Pan-PGR and PGRB were both expressed in TS. Associations with patient outcomes did not reach significance in multivariate analysis **(Paper III)**.
- PGRA was solely expressed in TS and not associated with event-free survival **(Paper III)**.
- ERα was predominantly expressed in TS where it was an independent positive prognosticator for CF and PCD **(Paper II)**.
- ERβ was an independent negative prognosticator for BF in TE, but not in TS, and was not associated with CF or PCD **(Paper II)**.
- Aromatase was an independent positive prognosticator for CF in TE and for BF in TS **(Paper II)**.

The observations in this thesis indicate that evaluation of sex steroid hormone associated receptors and enzymes can be applied to assess prognosis, and to risk stratify prostate cancer patients. These results demonstrate different prognostic roles of the PGRB, ERα, ERβ and aromatase in patients with localized cancers, naïve to adjuvant hormone treatment, radiotherapy and chemotherapy. However, several studies describe variations in receptor expressions through the course of the prostate carcinogenesis, this applies for both ERα[350,354], ERβ[347,350,359,361] and PGR[324,328,335]. Implying that the receptors can exert different functions in early stages of cancer development, compared to advanced and metastatic stages. Further, the receptors can adapt, mutate and acquire additional abilities in advanced cancer exposed to different treatment regimes. Thus, lack of confirmation from studies in patients with castrate resistant disease, or in patients receiving advanced treatment, is not surprising. Considering

that our results come from hormone naïve cancers. This indicates a highly complex role of these SHRs in PC, which is further complicated by independent actions of the receptors' isoforms.

Different reports have been made from observations *in vivo and in vitro* regarding the role of these SHR in PC. An important consideration is, to which extent, the PC model systems (e.g. xenografts, cell cultures, genetically altered mouse) represents the environment where the human PC develops. Presumably, a balance between the SHRs mediated effects is required to maintain tissue integrity, and a disruption of this balance facilitates cancer development and progression. Further, the SHR and their interactions comprises a regulatory network that can affect the pharmaceuticals targeting SHR. Thus, of great importance is the stromal-epithelial interplay and the co-expression and cross reactivity which is expected from the different SHRs. Naturally, experimental settings lacking this complexity and the *in vivo* hormonal milieu will have challenges in finding reproducible results in humans. Finally, different modes of intracellular signaling, effects of receptor splice variants and acquired mutations must be taken into account when deciphering the roles of SHRs in cancer progression.

6 CONCLUDING REMARKS

6.1 Clinical implications

The executive aim of the thesis was to evaluate the prognostic value of specific sex-SHR in PC patients with localized, hormone naïve disease. This thesis includes three independent studies evaluating the tissue specific expression and prognostic value of ERα, ERβ, and aromatase **(Paper II)** and the pan-PGR **(Paper I)**, including in depth studies of its isoforms PGRA and PGRB **(Paper III)**. Risk evaluation and choice of therapeutic strategies for patients with localized PC have been a major challenge, and a much-contemplated topic in modern cancer research. A vast amount of energy and resources have been poured into the search for prognostic biomarkers. The goal is to stratify patients into risk groups based on biomarker expression, translating into improved prognostication and the facilitation of personalized and more optimal therapy. This would hopefully reduce overtreatment and hence save patients from the unwanted side effects of invasive treatment. So far, several potential biomarkers with promising results have emerged. However, very few have made it to the clinic, and none have added the necessary improvements to, or been able to replace, the traditional prognostication regime[t,139]. So, what motivated this continued search? In our time, overwhelming technical progress has been made in all aspects of science. This is giving us the possibility to re-examine previous theories and investigate new hypothesis in ways that where unthinkable only a decade ago. To be more specific with regards to this thesis; Initially, estrogen agonists (e.g. diethylstilbestrol) or orchiectomy were the standard hormonal treatment for symptomatic metastatic prostate cancer[5,343]. The prognostic role of different sex-SHR in PC have been in the searchlight for more than two decades. However, as outlined in "discussion of main results", studies investigating the prognostic significance of PGR isoforms and aromatase in PC are few, and the current results regarding ERα and ERβ are highly diverging. Additionally, only a fraction of the research separates between stromal and epithelial tissue compartments. Our work makes a novel contribution to this research field by describing the tissue distribution of the PGRs, ERs and aromatase in addition to their correlation to clinical outcome in a large PC cohort (n = 535) from the PSA-era, with long follow up time (mean 12.4 years). The previous divergence in results can to a large extent be contributed to differences in methodology and underpowered studies. Highly likely is also, to some extent, reporting of selective outcomes and publication bias. The PGR and ERα are

[t] **Conventional prognostication regime:** PSA-level, Gleason score and TNM stage

already well-established biomarkers in breast- and uterine cancer. Today, they provide an economic and efficient tool in treatment stratification for cancer patients word-wide. The prostate gland, similar to the breast and uterus, is a highly hormone responsive organ. In PC, the AR is not applied as a prognostic marker, but it is well-recognized as a therapeutic target. Considering this, a value of the other sex-SHR as biomarkers in PC is highly likely, and thus worth investigating further.

Our description of the tissue distribution of the investigated sex-SHR, and our confirmation of their prognostic value in PC, contributes to new insight into the role of steroid hormones in PC. For our biomarkers, we detected additional prognostic information, supplementing the conventional prognostic markers[r] in PC. Given the association with clinical outcomes, these sex-SHR and the aromatase enzyme could prove useful in aiding the decisions regarding active surveillance and also in directing the need for adjuvant therapy. There is further potential of inclusion in prognostic models alongside other promising biomarkers. After the initial use of estrogens in PC, treatment was discarded due to unwanted side effects[343], investigations of targeting other sex-SHR than AR in PC has yielded few results. Nonetheless, the sex-SHRs remain valuable therapeutic targets in cancer treatment. This is another topic that warrants future investigation. The goal must be to identify the optimal condition where the impact of these sex-SHRs can be best exploited. Further, a better understanding of the dynamics in sex-SHR expression that occurs during ADT and development of CRPC is necessary. This warrants further functional studies with the aim to investigating the underlying mechanisms.

6.2 Future perspectives

Our research has been exploratory, meaning that we have investigated potential biomarkers and associations with certain outcomes. The major strengths are the large, multicenter cohort and the long follow-up time. A majority of comparative studies have smaller cohorts and shorter follow-up time. The use of standardized cut-off values and the separate focus on stromal and epithelial tissue compartments, also adds quality to our findings. Due to PCs protracted nature, the number of events for clinically valuable endpoints, such as PCD, remains low despite long follow-up. This challenge the statistical analyzes. BF is associate with cancer recurrence, but it does not necessarily indicate further cancer progression.

Nonetheless, by investigating BF and CF as surrogate endpoints for PC advancement, we were able to obtain solid statistical results indicating a prognostic pattern.
In line with PROGRESS[376,377] [u], an exploratory study needs replication and confirmation under transparent and standardized conditions. The potentials for clinical implementations are many. However, before implementation in the clinic can be considered, further validation of our observations, and well-designed prospective studies examining their prognostic value, is necessary. It would also add value to investigate the biomarkers in biopsy material, in addition to prostatectomy specimens. Especially to explore their potential value in directing the decision-making between radical treatments and active surveillance. Finally, these confirmatory studies must be investigated in meta-analyzes and systematic reviews so that a final conclusion can be drawn[376,377].

In conclusion, this thesis contributes additional, high quality results regarding the tissue distribution, and prognostic value, of steroid hormone related receptors and enzyme in localized PC. Thereby bringing new insight to the role of steroid hormones in PC. We hope this contribution will aid the development of patient risk stratification and contribute to a foundation for future PC treatment.

[u] **PROGRESS**: The PROGnosis RESearch Strategy. A collaboration working towards improving the quality of prognostic research

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PAPER I

PAPER II

PAPER III