The prognostic and predictive value of *TMPRSS2-ERG* gene fusion and ERG protein expression in prostate cancer biopsies

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This review has been accepted as a thesis together with three previously published papers by University of Copenhagen 18th of June 2015 and defended on 25th of September 2015

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Dan Med J 2016;63(12):B5319

THE THREE INCLUDED ORIGINAL PAPERS ARE:

1. Berg KD, Brasso K, Thomsen FB, Røder MA, Holten-Rossing H, Toft BG, Iversen P, Vainer B. ERG protein expression over time: from diagnostic biopsies to radical prostatectomy specimens in clinically localised prostate cancer. J Clin Pathol. 2015 Oct;68(10):788-94.

2. Berg KD, Vainer B, Thomsen FB, Røder MA, Gerds TA, Toft BG, Brasso K, Iversen P. ERG protein expression in diagnostic specimens is associated with increased risk of progression during active surveillance for prostate cancer. Eur Urol. 2014 Nov;66(5):851-60.

3. Berg KD, Røder MA, Thomsen FB, Vainer B, Gerds TA, Brasso K, Iversen P. The predictive value of ERG protein expression for development of castration-resistant prostate cancer in hormone-naïve advanced prostate cancer treated with primary androgen deprivation therapy. Prostate. 2015 Oct;75(14):1499-509.

INTRODUCTION

The clinical course of prostate carcinoma (PCa) is heterogeneous. It ranges from purely indolent, where the cancer causes no symptoms and is not a threat to the patient's well-being, to highly aggressive, with dreadful complications and ultimately death.

Unfortunately, standard clinicopathological parameters can only explain some of the heterogeneity. Moreover, currently used parameters are insufficient in selecting the optimal treatment for an individual patient and have shortcomings in identifying patients who will benefit from a given treatment. Consequently, novel prognostic and predictive tools are needed to differentiate between truly indolent and aggressive disease and to tailor personalised treatments.

During the last decades, it has become evident that PCa, also at the genomic level, is a heterogeneous disease. One common genetic alteration is the fusion between the transmembrane protease serine 2 (*TMPRSS2*) gene and the v-ets avian erythroblastosis virus E26 oncogene homolog (*ERG*) gene resulting in expression of the transcription factor ERG. The gene fusion occurs early in PCa pathogenesis and divides PCas into two distinct subgroups with marked differences in their genomic signatures. This PhD thesis focuses on the prognostic and the predictive value of ERG protein expression in diagnostic biopsies from PCa patients. More specifically, the thesis examines changes in ERG expression over time, the prognostic value of ERG protein expression for disease progression in an active surveillance programme, and the predictive value of ERG expression for treatment response and the risk of developing castration-resistant PCa in patients undergoing first-line castration-based androgen deprivation therapy.

BACKGROUND

The prostate

The prostate is an exocrine glandular organ located caudal to the bladder surrounding the bladder neck and the proximal urethra. It is part of the male reproductive system and the normal differentiation, growth, and biological function depend on androgenic stimulation[4].

According to different physiological and pathological features, the gland is divided into peripheral, central, transitional, and periurethral zones, with the peripheral zone being the most susceptible to cancer[5]. At the cellular level, the prostate is composed of stromal and glandular components; the latter is subdivided into ducts and acini consisting of secretory cells, basal cells, and a few neuroendocrine cells[5]. Basal cells separate the secretory cells from the basement membrane by forming a continuous layer in the normal prostate gland, and they are thought to represent a multipotent cell population[5]. The luminally placed secretory cells produce and excrete a proteolytic secrete, which is mixed into the male ejaculate to liquefy the semen, improve sperm mobility, and modify the vaginal environment to enhance fertilisation[6,7].

Carcinoma of the prostate

Prostate carcinoma (PCa) develops from the glandular component and is, apart from non-melanoma skin cancer, the most commonly

diagnosed male malignancy in the Western world[8]. During the past decades, the incidence of PCa has increased dramatically, and the cumulative incidence of Danish men younger than 75 years who are diagnosed with PCa has increased from 3.1% in 1995 to 9.8% in 2012. This corresponds to 4,315 new cases in Denmark in 2012[9].

The exact aetiology of PCa is not fully established, but known risk factors are increasing age, family history of PCa, and African ancestry[10]. Moreover, environmental factors are important, and genome-wide association studies have identified nearly 100 single-nucleotide polymorphism variants associated with the risk of PCa[11]. Finally, androgen stimulation is required for PCa development[12], but no clear association between serum levels of androgens and risk of PCa has been established[13].

Studies investigating the natural course of clinically localised PCa have shown that the cumulative incidence of PCa-specific mortality is 29% after 20 years of observation[14]. Still, the prognosis is very heterogeneous and the long-term mortality rates ranged from 7% in the best prognostic group to 66% in the worst prognostic group[14]. Comparable results have been published from a Swedish register-based study[15]; thus, there is a huge discrepancy between histopathologically verified PCa and clinically significant PCa.

Diagnosis of prostate cancer

Prostate cancer is most often diagnosed using transrectal ultrasound (TRUS)-guided core needle biopsies triggered by an elevated prostate-specific antigen (PSA) level or an abnormal digital rectal examination (DRE) [16].

Prostate-specific antigen

The luminal cells in benign as well as most PCa lesions produce the serine protease PSA. The enzyme is part of the proteolytic secretion from the prostate gland[17]. PSA can leak into the blood and, as such, can be used as a serum biomarker. Low PSA levels can be detected in sera from healthy men, but abnormalities in the glandular architecture, vascularisation, and disruption of the basement membrane result in elevated levels[18]. These abnormalities include PCa, and men with PCa generally have higher PSA levels than non-PCa men[19]. This has led to the use of PSA as a marker in PCa management. Still, several benign conditions, including benign prostatic hyperplasia and prostatitis, can cause elevated PSA values, and individual PCa cells actually have lower PSA expression levels than their benign counterpart[18,20]. In addition, certain aggressive forms of PCa do not express PSA, probably because of the loss of cellular differentiation[21]. Thus, although low PSA expression levels have been reported in other tissue types[22], PSA can, for clinical and practical purposes, be regarded as organ but not PCa specific.

The risk of PCa is positively correlated with PSA levels in patient cohorts[23], and a cut-off value of 4 ng/mL has been chosen as the threshold level for PCa suspicion and trigger for taking biopsies[16]. However, there is no safe cut-off level that confers a zero risk of PCa or a certain cancer diagnosis[24,25], and PSA seems to have a limited ability to predict the pathological tumour stage for an individual patient[16,22]. Despite these shortcomings, PSA harbours prognostic value[26,27], and is incorporated into various risk calculators and nomograms for risk stratification[28–31].

Digital rectal examination, transrectal ultrasound imaging, and clinical staging

A DRE is an essential part of the clinical assessment of PCa patients, and TRUS is the standard imaging modality for determining prostate volume and guiding the biopsy session[16]. Most PCas are found in the peripheral zone and are, at least in theory, ultrasonically visible and palpable as hard and irregular nodules when the tumours reach a certain size[32]. Both modalities are, however, subject to significant interobserver variations, and cancer lesions can appear both hypo-echoic and iso-echoic on TRUS[33–35].

A central part of the examinations is to assess the clinical tumour (cT) category according to the TNM classification [36]. The cT category describes the local extent of the tumour and is based on four main categories with subgroups. It harbours prognostic value for PCa aggressiveness and the risk of recurrence after curatively intended therapy[28,37]. For therapeutic and prognostic purposes, it is crucial to determine whether the tumour is organ-confined (T1-2) or has become extraprostatic (T3-4) due to the penetration of the fibromuscular layer surrounding the prostate. Although it is not a well-defined structure, especially at the anterior and apical surfaces, the fibromuscular layer is usually referred to as the prostatic capsule[5].

Histopathological assessment and prostate cancer grading using the Gleason score

The current gold standard for PCa diagnosis is a histological examination of a 10 to 12 core TRUS guided biopsy set[16]. The diagnosis is based on architectural and cellular atypia including a random glandular pattern, absence of basal cells, and atypical nuclei with prominent nucleoli[38]. Most often, the diagnosis can be made based on morphology alone using haematoxylin and eosin (H&E) stains, but additional techniques can be applied to distinguish between PCa and atypical benign foci. Lack of basal cells can be established by a negative immunohistochemical staining for basal cells using high molecular weight cytokeratin or p63 as basal cell markers, either individually or in combination[38,39]. However, some benign lesions, including partial atrophy and adenosis, may show a disrupted basal cell layer[39]. Accordingly, basal cell markers are often used in combination with positive markers for neoplastic epithelial cells such as alpha-methylacyl-CoA racemase (P504S), which is overexpressed in most (82-100%) tumours[38].

PCas are most often (95-99%) acinar. A proportion of acinar and non-acinar variants have been classified, which are typically intermingled with conventional acinar adenocarcinoma[40,41]. Non-acinar variants constitute a small percentage of PCas and comprise among others of ductal adenocarcinomas and neuroendocrine tumours including small-cell carcinomas[40,41].

During the past decades, the purpose of prostate biopsies has changed from being mainly diagnostic to being a central part of PCa

management. Accordingly, the perseverative task of uropathologists is, besides establishing diagnoses, estimating the tumour's malignant potential. For PCa, the histopathological evaluation includes a description of the tumour's differentiation[39,42]. The most widely accepted grading system was established in 1966 by Donald Floyd Gleason[43]. The Gleason grading system is based on the glandular pattern of cancerous glands and uses a five-tier grading scale ranging from grade 1 for the most well-differentiated pattern resembling benign glands to grade 5 for the most undifferentiated pattern with the lack of glandular differentiation including formation of solid tumour sheets, single tumour cells, and comedonecrosis[43]. Historically, a primary (predominant) and a secondary (second most prevalent) pattern were identified and a Gleason score (GS) was obtained by adding the grades. If only one pattern was present, the same grade was given to both patterns[43].

The Gleason grading system underwent changes in 1974[44] and again in 2005 as the International Society of Urological Pathology (ISUP) revised the interpretation of Gleason grades [45]. Importantly, histopathological features such as ill-defined glands with poorly shaped lumina and regular cribriform glands, which were considered Gleason grade 3 by the classical scoring system, were changed to Gleason grade 4. Moreover, the ISUP 2005 consensus report established that the GS for biopsies should always include the worst pattern, and lower-grade patterns occupying < 5% of the tumour volume should be ignored. Thus, the GS for biopsy specimens was altered to the sum of the most common Gleason grade and the highest grade presented by non-primary patterns[45].

To be useful, a grading system must be reproducible and provide a reasonable accordance between matched biopsy and prostatectomy specimens. The implementation of the ISUP 2005 consensus guidelines have increased this concordance[46–53], and studies have repeatedly shown that the GS is one of the strongest prognostic markers in PCa patients managed both expectantly and with curative intent[14,28,54–56]. In biopsies, the GS has been demonstrated to be positively correlated with pathological tumour stage[57] and biochemical recurrence following radical prostatectomy[57–59], as well as the development of metastases and PCa-specific death[60]. Consequently, the GS is a cornerstone in therapeutic decision making[16]. Still, it is subject to inter-observer variations, which result in problems with exact and reproducible prognostication[46]. Implementation of new and objective biomarkers might help to define PCa aggressiveness.

Risk assessment of prostate cancer

Several PCa nomograms and risk-stratifications have been developed based on combinations of cT category, GS, and PSA[28–31]. The most frequently used pre-treatment risk-stratification for localised PCa was published by D'Amico *et al.* (Table 1), and it has subsequently been externally validated[28,61]. Several other risk-stratifications have been published, whereof the National Comprehensive Cancer Network's guidelines on PCa risk groups (Table 1) includes locally advanced and metastatic disease[62].

Unfortunately, the clinical and histopathological factors can only stratify men into broad prognostic groups. Consequently, these factors can only describe some of the variation observed in the clinical course from patient to patient[63]. Thus, additional and robust tools for prognosis are needed if we are to treat each patient according to his individual prognosis.

Treatment options

Therapy planning for PCa is based on a trinity of (1) tumour characteristics such as PSA, GS, and TNM stage; (2) patient characteris-

Table 1: Risk classification of prostate cancer

D'Amico risk classification for localised prostate cancer [28]

	PSA (ng/mL)	Gleason score	cT category
Low risk*	≤ 10	≤ 6	≤ cT2a
Intermediate risk†	> 10 and ≤ 20	7	cT2b
High risk‡	> 20	≥ 8	cT2c

National Comprehensive Cancer Network's guidelines on prostate cancer risk groups [62]

	PSA (ng/mL)	Gleason score	cT category	Positive biopsies	Tumour volume¥	PSA density	N category	M category
Very low risk*	< 10	≤ 6	T1c	< 3	≤ 50%	< 0.15	N0/x	M0/x
Low risk†	< 10	≤ 6	≤ T2a	-	-	-	N0/x	M0/x
Intermediate risk†	≥ 10 and ≤ 20	7	T2b-T2c	-	-	-	N0/x	M0/x
High risk†	> 20	≥ 8	T3a	-	-	-	N0/x	M0/x
Very high risk†	Any	Any	T3b-T4	-	-	-	N0/x	M0/x
Metastatic‡	Any	Any	Any	-	-	-	N1	M0/x
	Any	Any	Any	-	-	-	Any	M1

Abbreviations: cT category: clinical tumour category; PSA: prostate-specific antigen; PSA density: PSA value per gram prostate. * Fulfil all criteria and no higher risk features; † Fulfil at least one criterion and no higher risk features; ‡ Fulfil at least one criterion; ¥ Amount of tumour in any single biopsy.

tics including age, co-morbidity, body proportion, and life expectancy; and (3) patient preferences and availability of treatment modalities. It is not within the scope of the thesis to elaborate on all treatment options for PCa patients. Rather, this section will provide a brief overview of the treatments offered to patients included in the thesis, which are first-line therapeutic opportunities for low-risk PCa patients as well as men with advanced PCa.

Treating low-risk prostate cancer

It is possible to cure localised non-disseminated PCa by either surgery or radiation therapy[16]. However, both treatment modalities entail a significant risk of side effects which can affect the quality of life negatively[64–66].

Two randomised trials (PIVOT and SPCG-4) have compared radical prostatectomy to an observational strategy for clinically localised PCa[67,68]. An overall survival benefit was observed in the SPCG-4 study favouring surgery over observation[68], but no survival difference was detected in patients with low-risk tumour characteristics in either of the studies[67,68]. This indicates that low-risk patients will risk the treatment-associated side effects and gain little or no survival benefit.

Given the excellent prognosis of low-risk PCa with a reported PCa-specific mortality of 9% after 15 years of observation[15], the risk of overtreatment is substantial. Consequently, the concept of active surveillance (AS) has emerged as an initially non-curative observational strategy with the opportunity to switch to curative intervention, if later assessments show either disease progression or an initial undergrading of the tumour due to sampling error[69,70]. AS is considered a treatment option for selected low-risk patients, and these men are followed with regular PSA measurements, clinical examinations, and re-biopsies[70]. During surveillance, approximately 30% of patients will be offered curative treatment[71], and one of the greatest shortcomings of AS is the lack of reliable prognostic markers that at time of diagnosis can identify patients at greatest risk of progression[72]. A reliable prognostic marker could provide patient-tailored AS programmes according to the risk of progression and might increase the acceptance of this observational strategy among both patients and physicians.

Treating advanced prostate cancer

Benign as well as malignant prostate cells depend on androgen stimulation for growth, proliferation, and survival, and androgen deprivation therapy (ADT) is the recommended treatment for advanced PCa[73]. Testicular testosterone expression is under the regulation of the hypothalamic-pituitary-gonadal axis. The hypothalamic hormone luteinising hormone-releasing hormone (LHRH) induces the secretion of luteinising hormone from the pituitary gland, which subsequently stimulates the testicular Leydig cells to express testosterone[73]. The ablation of testicular testosterone secretion can be obtained by either surgical bilateral orchiectomy or treatment with LHRH agonists or antagonists, and androgens can be inhibited at their receptors by applying anti-androgens[73]. In patients with advanced or metastatic PCa, ADT is recommended to alleviate symptoms and defer progression to a symptomatic stage in asymptomatic men. Moreover, ADT reduces the risk of dreadful complications including spinal cord compression, pathological fractures, and urethral obstruction[73,74].

ADT results in cellular apoptosis, and even though most tumours initially respond to ADT, the majority of PCas will subsequently acquire the ability to proliferate even though serum testosterone values are kept in castration levels, and castration-resistant PCa (CRPC) emerges[75,76]. The advanced and metastatic PCa population is, however, very heterogeneous, and the duration of ADT response is widely variable, resulting in great variation in the time to disease progression, CRPC, and ultimately death[15,77,78]. Currently, no reliable marker can safely distinguish between patients who will benefit most from ADT and those who will progress rapidly and need additional therapy.

By combining the information regarding PCa incidence and mortality with the heterogeneity in the clinical course of the disease - both between and within risk groups - illustrates some of the limitations of current risk stratifications. If we are to obtain more patient-directed treatment recommendations, we need new optimal prognostic and predictive tools. The implementation of novel biomarkers might overcome some of these issues.

Biomarkers

The National Cancer Institute defines a biomarker as '*a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease*' and '*a biomarker may be used to see how well the body responds to a treatment for a disease or condition*'[79].

Biomarkers are typically proteins, metabolites, RNA transcripts, DNA, or epigenetic modifications obtained from tumour samples, blood, or other body fluids and can provide important information in different stages of a disease (Table 2) [21,80,81]. An ideal biomarker should be safe and easy to measure objectively, have high sensitivity and specificity for the outcome of interest, and improve decision making alone or in combination with standard clinicopathological parameters[81]. Biomarkers can potentially improve disease prognostication and thereby be used to personalise treatment regimens.

Biomarkers in prostate cancer

During the last decades, the diagnosis and management of PCa has hugely relied on biomarkers. Prostatic acid phosphatase (PAP), an enzyme produced by the luminal cells, was the first biomarker to be introduced[82]. In the 1970s and 1980s, PAP was replaced by the human kallikrein PSA[83,84], as PSA was demonstrated to be more sensitive when monitoring PCa patients[19].

The PSA-test has provided advancements in the management of PCa, but it has shortcomings, including its lack of specificity for PCa and the deficiency of a safe lower level that confers to a zero risk of PCa[24,25]. Thus, PSA is not an ideal biomarker for all aspects of the disease (Table 2). Due to these shortcomings, efforts have been made to enhance its performance[85], including the investigation of age-specific PSA references, the ratio of free to total PSA (percentage-free PSA), normalisation of the PSA value to the size of the

Table 2. The application of biomarkers		
Type of biomarker	*Patients*	*Use of biomarker*
Screening/disposition	Person at risk of developing a disease	Assess a person's risk of developing a disease
Diagnostic	Person with risk factors or symptoms compatible with a disease	Help in assessing presence or absence of a disease – who has the disease?
Prognostic	Patient who is currently not being treated	Predict patient outcome based on risk of progression or recurrence – what is the outcome without therapy?
Predictive	Patient who is currently being treated	Predict or monitor effectiveness of treatment – how effective is the therapy?
Therapeutic	Patient with a disease	Identify patients who will benefit from a given treatment – will therapy be effective or cause adverse reactions?
Surrogate	Patient with a disease	Substitute for a clinical endpoint
Adapted from Shariat SF *et al.*, Schalken J *et al.*, and Prensner JR *et al.* [21,80,81].		

prostate (PSA density), PSA dynamics (velocity and doubling time), and different isoforms of PSA (e.g. proPSA) [80,81]. However, none of these tests are able to truly answer two of the most crucial questions in PCa management: which patients need aggressive treatment in contrast to observation, and which patients will benefit most from the treatment?

Since the introduction of PSA, there has been a tremendous research activity in the field of PCa biomarkers and a considerable number of prognostic biomarkers have been proposed. Still, the only prognostic biomarker used routinely is PSA[86], and only two other biomarkers, PCA3 and proPSA, have been approved by the Food and Drug Administration. To some extend this is due to a lack of standardised methods for performing and interpreting biomarker studies, and consequently, the REMARK guidelines (REporting recommendations for tumour MARKer prognostic studies) have been published to enhance the transparency and uniformity of biomarker studies[87].

The need for new biomarkers in prostate cancer

Currently, PCa is regarded as a heterogeneous disease with a wide range of clinical courses. It can, however, be presumed that a better characterisation of the genomic and molecular diversity may lead to a transition in this perception. Hopefully, in the future, PCa will be regarded as an assembly of homogenous subtypes with individual risk profiles characterised by molecular criteria[88].

Implementation of new biomarkers is required for several reasons. First, PCa is a clinically heterogeneous disease, even within risk groups[28], and better treatment stratification of patients is needed[15,42]. Second, there is substantial inter-observer variability both among uro-pathologists in Gleason grading and among urologist in tumour staging, which have influence on the therapeutic decision-making[46,89,90]. Consequently, treatment recommendations in part depend on subjectivity, and objective markers might improve the risk assessment. Finally, PCa is characterised by spatial heterogeneity, and a standard biopsy set only samples 0.05-0.5% of the gland[42]. This may cause tumour undersampling, and novel biomarkers might predict the presence of unsampled significant tumour[42].

One of the most essential lessons learned from PSA studies is that the implementation of a biomarker needs *a priori* consideration regarding its clinical utility (Table 2) [81]. Although a significant amount of effort has been put into identifying new biomarkers, none have been able to outperform PSA. Hence, PSA remains the first choice among biomarkers for PCa management, as it is easy to assess and it is inexpensive, quantitative, and sensitive for monitoring post-therapy progression and recurrence[28]. Consequently, novel biomarkers will most likely complement rather than replace PSA.

An important requisite for new biomarkers is focusing on currently unmet clinical needs, and in order to affect therapeutic decision-making, the validation of biomarkers in pre-treatment biopsies is crucial[42]. Two of the most important clinical issues are: (1) which patients need treatment due to aggressive disease, and which patients can be followed expectantly due to indolent disease; and (2) which men will respond to a given therapy, and which treatment is the best for the patient. The identification and validation of such prognostic and predictive biomarkers would aid patients and clinicians in achieving personalised therapy.

Genetic alterations in prostate cancer

Cancers are characterised by genetic alterations, either germline variations present in all cells or somatic alterations arising in differentiated cells[91], leading to the activation of oncogenes or the inactivation of tumour suppressors. The alterations driving these changes include point mutations, gene copy number alterations, and promoter hypermethylation[92].

During recent years, it has become increasingly evident that PCa harbours an exceptional genetic heterogeneity, which may to some extent explain the clinical heterogeneity[88,93,94]. Whereas the PCa genome displays a low mutation rate and few chromosomal gains and losses compared with other cancers[88], gene fusions caused by chromosomal rearrangements are found in approximately half of PCas[95,96]. Gene rearrangements are generally of two types: (1) a promotor or enhancer element of one gene is placed next to a proto-oncogene, resulting in altered expression of the oncogenic protein, and (2) a fusion protein with altered activity arises from the fusion of two genes[95]. These structural alterations occur due to the imperfect restoration of double-strand DNA breaks and arise as either inter-chromosomal rearrangements, where genetic material is exchanged between different chromosomes with little or no loss, or intra-chromosomal rearrangements, where genetic material is exchanged within a single

chromosome, often resulting in deletion of genetic material[92]. As a result, genomic translocations, deletions, or amplifications occur, which can activate oncogenes or inactive tumour suppressors.

TMPRSS2-ERG *gene fusion*

Gene fusions are known to be important in the initial steps of PCa tumourigenesis[97]. Typically, the fusions consist of the untranslated region of an androgen-regulated gene as the 5' partner and an oncogenic member of the erythroblast transformation-specific (*ETS*) transcription factor gene family as the 3' partner[95,96,98]. The gene fusions result in the overexpression of an oncogene under transcriptional control of a tissue-specific androgen-regulated promotor[95,98].

Gene fusions between *ETS* transcription factor genes and the transmembrane protease serine 2 (*TMPRSS2*) gene were first described by Tomlins *et al.* in 2005[95]. Before their discovery, gene rearrangements were considered to exist mainly in haematological cancers and sarcomas and were thought to play only a minor role in the pathogenesis of carcinomas[92]. Now, gene fusions are known to occur in approximately half of PCas[95,99,100], with the most frequent (>90%) involving the 5' untranslated promoter region of *TMPRSS2* and the coding region of the transcription factor v-ets avian erythroblastosis virus E26 oncogene homolog (*ERG*) of the *ETS* gene family[95,96,99]. The resulting *TMPRSS2-ERG* gene fusion is found in 40-60% of PCa patients, with the highest prevalence among Caucasians and the lowest prevalence among Asians[100,101].

The *TMPRSS2* gene codes for an apical located, prostate-specific, constitutively expressed serine protease. The gene is under transcriptional control of androgens due to androgen response elements in the promotor region[102,103]. The human *ETS* transcription factor gene family consists of 28 members[104] that act as repressors and activators of distinctive transcriptional programmes by regulating the expression of downstream target genes[96]. By these means, ETS transcription factors regulate various biological processes including angiogenesis, cell proliferation, differentiation, tissue remodelling, invasive growth, and apoptosis[96,105–109]. The human *ERG* gene was discovered in 1987[110], and its specific downstream effects include the regulation of cell differentiation, proliferation, and apoptosis[111,112], which are all oncogenic hallmarks. ERG is expressed in adult and embryonic endothelial cells with effects on angiogenesis and maintenance of endothelial cell junctions[113] as well as in haematopoietic progenitor cells contributing to the normal haematopoiesis[114]. In contrast, ERG is not expressed in prostatic cells unless *ERG* rearrangements have occurred.

Both *TMPRSS2* and *ERG* are found on the long arm of chromosome 21 (21q22.2) approximately three megabase pairs apart[96]. The rearrangement breakpoints are located near androgen receptor binding sites, and androgen signalling facilitates chromatin looping leading to the juxtaposition of the *TMPRSS2* and *ERG* gene loci[94,115,116]. This enables the formation of *de novo* gene fusions when the prostate cells are exposed to genomic stress causing double-strand DNA breaks, which subsequently are incorrectly repaired[116]. *TMPRSS2-ERG* gene fusions are generated by either an interstitial deletion or an inter-chromosomal insertion (Figure 1) of the intervening 3 megabase pair region[99,117–122]. As a result, the promoter region that normally controls the expression of *TMPRSS2* drives the expression of an 5' truncated ERG oncoprotein in the presence of androgens, and ERG can then induce its target downstream effects[96].

Several transcriptional profile studies have demonstrated that PCas differ in their genomic signature according to gene fusion status[123–125]. Certain alterations, including deletions of the tumour suppressor phosphatase and tensin homolog (*PTEN*) and tumor protein p53 (*TP53*), are enriched in *ETS* fusion-positive tumours[88,126]. In contrast, mutations of speckle-type POZ protein (*SPOP*), deletion of chromodomain helicase DNA binding protein 1, and the overexpression of serine peptidase inhibitor, Kazal type 1 (*SPINK1*) is accumulated in *ETS* fusion-negative tumours[88,94,127]. Interestingly, genes, which are highly overexpressed in fusion-positive PCa compared to fusion-negative PCa, are strongly related to promoting cell proliferation[125]. Moreover, *ERG* rearrangements are more frequently found in early onset PCa, which might be caused by higher testosterone values in younger men[128,129]. Overall, these results suggest that *TMPRSS2-ERG* fusion-positive and fusion-negative tumours represent two different subtypes of PCa with potentially different clinical courses[117].

Several studies have analysed the functional role of *ERG* gene-fusions in PCa pathogenesis, and both *in vitro* and *in vivo* studies support a causal role for *ERG* rearrangements in initiating carcinogenesis. *In vitro* studies have demonstrated that *TMPRSS2-ERG* gene fusions drive a number of malignant processes including increased

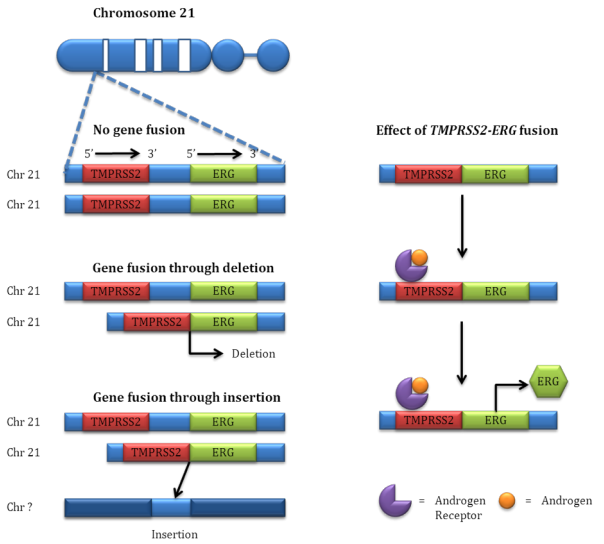

Figure 1: *The prostate-specific androgen-induced* TMRPSS2 *gene and the* ERG *gene are both located on chromosome 21 approximately three megabases apart. The* TMPRSS2-ERG *gene fusion can occur by one of two mechanisms: deletion of the intervening region or insertion of the intervening region into another chromosome. As a result,* ERG *expression comes under transcriptional control of the androgen sensitive promoter of* TMPRSS2. *Thus, when androgen-bound androgen receptors bind to the* TMPRSS2 *promoter region in fusion positive tumours,* ERG *is overexpressed and can induce its target downstream effects.*

cell proliferation, invasive growth, and cell motility[130–132]. *ETS* expression can induce the formation of hyperplasia and focal high-grade prostatic intraepithelial neoplasia (HGPIN) *in vitro* and *in vivo*, but is insufficient on its own to cause the development of carcinoma[123,132,133]. However, the addition of other genetic alterations including androgen receptor overexpression or an aberrant phosphoinositide 3-kinase signalling pathway can lead to the formation of invasive PCa[133,134]. These results suggest that *ERG* gene fusion facilitate the transition from HGPIN to PCa.

In PCa patients, the presence or absence of the *TMPRSS2-ERG* gene fusion can be determined by fluorescence *in situ* hybridisation (FISH) assays applied to sections from formalin-fixed, paraffin-embedded (FFPE) tissue. This technique visualises the gene fusion at the chromosomal level, as specific DNA sequences surrounding the *TMPRSS2* and *ERG* loci are detected using direct fluorochrome-labelled probes[95,99]. Moreover, the fusion transcript can be demonstrated by polymerase chain reaction techniques[135]. Several transcript variants have been described differing in the *TMPRSS2* and *ERG* exons included. The most common transcripts involve fusions between exon 1 or 2 of the *TMPRSS2* gene and either exon 2, 3, 4, or 5 of the *ERG* gene[95,118,136–138]. Importantly, the *ETS*-domain responsible for the DNA binding and the C-terminal transactivation domain are retained in all fusion transcripts, and the pointed PNT domain involved in homo-oligomerisation, hetero-dimerisation, and transcriptional alteration through phosphorylation is conserved in all but one of the transcript variants[137].

The gene fusion causes the expression of an ERG oncoprotein, which is truncated at the N-terminus due to the loss of the 5' exons of the *ERG* gene. In contrast, the functionally important DNA binding ETS-domain is conserved in all variants as it is located in the C-terminus[137,138]. The ERG protein is detectable by immunohistochemistry (IHC) techniques, and IHC has been shown to have high sensitivity (95.7-97.5%) and specificity (96.5-99.4%) for *ERG* rearrangements assessed by FISH[138,139]. In addition, ERG protein expression has been demonstrated to be equally high, regardless of the 5' partner, and protein expression levels are independent of the transcript variant[138]. Hence, ERG IHC can be applied as a surrogate for *ERG* rearrangement, which enables its routine use in clinical practice[38].

ERG rearrangements are never or very rarely found in benign prostatic cells and then only in close proximity to *ERG* rearranged malignant cells[140–142]. In contrast, approximately 15-20% of HGPIN lesions and 40-60% of PCa lesions harbour *ERG* rearrangements[95,117,137–140,143]. Importantly, all patients with *ERG* rearranged HGPIN lesions have been demonstrated to have PCa foci with *ERG* rearrangement as well[122,144–146]. Finally, *TMPRSS2-ERG* gene fusions have not yet been found in any other common tumour forms[147], and, as such, is a PCa-specific genetic alteration that occurs as an early-stage event during carcinogenesis[88,122].

Although numerous studies have been published, the prognostic value of *TMPRSS2-ERG* is still debatable. In brief, population-based studies have demonstrated that PCa patients with the gene fusion have a worse prognosis in terms of risk of developing metastases and PCa-specific death when managed observationally[148–150]. In contrast, a recent meta-analysis demonstrated that although the gene fusion was associated with increased pathological tumour (pT) category, (risk ratio [RR] ≥ pT3 versus pT2 = 1.23, [95% confidence interval (CI): 1.16-1.30]), the gene fusion was not associated with risk of PSA recurrence (RR = 1.00, [95% CI: 0.86-1.17]) or fatal disease defined as distant metastasis or PCa-specific death (RR = 0.99, [95% CI: 0.47-2.09]) after radical prostatectomy[100]. These contradictory observations may have several explanations, which will be discussed later.

The prevalence of the *TMPRSS2-ERG* gene fusion, the distinct genomic features observed between fusion-positive and fusion-negative tumours, and the possibility to indirectly detect the gene fusion using IHC provides an idea of its potential as a prognostic and predictive biomarker in selected PCa patients[117].

Immunohistochemistry

The thesis is mainly based on IHC detection of ERG protein, and the basic principles of the method are outlined in this section. Slightly simplified, IHC is founded on immunological techniques[151,152], where antigens can give rise to complimentary antibodies. These properties are usually located in one or a few epitopes of the antigen, and the complimentary antibodies have a high affinity for these epitopes[152]. Hence, IHC seeks to demonstrate the presences or absence of a specific protein, the antigen, in cells and tissues by applying epitope-specific antibodies to tissue sections.

Immunohistochemical detection of ERG protein

A commercially available anti-ERG rabbit monoclonal primary antibody was first described in 2010[138]. The antibody can be combined with the Ventana BenchMark XT's OptiView DAB kit, which uses an enzymatic peroxidase detection system to identify the binding of the primary antibody and thereby indirectly the presence of ERG (Figure 2).

During routine tissue preservation, the tissue samples are put into a fixative medium[152]. Fixation can result in the masking of epitopes, and epitope retrieval is often necessary for regaining immune reactivity. Consequently, an important step during the IHC procedure is the application of proteolytic enzymes or heat-induced epitope retrieval (HIER) for antigen retrieval to increase the likelihood of antibody binding[153,154].

The primary antibody binds to the target epitope of the ERG protein. Subsequently, a haptenated secondary antibody binds to the primary antibody. Finally, an anti-hapten horse-radish peroxidase (HRP) multimer reacts with the secondary antibody and its binding is detected indirectly by adding hydrogen peroxide as a substrate and the chromogen 3-3'-diaminobenzidin (DAB) as the electron donor (Figure 2). DAB becomes insoluble and brown after oxidation by HRP, and this product is visible in a bright-field microscope[152].

The monoclonal anti-ERG primary antibody is targeted at the C-terminus of ERG corresponding to the conserved 3' end of the gene. It is capable of detecting wildtype as well as truncated ERG and has

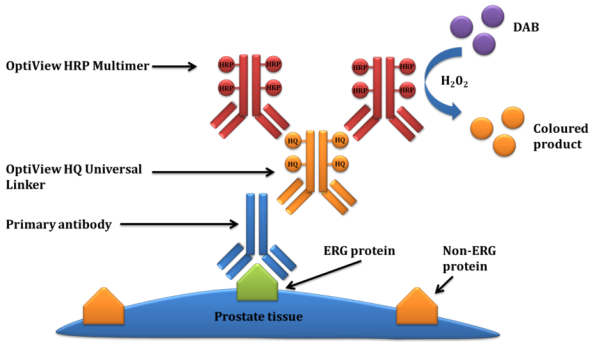

Figure 2: *The IHC assay for ERG protein detection. The anti-ERG rabbit monoclonal primary antibody binds to ERG in the tissue section. The OptiView HQ Universal Linker binds the primary antibody and provides numerous non-endogenous HQ haptens facilitating the binding of OptiView HRP Multimer. Finally, the DAB chromogen reacts with HRP and H_2O_2 to generate a coloured product, which can be visualised in a bright-field microscope.*
Abbreviations: DAB: 3-3'-diaminobenzidin; HRP: horse-radish peroxidase.

been demonstrated to be highly specific for the ERG protein in immunoblotting analyses. Moreover, a high concordance with *ERG* rearrangement has been demonstrated irrespective of the 5' fusion partner[138]. Lastly, ERG expression is confined to the nuclei of malignant prostate cells harbouring *ERG* rearrangement but can also be demonstrated in endothelial cells of the small vessels (Figure 3); the latter can serve as an internal control for the staining procedure.

OBJECTIVES AND HYPOTHESES

Prognostic molecular markers defining PCa aggressiveness and predictive molecular markers predicting treatment response are needed if personalised treatment is to be implemented in the management of PCa. To analyse the true prognostic value of a biomarker necessitates the use of a non-interventional study cohort like an AS patient cohort. Moreover, as treatment decisions are mainly based on the histopathological assessment of biopsies, it is desirable to identify and validate prognostic as well as predictive biomarkers in diagnostic tissue samples.

Objectives

The main objectives of the studies included in this PhD thesis were to assess the prognostic value and the predictive value of ERG protein expression in PCa patients. Specific objectives for the thesis and the three included papers were:

I. *Study 1:* Assess the concordance in biopsy specimens between FISH analysis for *TMPRSS2-ERG* rearrangement and IHC analysis for ERG protein expression[1].
II. *Study 1:* Describe changes in ERG protein expression between biopsy sets and between biopsies and radical prostatectomy specimens in patients managed on AS and assess the reliability of ERG expression status in biopsies[1].
III. *Study 2:* Assess the association between ERG protein expression and the risk of disease progression during an AS programme[2].
IV. *Study 3:* Assess the association between ERG protein expression and response to primary castration-based ADT and risk of development of CRPC for patients undergoing first-line castration-based ADT for advanced PCa[3].

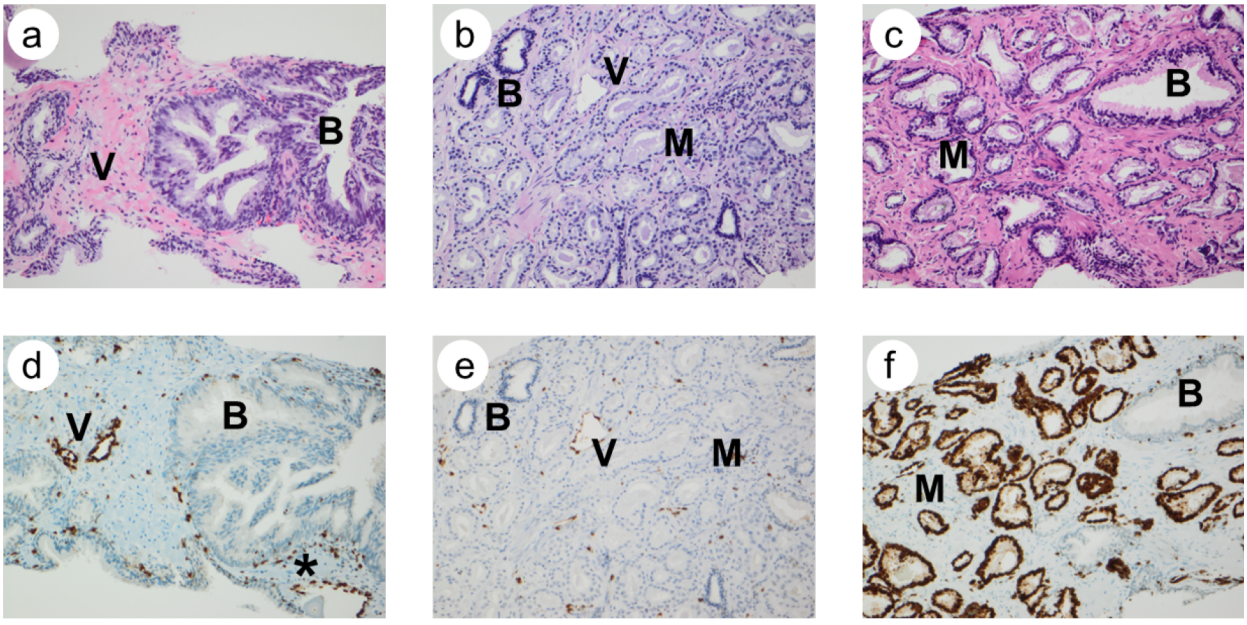

Figure 3: *H&E staining (a - c) and IHC staining for ERG protein (d - f) in three matched biopsy cores from the active surveillance population. (a+d) Matched H&E stain and negative IHC stain of benign glands. (b+e) Matched H&E stain and negative IHC stain of malignant glands. (c+f) Matched H&E stain and positive IHC stain of malignant glands. Endothelial cells and immune cells stain positive for wild-type ERG and serve as internal controls for the IHC staining procedure. Original magnification: x100.*
Abbreviations: B: benign glands; M: malignant glands; V: vessel; asterisk: immune cells[2].

Hypotheses

I. *Study 1:* IHC analysis for ERG protein expression and FISH analysis for *TMPRSS2-ERG* rearrangement show a high (>95%) correlation enabling the use of IHC in clinical practice.
II. *Study 1:* Assessment of ERG protein expression in serial biopsies and radical prostatectomy specimens from patients initially managed on AS show a low degree (<10%) of qualitative variation supporting the potential use of ERG IHC in needle biopsies for prognostication and prediction.
III. *Study 2:* AS patients expressing ERG protein at the time of diagnosis have an increased risk of disease progression during surveillance and thereby being recommended to change treatment strategy to a curatively intended therapy.
IV. *Study 2:* ERG protein expression adds prognostic value to standard clinical and pathological variables for disease progression during AS.
V. *Study 3:* Advanced PCa patients, who express ERG protein and undergo first-line castration based ADT, have a prolonged response to ADT therapy and thereby time to CRPC when compared to patients without ERG expression.
VI. *Study 3:* ERG protein expression adds prognostic value to standard clinical and pathological variables for predicting the risk of CRPC development following first-line ADT.

PATIENTS AND METHODS

This section provides an overview of the patient cohorts included in the thesis (Table 3). Moreover, a summary of the IHC and the FISH assays as well as the study designs and statistical methods for each of the three included papers are presented. For more detailed descriptions, see the three included papers.

Study populations

Rigshospitalet is a large university hospital with PCa patients referred from the eastern part of Denmark, i.e. Zealand and Bornholm, but also from Greenland and The Faroe Islands. Thus, the included patients were referred from these areas to the Department of Urology at Rigshospitalet for the treatment of PCa. The diagnostic work-up followed the institutional guidelines and national guidelines published by the Danish Urological Society and the Danish Urological Cancer Group[155].

The active surveillance cohort

Since the institutional AS protocol was established in 2002, patients have been prospectively enrolled and registered. Selection criteria for enrolment were: PSA ≤ 10 ng/mL, cT category ≤ cT2a, diagnostic GS ≤ 6, maximum 3 cores with cancer involvement, and ≤ 50% tumour in any single core[156]. Moreover, well-informed patients who did not fulfil all of the selection criteria and who had a strong request for AS were accepted onto the programme. The follow-up protocol consisted of quarterly PSA measurements and

Table 3. Study populations included in the thesis

Study number	*Patient cohort (Period)*	*Number of patients*	*Tissue type and number of specimens with tumour*	*IHC or FISH*	*Endpoints*
Study 1 [1]	Active surveillance cohort (Oct. 2002 to Oct. 2012)	Total number of patients: *n = 282* Eligible patients: *n = 265* Final analysis: *n = 265*	Diagnostic specimens *n = 459* Re-biopsies *n = 402* Radical prostatectomies *n = 86*	IHC: all specimens with tumour FISH: 74 selected biopsies	Primary endpoint: Concordance in ERG-status between biopsies and prostatectomy Secondary endpoint: Concordance between IHC and FISH
Study 2 [2]	Active surveillance cohort (Oct. 2002 to Oct. 2012)	Total number of patients: *n = 282* Eligible patients: *n = 265* Final analysis: *n = 217*	Diagnostic specimens *n = 459*	IHC: all specimens with tumour	Primary endpoint: Risk of AS progression Secondary endpoints: Risk of histopathological progression; Risk of PSAdt progression; Risk cT progression
Study 3 [3]	Advanced PCa cohort (Jan. 2000 to Dec. 2011)	Total number of patients: *n = 213* Eligible patients: *n = 194* Final analysis: *n = 194*	Diagnostic specimens *n = 968*	IHC: all specimens with tumour	Primary endpoint: Risk of CRPC Secondary endpoints: Time to PSA nadir; Proportion achieving PSA nadir ≤ 0.2 ng/ml; Risk of PCa-specific death

Abbreviations: AS: active surveillance; CRPC: castrate-resistant prostate cancer; cT: clinical tumour category; FISH: fluorescence *in situ* hybridization; IHC: immunohistochemistry; PCa: prostate cancer; PSA: prostate-specific antigen; PSAdt: prostate-specific antigen doubling time;

DREs, and a 10 to 12 core TRUS-guided re-biopsy after 12 months[156,157]. During the AS programme, patients were risk-stratified according to changes in cT category, histopathological assessments, and PSA values (Table 4). Subsequent follow-up and treatment recommendations were according to the protocol[156,157].

The advanced prostate cancer cohort

The cohort was established retrospectively by a standardised extraction of patient data from the institutional patient register (GS-Åben database). The register consists of complete data regarding institutional patient contacts, diagnose codes, procedure codes, and dates of visits, and all data are coupled to the unique 10 digit civil registration number assigned to all Danish citizens.

Data extraction included names and civil registration numbers for patients fulfilling the inclusion criteria for *Study 3*: men diagnosed with PCa (International Classification of Diseases 10 code: DC619) during January 1st 1995 to December 31st 2012 who had undergone endocrine therapy (Sundhedsvæsenets Klassifikations System [SKS] procedure code: BWHC and subgroups) between January 1st 2000 and December 31st 2012. Only patients undergoing first-line ADT were of interest; thus, exclusion criteria were prior radical prostatectomy (SKS procedure code: KKEC and subgroups) and external beam radiation therapy (SKS procedure code: BWGC and subgroups). The data extraction resulted in a total of 427 patients for which patient records were reviewed. Prior to *Study 3*, a total of 214 patients were excluded. Exclusions were caused by prior radical prostatectomy (n=7), radiotherapy (n=82), or other curatively intended therapies (n=8) at other institutions, first-line anti-androgen (n=39) or MDV-3100 therapy (n=1), PCa diagnosis before January 2000 or after December 2011 (n=29), missing patient records (n=14), and no relevant PCa tissue sampled prior to ADT (n=34). Patient records from the remaining 213 patients undergoing primary castration-based ADT were thoroughly reviewed for relevant patient and tumour characteristics plus follow-up information.

According to local guidelines, patients eligible for primary ADT include PCa patients with lymph node metastases diagnosed by lymphadenectomy or suspected due to enlarged lymph nodes visualised by abdominal imaging, and patients with symptomatic or asymptomatic distant PCa metastases visualised by bone scans, computed tomography (CT) scans, and/or magnetic resonance imaging (MRI) [155]. Moreover, ADT could be initiated in men with locally advanced PCa and a suspicion of tumour dissemination due to highly elevated PSA values, even though metastases had not been visualised. ADT modalities included bilateral orchiectomy and LHRH analogues with 4-week anti-androgen treatment for flare protection. Maximal androgen blockade was rarely considered a first-line treatment option.

Patients visited the outpatient clinic once per quarter and were followed with PSA-measurements and clinical examinations. Serum testosterone was measured when PSA-values increased to ensure that the patient was sufficiently castrated (serum testosterone < 50 ng/dl) before considering a change of treatment. Patients were recommended to undergo bilateral orchiectomy in case of a non-sufficiently reduced testosterone level.

Histopathological assessment and laboratory procedures

Histopathological assessment of tissue samples

All available slides were collected from referral hospitals and the institutional archive. The slides were reviewed by two highly experienced uro-pathologists to confirm the PCa diagnosis and re-evaluate the GS according to the ISUP 2005 guidelines[45]. Moreover, biopsies eligible for FISH analysis in *Study* 1 were identified. To ensure the availability of sufficient tumour tissue, eligible biopsies had to contain a minimum of 2 mm of consecutive cancerous tissue not intermingled with benign glands or inflammatory cells.

For *Study 1*, H&E sections from the prostatectomy specimens were re-evaluated and cancer regions were marked for the construction of tissue microarrays (TMA). From all cancer-containing sextants (apex, middle, and base; right versus left), 2.0-mm cores were collected from tumour foci and one or two benign cores were collected from the specimen. The TMA cores were transferred into one of seven recipient blocks.

Tumour-containing FFPE tissue blocks were collected from relevant archives, and new sections were cut for H&E, IHC, and FISH analysis. One or two consecutive sections were mounted on each of three glass slides. The first slide was used for H&E staining, whereas the following two slides were saved for IHC. Furthermore, for the selected biopsies, an additional section was cut for FISH analysis and was mounted on a Super Frost Plus glass slide next to a freshly cut tonsil section from a healthy male.

Table 4. Risk stratification of active surveillance patients

*Risk group**	*PSAdt (years)*	*Histopathological assessment of re-biopsies*	*cT category*	*Treatment recommendation*
High-risk	< 3	GS ≥ 7 (4+3) > 3 cores with cancer involvement Bilateral tumour on re-biopsies	≥ cT2c	Undergo curatively intended treatment
Intermediate-risk	3 – 5	GS = 7 (3+4)	cT2b	Discuss active surveillance versus curatively intended treatment with patient
Low-risk	> 5	No progression	≤ cT2a	Continue active surveillance

*High-risk patients fulfilled at least one high-risk criterion; intermediate-risk patients fulfilled at least one intermediate-risk criterion and no high-risk features; low-risk fulfilled all low-risk critertia.
Abbreviations: cT: clinical tumour category; GS: Gleason score; PSAdt: prostate-specific antigen doubling time.
Adapted from Thomsen FB *et al.*[156].

The immunohistochemical assay for ERG protein expression

For IHC, freshly cut 3-4 µm sections were stained using an automated staining system (Ventana Benchmark, Ventana Medical Systems, Tucson, USA). Sectioning and IHC procedures were performed by experienced lab-technicians. For all batches of the primary antibody, one ERG-positive and one ERG-negative section were stained together with the study tissue as a control for the procedure.

A ready-to-use kit from Ventana was applied with buffers and reagents from Ventana (Figure 2). Slides were heated to 72°C for paraffin removal. Afterwards, the slides were rehydrated and Cell Conditioning 1 (pH 8.5) was applied for HIER at 100°C (32 min). A peroxidase inhibitor was applied to inhibit the endogen peroxidase activity succeeded by application of the anti-ERG rabbit monoclonal primary antibody (clone: EPR3864, dilution 23 µg/mL; 32 min at 36°C). Afterwards, a universal linker was added followed by the HRP multimer, addition of H_2O_2, and one drop of the amplifier for signal amplification. Then, the DAB chromogen was applied followed by copper-enhancement to increase the signal and give it a reddish-brown colour. Finally, the slides were counter-stained with haematoxylin before coverslips were mounted.

Slides were assessed in a bright-field microscope (Olympus BX51) for ERG expression. The staining intensity in the nuclei of PCa cells was scored separately for each tumour focus using a four-tier semi-quantitative grading system: negative (no staining = 0), weakly positive (only evident at high magnification [x10 objective magnification] = 1+), moderately positive (evident at low magnification [x4 objective magnification] = 2+), and strongly positive (striking at low magnification = 3+) [138,158]. A patient was labelled as being ERG-positive if all foci demonstrated ERG expression, whereas patients with exclusively negative foci were labelled ERG-negative. If both ERG-positive tumour foci and ERG-negative tumour foci were evident, the patient was labelled ERG-heterogeneous. Nuclear staining of endothelial cells served as a positive control for the staining procedure.

The fluorescence in situ *hybridisation assay for* TMPRSS2-ERG *gene fusion*

Fresh 3-4 µm sections mounted on Super Frost Plus slides were used for FISH analyses for selected biopsies in *Study 1*. The sectioning and the FISH procedure were performed by experienced and dedicated lab-technicians. The FISH analysis consisted of a triple-colour assay with labelled probes hybridising to the 21q22.13-21q22.3 region at 3' *ERG* (orange), 5' *ERG* (green), and 5' *TMPRSS2* (aqua) regions (Figure 4).

Sections were heated for 1h at 60°C, deparaffinised in xylene, and rehydrated in ethanol. Subsequently, pepsin solution (5°C) was applied and the sections were dehydrated in ethanol washes. The tumour area of interest was marked on the back of the slides and according to tumour size, 3-10 µl of the FISH ZytoLight® TriCheck™ Probe, SPEC *ERG/TMPRSS2* (Zytovision) was applied in darkness. Coverslips were mounted and sealed, and sections were denatured (75°C) and hybridised at 37°C overnight. The following day, coverslips were demounted, sections were washed and rehydrated, and finally 10 µl of 4',6-diamidino-2-phenylindole was applied for cell nuclei staining before coverslips were mounted.

FISH slides were screened for *ERG* rearrangement by an experienced lab-technician using a Leica DM6000B fluorescence microscope with proper filters. Subsequently, the slides were scanned in a Zeiss Axio Scan.Z1 fluorescence whole slide scanner (Carl Zeiss Microscopy Gmbh) using the appropriate filters for further assessment. Presences of ≥20% rearranged cells was considered to indicate the presence of *ERG* rearrangement[159,160].

Study designs

An overview of the three included studies is provided in this section and in Table 3. Detailed descriptions are provided in the three included papers.

Study 1 – Agreement in ERG status over time

All tumour-containing diagnostic specimens, re-biopsies, and prostatectomy specimens from the AS cohort were included[1]. ERG expression was assessed by IHC in all specimens, and for 76 patients, a diagnostic biopsy or a re-biopsy was selected for FISH analysis. ERG status was treated both as a dichotomised (ERG-negative versus ERG-positive or ERG-heterogeneous) and as a three-tier category.

The objectives of *Study 1* were to describe changes in ERG expression between biopsy sets and between biopsies and radical prostatectomy specimens, and furthermore assess the concordance between FISH analysis for the *TMPRSS2-ERG* rearrangement and IHC

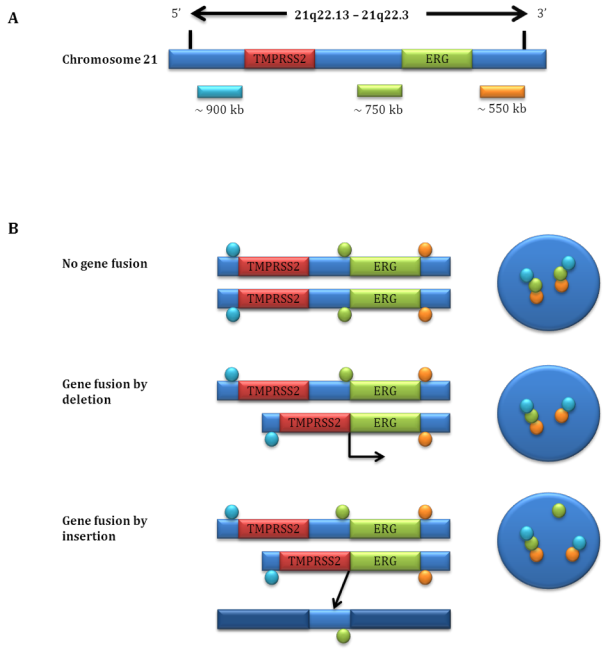

Figure 4: *A: Schematic map of the* TMPRSS2 *and* ERG *gene regions on chromosome 21. Aqua, green, and orange signals represents the 5'* TMPRSS2*, 5'* ERG*, and 3'* ERG *regions, respectively. B: The assay allowed identification of nuclei without* TMPRSS2-ERG *rearrangement (one triple signal for each allele composed of juxtaposed orange, green, and aqua signals), gene rearrangement through deletion (loss of the green signal for the rearranged allele), and rearrangement through insertion (a fused orange-aqua signal and a separated green signal for the rearranged allele).*

analysis for ERG protein expression. Finally, the association between ERG protein expression and histopathological features in the prostatectomy specimens was analysed. Histopathological features that indicate a poor outcome, henceforth referred to as 'unfavourable outcome', was defined as the presence of minimum one of the following three features: (1) extraprostatic tumour growth (≥ pT3a), (2) GS ≥ 7 (4+3) in the prostatectomy specimen, and (3) node positive disease (i.e. spreading to regional lymph nodes).

Reclassification of ERG status was analysed using descriptive statistics. The concordance between IHC and FISH was analysed using the 2-tailed Fisher's exact test, and the sensitivity and specificity for the IHC procedure was calculated using FISH as the gold standard. Finally, the association between ERG status and an unfavourable outcome was analysed using univariate logistic regression.

Study 2 - ERG expression and risk of active surveillance progression

Study 2 sought to analyse the association between ERG expression in diagnostic specimens and the risk of disease progression during the AS programme[2]. All tumour-containing diagnostic specimens from the AS cohort were included and ERG protein expression was assessed using IHC. In *Study 2*, patients were dichotomised according to ERG status.

The primary endpoint was overall progression during AS defined as at least one of (1) clinical progression (increase in cT category ≥ cT2b), (2) PSA progression (estimated PSA doubling time [PSAdt] < 3 years), and (3) histopathological progression on re-biopsies (GS ≥ 7 (3+4) and/or more than three cores with tumour and/or bilateral positive re-biopsy cores). Secondary endpoints were the risk of progression in any of the three criteria alone.

The final analyses included the 217 patients with complete diagnostic information, who had undergone at least one re-biopsy session, and had a minimum of three valid PSA measurements after AS enrolment. Cumulative incidences of the primary and secondary endpoints were analysed with competing risk models using the Aalen-Johansen method. Death and treatment change to either watchful waiting or curative intended therapy were treated as a single competing event. Gray's test was applied to analyse for differences in the cumulative incidences between ERG positive patients and ERG negative patients. Finally, multiple cause-specific Cox proportional hazard regression analyses, reclassification diagrams, calibration plots as well as plots for changes in the discriminative ability and prediction error obtained by adding ERG status to the model were performed to evaluate the prognostic ability of ERG status for the risk of overall progression.

Study 3 - ERG expression and risk of castration-resistant prostate cancer

The objective of *Study 3* was to assess the association between ERG protein expression and response to primary castration-based ADT[3]. All tumour-containing diagnostic specimens from the advanced PCa cohort were included and examined for ERG expression using the IHC assay. In *Study 3*, patients were dichotomised according to their diagnostic ERG status.

The primary endpoint was risk of CRPC development defined according to the European Association of Urology (EAU) guidelines[73]. Secondary endpoints were the percentage of patients reaching a PSA nadir ≤ 0.2 ng/mL, time to achievement of a PSA nadir, and risk of PCa-specific mortality. The cumulative incidences of CRPC, PSA nadir, and PCa-specific mortality were investigated by the use of the Aalen-Johansen method for competing risks. Further, differences in the cumulative incidences between the ERG subgroups were analysed using Gray's test. Differences in the proportion of patients with ERG-positive and ERG-negative tumours reaching a PSA nadir ≤ 0.2 ng/mL were analysed using the chi-square test.

In order to analyse the risk of developing CRPC, *Study 3* applied an ERG-stratified multiple cause-specific Cox proportional hazard regression model. This method was used due to the rejection of the proportional hazard assumption for ERG status. The predictive value of ERG protein expression for developing CRPC was investigated by comparing predictions obtained from the stratified model with those obtained from a model omitting ERG status. Finally, the discriminative ability of the two models was compared using time-dependent area under the ROC curves (AUC).

RESULTS

According to the objectives and hypotheses of the thesis, core results from the three included studies are presented in the following section.

Core results

Study 1 - Agreement in ERG status over time [1]

Assessment of the diagnostic specimens categorised 101 patients (38.1%) as being ERG-positive, 22 (8.3%) as being ERG-heterogeneous, and 142 (53.6%) were labelled ERG-negative. The concordance between the FISH assay and IHC assay was 97.3% (95% CI: 89.7 - 99.5) when FISH results and IHC results were dichotomised (Table 5). This corresponded to a sensitivity of 100% and a specificity of 95.5% for the IHC assay.

During the four rounds of re-biopsies, 27 patients experienced ERG reclassification (Table 6). For 22 of these patients (81.5%), the reclassification was caused by an ERG-positive PCa focus demonstrated in a re-biopsy from a novel sextant of an otherwise previously entirely ERG-negative prostate, or vice versa.

Table 5: Concordance between *ERG* rearrangement by FISH and ERG protein expression by IHC [1]

	***ERG* rearrangement**		
	Positive	Negative	Total
ERG protein expression			
Positive	42	2	44
Negative	0	30	30
Total	42	32	74

ERG rearrangement positive cases included both deletions and translocations. Two-tailed Fischer exact test: $p<0.001$.
Abbreviations: FISH: fluorescence *in situ* hybridisation; IHC: immunohistochemistry.

Table 6: Concordance in ERG protein expression between diagnostic specimens and re-biopsies [1]

	Re-biopsies						
Diagnostic specimens	ERG-negative	ERG-positive	ERG-heterogeneous	No cancer	Not collected	Total	Agreement (95% CI)
ERG-negative	49 (34.5%)	7 (4.9%)	8 (5.6%)	62 (43.7%)	16 (11.3%)	142	88.2% (83.1-91.9)
ERG-positive	7 (6.9%)	42 (41.6%)	5 (5.0%)	29 (28.7%)	18 (17.8%)	101	93.4% (89.2-96.1)
ERG-heterogeneous	4 (18.2%)	6 (27.3%)	5 (22.7%)	4 (18.2%)	3 (13.6%)	22	
Total	60	55	18	95	37	265	

Light-grey and dark-grey-shaded boxes indicate disagreement in ERG status between biopsy sets, when ERG status was assessed as a three-tier category. Moreover, dark-grey-shaded boxes indicate disagreement in ERG status between biopsy sets, when ERG status was dichotomised.
Agreement in ERG status is presented in two ways: ERG status assessed as either a three-tier category (the uppermost percentage) or dichotomised (the lowermost percentage).

Table 7: Concordance in ERG protein expression between biopsies and prostatectomy specimens [1]

	Radical prostatectomy specimens					
Diagnostic specimens and re-biopsy no. 1-4	ERG-negative	ERG-positive	ERG-heterogeneous	NA	Total	Agreement (95% CI)
ERG-negative	29 (78.4%)	0 (0%)	5 (13.5%)	3 (8.1%)	37	81.4% (71.2-88.7)
ERG-positive	2 (5.7%)	24 (68.6%)	9 (25.7%)	0 (0%)	35	94.2% (86.3-97.8)
ERG-heterogeneous	5 (29.4%)	0 (0%)	12 (70.6%)	0 (0%)	17	
Total	36	24	26	3	89	

Light-grey and dark-grey-shaded boxes indicate disagreement in ERG status between biopsies and prostatectomies, when ERG status was assessed as a three-tier category. Moreover, dark-grey-shaded boxes indicate disagreement in ERG status between biopsies and prostatectomies, when ERG status was dichotomised.
Agreement in ERG status is presented in two ways: ERG status assessed as either a three-tier category (the uppermost percentage) or dichotomised (the lowermost percentage).
Abbreviation: NA: not assigned (due to no cancer in RP specimen [n=1] or no tissue available for ERG IHC analysis [n=2]).

After radical prostatectomy, a total of 24 patients (27.9%) were ERG-positive, 36 patients (41.8%) were ERG-negative, and 26 patients (30.2%) were ERG-heterogeneous. When ERG status in biopsies was compared to ERG status in the prostatectomies, 16 patients were reclassified after surgery (Table 7). Also in this setting, the majority of reclassifications (81.3%) were caused by finding an ERG-positive PCa focus in a novel sextant of an otherwise ERG-negative gland, or vice versa.

ERG status in prostatectomy specimens was not associated with GS, pT category, pN category, or resection margin status, but a trend for an increased odds of having an 'unfavourable outcome' was found for patients with ERG-positive tumours compared to patients with ERG-negative tumours (odds ratio [OR]: 2.14; 95% CI: 0.74 - 6.22; $p = 0.16$).

Study 2 - ERG expression and risk of progression during active surveillance[2]

The analyses included 459 tumour containing diagnostic specimens from 265 patients followed for a median of 4.1 years on AS. Patients were dichotomised according to ERG status at diagnosis. A total of 123 patients (46.4%) were categorised as ERG-positive, whereas 142 patients (53.6%) were categorised as ERG-negative (Table 8).

Prior to the main analyses, 48 patients were excluded from the study, whereof 21 (43.7%) were ERG-positive and 27 (56.3%) were ERG-negative. Reasons for exclusions were: no re-biopsies performed (n=32), less than three PSA measurements obtained during AS (n=4), missing diagnostic information (n=7), or combinations hereof (n=5). Thus, 217 patients were included in the main analyses.

A significantly higher cumulative incidence of overall progression was demonstrated in patients with ERG-positive tumours compared to patients with ERG-negative tumours ($p < 0.0001$) corresponding to a 2-year cumulative incidence of progression of 58.6% (95% CI: 48.7-68.5) in patients with ERG-positive PCas and 21.7% (95% CI: 14.3-29.1) in patients with ERG-negative PCas (Figure 5a). Moreover, ERG-positive patients had a higher cumulative incidence of histopathological progression (Figure 5b, $p < 0.0001$) and PSA progression ($p < 0.0001$) when these criteria were examined separately, whereas no significant difference was observed in terms of cT progression ($p = 0.13$). In the multiple cause-specific Cox analyses, ERG positivity was a strong and independent predictor for both overall progression (Hazard ratio [HR]: 2.45, 95% CI: 1.62-3.72) and progression on re-biopsies (HR: 3.06, 95% CI: 1.78-5.26), (Table 9). Finally, the inclusion of ERG status into the model

Table 8. Baseline characteristics of the active surveillance cohort [2]			
	ERG-negative n=142	**ERG-positive n=123**	***p* value**
Age at AS enrolment, years, median (IQR)	65.8 (63.5-68.5)	65.8 (63.5-67.8)	0.55
PSA, ng/mL, median (IQR)	6.4 (4.6-8.1)	6.5 (5.0-8.5)	0.36
TRUS prostate volume, mL, median (IQR)*	44 (33-61)	46 (35-66)	0.48
PSA density, median (IQR)*	0.13 (0.09-0.19)	0.13 (0.09-0.20)	0.79
Diagnostic cores taken, no., median (IQR)	10 (10-20)	10 (10-12)	0.38
Diagnostic positive cores, no., median (IQR)†	1 (1-2)	2 (1-2)	0.18
Percentage of positive biopsies, median (IQR)	10.0 (5.0-20.0)	12.5 (10.0-20.0)	0.018
Max tumour involvement, %, median (IQR)	7.0 (5.0-10.0)	10.0 (5.0-20.0)	0.003
Diagnostic Gleason score, n (%)			0.54
GNA	11 (7.7)	11 (8.9)	
GS 6 (3 + 3)	121 (85.2)	99 (80.5)	
GS 7 (3 + 4)	10 (7.1)	13 (10.6)	
Clinical tumour category, n (%)			<0.001
cT1	135 (95.1)	102 (82.9)	
cT2	7 (4.9)	21 (17.1)	

Abbreviations: GNA: Gleason score not assigned due to low tumour burden; IQR: Interquartile range; PSA: prostate specific antigen; TRUS: Transrectal ultrasound.
* Number of missing values = 9.
† Twenty-five patients were diagnosed with transurethral resection of the prostate without any positive biopsy cores prior to enrolment in AS; in 10 patients the number of positive cores was ≥ 4 due to more than one biopsy-set.

altered the predicted risk of overall disease progression significantly, lowered the prediction error, and increased the discriminative ability of the model (Figure 6).

Study 3 - ERG expression and risk of castration-resistant prostate cancer[3]

This study was comprised of 968 tumour-containing diagnostic specimens from 194 men diagnosed with advanced PCa. A total of 105 patients (54.1%) had ERG-positive tumours and 89 patients (45.9%) had ERG-negative tumours.

Median time of follow-up was 6.8 years, during which 114 patients (58.7%) developed CRPC. Forty-two patients (21.6%) died before they developed CRPC, and the remaining patients (19.6%) were censored at data analysis. Time to CRPC (ERG-positive versus ERG-negative patients: median 3.9 years [95% CI: 3.2-5.1] versus 4.6 years [95% CI: 2.3-not reached]) and the risk of CRPC (Gray's test: $p = 0.51$) did not differ between the ERG subgroups (Figure 7).

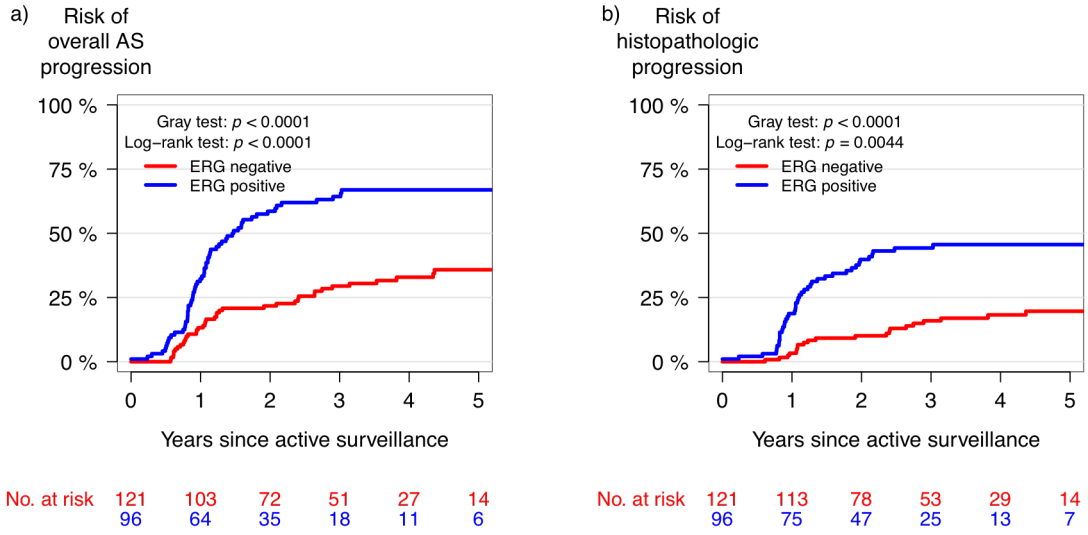

Figure 5: *The cumulative incidence of (a) overall progression and (b) histopathological progression during active surveillance. Competing events are change in treatment strategy to curatively intended treatment or watchful waiting, or death without prior progression*[2].

Table 9. Adjusted cause-specific proportional hazard models for progression during AS [2]				
Variables	**Overall progression**		**Histopathological progression**	
	Hazard ratio (95% CI)	*p* value	Hazard ratio (95% CI)	*p* value
Age; 5 years diff.	1.00 (0.75-1.33)	0.99	1.05 (0.73-1.51)	0.80
PSA density; 2-fold diff.	1.19 (0.92-1.53)	0.19	1.32 (0.94-1.84)	0.10
PPB; 5 %-points diff.	1.10 (0.98-1.23)	0.12	1.20 (1.05-1.38)	0.0089
Max tumour involvement; 2-fold diff.	1.21 (0.99-1.48)	0.067	1.26 (0.97-1.63)	0.078
Diagnostic Gleason score				
GNA	1.00 (Ref)		1.00 (Ref)	
GS 6 (3 + 3)	0.58 (0.29-1.14)	0.11	0.52 (0.21-1.24)	0.14
GS 7 (3 + 4)	1.23 (0.53-2.87)	0.63	1.13 (0.38-3.33)	0.83
Clinical tumour category				
cT1	1.00 (Ref)		1.00(Ref)	
cT2	1.35 (0.72-2.50)	0.35	1.11 (0.52-2.39)	0.79
ERG status				
ERG-negative	1.00 (Ref)		1.00 (Ref)	
ERG-positive	2.45 (1.62-3.72)	<0.0001	3.06 (1.78-5.26)	<0.0001
Abbreviations: AS: Active Surveillance; CI: Confidence intervals; GNA: GS not assigned; GS: Gleason score; PPB: Percentage of positive biopsies.				

In the ERG stratified multiple cause-specific Cox analysis (Table 10), doubling of baseline PSA values were found associated with a 33% increased hazard of CRPC development (HR: 1.33, 95% CI: 1.18-1.49). The discriminative ability for predicting the risk of CRPC within the first 8 years of ADT was not increased by adding ERG status to the multiple model (Figure 8).

During the study period, most patients (92.3%) achieved a PSA nadir, and 111 men (57.2%) died, with 58 (52.3%) dying as a result of PCa. No differences between ERG subgroups were observed for these secondary endpoints. Moreover, the proportion of patients with ERG-positive and ERG-negative tumours reaching a PSA nadir ≤ 0.2 ng/mL did not differ (35.2% versus 30.3%; $p = 0.89$).

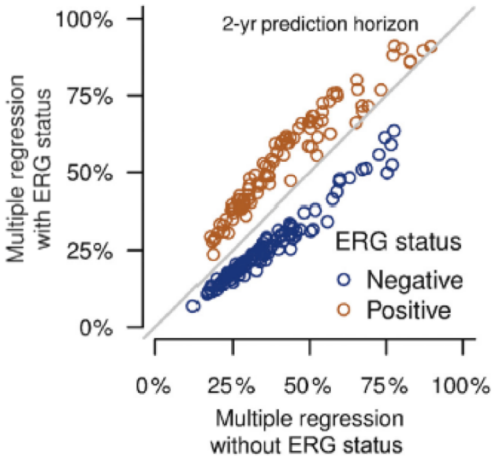

Figure 6: *The impact of ERG status for predicting the risk of overall progression within two years of AS, based on a multiple model without ERG status (x-axis) versus an expanded multiple model including ERG status (y-axis) [2].*

DISCUSSION

The focus of the thesis was to evaluate the feasibility of IHC assessment of ERG protein expression in biopsy specimens and to evaluate the prognostic value and the predictive value of ERG expression in PCa patients. Overall, the thesis demonstrated that ERG status in biopsies can be used reliably to characterise men's ERG status. Moreover, ERG status harbours important prognostic value in terms of tumour progression for patients managed on AS, whereas ERG status seems to have no predictive value for ADT response in patients with advanced PCa.

General considerations

The implementation of new technologies requires several important considerations. For a biomarker, an *a priori* consideration

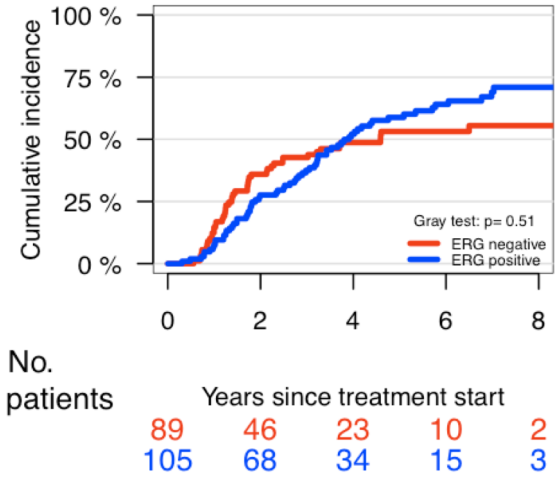

Figure 7: *Cumulative incidences of CRPC. The competing event was death without reaching the endpoint [3].*

Table 10. Risk of developing CRPC - an ERG-stratified multiple Cox regression model [3]		
Variables	**Risk of CRPC**	
	Hazard ratio (95% CI)	*p* value
Age; 5 years diff.	0.98 (0.87-1.11)	0.75
PSA; 2-fold diff.	1.33 (1.18-1.49)	<0.0001
Diagnostic Gleason score		
GS ≤ 7	1.00 (Ref)	
GS 8	1.10 (0.60-2.02)	0.75
GS 9-10	1.74 (1.00-3.03)	0.050
Clinical tumour category		
≤ cT2	1.00 (Ref)	
cT3a	0.99 (0.52-1.90)	0.97
cT3b	1.20 (0.64-2.26)	0.57
cT4	1.12 (0.56-2.27)	0.74
Metastatic stage		
No apparent metastases	1.00 (Ref)	
Lymph node metastases only	1.18 (0.59-2.36)	0.64
Bone metastases +/- lymph node metastases	1.12 (0.68-1.85)	0.65
Abbreviations: CI: confidence interval; CRPC: castration-resistant prostate cancer; GS: Gleason score; PSA: prostate-specific antigen.		

regarding its clinical utility is essential, since it must be expected that a single biomarker cannot contribute with important information in all disease stages[81]. Furthermore, it is crucial that methods for performing and interpreting a biomarker are standardised and based on appropriate studies with clinically meaningful end-points[87]. Finally, external validation of study results is important, as selection biases have impact on the results and the conclusions. If the study cohort is not representative of the background patient population, the generalisability of the results will be affected. Thus, the initial selection of patients and losses to follow-up affects the results' representativeness and generalisability and thereby also the implementation of the biomarker. Major sources of biases for *Studies 1-3* will be discussed in this section.

First, tumour characteristics will be affected by the diagnostic strategy in both the general population as well as the clinical work-up in hospitals. Post-hoc analyses of the European Randomised Study of Screening for Prostate Cancer have demonstrated that PSA screening induces a 6-7 years lead-time in PCa diagnosis, causing a significant reduction of tumour stage[161,162]. PSA-based screening is not recommended in Denmark; still, reports have shown an increased non-systematic use of PSA testing during the last decades[163]. Consequently, tumour characteristics might

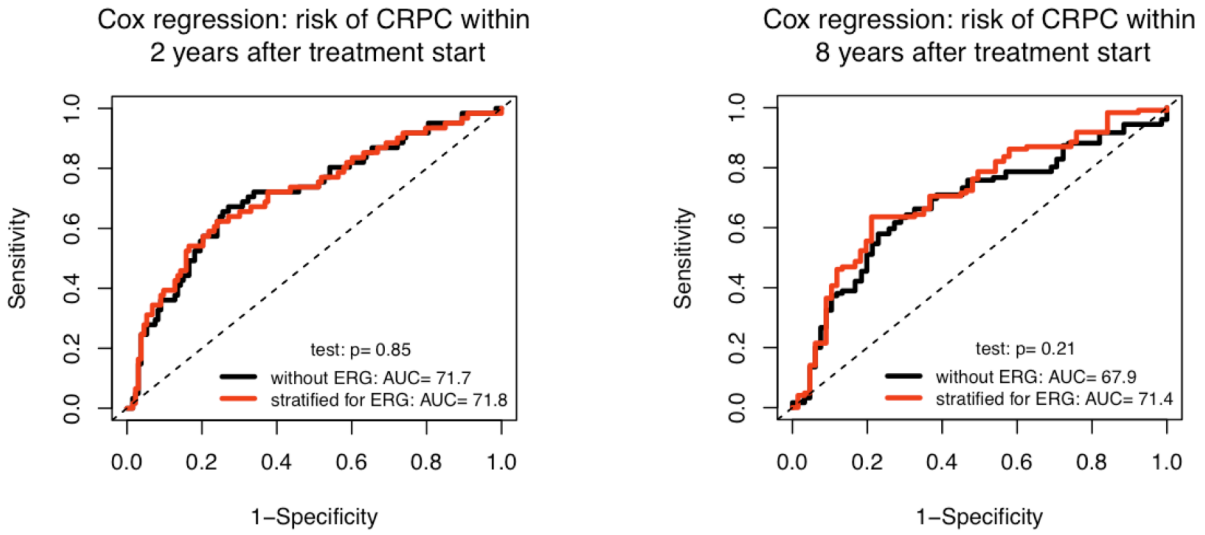

Figure 8: *The discriminative ability of ERG status was analysed using time-dependent area under the ROC curves (AUC) comparing predictions obtained with an ERG stratified multiple model (red) and a second multiple model omitting ERG status (black). Results from two and eight years of follow-up from ADT start are presented [3].*

have shifted during the study periods, and contemporary patient cohorts might have more favourable tumour characteristics than a historical cohort, from which the patients enrolled in the present thesis derive. This can hamper the direct transferability of the results to modern practice.

Second, the biopsy strategy will affect tumour characteristics of newly diagnosed patients. According to national guidelines, indications for prostate biopsies include a PSA value > 4.0 ng/mL and a suspicious DRE[155]. These indications have remained unchanged during the study periods. However, a standard biopsy set has changed gradually from six cores to a 10-12 core approach. An increased cancer detection rate has been demonstrated when comparing sextant biopsy protocols with 10-12 core protocols, which by itself can induce stage and grade migration[164]. Another consideration is the GS migration induced by the modifications of the Gleason grading system[45]. This is exemplified by the observed shift in GSs following the 2005 ISUP modifications[165], resulting in some men diagnosed with GS 6 tumours according to the 1974 Gleason grading system[44] being assigned GS 7 (3+4) or higher[45,47,166]. For AS cohorts, this can impede the direct comparison of a contemporary AS cohort with historical cohorts, as patients with a higher GS are most often not considered candidates for AS[70]. In this thesis, the effect of the modification of the Gleason grading system is limited as the majority of the AS patients (87.2%) were included from 2007 and onwards, and most importantly all tissue samples were re-graded according to the ISUP 2005 guidelines.

Third, Rigshospitalet serves as a large tertiary referral hospital. Thus, an important source of selection bias lies in the referral pattern to Rigshospitalet. For PCa, there are no unambiguous data illustrating the impact of referral bias on patient, tumour, or socioeconomic characteristics. It has been shown for other cancers that patients referred to tertiary university hospitals tend to be younger[167] and live closer to the referral hospital[168]. Differences in health care systems and referral patterns between countries as well as disparities among diseases make it problematic to translate these results into a Danish PCa setting. However, it can be assumed that PCa patients referred to Rigshospitalet differ from the general PCa population, which will affect the representativeness of the study populations included in the thesis.

Finally, the indications for AS enrolment and treatment with ADT can change over time, as can the definition of study endpoints. The indications for AS and ADT have not changed at our institution during the study periods[155,156]. However, it has recently been shown that PSAdt is not associated with histopathological features that indicate poor outcome in patients initially managed on AS[169], and the definition of CRPC was recently changed[73]. This demonstrates that future patient cohorts will be monitored differently than the ones in the present thesis.

These considerations illustrate the most important biases that need to be recognised, when the study results are attempted transferred to contemporary cohorts. Moreover, they underline the necessity of validation studies before ERG IHC can be implemented in clinical practice.

Implementation of ERG as a biomarker for prostate cancer

To optimise prognostication and prediction in PCa management, it is desirable to identify accurate and reproducible markers that at time of diagnosis can help to differentiate between insignificant, curable, and lethal PCa. For a tissue biomarker, this implies that the marker should be assessed in diagnostic biopsy specimens since treatment planning is most often based on biopsy assessments[38]. The intratumoral heterogeneity of PCa and sampling bias during the biopsy session can, however, hamper the reliability of biomarker assays in biopsy specimens.

Most PCas are multifocal at the time of diagnosis, and individual tumour foci present with different GSs and histopathological features in more than 90% of multifocal tumours[170]. Recognition of the heterogeneity is important in biomarker research especially when biopsy samples are used. For *ERG* rearrangement, intraprostatic heterogeneity can be reflected in discordance in fusion mechanism (gene fusion through deletion or insertion) or discordance in fusion status (fusion positive or fusion negative) between and within tumour foci[120]. Interfocal heterogeneity has been demonstrated in 41-67% of multifocal prostatectomy specimens, most often due to discordance in fusion status[120,140,171,172]. In contrast, when *ERG* rearrangement is present, almost all PCa cells in a given focus are positive and intrafocal heterogeneity is only demonstrated in a minority of cancers. One study has, however, demonstrated intrafocal heterogeneity in 72% of ERG-positive patients[173], which might represent collision of ERG-positive and ERG-negative foci rather than true intrafocal heterogeneity. ERG heterogeneity in biopsy samples has only been scantly studied and is generally found at lower frequencies (12-47%) compared to prostatectomy cohorts[174,175].

The marked difference in the prevalence of ERG heterogeneity between radical prostatectomy studies and biopsy studies can in part be explained by sampling bias[42]. Moreover, patient selection and selection criterion of the included tissue is important. Whereas one of the biopsy studies included AS patients with low-volume cancers[174], multifocal and extensive PCas were included in most of the prostatectomy series[120,140,171,172], which most likely leads to differences in the observed prevalence of ERG heterogeneity between the studies.

Taken together, the literature supports the notion of a multiclonal nature of PCa and suggests that at least two different pathways for PCa pathogenesis can coexist in the same gland leading to ERG-positive and ERG-negative foci. Although the underlying mechanisms of ERG heterogeneity are not fully elucidated, one can hypothesise that heterogeneous foci appear due to proliferation of independent cancer clones or development of secondary rearrangements during PCa progression. Moreover, differences in zonal origin can explain some of the observed heterogeneity, as tumours arising from the transition zone less frequently harbour *ERG* rearrangements compared to tumours from the peripheral zone[176,177].

The prevalence of ERG heterogeneity in prostatectomy specimens has led to the assumption that ERG status cannot be assessed reliably in biopsy specimens due to sampling bias[171,173]. *Study 1*

was conducted to analyse whether this indeed is correct. Based on a dichotomisation of ERG status, *Study 1* demonstrated that only 5.8-10.5% of patients will be ERG misclassified in biopsy specimens, and the degree of misclassification could be reduced by inclusion of more biopsy specimens. Importantly, ERG reclassification was almost exclusively caused by sampling differences due to the finding of an ERG-positive focus in a novel part of an otherwise entirely ERG-negative gland, or vice versa. Overall, *Study 1* supports that ERG expression is often heterogeneous, and as a result of the minimal ERG reclassifications over time, the study also support the notion that *ERG* rearrangement occurs early in the pathogenesis of a tumour focus. However, *Study 1* challenges the assumption that ERG expression in biopsies cannot reliably assess the ERG status of the entire prostate.

Still, implementation of ERG as a biomarker in PCa biopsies is challenging, as it is important to understand its potential use, implications, and limitations. First, ERG assessment must be accurate and reproducible. Since ERG expression is used as a surrogate indicator of *ERG* rearrangement, the high concordance between the FISH assay and the IHC assay is crucial[138,139,160,178,179]. *Study 1* confirmed these previous findings and demonstrated that ERG IHC was applicable in biopsy sections. The non-perfect conformity between the assays might have several explanations. ERG expression without *TMPRSS2-ERG* rearrangement could be explained by poor tissue preservation and other technical factors, the presence of 5' fusion partners other than *TMPRSS2*[143,180], gene copy-number gain[181], or insertion of the complete *ERG* locus into a genomically active region[138]. The presence of a *TMPRSS2-ERG* rearrangement in the context of no detectable ERG expression can be caused by ERG expression levels below the detection threshold[139], and alterations in androgen signalling or posttranscriptional mechanisms[118,138].

Second, prognostic and predictive biomarkers are often used in cooperation with standard clinical parameters[182]. Still, it is insufficient to simply show that a biomarker is associated with the endpoint of interest in a multiple adjusted model, as the biomarker might not improve the predictive accuracy[183,184]. Therefore, the marker's impact on the discriminative ability needs to be analysed as shown in *Study 2* and *Study 3*. For example, if ERG should be implemented as a prognostic or predictive biomarker, the magnitude of disease progression, choice of treatment modality, and/or treatment effect must be sufficiently different between ERG-positive and ERG-negative patients, so clinicians and patients will accept a change of treatment strategy based on the ERG status. Moreover, the change of treatment caused by the ERG status should ultimately benefit the patient[185].

Third, as previously noted, due to selection biases, results must be externally validated to allow for generalisability. A structured approach for biomarker discovery, evaluation, and validation has previously been proposed. This approach ranks studies into five phases: (a) preclinical biomarker discovery, (b) assay development and optimisation, (c) retrospective studies to clarify target patients, (d) prospective validation studies to determine efficacy and generalisability, and (e) post-approval analyses of biomarker impact on cost-effectiveness and patient outcomes[81,186,187]. This implies that the prognostic value found for ERG in *Study 2* needs to be tested in large, prospective, and preferably multi-institutional studies.

Finally, a major challenge is the long natural history of PCa and the opposing interests regarding treatment optimisation and robust endpoints. Consequently, surrogate endpoints such as AS progression and development of CRPC will often have to be accepted while follow-up is pending[88,117]. Limitations in study end-points are discussed later.

The diagnostic, prognostic, and predictive values of ERG

The clinical utility of *ERG* rearrangement and ERG expression has been investigated in several settings. ERG expression is highly specific for PCa, and ERG expression can be used to differentiate small PCa foci from atypical benign foci[142]. However, although rare, benign prostatic glands have been found to harbour *ERG* rearrangements, and the premalignant HGPIN lesions are ERG-positive in approximately 20% of cases[140–142]. This may limit the use of ERG as a PCa-specific marker, but it must be kept in mind that ERG positivity in normal tissue and HGPIN lesions is only found adjacent to ERG-positive tumour foci. Thus, ERG-positive glands in the context of no tumorous cells might indicate the presence of unsampled PCa. This theory has been confirmed, as patients with isolated ERG-positive HGPIN lesions were found to be more likely subsequently to be diagnosed with PCa compared to ERG-negative HGPIN patients (53% versus 35%) in a study with yearly re-biopsies and a three year follow-up[145].

Since PCa is the only common cancer harbouring *ERG* rearrangement[147], ERG expression in a metastatic lesion of unknown origin, especially with concurrent cytokeratin expression indicating an epithelial neoplasm, is strongly indicative of PCa. This even holds true for small cell carcinomas[38,188]. While ERG is highly specific for PCa, an obvious limitation to its diagnostic utility is the low sensitivity reflected by its prevalence of 15-72% depending on the cohort design[176], the zonal origin of the tumour[176,177], and patient ethnicity[101].

Whereas the overall diagnostic value of ERG expression seems indisputable, the prognostic and predictive impact is less clear. Current data are mainly based on retrospective studies or retrospective analyses of prospectively managed cohorts, and no definitive conclusions can be reached at present.

ERG and observational strategies for prostate cancer

The prognostic value of *ERG* rearrangements and ERG expression in conservatively managed PCa patients have been studied in four independent watchful waiting cohorts. Overall, these studies have demonstrated an association between *ERG* rearrangement or ERG expression and lethal PCa[148–150,189]. The prevalence of ERG positivity in these studies is low (15-34%), as the patients were diagnosed by transurethral resections of the prostate and most likely have PCa originating from the transition zone.

In the Örebro Watchful Waiting cohort, *TMPRSS2-ERG* rearrangement was found to be associated with a 2.7-fold increased cumulative incidence of lethal PCa, defined as development of distant metastasis or PCa-specific death[149]. However, probably due to the low number of ERG-positive cases, the association was not significant after adjustment for GS. These promising results were later confirmed in three larger watchful waiting cohorts[148,150,189]. In the study of Attard *et al.*, *ERG* rearrangement was demonstrated to be positively associated with GS, tumour stage, and PSA, and *ERG* rearrangement as a result of deletion emerged as an independent marker of poor PCa-specific survival (HR: 1.72, $p = 0.042$) and overall survival (HR: 1.43, $p = 0.028$) after adjustment for age, GS, and PSA[148]. Interestingly, patients with two or more copies of an *ERG* rearrangement through deletion had the poorest prognosis. Whether this is due to higher levels of ERG expression or is caused by a general genomic instability with gene copy number alterations and/or aneuploidy is not fully understood, but it implies that mechanisms other than the oncogenic potential of the *ERG* rearrangement on its own are important[148]. The interstitial deletion results in the down-regulation of at least 13 genes located at this intervening genomic region separating the *TMPRSS2* locus from the *ERG* locus. Two of these genes, *ETS2* and *HMGN1,* are known tumour suppressors, and the loss of these genes may to some extent explain the different course observed between patients with *ERG* rearrangement through deletion and insertion[99,190,191].

In a Chinese watchful waiting cohort, *ERG* rearrangement was demonstrated to be associated with an increased hazard of PCa-specific mortality (HR: 2.1, $p = 0.022$) after adjustment for known prognostic tumour characteristics[189]. Similar results were demonstrated in the Västerås Watchful Waiting cohort, showing that ERG-positive patients had a significantly reduced survival compared to ERG-negative patients after adjustment for GS and tumour stage (RR: 1.9; $p = 0.019$) [150]. Of special interest, ERG expression was found to be associated with high stromal expression of hyaluronan, PDGFRβ, and von Willebrand factor, as well as low stromal Caveolin-1 expression, which have all been found associated with poorer PCa prognosis[150]. However, the temporal development of these genetic and expressional alterations in the context of ERG expression is not fully understood, and whether these markers add prognostic information independently needs further studying.

The concept of AS is to some extent related to watchful waiting, given that both treatment modalities involve an initial observational approach. However, whereas watchful waiting actively excludes curatively intended therapy and instead offers delayed application of palliative therapies, AS is only a treatment option for selected low- and intermediate-risk PCa patients, who could also be candidates for surgery or radiotherapy in the future[70]. Thus, AS includes an initial active non-treatment decision, whereas therapy is prompted if predefined thresholds of specified progression criteria indicative of a possible life-threatening condition are reached. An important aim of AS is to designate curative treatment with the proper timing to patients, who are believed to benefit from the intervention, and thereby eliminate overtreatment and side-effects from the intervention for the rest of the patients. Approximately 30% of AS patients will experience disease progression or reclassification during surveillance and will be offered curatively intended treatment[71]. Currently, progression is determined by frequent clinical examinations, PSA measurements, and re-biopsies with a significant risk of post-biopsy infections and sepsis[157]. Based on the results from the watchful waiting cohorts[148–150], *Study* 2 was conducted to analyse whether ERG expression at diagnosis was associated with risk of AS progression. The data demonstrated that ERG was a strong and independent prognostic marker for overall AS progression and progression on re-biopsies. Importantly, implementation of ERG status significantly changed both the discriminative ability of the multivariable model and the prognostication for the individual patient. Thus, ERG-positive patients have an increased risk of disease progression or reclassification, and maybe they should be offered more intense surveillance or immediate curative therapy at time of diagnosis. This may create a basis for a stratified AS programme with differentiated selection criteria and follow-up protocols for future AS patients depending on their ERG status.

ERG and radical prostatectomy

Numerous studies have analysed the association between ERG status in prostatectomy specimens and histopathological features, risk of biochemical recurrence, and PCa-specific mortality. As this thesis focuses on the prognostic and predictive value of ERG status in biopsy specimens, those data are not within its scope. Hence, only an overview of the many and conflicting results will be presented.

Data are conflicting regarding the association between the gene fusion and histopathological features that indicate a poor outcome[100,192]. Whereas some studies have found an association between ERG and higher GS[140] and tumour stage[99,100,179,180,193], several studies have found no or even a negative association between the presence of ERG and tumour stage[158,194–196] and/or GS[100,139,158,178–180,195,196]. Based on information from 8,003 patients, a recent meta-analysis concluded that ERG-positive PCa had a modest but significant higher risk of extraprostatic tumour extension (RR: 1.23; 95% CI: 1.16-1.30) compared to ERG-negative patients, whereas a non-significant trend towards lower GS (RR: 0.85; 95% CI: 0.72-1.01) was found in the ERG-positive group[100]. Although the number of cases is limited in *Study 1*, our institutional data support these findings, but it is important to note that we only found a trend for an association between ERG status and pathological tumour stage.

The weak or lacking association between ERG expression and other prognostic tumour characteristics might be seen as a potential strength, as this might enable ERG to differentiate between aggressive and indolent tumours based on established risk factors. In other words, ERG might designate the patients with the greatest risk of recurrence within existing risk-groups and thus provide additional prognostic information. However, only a few, generally small studies have found a positive association between ERG and

biochemical recurrence following radical prostatectomy[196,197], whereas most larger studies have reported no such association[100,129,158,179,194]. Based on information from 5,074 patients, the aforementioned meta-analysis found no difference in the risk of PSA recurrence between ERG subgroups (RR: 1.00; 95% CI: 0.86-1.17) [100]. Fewer studies have analysed the association between ERG and survival in the context of radical prostatectomy, and no such association has been established to date (RR: 0.99; 95% CI: 0.47-2.09) [100].

Biochemical recurrence is often used as indicator for later PCa-specific mortality, but the endpoint has its limitations. First, there is no generally accepted definition of biochemical recurrence[73], and studies often differ in this aspect. Moreover, while biochemical recurrence is associated with PCa-specific death, a large proportion of patients with biochemical recurrence will not develop metastases nor die from PCa with 10-15 years of follow-up even in the absence of adjuvant or salvage therapy[198], which weakens the relevance of the endpoint. Finally, PSA progression might not be an optimal endpoint in the context of ERG expression, as ERG has been shown to suppress PSA levels through the co-expression of EZH2 and HDACs[199]. A further caveat in most studies is the multifocal nature of PCa[120,140,171,172]. Often, only one or two TMA cores from the largest tumour focus or the lesion with the highest GS is included, which might skew the results. With these limitations in mind, ERG-positive PCa does not seem to run a more aggressive course following radical prostatectomy when compared to ERG-negative PCa, and current data indicate that ERG is not a valuable prognostic biomarker for prostatectomised patients[100].

ERG and advanced prostate cancer

The increased diagnostic activity has caused a significant reduction in tumour stage at time of diagnosis[162,163]. Still, recent Danish figures have demonstrated that 13.7% of newly diagnosed PCa patients have bone metastasis at the time of diagnosis[200].

ERG rearrangements are found in approximately 50% of PCa patients with no apparent accumulation in more advanced stages of the disease[96,122,148,149,201–204]. In an autopsy study, all metastases from individual patients harboured the same gene fusion subtype[205], suggesting that distinct metastases are derived from a single clone. Moreover, all metastases harbouring *TMPRSS2-ERG* gene fusion showed *ERG* rearrangement through deletion[205], supporting the aggressive behaviour of this subtype[148]. Furthermore, *ERG* rearranged foci have been demonstrated to have a predilection for lymph node dissemination in patients with multifocal PCa with interfocal discordance in *ERG* status[206], suggesting that ERG-positive PCa foci are particularly aggressive.

The androgen response element in the promoter region of the 5' gene is fused with the *ERG* loci in *ERG* rearranged tumours. Consequently, androgen receptor signalling results in the increased expression of the oncogenic transcription factor ERG in fusion positive tumours[207], and *Study 3* was initiated to investigate whether response to first-line ADT was dependent on ERG status. ERG expression was found not to be associated with the risk of CRPC development, nor with time to PSA nadir, maximum PSA response, or risk of PCa-specific mortality. Furthermore, the addition of ERG status to the multivariable predictive model did not change the discriminative ability, indicating that ERG protein expression should not be used as a predictive biomarker in ADT-treated patients.

This notion is supported by two previous studies consisting of 71 node-positive and 178 hormonally treated patients[202,208]. Whereas *Study 3* only included patients treated with first-line castration-based ADT, the two previous studies also included men undergoing anti-androgen monotherapy, which causes a different gene expression pattern compared to castration-based ADT[125]. In addition, an important distinction is the attempt to limit the impact of tumour heterogeneity in *Study 3* by including all available biopsy specimens as opposed to the previous studies.

One plausible explanation for the missing prognostic and predictive value of ERG expression in late stage PCa is that ERG protein expression suppresses downstream target genes of the androgen receptor by competing with its binding to DNA and by direct protein-protein interactions with the receptor[209]. Thus, ADT lowers ERG expression in fusion positive cancers and thereby diminishes the oncogenic potential of ERG, but also eliminates the attenuation of the androgen signalling. Another explanation is the accumulation of other genomic alternations in later stage PCa, making *ERG* rearrangements redundant[91]. For example, *PTEN,* 5q21, and 6q15 deletions, *SPOP* and *TP53* mutations, as well as androgen receptor and *SPINK1* overexpression accumulate during PCa progression, and whereas *PTEN* deletions are enriched in ERG-positive tumours, ERG-negative tumours accumulate 5q21 and 6q15 deletions and *SPOP* mutations[129,179,201,204,210,211]. Moreover, not only ERG expression but also the presence of a functional androgen receptor axis is important for ADT response. Androgen receptor expression levels have been found to be higher in ERG-positive patients compared to ERG-negative patients[179], and patients with both ERG and androgen receptor overexpression have been demonstrated to have an increased risk of PCa-specific mortality compared with ERG-negative men with low androgen receptor expression[212]. Finally, in the absence of androgen receptor signalling, ERG expression can be regulated by androgen-independent mechanisms including the oestrogen receptor and ERG itself[213].

Taken together, current data indicate that ERG in itself is not a predictive biomarker for ADT response in patients with advanced PCa. However, this conclusion is based on cross-sectional studies, and it is not clear whether dynamic changes in biomarker levels could reflect treatment response. The EFFECT trial (EudraCT 2012-000101-69) is an open-label, multicentre study assessing the impact of a 6-month formulation of leuprorelin on PCa biomarker levels including *TMPRSS2-ERG* mRNA in blood. Results from the study and the prognostic value of biomarker changes over time are pending.

The controversial role of ERG expression

Despite an enormous research activity and enthusiasm regarding the clinical utility of ERG in PCa management, no definitive conclusions have been reached. In consistency with previous reports[214], *Study 3* demonstrated that patients with ERG positive

tumours had lower PSA values and GSs compared to men with ERG negative tumours. Reasons for the lack of prognostic and predictive value of the ERG status in patients undergoing treatment[100,202,208] despite associations with well-known prognostic clinicopathological parameters, and reasons for conflicting prognostic associations (ERG positive tumours are associated with lower GSs and PSA values but higher tumour stage) requires additional research. One can hypothesise that subtyping PCa lesions based on ERG status plays a more important role in terms of tumour initiation and local tumour growth, as *Study* 2 and previous papers indicate that ERG status harbours prognostic value for aggressive disease in PCa managed conservatively[148–150,189]. However, the opposing results might have several other explanations.

Obviously, significant differences exist between the study populations. First, the watchful waiting cohorts consist of patients diagnosed incidentally in the pre-PSA era, whereas patients included into the surgical cohorts are primarily diagnosed in the PSA era. Second, the watchful waiting cohorts are enriched with tumours originating from the transition zone, which contrasts with tumours from the peripheral zone in the biopsy and prostatectomy cohorts. Third, observational studies, including *Study 2,* investigate the natural history of PCa and thereby the true prognostic value of *ERG* rearrangement or ERG expression. In contrast, radical prostatectomy and ADT studies introduce an intervention in terms of the treatment applied, and the prognostic value of ERG could potentially be lost due to a successful intervention. Ideally, the prognostic value of ERG in the context of interventions should be analysed in a two armed randomised trial comparing observation with intervention. Examples of such trials are the PIVOT and the SPCG-4 studies, which compared radical prostatectomy to an observational strategy[67,68]. This would allow analysis of the impact of the intervention stratified on ERG status, as ERG-positive and ERG-negative patients might not achieve the same degree of benefit from therapy. The observed increased mortality in ERG-positive patients managed observationally which seems to be balanced in patients undergoing surgery could, at least in part, be explained by a greater benefit of the intervention among the ERG-positive patients. Finally, the endpoints reported in the observational studies and interventional studies are very distinct (PCa-specific survival and overall survival versus biochemical recurrence), which hampers any attempt to compare the results.

Beside these inter-study differences, most studies are also subject to universal caveats, which contribute to the controversies regarding the prognostic value of ERG. As previously stated, both observational and intervention studies have not fully addressed the multifocality of PCa, and it is not possible to determine whether the foci used for ERG assessments are the aggressive and lethal ones. Furthermore, most studies (including *Studies 1-3*) have not stratified patients according to the fusion mechanism, which might be clinically relevant[148].

In summary, the value of ERG expression in biopsy specimens seems to be as a prognostic marker in early and conservatively managed PCa, whereas it currently appears without value in later disease stages, including following prostatectomy and ADT treatment.

Overall strengths and limitations of the studies

An overall shortcoming of the included studies is the observational design with retrospective IHC assessments of archived tissue samples. A prospective randomised clinical trial is the gold standard for establishing the clinical utility of new techniques including biomarkers[185]. However, it is widely accepted to use archived specimens when these are derived from prospectively managed cohorts – so-called 'prospective-retrospective' studies[185], and retrospective studies are important for identifying target patients[81,186,187]. As such, *Studies 1-3* provide important information towards the clinical implementation of ERG.

A limitation regarding the advanced PCa cohort is its retrospective origin. Moreover, the cohort is heterogeneous in terms of tumour characteristics, which can be considered as both a limitation and a strength regarding the generalisability of the results. In contrast, the AS cohort is more homogeneous with the patients being followed prospectively, and thus *Study 1* and *Study 2* both represent true 'prospective-retrospective studies'. For both cohorts, an additional strength is the strictly consecutive enrolment of patients, and only a few patients were excluded due to missing biopsy samples (4-5%), which minimised the risk of selection bias.

The selection of tissue samples for IHC is a trade-off. On the one hand, prostatectomy specimens provide sufficient tissue for several analyses but include a significant drawback by means of the surgical intervention. On the other hand, biopsies provide a limited amount of tissue, but are collected prior to treatment initiation. To mimic a clinical setting, the thesis included diagnostic biopsy samples, as biopsies are the only tissue samples available for AS cohorts and patients treated with first-line ADT. Thus, the prognostic and predictive utility of ERG expression in these settings needs to be validated in biopsy samples, which is a strength of the thesis. Still, one cannot be certain whether all relevant tumour foci have been biopsied due to sampling error and tumour heterogeneity, but *Study 1* suggests that in terms of ERG status this issue seems to be of minor importance.

Beside the inclusion of biopsy samples and the prospectively managed AS cohort, the strengths of the thesis are the very few losses to follow-up, the re-evaluation of the GS in all tissue samples by expert uro-pathologists, and the fact that all ERG readings were assessed blinded to both clinical data and patient outcomes.

Pitfalls in the immunohistochemical assessment of ERG expression

Immunohistochemistry is an antibody-based procedure used for detection of protein expression and protein localisation in cells and tissue sections. In general, the primary antibody is applied to a tissue section mounted on a slide, and the bound primary antibody is subsequently detected by a secondary antibody. Finally, a chromogen is added, which is oxidised by antibody-bound HRP and the chromogen becomes insoluble (Figure 2). This product is visible in a bright-field microscope and indirectly demonstrates the presence of the protein of interest[151,152].

As with other techniques, IHC has caveats, pitfalls, and limitations[215]. Staining quality varies between laboratories due to differences in methods and technical expertise, which result in a lack of reproducibility of results between laboratories[216]. A major factor is the difficulties in standardising the procedural steps, and readings are subjective and most often semi-quantitative with an inherent inter-observer variability[217].

In general, the IHC procedure can be stratified into pre-analytical, analytical, and post-analytical phases. Important pre-analytical factors with potential impact on staining quality and readings include tissue processing, fixation medium, fixation time, and tissue sectioning. During the fixation procedure cross-links are made to minimise the degradation of proteins and to immobilise and stabilise the constituents of the tissue. These cross-links result in masking of antigenic epitopes and impede antigen recognition by the primary antibody. Thus, the choice of fixation medium and fixation time can affect immune reactivity and IHC readings[153,154]. The tissue samples included in *Studies 1-3* originated from eight different pathological departments, and important limitations of the studies are thus interlaboratory variations in the pre-analytical methodology.

During the analytical phase, significant pitfalls comprise the selection and dilution of the primary antibody clone and the pre-treatment method of epitope retrieval to eliminate the effect of fixation. Epitope retrieval is applied prior to incubation with the primary antibody. The process seeks to de-masks the antigenic epitopes by applying either proteolytic enzymes or by boiling the tissue in specific buffers[151,152]. For HIER, the combination of optimal temperature and heating time determines the staining quality, and the correct setting is epitope-dependent[218]. Also, the optimal pH level for HIER is epitope dependent. Still, most antibodies prefer a slightly alkaline medium[219,220].

Finally, post-analytical pitfalls include the definition of positivity and negativity, false-positive and false-negative stains, and if an internal staining control is missing.

These analytical- and post-analytical caveats were attempted to be minimised by optimising and validating the ERG IHC procedure in our laboratory prior to conducting the studies. Consecutive sections from radical prostatectomy specimens with known ERG status were processed for IHC according to the optimised protocol, and the sensitivity and the specificity of the anti-ERG antibody were analysed by replacing the primary antibody with non-ERG antibodies and by omitting the anti-ERG antibody. In addition, the applied primary antibody is directed at the conserved C-terminus region, and previous validation studies have demonstrated it to be highly specific and sensitive for all known transcript variants[138]. Finally, all sections included in *Studies 1-3* used endothelial cells as internal positive controls for the staining procedure, tissue sections with known ERG status were stained before the deployment of all primary antibody batches to assure uniformity of the procedure, and the staining was performed in the same laboratory by an experienced lab-technician and by use of an automated staining system.

Although the applied primary antibody is monoclonal and highly specific for ERG, cross-reactivity with friend leukaemia virus integration 1, an ETS protein expressed by lymphocytes, has been demonstrated and might hamper the use of ERG IHC[140,142]. However, the high accuracy between *ERG* rearrangements and ERG expression observed in *Study 1* indicates that the impact of such cross-reactivity on the validity of the applied ERG antibody is limited.

It is possible to estimate the expression level of a protein by counting the number and percentage of positive malignant cells in a tumour focus, and by calculating an H-score combining the staining intensity and fraction of cells with a given staining intensity[221]. Often, this is not feasible in clinical practice. Consequently, IHC is frequently semi-quantitative with both inter- and intra-observer variations as important caveats[217]. Although the thesis has not studied these variations, one may expect them to be minor in the context of ERG expression, as the nuclear staining most often is strong and uniform and thus easily recognisable.

A final shortcoming of the applied IHC assay is the lack of differentiation between *ERG* rearrangements through insertion and through deletion. A FISH-based assay can determine the rearrangement mechanism[148], but FISH has limitations, making it unsuitable for clinical practice. The main limitations of FISH are that it is costly, time-consuming, and a less automated procedure, and it requires both experienced lab-technicians and pathologists to obtain reliable results. Furthermore, the morphology is not visible in the fluorescence microscope, which makes the readings difficult. In contrast, IHC is cheaper, the procedure can more easily be automated resulting in minimal intra-laboratory variations, and IHC stains can be read in the context of prostate morphology. Therefore, despite its shortcomings, ERG IHC seems more suited for clinical practice.

Limitations in study endpoints

The relatively short follow-up (median 4.1 years in *Study 2* and 6.8 years in *Study 3*) is a limitation of the included studies. Consequently, the applied endpoints are surrogates for more robust endpoints such as PCa-specific survival and overall survival.

A shortcoming of biomarker studies in the context of AS is the designation of an appropriate endpoint. For *Study 2*, the primary endpoint was overall AS progression defined as either clinical progression, PSA progression, and/or histopathological progression. The risk of AS progression is largest during the first 2-3 years of surveillance[71,222–224], and, as such, the follow-up in *Study 2* is sufficient for evaluating the impact of ERG on AS progression. Although the study endpoint has great clinical impact for the individual patient, it is still only a surrogate for meaningful tumour progression, and its shortcomings have previously been demonstrated. Neither a short PSAdt nor cT progression is consistently predictive of higher pT category, higher GS, or biochemical recurrence following deferred radical prostatectomy after an initial AS strategy[169,222,225,226]. Most likely, these observations reflect the fact that PSA levels are affected by changes in both the malignant and the benign component of the prostate[18]. Consequently,

PSAdt estimates are subject to a substantial uncertainty[169]. Moreover, DREs are flawed by inter-observer variations[33] leading to false-positive and false-negative results. Most AS protocols agree that tumour progression on re-biopsies, irrespective of whether this represents actual progression or merely sampling of novel areas of an otherwise stable tumour, should result in treatment change[70,227]. Moreover, histopathological progression is the only progression criterion found positively associated with pT category and GS in prostatectomy specimens[222,224]. Both pT category and GS are known risk factors for biochemical recurrence, metastases, and PCa-specific mortality following radical prostatectomy[55,198]; thus, it is highly interesting that the prognostic value of ERG status in *Study 2* favours the ERG-negative group in terms of both overall AS progression and histopathological progression.

In both *Study 2* and *Study 3*, the endpoints include PSA progression. As previously stated, one potential caveat of PSA progression in this context is that ERG can suppress PSA expression through EZH2 and HDAC co-expression[199]. Thus, for ERG-positive patients the estimated PSAdt may be falsely elevated in the AS setting and time to CRPC may be falsely prolonged in the advanced PCa setting. Moreover, the PSA measurements were analysed at different laboratories. Although the laboratories applied the same immunoassay kit, PSA values are potentially subject to both intra- and inter-laboratory variations but on the other hand represent daily clinical practice. *Study 2* also included histopathological progression in the endpoint, which is a strength in this context. A more robust primary endpoint such as radiographic progression or PCa-specific survival would have been desirable for *Study 3*, but radiographic imaging was not part of the regular follow-up regime in our department. Moreover, the patients in the advanced PCa cohort were diagnosed and treated at a time where numerous new treatment modalities have entered into clinical practice for CRPC patients. Thus, the use of PCa-specific survival as a primary endpoint would necessitate the implementation of subsequent life-prolonging treatments into the statistical models.

Despite these shortcomings, both AS progression and CRPC development represent clinically important endpoints, as they both warrant change in treatment modalities. Moreover, it is a strength that the endpoints are defined according to the institutional AS protocol and EAU guidelines, respectively.

CONCLUSIONS

For a clinically heterogeneous disease such as PCa, it is highly desirable to pursue tailored and individualised clinical workup and treatment. The thesis evaluated the clinical utility of ERG protein assessment in biopsy samples from low-risk, intermediate-risk, as well as advanced PCa patients. It demonstrated that IHC for ERG protein expression was practicable in core needle biopsies using a setup reflecting the daily clinical practice.

The concordance between the IHC assay and the FISH assay was demonstrated to be high (97.3%), suggesting that the much cheaper and less time consuming IHC assay can be used to assess the presence or absence of *ERG* rearrangement in biopsy specimens. Furthermore, a key result described in *Study 1* was the strong agreement in ERG status between biopsies and the prostatectomy specimens for AS patients who underwent deferred radical prostatectomy. This implies that assessment of ERG status in biopsy specimens can provide reliable information regarding the "true" ERG status in the entire gland despite of tumour heterogeneity and the limited amount of tissue available for analyses.

A strong and independent prognostic value for disease progression during AS was established for ERG expression in diagnostic specimens. The hazard ratio for overall AS progression and histopathological progression was 2.5-3.1 in the ERG-positive group compared to the ERG-negative group, and inclusion of ERG status in the prognostic model increased the discriminative ability significantly. On the other side of the disease spectrum, ERG status was demonstrated not to provide predictive information regarding response to primary castration based ADT for patients with advanced PCa.

The overall conclusion of the thesis is that ERG expression assessed in biopsy specimens harbours prognostic value for PCa patients managed on AS. Moreover, implementation of ERG IHC in this therapeutic setting may be used to tailor individualised surveillance regimes for future patients based on their ERG status.

PERSPECTIVES FOR FUTURE RESEARCH

PCa is, and will most likely continue to be, a major health concern. The focus during recent years has been on establishing personalised therapy, which can provide important benefits for both patients and healthcare services, as it allows for targeted therapy and a reduction in overtreatment. The path to individualised treatment is, however, not without challenges and obstacles, as the optimal use of the new technologies has not been fully elucidated. One approach is to establish the temporal sequence of genomic alterations in PCa. *ERG* rearrangement is believed to occur early in the pathogenesis[88,122], however, the sequence of subsequent alterations, which are largely determined by ERG status, might provide valuable insight into tumour aggressiveness and improve risk stratification[88].

The limitations of IHC in its current form have been described. Recent developments in computed image analysis and digital pathology have shown a high correlation (Spearman's rank correlation coefficient: 0.94) between manual scorings and computer supported image analyses for ERG expression[228]. This technology has the potential to objectivise IHC readings, eliminate inter-observer variation, and provide more precise protein quantitation. The expression level of ERG may be valuable in terms of determining PCa aggressiveness and staging[229,230], and future studies should focus on the prognostic value of an objective quantitation of ERG.

Currently, AS protocols require frequent and close monitoring of the patients using uncomfortable re-biopsy procedures with significant risks of serious complications[157]. Therefore, it is desirable to obtain individualised and risk-stratified follow-up regimens to reduce the need for re-biopsies. If the results from *Study 2* could be externally validated in a prospective multicentre setting, the results could lay the basis for stratified AS programmes based on men's ERG status. Additional high-risk features such as high PSA-

density and extensive tumour load in biopsy specimens might be used to exclude ERG-positive patients from AS due to a high risk of initial understaging or early tumour progression. These patients might benefit from early intervention in a disease stage where nerve sparing treatment is possible thereby minimising the risk of side-effects from the surgical intervention. In contrast, higher-risk features could be acceptable in ERG-negative patients who are candidates for AS, as their *a priori* risk of progression is lower. Moreover, ERG-negative patients might need less frequent examinations as compared to ERG-positive patients whereby the number of visits and ultimately the number of re-biopsies could be reduced.

In the context of AS, the performance of ERG could possibly be further improved by combining ERG IHC with additional novel technologies. Advances in MRI have enhanced the possibility of detecting tumour lesions by imaging and the ability to collect targeted biopsies[231]. Besides improving the detection of significant PCa, MRI can be expected to increase the accuracy of the 'estimated' ERG status in biopsies by lowering the sampling error, which would allow for more accurate risk stratification at AS enrolment. Moreover, recent advances in the use of *TMPRSS2-ERG* as a urinary mRNA-based biomarker could provide a non-invasive approach for determining the aggressive potential[232,233] and risk of tumour progression in AS patients[234,235]. Lin *et al.* have demonstrated that the median *TMPRSS2-ERG* score increased significantly with higher volume PCa (negative, 1-10% positive cores, 11-33% positive cores, ≥ 34% positive cores; $p < 0.0001$) and higher-grade PCa on re-biopsies (negative, GS 5-6, GS ≥ 7; $p = 0.001$) [234]. When combined with a baseline ERG status assessed by IHC in MRI-targeted biopsies, serial urinary measurements of *TMPRSS2-ERG* could potentially provide a new, dynamic, and non-invasive risk-stratification of AS patients and might lead to individualised protocols where re-biopsies are performed only in patients with elevated *TMPRSS2-ERG* scores.

In advanced PCa, accumulation of alterations in other genes and signalling pathways is partly explained by ERG status. Androgen receptor expression levels[212], PI3K signalling pathway alterations, and *PTEN* deletions[204,236] are associated with aggressive behaviour in ERG-positive tumours, whereas *SPINK1* overexpression and *SPOP* mutations can designate aggressive ERG-negative tumours[201,208]. Data indicate that ERG status has an impact on the prognostic value of these other biomarkers, and ERG should be taken into consideration when other genomic alterations are investigated for their prognostic value[237]. Future studies might benefit from assessing additional genomic alterations and thereby divide ERG-positive and ERG-negative patients into subgroups.

During the past decade, several new treatment modalities have been approved for treating CRPC patients. Currently, one of the largest unmet needs in this stage of the disease is to sequence the new treatments optimally for the individual patient. Data suggest that ERG-positive CRPCs are particularly hormone-sensitive, as these cancers are enriched in the group of patients with the largest radiographic progression-free survival[238] and the biggest PSA response to the potent CYP17 inhibitor abiraterone acetate[239]. Moreover, ERG-positive PCas seem to have a prolonged response to the androgen receptor signalling inhibitor enzalutamide[240]. In contrast, ERG-positive CRPC patients seem to have a less favourable response to the chemotherapeutic drug docetaxel, probably due to a direct effect of ERG on the microtubule dynamics[241]. Whether ERG status can be used as a predictive biomarker for patient tailored therapy in this late stage of the disease needs further investigation. Such future studies can be further enhanced by the possibility of obtaining a "liquid biopsy" by means of circulating tumour cells to determine the *ERG* rearrangement status of the metastatic PCa clone[239].

Finally, ERG might serve as a therapeutic biomarker. ERG has several downstream molecular targets including the enzyme poly (ADP-ribose) polymerase 1 (*PARP1*), and ERG-mediated cell invasion requires *PARP1* as a co-factor. Tumour growth is inhibited in ERG-positive but not ERG-negative PCa xenografts when treated with *PARP1* inhibitors[242]. Ongoing phase 2 studies in metastatic CRPC patients (ClinicalTrials.gov identifier: NCT01972217 and NCT01576172) investigate the effect of adding an oral *PARP* inhibitor, either olaparib or veliparib, to abiraterone acetate, and the association between drug efficacy and ERG status will be assessed. The predictive value of *TMPRSS2-ERG* in terms of treatment response is pending.

SUMMARY

Background

The clinical course of prostate carcinoma (PCa) is very heterogeneous. Consequently, a personalised approach for risk stratification and treatment planning is important. Recently, it has become evident that PCa, also at the genomic level, is heterogeneous. An early and common alteration is the gene fusion between the transmembrane protease serine 2 (*TMPRSS2*) gene and the v-ets avian erythroblastosis virus E26 oncogene homolog (*ERG*) gene resulting in expression of the oncoprotein ERG. The gene fusion is present in approximately half of PCa patients and the resultant two subgroups demonstrate marked differences in their genomic signatures. It has been hypothesised that genomic alterations can explain some of the observed heterogeneity in the clinical course of PCa. In order to conduct an analysis of the prognostic and predictive value of ERG protein expression in PCa biopsies, the thesis sought to evaluate (1) the concordance in ERG expression between biopsies and radical prostatectomies, (2) the association between expression of ERG protein and the risk of PCa progression during active surveillance (AS), and (3) the association between ERG protein expression and response to primary castration-based treatment for advanced PCa.

Material and methods

The included patients derived from the institutional AS cohort and an institutional cohort of advanced PCa patients undergoing first-line castration-based androgen deprivation therapy (ADT). The 265 patients in the AS cohort were enrolled prospectively between October 2002 and October 2012 and were followed with regular dig-

ital rectal examinations, PSA measurements, and repeated biopsies. The advanced PCa cohort comprised of 194 patients diagnosed between January 2000 and December 2011 and was established retrospectively by a standardised extraction of patient data. Immunohistochemical (IHC) assessment for ERG protein expression was performed in all tumour containing diagnostic specimens (AS cohort: n = 459; advanced PCa cohort: n = 968), re-biopsies during AS (n = 402), and deferred radical prostatectomy specimens following AS (n = 86). An anti-ERG rabbit monoclonal primary antibody (clone: EPR3864, dilution 23 μg/mL) was used. Fluorescence *in situ* hybridisation (FISH) for the *TMPRSS2-ERG* gene fusion was performed in 76 selected biopsies from the AS cohort using the ZytoLight® TriCheck™ Probe, SPEC *ERG/TMPRSS2*.

Results

Based on the AS cohort, *Study 1* found a 97.3% concordance between the FISH assay and the IHC assay. The IHC assessments of ERG expression in diagnostic biopsies, re-biopsies, and radical prostatectomy specimens demonstrated a low proportion of temporal ERG reclassification. During four rounds of re-biopsies, 6.6% of the patients experienced ERG reclassification, and depending on the number biopsy specimens included 5.8-10.5% of the patients were ERG reclassified after radical prostatectomy.

In *Study 2*, 46.4% of the AS patients were categorised as ERG-positive, whereas 53.6% were categorised as ERG-negative. After median 4.1 years follow-up, a significantly higher risk of disease progression was observed in men with ERG-positive tumours corresponding to a two-year cumulative incidence of 58.6% (95% CI: 48.7-68.5) and 21.7% (95% CI: 14.3-29.1) in the ERG-positive and the ERG-negative group, respectively. In the multiple cause-specific Cox analyses, ERG expression was a strong and independent predictor of overall disease progression (HR: 2.45, 95% CI: 1.62-3.72) and histopathological progression in repeated biopsies (HR: 3.06, 95% CI: 1.78-5.26), and ERG status increased the discriminative ability for predicting disease progression significantly.

Study 3 included 194 patients with advanced PCa treated with first-line ADT. In total, 54.1% had ERG-positive tumours and 45.9% had ERG-negative tumours. With a median of 6.8 years of follow-up, the risk of developing castration-resistant PCa (CRPC) did not differ between the ERG subgroups (p = 0.51). Finally, inclusion of ERG status in a multiple cause-specific Cox model did not increase the discriminative ability for predicting CRPC development during the first 8 years of ADT.

Conclusion

The thesis has demonstrated that assessment of ERG protein expression is feasible in biopsy specimens, and a high concordance was found between the IHC assay and FISH assessment of *ERG* rearrangement. The low proportion of ERG reclassification between biopsies and prostatectomies supports the use of ERG assessment in biopsies to characterise the individual patient's ERG status. ERG status harbours important prognostic value in terms of tumour progression for patients managed on AS, whereas ERG expression has no predictive value for ADT response in men with advanced PCa undergoing first-line castration-based ADT. The overall conclusion of the thesis is that ERG protein expression provides valuable prognostic information in low-risk PCa managed observationally, and ERG expression might be used to personalise follow-up regimens in future AS programmes.

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