Prostate Cancer Progression and Mortality-

Focus on β-blocker use and β$_2$-adrenergic receptor level

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Preface

Acknowledgements

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Abbreviations

ACE	Angiotensin-converting enzyme
ADRB	β-adrenergic receptor
ADRB2	β$_2$-adrenergic receptor
ADT	Androgen deprivation therapy
AJCC	American Joint Committee on Cancer
AR	Androgen receptor
ARE	Androgen responsive element
ASA	Acetylic salicylic acid
BPH	Benign Prostatic Hyperplasia
C42-cells	LNCaP C4-2 cells
cAMP	cyclic adenosine monophosphate
CRPC	Castration resistant prostate cancer
CREB	cAMP response element binding protein
DHT	Dihydrotestosterone
EMT	Epithelial-mesenchymal transition
EPAC	Exchange protein directly activated by cAMP
ERK	Extracellular signal-regulated kinase
ETS	E-twenty six
EZH2	Enhancer of zeste homolog 2
GPCR	G-protein coupled receptor
HR	Hazard ratio
IL	Interleukin
ISUP	International Society of Urological Pathology
LHRH	Luteinizing hormone releasing hormone
LNCaP-cells	Lymph node cancer prostate- cells
MET	Mesenchymal-epithelial transition
MMP	Matrix metalloproteinase
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homolog
RP	Radical prostatectomy
RT	Radiotherapy
SHR	Sub hazard ratio
TNM	Tumor, node and metastasis
TUR-P	Transurethral Resection of the Prostate
UICC	Union Internationale Contre le Cancer
VCAP-cells	Vertebral cancer of the prostate- cells
VEGF	Vascular endothelial growth factor

List of papers

I. Use of β-blockers is associated with prostate cancer-specific survival in prostate cancer patients on androgen deprivation therapy (Prostate, 2012)

II. Association between use of β-blockers and prostate cancer- specific survival; a cohort study of 3561 prostate cancer patients with high risk or metastatic disease (submitted)

III. The level of β_2-adrenergic receptor in prostate cancer tissue is associated with development of castration resistant prostate cancer: a pilot study (manuscript)

Introduction

The Normal Prostate Gland

The prostate is a walnut- sized endocrine gland of the male reproductive system, localized just below the bladder, surrounding the urethra. The gland is made up of canals and follicles that are lined with epithelial cells. The fibromuscular stroma surrounding the prostate consists of connective tissue and smooth muscle [1].

Being a gland of approximately one gram at birth, the prostate grows very slowly in childhood, with a rapid increase in weight to about 20 grams during puberty; as the growth is controlled by male sex hormones. After puberty, the gland stays in this size range for approximately 20 to 30 years [2]. With time, an increasing number of men develops benign prostate hyperplasia (BPH), represented by non-malignant growth of the prostate gland. It has been estimated that the clinical prevalence is around 45% for men in their fifties and around 62% for men in their seventies, with an even higher number of histological evident BPHs [3].

The main function of the prostate gland is to produce and secrete components of the seminal fluid [1]. Specifically, secretion from the prostate provides optimal pH and nutrients for sperm cells, and aids in maintaining motility by the actions of proteases [4].

The prostate is innerved with nerve fibers. Both muscarinic and adrenergic receptors are abundant in the prostate, and are suggested to be involved in growth maturation and normal prostate function [5]. Noradrenergic nerves are shown to be innerved mainly in the prostatic stroma, and stimulation of these nerves leads to contraction of prostate smooth muscle [6].

Prostate Cancer

Epidemiology

Prostate cancer is the most frequently diagnosed non-skin cancer in men, with 4299 new cases in Norway in 2009; representing 29.1% of all diagnosed cancers in men [7]. Almost 0.9 million new cases were recorded worldwide in 2008 [8]. Known risk factors are increasing age, ethnicity, and a family history of prostate cancer [9]; the risk of prostate cancer is reported to be two times higher for men with a first-degree relative with a prostate cancer diagnosis, and the risk increases with the number of relatives affected [10].

Androgens and the Androgen Receptor (AR)

Androgens are the omnibus designation of the male sex hormones, including testosterone and its metabolite dihydrotestosterone. Androgens are synthesized mainly in the testes' Leydig cells, accounting for about 90% of the total androgen produced. A low proportion of androgens is also secreted by the adrenal glands [11;12]. The production of testosterone is regulated by luteinizing hormone (LH) and luteinizing hormone-releasing hormone (LHRH), via the hypothalamus- pituitary-gonadal axis. Inside prostate cells, testosterone is converted to the more potent dihydrotestosterone (DHT) by the enzyme 5α-reductase [12].

The significance of androgens in male development was recognized already back in 1849, when an association between a substance secreted from the testes into the blood and castration-induced changes in male psychology and behavior was first observed [12]. Testosterone was first isolated in 1935, and successfully synthesized artificially in 1935 [13].

The androgen receptor (AR) is a ~110 kDa nuclear transcription factor in the steroid receptor family. When bound by androgens, the AR undergoes a conformational change that causes AR dislocation from heat shock proteins, with subsequent phosphorylation of the receptor and translocation to the nucleus [14]. Once in the nucleus, AR binds to androgen response elements (AREs) on target genes, regulating their transcription [14;15]. Some well-characterized androgen regulated genes include the prostate specific antigen (PSA) [16] and the homeobox protein NKX3.1 [17]. Via the regulation of androgen regulated genes, the AR regulates several important functions in the prostate including metabolism, secretory function, morphology, proliferation and survival [18]. The AR is also crucial for the lineage-specific differentiation of the prostate [4;19].

Prostate Carcinogenesis

The first detectable event in prostate cancer development is thickening of the epithelial layer and loss of the basal cells, as illustrated in Figure 1 [20]. This precursor of prostate cancer is referred to as prostatic intraepithelial neoplasia (PIN), and is currently the only widely accepted precursor of prostatic carcinoma. Atrophy and malignancy-associated changes without any morphological changes are also proposed as prostate cancer precursors [21]. Loss of polarity and glandular structure is observed in less differentiated prostate cancer. These de-differentiated cancer cells have an increased migration capacity and may metastasize to other organs.
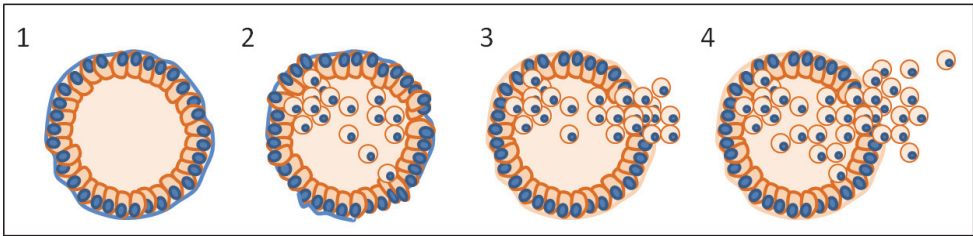
Figure 1: Schematic drawing of prostate cancer development. (1) Normal prostate glands consist of two cell layers: one basal cell layer, and columnar secretory luminal cells interspread with neuroendocrine cells. (2) Reduced number of basal cells and luminal epithelial hyperplasia are among the characteristics of PIN areas, representing a precursor form of prostate cancer. (3) Lack of basal cells is a diagnostic criterion for prostate cancer, besides abnormal nuclear structures. (4) The glandular structure becomes more and more distorted as the differentiation grade of the cancer decreases. De-differentiated cells have increased migratory and invasive potential and may give rise to metastasis.

Androgens and AR signaling are considered critical for the development of both the normal prostate and prostate cancer progression [11;22;23]. Androgen signaling have been demonstrated to be involved in regulation of oncogenic pathways, resistance to apoptosis, and pro-metastatic signaling pathways [11].

The fusion gene TMPRSS-ERG has received increasing interest in the prostate cancer research field, as this rearrangement has been shown to be present in around 50% of prostate cancers [24;25]. AR protein levels and AR mediated transcription have been shown to be disrupted in prostate cancer tumors and cell lines expressing the TMPRSS2-ERG fusion gene [26]. The down-regulation of AR and repression of AR induced transcription by TMPRSS2-ERG were suggested to be a mechanism for the induction of cellular dedifferentiation. The loss of the androgen regulated homeobox gene NKX3.1 is also suggested as a mechanism for cellular dedifferentiation. The gene is located at chromosome band 8p21, a region which deletion is reported to be associated with dedifferentiation and loss of androgen dependence [27]. NKX3.1 is shown to be one of the earliest markers of differentiated prostate epithelium, and has been suggested as a tumor suppressor gene as mutations in NKX3.1 were shown to induce prostatic epithelial hyperplasia and dysplasia in mice [28]. Furthermore, NKX3.1 is suggested to mark a sub-population of prostate stem cells [29]. Another gene which is believed to be of significance in prostate cancer carcinogenesis is the tumor suppressor phosphatase and tensin homolog (PTEN). Deletions in this gene are present in >60% of prostate cancers, and lead to loss of inhibition of downstream oncogenic pathways such as AKT and mTOR signaling [24]. Environmental factors, such as diet and infections, have also

emerged as potential mediators of prostate carcinogenesis; chronic inflammation has been suggested as the driver of the observed effects, as it may be induced by a number of different environmental factors [30].

Symptoms and Diagnosis

The symptoms of prostate cancer are often associated with the increasing size of the prostate, leading to obstruction of the urethra with subsequent symptoms such as urine retention, hesitancy, poor and / or intermittent urine flow, and incomplete emptying of the bladder. Other symptoms are nocturia, urgency and frequent urinary needs [31]. In later stages of the disease, the malignancy may give rise to systemic symptoms like anemia, fatigue, weight loss and loss of appetite.

The diagnosis of prostate cancer relies on tissue assessment after biopsy. The most widely used tools for elucidating prostate cancer are digital rectal exploration (DRE), serum concentration of prostate specific antigen (PSA), and transrectal ultrasound (TRUS) guided biopsies which ultimately are examined pathologically [9].

PSA testing

PSA, first identified in 1979 [32], is a serine protease whose main function is to liquidate the semen [18]. The serum levels of PSA are frequently increased in both prostate cancer and BPH [33], and is also commonly elevated in other prostatic diseases.

PSA testing was originally developed as a surveillance tool, but was adopted for prostate cancer diagnostics in the late 1980's [34]. Compared to digital rectal examination, which earlier was the primary test for prostate cancer, PSA testing has been found to detect a higher proportion of low grade prostate cancer [34]. The PSA test is not specific for prostate cancer, however, as non-cancerous conditions such as BPH and urinary tract infections also can lead to elevated serum PSA levels [33;35]. In addition, the PSA test is not considered to be sufficiently sensitive, as an evident prostate cancer will not always give rise to elevated PSA [36]. As sensitivity and specificity are inversely correlated, there is no certain cut-off value of PSA that yields sufficient specificity and sensitivity to be used for prostate cancer screening [37]. Alternative measurements of PSA are suggested and in some clinical use; namely PSA density, PSA velocity, age-adjusted PSA, and free PSA [35;38].

The use of PSA testing for screening purposes is under constant evaluation, mainly due to concerns of over-diagnosing [39]. The U.S. Preventive Services Task Force's (USPSTF) most

recently updated recommendations argue against PSA screening for prostate cancer, regardless of age [40]. Screening is also not recommended by the European Association of Urology [41], and routine PSA screening is not used in Norway per 2012 [42]. However, PSA testing is widely used as a surveillance tool, as PSA functions as a marker of treatment efficiency for patients with confirmed prostate cancer [9;43]. Intensive work is being conducted to identify more specific diagnostic markers and prognostic biomarkers that differentiate between indolent and aggressive prostate cancers [35]. Currently, several novel biomarkers are under investigation, some showing promising results (reviewed in [35;38]).

Gleason Grading

In the prostate cancer tissue, the cell morphology and hierarchy are evaluated by the Gleason grading system. The system was first described by the pathologist Donald Floyd Gleason in 1966, and has been refined several times since then [44]. The use of Gleason grading is under constant evaluation, and the latest agreement per fall 2012 is from the 2005 International Society of Urological pathology Consensus (ISUP) conference [45;46].

The Gleason system assesses the degree of cell differentiation and translates it into a common scale of 1 through 5; 5 being the least differentiated and hence most aggressive pattern (Figure 2). The Gleason score from a radical prostatectomy is calculated adding the two most dominating tissue structures in the preparation, yielding a scoring system that ranges from 2 to 10. During the last decade, there have been debates as to whether or not to include a tertiary grade if one is present [44].
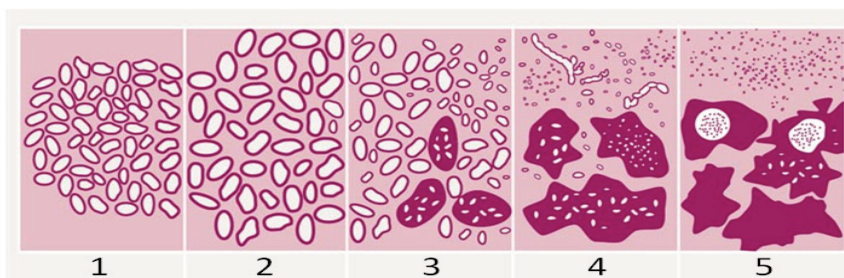
Figure 2. An illustration of the Gleason grading system. The Gleason grading system is based on the growth pattern of the luminal cells of the prostate. Gleason grade 1 and 2 closely resembles normal prostate glands. The glandular structures are also easily recognized in Gleason grade 3, but they are smaller and the cells darker. In Gleason grade 4, the glands are starting to fuse, whereas in Gleason grade 5 the glandular structure is lost. The figure is from Taskén et al., Tidsskrift for Norsk Legeforening [47], with permission.

Staging

The TNM staging system is used to describe the extent of cancer spread with regard to tumor, nodes and metastasis (Table 1). The use of the TNM staging system for prostate cancer was first started in 1992 [48]. Since then, the system has been evaluated and revised a total of seven times by the American Joint Committee on Cancer and the Union Internationale Contre le Cancer (AJCC/UICC), latest with validity from January 1.st, 2010 [49;50].

Table 1: The TNM classification of prostatic tumors according to the AJCC/UICC, 2010. Modified from Cheng et al., Histopathology [49].

Stage	Description of Tumor
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
T1	Clinically unapparent tumor; neither palpable nor visible by imaging
T1a	Tumor incidental, histological finding in ≤5% of tissue resected
T1b	Tumor incidental, histological finding in >5% of tissue resected
T1c	Tumor identified by needle biopsy
T2	Tumor confined within prostate
T2a	Tumor involves ≤ one half of one lobe
T2b	Tumor involves > one half of one lobe but not both lobes
T2c	Tumor involves both lobes
T3	Tumor extends through the prostate capsule
T3a	Extra capsular extension (unilateral or bilateral)
T3b	Tumor invades seminal vesicle(s)
T4	Tumor is fixed or invades adjacent structures other than seminal vesicles
N0	No regional lymph node metastases
N1	Regional lymph node metastases
Nx	Regional lymph node status not assessed
M0	No distant metastases
M1	Distant metastases

Treatment and Prognosis of Prostate Cancer

The current guidelines for prostate cancer treatment in Europe are drawn up by the European Association of Urology (EAU), and are presented separately for clinically localized disease [9] and advanced, relapsing, and castration resistant prostate cancer [43]. Given the heterogeneity of prostate cancer, urologists are faced with the challenge to predict whether or not a given tumor is likely to give rise to symptomatic disease. Identification of patients at highest risk of cancer progression is crucial to aid the physician and the patient towards the most beneficial treatment.

For treatment of patients with localized disease, a summary of the current treatment recommendations are given in the EAU guidelines, part I [9]. For some patients, the combination of high age and the outlook of a localized, non- aggressive tumor make the potential side-effects of treatment likely to surpass the expected symptoms of the disease. In this situation, the recommended treatment is so-called watchful waiting; the cancer is monitored, and potential symptom onset and disease progression is caught and palliatively treated if necessary. Active surveillance, on the other hand, is used for patients diagnosed with low-risk prostate cancer, who might become candidates for curative therapy if the disease progresses. The benefits of the curative surgery radical prostatectomy (RP) versus active surveillance and watchful waiting remain controversial, and more studies are needed to ensure that patients are offered the right treatment [51-57]. Other treatment options for this group of patients include androgen deprivation therapy (ADT), and radiotherapy (RT); mainly external beam or brachytherapy. Over the last few years, cryosurgical ablation of the prostate and high-intensity focused ultrasound have been increasingly used in patients with localized disease that are not candidates for RT [9].

Since the discovery of the beneficial effects of castration on advanced prostate cancer by Huggins *et al.* in 1941 [58], a discovery for which Huggins and Hodges were awarded the Nobel Prize in 1967, androgen deprivation therapy (ADT) has been widely accepted as a first line treatment for patients with advanced prostate cancer [59]. Aiming at reducing the effects of androgen on prostate cancer cells, ADT is achieved either by castration or by administration of nonsteroidal anti-androgens, which inhibit the binding to and activation of AR by androgens. Castration aims at reducing the production of androgens in the testes, and is achieved either by surgical castration or by administration of a luteinizing hormone-releasing hormone (LHRH) analogue or antagonist. To achieve total androgen blockade, castration and use of anti-androgens are combined.

Castration Resistant Prostate Cancer

Although initially effective in most patients, prostate cancer cells develop resistance against traditional ADT in the majority of cases, in a median time of two to three years [60]. The exact mechanisms behind castration resistant prostate cancer (CRPC) development are not fully understood, although several potential pathways have been suggested; a common denominator being that the AR is believed to be involved also in the castration resistant state of the disease. The multiple molecular mechanisms by which the AR may contribute to

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disease progression despite castration levels of androgens have been described in several recent review articles [22;61-63], and include amplification and/or overexpression of the AR, up-regulation of AR transcriptional co-activators, transcription of AR splice variants lacking the ligand-binding domain, *de novo* synthesis of androgens, increased transport of androgen into the cells, and AR interaction with other oncogenic signaling pathways. Furthermore, new insight into the molecular landscape is expected to come from genome wide RNA sequencing studies, as exemplified by Grasso *et al.* [64].

If a patient has developed CRPC, the anti-mitotic chemotherapeutic Docetaxel in combination with prednisone is the preferred cytotoxic treatment regime [43]. Docetaxel has been shown to improve survival, pain and quality of life in clinical studies, independently of age, pain, performance status or the presence of metastatic disease at treatment initiation [43]. For patients with bone metastases, the bisphosphonate zoledronate has been shown to relieve metastasis-associated symptoms [65].

In the later years, secondary hormonal therapies for additional inhibition of AR activation and other compounds aimed at patients with CRPC have been developed (reviewed in [66-70]). The Cytochrome P450 (CYP) 17 inhibitor abiraterone acetate, the AR antagonist MDV3100/enzulatamide, and Sipuleucel-T, an antigen-presenting cell vaccine, have recently been approved by the United States' Food and Drug Administration (FDA) for the treatment of CRPC. Abiraterone acetate is also approved by the European Medicine Agency (EMEA) [66;67]. In addition, several novel compounds including hormonal agents, immune-based therapy and cytotoxica are currently under investigation and development [66;67].

Evidence of resistance against some of the recently approved drugs have already started to emerge [71;72]. Hence, although new drugs targeting CRPC are currently being developed, there is a continuous need for additional treatment options for this group of patients.

The β_2- Adrenergic Receptor

The β_2-adrenergic receptor (ADRB2) is part of the β- adrenergic receptor family consisting of β_1-, β_2- and β_3 –adrenergic receptors. These are seven trans-membrane G-coupled receptors, which bind the natural catecholamines noradrenaline and adrenaline (alternatively norepinephrine and epinephrine, respectively). Catecholamines are synthesized in axon terminals and in the adrenal medulla, and their synthesis and release are controlled by autoreceptors on the presynaptic terminals and by sympathetic preganglionic nerve fibers [73]. Indeed, nerve fibers are shown to be abundant in the prostate [6]. Catecholamines may

also be synthesized by macrophages [74]. Upon ligand binding, ADRB2 activates the G-protein G_s, leading to activation of adenylyl cyclase and generation of cyclic AMP (cAMP) (Figure 3). Most effects of cAMP are mediated through protein kinase A (PKA), although other mechanisms such as activation of exchange protein directly activated by cAMP (Epac) also are involved [75]. The ADRB2 also activates class C L-type calcium channels [76]. It has also been shown that the ADRB2 signaling pathway may involve activation of the G-protein G_i, initiating a signaling pathway involving PI3K and Akt [77], and G-protein independent activation of extracellular signal-regulated kinase (ERK) [78]. There is evidence that the β-adrenergic receptors have some intrinsic activity [79]; indeed, in mouse models, over-expressing the β2-adrenergic receptor has been shown to yield the same increase in cardiac contractility as stimulation by agonist [80].
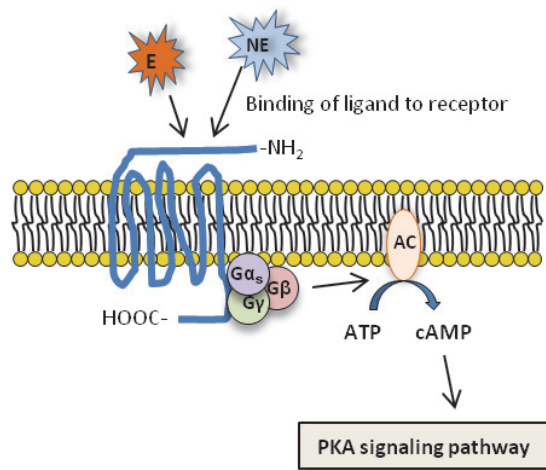
Figure 3. The classical ADRB2 pathway. Beta2-adrenergic receptor is a seven-transmembrane G-protein coupled receptor that binds epinephrine and norepinephrine. Ligand binding induce a conformational change that via a G-protein complex stimulates adenylyl cyclase activity, resulting in increased levels of cAMP. The major intracellular effector of cAMP is protein kinase A (PKA) or cAMP-dependent protein kinase. E, epinephrine; NE, norepinephrine; AC, adenylyl cyclase; PKA, protein kinase A

The ADRB2 is expressed in many tissues in the body, including lung, pancreas, the lymphoid, digestive and reproductive systems [81], the eyes [82] and bone [83]. ADRB2 is also highly expressed in the prostate gland [84]. The functions of the ADRB2's are dependent on their

location, but their stimulation are mostly associated with relaxation of smooth muscle and arterioles [73].

25 years ago, Thompson *et al.* showed that catecholamines were involved in normal growth and differentiation of the prostate gland in rats [85]. McVary *et al.* later showed that symphatectomy lead to decrease in rat prostate weight [86]. The role of ADRB2 in the prostate is not fully understood [87]; however, ADRB2 activation has been shown to inhibit α-adrenoceptor mediated, field stimulation induced or receptor independent contraction of the prostate gland in several species, including humans [84]. It has also been suggested that the ADRB2 contributes to the maintenance of a differentiated phenotype in the prostate cells, as silencing of the ADRB2 in the benign prostate cell line RWPE-1 has been shown to induce epithelial-to-mesenchymal transition (EMT) and a tumorigenic phenotype [88]. Stimulation of ADRB2 with agonist has also been shown to be involved in the expression of the organ-specific protein prostatic binding protein (PBP), and to influence prostatic morphology in rat prostate [89]. Interestingly, ADRB2 signaling has also been implicated in steroidogenesis in granulosa cells [90] and testosterone secretion from rat Leydig cells [91;92].

The β$_2$- Adrenergic Receptor in Cancer

The first evidence of a role for ADRBs in cancer was in 1989 by Schuller *et al.*; *in vitro* activation of ADRB's by the agonist isoproterenol was shown to increase the proliferation of human lung adenocarcinoma cells. This effect was inhibited by addition of the β-blocker propranolol [93]. Since then, an increasing body of pre-clinical findings has indicated a role for the β-adrenergic signaling pathway in cancer (reviewed by Cole *et al.* in [94]). It has been observed that stress-levels of β-adrenergic agonists epinephrine and norepinephrine increase the invasiveness and migration potential in ovarian cancer cells [95]. This induction of invasive and migratory properties has also been observed in human breast- and prostate cancer cell lines [96;97].

Indeed, the ability of adrenergic stimulation to initiate metastasis development in animal models has been reported for several cancer forms, including prostate [98], breast [99;100] and ovarian [101] cancer. The metastatic growth initiated with adrenergic stimulation was shown to be prevented by treatment with the β$_1$/β$_2$-adrenergic receptor antagonist propranolol. One of these studies has identified the ADRB2 as the main mediator of these effects, with little or no effects mediated through the ADRB1 [101]. Thaker *et al.* also showed, by use of siRNA targeting the human ADRB2, that the effects on tumor progression and metastasis

formation were mediated through the ADRB2 present on the tumor cells, and independent of host cells ADRB2 levels.

A link between the ADRB2 and AR signaling was reported in 1988, when Marchetti *et al.* showed that the ADRB2 was regulated by testosterone levels in rat prostate [102]. Since then, this regulation has also been observed in prostate cancer cell lines and human RP-specimens [103]. In addition, the ADRB2 signaling pathway has been shown to be able to activate the AR in the absence of androgens [104], indicating a potential role for ADRB2 in the development of CRPC. The downstream mediators of ADRB2 signaling, cAMP and PKA, are also reported to be involved in cross-talk with the AR signaling pathway (reviewed in [105]), and a number of target genes are common between the androgen and PKA signaling pathways [106]; further supporting the potential significance of the ADRB2 signaling pathway in the progression of prostate cancer to castration resistance.

Interestingly, down-regulation of the ADRB2 has been suggested to be one of the main mediators of Enhancer of zeste homolog 2 (EZH2) induced prostate malignancy [88]. EZH2 is a histone methyl transferase, which expression is shown to be associated with several cancer forms, including lymphoma, breast cancer and prostate cancer [107-109]. EZH2 was later shown by Yu *et al.* to be activated by ERG, an oncogenic E-twenty six (ETS) transcription factor that is believed to be a driver of oncogenesis in prostate cancer tumors with the ERG-TMPRSS2 fusion gene. ERG binding directly to the ADRB2 gene was also observed [26]. Furthermore, EZH2 up-regulation is also observed in fusion negative cells, and is shown to be caused by altered methylation patterns in the cancer cells [110].

The Expression and Prognostic Value of ADRB2 in Cancer
In the work by Yu *et al.*, low expression of ADRB2 in prostate cancer tissue from prostatectomies was found to be associated with poor prognosis [88]. Also in oral squamous carcinoma, low levels of ADRB have been associated with poor prognosis [111]. In contrast, findings by our group indicated increased expression levels in malignant versus benign prostate tissue [103]. In breast cancer, strong expression levels of ADRB2 have been found to be correlated with small size, luminal-like estrogen receptor positive tumors of low grade; however, these patients showed poor prognosis when hormonal treatment was withheld [112].

β-blockers
One of the first drugs developed on the basis of knowledge on receptor-driven cell signaling, β-blockers were first synthesized in the early 1960's by Sir James Black *et al.* [113]. In 1988,

Black received the Nobel Prize for this work [114]; and the β-blocker atenolol have since become one of the most selling drugs in medical history [113]. Propranolol, being the first clinically useful β–blocker synthesized [115], was utilized clinically from 1968 for the treatment of angina [116]. While initially designed and developed to prevent and treat angina and myocardial infarction, β-blockers have since been found useful in treating diseases as divert as hypertension, cardiac heart failure, glaucoma, and migraine, to name a few. Later studies have also implied a use for β-blockers in diseases like osteoporosis, malaria and cancer [113].

Second generation β-blockers were introduced in the 1970's; the rationale being that avoiding the blockade of β_2-adrenergic receptors would reduce side-effects in the periphery and in the lungs, which are believed to be mediated by lack of ADRB2 mediated dilation. Since then, several new compounds in the β–blocker family, with different specificities and pharmacokinetics, have been developed [117].

Table 2: The different β-blockers in clinical use in Norway today, according to class, and their indications [118]

Class	Substance	Indications for use in Norway
$\beta_{1/}\beta_{2}$ blockers		
	Propranolol	Angina pectoris, hypertension, arrhythmias, migraine, essential tremor, thyrotoxicosis, feocormocytoma
	Sotalol	Arrhythmias
β_1- selective blockers		
	Metoprolol	Angina Pectoris, hypertension, arrhythmias, migraine, thyrotoxicosis
	Atenolol	Hypertension, arrhythmias, angina pectoris, migraine, thyrotoxicosis
	Bisoprolol	Hypertension, angina pectoris
	Esmolol	Tachycardia, perioperative hypertension, non- compensatory sinus tachycardia
α-and β- blockers		
	Labetalol	Not yet specified
	Carvedilol	Hypertension, angina pectoris, cardiac failure

Being used for such a wide range of indications, β-blockers are used by a large number of patients today. As β-blockers are among one of the first lines of therapy in the treatment of hypertensive, anginal and cardiac heart failure patients, the prevalence of use increases with age. The prevalence of use among Norwegian men has been increasing for the age groups 70-79, 80 - 89 and 90+ years, while being quite stable for younger age groups during the last eight years (Figure 4).
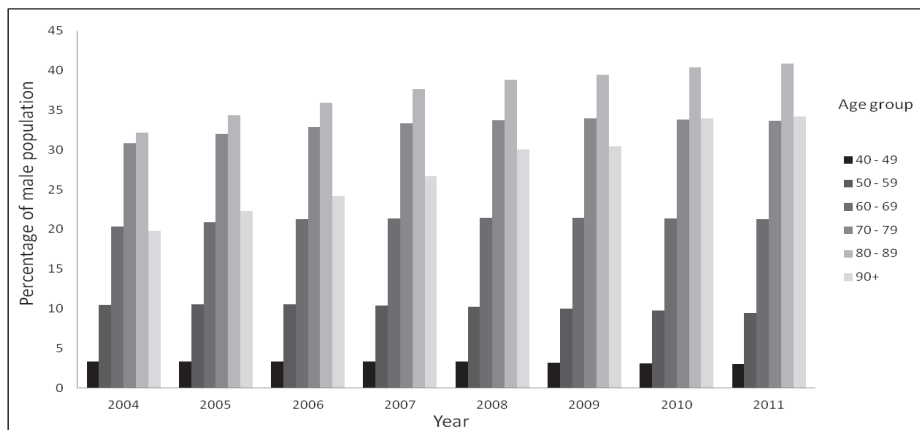
Figure 4. The prevalence of β-blocker use among men in Norway, 2004-2011. The percentage of the male population that has filled one or more prescriptions of β-blockers in different age groups is shown for the period 2004 to 2011 *[119]*.

Use of β-blockers and Cancer

On the background of the pre-clinical findings of the role of ADRB2 signaling in cancer mentioned above, several epidemiologic studies have been initiated to investigate possible associations between β-blocker use and cancer incidence, progression and survival. For prostate cancer, some studies have reported a possible negative association between use of β - blockers and prostate cancer incidence [120;121]. This has not been reproduced, however, and the total evidence points toward no benefit of β-blocker use regarding prostate cancer risk [122-127].

In the later years, increasing research has been focused on the possibility of exploiting β-blockers to slow down cancer progression. A beneficial association between β-blockers and cancer progression and/or survival has been reported in breast cancer [128;129], malignant melanoma [130;131], and ovarian cancer [132] patient cohorts.

One of the first studies looking into the link between β-blocker use and prostate cancer in particular , was conducted using a UK primary care database and published in 2011 [133]. Interestingly, the findings of this study were poorer overall survival in prostate and pancreatic cancer patients receiving β-blockers. For the other cancer forms investigated, namely breast, lung and colon cancer, no effect on overall survival was seen. Cancer specific survival was not assessed.

Aims

The overall aim of this study was to examine the role of β_2-adrenergic receptors in aggressive prostate cancer. The following questions were addressed by utilizing epidemiological data, clinical information and biological material from prostate cancer patients, and *in vitro* studies using prostate cancer cell lines:

- Does the use of β-blockers influence prostate cancer incidence or mortality?
- Is the potential effect of β-blockers independent on tumor characteristics at diagnosis?
- Does the expression level of ADRB2 affect androgen receptor activity?
- Does the expression level of ADRB2 influence the progression to CRPC?

Summary of Papers

Paper I: The use of β-blockers improves prostate cancer-specific survival in men treated with androgen deprivation therapy

We used the coupling of data from the Oslo II survey from 2000, the Cancer Registry of Norway and Statistics Norway to assess potential effects of β-blockers on prostate cancer risk and survival. Information on β-blocker use was obtained from self-reported questionnaires collected in the Oslo II study.

The use of β-blockers did not have any impact on prostate cancer risk in this cohort. However, when looking at survival, we found a reduction in prostate cancer specific mortality of almost five fold in patients treated with ADT (sub hazard ratio (SHR) 0.14, 95% CI 0.02-0.85, p-value 0.032; analyzed by competing risk regression). For patients not treated with ADT, no association was observed. Survival analyses were adjusted for age and metastasis status at diagnosis, as well as educational level reported at baseline in 2000. Because many patients were diagnosed before the routine reporting of prognostic markers to the Norwegian Cancer Registry was started in 2004, information on prognostic factors was missing for a large proportion of cases. Hence, we were not able to rule out potential confounding from PSA level, Gleason score, and clinical T-stage in this study.

Paper II: Association between use of β-blockers and prostate cancer-specific survival; a cohort study of 3561 prostate cancer patients with high risk or metastatic disease

By use of data from the Norwegian Cancer Registry, we coupled clinical data on all prostate cancer patients reported to be diagnosed with prostate cancer in Norway between 2004 and 2009 with data from the Norwegian Prescription Database, to identify β-blocker users. From the Norwegian Prescription Database we received information on drug type, dosage, and dates of prescription fillings for β-blockers, statins and acetylic salicylic acid (ASA), as well as LHRH agonists and anti-androgens. We focused our analyses on patients diagnosed with high risk or metastatic prostate cancer, and only patients with a complete set of covariates were included in the analyses.

When comparing prostate cancer-specific mortality rates between β-blocker users and non-users, β-blocker use was associated with a reduction in prostate cancer-specific mortality of 21% (SHR=0.79, p-value 0.001; analyzed by competing risk regression). We observed no effects of β-blockers on all-cause mortality in this cohort; however β-blocker users had significantly higher other-cause mortality. All survival analyses were adjusted for age, PSA-level, Gleason score, T-stage, metastases, and performance status at diagnosis, as well as ADT within six months of diagnosis.

The use of ASA and statin were each individually associated with prostate cancer-specific mortality. Still, adjusting for either ASA or statins in the multivariable analysis did not reduce the estimated effect of β-blockers substantially. We observed a potential additive or synergistic effect of β-blockers and ASA, however no such observation was made for β-blockers and statin.

Together, our results indicate a possible beneficial effect of β-blockers on prostate cancer survival. However, the result may have been influenced by outside confounders, and our results need validation from further observational studies.

Paper III: The level of β₂-adrenergic receptor in prostate cancer tissue is associated with development of castration resistant prostate cancer: a pilot study

Stimulation of prostate cancer cell lines with the ADRB2 agonist isoproterenol caused activation of AR, as shown by an increased luciferase expression from androgen responsive reporters. This effect was inhibited by anti-androgens, AR siRNA and mutations in the androgen response elements. In addition, basal luciferase expression was reduced in LNCaP cells with stable knockdown of the ADRB2.

To look at a potential impact of the ADRB2 on the development of CRPC, prostate cancer patients treated with hormonal therapy and operated with TUR-P at Oslo University Hospital, Aker, were retrospectively identified from medical records. 40 patients were found eligible and included in the analyses. Tissue from these patients was included on a TMA, which was immunohistochemically stained with an anti-ADRB2 antibody. The staining intensity was scored by two independent uro-pathologists, who also designated a Gleason grade for each spot. Clinical information was coupled with staining intensities and Gleason grade from the TMAs, and the impact of ADRB2 levels on disease progression and survival was analyzed. We found a significant association between high ADRB2 expression levels and longer time to development of CRCP (hazard ratio (HR) 0.60, 95% CI 0.38-0.95, p-value 0.028). There was a trend towards reduced prostate cancer-specific mortality; however this association was statistically non-significant.

To look for potential mechanisms by which ADRB2 may affect progression to CRPC, we used LNCaP cell lines with stable ADRB2 knockdown. Compared to control, we observed that ADRB2 knockdown cell lines had lower secretion levels of PSA and lower expression of the NKX3.1 protein. In addition, we observed a significant correlation between ADRB2 and NKX3.1 mRNA levels in RNA extracts from 22 radically operated prostate cancer patients. We hypothesize that an involvement in prostate cancer cell differentiation might be a mechanism by which the ADRB2 may influence CRPC development.

Methodological considerations

Observational Studies

The basis for papers I and II in this thesis is survey data and registry data, meaning that the information obtained was strictly observational. Observational data are an important source of information in the field of epidemiology, and gives the opportunity to conduct studies that may uncover associations between given exposures and potential outcomes which would otherwise go undetected. Prospective follow-up for disease outcomes after screening gives the best evidence for the directionality required in terms of uncovering a causal relationship between risk factor and disease. However, the use of observational data is prone to bias, as one cannot make sure that all potential confounders have been accounted for. Known confounders can be controlled for in the statistical analyses. The validity of observational studies in determining the effect of an exposure is also challenged by selection bias, meaning systematic differences between exposed and non-exposed subjects that may influence the outcome. When comparing treatment effects in different treatment groups, confounding by indication – systematic differences in patient characteristics between treated and non-treated patients - is an important limitation that may threaten the validity of the study [134]. As a result, it is not possible to fully determine causal relationships based on observational studies. To definitely establish a causal relationship, randomized studies are needed. Nevertheless, observational studies are an important tool for discovering associations and developing hypotheses to be tested in randomized controlled trials.

Because of the observational nature of the data used in paper I and II, the results of the two studies reported herein are not suited to be interpreted as a causal relationship between β-blocker use and prostate cancer mortality. Further studies are needed to potentially establish this relationship.

Use of Survey Data

The patients' baseline data used in paper I is based on the self-reported questionnaires filled in by study subjects participating in the Oslo II study. The Oslo study from 1972/73 was a health survey conducted to identify risk factors for cardiovascular disease [135]. Participants from the Oslo study were invited to a second screening in 2000, the Oslo II study, which consisted of self-filled questionnaires and measurements of height, weight, blood pressure, cholesterol, triglycerides and glucose [136]. A thorough description of the execution of the Oslo I- and the Oslo II studies is given at the web-pages of the Norwegian Institute of Public Health [137].

Self-reported information is an important source of information when performing prospective cohort studies; however, there are some limitations that need to be considered when interpreting the results. First, this kind of reporting is prone to recall bias; the memory of study subjects may be influenced by the study purpose and /or the subject's own expectations of causal mechanisms between different exposures and the outcome(s) of interest. Patients with a severe diagnosis might reflect more about their general health and lifestyle habits than otherwise healthy subjects, and hence give more accurate information. However, as the main focus of the Oslo II study was cardiovascular disease, we do not believe that any recall bias related to the development or progression of prostate cancer has occurred. Still, any potential risk factors common to heart disease and prostate cancer may be influenced by this potential bias. Second, there might be a skewed selection regarding which patients are willing or capable to respond; so-called non-response bias. This is a form of selection bias, potentially making the sample non-representative of the general population. Hence, the external validity of the study may be reduced. A third limitation to this kind of survey data is that the information on exposure and potential confounders is only recorded at baseline. The information provided at the time of the survey is likely to have changed during the course of follow-up, and hence the observed associations might be different from what would have been observed if continuous information on exposures and covariates was recorded.

Use of Registry Data

Data from the Norwegian Prescription Database (paper II), the Cancer registry of Norway (papers I + II), and Statistics Norway (papers I + III) have been utilized in the work of this thesis. The Norwegian population is well suited for epidemiological studies, as we have several different population based registries which can easily and dependably be combined by the use of the Norwegian national identity number. In addition, we have a widely employed public health care system, which means that the recording of information can be performed in a widespread and controlled manner.

An important limitation of the use of registry data for the studying of disease is that the information obtained is merely observational. Hence, it is difficult to determine causal mechanisms as long as all potential confounders are not known.

In addition, failure to accomplish complete datasets in registries will always raise the issue of compliance bias; there may be fundamental differences between the baseline characteristics of patients that are reported with complete information to the registries and the patients for

whom clinical characteristics are not reported. If this compliance bias is in any way associated with β-blocker use, which is the exposure of interest in papers I + II, this may lead to biased estimates of the effect of β-blocker use on prostate cancer mortality. However, we observed no large differences between the level of reporting of baseline clinical and pathological data for β-blocker users and non-users, indicating that compliance bias is not a severe issue in our studies.

The Cancer Registry of Norway

The Cancer registry of Norway was established in 1951. The main purposes of the registry are registration, research and information about cancer in Norway [138]. The reporting of new cancer diagnoses to the registry is required by law, as stated in the Cancer Registry Regulation [139]. The data quality of the information available from the Cancer Registry of Norway has been validated by comparing the information with information from the Norwegian Patient Registry, and was found to be satisfactory (97% correspondence for prostate cancer diagnosis) [140].

In 2004, the quality register "The Prostate Cancer Registry" was established as a sub-registry of the Norwegian Cancer Registry. This registry includes clinical characteristics at diagnosis, such as PSA-level, Gleason score, clinical T-stage, and the presence and basis of metastases. The information regarding clinical parameters at prostate cancer diagnosis used in papers I and II comes from this quality registry. The compliance of this part of the Norwegian Cancer Registry was found to be 96% for the first year of registration [141].

The Norwegian Prescription Database

The Norwegian Prescription Database was established in 2004, as a sub-division under the Norwegian Institute of Public Health. The aim of the registry is "*to collect and process data on prescribed drug use in humans and animals*" [142]. The registry collects information on prescription drug dispensing from all Norwegian pharmacies to patients, doctors and institutions. Information on over-the-counter drugs (OTC's) is not registered.

Statistics

The statistical software packages used for the work in this thesis are Statistical Software Package for the Social Sciences (SPSS) version 18 (paper I-III), and STATA version 8 (paper I) and 12 (paper II and III).

Comparisons of Categorical and Continuous Variables Between Groups

Student's t-test has been used for comparison of continuous variables between groups of patients (paper I+II), and for comparison of effects after different treatment conditions in cell line studies (paper III). Student's t-test requires a distribution of the different observations that resembles the normal distribution [143]. Variables compared with Student's t-test in the work of this thesis have been found to fulfill this requirement, with the potential exception of age at prostate cancer diagnosis, which was slightly skewed. Categorical variables with two exposure variables and two outcome variables were analyzed by means of a 2x2 table, with statistical significance calculated with the Pearson's chi^2 test. The Pearson's chi^2 test measures the probability that the distribution of the outcome variables observed are random based on the two exposure variables [143]. This test, however, does not take into account that the variables may be ordinal; that is, if there is a natural order of increase or decrease in the variable measured. For ordinal variables with more than two levels, i.e. categorized Gleason scores and PSA-levels, the Wilcoxon Mann-Whitney test was therefore used.

Survival Analyses

Cox Proportional Hazards Modeling

Cox proportional hazards modeling (Cox regression) was proposed by Shehee in 1962 [144]. Generally known as the "Cox model", it is a commonly used way of analyzing time to an event of interest. The model allows controlling for several co-variables, to generate a model where potential confounding can be addressed. The hazard function measures the probability that the event of interest will occur within a given period of time, and is a central concept in the context of Cox regression modeling [145].

The Cox regression model assumes constant hazard ratios with time when comparing different exposure groups. This assumption can be tested by visually examining log-log plots of survival probabilities versus time [143]. The graph should resemble a straight line, and the lines representing different exposure groups should be parallel during follow-up. The proportional hazards assumption may also be also tested by Schoenfeld residuals [146]. The variables examined in the different Cox proportional hazard models presented in this thesis was found to adhere satisfactory to the proportional hazards assumptions.

Competing Risk Analysis

The Kaplan-Meier and Cox proportional hazards survival analysis methods were originally developed for the studying of all-cause mortality, assuming that any censoring events are

independent of the outcome of interest [147]. In the case of cause-specific mortality, this assumption does not hold, as death from other causes during follow-up excludes the chance of dying from the cause of interest. In this case, estimation of hazard ratios by Kaplan-Meier or Cox regression analysis may lead to biased results. Hence, methods taking into account the competing risk of other-cause mortality are considered more appropriate [148;149]. This issue of competing risk in survival analysis was addressed by Fine and Grey [150], who calculated a model taking into account the competing risk of non-outcome events when performing cause-specific survival analyses. The model calculates the hazard of the subdistribution for the failure of interest, the so-called subhazard. Like the Cox proportional hazards model, the competing risk regression model allows for the inclusion of covariates [147]. It has been recommended that the competing risk should be taken into account whenever the outcome of interest is cause-specific mortality [151], and particularly when studying disease in the elderly [147]. This is indeed of great importance when analyzing prostate cancer-specific mortality, as prostate cancer patients are at large risk of other-cause mortality due to a combination of high age and the often slow progression of the disease. When studying patients groups with co-morbidities, such as heart disease or hypertension, this concern becomes increasingly important.

Clinical Samples

In paper III, we identified patients meeting the eligibility criteria from patient records at Oslo University Hospital, Aker. Prognostic information and clinical follow-up data were retrieved from the same source. The registration of important clinical parameters was highly variable between records; hence, we did not achieve complete datasets to address all potential confounders when analyzing progression-free survival and mortality. Also, as some patients were followed also by their primary physician, we did not have sufficient follow-up information to precisely identify dates of progression for all patients, as this was not always noted in the records. As a substitute, we used the middle date between two known consultations before and after progression occurred. This may have lead to incorrect survival times used in our analyses; however, we have no reason to believe that this potential error was anything but completely random between study subjects with different levels of tissue ADRB2.

All use of clinical information and use of patient tissue were approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Protection Authority. An

informed written consent was obtained from all living patients, while an exception from the professional secrecy was approved for the use of information from deceased patients.

Immunohistochemistry

In immunohistochemistry, tissue samples are stained with an antibody targeting the protein of interest. The quality of the tissue is of major importance, as this may have implications for the antigen retrieval and binding of the antibody to the target protein during the staining procedure. Tissue quality may be compromised both by poor fixation and by sub-optimal storage.

The patient tissue utilized in paper III in this thesis was collected over a long period of time (1992-2008), and changes in fixation protocols and storage conditions are likely to have occurred. To assure that this did not induce differences in the staining intensities observed, we plotted staining intensity versus year of TUR-P surgery. We observed no trends with time regarding the staining intensity (Pearson's correlation coefficient 0.009, p-value 0.96), hence we believe that the capability to bind antibody were similar across tissue samples from different periods.

To achieve dependable results, one needs to make sure that the antibody used for the immunohistochemical staining recognizes the specific target protein of interest (sensitivity), and that there is no untargeted binding (specificity). The anti-ADRB2 antibody from Santa Cruz, which has been previously used by Yu *et al.* [88], has been reported to lack sufficient specificity [152;153]. Therefore, we tested this and three additional antibodies on a western immuno-blot containing protein extract from patient tissue samples and from ADRB2 expression vector- transfected HEK 293- cells. The rabbit anti -ADRB2 antibody from Nordic Biosite was chosen for the immunohistochemical staining, as it recognized the correct protein band in extracts from ADRB2 expressing HEK293 cells as well as only one band in patient tissue extracts. This band corresponded to the theoretical size of ADRB2 (Figure 5).
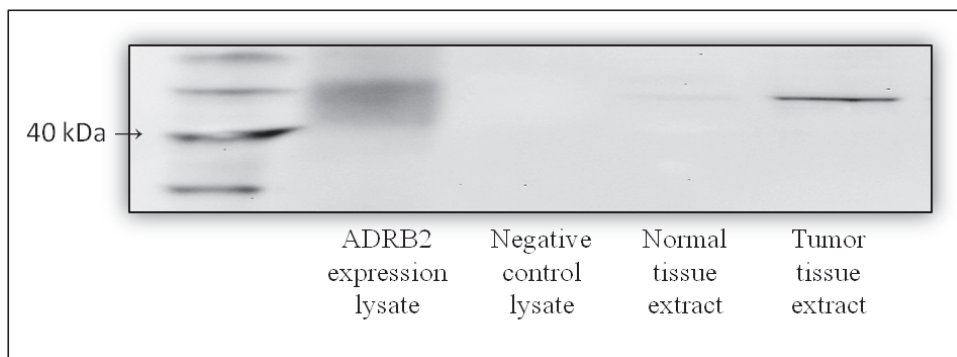
Figure 5: Western blot of protein extracts from ADRB2 expression vector-transfected HEK293 and control HEK293 cells, plus normal and tumor patient tissue protein extracts.

This antibody gave minimal background and correct sub-cellular localization in the immunohistochemical staining. Western analysis and immunohistochemical staining may, however, modify the ADRB2 epitope differently. Thus, competition experiments with synthetic peptides representing the epitope, which has undergone the same chemical modifications as endogenous ADRB2, would have been a better control; however this was not available for our experiments.

In Vitro Models for Studying the β₂- Adrenergic Receptor in Prostate Cancer

The use of cell lines as models of neoplastic disease is a powerful tool to identify mechanisms of malignant behavior and progression of cancer cells. The use of immortalized human cancer cell line models permits standardization of experiment procedures, which aids in avoiding reporting of random observations. However, there are several limitations to this kind of *in vitro* models that needs to be taken into account when interpreting the results.

First, the use of an isolated cancer cell line prevents any influence from the tumor microenvironment, which is increasingly recognized as an important mediator of cancer development and progression [154;155]. And, importantly, tumors are often heterogeneous; different cell types within the tumor might communicate *in vivo*, and such cell-cell interactions are lost when cells are isolated and grown in culture. In addition, the influence on the tumor cells from the immune system and the nervous system are not easy to reproduce, and all is not known of the importance of interactions between the tumor and its surroundings.

Second, one of the hallmarks of cancer development is genetic instability. Cultivation of cancer cells in the laboratory may over time lead to changes in the baseline traits of the cell line. Indeed, a comparison between different batches of the MCF-7 breast cancer cell line cultivated at different laboratories showed that there were significant differences in both chromosomal alterations, growth rates, hormonal receptor status, tumorigenicity in mice, and treatment response between the four cell lines tested [156]. Third, many cell lines in use today are derived from cancer metastases and not the primary tumor. This makes the interpretation of findings and generalization to the clinical setting less straight forward.

Cross contamination between different cell lines used in the laboratory has also been reported [157], and constitutes a large threat to the validity of the results obtained with cell line studies. To address this issue, short tandem repeat (STR)-profiling [158] has been applied to authenticate the origin of all cell lines used in this thesis.

Cell Lines

The cell lines used in this work was LNCaP cells, LNCaP C4-2 cells, VCaP cells, RWPE-1 cells, and DU145 cells.

The LNCaP cell line has been most extensively used in this thesis. This cell line was isolated from a lymph metastasis (hence the name LNCaP; Lymph Node Cancer of the Prostate) in 1977 [159;160]. The LNCaP cell line is hormone sensitive, and responds readily to androgen deprivation. The cell line expresses a mutated version of AR [161]. The LNCaP C4-2 cell line is derived from the LNCaP cell line by growing LNCaP cells as xenografts for two rounds in castrated mice [162]. This cell line is not dependent on androgens for growth and survival like the LNCaP cells, and has metastatic potential [163]. The difference in gene expression profile between these two cell lines is found to correspond with changes observed in prostate cancer progression in vivo [164]. VCaP (Vertebral Cancer of the Prostate) cells are derived from a vertebral prostate cancer metastasis. The cells are hormone sensitive, and express wildtype AR [165]. DU145 cells are isolated from a brain metastasis of prostate cancer [166]. This cell line is known not to express PSA. DU145-cells are considered hormone insensitive, and have traditionally been believed to be an AR negative prostate cancer model; however, in a 2006 study by Alimirah *et al.*, they were able to detect AR protein levels in this cell line. Still, the protein levels were substantially lower than in the androgen responsive LNCaP-cells [167]. The RWPE-1 cell line is a papillomavirus 18- immortalized benign prostate cell line derived

from non-neoplastic prostate epithelial cells. The cells are hormone sensitive, and express AR [168].

Reporter Assay as a Measure of Androgen Receptor Activity

In a reporter assay, a vector containing a promoter region with response elements for the transcription factor of interest in front of the gene encoding the reporter (i.e. luciferase) is transfected into the cells. Manipulations can then be performed to examine the effects of different treatment conditions on the transcriptional activity. In paper III of this thesis, we have performed several reporter assays using the rat prostate promoter probasin, which contains androgen response elements (AREs) [169]. Binding of androgen leads to increased transcriptional activity of the AR, which binds to the promoter region of probasin and initiates transcription of the luciferase gene. One problem that may arise when performing reporter assays is that the promoter sequence in question may harbor response elements for other transcription factors than the one in question. In the context of studying effects of ADRB2 stimulation effects on AR activity, a specific issue was the potential harboring of CREs; response elements for the transcription factor cAMP response element binding protein (CREB). CREB is a transcription factor downstream of the classical ADRB2 signaling pathway. To assure that the increased level of luciferase expression after ADRB2 stimulation was not caused by CREB binding to regions on the probasin gene, we introduced point mutations in two known ARE's in the probasin promoter [169]. Both basal and isoproterenol induced luciferase expression were decreased by these point mutations, indicating that the effects observed are indeed mediated through the AR.

Generation of Stable ADRB2 Knockdown Cell lines

When studying the function of a protein in cell systems, manipulation of protein amount is a useful tool to investigate the role of the respective protein. This can be achieved by transfecting a vector expressing the protein of interest into the cells (over-expression), or by transfecting a RNA interference molecule into cells to silence translation or induce mRNA degradation of the mRNA in interest. This can be achieved by introducing a small interfering RNA sequence (siRNA) into the cells (transient transfection), or by introducing a plasmid vector containing a short hairpin- RNA (shRNA) into the genome of the cells (stable transfection). The vector carrying the shRNA usually also contains a resistance gene for an antibiotic, which allows for selection of successfully transfected cells.

There are some challenges when attempting to silence gene expression by RNA interference. First, there is a possibility of off-target effects, as RNAi molecules do not need to be completely complementary to the target mRNA to achieve silencing effects. Introduction of a foreign siRNA/shRNA molecule may also induce immune responses in the target cells [170], which may initiate unforeseen responses. In addition, the use of liposomal delivery systems may also initiate off-target effects [171]. Together, these limitations highlight the need to include control cells transfected with a non-targeting RNAi in all functional experiments.

The study of differentiation and morphologic changes can be difficult by use of transient transfection, as these traits may develop over time. In addition, the LNCaP cell line, which has been used in most of our experiments, can be hard to transfect; hence it can be difficult to achieve sufficient and reproducible knockdown results. Therefore, we established stable ADRB2 knockdown clones to study the effects of low levels of ADRB2. We also established a control cell line by transfecting LNCaP cells with a non-targeting shRNA.

To assure knockdown of ADRB2, the ADRB2 protein levels were quantified by radioactive ligand receptor binding assay, measuring the active receptor levels in membrane fractions from the different cell clones. Binding activity was normalized by the amount of total protein in the membrane fractions.

Discussion of Results

Use of β-blockers is Associated with a Reduced Risk of Prostate Cancer-specific Mortality

In this research project, we have sought to investigate the role of the ADRB2 in prostate cancer development and progression, while simultaneously looking into the possible effects of β-blocker treatment on the incidence and progression of prostate cancer in prostate cancer patient cohorts.

Among the 6515 men included in the Oslo II cohort, 6303 men were not diagnosed with prostate cancer at baseline in year 2000. No effects of β-blocker use on prostate cancer risk were observed. This finding is in accordance with several previous reports, in which no association between β-blocker use and prostate cancer risk has been observed [122;123;125-127], although two studies have reported a possible reduced risk [120;121].

Although no effect on prostate cancer-specific mortality was observed when analyzing all patients, use of β-blockers yielded a SHR of 0.14 (95 % CI 0.02-0.85) when we analyzed men who were reported to be planned for ADT to the Cancer Registry of Norway only. The choice to analyze this sub-group separately was based on a wish to analyze patients with aggressive disease. The routine reporting of prognostic parameters was not initiated at the Cancer Registry of Norway until 2004, meaning this information was missing for a large proportion of the prostate cancer patients in the Oslo II cohort. Planned ADT was therefore used as a surrogate marker for aggressive disease, as ADT is first line therapy for patients presenting with locally advanced or metastatic prostate cancer not eligible for curative treatment [43]. However, ADT is also commonly used as an adjuvant to RP or RT, of which we had no information, meaning that some patients in this ADT-subgroup might have undergone curative treatment. Furthermore, due to the limited amount of information on baseline clinical and prognostic factors, the survival analyses in this study were not adjusted for Gleason score, PSA, or clinical T-stage at diagnosis.

This study had several additional limitations, together causing limited ability to draw conclusions from our findings. Besides lacking information on planned curative treatment and baseline clinical characteristics, this study included a rather small patient material; only 35 patients reported the use of a β-blocker at baseline among a total of 263 prostate cancer patients planned to receive ADT. Additionally, there was no information regarding the

duration of β-blocker use besides the reported use for less or more than one year before the Oslo II survey. Furthermore, baseline information regarding use of prescription drugs in year 2000 was self-reported, hence this information may not have been correct for all patients. Finally, as the source of information on drug use was only collected at baseline, some non-users at baseline are likely to have initiated β-blocker use after the survey, and vice versa.

To address some of the limitations in paper I, we initiated a study where we had access to information from all men being reported to the Cancer Registry of Norway with a prostate cancer diagnosis between years 2004 and 2009. In 2004, the routine reporting and registration of clinical information such as Gleason score, PSA level, clinical T-stage, and metastasis were initiated at the Cancer Registry [141]. At the same time, the Norwegian Prescription Database was established [172], with routine and electronic reporting on all filled prescriptions from Norwegian pharmacies.

We received data from 24 571 men, of which we excluded 4529 due to coinciding date of diagnosis and death, emigration prior to diagnosis, or the initiation of β-blocker treatment after diagnosis. The latter exclusion criterion was applied to avoid positive survival bias.

As we only had data from 2004 and forward, we had limited follow-up to study survival effects for patients with localized prostate cancer, as these patients have a relatively long remaining life expectancy. Therefore, we only included patients with high risk disease according to the European Association of Urology (EAU) [9] or reported metastases at the time of diagnosis. In addition, we excluded all patients planned to receive treatment with radical prostatectomy, as we had no secure information as to whether these patients actually ever received the planned treatment or not. Patients reported to be planned for radiation were also excluded for the same reason, and also because we lacked information on the curative or palliative nature of the planned RT.

The reported information on clinical characteristics was not complete, and we received information on PSA-levels, Gleason score, clinical T-stage and performance status for 71.2, 68.4, 66.9 and 70.3 % of the 24 571 men, respectively. To be able to adjust for these clinical parameters in the survival analyses, we excluded all patients with missing information on one or more of these covariates. All survival analyses were hence controlled for age, PSA-level, Gleason score, clinical T-stage, performance status, presence and type of metastases (regional or distant) at diagnosis, as well as ADT initiated within the first six months after diagnosis. β-blocker use was associated with a 21% reduction in the SHR of prostate cancer-specific

mortality. All covariates included in the analysis were significantly associated with prostate cancer-specific mortality.

Because the information on planned RT and/or RP was from time of diagnosis, we had no information about later initiated RP or RT, which may have been offered to some patients who were not planned for curative treatment at diagnosis. To our knowledge, there have not been any studies investigating whether the use of active surveillance and the potential subsequent decision to curatively treat the patient are influenced by co-morbidities. Although we believe the chance of later initiated curative treatment to be low due to the inclusion of patients with high risk or metastatic disease only, we cannot exclude that there might be some bias in this respect that we have not been able to address.

We found that the observed effect of β-blockers was at least partly mediated by $β_1$-selective blockers, as use of $β_1$-selective blockers was independently associated with prostate cancer-specific mortality. A potential mediation of effects through $β_1$-selective blockers has also been observed in some breast cancer cohorts [129;173;174]; as 74 - 89% of the women studied in these cohorts were users of $β_1$-selective blockers. In a review of the epidemiological evidence for an effect of β-blockers in breast cancer by Ganz and Cole, the authors suggest that the effects observed may be caused by off-target $β_2$-affinity of the $β_1$-selective blockers atenolol and metoprolol [175]; as this have been shown in recombinant cell lines expressing human ADRB2's [176]. However, in a study by Barron *et al.* they observed no effects in patients treated with atenolol [128].

We performed a sub-analysis controlling for the use of acetylic salicylic acid (ASA) or a statin, as these drugs are used by many β-blocker users due to coinciding co-morbidities. In addition, use of both drug classes have each been found to be associated with prostate cancer-specific mortality [177-179]. Both ASA use and statin use were individually associated with reduced prostate cancer-specific mortality when analyzed separately in multivariate analyses. Adjusting for either of these drugs did not influence the estimated effect of β-blockers substantially; there were a <10% change in the SHR for β-blocker use, and p-values remained <0.05. Adjusting for both drug classes simultaneously increased the estimated SHR by 10%, and β-blocker use was no longer statistically significant (SHR 0.89, p-value 0.17). However, we believe that this is at least partly caused by a loss of statistical power, as this analysis included only 3000 patients (compared to the 3561 patients included in the original analysis). We observed a potential additive or synergistic effect of β-blockers and ASA, as the

combination of these two drug classes yielded a lower SHR than use of each of the drugs alone. However, there seemed to be no such association between β-blockers and statins, suggesting either a common biological mechanism or a common outside confounder. On this notion, regulation of AR activity and AR protein levels have been reported for β-adrenergic signaling [104] and for statins [180], respectively; suggesting altered AR activity as a potential common mechanism by which β-blockers and statins may act to influence prostate cancer progression.

Many β-blocker users are likely to have morbidities that fit with the definition of metabolic syndrome. The metabolic syndrome is a clustering of different conditions associated with increased risk of coronary heart disease, atherosclerosis, and diabetes mellitus type II; including hypertension, dyslipidemia, dysregulation of glucose homeostasis, insulin resistance, and abdominal obesity, depending on the definition [181]. Indeed, among β-blocker users in the Oslo II study we observed significantly increased BMI, waist-hip ratio and fasting blood sugar levels (appendix I; the latter two not shown in paper I). An association with prostate cancer mortality has been reported for several drug classes which are commonly used by patients with metabolic syndrome-associated conditions: Reduced prostate cancer progression or mortality have been observed for use of angiotensin-converting enzyme (ACE)-inhibitors [182], metformin and thiazolidinediones [183], statins [178;179], and ASA [177]. Interestingly, some of the underlying conditions included in the metabolic syndrome have also been associated with aggressive disease: high cholesterol levels [184-186], hypertension [187] [188], and obesity [188-190]. In addition, the presence of metabolic syndrome has been reported to be associated with aggressive prostate cancer [188;191;192]. Recently, a review article focused on the correlation between the metabolic syndrome and prostatic diseases concluded that it seems to be an association between metabolic syndrome and prostate cancer progression, however any causal relationship has yet to be proven [193].

When looking at disease aggressiveness in the cohort of all patients diagnosed with prostate cancer in Norway between years 2004 and 2009, we found a higher proportion presenting with aggressive disease among β-blocker users (data not shown in Paper II; appendix II). A possible explanation for this, could be that the group of β-blocker users are more likely to suffer from obesity (which were also observed in the Oslo II cohort), a condition that is suggested to reduce the chance of detecting early prostate cancer due to lower PSA levels and larger prostates in these patients [194], potentially making detection of cancer by needle biopsy more difficult. Hence, prostate cancer in obese patients may be diagnosed at a later

stage, allowing the disease to grow more aggressive before diagnosis. As with obesity, a larger proportion of β-blocker users compared to non-users reported having diabetes in the Oslo II study. Type-2 diabetes has been shown to be associated with a reduced risk of prostate cancer, an association that is suggested to be caused by lower PSA levels and potentially delayed diagnosis in these patients [195]. Together, these suggestions are in accordance with the observation in papers I and II that β-blocker users were significantly older at diagnosis than non-users, even though the age-adjusted risk of prostate cancer was not found to be reduced for β-blocker users in the Oslo II cohort.

There might have been a skewed distribution of patients with long life expectancy in favour of the β-blocker group in this study, as a relatively larger proportion of patients with assumed long life expectancy in the control group was excluded due to planned RP (see appendix II). This might simply reflect the increased aggressiveness observed among β-blocker users, however there is a possibility that β-blocker users are offered curative treatment less often due to co-morbidity. By including patients with high risk or metastatic disease only, we have sought to minimize this potential bias as the proportion of patients excluded due to planned curative surgery among high risk and metastatic patients was only 7.5% (versus 19.2% in the whole cohort).

In a previous study by Shah et al, increase in all-cause mortality was observed for prostate- and pancreatic cancer patients taking β-blockers [133]. In their study, patients with coronary heart disease, arrhythmias, stroke or heart failure before cancer diagnosis were excluded to avoid excess morbidities in this group. However, there was no information about morbidities presenting after cancer diagnosis, which may have caused bias due to an elevated risk of developing cardiovascular disease for hypertensive patients. In addition, the authors did not have access to prognostic factors or treatment plans for the patients. As β-blocker users hence may have presented with more aggressive disease, this study may have been biased by both higher other-cause mortality and higher prostate cancer aggressiveness for β-blocker users.

Together, the findings presented in Paper I and II support that there might be a beneficial effect of β-blockers on the progression of prostate cancer. However, as the studies performed were merely observational, we cannot advocate any causal mechanisms behind this finding. Because an association is reported for so many different drug classes used by the same patient group, a common outside confounder may be at least partly responsible for the results observed.

The Expression Level and Activation of ADRB2 in Prostate Cancer Cell Lines are Associated with CRPC Development

We observed an increase in the transcriptional activity of AR after stimulation of prostate cancer cell lines with the ADRB agonist isoproterenol, shown by increased luciferase expression from the ARE-containing promoter probasin. This effect was shown to be mediated through the AR both by siRNA knockdown of AR and by the addition of anti-androgens prior to stimulating with isoproterenol. In addition, the isoproterenol-induced transcriptional activity was reduced by introducing point-mutations in two well characterized ARE's in the probasin promoter [169]. The baseline luciferase expression was also reduced in cell lines with a stable knockdown of ADRB2, an effect which was contradicted by ADRB2 rescue experiments. Together, these results indicate that activation of the ADRB2 signaling pathway increases the transcriptional activity of the AR. This has also been shown previously [104], and proof that the transcriptional activity of AR can indeed be activated by the cAMP/PKA signaling pathway has been presented by means of experiments performed with an AR with mutation in the DNA-binding domain [196].

Interestingly, while the expression and activation of ADRB2 leads to increased AR transcriptional activity in prostate cancer cell lines, a high level of the ADRB2 may be protective against the development of CRPC. In a retrospective cohort of hormonally treated prostate cancer patients, low levels of ADRB2 were correlated with poor prognosis and shorter time to CRPC development. In this study, limited information in medical records caused a lack of ability to define CRPC according to the most recent recommendations [41], and hence surrogate criteria were used. Although not completely correct, we believe that our surrogate definition is sufficiently accurate to defend its use in this kind of pilot study.

In an LNCaP ADRB2 knockdown cell line, we observed a lower level of secreted PSA than in the control transfected LNCaP-cells. Furthermore, there was a significant correlation between the mRNA levels of ADRB2 and the androgen regulated differentiation marker NKX3.1 in RNA extracts from prostatectomy specimens. NKX3.1 protein levels were also lower in the ADRB2 knockdown clones when compared to control. Together, these findings suggest a lower degree of differentiation in cells with low levels of ADRB2 as a potential mechanism behind the observed association between ADRB2 expression level and development of CRPC.

We hypothesize that a lower activation level of AR could be the mechanism by which low ADRB2 levels might influence prostate cancer cell differentiation and progression to a castration resistant state. Indeed, low testosterone levels have been associated with higher Gleason score [197;198] and tumor grade [198;199] in patients with localized or newly diagnosed prostate cancer, and with poor prognosis in hormonally treated patients [200-204]. A proposed mechanism behind this observation is that the prostate cancer cells in these patients are less androgen dependent [203], but a non-causal mechanism has also been suggested [202]. Schatzl *et al.* showed that AR expression levels were higher in prostate cancer tissue from patients with low testosterone levels [197]. Together with reports of AR up-regulation as a potential mechanism behind the development of CRPC [205;206], this strengthens the suggested hypothesis that prostate cancer in patients with low testosterone levels are able to adopt more readily to castration. Similarly, as ADRB2 signaling has been shown to activate the AR, low levels of ADRB2 may contribute to lower androgen dependence and hence more rapid progression following ADT treatment.

The ADRB2 in Prostate Cancer: Friend or Foe?

Epidemiologic studies point towards a beneficial effect of ADRB blockade in cancer progression and survival. In addition, the ADRB2 signalling pathway have been implicated in metastasis development and cell proliferation in xenograft and cell line studies for several cancer forms [94;98-101]. Here, we report reduced prostate cancer specific-mortality among β-blocker users in two independent cohorts. However, we have also observed that high expression levels of the ADRB2 in prostate cancer are correlated with longer time to development of CRPC, and that high levels of ADRB2 might contribute to the maintenance of a differentiated phenotype of prostate cancer cells.

These individual findings might seem paradoxical at first glance. However, it has been suggested that while cell de-differentiation may be important for the cells' ability to detach from the primary site and migrate to distant organs, the reverse process might be necessary for the cells to be able to settle at the new site. This has been described in the context of epithelial-to-mesenchymal (EMT) plasticity; the reverse process of MET has been increasingly acknowledged as a possible key event in the formation of metastasis (reviewed in [207-209]. An E-cadherin to N-cadherin-switch that may reflect EMT has been observed , and was found to be correlated with poor prognosis in prostate cancer specimens [210]. Evidence of MET events in prostate cancer has been observed in lung metastases from prostate tumors in a rat pre-clinical model [211], and a higher proportion of cells expressing the epithelial

marker E-cadherin has been observed in prostate cancer bone metastases compared to primary tumor [212]. A potential role of ADRB2 in EMT/MET plasticity is supported by findings of Yu *et al.*, who showed that knockdown of the ADRB2 in a benign prostate cancer cell-line induced a malignant and migratory phenotype with mesenchymal properties [88]. Interestingly, up-regulation of ADRB2 in metastatic prostate cancer have previously been reported by our group [103].

In this respect, the herein observed association of ADRB2 levels in prostate with CRPC development might be mediated by an involvement in keeping the cells in a differentiated state, and thereby preventing migratory events and castration resistant growth. Suppression of ADRB2 signalling by use of β-blockers may have beneficial effects via prevention of the re-differentiation process which might be necessary for circulating tumour cells to establish at the metastatic site (Figure 6).
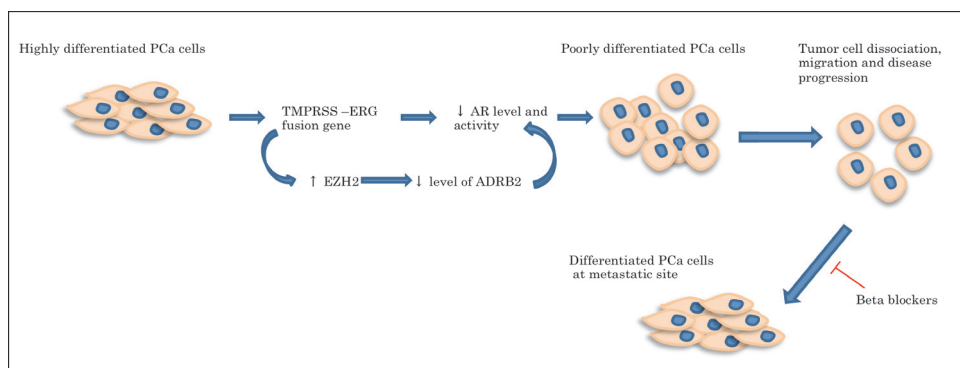

Figure 6. A hypothetical model of the role of ADRB2 and β-blockers in prostate cancer progression and metastasis. ADRB2 is suggested as a marker of differentiated prostate cancer cells and a regulator of AR activity. ERG over-expression has been shown to induce EZH2 expression and to suppress AR activity. ADRB2 is a target gene of EZH2, and high expression of EZH2 may correlate to low level of ADRB2. Down-regulation of ADRB2 levels promotes decreased AR activity and de-differentiation of the prostate cancer cells. These cells have increased migratory and invasive potential. The dissociated cancer cells migrate to metastatic sites, and may re-differentiate to form metastases. This latter process is inhibited by β-blockers.

There might be other explanatory-models for our combined results. The observed effect of β-blockers may have been caused by inhibition of some of the systemic processes suggested to be induced by the adrenergic mediated stress response, and be independent of ADRB2 expression on tumor cells. Indeed, in the study by Campbell *et al.*, they identified that

adrenergic stimulation of the host bone marrow stromal cells was the main mediator of adrenergic stimulation of metastasis to bone [99]. Furthermore, Sloan *et al.* found that the pro-metastatic effect of adrenergic signalling could be attributed to increased macrophage infiltration into the primary tumour after adrenergic stimulation [100]. Under this hypothesis, the reduced levels of ADRB2 seen in patients with poor prognosis could merely be a consequence of agonist-induced ADRB2 desensitization, which has been previously described [213;214]. However, involvement of ADRB2 expressed by the tumour cells themselves was observed by Thaker *et al.*, who showed by use of a ovarian cancer xenograft model that stimulation of ADRB2 on tumour cells increased the expression of vasculature endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) 2 and 9, as well as stimulating tumor growth and angiogenesis [101]. ADRB2 induced expression of VEGF and interleukins 8 and 6 (IL-6 and IL-8) have also been shown in melanoma cell lines [215].

Conclusions

In this study, we have shown that

- The use of β-blockers was not associated with prostate cancer risk among the 6303 men in the Oslo II cohort who did not have prostate cancer at baseline.
- The use of β-blockers was associated with prostate cancer specific mortality in two independent cohorts, including: i) 263 men who were planned to be treated with ADT, and ii) 3561 men with high risk or metastatic disease. All-cause mortality was not significantly affected by β-blocker use in either cohort.
- The observed association between β-blocker use and prostate cancer-specific mortality seemed to be independent of tumor- and clinical characteristics at diagnosis.
- The expression level of ADRB2 influence the transcriptional activity of AR, as measured by luciferase reporter assay, PSA secretion, and NKX3.1 protein expression level.
- The expression level of ADRB2 was associated with time to CRPC development. Prostate cancer-specific and all-cause mortality was not significantly associated with ADRB2 expression.

Future Perspectives

Regarding the ADRB2 in prostate cancer, much is still unknown regarding function and the contribution to cancer development and progression. Additional work is needed to determine whether ADRB2 may simply be a marker for dedifferentiation, or if it is an actual causal contributor to prostate cancer carcinogenesis. Looking forward, we would like to further investigate the effects of ADRB2 knockdown in prostate cancer cell lines, presumably by adding more knockdown cell lines to our established LNCaP ADRB2 knockdown lines. We will then focus on the effects of ADRB2 knockdown on functional properties of the cells, such as migration and invasion. Furthermore, it would be interesting to look into expression of additional differentiation markers and potential EMT markers. It would also be interesting to perform animal experiments with ADRB2 knockdown xenografts to assess whether ADRB2 expression levels have any independent impact on prostate tumor growth, CRPC development and metastasis.

Although we have shown an association between β-blocker use and survival in two independent prostate cancer patient cohorts, further work is needed to establish a potential causal relationship between β-blocker use and prostate cancer-specific mortality. More epidemiologic studies are needed to further investigate this potential association, to determine possible sub-group effects and potential dose-response relationships. Further studies may also determine if β-blockers may be associated with prostate cancer mortality also in patients with low risk disease. However, only a randomized controlled trial is suited to finally establish an actual causal relationship between β-blocker use and prostate cancer-specific mortality, as all potential confounders are not known. Meanwhile, we would like to test β-blockers in animal xenograft models, to investigate if there is any direct effect of β-blockers also on non-stress induced metastases or progression to CRPC. This study could ideally be performed in combination with the planned ADRB2 knockdown study, to examine whether a potential effect of β-blockers is dependent on the expression level of ADRB2 in prostate cancer cells or not.

If eventually proven to have a direct effect on prostate cancer mortality, β-blockers have the potential to be used as a cheap and well tolerated supplement to current treatment strategies for prostate cancer.

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Papers I-III

I

II