PROGNOSTIC FACTORS IN PROSTATE CANCER BIOPSIES:
A POPULATION-BASED COHORT STUDY

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<th>Abbreviation</th>
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<td>3D-CRT</td>
<td>3-dimensional conformal radiation therapy</td>
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<td>ADT</td>
<td>Androgen deprivation therapy</td>
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<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>AS</td>
<td>Active surveillance</td>
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<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
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<tr>
<td>CAB</td>
<td>Complete androgen blockade</td>
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<tr>
<td>CAF</td>
<td>Carcinoma-associated fibroblast</td>
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<td>CI</td>
<td>Confidence interval</td>
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<td>CSC</td>
<td>Cancer stem cell</td>
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<td>CT</td>
<td>Computer assisted tomography</td>
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<td>DRE</td>
<td>Digital rectal examination</td>
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<tr>
<td>DT</td>
<td>Diphteria toxin</td>
</tr>
<tr>
<td>EAU</td>
<td>European Association of Urology</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EORTC</td>
<td>European Organization for Research and Treatment</td>
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<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
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<tr>
<td>GS</td>
<td>Gleason score</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
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<tr>
<td>HG-PIN</td>
<td>High grade intraepithelial neoplasia</td>
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<tr>
<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>IDC-P</td>
<td>Intraductal carcinoma of the prostate</td>
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<tr>
<td>IMRT</td>
<td>Intensity modulated radiation therapy</td>
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<tr>
<td>ISUP</td>
<td>International Society of Urological Pathology Consensus Conference on Gleason Grading of Prostatic Carcinoma</td>
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<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
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<tr>
<td>LHRH</td>
<td>Luteinizing-hormone-releasing-hormone</td>
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LVI: Lymphovascular invasion
MET: Mesenchymal-epithelial transition
MRI: Magnetic resonance imaging
PC: Prostate cancer
PIA: Proliferative inflammatory atrophy
PIN: Prostatic intraepithelial neoplasia
PI-RADS: Prostate imaging reporting and data system
PIVOT: Prostate cancer intervention or observation trial
PNI: Perineural invasion
ProtecT: Prostate testing for cancer and treatment
PROGRESS: The The PROGnosis RESearch Strategy partnership
PSA: Prostate-specific antigen
REDEEM: Reduction by Dutasteride of clinical progression Events in Expectant management study
REMARK: The Reporting recommendations for tumor marker prognostic studies
RP: Radical prostatectomy
RSG: Reactive stromal grade
RT: Radiotherapy
SPCG: Scandinavian prostate cancer group
TAM: Tumor-associated macrophage
TGF-β: Transforming growth factor-β
WHO: World Health Organization
WW: Watchful waiting
LIST OF ORIGINAL PAPERS

1. THE PROGNOSTIC VALUE OF REACTIVE STROMA ON PROSTATE NEEDLE BIOPSY: A POPULATION-BASED STUDY

Sæter T, Vlatkovic L, Waaler G, Servoll E, Nesland JM, Axcrona K, Axcrona U.

Prostate 2015; 75:662-671

2. THE RELATIONSHIP BETWEEN PERINEURAL INVASION, TUMOR GRADE, REACTIVE STROMA AND PROSTATE CANCER-SPECIFIC MORTALITY: A CLINICOPATHOLOGIC STUDY ON A POPULATION-BASED COHORT

Sæter T, Bogaard M, Vlatkovic L, Waaler G, Servoll E, Nesland JM, Axcrona K, Axcrona U.

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3. COMBINING LYMPHOVASCULAR INVASION WITH REACTIVE STROMAL GRADE PREDICTS PROSTATE CANCER MORTALITY

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4. INTRADUCTAL CARCINOMA ON DIAGNOSTIC NEEDLE BIOPSY PREDICTS PROSTATE CANCER MORTALITY: A POPULATION-BASED STUDY

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Unpublished manuscript
PART I: BACKGROUND

CHAPTER 1 – PROSTATE CANCER

1.1 EPIDEMIOLOGY

In 2011, 4978 men were diagnosed with prostate cancer (PC) in Norway (1). The PC-specific mortality in Norway is one of the highest globally (2). In addition to the mortality of PC, serious morbidity affects men with metastatic PC such as cancer-related pain and pathological fractures (4). Men who undergo potentially curative treatment also face quality of life impairing adversities in the form of urinary and sexual side effects (5, 6). PC is a heterogeneous disease with a broad spectrum from slow-growing tumors with low risk of morbidity and mortality to aggressive and ultimately metastatic and lethal disease. Thus, accurate identification of low-risk and high-risk patients is necessary to ensure that patients that don’t need treatment are managed conservatively whereas patients with potentially lethal disease are treated curatively.

1.2 THE DIAGNOSIS OF PROSTATE CANCER

In contemporary practice, the diagnostic investigation for PC is commonly initiated due to an elevated serum prostate-specific antigen (PSA) - level and/or abnormal digital rectal examination (DRE) (7).

1.2.1 SYMPTOMS

Direct symptomatic PC is uncommon in contemporary diagnostic setting since the majority of PC cases are detected early due to opportunistic PSA – testing. Nonetheless, urinary problems are common in PC patients due to concomitant benign prostatic hyperplasia (BPH) (8).
1.2.2 PROSTATE-SPECIFIC ANTIGEN

PSA is an enzyme produced by the epithelial cells of the prostate. An elevated serum PSA-level is an established indicator of prostatic adenocarcinoma (9).

To date, no country has initiated a national PSA-based screening program (10). Thus, PSA-measurement in an asymptomatic patient represents opportunistic screening (10,11). Systematic PSA-screening has been demonstrated to reduce PC-specific mortality, but is accompanied by a risk of overdiagnosis (12, 13).

1.2.3 DIGITAL RECTAL EXAMINATION

Although PSA-testing has become the most common cause for investigating a patients’ prostate for PC, DRE is still a valuable and necessary tool (14). Importantly, a positive DRE may indicate aggressive disease (15).

1.2.4 MAGNETIC RESONANCE IMAGING

In recent years, there has been an increasing interest in the application of magnetic resonance imaging (MRI) for the detection, staging and localization of PC (16). The sensitivity and specificity of MRI for detecting PC varies considerably between studies (17). Small and low grade tumors that grow diffusely within the prostate are particularly difficult to detect (17). With regard to staging for organ –confined versus extracapsular disease, an accuracy of about 90% has been reported by the American College of Radiologists (18). Other applications for MRI include targeting biopsies, assessing disease aggressiveness and monitoring conservatively managed patients (17).

1.2.5 PROSTATE BIOPSY

Although PSA, DRE, symptoms and MRI provide the clinician with a basis for suspecting PC, a prostate biopsy confirming the presence of prostatic adenocarcinoma is mandatory for establishing the diagnosis. A prostate biopsy is obtained by perforating the prostate with a thin needle and thereby collecting a sample of prostatic tissue. The procedure can be performed
through the transrectal or the transperineal route. The transrectal approach is the most common (19). One of the major disadvantages of this approach is bacterial contamination which can result in infectious complications (20). Prior to the introduction of PSA and transrectal ultrasound technology, biopsies were obtained finger-guided towards a suspicious palpable finding (49). Following the introduction of transrectal ultrasound, biopsies would also be targeted towards hypoechoic lesions (21). Eventually a systematic approach using a sextant biopsy scheme was adopted (21). In contemporary practice, transrectal ultrasound is used to steer the biopsy needle towards the prostate and biopsies are taken according to a predefined template. The number of and the locations of biopsies is subject to controversy. However, it is recommended to obtain 12 biopsies, sampling both sides far laterally from basis to apex (7, 22). Technology that fuses MRI and ultrasound images is becoming increasingly available and allows for targeting MRI suspicious lesions in the prostate (21).

1.3 CLINICAL STAGING OF PROSTATE CANCER

Upon establishing the diagnosis of PC, patients are assigned a clinical stage. The clinical stage describes the extent of the disease and is a prerequisite for future decisions on treatment.

Clinical stage is based on the following axes:

-T-stage: tumor extent

-N-stage: lymph node involvement

-M-stage: metastases to distant organs

Clinical T-stage is determined by DRE. However, accuracy is low and 40%-50% of men considered to have organ-confined disease turn out to have extraprostatic extension on surgical specimens (23). MRI has in recent years become a helpful adjunct for more precise determination of clinical stage (18). A clinical stage T1 means that the cancer is detected in a
transurethral resection specimen (T1a/b) or in a needle biopsy on the basis of an elevated PSA(T1c). There is no palpable finding in clinical stage T1 (59). When there are suspicious findings by DRE, but these appear to be confined within the prostate, clinical stage T2 is assigned (24). If the tumor extends beyond the prostate, clinical stage T3-T4 is present (24).

N-stage is determined in patients eligible for curative treatment. The assessment is based on nomograms that take into account clinical information such as T-stage, Gleason score (GS) and PSA (23). An estimated risk of lymph node involvement is obtained and is used to guide treatment decisions. Radiological investigations such as computer assisted tomography (CT) and MRI can also be performed to determine N-status. However, the ability to detect malignant lymph nodes is low (25). In selected patients, lymph node status is assessed surgically by pelvic lymphadenectomy. Although lymphadenectomy is the best approach currently available, it is still associated with a risk of undersampling (25).

M-stage is determined radiologically. Most commonly, a bone scan is performed to assess skeletal metastasis. CT or MRI can be used as adjuncts to further assess suspicious areas on bone scan if needed. (26).

Based on the PSA-level, clinical stage and GS, patients can be assigned to risk categories ranging from low - intermediate - high and very high. The appropriate treatment for a patient depends on which risk category he belongs (7).
1.4 TREATMENT OPTIONS FOR PROSTATE CANCER

1.4.1 WATCHFUL WAITING
Watchful waiting (WW) refers to deferring treatment until symptoms occur (7). The purpose of the strategy is to minimize treatment related side-effects and provide palliative treatment when needed. Thus, it is most commonly selected for elderly men with comorbidity and a short life expectancy (27). These patients, particularly in the presence of low grade disease, have low risk of dying from PC (28).

1.4.2 ACTIVE SURVEILLANCE
Active surveillance (AS) also refers to deferring treatment. The purpose is to avoid side effects of treatment, reduce overtreatment and initiate curative treatment when the disease shows signs of progression (27). Thus, it is restricted to patients with low risk disease and involves monitoring of the disease and transition to an active treatment protocol when certain criteria are fulfilled. A long-term study on patients enrolled in AS demonstrated a 15-year cancer-specific survival > 90% (28).

1.4.3 RADICAL PROSTATECTOMY
Radical prostatectomy (RP) means surgically removing the prostate. Different approaches for RP are available. Traditionally the procedure was performed through open incision, either retropubic or transperineal. In recent years, the advent of mini-invasive surgery has led to the development of laparoscopic RP and ultimately robot-assisted laparoscopic RP. The aim of RP is to cure the patient of PC. The Scandinavian Prostate Cancer Group (SPCG)-4 trial evaluated the effect of RP by randomizing men to either RP or WW. The relative risk of PC-specific death was 0.56 (95% confidence interval [CI] 0.41-0.77), thus demonstrating the curative potential of surgical treatment for PC (29). However, the trial was carried out prior to the surge in low-volume and low-grade PC that followed opportunistic PSA-screening. Thus, critics argue that the findings of SPCG-4 may not be applicable in current practice.
Prostate cancer Intervention or Observation Trial (PIVOT) study intended to amend these shortcomings by including a more contemporary patient cohort in a randomized trial between RP and WW. After a median follow-up time of 10 years, the study did not detect statistically significant reductions in PC-specific mortality or all-cause mortality (30). Although significant reductions in all-cause mortality among men with PSA > 10 and intermediate risk tumors were found (30). The PIVOT study has been criticized for the fact that it did not accrue the necessary number of patients needed to obtain statically significant results (31). Furthermore, a substantial number of the enrolled men died within 10 years, suggesting that these patients had too short life expectancy to benefit from surgery (31).

1.4.4 RADIOTHERAPY

Radiotherapy (RT) aimed at the prostate causes DNA damage and ensuing cellular death. To optimize the delivery of radiation to the target organ rather than to the surrounding tissue, conformal techniques are employed (32). The previous standard technique was 3-dimensional conformal radiation therapy (3D-CRT), in which multiple radiation beams from various angels result in higher radiation dose in the prostate compared to surrounding tissue (32). The current gold standard is intensity modulated radiation therapy (IMRT), in which dynamic radiation fields with varying intensity result in improved adaptation of the radiation to the patient’s anatomy (7,32). RT is delivered in small daily fractions over several weeks, leading to large cumulative doses. According to the European Association of Urology (EAU) guidelines, an accumulated minimum dose of ≥ 74 Gy is recommended (7). RT is combined with neoadjuvant and adjuvant hormone therapy, as the superiority of this strategy has been documented in randomized controlled trials (33). The European Organization for Research and Treatment of Cancer (EORTC) 22863 study randomized patients with high metastatic risk PC to either RT alone or combined RT and androgen suppression. The 10-year PC-specific mortality was 30.4% for the RT alone group and 10.3% for RT and androgen suppression.
group (33). The SPCG-7 trial examined the impact of radiotherapy combined with hormone therapy versus hormone therapy alone in a randomized fashion (34). The study demonstrated that radiotherapy halved the 10-year PC-specific mortality compared to hormone therapy alone and thereby proving the efficacy of radiotherapy in the management of PC.

1.4.5 ANDROGEN DEPRIVATION THERAPY
PC is dependent on androgen receptor (AR) activation for its survival and growth (35). Thus, androgen deprivation therapy (ADT) is commonly applied in selected patients. Even though hormonal treatment provides effect on the tumor initially, PC cells ultimately become able to reactivate AR signaling through various mechanisms (35). The clinical result of these processes is termed castration-resistant PC.

ADT can be achieved by either lowering serum androgen levels or by blocking the AR (35). The former is referred to as castration and can be obtained surgically by bilateral orchiectomy or medically by manipulating the hypothalamo-pituitary gonadal axis with luteinizing-hormone-releasing-hormone (LHRH) agonists/antagonists (35).

In recent years, newer ADT compounds have become available. Abiraterone acetate is an inhibitor of cytochrome P-450 17A1, an enzyme involved in the synthesis of androgens at extra-gonadal sites (35). Enzalutamide inhibits AR signaling more potently than traditional anti-androgens due to inhibition of AR nuclear translocations, DNA binding and coactivator recruitment (35).

ADT has been shown to increase cancer-specific and overall survival if added to radiotherapy (33). Moreover, ADT improves quality of life and reduces tumor-related morbidity in symptomatic metastatic PC (35). The new ADT compounds, abiraterone and enzalutamide, have been shown to increase overall survival in castration-resistant PC (35).
1.5 HISTOPATHOLOGY

1.5.1 THE HISTOLOGY OF THE PROSTATE

Histologically, the prostate is composed of epithelium and a fibromuscular stroma (36). The epithelium forms glands consisting mainly of secretory cells, but also a few neuroendocrine cells and basal cells that rest on a basement membrane (37). The produce of the secretory cells is released into luminal spaces which ultimately enter the ejaculatory ducts (37). Surrounding the epithelial glands lies the fibromuscular stroma composed of fibroblasts, smooth muscle cells and extracellular matrix (37).

The secretory epithelial cells receive innervation from parasympathetic nerves which stimulate secretory production through muscarinic signaling (38). The smooths muscle cells of the fibromuscular stroma is stimulated to contract by β-adrenergic sympathetic nerves, which results in expulsion of secretions into the luminal space (38).

1.5.2 PROSTATE CANCER PATHOPHYSIOLOGY

1.5.2.1 PRECURSORS OF PROSTATE CANCER

The cell of origin for PC is debated. There is evidence supporting the concept of luminal cells of the prostatic glands as the originating cells (39). However, there is also data suggesting that PC arise from the basal cells of the glands (39). The postulated earliest precursor lesion of PC is proliferative inflammatory atrophy (PIA), which is characterized by focal atrophic lesions associated with inflammation (40). Following PIA, prostatic intraepithelial neoplasia (PIN) may develop. The characteristics of PIN include dysplasia and nuclear atypia, but a preserved basal lamina – which distinguishes it from carcinoma (37). The role of PIN as a precursor for PC is well established (41). For instance malignant glands can be seen as buds arising from high-grade PIN lesions (41). Subsequent to PIN formation, the basal lamina may be breached by the dysplastic cells signifying the development of prostatic carcinoma (37).
1.5.2.2 INFLAMMATION
Inflammation is characterized by the presence of inflammatory cells, cytokines, chemokines, reactive oxygen species and prostaglandins (42). The inflammatory response may trigger tumor initiation and progression through genetic damage, promotion of restorative cell proliferation and angiogenesis (42). There is an increasing amount of data corroborating a link between inflammation and PC (for review see 43).

1.5.2.3 GENETIC ALTERATIONS IN PROSTATE CANCER
Changes in the genetic material of epithelial cells have been considered a key mechanism for formation of carcinoma. Several molecular alterations in the genome have been found to contribute to PC pathogenesis (44). Particularly significant genetic alterations include myc amplification, TMPRSS2:ERG fusion, PI3K/PTEN/AKT alterations and modifications of the AR (44).

1.5.2.4 EPITHELIAL PLASTICITY
PC cells have the ability to transit between epithelial and mesenchymal states, which facilitate metastatic development (45). Firstly, PC cells may undergo epithelial-mesenchymal transition (EMT) which is characterized by loss of adhesion molecules and increased migratory capacity (45). Thus, cells undergoing this transformation have the ability to leave the primary tumor site, enter the circulation and invade a distant organ. Upon arrival at the metastatic site, the tumor cells may reverse EMT and undergo mesenchymal-epithelial transition (MET) (45). The plasticity of PC cells is related to stem cells features and is critical for the ability to create viable metastatic lesions (45).

1.5.2.5 CANCER STEM CELLS
Cancer stem cells (CSCs) are tumor cells that have the ability to self-renew and form new tumors (39). These cells are considered to constitute a small fraction of the tumor. In recent years, there has been increasing data on CSC in PC. The role of CSC in PC pertains to the
development of metastatic disease. For instance, CSCs have been shown to be more frequent in metastatic lesions than in primary tumors (39).

1.5.3 ANDROGENS AND THE ANDROGEN RECEPTOR

Androgen signaling is a critical factor for prostate cancer growth. However, it is controversial whether androgens induce PC carcinogenesis. The effects of androgens on PC cells include induction of proliferation, regulation of apoptosis and EMT (46).

1.5.4 THE TUMOR MICROENVIRONMENT

The main theory of carcinogenesis has been based on cells accumulating mutations that lead to uncontrolled proliferation. However, recent discoveries have underscored the importance of cells and tissue surrounding the tumor. An increasing amount of evidence suggests that these components are not passive by-standers, but rather initiators and propagators of cancer development (47). The components of the microenvironment that contribute to PC development include stromal fibroblasts, macrophages and other immune cells, extracellular matrix and integrins, and nerves (47).

1.5.4.1 STROMA

The stroma of the benign prostate consists of smooth muscle cells and extracellular matrix. During carcinogenesis, the stroma may become populated by carcinoma-associated fibroblasts (CAFs) and there is increased deposition of extracellular matrix (36, 37). This stromal transformation is schematically illustrated in Figure 1. The process is similar to the wound healing process where fibroblast become activated as myofibroblasts (37). Whereas wound healing is a finite process and myofibroblasts eventually undergo apoptosis, the stromal changes taking place in carcinomas is perpetuated and play a key role in supporting tumor development (36, 37). The term “reactive stroma” has been coined to describe these stromal changes in carcinomas. CAFs are characterized by the absence of cytokeratin and expression of smooth muscle α- actin and vimentin (48). The role of CAFs in PC carcinogenesis has been
demonstrated by the seminal works of Olumni et al and Hayward et al (49, 50). Olumni et al found that when BPH-1 cells (a non-tumorigenic prostatic epithelial cell line) were combined with CAFs *in vivo*, malignant transformation of the benign epithelial cells ensued (49).

**FIGURE 1**

*Schematic illustration of normal and reactive stroma. The image on the left shows normal prostatic stroma consisting of smooth-muscle cells and fibroblast in a benign prostate. On the right, the stroma of prostate cancer is depicted, showing influx of carcinoma-associated fibroblasts and increased extracellular matrix deposition.*

The influence of stroma and CAFs on PC pathogenesis is multifaceted and is not confined to the primary site. Stromal cells are important contributors to metastatic disease by releasing soluble factors that result in increased vascular permeability and penetration of blood vessels by tumor cells (51). Moreover, it appears that stromal cells may co-metastasize with tumor cells and settle in distant sites to create a metastatic niche that allows for survival of the tumor cells (51). The interaction between tumor and stroma is mediated through production of various soluble factors. Of particular interest, is the TGF–β superfamily. The role of TGF-β is complex and paradoxical, but contributes to PC pathophysiology. For instance, TGF-β can
induce EMT, stimulate angiogenesis, alter immune surveillance and induce secretion of growth factors (37). Other factors involved in paracrine signaling between stroma and tumor include insulin like growth factor 1, hepatocyte growth factor/scatter factor (48, 51). The origin of CAFs is poorly understood. Local fibroblasts are considered to be the main source (48). However, recent data suggest that bone-marrow derived mesenchymal stem cells are important contributors to the CAF population (48). It has also been suggested that epithelial cells undergoing EMT represent a source of CAFs (48). Furthermore, local endothelial cells can contribute in a mechanism termed endothelial to mesenchymal transition. (48).Whether CAFs are subject to genetic changes is debated as the data is conflicting (52) However, there is data demonstrating epigenetic alterations in tumor stroma in the form of silencing of various genes through hypermethylation (48, 52).

1.5.4.2 TUMOR - ASSOCIATED MACROPHAGES
Tumor cells are able to recruit bone marrow derived cells including macrophages. Once recruited, macrophages differentiate into tumor-associated macrophages (TAMs) (53). Phenotypically, macrophages can be classified as M1 and M2. M1 macrophages are pro-inflammatory, involved in the immune response and tumoricidal (53). M2 macrophages are involved in wound healing by scavenging debris and influencing tissue remodeling. TAMs show characteristics akin to M2 macrophages and provide a pro-tumoral influence in processes like growth, angiogenesis, migration, invasion, intravasation and extravasation (53). TAMs and CAFs are functionally associated and can be viewed as co-conspirators in the tumor microenvironment (37). The clinical significance of TAMs in PC has been investigated in two studies, which demonstrated a relationship with prognosis (54, 55).

1.5.4.3 NEURO-EPITHELIAL INTERACTIONS
The significance of neural signaling for PC development is underscored by the fact that spinal cord injury patients rarely develop PC (56). Moreover, use of β-blockers is associated with
reduced risk of PC-specific – mortality (57). A recent landmark study elucidated the mechanisms underlying neural signaling and PC development (58). Magnon et al demonstrated in an orthotopic mouse model of PC that sympathetic signaling via stromal adrenergic receptors was critical for the formation of PC tumors and parasympathetic signaling via stromal muscarinic receptors was related to progression and metastasis (58). The most commonly recognized form of interaction between nerves and tumor is perineural invasion (PNI). The invasion of nerves by tumor cells is a bilateral process (59). It involves growth of tumor cells towards nerves, which in turn develop axonal sprouts that seek towards the tumor (59). Thus, neurogenesis/axonogenesis precedes PNI (60). Moreover, neurogenesis/axonogenesis is involved in the development of aggressive PC (60). Prostatic stromal cells have been shown to facilitate the development of PNI. In turn fibroblasts can increase their fibrogenic activity in response to neurotransmitters (61, 62). Thus, a multidirectional signaling network between nerves, stroma and tumor can be postulated to influence PC pathogenesis.
1.5.5 HISTOPATHOLOGICAL EVALUATION OF PROSTATE BIOPSIES

After obtaining prostate biopsies, the tissue cores are formalin fixed and then placed in cassettes and embedded in paraffin. Then, sections are cut from the paraffin blocks and the sections are stained with hematoxylin and eosin (H&E).

After processing, the pathologist evaluates the biopsy sections for the presence of prostatic adenocarcinoma. The major criteria for prostatic adenocarcinoma include (63):

- Infiltrative growth pattern of the glands

- Absence of basal cells

- Nuclear atypia: nuclear or nucleolar enlargement.

Minor criteria may also indicate the presence of adenocarcinoma, although these are not specific for cancer. These criteria include: intraluminal blue mucin, pink amorphous secretions, mitotic figures, intraluminal crystalloids, amphophilic cytoplasm, nuclear hyperchromasia and adjacent high grade (HG)-PIN (63).

Immunohistochemistry is a valuable adjunct to establishing the diagnosis of prostatic adenocarcinoma. Staining with antibodies against basal cells may aid in assessing the absence of a basal layer. The most commonly used antibodies in this regard include 34βE12/CK903 which binds to high molecular – weight cytokeratin (HMWCK) expressed in basal cell cytoplasm and p63 which detects proteins in the nucleus of basal cells (63). Moreover, alpha-methylacyl-CoA-racemase is upregulated in PC and antibodies against its gene product P504S can be used to detect prostatic adenocarcinoma with a sensitivity of 82-100% (64).
The Gleason grading system for PC was developed in 1966 (65). It has been modified since its inception and currently PC is graded according to the 2005 International Society of Urological Pathology Consensus Conference on Gleason Grading of Prostatic Carcinoma (ISUP 2005) (66). Even though almost 50 years has passed since its initial development, the Gleason grading system remains the best predictor of the pathological and clinical outcome of PC.

The Gleason grading system is based on evaluating the architectural pattern of the tumor which in contemporary practice is graded from 3 to 5 (the initial system also included grade 1 and 2, but these are rarely reported in current pathology reports) (66). Moreover, the most common and the second most common pattern grade is summed to obtain the Gleason score (GS) (66). If there is only one grade present, that grade will be doubled. In some cases, three grades are present (67). In this situation it is recommended to assign the most common grade plus the highest grade (67).

Below follows a brief description of the currently used Gleason grade patterns (66):

- Gleason grade 3: Discrete glandular units with variation in size and shape
- Gleason grade 4: Fused small glands with ill-defined lumina. Cribriform pattern
- Gleason grade 5: No glandular differentiation. Solid sheets, cords or single cells. Comedonecrosis can be present.

A GS of 3+3= 6 corresponds to a highly favorable prognosis (68). GS 7 can be divided into two subcategories, namely 3+4=7a and 4+3=7b. A GS 7a also corresponds to a good prognosis, although not as good as that of GS 6 (68). However, the step up to GS 4+3=7b equates a considerable worsening of the outcome (68). GS 8-10 have the highest risk of
unfavorable outcome and there is data suggesting that there is a prognostic distinction between 8 and 9-10 (68).

In addition to the presence of carcinoma and its accompanying GS, the pathology reports for cancer-bearing biopsy specimens also include number of positive cores, tumor quantification per positive core (either millimeters or percentage), the presence of perineural invasion, atypical small acinar proliferation, HG-PIN and intraductal carcinoma (67).

In 2014, ISUP convened and proposed a new grading system. The new grading system is based on the GS, but uses the term Grade Group instead. There are five Grade Groups, 1: corresponds to GS 3+3=6; 2: GS 3+4=7a; 3: GS 4+3=7b; 4: GS 8; 5: GS 9-10 (69). It has been argued that this simplified PC grading system is more accurate (70). Moreover, the conversion of the lowest grade from 6 (GS 3+3=6) to 1 may have the potential to reduce overtreatment (70, 71).

1.5.7 HISTOPATHOLOGICAL FACTORS OF PROGNOSTIC SIGNIFICANCE

The clinical outcomes of PC are distributed across a wide range (72). On one hand, patients may live for several years with PC and die of unrelated causes. In contrast, other patients are managed with a curative intent, but still experience disease relapse and ultimately die from metastatic PC. Thus, it is of paramount importance to distinguish between these patient groups and tailor the intensity of the disease management accordingly (72). Commonly, clinical factors such as stage, GS and PSA are used to stratify patients into low-, intermediate- and high risk categories (73). However, these factors can only account for a fraction of the variation in the prognosis of PC patients (74). For instance, Vickers et al used data from the SPCG-4 trial and found that stage, grade and PSA resulted in a concordance index of 0.69 (75). The concordance index is a measure of predictive accuracy and a value of 0.5 implies predictive capability equal to chance. Thus, it is apparent that prognostic factors are necessary in PC management and there is a need for factors in addition to those currently in clinical use.
1.5.7.1 REACTIVE STROMAL GRADING

The increasing awareness of stromal influence on PC pathogenesis has led to attempts to detect stroma based prognostic factors. These include evaluating the immunohistochemical expression of various CAF related molecules such as vimentin, desmin and Mallory trichrome staining (76, 77). However, the stromal process influencing PC, termed “reactive stroma”, can also be assessed in light microscopy using H&E staining. The smooth muscle cells of the normal prostatic stroma show dense eosinophilia on H&E stained sections, and are uniform in size and shape with round nuclei. The reactive stroma is less eosinophilic and becomes blue-gray on H&E-stained sections. The extracellular matrix of reactive stroma is loose and fibrillar. The reactive stromal cells have elongated nuclei and a disorderly growth pattern. Thus, in 2007 Yanagisawa et al described a grading system for reactive stroma in prostatic needle biopsies using H&E stains (78). According to Yanagisawa et al, the percentage amount of reactive stroma is semi-quantitatively assessed and a reactive stromal grade (RSG) is set as follows, RSG 0, 0-5 % reactive stroma; RSG 1, 6-15 %; RSG 2, 16-50%; RSG 3, 51-100 % (78). The grading of reactive stroma was found to predict biochemical recurrence in patients undergoing RP (78). Moreover, RSG 3 on prostatectomy specimens has been found to correspond with increased risk of PC-specific mortality (79).

FIGURE 2

Schematic illustration of reactive stromal grade 0 (left) and reactive stromal grade 3 (right)
1.5.7.2 PERINEURAL INVASION
Tumor invasion of nerves is an established adverse prognostic factor in several malignancies. PNI in prostate cancer has in several studies been found to correlate with non-organ confined disease (80-82) and to predict poor prognosis in terms of rising PSA-levels after treatment (83-86) and PC-specific mortality (87-90). However, other researchers have found that PNI is not an independent predictor of advanced disease (91-93) and prognosis (94-97). The prognostic value of PNI is therefore considered to be uncertain. It is of note that PNI is usually associated with adverse prognosis in univariable analysis and the association is rendered statistically non-significant when adjusting for other prognostic factors.

1.5.7.3 LYMPHOVASCULAR INVASION
Lymphovascular invasion (LVI) describes the presence of tumor cells within an endothelial-lined structure, either a lymphatic or blood vessel. It represents one of the early steps of metastatic disease (51). However, its prognostic significance is variable and therefore uncertain (98). Some investigators have detected a negative influence of LVI on the rate of PSA-relapse after surgery, whereas others have not found data to support this (99-105). There are few studies evaluating the effect of LVI on more definite endpoints such as metastatic disease and PC-specific mortality. However, the findings of these studies suggest a negative influence of LVI (106-108).

1.5.7.4 INTRADUCTAL CARCINOMA OF THE PROSTATE
According to the Epstein criteria, intraductal carcinoma of the prostate (IDC-P) is defined as malignant cells filling prostatic ducts with preserved basal cell layer (109). IDC-P is mainly considered to be cancerous invasion of prostatic ducts (110). However, there is data suggesting that IDC-P may be a precursor lesion in some cases (110). It is of crucial importance to distinguish between IDC-P and HG-PIN, which is also characterized by cellular atypia and intraductal localization (111). The latter represents a benign phenomenon, but it is
indeed potential precursor lesion of PC (111). HG-PIN does not warrant measures other than repeat biopsy when it is abundantly present in the absence of actual carcinoma on biopsy (35). IDC-P on diagnostic prostate biopsy is most commonly found with concurrent invasive carcinoma on the outside of the invaded ducts (112). Although rare, there exist cases where IDC-P is the only evidence of carcinoma (112). In either situation, IDC-P is associated with high grade and advanced stage disease (112-115). Moreover, IDC-P has been found to predict disease relapse, either defined as rising PSA-level or clinical signs of recurrence, following surgery or radiotherapy (116-119). Kimura et al demonstrated that IDC-P on RP specimens predicted death from PC in patients with surgically treated high-risk disease (115). Similarly, it has been shown that IDC-P is a predictor of overall survival in patients with metastatic PC (120). Thus, there is an increasing amount of evidence highlighting the negative prognostic effect of IDC-P. However, there is lacking a full understanding of the pathophysiology of IDC-P and how to properly manage patients with this apparently unfavorable histopathologic feature.

CHAPTER 2 - PROGNOSTIC RESEARCH

2.2. PROGRESS

The PROGnosis RESearch Strategy (PROGRESS) partnership is a collaboration aimed at improving the quality of prognostic research. The definition of prognostic research is: “the investigation of the relations between future outcomes among people with a given baseline health state in order to improve health” (121).
According to PROGRESS, prognostic research can be stratified into four levels (121):

1. Fundamental prognostic research
   - Studies the prognosis of various diseases, e.g. rate of disease-specific death among patients with PC.

2. Prognostic factor research
   - Identify factors that are associated with prognosis

3. Prognostic model research
   - Building a model that include several prognostic factors to accurately predict outcomes

4. Stratified medicine research
   - Developing therapeutic options based on individual risk profiles

2.3 PROGNOSTIC FACTOR RESEARCH

Prognostic factor research aims to determine whether a measure is associated with a clinical outcome. Establishing prognostic factors have several implications for disease management such as; changing how diseases are defined, aid in patient information and treatment selection, and identify future therapeutic targets (122).

A prognostic factor is any measure that can be connected to an outcome, ranging from tumor grade to socioeconomic characteristics (122). Most commonly, prognostic factors are biomarkers, for instance histopathologic variables (122).

It should be noted that the terms prognostic and predictive factor are frequently used interchangeably. However, a prognostic factor is a characteristic that is associated with the
outcome when no therapy or standard therapy is given. A predictive factor is a characteristic that is associated with response or lack of response to a specific treatment (123).

**PROGRESS** points out that there are two steps in prognostic factor research (122):

1. **Exploratory**
   
   These studies investigate several potential factors to assess whether there is an association with outcome

2. **Replication and confirmation**
   
   These studies aim to reproduce the findings reported in the first step. This step requires multiple and independent studies. It is important that these studies are transparent and thorough in their reporting since they form the basis of meta-analyses.

Ideally, prognostic research should be performed as registered prospective trials (122). However, a prospective design is frequently unfeasible due to long latency between disease and outcome combined with a continuously evolving medical field that introduces new potential prognostic factors. Hence, data from retrospective studies remains an important source for knowledge in prognostic research.

Cohort studies are recommended for evaluating prognostic factors. Moreover, the cohort should include the entire spectrum of the disease at an appropriate time of the disease, that is all patients in a given area and time period, preferably at diagnosis (123, 124). Selecting patients may lead to estimates that are not representative for the clinical setting in which prognostic factors are intended to be used. For instance, prognostic factors in PC are used to stratify patients with regard to active treatment or conservative approaches. However, prognostic research studies in PC commonly employ radical prostatectomy cohorts, i.e. patients that have undergone curative surgery. A considerable uncertainty must be expected
when inferring the results of these studies to patients with newly diagnosed PC of whom only a fraction will undergo surgery.

In prognostic research, causality is a debated matter. Since the aim is to identify a factor that foretells outcome, it is an obvious conclusion that factors that produce the outcome have value as prognostic determinants. However, it is not given that it is mandatory to accurately distinguish causal relationships from confounded associations as it is in other forms of medical research. When evaluating a potential treatment it is absolutely necessary to determine whether the treatment itself actually produces the observed disease remission. Similarly, when addressing potential factors for disease prevention, one must know with certainty that these factors produce the disease or else preventive measures will be futile. Thus, these forms of research need to eliminate the role of confounding bias to isolate a causal relationship. The view on causality and confounding in prognostic research differs between researchers. Mak et al argue that prognostic research must take into account potential confounders and adjust for them statistically (123). However, Grobbee and Hoes takes an opposite stand and states that confounding bias is of no concern in prognostic research (125). When considering the role of causality and confounding bias in prognostic research one must first focus on the purpose of prognostication. The aim of prognostication is to predict an outcome, thus any measure that carries this ability could be a valid prognostic factor. Whether a prognostic measure actually produces the outcome is irrelevant as long as it satisfies the primary purpose.

However, it is still crucial to include other variables in statistical analysis of prognostic factors. The purpose is to determine whether the factor being investigated has prognostic value over established factors. (122, 126)
Identification of prognostic factors should take into account practical considerations. A prognostic factor should be easily available at the time of its intended application. Moreover, it is *prima facie* that obtaining prognostic data should not jeopardize the health of the patient to the extent that it outweighs the potential benefits.

One could for the sake of argument assume that lymph node status is a better prognosticator than tumor grade in any given cancer. However, tumor grade is readily available in all patients and requires no further measures than a diagnostic specimen. In contrast, lymph node status usually requires invasive procedures and is only obtained in selected patients. Thus, tumor grade would in most circumstances be a more practically suitable prognostic factor.

It has been pointed out by investigators that prognostic research is riddled by several methodological faults which result in variations in reported findings (127). These weaknesses include underpowered studies, missing data, selective outcome reporting and publication bias (127). Systematic reviews and meta-analysis of numerous confirmatory studies are needed to reach a final recommendation regarding the utility of a prognostic marker (122). Thus, it is important to report these studies in a manner which facilitate meta-analyses. The Reporting recommendations for tumor marker prognostic studies (REMARK) should be adhered to when reporting prognostic factors studies (122, 127).

2.4 POPULATION-BASED STUDIES

A population-based cohort consists of either a sample or the entirety of a population, usually within a defined geographic region (128). By employing a population-based approach, estimates of disease frequency in the population can be obtained and it is valuable for assessing relations between exposures and outcomes (128). The primary justification for population-based studies is their high degree of generalizability (also referred to as external validity), i.e. the findings from the studies are applicable to the background population (128).
Nonetheless, there are issues that may limit generalizability. The main premise for external validity is that the study population is representative of the background population (128). Due to issues with recruitment and follow-up, study populations may differ from the background population with regard to important characteristics such as age, race and disease-specific features. However, this issue pertains mostly to estimates of disease frequency (128). When it comes to detecting associations, differences between the study and the background population are of less importance (128). In fact, it has been proposed that unbiased estimates of associations can be obtained even if the distribution of disease characteristics is not representative of the background population (128). It has been pointed out that it is more important with sufficient variation in characteristics in order to detect associations (128). For instance, within-country studies of salt intake show no effect on hypertension whereas between-country studies detect an impact of salt intake on hypertension (the example is adopted from Szlko [128]). The disparity is attributed to the fact that there is little variation in salt consumption within countries whereas it is considerable between countries. Similar reasoning can be applied to studies on prognosis of PC; studies limited to patients undergoing surgery will have limited variation in patient and disease characteristics, potentially resulting in underestimation of associations between variables and outcome.
PC warrants accurate determination of each individual patient’s risk of dying from the disease in order to optimize treatment stratification. Although several risk factors have been identified and incorporated into various nomograms, there is still a need for additional factors that improves prognostic discrimination. Several prognostic factors have been proposed in this regard, but we would claim that there are three limitations to the body of evidence regarding these factors:

1. There is variation among studies regarding the prognostic value of these factors.

2. Most studies use surrogate end-points such as PSA-relapse.

3. Most studies are limited to patients undergoing surgery or in some cases radiation therapy.

Prognostic factors based on histopathologic evaluation of diagnostic biopsy specimens have several advantages. Firstly, specimens are routinely available since the diagnosis of cancer cannot be made without it. Secondly, histopathology allows direct insight into the malignant disease and thereby reflects disease processes that influence prognosis. Thirdly, histopathologic data from diagnostic biopsy specimens are obtained without any additional cost or burden for patients or healthcare providers.

Thus, we considered that the present study was thoroughly justified as it aimed to be a prognostic factor study using histopathologic data from diagnostic specimens in a population-based cohort with PC-specific death as end-point.

The main purpose of the current study was to evaluate prognostic factors on diagnostic needle biopsy in a heterogeneous and population-based cohort using PC-specific death as endpoint.
Increasing awareness of the role of reactive stroma in PC pathogenesis, led us to test whether RSG could be used as a prognostic factor. Moreover, we aimed to test more known factors such as PNI, LVI and IDC-P. Given the interaction between these factors and the tumor stroma, we also addressed how this relationship influenced prognostic effect in order to generate hypotheses to explain the variability and uncertainty surrounding the predictive value of these factors.

CHAPTER 4 - SPECIFIC AIMS

1. To test whether RSG when evaluated in diagnostic prostate needle biopsies predicts prostate cancer-specific death in a population-based cohort of PC patients.

2. To test whether PNI when evaluated in diagnostic prostate needle biopsies predicts prostate cancer-specific death in a population based cohort of PC patients. Moreover, to assess the relationship between PNI and reactive stroma and how this relationship influences the prognostic effect of PNI. Additionally we tested how the percentage number of biopsy cores with PNI influenced prognosis.

3. To test whether LVI when evaluated in diagnostic prostate needle biopsies predicts prostate cancer-specific death in a population based cohort of PC patients. Secondly, to assess the relationship between LVI and reactive stroma and how this relationship influences the prognostic effect of LVI.

4. To test whether IDC-P on diagnostic needle biopsy predicts PC-specific death. In addition we assessed the relationship between IDC-P and other adverse factors such as reactive stroma.
CHAPTER 5 - MATERIALS AND METHODS

5.1 STUDY POPULATION

5.1.1 AUST-AGDER COUNTY

Aust-Agder County is situated in the south-east part of Norway. Arendal is the largest city and the administrative center in the county. There are approximately 100,000 inhabitants in Aust-Agder County.

During the study period, Aust-Agder Central Hospital (also called Arendal Hospital) was the only hospital serving the population of Aust-Agder County. The urological department consisted of two urology consultants and a varying number of residents.

5.1.2 STUDY PARTICIPANT RECRUITMENT AND DATA ACQUISITION

The Cancer Registry of Norway receives and registers detailed information on all cancer cases in Norway (129). Medical doctors in Norway are legally obliged to send information on cancer cases, thus ensuring the completeness of the registry (129).

Through the Cancer Registry of Norway, all cases of PC in Aust-Agder County from 1980 to 1999 were identified and registered in a local quality assurance database. Clinical data were collected retrospectively by reviewing medical journals. Follow-up data were also available in the hospital charts for most patients. In the remaining cases, general practitioners had to be contacted in order to obtain follow-up information. Accordingly, the quality assurance database, henceforth referred to as the Aust-Agder Prostate Cancer Database, contains data on 1131 patients with follow-up until 31. December 2012. The database has been the basis for several publications (130-132).

When initiating the present study, we found it necessary to limit the study to patients diagnosed by needle-biopsy. The rationale being that we wanted the data to be applicable on contemporary PC patients, which is mainly diagnosed by needle biopsies (7). It is of interest
to determine whether a prognostic factor is independent of other well-established factors. Thus, multivariable analysis with adjustment for PSA, GS and clinical stage is mandatory in a study such as the present. Hence, we restricted the study to the time PSA-measurements were available in Aust-Agder County, which is from 1991. We also applied a third limitation; exclusion of patients with highly probable metastatic disease (M1 or Mx and PSA ≥100 ng/mL[133]). This limitation requires further elaboration since one can either make the argument that these patients should have been included or the opposite argument that more strict criteria should have been applied. We cannot claim that our criteria effectively exclude all cases with metastatic disease, since there are patients with likely lymph node metastasis, circulating tumor cells and even undetected skeletal metastasis included in the cohort.

Conversely, a recent study demonstrated that a substantial number of patients with PSA ≥ 100 ng/mL do not have metastatic disease, thus suggesting that our criteria of Mx and PSA ≥ 100 ng/mL may exclude patients with non-systemic disease (134). We would argue that the criteria we have applied are ones that with high likelihood identify patients with metastatic disease and usually these patients have a dismal prognosis (133,134). Accordingly, we would make the argument that these patients constitute a separate clinical entity which is subject to other considerations compared to patients with localized, locally advanced or uncertain metastatic disease. The former is considered beyond cure and only life prolonging measures are available, whereas a notable proportion of the latter group may be considered for curative therapy. Hence, we concluded that that these groups should be studied separately.

We did not impose any restrictions according to lymph node status. The reason was that accurate lymph node status, which is based on lymphadenectomy, is not available at the time of diagnosis and is only obtained in selected patients. Hence, lymphadenectomy status is not a commonly employed prognostic factor upon diagnosis and multivariable adjustment for lymph node status cannot be considered mandatory in our study.
A total of 623 patients were diagnosed with PC in Aust-Agder County between 1991 and 1999. Out of these, 318 patients fulfilled the inclusion criteria. In 20 cases, the pathology specimens could not be located for re-examination. Furthermore, 12 patients were found to have no signs of cancer in their biopsies upon reexamination. Two patients declined participation in the study. Thus, 284 patients were finally included.

The primary endpoint of the study was death from PC. Determining cause of death is difficult and is subject to misclassification bias (135). Well-designed prospective trials frequently employ independent committees of experts as the gold standard in this regard (135). The cause of death may be determined through the use of a flow-chart and consensus between the committee members is necessary (136). In the present study, the evaluation of cause of death was performed by a urologist with extensive clinical and research experience (Gudmund Waaler). The cause of death was based on data extracted from medical charts. In cases of uncertainty, the cause of death was determined in cooperation with two other urologists. A death was registered as having been caused by PC when there was documented hormone-refractory metastatic disease with biochemical and/or clinical progression despite treatment and no other probable unrelated cause of death. The researchers involved in determining cause of death were blinded to the pathology review. The stringent definition of PC-specific death in the present study may misclassify patients as not dying from PC. Since the researchers were blinded to the pathology review, this potential misclassification bias is most likely non-differential (137). Thus, estimates could be biased towards null, resulting in an underestimation of the effect on PC-specific death (137).
5.1.3 CHARACTERISTICS, MANAGEMENT AND DISEASE COURSE OF THE STUDY POPULATION

The following sections aim to describe the study cohort of 284 patients. However, in certain segments we have included data from the Aust-Agder Prostate Cancer Database in order to highlight aspects of PC epidemiology in Aust-Agder County that are essential for comprehending the study cohort.

The patients in the study cohort were referred from the community physician based on opportunistic PSA screening, urinary tract symptoms or generalised disease. One-hundred and nine (38%) patients were referred without any symptoms, 163 (57%) patients had urinary bother at the time of diagnosis and 11(4%) had general symptoms.

Serum PSA – testing became available as a tool for early detection of PC in Aust-Agder County in 1991. At the time, high quality data on the effect of screening was lacking (138). The recommendation of the Norwegian health authorities was to abstain from testing in asymptomatic men (139). Nonetheless, this view did not harmonize with the recommendations made by the American Cancer Society and the American Urological Association, which favored a more aggressive approach with testing in men 50 years of age and older as part of an annual prostate exam (138). Consequently, discrepant views existed within the Norwegian medical community, resulting in variations between counties regarding the extent of PSA-testing (139). In 1994, the Urological department of Arendal Hospital issued a letter to the general practitioners in the county, recommending PSA - testing as part of a general a health check up, evaluation of symptomatic BPH and in patients with close relatives diagnosed with PC (140). PSA- testing was reserved for patients aged 50-70 years with a life expectancy > 10 years (40-70 years if family history of PC). Age-specific reference values were used according to Oesterling et al (141). A survey performed in 1999 demonstrated that opportunistic PSA-screening was performed more frequently in Aust-Agder
than in other Norwegian counties and Aust-Agder had one of the highest incidences of PC in Norway in the period 1992-1996 (139, 142). As can be seen in Figure 3, the number of cases with PC increased from the end of the 1980s and during the 1990s. A particularly steep increase is noted after 1994, coinciding with the urological department of Arendal Hospital encouraging general practitioners to use PSA-testing. Thus, the greatest increase occurred after the introduction of PSA, which is in accordance with other studies (143).

FIGURE 3

*The incidence of prostate cancer in Aust-Agder County from 1986-1999. Total number of cases and the percentage distribution of clinical stage T1a/b-T3. Based on data extracted from the Aust-Agder Prostate Cancer Database. 5-years moving averages to smooth the lines.*
The diagnostic work-up consisted of PSA measurement, digital rectal examination, transrectal ultrasound, transrectal biopsies and radionuclide bone scan.

Clinical stage was assigned according to the 1992 International Union Against Cancer TNM classification (144). Table 1 details the distribution of age, PSA-level, stage, tumor grade and primary treatment modality.

**TABLE 1**

*Characteristics of the study cohort*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients (%)</th>
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<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>28(10%)</td>
</tr>
<tr>
<td>60-69</td>
<td>87(30%)</td>
</tr>
<tr>
<td>70-79</td>
<td>118(42%)</td>
</tr>
<tr>
<td>≥80</td>
<td>51(18%)</td>
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<tr>
<td><strong>PSA (ng/mL)</strong></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>61(22%)</td>
</tr>
<tr>
<td>10-&lt;20</td>
<td>84(30%)</td>
</tr>
<tr>
<td>≥20</td>
<td>133(47%)</td>
</tr>
<tr>
<td>Missing</td>
<td>6(2%)</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
<td></td>
</tr>
<tr>
<td>T1c</td>
<td>54(19%)</td>
</tr>
<tr>
<td>T2</td>
<td>56(20%)</td>
</tr>
<tr>
<td>T3</td>
<td>174(61%)</td>
</tr>
<tr>
<td><strong>Lymph node status</strong></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>93(33%)</td>
</tr>
<tr>
<td>N1</td>
<td>6(2%)</td>
</tr>
<tr>
<td>Nx</td>
<td>185(65%)</td>
</tr>
<tr>
<td><strong>Metastasis status</strong></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>253(89%)</td>
</tr>
<tr>
<td>Mx</td>
<td>31(11%)</td>
</tr>
<tr>
<td><strong>Primary treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Radical prostatectomy</td>
<td>39(14%)</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>34(12%)</td>
</tr>
<tr>
<td>Androgen deprivation therapy</td>
<td>98(34%)</td>
</tr>
<tr>
<td>Watchful waiting</td>
<td>110(39%)</td>
</tr>
<tr>
<td>Other</td>
<td>3(1%)</td>
</tr>
</tbody>
</table>

PSA: Prostate - specific antigen
A considerable number of patients in the study cohort had locally advanced disease (clinical stage T3). When comparing the distribution of clinical stages in the Aust-Agder Prostate Cancer Database with similar published datasets from the same time period, one will notice that the number of T1-tumors in Aust-Agder County was comparable to other populations (145-147). Table 2 shows that in 1996 the number of T1-tumors in Aust-Agder County was comparable to that of the Swedish Prostate Cancer Registry in 1996 (146). However, there is a peculiar discrepancy regarding the distribution of T2 and T3/T4 tumors, suggesting that the latter occurred more frequently in Aust-Agder (146). The pattern remains the same when comparing with other datasets from Sweden and the Netherlands from the same time period (145, 147). As can be seen in Figure 3, the incidence of T3-tumors began to rise at the end of the eighties and it coincided with a decrease in the number of T2-tumors. A possible explanation could be a shift from a tendency to classify palpable nodules as organ-confined to extracapsular. This development occurred after the introduction of surgical treatment of PC at Arendal Hospital in 1985 (148). Following the introduction of surgical treatment, detailed pathological review of prostatectomy specimens became available, which led to the realization that many cases considered to be organ-confined were indeed pathological T3 and accompanied by high rates of positive surgical margins (140, 148). It is unlikely that the high frequency of T3 is due to higher rates of biologically aggressive PC in Aust-Agder or due to delayed detection of PC since the number of T1-tumors in comparable to other populations and a tradition for early detection of PC was well-established in the county. Thus, in Aust-Agder County there were a high number of PC cases considered as T3-tumors. This is then reflected in the study cohort. Moreover, when applying the criteria established for the study, a substantial number of patients from the population (including all cases with T1a/T1b) were excluded, resulting in an accentuation of the skewed distribution of T-stages. Not surprisingly, the number of cases with stage T1c increased during the study period which is
consistent with other reports from this period and reflects the increasing use of PSA-testing (143).

TABLE 2

*Data extracted from the Aust-Agder Prostate Cancer Database

**the Swedish National Prostate Cancer Registry in 1996 included four of six regions in Sweden: the north, south-east, south and Uppsala/Örebro regions (Sandblom et al)

Almost 90% of the patients in the study cohort underwent bone scan and showed no signs of metastatic disease. The remainder of the patients (31 cases) had a PSA-level < 100 ng/mL. According to Rana et al, a PSA greater than 100 ng/mL has a predictive value of 100% and correctly identifies 83.3% of cases with metastatic disease (133). It is not unlikely that there are patients within the Mx and PSA <100 ng/mL group that indeed had metastatic disease at the time of diagnosis. Twenty-six percent (10 cases) of these patients had a PSA-level ≤ 10 ng/mL, which according to Haukaas et al would suggest that it is unlikely that these patients had metastatic disease (149). An additional 6 cases had PSA level ≤ 20 ng/mL, which also
implies a low probability of metastatic disease (149). A remainder of 10 patients had PSA >20 ng/mL and the metastatic status of these patients could be considered uncertain. Nonetheless, a recent study by Stattin et al found that one-fourth of men with PSA \( \geq 100 \) ng/mL do not have metastatic disease (134).

The primary treatment modalities were RP, RT, ADT or WW. Radical prostatectomy was performed with an open retropubic approach at Arendal Hospital. Neoadjuvant or adjuvant ADT was not employed in conjunction with surgical treatment. Patients selected for RT were referred to the Norwegian Radium Hospital. The present study did not register the details of RT. Until early 1996, RT was usually administered as monotherapy with radiation doses of 66-70 Gy using a four-field box technique (150). Four of our patients received RT within this time frame. From 1996, patients usually received neoadjuvant and adjuvant ADT in addition to a standard radiation dose of 70 Gy applied with 3D-CRT (34, 151). Patients considered for curative therapy, either surgery or radiation, underwent staging lymphadenectomy and were excluded from curative treatment if nodal positive disease (34, 150, 148). As can be seen in Table 1, a total of 74 patients were initially managed with active curative treatment, constituting approximately one-quarter of the study cohort. Of the remainder, comparable fractions were managed expectantly or hormonally. Figure 4 depicts the percentage distribution of treatment options according to year of diagnosis. As can be seen, there was a steep increase in the use of curative treatment options coinciding with the increase in T1c cases. The use of WW remained fairly stable throughout the study period, whereas the use of primary ADT declined.
Table 3 describes the characteristics of the patients according to primary treatment modality. Surgery was usually offered to younger patients with localized and low grade disease. Patients receiving RT were slightly older and with more advanced disease. Hormone therapy was offered to elderly patients with advanced and high grade disease. Watchful waiting was also commonly opted for in elderly patients, however; this category of patients span from localized disease with low GS to advanced high-grade disease.
### TABLE 3

**Patient characteristics of the study cohort stratified by primary treatment**

<table>
<thead>
<tr>
<th></th>
<th>RP (Age, median(IQR), years)</th>
<th>RT (Age, median(IQR), years)</th>
<th>ADT (Age, median(IQR), years)</th>
<th>WW (Age, median(IQR), years)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age, median(IQR), years</strong></td>
<td>59(54-65)</td>
<td>66(62-69)</td>
<td>77(70-81)</td>
<td>73(70-78)</td>
</tr>
<tr>
<td><strong>PSA median(IQR), ng/mL</strong></td>
<td>18.1(6.6-12.6)</td>
<td>15.7(12.2-26.4)</td>
<td>32.3(17.2-54.9)</td>
<td>18.3(10.5-31.9)</td>
</tr>
<tr>
<td><strong>Clinical stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>T1c</strong></td>
<td>23(59%)</td>
<td>4(12%)</td>
<td>6(6%)</td>
<td>20(18%)</td>
</tr>
<tr>
<td><strong>T2</strong></td>
<td>16(41%)</td>
<td>2(6%)</td>
<td>7(7%)</td>
<td>30(27%)</td>
</tr>
<tr>
<td><strong>T3</strong></td>
<td>0</td>
<td>28(82%)</td>
<td>85(87%)</td>
<td>60(55%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>39</td>
<td>34</td>
<td>98</td>
<td>110</td>
</tr>
</tbody>
</table>

IQR: Interquartile range, RP: Radical prostatectomy, RT: Radiotherapy curative, ADT: androgen deprivation therapy, WW: Watchful waiting

(A similar table is presented in Paper I)

Figure 5 details a flowchart of primary treatment modality and progression to secondary modalities. As can be seen, only a small number of the patients that were initially managed conservatively were later offered curative treatment. The limited use of curative treatment can to some extent be attributed to the fact that a notable proportion of the patients were fairly old with advanced disease. At the time, there were limited data on the effect of curative treatment for PC (152). Moreover, surgery was considered an option primarily for localized PC, whereas RT could be considered both in localized and locally advanced disease (152).
FIGURE 5

Flowchart of treatments within the study cohort

Cohort (n=284)

- RP (n=39)
  - Salvage RT (n=17)
  - ADT (n=6)

- RT (n=34)
  - Other (n=1)
  - ADT (n=12)

- ADT (n=98)
  - RP (n=1)
  - RT (n=1 curative/n=4 palliative)

- WW (n=109)
  - RT/RP (n=1/n=2)
  - ADT (n=75)

- Other (n=3)
  - RT (n=1)
  - ADT (n=1)

RP: Radical prostatectomy, RT: Radiotherapy curative, ADT: androgen deprivation therapy, WW: Watchful waiting

(A similar figure is presented in Paper I-III)
Table 4 compares the distribution of treatment options in Aust-Agder County in 1996 with that reported by Sandblom et al from the Swedish Prostate Cancer Registry (146). It appears that the use of various treatment modalities were relatively similar to Sweden.

**TABLE 4**

*Distribution of treatment options in Aust-Agder County and the Swedish National Prostate Cancer Registry in 1996*

<table>
<thead>
<tr>
<th>Treatment option</th>
<th>Aust-Agder county 1996*</th>
<th>Swedish National Prostate Cancer Registry 1996 **</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curative</td>
<td>15.2%</td>
<td>10.9%</td>
</tr>
<tr>
<td>WW</td>
<td>43.0%</td>
<td>34.1%</td>
</tr>
<tr>
<td>ADT</td>
<td>35.4%</td>
<td>53.5%</td>
</tr>
</tbody>
</table>

*Data extracted from the Aust-Agder Prostate Cancer Database

**Swedish National Prostate Cancer Registry in 1996 included four of six regions in Sweden: the north, south-east, south and Uppsala/Orebro regions (Sandblom et al)

WW: Watchful waiting, ADT: Androgen deprivation therapy

The patients were initially followed by a urologist at regular intervals - usually at 3-6 months - and thereafter by their general practitioner in cooperation with the hospital. The median (IQR) observation time was 109(51-171) months (approximately 9 years). At the end of the follow-up, 203 patients were registered as dead. We determined that 58 deaths were attributable to PC. Post et al reported on a population-based cohort of PC patients diagnosed with PC stage T1-T3 and M0 from 1993-95 in the Netherlands and found a 3-year overall survival of 85% (147). Similarly, the 3-year overall survival in our study cohort was 84%. Fall et al used the Regional Prostate Cancer Registry in the South-East region of Sweden to identify 5980 PC patients who were diagnosed between 1987 and 1999 and deceased by the
end of 2003 (153). They reported that according to the Swedish Death Register and the Regional Prostate Cancer Registry, the cause of death was PC in 48.2% and 44.9% of the cases, respectively (153). When we perform the same data extraction from the Aust-Agder Prostate Cancer Database, we identify 508 men who were diagnosed between 1987 and 1999 and deceased by the end of 2003. We found that of these patients, 39.8% (202 cases) were dead due to PC. Thus, the numbers of PC deaths in our study is somewhat lower, but appears to be comparable to that of a Swedish population from the same time period. As mentioned previously, there is a possibility of underreporting of PC-deaths in the current study due to its stringent cause of death - criteria.

5.2 PATHOLOGY

5.2.1 PATHOLOGY EVALUATION

During the study period, histopathological specimens obtained at Arendal Hospital were transported to the Norwegian Radium Hospital in Oslo for evaluation. At the time, the grading of specimens was based on the World Health Organization (WHO) classification of prostatic tumors proposed by Mostofi et al (154). The grading reflects the degree of nuclear anaplasia and glandular differentiation ranging from I (well differentiated), II (moderately differentiated) and III (poorly differentiated). Table 5 depicts the distribution of WHO–grades within the Aust-Agder Prostate Cancer Database compared to patients in the Swedish National Prostate Cancer Registry (146). It appears that the distribution of histological grades in Aust-Agder was similar to that of a comparable Swedish population. Following processing and evaluation, the specimens were stored at the Norwegian Radium Hospital.

When initiating the present study, histopathological specimens from the patients included in the study cohort were retrieved and re-evaluated. The re-evaluation was performed by two dedicated uropathologists at the Norwegian Radium Hospital blinded to the clinical data. The conclusion of the histopathologic evaluation was based on consensus between the examining
uropathologists. The Norwegian Radium Hospital is a tertiary referral center in Norway dedicated to the management of malignant diseases. The pathology department has the expertise to provide central pathology review. Thus, the pathology evaluation in the present study ensures unified diagnosis and classification (155).

### TABLE 5

**Distribution of World Health Organization grades within Aust-Agder County and the Swedish National Prostate Cancer Registry in 1996**

<table>
<thead>
<tr>
<th>WHO-grade</th>
<th>Aust-Agder county 1996*</th>
<th>Swedish National Prostate Cancer Registry in 1996 **</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>25.3%</td>
<td>27.5%</td>
</tr>
<tr>
<td>II</td>
<td>53.2%</td>
<td>43.2%</td>
</tr>
<tr>
<td>III</td>
<td>21.5%</td>
<td>23.8%</td>
</tr>
</tbody>
</table>

*Data extracted from the Aust-Agder Prostate Cancer Database

** the Swedish National Prostate Cancer Registry in 1996 included four of six regions in Sweden: the north, south-east, south and Uppsala/Örebro regions (Sandblom et al)

WHO: World Health Organization
5.2.2 BIOPSIES

The number of biopsy cores obtained per patient in the study cohort ranged from 1 to 10, with a median of 4 biopsy cores per patient. Obtaining up to 10 biopsy cores occurred in some cases, but as shown in the histogram in Figure 6 this was rare.

FIGURE 6

*Histogram of the number of biopsy cores per patient*

However, as can be seen in Figure 7, the median number of biopsy cores increased up to 6 in the later years of the study period. Moreover, as seen in Figure 8, a low median number of biopsy cores was more common in cases with palpable findings. The median number of biopsies was 6 in cases with only an elevated PSA-level.
The sextant systematic biopsy scheme was introduced in 1989 and considered standard practice in Norway by the end of the nineties (156, 139). In the current study, the number of biopsies was at the discretion of the urologist and beyond the influence of the investigators due to the retrospective nature of the study. It was not registered whether the urologist...
performing the biopsies opted for a systematic approach such as the sextant scheme or used a more directed approach towards palpable or ultrasonic findings. It is plausible that a more direct approach was more common early in the study period, which was characterized by more frequent cases with palpable disease. In contrast, later in the study period a sextant approach would likely become more common due to increasing number of patients without guiding findings.

5.2.3 PREPARATION
All specimens were fixed in 10% buffered formalin, paraffin-embedded, cut at 4-μm thickness, and routinely stained with H&E. The histopathological evaluation was based solely on morphological assessment. Supplemental analyses with immunohistochemical staining were not performed.

5.2.4 GLEASON GRADING
Since the initial grading was based on an obsolete system, the specimens were re-graded using the Gleason system as described by the 2005 International Society of Urological Pathology Consensus Conference on Gleason Grading of Prostate Carcinoma (66).

Table 6 details the concordance between the original WHO-grade and the GS based on pathology re-evaluation. It is notable that 16% of patients with low WHO-grade were upon review considered as to have a GS equal to or higher than 4+3=7b.
**TABLE 6**

*The concordance between Gleason score and World Health Organization-grade*

<table>
<thead>
<tr>
<th>Gleason score</th>
<th>WHO I</th>
<th>WHO II</th>
<th>WHO III</th>
</tr>
</thead>
<tbody>
<tr>
<td>3+3=6</td>
<td>38(42%)</td>
<td>9(6%)</td>
<td>0</td>
</tr>
<tr>
<td>3+4=7a</td>
<td>38(42%)</td>
<td>39(26%)</td>
<td>3(7%)</td>
</tr>
<tr>
<td>4+3=7b</td>
<td>3(3%)</td>
<td>26(17%)</td>
<td>2(5%)</td>
</tr>
<tr>
<td>≥8</td>
<td>12(13%)</td>
<td>78(51%)</td>
<td>36(88%)</td>
</tr>
<tr>
<td>Total</td>
<td>91</td>
<td>152</td>
<td>41</td>
</tr>
</tbody>
</table>

WHO: World Health Organization

---

### 5.2.5 REACTIVE STROMAL GRADING

The RSG was evaluated on the basis of a scoring system previously described by Yanagisawa et al (78). In brief, RSG was evaluated according to the percentage of reactive stroma/epithelium ratio as follows, grade 0, 0 up to 5% reactive stroma; grade 1, 6 to 15%; grade 2, 16 to 50%; grade 3, 51 to 100%. RSG was evaluated within the tumor area with the highest GS in the biopsy set. The highest RSG found in that tumor focus was reported and the specimen was categorized accordingly. There are some discrepancies between the evaluation of RSG in the present study and that of other studies. Firstly, prior reports have used the largest lesion as the basis of RSG evaluation (78). Secondly, we considered absence of reactive stroma as RSG 0 regardless of other tumor characteristics. In contrast, Yanagisawa et al stated that small foci of Gleason grade 3 without stroma was to be considered as RSG 1 (78). Thirdly, we found that high GS tumors could have varying amount of reactive stroma whereas previous reports found that these tumors were exclusively RSG 0 (78).
5.2.6 PERINEURAL INVASION

PNI was defined as growth of cancer in the surrounding perineural space of nerves. This definition of PNI is in accordance with Liebig et al. According to Liebig et al PNI is “tumor cell invasion in, around and through the nerves” (157). Although other more stringent criteria have been proposed, these might classify obvious cases of PNI as false negative (157). The presence or absence of PNI was registered for the entire biopsy set for each patient. In addition we registered the number of biopsy cores with PNI per biopsy set and used this to calculate the percentage of biopsy cores with PNI according to the following formula: (Number of biopsy cores with PNI/Total number of biopsy cores obtained within that biopsy set)*100. Immunohistochemical staining for S-100 may be applied to detect nerves and assess PNI (158). However, this method is not routinely employed and there is data indicating that routine examination with H&E is sufficient (159). It has been proposed that studies on PNI should restrict analyses to cases in which nerves are detected, since cases without any nerves present may be false negative (160). However, other researchers claim that absence of PNI represents true absence of PNI (159). Regardless, in most studies and clinical practice, the absence of PNI on biopsy is considered as true negative and the presence of nerves is not routinely accounted for.

5.2.7 LYMPHOVASCULAR INVASION

LVI was defined as tumor cells within an endothelial-lined structure. Immunohistochemical analyses to distinguish between blood and lymphatic vessels were not performed. It is not considered necessary to determine the nature of the invaded vessel and therefore immunohistochemistry in this regard is not warranted (161). Although LVI is routinely identified with the use of H&E staining, there are potential mimickers of LVI that must be considered. Especially retraction artifacts, which occur frequently in prostatic specimens, may mimic LVI (161). Other mimickers include displaced benign or malign epithelium within a vascular lumen, cancer glands in atrophic ducts and myofibroblast in large thrombosed
vessels (161). There is a risk of overestimating the incidence of LVI since the number of foci mimicking LVI may outnumber the number of actual LVI foci (161). However, Kryvenko et al showed that meticulous evaluation of H&E slides by experienced pathologists is reliable and immunohistochemical analyses are usually not necessary (161).

5.2.8 INTRADUCTAL CARCINOMA OF THE PROSTATE

IDC - P was evaluated morphologically according to Epstein criteria. The main criterion is malignant cells filling large acini and ducts with a preserved basal cell layer (109). Additional criteria are as follows; 1) a solid or dense cribriform pattern, or 2) loose cribriform or micropapillary pattern with either marked nuclear atypia or non-focal comedonecrosis (109). Immunostaining against HMWCK or p63 to evaluate the presence of basal cells was not performed. Thus, the presence of basal cells was evaluated on the basis of routine H&E stained sections. The differential diagnoses of IDC-P include cribriform acinar adenocarcinoma, ductal carcinoma, intraductal spread of urothelial carcinoma and HG-PIN (109). Immunostaining for basal cells may be particularly useful when distinguishing IDC-P from cribriform adenocarcinoma. According to Guo & Epstein, assessment of basal cells can be performed on H&E sections (109). However, in their study many cases of IDC-P would have been misclassified as cribriform adenocarcinoma without additional immunostaining (109). Distinguishing IDC-P from ductal carcinoma and urothelial carcinoma spreading intraductally within the prostate may be aided with the use of immunohistochemistry. However, the morphological features of these entities are more distinct and identification without immunostaining can be done more readily (109). The most critical differential diagnosis is HG-PIN. IDC-P signifies invasive carcinoma whereas HG-PIN is a benign precursor lesion not warranting treatment (111, 7). To make the distinction between IDC-P and HG-PIN, the pathologist relies on morphological criteria since the basal cell layer is preserved in both entities (109). Expression of ERG and loss of PTEN can be used to
differentiate between IDC-P and HG-PIN. However, these ancillary investigations are not used in routine practice (111).

5.3 STATISTICAL ANALYSIS

5.3.1 SAMPLE SIZE

We had little influence on sample size due to design of the study, meaning that we had to settle with the number of patients that were diagnosed in our geographic region during the study period. However, assuming an alpha of 0.05 and 80% statistical power, we calculated that a hazard ratio (HR) >2.0 in Cox regression analyses would become statistically significant.

The critical aspect in this regard is that a true effect with a HR less than 2.0 would be rendered statically non-significant in our study and thereby falsely concluding that there is no negative prognostic effect, i.e. type II error. A counterargument could be that effects with a HR less than 2.0 are so small that they are not clinically significant. However, the HR is a relative measure and is difficult to translate to clinically meaningful terms (162). Therefore, one cannot make definitive statements on whether a HR is clinically significant or not.

One could argue that inclusion of more patients would have been feasible by including patients diagnosed after 1999. However, reorganization of the Norwegian health system in accordance with the Hospital Act of 2001 and the Norwegian Hospital Reform of 2002 resulted in changes in the delivery of specialized medical services to Aust-Agder county (163). Consequently, it would not be feasible to obtain detailed data and central pathology review on all PC patients within Aust-Agder county following the millennial shift.
5.3.2 ASSESSMENT OF ASSOCIATIONS

We evaluated whether the prognostic factors investigated were associated with other known adverse features such as PSA-level, clinical stage and GS. Mainly the Pearson’s Chi-squared test was applied to test for differences between categorical variables. We also applied the Spearman’s rank order correlation coefficient to obtain a measure of correlation between discrete ordinal variables.

5.3.3 SURVIVAL ANALYSIS

For survival analysis, time to event was between the date when the prostate needle biopsy was taken and the date of death from PC. The Kaplan-Meier method and the log rank test were applied to evaluate survival rates. Univariable and multivariable Cox proportional hazards regression analyses were performed to obtain HRs with 95% confidence intervals. Log-log plots were used to assess the assumption of proportional hazards. Patients with incomplete follow-up (n=3) were censored at the last date of observation and patients with no follow-up information were excluded from survival analyses (n=3).

5.3.4 COMPETING RISKS

Patients who died from other causes than PC were treated as censored and the survival analyses were carried out using standard methods; Kaplan-Meier curves and Cox regression. The choice of these analytical procedures requires further elaboration since the study participants were subject to competing risks.

A straightforward survival analysis studies the time until an event occurs. The study subjects either experience the event or don’t. The latter are followed in the study, but when they ultimately become unavailable for further follow-up, they are censored (164). A crucial prerequisite in this regard is that the censoring is independent (164). This means that the patients who are censored should ideally have the same risk of experiencing the event as those who remain in the study.
still present in study. However, this criterion is not satisfied when the reason for censoring influences the risk of experiencing the event. For instance, in PC research, patients are ideally followed until they die from PC (the event) or are censored due to loss to follow-up or end of study. However, in PC research patients may die from other causes and these patients cannot meet the ideal criteria of having the same risk of the event as those still in the study; a patient cannot die from a heart attack and still be at risk of dying from PC. This is the concept of competing risk which is defined as follows “an event that either hinders the observation of the event of interest or modifies the chance that this event occurs” (164). Different analytical approaches may be taken to remedy the problem of competing risks in survival analysis and the two most common ones will be described briefly herein.

Cumulative incidence analysis accounts for all of the potential events that the study subjects may experience and the estimates for the events are dependent on each other (164). Thus, the occurrence of one event reduces the estimates of the other events. Cumulative incidence analysis can be depicted as incidence curves or modeled in regression analysis described by Fine and Gray in order to handle adjustment for other covariates (164).

A second approach is to focus on the event of interest and handle other competing events with censoring. In this approach one can use traditional Kaplan-Meier curves or Cox regression and simply censor patients that experience competing risks (164). Critics of this approach argue that since one does not account for competing events, the estimate of the event in interest is inflated (164). This approach is referred to as cause-specific (165).

The important question in a setting with competing risk is which approach to use. Dignam et al points out that “the different approaches may only illuminate one important aspect of the data while possibly obscuring others” (165). Thus, the choice of approach depends on the research question one intends to answer.
An illuminating example in this regard has been put forward by Chappell and will be referred here (166). One imagines a disease where 80% of those afflicted die within one year. Of those of who survive, 100% experience late toxicity by 3 years. Using a cumulative incidence approach the risk of death would be 80% and the risk of late toxicity 20% in a joint analysis. If one were to use a cause-specific approach, the risk of death would be 80% in a separate analysis with death as the event of interest. The risk of late toxicity would be 100% in another separate analysis with toxicity as event of interest and deaths being censored events. Neither of the estimates can be said to be wrong. If one is interested in obtaining data on the disease course with the purpose of counseling an afflicted patient, it is correct to say the risk of death and late toxicity is 80% and 20%, respectively (166). If one were interested in solely assessing the risk of late toxicity in successfully treated patients, it is correct to say that the risk of toxicity is 100% (166).

Dignam et al have elaborated further on this matter using various simulated scenarios. Accordingly, they concluded that if one is studying a prognostic factor and is interested in knowing whether this factor is indeed associated with the outcome of interest, the cause-specific approach is recommended (165). However, if one is creating a prognostic nomogram to counsel patients one their risk of dying from a disease when taking into account other possible events, a cumulative incidence approach is more useful (165).

Since the purpose of the present study was to examine whether various histopathologic factors were associated with PC-specific death, a cause-specific approach was considered appropriate.
In the Cox regression analyses, multivariable adjustment for other covariates that may influence the risk of PC-specific death was performed.

Univariable survival analysis tells the researcher whether factor A is associated with an outcome. However, this association may be due to the fact that factor A is also associated with factor B, which in turn is associated with the outcome. Factor B may be a mediator for the effect of Factor A on outcome, e.g. genomic mutations (A) cause high grade tumors (B) which in turn causes tumor-related death (167). On the other hand, factor B may be a confounder for factor A, e.g. metastatic PC (B) causes increased use of palliative radiotherapy (A) and increased risk of PC-death (outcome) leading to a statistical (but causally false) association between palliative radiotherapy and PC-death (167). In multivariable analysis, Factor B would render Factor A statistically insignificant (167). As mentioned in section 2.3, determining causal relationships is less important in prognostic research than in other forms of biomedical research. Thus, determining whether factor B is a mediator or confounder is irrelevant in a study such as ours. However, multivariable analysis serves an important function in prognostic research to determine whether the prognostic information obtained by a new factor under study is independent of other commonly applied factors. E.g. if factor B is already available and in clinical use whereas Factor A is an expensive and invasive new method, it would not be rational to implement factor A in clinical use since the same prognostic information is actually provided by factor B.

The number of PC-specific deaths in the present study was 58. A rule of thumb is that multivariable analysis should not include more than one covariate per 10 events (168). Thus, the multivariable analyses in the present study could include a maximum of 5-6 covariates. Since the purpose of the study was to assess the prognostic impacts of specific histopathologic factors individually, it was reasonable to build a multivariable model that accounted for the
The most commonly used prognostic factors. The intent of this study was not to build a model to be the basis of a prognostic nomogram. Hence, we did not remove or add covariates according to how well the model fitted the data. Rather covariates were added and removed to see how they influenced the histopathologic factor under scrutiny. However, the full model always included all covariates that were considered relevant a priori.

The covariates included in the model were PSA, clinical stage, GS and curative treatment. The three first covariates are the most commonly used prognostic factors and form the basis for risk stratifying PC patients (73). The cohort was not homogenous in terms of treatment modality. Curative treatment in the form of surgery or radiation has been proven to influence PC-specific mortality. Consequently, it was necessary to include curative treatment as a covariate (29, 34). We did not include adjustment for ADT since its effect on PC-specific mortality has not been clearly proven (169).

Initially, the a priori statistical analysis plan was to categorize the covariates as follows:

- PSA: continuous
- Clinical stage: T1c, T2, T3
- GS: 3+3=6, 3+4=7a, 4+3=7b, ≥8
- Curative treatment: Yes or no

However, this resulted in wide confidence intervals for each category which meant that the estimates for each category was shrouded by considerable uncertainty and made little sense (170). The basis for this uncertainty was few events relative to the number of categories.

Univariable analyses of the covariates showed that there were small variations in the risk of PC-specific death between some of the categories. If one takes GS as an example, there was
only a minor difference in risk between GS 7b and GS ≥8, whereas there was a great leap in risk from 7a to 7b. Thus, it had little bearing on prognosis whether the GS was 7b or ≥8, the most important distinction was between 7a and 7b. Hence, adjusting for GS ≥8 seemed needless and only resulted in imprecise estimates. Similar patterns were observed for the other covariates and justified the way we collapsed categories as shown below:

PSA: <10, ≥10

Clinical stage: T1c-T2, T3

GS: ≤3+4=7a, ≥4+3=7b

This resulted in estimates with higher precision (narrower confidence intervals) (170).

The overall purpose of a prognostic factor is implementation in a prognostic system which accurately determines whether a patient will experience an event of interest. The ability of a prognostic model to distinguish between patients who will have the event and those who won’t is referred to as discrimination and can be assessed with so-called C-statistics, such as the C-index proposed by Harrell (171, 172). Thus, when introducing a new prognostic factor it is of interest to determine whether it improves discrimination when added to an established prognostic system. In this regard we assessed whether RSG, which is a novel prognostic factor, increased the discrimination of the multivariable model by calculating Harrell’s C-index.

5.3.6 SUBGROUPS AND INTERACTIONS

In some circumstances, the effect of a prognostic factor or a treatment may vary between different patient groups. For instance, a new treatment may have effect in women, but no effect in men. This variation is referred to as “effect modification”, i.e. the effect of one variable is modified by another variable (173). A common, but erroneous, approach to test for
effect modification is to assess the effect within each category of the modifying variable (173). E.g. one tests whether the treatment has effect in women and then tests whether there is an effect in men. If the effect of the treatment is statistically significant in women and not in men, one might infer that there is an effect in women, but not in men. However, this is a fallacy since this approach really just tests whether the treatment has an effect and does not provide any data on the difference in the treatment effect between the sexes. Rather the correct approach is to include an interaction term in say a Cox regression model; treatment x sex (174).

Testing for effect modification is subject to pitfalls. Most importantly, studies usually lack power to obtain a statistically significant result in this regard (174). Secondly, which may appear somewhat contradictory to the first point, with an increasing numbers of subgroup analysis there is an increased risk of false positive results (174). Thus, subgroup/interaction analysis should be carried out according a pre-specified and biologically sound hypothesis (173).

In the present study we hypothesized that reactive stroma may influence the effect of LVI on prognosis and thus carried out a test of interaction between RSG and LVI. In this regard we also performed separate tests for the effect of LVI within the categories of RSG. This was not the basis of inference, but to illustrate the relationship between LVI and reactive stroma with regard to prognosis.
CHAPTER 6 - MAIN RESULTS

6.1 PAPER I

Reactive stroma could be assessed and graded in the diagnostic needle biopsies of 278 patients. The RSG was positively correlated with the patients’ PSA-level, clinical stage and GS. Moreover, the risk of dying from PC increased as the RSG increased. The effect of RSG on PC-specific death was independent of PSA-level, clinical stage, GS and curative treatment in multivariable adjustment. Furthermore, addition of RSG to the multivariable model resulted in increased predictive power.

6.2 PAPER II

Perineural invasion could be determined in 281 patients. The presence of PNI on diagnostic needle biopsy was associated with advanced clinical stage, high GS and intense RSG. Patients with PNI on their needle biopsies had poorer PC-specific survival compared to patients without PNI. The relationship between PNI and prognosis was independent of clinical factors such as PSA, stage and curative treatment in multivariable analysis. However, it was attenuated when adjusting for GS and RSG. When addressing PNI quantitatively, we found that a high percentage number of biopsy cores with PNI was strongly associated with death from PC independently of other prognostic factors.

6.3 PAPER III

Lymphovascular invasion on diagnostic needle biopsy could be evaluated in 283 patients. The presence of LVI was significantly associated with high PSA-level, locally advanced disease and high GS. There was a statistically significant association between the presence of LVI and RSG in the prostate needle biopsies. LVI was an independent and statistically significant predictor of PC-specific death. Moreover, there was a statically significant interaction
between LVI and high RSG. Thus, the already negative effect of LVI on PC-specific mortality was further enhanced if an intensely reactive stroma was present.

6.4 PAPER IV

Intraductal carcinoma of the prostate could be assessed in 283 patients. The presence of IDC-P was associated with increased risk of dying from PC independently of other adverse prognostic factors and curative treatment. Patients with IDC-P tended to have higher grade tumors with more reactive stroma compared to patients without IDC-P.
CHAPTER 7 - DISCUSSION

In the following sections, the main findings will be discussed in relevance to previous reports and with regard to clinical implications. Lastly, an elaboration on the strengths and limitations of the study will be given.

7.1 REACTIVE STROMAL GRADING

7.1.1 OUR FINDINGS COMPARED TO PREVIOUS REPORTS

In Paper I it was shown that RSG was associated with PC-specific death in such a manner that as the RSG increased so did the risk of dying from PC. This relationship was independent of traditional prognostic factors and RSG improved predictive power. Thus, we corroborated previous findings of RSG being related to the prognosis of PC patients and hence could be used as a prognostic tool. Although it has been shown by Ayala et al that the percentage amount of RSG 3 in RP specimens correlates with PC-specific death, our study is the first to demonstrate a relationship between reactive stroma and PC-specific death using the full grading system on biopsy specimens (79). However, there are some discrepancies between our study and previous reports. In the following sections, we will discuss our findings and that of Ayala et al, Yanagisawa et al, Billis et al and Wu et al (77, 78, 175, 176). To the best of our knowledge, these are the studies that have investigated the prognostic effect of RSG.

Firstly, one needs to address variations in the evaluation of RSG. Yanagisawa et al stated that Gleason grade 3 without any evaluable reactive stroma should be considered as RSG 1 (78). Moreover; they found that high GS tumors did not have any stroma and designated these tumors as RSG 0 (78). Furthermore, it appears that Yanagisawa et al considered RSG 0/3 to be high stromal grades and RSG 1/2 to be low stromal grades; “the highest stromal grade (RSG 0 or 3)…categorized the patient as having that RSG” (78).
We found it more reasonable that absence of reactive stroma should be designated as RSG 0, irrespective of the GS. Secondly, we also found variable amounts of reactive stroma among high GS tumors. Lastly, we evaluated the RSG in the tumor with the highest GS.

Both we and Billis et al found that RSG 0 was a common finding, occurring predominantly in low GS tumors (175). In contrast Ayala et al, Yanagisawa et al and Wu et al found RSG 0 mainly in high GS tumors and it was an infrequent finding in the two former studies (only one patient with RSG 0 in the study of Yanagisawa et al) (77, 78, 176).

The observation of various stromal grades within the high GS category in our study and that of Billis et al falsifies the claim that high GS tumors do not have a reactive stromal component (175). The fact that two independent studies on two different populations report a similar finding, strengthen the validity of the finding. However, Wu et al reported that although a stromal component was lacking in the tumoral area, the peritumoral areas of high GS tumors showed various stromal reaction grades (176). Thus, one could claim that we and Billis et al have misinterpreted what constitutes the stroma of high GS tumors and inadvertently reported the stromal grade of the peritumoral stroma. However, Billis et al pointed out specifically that their evaluation of RSG was performed on the stroma surrounding the tumor (175). Moreover, in Figure 9 we show a high GS tumor with clearly abundant reactive stroma in the immediate surroundings of the tumor cells.
The discrepant findings regarding stromal grades open up for one of the main criticisms raised against RSG; the subjective and uncertain nature of semiquantitatively assigning a percentage based grade. When discussing this matter it is valuable to compare the distribution of stromal grades between Yanagisawa et al and Billis et al since these studies are based on similar materials and represent fairly similar populations, i.e. needle biopsies stained with H&E and obtained from patients treated with RP (78, 175). One would expect that these two studies had relatively similar distributions of stromal grades. This expectation holds true for RSG 3 and RSG 2 if one look at the graphical distribution in Figure 10. However, the occurrences of RSG 0 and RSG 1 are virtually inverted when comparing the two studies. Thus, it appears that the distinction between RSG 0 and RSG 1 may be subject to variations. RSG 0 is defined as 0-5% reactive stroma and RSG 1 as 6-15% reactive stroma. It is reasonable to imagine that distinguishing between these two categories is subject to interobserver variation. Regarding the distribution of RSG 0 and RSG 1, it should be remembered that Yanagisawa et al designated low GS cancers with no stroma as RSG 1 instead of RSG 0 (78). Billis et al made no comment on whether they adhered to this rule (175).
Ayala et al and Yanagisawa et al found that RSG 0 and RSG 3 were associated with poor prognosis whereas RSG 1 and RSG 2 represented a favorable outcome (77, 78). Billis et al found a relationship between RSG and prognosis that was similar to that reported in our study, i.e. the risk of progression increases as the RSG increases (175). It is a possible scenario that in our study and Billis et al the RSG 0 category contained several low GS tumors. In contrast, Yanagisawa et al and Ayala et al would have had more high GS cases in the RSG 0 category and more low GS cases in the RSG 1 category. Given the strong influence of GS on prognosis, a discordant distribution of GS within the RSG categories could contribute to the discrepant findings regarding the prognostic impact of RSG.

It should be noted that our study is the only study that evaluated RSG in the tumor with the highest GS, whereas other studies used the most extensive carcinoma as basis for evaluating RSG. This might explain the correlation between RSG and tumor grade observed in our study. However, our findings are fairly comparable to that of Billis et al, which suggests that our approach did not bias results. Of the patients included in Paper I, 122 (44%) had a tumor
focus in addition to that with the highest GS. Out of these patients, 46(26%) had a carcinoma focus that was more extensive than that with the highest GS. Thus, only a limited number of patients could have been incorrectly (if one considers the approach applied in the other studies as gold standard) evaluated with regard to RSG.

We would argue in favor of the approach and findings reported in our study since they reflect a biologically sound relationship between the RSG and outcome. i.e. the prognosis becomes worse as the amount of reactive stroma increase. Sleeman et al proposed a concept termed “the stromal progression model” which aimed to incorporate current observations on tumor development and progression in a unified theory (177). They suggested that tumor and the surrounding stroma co-evolve; the tumor undergoes genetic and epigenetic changes which are paralleled by stromal changes such as extracellular matrix remodeling and influx of CAFs and TAMs (177). Without any parallel evolution of the stroma, the tumor is not viable and unable to metastasize (177). It is precisely the stromal changes described by Sleeman et al which is morphologically described as reactive stroma on histopathologic specimens. Thus, it stands to reason that a low amount of reactive stroma should correspond to a well-differentiated tumor with good prognosis and vice versa. However, it should be taken into consideration that Ayala et al and Wu et al postulated that high GS tumors become stromal independent and thus these tumors will have absent stromal reaction (77, 176).

In conclusion, it is becoming increasingly apparent that reactive stroma is associated with the prognosis of PC patients. Particularly, the occurrence of RSG 3 appears to be consistent across studies and unequivocally associated with poor prognosis. However, it is also clear that further studies are needed to investigate the conflicting aspects of RSG.
In contrast to previous studies, there were a considerable number of patients managed with ADT in our study. The AR is expressed both in epithelial and stromal cells of the prostate (178). The development of the prostate is dependent on androgen signaling through stromal AR positive cells (178). However, during carcinogenesis there is a gradual loss of AR in stromal cells as the cancer progresses (179). It appears that in PC, epithelial PC cells rely mainly on autocrine dependent androgen signaling (179). Myofibroblasts, which are a principal component of reactive stroma, can be AR negative or AR positive. Both AR negative and AR positive myofibroblasts promote cancer growth in the absence of androgens (179). However, when androgens are present and interact with AR positive myofibroblasts, these cell produce paracrine factors that inhibit tumor growth (179). Thus, it is reasonable to hypothesize that high reactive stromal grade tumors have abundant myofibroblasts, these myofibroblasts may respond to androgen signaling in a fashion that inhibits tumor growth (this could be viewed as a counterweight to the tumor promoting effect of epithelial AR signaling). If patients with high reactive stromal grades are managed with ADT, they may lose this counterweight leading to accelerated tumor progression. Thus, one could claim that the use of ADT in this cohort may have accentuated the negative impact of reactive stroma on prognosis. If this relationship between reactive stroma and response to ADT is confirmed in clinical studies, one could postulate that reactive stroma assessment could be used to stratify metastatic PC patients for hormonal treatment, i.e. patients with low RSG could respond favorably to ADT whereas patients with high RSG should not be managed with ADT. Possibly, the latter group should rather be considered for exogenous androgen supplementation. Recently, clinical trials have been initiated to assess the safety, tolerability and efficacy of exogenous testosterone therapy in patients with castration resistant PC (180). Preclinical studies have shown that supraphysiological levels of testosterone have deleterious
effects on castration resistant PC cells (180). In this regard one should evaluate the role of the tumor stroma and assess whether RSG could be a predictive factor for treatment response.

7.2 PERINEURAL INVASION

7.2.1 OUR FINDINGS COMPARED TO PREVIOUS REPORTS

In Paper II we found that patients with PNI on diagnostic needle biopsy specimens had a higher risk of PC-specific mortality than patients without PNI. This association was independent of clinical factors, but was rendered non-significant when adjusting for RSG and GS. However, we also found that the risk of PC-specific mortality increased as the percentage of biopsy cores with PNI increased. When more than 50% of the biopsy cores were involved with PNI, the risk of PC-specific mortality was independent of all histopathologic factors, including the percentage of biopsy cores with cancer. Thus, PNI is associated with dying from PC and the risk increases with increasing extent of PNI.

There are only few articles examining the impact of diagnostic biopsy PNI on PC-specific mortality. These studies have been limited to patients undergoing either surgery or radiation treatment (87-90). However, they all confirm a detrimental effect of PNI on prognosis which is independent of other relevant prognostic factors (87-90).

The non-significant effect of PNI (when evaluated as present or absent) in multivariable analysis can be understood in light of the relationship between prognosis and extent of PNI. When PNI is evaluated only as absent or present, the group of patients labeled as PNI positive is indeed a heterogeneous group in terms of risk. On one hand there are patients with sparse PNI and corresponding low risk, whereas patients with extensive PNI have high risk. Thus, one can say the risk becomes diluted. In our relatively small study with limited number of events, it is reasonable that the diluted effect of PNI was not significant after multivariable adjustment due to lack of statistical power. In contrast, when one evaluates PNI based on
extent, the effect of extensive PNI is so large that less statistical power is required for a significant finding (181). This reasoning assumes that the relationship between PNI biopsy cores and prognosis is a valid finding. The variable number of biopsy cores and lack of a systematic biopsy scheme are factors that make the validity of this relationship uncertain and in need for external validation. Maru et al studied patients undergoing RP, and found that the extent of PNI in millimeter corresponded with prognosis in terms of PSA-relapse (182). Interestingly, they found that PNI was not an independent predictor of prognosis when PNI was evaluated as absent or present. However, the quantitative extent of PNI was independent of other adverse prognostic factors. Thus, their findings regarding the extent of PNI are similar to ours, even though we used a cruder method for quantitative assessment of PNI. This raises the pertinent question whether our approach to assessing the extent of PNI could be a simple and useful prognostic method that should be evaluated in further studies and possibly implemented in clinical practice.

7.2.2 PERINEURAL INVASION AND REACTIVE STROMA

In Paper II we also studied the relationship between reactive stroma, PNI and prognosis. This might seem unnecessary since RSG is not a routinely applied prognostic system. However, neuro-epithelial interactions such as PNI are closely interlinked with stromal processes, and assessing this aspect in a clinical study provides important translational evidence. For instance, stromal cells have been found to promote PNI in \textit{in vitro} models (61). Thus, it is an interesting finding in our study that PNI became more frequent with increasing stromal grades. Although, our study cannot make any conclusions regarding the causal direction of this relationship, it is reasonable to interpret it as a histopathologic confirmation of the previously mentioned \textit{in vitro} findings.
A debated topic is whether the presence of PNI should be an ineligibility criterion for AS. Al-Hussain et al and Trpkov et al investigated patients who were eligible for AS, but still opted for RP (92, 93). Both research groups found that patients with PNI did not differ from patients without PNI in terms of adverse prostatectomy findings. Hence, these investigators concluded that patients with PNI should not be excluded from AS. However, we propose that there are two aspects of these studies that render this conclusion uncertain. Firstly, the studies do not report why these patient were given radical treatment rather than AS. It may be due to the patients’ choice, or there may be unregistered disease-related factors that led the physicians to conclude that these patients were at risk of progression and not suitable for AS. Thus, the patients without PNI in these cohorts may have been at higher risk of adverse findings than patients without PNI that are actually enrolled in AS. This would impair theses studies’ ability to detect differences between patients with and without PNI. Secondly, these studies ignore the chronological aspect of PC. Even if one accepts that patients with PNI do not differ from patients without PNI upon surgery, it does not mean that these two patient groups would not have diverged from each other in terms disease development if enrolled in AS. Cohn et al investigated a cohort of patients being considered for AS and found that PNI was associated with higher risk of GS upgrading on confirmatory biopsy (183). Thus, it possible that patients with PNI eligible for AS have undetected foci of higher GS due to sampling error or are at higher risk of progression. A strong argument in favor of the latter can be found in the study by Moreira et al (184). They studied patients on AS enrolled in the REduction by Dutasteride of clinical progression Events in Expectant Management (REDEEM) study. In this well-conducted study of 302 men on AS, 11(4%) patients had PNI on their diagnostic baseline biopsy. Patients with PNI were at higher risk of clinical progression (defined as worsened pathology on re-biopsy or initiation of therapeutic measures) in multivariable analyses (184). Our study, which consists of predominantly conservatively patients and demonstrates a
negative prognostic effect of PNI, also supports the view that patients with PNI are not suitable for expectant management. Nonetheless, it should be kept in mind that our patients were not actually enrolled in AS and frequently had locally advanced disease.

7.3 LYMPHOVASCULAR INVASION

7.3.1 OUR FINDINGS COMPARED TO PREVIOUS REPORTS

In Paper III, we found that LVI on diagnostic needle biopsy predicted PC-specific mortality independently of PSA, cT, GS and curative treatment. The presence of LVI has previously been studied as a finding on RP specimens. The impact of LVI on PSA-relapse has been variable and a review concluded that the results are conflicting. Regarding long-term outcomes there is sparse data. Cheng et al reported that LVI on RP specimens predicted PC-specific survival independently of other factors and Shariat et al found that LVI was associated with overall survival (106, 107). Hence, Paper III provides important findings regarding the prognostic value of LVI.

7.3.2 INTERACTION BETWEEN REACTIVE STROMA AND LYMPHOVASCULAR INVASION

In Paper III, we also examined the relationship between LVI and reactive stroma. Not surprisingly, increasing stromal grades were associated with increased occurrences of LVI. Cellular components of reactive stroma appear to increase vascular permeability and penetration of blood vessels, thus facilitating LVI (51). A gene signature for vascular invasion has been developed in breast cancer. This gene signature is associated with wound response genes, which is interesting since reactive stroma and the wound response have several similarities (185, 37).

In Paper III, we proposed a possibly controversial hypothesis, namely that reactive stroma interacts with LVI and enhances the negative effect of LVI on prognosis. In detail, we suggest that CAFs, the principal cellular component of reactive stroma, joins the metastatic PC cells
into the vasculature structures and travel along to the distant metastatic site. Upon arrival at the distant site, the co-metastasizing CAFs provide the stromal niche necessary for metastatic PC cells to survive and develop into disseminated and lethal disease. When there is large amount of reactive stroma with high numbers of CAFs and increased tendency for invasion of vascular structures, it is conceivable that this process takes place more frequently, resulting in more metastasis per unit of time and ultimately more rapid progression to death. Statistically, we conceived that this process would manifest itself in the form of an interaction between high RSG (RSG 3) and LVI; i.e. the presence of RSG 3 modifies the effect of LVI on PC-specific mortality in a Cox-regression analysis. The basis for this hypothesis was found in the work of Duda et al, titled “Malignant cells facilitate lung metastasis by bringing their own soil” (186).

Using experimental models with Lewis lung carcinoma cells LLC1 and GFP-expressing mice, Duda et al found the following (206):

1. The efferent blood from tumors contains not only single tumor cells, but also tumor clumps containing both tumor cells and host stromal cells. The tumor cells of the clumps were more viable than single tumor cells.
2. The host stromal cells were detectable in lung metastasis.
3. The host stromal cells in lung metastasis expressed CAF-markers.
4. Depletion of CAFs in lung metastasis reduced the number of metastasis.

The data in our study corroborate our hypothesis as it was apparent that RSG 3 further enhanced the negative effect of LVI on prognosis. However, the finding needs validation as the low number of events in our small study increases the risk of a spurious false positive results when conducting subgroup and interaction analyses (174).
If one accepts the finding of the statistical interaction as valid, it could have ramifications for the interpretation of previous reports on LVI and prognosis. The predominant view on LVI as a prognostic factor is that the evidence is conflicting (98). This view is based on the fact that there is variability between studies regarding the magnitude of the prognostic effect and independence from other relevant factors (98). However, if reactive stroma functions as an effect modifier of LVI, than it stands to reason that in cohorts with predominantly low stromal grade tumors the effect of LVI would be weaker whereas it would be stronger in cohorts dominated by high stromal grades. This could possibly explain the discrepant results between studies on LVI.

7.3.3 CLINICAL IMPLICATIONS

The subgroup of patients with concomitant RSG 3 and LVI, experienced rapid progression to death from PC. It is reasonable that this rapid progression indicates systemic disease that was not otherwise clinically apparent at the time of diagnosis. Thus, combined evaluation of RSG and LVI could serve as a histopathologic mean to identify patients with occult metastatic disease and warrant systemic treatment.

7.4 INTRADUCTAL CARCINOMA OF THE PROSTATE

7.4.1 OUR FINDINGS COMPARED TO PREVIOUS REPORTS

In Paper IV, we found that IDC-P on diagnostic needle biopsy predicts PC-specific mortality independently of PSA, clinical stage, GS and curative treatment. To the best our knowledge, this is the first study to assess IDC-P on biopsy in relation to PC-specific mortality. The finding is not unexpected since several studies have established that IDC-P is a predictor of treatment failure following surgery of radiation treatment (113-120). Moreover, a recent study on high risk patients undergoing RP, demonstrated that IDC-P on prostatectomy specimen was associated with increased risk of death from PC (115). Along the same line, it has been
shown that patients with metastatic PC and IDC-P on biopsy have reduced overall survival compared to their IDC-P negative counterparts (120).

The incidence of IDC-P in our study was considerably high. Little over one-third of the patients had IDC-P on diagnostic needle biopsy. In comparison, a prospective study by Watts et al reported an incidence of 10.6% in daily contemporary practice (187). Kimura et al studied a cohort of high risk PC patients and found an incidence of 36.9% (115). Thus, it is most likely that our high number of IDC-P reflects the advanced stage and grade within our cohort. However, a weakness in this regard is the lack of ancillary immunohistochemical staining to assess the presence of basal cells. This might have reduced the pathologists’ ability to distinguish IDC-P from cribriform adenocarcinoma (109). Nonetheless, it appears from the work of Guo & Epstein, that the problem in this regard is that IDC-P is misclassified as cribriform carcinoma (109). Hence, the lack of immunohistochemistry will introduce a misclassification bias that would give a falsely lower estimate of the incidence of IDC-P. If this misclassification bias is present, our results regarding the effect of IDC-P on prognosis would be biased towards null. Given the incidence of IDC-P and its significant effect on prognosis, we find it improbable that lack of immunohistochemical staining introduced any misclassification bias of significance.

7.4.2 INTRADUCTAL CARCINOMA OF THE PROSTATE AND REACTIVE STROMA

A novel finding in Paper IV was the association between IDC-P and reactive stromal grade. High stromal grade occurred more frequently among patients with IDC-P compared to patients without IDC-P. Again, this is not unexpected since an association between IDC-P and various adverse clinicopathologic features has previously been demonstrated. However, it is believed that IDC-P is the result of carcinoma invading ductal structures (110). Since reactive stroma has been shown to facilitate invasion of nerves and lymphovascular structures, an immediate hypothesis would be that reactive stroma also facilitate invasion of ductal
structures. An approach to study this further in an experimental setting could be to use patient-derived xenografts from patients with various stromal grades in prostatectomy specimens (188). Following implantation in mice, a histopathological evaluation to assess the presence of IDC-P in the xenografts would be carried out. One would expect to find more IDC-P in xenografts with high RSG than in xenografts with low RSG. To further isolate the causal role of reactive stroma, one could treat implanted mice with diphtheria toxin (DT) which results in depletion of human CAFs (186). One would expect that high RSG xenografts in mice treated with DT would have lower frequency of IDC-P compared to their non-treated counterparts.

7.4.3 CLINICAL IMPLICATIONS

Given the increasing evidence of IDC-P as a harbinger of lethal PC, there should be debate on how to manage patients with this adverse histopathological feature on diagnostic biopsy specimens. Since IDC-P is commonly associated with other adverse features, it is likely that a large proportion of these patients are considered as high risk irrespective of IDC-P and managed accordingly. However, one could picture a situation where IDC-P is present and other features such as high GS, high PSA or advanced stage is absent. In this setting one could suggest that IDC-P is a criterion for bumping these patients up to a higher risk category than what they would normally belong to. The recent ISUP 2014 conference concluded that when IDC-P is present on biopsy, a comment regarding its association with poor prognosis should be made (69).
7.5 STRENGTHS AND LIMITATIONS

7.5.1 STRENGTHS

We would claim that the principal strength of the present study is its follow-up which allowed us to use PC-specific death as an end-point. The median follow-up time was approximately 9 years. The longest and shortest potential follow-up times were 21 and 13 years.

Another strength of the study is its population-based design. As outlined in section 2.5.3, this provides a wide range of patient categories which facilitate detection of associations.

An important beneficial aspect of this study is the low loss to follow-up, which minimizes bias by attrition which can severely distort findings of prognostic studies (123, 137).

Furthermore, our cohort represents the patient population diagnosed with PC in a urologic outpatient practice. To understand how this is an advantage, it is necessary to consider the fact that a considerable number of studies on PC is conducted on patients undergoing curative treatment at high-volume referral center. This may not accurately reflect the clinical setting in which prognostic factors are intend to be used. Rather, it is more likely that a patient with newly diagnosed PC is being evaluated in an outpatient clinic at a local hospital, his future regarding treatment is not determined yet and his prognosis is uncertain. It is in this setting that prognostic factors can be of use to guide treatment choices and inform patients on their future disease course. Grobbee et al have pointed out that prognostic accuracy may vary among clinical settings (189). Thus, it is necessary to study prognostic factors in a setting that is similar to the one where the factor is intended to be used.

An important strength of our study is the use of central pathology review. All specimens were evaluated by two uropathologists with several decades of combined experience. This ensures high accuracy of diagnosis and classification.
Even though there are meritable features to our study, the most pertinent questions that must be answered are as follows: are the findings valid and can they be generalized? The validity of a finding refers to whether the results are true and not the product of various biases (190). Generalizability means that the findings can be extrapolated to the whole population (190). Evaluating validity and generalizability cannot be reduced to statistical procedures with clear-cut answers; rather it is a qualitative process based on reasoning and knowledge of the field being studied (190). In the following sections, possible threats to the validity of the present study will be addressed and then the generalizability of the findings will be discussed.

7.5.2 VALIDITY

7.5.2.1 THE RETROSPECTIVE STUDY DESIGN

The present study was based on data collected from patients’ medical charts. Thus, it can be classified as a retrospective, or more precisely a historical cohort study (191). A retrospective design is frequently cited as a weakness and the findings are considered uncertain. However, again the pertinent question is whether the retrospective design is a threat to the validity of the findings. The answer is in fact that in most circumstances the findings of a retrospective study should be considered as valid unless one can point out specific biases that follow from its retrospective nature (191). In the present study, the retrospective design resulted in a lack of influence on various relevant factors such as which patients were given treatment, how many biopsies were taken per patient and the accuracy of clinical stage assignment. If the study had been prospectively carried out, these factors could have been pre-specified and standardized, thus leading to less heterogeneity. If these factors vary across categories they may influence results. Even though we will address these factors’ effect on our findings in later sections, it appears that a prospective design could have remedied some potential limitations of our study. However, if we hypothetically were to carry out the present study in a prospective fashion and we did not for various reason address the aforementioned factors prior to study start, then the
hypothetical prospective study would be equal to the actual retrospective study in terms of validity. Thus, a prospective design would not intrinsically reduce the number of threats to validity and therefore it follows that the retrospective nature of the present study is not in itself a limitation.

7.5.2.2 POPULATION-BASED OR NOT?
One of the intents of the present study was to make it population-based and unselected. This would provide the benefits detailed in section 2.4. It would also reduce risk of selection bias, which we posit is a potential problem for many prognostic studies in PC research since they are frequently based on prostatectomy cohorts from referral centers. However, we applied certain inclusion and exclusion criteria which are detailed and explained in section 5.1.2. The application of these criteria may constitute an argument against our claim that the present study is unselected and population-based. However, we would argue that these restrictions do not conflict with a population-based design. All studies must define the population to be studied. Accordingly, our restrictions serve to define the population we intended to study; namely patients diagnosed by needle biopsies without evident metastatic disease. When the population was clearly defined, we included all cases of this population within our geographic region. Thus, the restrictions did not select patients in a way that could have biased results.

7.5.2.3 SMALL SAMPLE SIZE
We have previously touched upon the issue of sample size in section 5.3.1. The findings in our study were positive, i.e. statically significant effects were detected. A small sample size may be a source of uncertainty when there is a lack of statically significant effect and the researchers conclude that there is no effect. In this situation, the lack of effect may be the results of low statistical power (181). This problem is well-known, but another issue is that low statistical power is associated with low positive predictive value and a tendency for inflated effects (181). When small studies report a statically significant result, the probability
of this positive result reflecting a true effect is low (181). Similarly, a statically significant finding in a small study will often demonstrate an inflated magnitude of the effect (181). This is an important issue concerning the validity of our findings. However, the majority of our findings are in line with that of previous reports and our study serve primarily to validate previous results. Secondly, in situations where we report a novel finding (such as the interaction between RSG 3 and LVI) we have clearly specified that these should be considered hypothesis-generating and in need of external validation. Nonetheless, a larger sample size would have allowed us to reach more definite conclusions regarding these findings.

7.5.2.4 THE NUMBER OF BIOPSY CORES
As described in section 5.2.2, the number of biopsy cores obtained in the present study was low compared to what is considered the routine approach today. One must ask oneself how this aspect influences the study results. Firstly, a tendency for few biopsies will most likely result in large and advanced cases being detected more readily than smaller non-palpable lesions. Thus, the distribution of patients will be skewed towards more advanced cases. This has important implications for generalizability which will be addressed in a later section. However, it is improbable that this skewed distribution would influence results in such fashion that false associations between the studied factors and outcome would be observed.

Another critical consequence of the low number of biopsy cores it that it increases the risk of sampling error (192). I.e. the findings in the biopsy set may not accurately reflect the true histopathologic state of the prostate. This pertains particularly to the GS which may be underestimated (192). Inaccurate GS would most likely impair the prognostic value of the GS, thus making the studied prognostic factors appear wrongfully independent of the GS and inflating improvements in predictive power in multivariable analyses. Sampling error also results in uncertainty whether absence of a prognostic factor represents true absence. If that
factor truly has a negative influence on outcome, the group of patients considered to be without that factor will have an increased risk estimate due to contamination with false negative patients. This could lead to underestimation of prognostic effects and type II errors.

7.5.2.5 PATIENTS WITH REGIONAL METASTATIC DISEASE
A considerable number of the patients in our cohort could have lymph node metastasis. The prognostic factors we studied may be associated with a higher risk of regional metastasis and the latter may be the crucial determinant whether patients die from PC. Thus, one could claim that any association between prognostic factors and outcome observed in our study is confounded by an association with lymph node metastasis. However, this claim can only hold true in a causal and etiological setting where one aims to identify the underlying mechanism of PC-specific death. Even in such a setting, one could make the argument that lymph node status is mediator for the histopathologic parameters we have studied, thus not undermining the importance of these parameters. Nonetheless, the purpose of the present study was to define prognostic factors. Hence, we were only interested in descriptive relationships. In this regard, confounding is not of importance (125).

7.5.2.6 TREATMENTS
As detailed in section 5.1.3, the patients in our cohort varied in terms of which treatment they received. Approximately one quarter of the patients were given treatment that has been shown to reduce PC-specific mortality. The group of patients managed curatively tended to have lower stromal grades and lower incidences of PNI, LVI and IDC-P. Conversely, patients with high RSG or any of the other adverse features were less likely to receive curative treatment. This distribution of curative treatment most likely reflects the association between the studied prognostic factors and other well-established adverse factors that were used to guide treatment selection. However, it has important implications for the validity of our findings since one could argue that the prognostic effects we observed actually reflect treatment effects. In this
regard it is crucial that our multivariable analysis included adjustment for curative treatment, demonstrating that the prognostic effects were indeed independent of whether the patients received active treatment or not. We consider variations in the use of ADT to be less threatening to validity since its effect on PC-specific survival has not been definitively been proven (169). Although, some reflections regarding the linkage between ADT and reactive stroma were addressed in section 7.1.2, this relationship must be considered in the light of absent clinical evidence and regarded as hypothetical.

7.5.3 GENERALIZABILITY

If one accepts that our findings are valid, the next step is to determine whether these findings are applicable to patients with PC in contemporary practice. A population-based approach should be ideal for obtaining data that can be generalized (128). However, the main problem in our work is the long study period. In order to obtain data on PC-specific mortality it is necessary to ensure a long-term follow-up. During that follow-up, dramatic changes in the management of the disease may take place which in turn affects the composition of the background population.

Our study originated in the 1990s and was concluded in present day. There have been several changes in the management of PC during this time period. These changes can be placed along three axes:
(I) DIAGNOSTICS

Increasing use of PSA-testing has led to earlier detection of PC (2). The number of biopsy cores has increased, leading to more sampling of the prostate (7). A systemic biopsy approach to sample the prostate is now routine (7).

(II) STAGING

PSA-testing has resulted in a shift towards more localized disease (193). Increasing use of MRI as a staging adjunct may improve staging accuracy (16).

(III) TREATMENT


Thus, these are the main aspects that separate our study population from the contemporary PC population. The next and probably most difficult step is to assess how these differences may modify the association between prognostic factors and outcome.

INCIDENCE

All the prognostic factors studied in the present study were associated with advanced disease. Since PSA-testing has led to earlier detection, advanced disease occurs less frequently in contemporary cohorts (194). Thus, the incidence of the adverse factors we have studied will be higher in our cohort than in current PC populations.
TIME TO PC-SPECIFIC DEATH

Earlier detection of PC results in longer time from diagnosis to death from PC, the basis for lead time bias (137). Our cases have most likely been diagnosed at a later stage, thus resulting in apparently shorter time to progression and lower rates of 10-year survival.

IMPROVED STAGING

If MRI improves staging accuracy, then stage will most likely reflect more accurately the disease process and lead to improved prognostic value of staging.

SAMPLING OF THE PROSTATE

Fewer biopsies lead to underestimation of the GS. In current practice, the biopsy GS may reflect more precisely the disease and be a stronger predictor of prognosis than in our study (195).

TREATMENT

Improved treatment and closer follow-up of conservatively managed patients may modify the effect of prognostic factors on outcome.

Based on the above one could claim that there are three modifications of the prognostic factor effects in our study relative to contemporary PC patients:

1. Lower incidence of these factors in contemporary PC populations
2. Weaker effect of these factors on prognosis in contemporary PC populations
3. Other prognostic factors have higher predictive value in contemporary PC populations

Thus, should one conclude that the results of the present study are of no value to contemporary PC patients? We would oppose such a conclusion and provide two arguments in favor of our stand:
1. **THE QUALITATIVE ARGUMENT**

   The potential differences between prognostic effects in our study and current PC patients, are quantitative ones. I.e. the incidence may be lower and the effect may be weaker. However, we find no basis for claiming that the associations themselves, namely the qualitative relationship between a factor and outcome, is not true for current PC patients. For instance, patients with PNI diagnosed in our study might have had shorter 10-year PC-specific survival than one would expect for current patients with PNI. However, when comparing patients with PNI to their PNI negative counterparts, there is no basis for claiming that the difference in outcome between these two groups does not exist in contemporary patients. We only find a basis for claiming that the magnitude of this difference in outcome is different between our study population and contemporary patient populations.

2. **THE BIOLOGICAL ARGUMENT**

   Even though the manifestations of PC and the management of PC have changed, there are no grounds for claiming that the biology of PC has been altered during the last twenty years or so. Its etiology and underlying mechanisms are with the highest probability unchanged. Any factor that reflects the disease process and is found to be associated with the final result of that process cannot become unconnected from each other unless the disease process itself changes.

Hence, we would conclude that the findings presented in our study are applicable to patients diagnosed with PC today in the sense that the presence of high RSG, LVI, PNI or IDC-P are harbingers of faster progression to death from PC. However, any statements in our study
regarding numerical aspects of this increased progression, i.e. the exact 10-year disease specific survival, will not be applicable to contemporary PC patients.

8. CONCLUSIONS

The present study demonstrates the following:

- Evaluation and grading of reactive stroma in diagnostic prostate needle biopsies can be used to predict death from PC.
- The presence of PNI on diagnostic needle biopsy indicates an increased risk of dying from PC and this risk is further increased when the number of biopsy cores with PNI increases.
- LVI on diagnostic needle biopsy predicts death from PC. Furthermore, the concurrent presence of an intense reactive stroma enhances the already lethal effect of LVI.
- Patients with IDC-P on diagnostic needle biopsy have increased risk of dying from PC independently of other prognostic factors.
9. FUTURE PERSPECTIVES

It is important to address reactive stroma and the grading of reactive stroma in further studies. Firstly, large multi-institutional studies to confirm that RSG can be used to predict prognosis are needed. For instance, the new PC grading system has been validated in a collaborative study between five academic institutions and included more than 20 000 men (70). Similar efforts to assess the prognostic value of RSG should be undertaken. Moreover, the discrepant aspects of the RSG system and how these discrepancies influence prognostic value should be addressed. If the RSG system is to be included in clinical practice, consensus conferences are necessary in order to ensure a unified approach to the grading of reactive stroma.

The finding that the number of PNI cores correlated with prognosis needs to be validated in additional and independent studies. Similarly, the prognostic interaction between LVI and reactive stroma needs to be validated.

Although the present study is based on clinical data, there are findings that can generate hypothesis for experimental studies. The associations between IDC-P, LVI, PNI and reactive stroma suggest that the tumor microenvironment promotes an invasive form of prostatic carcinoma. Experimental studies elucidating the causal mechanism underlying these associations could contribute significantly to our understanding of prostate cancer pathophysiology.
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