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Translational perspectives on MSMB and CRISP3 expression and regulation in prostate cancer

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Abstract

Prostate cancer is currently the most common form of cancer in Sweden. Currently, the only biomarker used in the clinic is serum PSA, and there is a great need for new biomarkers that may increase the diagnostic and prognostic information so that better predictions can be made, and treatment may be tailored. Here, we have investigated two proposed biomarkers microseminoprotein-β (MSMB) and cysteine-rich secretory protein-3 (CRISP3).

Firstly, we wanted to validate previous findings, that MSMB and CRISP3 are predictors of recurrence in patients undergoing radical prostatectomy for localized prostate cancer. Using a novel automated image analysis tool, *IHC-MARK*, we found that MSMB was an independent predictor of recurrence in a large patient cohort. Further, we showed that expression of both MSMB and CRISP3 was induced by androgen *in vitro*, and MSMB was decreased in patients receiving androgen deprivation therapy prior to radical prostatectomy. MSMB was virtually lost in advanced prostate cancer, in contrast to CRISP3 which was highly expressed. Inflammation has been suggested to be a primary aetiological event in prostate cancer and the presence of putative binding elements for inflammatory transcription factors in the promoter region of CRISP3 led us to hypothesise that CRISP3 may be regulated by inflammatory stimuli. Instead, stimulation of prostate cancer cells with interleukin (IL)-6 strongly induced *MSMB* expression but had no effect on *CRISP3* expression. A long-term IL-6 stimulated cell line, however, had no MSMB expression, probably due to DNA methylation. Finally, expression of MSMB in cell lines without endogenous MSMB resulted in decreased cyclin D1 expression and reduced proliferation.

In conclusion, MSMB is an independent predictor of recurrence, whereas the value of CRISP3 as a biomarker remains to be elucidated. *In vitro* studies show that MSMB may be important for prostate cancer proliferation, but more studies on MSMB and CRISP3 functions are warranted.

Key words: Prostate cancer, tissue biomarkers, tissue microarrays, androgens, IL-6, MSMB, PSP94, CRISP3

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List of Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Evaluation of the prognostic significance of MSMB and CRISP3 in prostate cancer using automated image analysis ***Anna Dahlman****, Elton Rexhepaj, Donal J Brennan, William M Gallagher, Alexander Gaber, Anna Lindgren, Karin Jirström and Anders Bjartell* Modern Pathology. January 2011, (e-pub).

II. Effect of androgen deprivation therapy on the expression of prostate cancer biomarkers MSMB and MSMB-binding protein CRISP3 ***Anna Dahlman****, Anders Edsjö, Christer Halldén , Jenny Liao Persson, Samson W Fine, Hans Lilja, William Gerald and Anders Bjartell* Prostate Cancer and Prostatic Diseases 2010 Dec;13(4):369-75.

III. Inflammatory Stimuli and Androgen Availability Determine the Expression of Prostate Cancer Biomarkers CRISP3 and MSMB ***Anna Dahlman****, Jörgen Olsen, Kristian Riesbeck, Anders Edsjö and Anders Bjartell* Manuscript.

IV. Prostate Cancer Cell Proliferation is abated by MSMB Expression ***Anna Dahlman*** *and Anders Bjartell* Manuscript.

No permissions were required for reprints of paper I and II.

Papers not included in this thesis

Galiellalactone is a novel therapeutic candidate against hormone-refractory prostate cancer expressing activated Stat3 *Rebecka Hellsten, Martin Johansson,* ***Anna Dahlman****, Nishtman Dizeyi, Olov Sterner and Anders Bjartell* Prostate. 2008 Feb 15;68(3):269-80

LRIG1 and the liar paradox in prostate cancer: A study of the expression and clinical significance of LRIG1 in prostate cancer *Marcus Thomasson, Baofeng Wang, Peter Hammarsten,* ***Anna Dahlman****, Jenny Liao Persson, Andreas Josefsson, Pär Stattin, Roger Henriksson, Anders Bergh and Håkan Hedman* International Journal of Cancer, December 2010 (e-pub)

Validation of CRISP-3 and β-MSP prognostic value on prostate cancer needle-biopsies in men treated by prostatectomy *Marije Hoogland,* ***Anna Dahlman****, Kees J. Vissers, Tineke Wolters, Fritz H. Schröder, Monique J. Roobol, Anders S. Bjartell and Geert J.L.H van Leenders* BJU International, in press.

Abbreviations

ADT androgen deprivation therapy
ALDH aldehyde dehydrogenase
AR androgen receptor
ARE androgen response element
BPH benign prostatic hyperplasia
CAP cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1
CI confidence index
CRISP3 cysteine-rich secretory protein-3
CRPC castration-resistant prostate cancer
CRT classification regression tree
CSC cancer stem cell
DHT dehydrotestosterone
ECM extracellular matrix
EMT epithelial to mesenchymal transistion
EZH2 enhancer of zeste homologue-2
GnRH Gonadotropin-releasing hormone
HR hazard ratio
Hsp heat-shock protein
IHC immunohistochemistry
IL-6 interleukin-6
JAK Janus kinase
KLK3 kallikrein-3
LUTS lower urinary tract symptom
MSMB microseminoprotein-β
NF-kappaB nuclar factor kappa beta
NMR nuclear magnetic resonance
PAP prostatic acidic phosphatase
PCR polymerase chain reaction
PIA proliferative inflammatory atrophy

PIN prostate intraepithelial neoplasia
PSA prostate specific antigen
ROS reactive oxygen species
SHBG sex-hormone binding-globulin
SNP single nucleotide polymorphism
STAT signal transducer and activator of transcription
TMA tissue microarray
TNM tumour, lymph node, metastasis
XMRV xenotropic murine leukemia virus-related virus

The Normal and Malignant Prostate

The prostate gland

The prostate is an exocrine walnut-shaped organ surrounding the urethra. Like other tissues of the male genito-urinary tract, the prostate depends on testosterone for growth and development, and rapidly increases in size at the onset of testosterone production during puberty. After puberty, the size of the prostate remains constant, but further benign enlargement may occur after 50 years of age, a process referred to as benign prostatic hyperplasia (BPH) [1, 2].

The function of the prostate is to produce and secrete a milky acidic fluid that contains several substances important for fertilization. The prostatic secretions constitute approximately 25% of semen, and contribute to sperm motility and viability. Prostatic fluid contains proteolytic enzymes which functions to break down the clotting proteins from the seminal vesicles [3]. By far, the most abundant proteins found in prostate secretions are prostate specific antigen (PSA), prostate acidic phosphatase (PAP) and microseminoprotein-β (MSMB) [4].

The prostate can be divided into three distinct zones: the peripheral, central and transitional zones (Fig. 1). This segmentation appears to be important, since the zones exhibit a considerable variation in their tendencies for prostatic conditions. For instance, the transitional zone constitutes approximately 70% of the prostate, and most BPH lesions occur there. Most tumours arise in the much smaller peripheral zone [2, 5, 6].

Histologically, prostatic tissue is made up of epithelial cells and surrounding stroma. The epithelial cells form glands with luminal secretory cells forming the glandular lumen, and basal cells in a single layer underneath. The luminal cells express androgen receptor and secrete components of the prostatic fluid such as PSA, PAP and MSMB [2, 7]. Basal cells secrete components of the basal membrane, but their functions remain somewhat abstruse. It is generally believed that this cellular compartment also harbours stem cells or progenitor cells that may differentiate and re-populate the luminal cell layer if needed [8-10]. Basal cells express low levels of androgen receptor but are not dependent on androgens for survival and growth [11]. Least common of the epithelial

cells are the neuroendocrine cells, which can be found scattered among both luminal and basal cells.

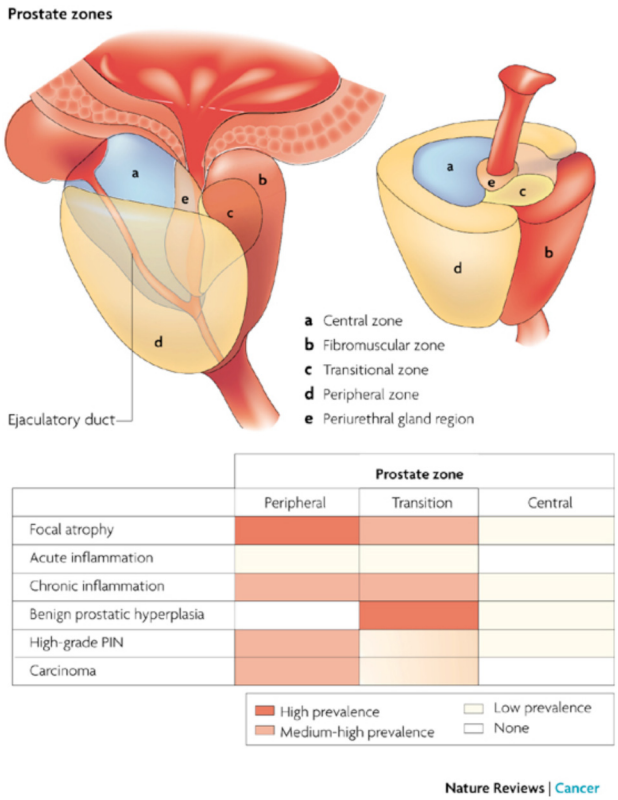

FIGURE 1. Zonal predisposition to prostate disease. The prostate consists of different zones, with varying predispositions to prostatic conditions. BPH is more common in the transitional zone, whereas prostatic intraepithelial neoplasia (PIN) and cancer are more common in the peripheral zone. Acute and chronic inflammation is equally common in peripheral and transitional zones. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Apr;7(4):256-69, ©2007.

Surrounding the epithelial glands is stroma, composed of fibroblasts and smooth muscle cells. Stromal cells support the epithelial cells with paracrine factors in a process called epithelial-stromal crosstalk, and they produce components of the extracellular matrix (ECM) [12]. Together, these cell populations constitute the prostate microenvironment. Several studies show that microenvironment is essential for cellular behaviour in the normal prostate and during tumour progression [13, 14].

Androgen signalling in the prostate

Androgens are required for development and maturation of the prostate, and for proliferation and survival of prostate epithelial cells [15]. Androgens include testosterone,

produced by the testes; dehydroepiandrosterone, made by the adrenal glands; and dihydrotestosterone (DHT), which is converted from testosterone within the prostate. Testosterone is the main circulating androgen, the majority bound to albumin or sex-hormone-binding-globulin (SHBG). A small fraction remains in free form, which may enter the prostate epithelial cell where it is converted to DHT by 5α-reductase (Fig. 2). Dihydrotestosterone is a more potent ligand for the androgen receptor, having a 5-fold higher affinity than testosterone [16, 17].

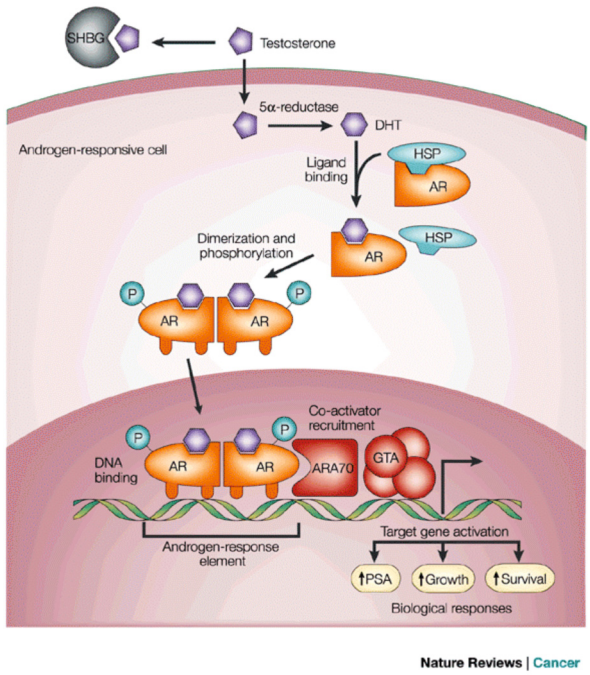

FIGURE 2. Androgen action in the prostate epithelial cell. Free testosterone enters the cell and is converted to DHT by 5α-reductase. DHT readily binds to the cytosolic androgen receptor, causing conformational changes resulting in homodimerization and entry into the nucleus. In the nucleus the androgen receptor can bind to specific DNA sequences, recruit co-factors, and induce transcription. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Oct;1(1):34-45, ©2001.

The androgen receptor is a member of the nuclear steroid-receptor family, and may act as a transcription factor upon activation. In absence of ligands the androgen receptor is kept inactive in a protein complex comprised of heat-shock proteins (Hsp), thus preventing DNA binding [18]. When DHT is available in the cell, the androgen receptor will bind to it, inducing a conformational change leading to dissociation from the Hsp-complex, receptor phosphorylation and ultimately receptor homodimerization. In the dimerized form, the receptors are able to bind androgen response elements (AREs), specific regions of DNA in the promoter region of androgen regulated genes (Fig. 2). The activated and DNA-bound androgen receptor complex then recruits co-activators, and initiates transcription [19].

Benign prostatic disorders

Benign prostatic hyperplasia

Also known as nodular hyperplasia or glandular and stromal hyperplasia, BPH is an extremely common condition in men. It is present in a significant number of men at the age of 40 years, and in a majority of men after 50 years of age [20]. Benign enlargement of the prostate involves active proliferation of both the epithelium and stroma [2]. Because the prostate surrounds the urethra, any enlargement of the gland, whether due to benign prostatic hyperplasia, acute or chronic inflammation, or a tumour, may block urine flow and cause lower urinary tract symptoms (LUTS).

Prostatic inflammation

Prostatitis, inflammation in the prostate, may be acute or chronic, and is common in males after puberty. In middle aged or older men, chronic prostatitis is the most common chronic infection in the male body [21].

Acute infection is normally caused by *Escherichia coli* (*E. coli*), the same bacteria associated with other urinary tract infections. Chronic prostatitis may follow acute prostatitis, or develop insidiously without previous episodes of acute inflammation. Chronic prostatitis is frequently present without visible evidence of bacteria, thus non-bacterial agents are believed to cause the condition. Non-bacterial agents include dietary components such as red or charred meat, harmful chemicals such as reactive oxygen species (ROS); or viral infection [21-23]. Recently, the implication of a prostate cancer-associated virus, xenotropic murine leukemia virus-related virus (XMRV) has gained serious attention as a causing factor [24], but this finding has been severely criticised for lack of experimental contamination controls [25].

There is an established connection between inflammation and cancer, and inflammation may be a primary aetiological agent for prostate cancer [6, 26, 27]. Invading neutrophils, lymphocytes, and macrophages will eradicate pathogens from the tissue by creating a harmful environment. In the process they will secrete harmful factors such as ROS, potentially leading to DNA damage in epithelial cells, and subsequent apoptosis; peptidases to break down the ECM facilitating immune cell invasion; and stimulatory factors such as cytokines and growth factors leading to proliferation and potentially dedifferentiation in the epithelial compartment. The harmful environment in combination with increased proliferation may promote genetic instability leading to increased mutation rate [28-32].

This state of proliferation and tissue destruction has been termed proliferative inflammatory atrophy (PIA) by De Marzo and colleagues (Fig 3) [33]. PIA is a likely precursor

to prostate intraepithelial neoplasia (PIN), which in turn is the most likely precursor to prostate cancer (Fig. 3) [6, 7, 34].

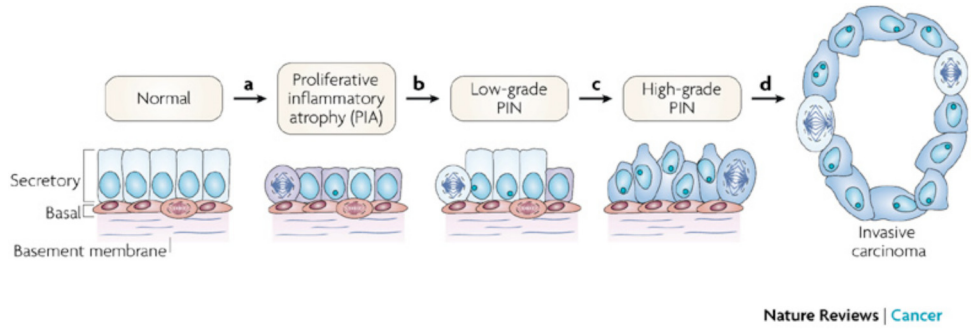

FIGURE 3. Model of the early cellular neoplastic progression. Inflammatory cells infiltrate the tissue, secreting factors that may cause DNA damage, cellular atrophy and proliferation. Down-regulation of tumour suppressor genes stimulates cell cycle progression (A). Subsequent silencing of more tumour suppressor- and stress response genes (such as glutathione-S-transferase P1 (*GSTP1)*) allows genomic instability (B-C). Genetically instable cells continue to proliferate (D). Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, Apr;7(4):256-69, ©2007.

Clinicopathological as well as experimental evidence links PIN to development of cancer [35-37]. Elkahwaji and colleagues showed that chronic inflammation resulted in dysplastic tissue areas mimicking PIN in a mouse model of prostate inflammation. The dysplastic tissue had increased proliferation and oxidative DNA damage, as well as decreased expression of androgen receptor and *GSTP1* [37].

Prostate cancer

Introduction to prostate cancer

Prostatic disorders, especially prostate cancer, draw large attention from the cancer research community. According to the Swedish National Board of Health and Welfare, prostate cancer has become the most common form of cancer in Sweden with 10317 Swedish men receiving the diagnosis in 2009 [38]. The corresponding life-time risk of approximately 18% is seen throughout the Western world [39]. Prostate cancer is predominantly a disease of the aging male, with a vast majority of diagnoses occurring in men over 60 years of age [40]. The number of prostate cancer diagnoses has increased during the last decades due to longer life span, but also due to the introduction of the PSA test in the clinic [41].

PSA tests have lead not only to increasing number of tumours being detected, but also many tumours are detected at early stages. Since prostate cancer is generally a latent

disease, most of these tumours will not develop into clinically relevant disease within the lifetime of the male [42, 43].

Importantly, PSA is not a good marker to predict aggressiveness of the prostate tumour, and it may be difficult to foretell which tumours will develop into clinically relevant, or remain indolent. Currently, many indolent cancers are diagnosed, causing anxiety and distress for the patient and possibly to (unnecessary) therapeutic interventions that may have severe side-effects. On the other hand, aggressive tumours are detected in an early stage, when they may still be manageable.

Diagnosis and treatment

Screening for prostate cancer

In most cases, primary prostate cancer does not present symptoms, and the cancer is detected by routine blood tests where elevated PSA levels may be detected and be indicative of cancer. There is an ongoing debate on whether PSA-based screening for prostate cancer is beneficial or not [44-46]. The PSA-test has been criticised for limited diagnostic specificity and predictive value, and the relationship between PSA and cancer risk remains subject to fundamental disagreements [47-49].

Traditionally, a PSA serum value of 3-4 ng/mL has been considered the upper limit of what is considered normal concentration in serum, but this is highly dependent on patient age and prostate volume. The risk for overdiagnosis is significant, and typically over 1400 men have to undergo screening, and 48 patients undergo treatment, in order to save one man from prostate cancer death [46]. The predictive value of PSA-tests must be enhanced before this method may be considered for population screening [50, 51].

On the other hand, population groups with increased risk for prostate cancer may benefit from screening. The most well established risk-factors include age, African ancestry, and family history [52]. As previously mentioned, prostate cancer is a disease of the aging male, and the risk for developing cancer increases with age. Furthermore, epidemiological studies show that African-American males have 2.5-fold higher risk for developing prostate cancer compared to the average Caucasian male, and twice as likely to develop fatal disease [39, 53, 54].

It is known that familial prostate cancer is associated with increased risk, hence hereditary factors does confer increased risk for prostate cancer. A study performed on Scandinavian twins showed that 42% of the risk could be attributable to familial risk [55]. In addition, specific small nucleotide polymorphisms (SNPs) in the genome may confer increased risk for prostate cancer [56, 57], and when combining familial risk

with specific SNPs, groups of individuals with 2- to 3-fold risk were identified [58, 59]. These high risk groups may benefit from PSA-based screening.

Furthermore, PSA may be a significant predictor of future prostate cancer development. A single PSA test taken before the age of 50 years could identify men at risk for developing prostate cancer 20 to 30 years later. Again, this could help identify men at high risk for prostate cancer, and would benefit from more frequent screening [60].

Staging and grading

Prostatic tumours are discovered by PSA-tests and digital rectal examination (DRE), but ultrasonography-guided biopsies collecting tissue for histological examination is required to verify the diagnosis. Classification of the tumour is essential to determine whether immediate or deferred treatment is the best course of action. The most common clinical classification system is the TNM system. The TNM (tumour, lymph node, and metastasis) classification system takes into account tumour volume, number of lymph nodes involved, and whether there are distant metastatic lesions present [61].

According to the TNM system, T1 and T2 stage tumours are still confined to the prostate. For localised prostate cancer, treatment methods such as surgery or radiation therapy may cure the cancer, or active surveillance may be an initial option. In Sweden, the majority of prostatic tumours are localised to the prostate at the time of diagnosis [40]. In stage T3 and T4, the tumours are locally advanced, and may have spread to organs outside the prostate [61].

Histological examination of the tissue derived from the biopsies will generate further information about the tumour grade. To grade tumours, the Gleason system is used, classifying tumours from 2-5 where 5 is the most malignant grade [62]. Prostate cancer being a very heterogeneous and multifocal tumour, normally the two most extensive tumour areas are graded, and summed in a Gleason score [63].

Treatment methods

Applying the clinical and pathological parameters to prediction models such as treatment nomograms, clinicians may select the most beneficial treatment method [64, 65]. A patient with a tumour that is likely to remain indolent may benefit from deferred treatment, during which PSA levels are monitored applying a protocol for active surveillance. If disease progression is detected, radical treatment is initiated. Most prostate cancers detected at an early stage will not pose a threat of progression within 15-20 years and therefore, active surveillance may be a suitable initial treatment option [66, 67]. Localised prostate cancer can be cured by radical prostatectomy or by radiation

therapy. However, surgery always poses a risk for the patient and adverse events include incontinence and erectile dysfunction.

For advanced prostate cancer, either recurring after surgery or radiation therapy, or when the disease has spread before the patient is diagnosed, there are no available cures. There are, however, treatments that will slow disease progression, and the mainstay therapy is androgen deprivation therapy (ADT). Primary prostate cancer cells are dependent on androgen for proliferation and survival, and depleting androgen levels will initially cause cell death, decreased proliferation, and tumour reduction [15, 68]. Androgen production can be regulated at different levels (Fig. 4). Unfortunately, the loss of testosterone confers significant side-effects in nearly all men [69].

The most common ADT drug target is the gonadotropin-releasing hormone (GnRH) receptor, for which both agonists and antagonists may be used for inhibition. Antagonising the GnRH receptor act via the pituitary to block testosterone production in the testes, and efficiently reduces circulating testosterone levels by 95% [70, 71]. However, steroid synthesis in the adrenal glands remains unaffected, and these steroids may be converted into DHT in the prostate. Therefore, despite inhibited testosterone production, DHT levels in the prostate may remain virtually unchanged [72, 73].

Therefore, treatment directed towards GnRH is frequently used in combination with antiandrogen treatment. Antiandrogens are antagonists that bind competitively to the androgen receptor to keep it in an inactive state [74]. Another therapeutic target is 5α-reductase, inhibitors of which inhibit the conversion of testosterone to DHT. Inhibition of 5α-reductase is an effective treatment for BPH, and their therapeutic value in prostate cancer prevention is being evaluated [75, 76].

Eventually, prostate cancer cells develop ways to escape the androgen blockade, and the tumour will progress again. This stage of advanced disease is referred to as castration-resistant prostate cancer (CRPC). At this stage the cancer frequently progress rapidly with metastatic lesions to bone. Unfortunately, most patients receiving ADT progress to CRPC within a median of 2 years [77, 78].

Chemotherapy is used for second-line therapy in patients with CRPC. For prostate cancer, chemotherapy is directed against classic targets such as cell division or DNA replication, and may be combined with anti-inflammatory drugs.

New therapies with better efficiency for depleting androgen production, or inhibiting the androgen receptor are in clinical trials [79]. Other components of the androgen signalling pathway are potential therapeutic targets, such as the Hsp-proteins, especially HSP90 [80].

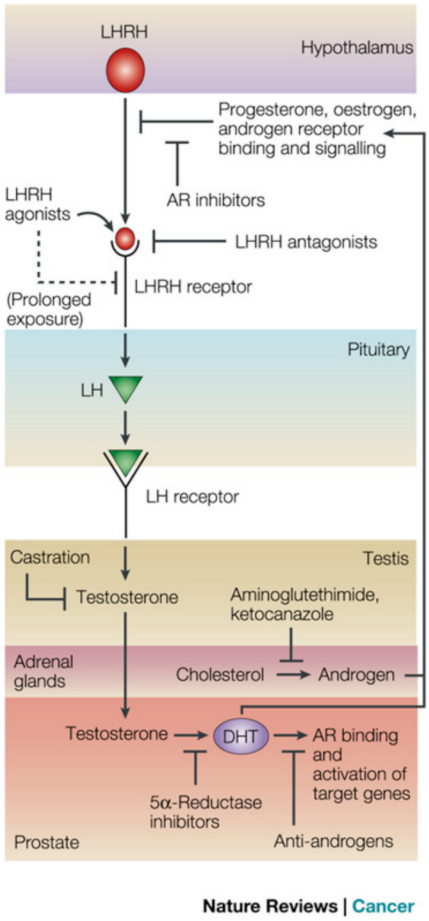

FIGURE 4 Drug targets for regulation of androgen production. Hypothalamic production of leutenizing hormone (LH) releasing hormone (also known as GnRH), increases production of LH from the pituitary. LH, in turn, acts on the testes to induce testosterone production. Testosterone is converted to DHT by 5α-reductase in the prostate epithelial cell. Androgen production may be pharmacologically inhibited in each of these steps. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer, May;2(5):389-96, ©2002.

Molecular mechanisms of androgen receptor dysregulation

Castration-resistant prostate cancer is characterized by tumour cell growth independently of androgens. Even though the androgen blockade therapy is no longer efficient, there is evidence that the androgen receptor is still activated in prostate cancer cells [81]. There are several ways by which the tumour cells may circumvent the androgen blockade. The androgen receptor may become hypersensitive to DHT, it may become activated by other ligands than DHT, or it may become activated in the absence of a ligand. Furthermore, androgen signalling pathway may be completely by-passed, or the tumour cells may begin to express enzymes enabling *de novo* synthesis of intratumoural androgens invoking an autocrine or paracrine mechanism for CRPC [72, 73, 82, 83]. It has been suggested that tumour cells with an androgen independent phenotype may be an early event in tumour progression, and that they are promoted by the selective pressure of androgen blockade [84]. Or, most thought provokingly, the cancer cells may be derived from a progenitor cell that was never androgen dependent (discussed in the next section) [85].

Hypersensitive androgen receptors enable androgen receptor signalling even at extremely low levels of DHT but are, strictly speaking, still dependent on androgens for activation. The hypersensitive pathway is made possible by *AR* gene amplification, mutations conferring increased androgen sensitivity, or increased levels of DHT in the tumour [82, 86].

Furthermore, although prostate cancer is a cancer of epithelial cells, it has been shown that tumour associated stroma is distinct from healthy stroma. The definition carcinoma-associated fibroblasts (CAFs) has been suggested to separate fibroblasts from normal stroma. CAFs may support the tumour by remodelling the ECM, thus enabling or contributing to angiogenesis and invasion, or by secretion of growth factors that act on the epithelial cells in a paracrine manner [87, 88].

Origin of prostate cancer

Prostate cancer stem cells

Cancer cells in tumours of the prostate are heterogeneous with regards to histology and response to therapies. This has generated the hypothesis that tumour cells are derived from multipotent stem cells, which would have the ability to give rise to such diverse progeny. This is referred to as the cancer stem cell (CSC) model, and is currently favoured among cancer researchers as the most probable initiation of prostate cancer [89-97]. The origin of such CSC could be by malignant transformation of normal tissue stem cells, believed to be present in most adult organs, or by transformation of differentiated cells to a more stem-like state, so called transiently amplifying cells [98, 99]. Differentiated cells may become more stem cell like by epithelial to mesenchymal transition (EMT) [100]. In connection, there is an ongoing debate regarding the properties and location of these CSC. Currently, the CSC phenotype is proposed to $CD133^{+}/\alpha2\beta1\text{-integrin}^{high}/CD44^{+}$ [89], although particularly the use of CD133 has been debated [101]. Recently, expression of aldehyde dehydrogenase (ALDH) was suggested to be an independent marker for CSC in prostate cancer [102].

Androgen receptor negative CSCs may be able to repopulate the tumour with both androgen dependent and androgen independent progeny, providing an explanation to the varying degree of sensitivity to ADT within the same tumour [103-105]. However, androgen receptor status in CSC is also debated [85, 106]. Importantly, the CSCs appear to be resistant to conventional cancer therapy and may therefore be involved in prostate cancer progression, and cause relapse and metastatatic disease [94, 107].

Inflammation as aetiology for prostate cancer

As previously discussed, there is an established connection between inflammation and cancer, and inflammation may be a primary aetiological agent for prostate cancer [6, 26, 27]. PIN lesions may develop in areas of inflammation, and PINs are in fact more closely related to carcinoma than to benign epithelium. These shared features include specific genomic alterations, phenotype, morphology, disrupted or lost basal cell layer, and increased rate of angiogenesis and proliferation [33, 35, 108, 109]. Fusion genes such as TMPRSS:ETS occur in PIN and in a majority of prostate cancers. It has been suggested that TMPRSS:ETS is involved in prostate cancer development and progression, and this genomic rearrangement may be an early event in the oncogenic process [110, 111]. Furthermore, the *GSTP1* gene, expression of which is frequently lost in prostate cancer, is also lost in a majority of PIN [112]. Finally, PIN and cancer both occur more frequently in the peripheral zone [6]; in older men; and in males of African ancestry [113].

As mentioned, inflammatory cells may cause DNA damage and genomic instability, and in combination with increased proliferation, this condition could be tumourogenic. Inflammation may also cause de-differentiation in epithelial cells by inducing EMT [114]. Stem cells from primary prostate cancer has a pronounced inflammatory phenotype compared to stem cells from benign tissue, including active cytokine signalling through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway [115].

Prostate Cancer Biomarkers

Introduction to prostate cancer biomarkers

A biomarker can be defined as a molecular test that provides information in addition to clinical data. The test could include detection of specific proteins or mRNA in blood, tissue, or urine; modifications of proteins, such as phosphorylation; or genomic modifications such as gene amplification, deletion, or fusion genes. The optimal biomarker has high disease specificity and high sensitivity.

In prostate cancer, there is a need for biomarkers for several reasons: to improve cancer detection and staging; to identify subclasses of prostate cancer; to predict outcome after treatment; and to select patients for different treatment strategies.

Furthermore, as we are moving towards a future where personalized medicine may converge with traditional risk prediction, there is a need to develop new strategies to assess risk and to accurately stratify patients into risk groups. Cancer research is increasingly focused on personalised medicine and methods to characterize the tumour cell phenotype in the individual patient are under development. For example, the MAMMAPRINT gene profiling test was recently approved for clinical use to aid diagnosis of breast cancer in the USA [116]. Potentially, this would comprise a systems biology approach, where genetic and proteomic profiling is assessed, and treatment is tailored.

A large number of tumour markers with prognostic information have been proposed (reviewed in [117-120]), but the incorporation of such markers into clinical practice has been largely unsuccessful [121]. Limiting factors include tissue availability, since diagnostic biopsy cores are all that is available for those patients that receive radiotherapy, ADT or active surveillance. Radical prostatectomy is the only treatment method generating plentiful tissue. Lack of standardised methods to perform and interpret immunohistochemistry, and tissue quality may also affect study result. In addition, a biomarker must be evaluated in the clinical context in order to fully asses the prognostic value. Specifically, a proposed marker must be included into current prediction models, and increase the current specificity and/or sensitivity [122].

It is beyond the scope of this thesis to review all proposed biomarkers for prostate cancer. Instead, the focus here will be on the proteins of interest in this thesis: PSA, androgen receptor, MSMB and cysteine-rich secretory protein-3 (CRISP3).

Prostate specific antigen

The human tissue kallikrein (*KLK*) gene locus consists of 15 genes on chromosome 19q13.4 [123]. *KLK3* is the best known of these genes, encoding the PSA protein. PSA is expressed in benign prostatic epithelial cells, BPH and in prostate cancer of all grades and stages [124, 125]. The function of PSA in the healthy male is believed to be liquefaction of seminal fluid [3]. *KLK3* is a well known androgen receptor target gene [126, 127].

Serum PSA levels has been the gold standard for detection and monitoring prostate cancer progression since it was incorporated into clinical practice in the 1990's. To this date, it remains the only biomarker used in the clinic.

Several markers have been proposed to be supportive in combination with serum PSA levels, by improving specificity of PSA. For instance, testing for PCA3 mRNA in urine maybe used as a complementary diagnostic test [128].

Very few studies have focused on the predictive ability of PSA expression in prostatic tissue, but a recent large study showed that tissue PSA was associated with adverse clinical features such as Gleason score and extraprostatic extension. It was not, however, a significant independent predictor of recurrence [129].

In contrast to its limitations as a diagnostic tool, PSA is of great value in screening for prostate cancer recurrence after radical prostatectomy, or ADT. Rising levels are indicative of recurrent disease and/or development of metastases.

The androgen receptor

As previously discussed, androgen receptor expression and signalling is present in benign prostate and all stages of prostate cancer [130, 131].

After the disease has progressed to an advanced stage, where cells are no longer dependent on androgen for survival and proliferation, the androgen receptor is frequently even more highly expressed. The increased expression level may be a result of *AR* gene amplification which is common [132, 133]. It has been proposed that increased androgen receptor expression is a hallmark for CRPC [81]. The increased expression in CRPC may indicate that ADT promotes a cell phenotype that is resistant to androgen blockade. It has been shown that ADT drives the amplification of the *AR* gene, and of enzymes involved in the conversion from adrenal steroids to DHT [130, 134].

The prognostic value of androgen receptor expression has been debated [135], whereas more recent studies show significant associations between androgen receptor expression and adverse outcome [136-139]

Microseminoprotein-β

One of the most predominant proteins in human seminal plasma is MSMB (other alias include prostate secretory protein of 94 amino acids (PSP94), and immunoglobulin binding factor (IgBF)) [4, 140]. Based on sequence homology, MSMB belongs to the immunoglobulin binding factor IgBF-family [141]. The function of MSMB in the healthy male is largely unknown, but evidence from guinea pig shows that MSMB may hinder spontaneous acrosomal reaction in the sperm cell [142]. In prostate cancer, on the other hand, MSMB has been attributed significant tumour suppressor functions (discussed in "Present investigation").

In the healthy human body, MSMB is expressed at high levels in the epithelium of the prostate, as well as in several tissues where mucous containing cells are present, such as the tracheobronchal epithelium, stomach, duodenum, colon, fallopian tubes and uterine cervix. In bodily fluids, the highest concentrations were found in seminal plasma (on average 0.89 g/L), and high levels are also found in tracheal and nasal secretions [143, 144]. In human seminal plasma, MSMB is bound to cysteine-rich secretory protein-3 (CRISP3) [145, 146], whereas in blood plasma, MSMB is bound to CRISP9 (also known as prostate specific protein of 94 amino acids binding protein (PSPBP)) but the ratios largely favour MSMB and a large amount of MSMB is therefore in free form [147].

MSMB – a prostate cancer suceptibility gene

In the past couple of years, the *MSMB* gene has become famed as one of the primary candidate prostate cancer susceptibility genes [56, 57]. The MSMB gene is located on chromosome 10q11.2, and several causal risk alleles were identified in the region upstream of the transcription start site, but the SNP known as rs10993994 had the highest association with prostate cancer risk [148, 149]. The polymorphism constitutes a change from CC or CT to TT, with the TT allele having only 13% of the activity of CC [150]. The low transcription level most likely depends on the formation of a CREB site [148]. Interestingly, a recent report show that the rs10993994 risk allele is common with a frequency of about 30-40% in Europeans and 70-80% in men of African ancestry [151].

Several reports show that the rs10993994 SNP has a detectable clinical effect since men with the TT allele has lower production of MSMB [152-155]. MSMB levels were lower

in urine from men with the TT allele, and this may be a useful clinical screening tool to find men that may be at higher risk for prostate cancer and would benefit from PSA-based screening [155]. There is an ongoing debate whether the risk allele confers risk for more or less aggressive prostate cancer, and whether there is a cumulative effect on prostate cancer risk with other SNPs. Whereas some groups find associations between the rs10993994 allele and less aggressive, low grade disease, and no additive effect with other risk SNPs [156], others report associations between aggressive prostate cancer and increasing risk when this SNP is combined with other risk alleles [58, 157].

So far, it is not known whether the decreased expression of MSMB seen in most prostate cancer cells is a reflection of less differentiated cells, or actively contributing to the carconogenic process. The loss of MSMB expression in prostate cancer is not likely due to gene deletion [158]. So far, the most likely mechanism by which MSMB expression may be silenced in prostate cancer, is by specific promotor methylation, mediated by enhancer of zeste homologue-2 (EZH2), a Polycomb group member which is often overexpressed in CRPC [159-161]. EZH2 has been shown to promote invasiveness and proliferation of prostate cancer cells, and may be considered an oncogene [162]. Interstingly, MSMB is the most down-regulated gene in the CWR22 cell line as it progressed into a castration-resistant state (the 22Rv1 cell line) [163].

Previous biomarker studies on MSMB

The suitability of MSMB as a biomarker for prostate cancer has been raised by several groups during the last two decades. It has been reported that MSMB mRNA and protein expression is reduced in malignant prostatic epithelium and in serum from men with prostate cancer compared to benign epithelium and healthy men [164-167]. In a recent microarray, *MSMB* expression was found to be the most down-regulated gene in prostate cancer tissue compared to benign [168].

Serum-levels of MSMB may be a discriminator between high and low grade disease [167]. In addition, MSMB expression, or the ratio free/bound MSMB, has been reported to be independent prognostic factors in both tissue and serum [147, 169-172].

Cysteine-rich secretory protein-3

Expression of CRISP3

Little is known regarding the function of CRISP protein family, and speculations must be based on sequence similarities and expression patterns. Human CRISP3 (also known as specific granule protein of 28 kD (SGP28)) was originally discovered at the protein level in neutrophilic granulocytes and was also cloned from a human bone marrow

cDNA library [173]. CRISP3 is also expressed in eosinophils and pre-B-cells, salivary glands, pancreas and prostate, where it is specifically expressed by the epithelium rather than prostatic stroma. CRISP3 was also found in less abundance in the epididymis, ovary, thymus and colon [174-177]. CRISP3 was present in many bodily secretions such as plasma, saliva, seminal plasma and sweat, with the highest levels detected in saliva (21.8 µg/mL) [178]. In human, the *CRISP3* gene is located on chromosome 6p12.3, and the CRISP3 protein is subjected to post-translational modification by glycosylation. So far, little is known about the function of CRISP3, but the expression in the male genital tract is indicative of a role in sperm cell maturation, or fertilisation [179].

CRISP3 is part of the CRISP family concisting of three members in human, whereas a fourth member has been found in mouse. The CRISPs are two domain proteins, with a CRISP domain, and a CAP (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1) domain. The CAP domain is evolutionaly conserved, with CAP proteins expressed and present in venom from poisonous snakes, lizards, and stinging insects, and involved in plant pathogenesis. This diversity suggests involvement in fundamental biological processes and highly conserved functions [180]. The CRISP domain, however, is not conserved, but the defining element of all CRISPs is that they contain 10 highly conserved cysteine residues, which forms 5 disulfide bonds (Table 1). Due to sequence homology, it is believed that all CRISP-members have ion channel regulatory activity, although this has only been shown for CRISP2 [181].

Table 1. CRISP3 amino acid sequence similarities between species

	Human CRISP2	**Equine CRISP3***	**Rat CRISP2**	**Mouse CRISP2**	**Rat CRISP1**	**Mouse CRISP1**
Human CRISP3	72%	66%	62%	60%	57%	56%

Sequence similarities between human, rat, mouse, and equine CRISPs were evaluated [182]. Mouse CRISP3, human CRISP1 and mouse CRISP4 all had less than 50% sequence homology to human CRISP3

*Equine CRISP3 was more similar (72%) to human CRISP2

CRISP3 in prostate cancer and inflammatory disease

In human seminal plasma, CRISP3 is bound in complex to MSMB (Fig 5). It has been suggested that this complex formation may inhibit the so far unknown function of CRISP3 in human seminal plasma [180]. Interestingly, one other CAP-domain protein, prostate secretory protein-binding protein (PSPBP, also known as CRISP9), binds to MSMB in serum. The relative serum levels of free MSMB to complexed MSMB: PSPBP has been suggested to be a serum marker for prostate cancer [147, 169, 170]. It remains to be elucidated whether these complex formations serve to prevent effects of the CRISP proteins, or of the MSMB protein.

In prostate cancer, CRISP3 was initially described to be up-regulated 21-fold compared to matched control tissue, and later studies by quantitative real-time PCR confirmed a 20 to 200-fold upregulation in prostate cancer [174, 183]. Since CRISP3 was the most upregulated gene in prostate cancer, it was suggested to be a prostate cancer biomarker [184]. The over-expression of CRISP3 seen in many prostate cancer tumours is not likely due to gene amplification [158].

We have previously reported that in a tissue microarray (TMA) with samples from 945 prostate cancer patients undergoing radical prostatectomy (RP), high CRISP3 was an independent predictor for poor outcome [171].

There is a notable bias for CRISP3 expression towards tissues involved in innate and adaptive immune responses. The localization of CRISP3 to the non-peroxidase granuled in neutrophils have rendered the suggestion that it may have a matrix-degrading role.

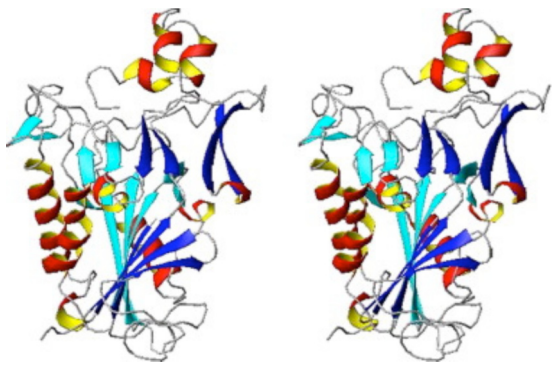

FIGURE 5. 3D-model of the MSMB:CRISP3 complex, based on multidimensional nuclear magnetic resonance (NMR). Reprinted from Biochemical and Biophysical Research Communications (378) Ghasriani, Fernlund, Udby and Drakenberg; *A model of the complex between human* β*-microseminoprotein and CRISP-3 based on NMR data*. 235-239, © 2009, with permission from Elsevier.

CRISP3 is dysregulated in several diseases, especially those with an inflammatory component. There is an upregulation of CRISP3 in chronic pancreatitis [185, 186], and prostate cancer [174, 183, 184, 187], whereas there is a down-regulation of CRISP3 in squamous carcinoma of the tongue [188]; in Sjögrens syndrome [189, 190]; and in asthmatic chronic rhinosinusitis [191]. Since Sjögren's syndrome is characterized by disturbed ion channel distribution and function (in particular aquaporin-5), and since CRISP3 may function as an ion channel regulator, it may have a direct role in the pathology of this disease.

Aims

- Validate the role of MSMB and CRISP3 to predict outcome after surgery for localised prostate cancer (Paper I)
- Explore the effects of ADT on MSMB and CRISP3 expression in patients with localised or advanced prostate cancer (Paper II)
- Investigate the transcriptional regulation of MSMB and CRISP3 (Paper III)
- Assess the anti-tumour effects of MSMB *in vitro* (Paper IV)

The Present Investigation

MSMB, but not CRISP3, is an independent predictor of biochemical recurrence after radical prostatectomy (paper I)

In **paper I**, we wanted to validate previous findings by our group and others, that MSMB and CRISP3 are independent predictors of recurrence after radical prostatectomy. We used a large independent tissue microarray (TMA) of 3268 patient samples and employed a new image analysis technique to evaluate staining intensity. We found that patients with tumours expressing high levels of MSMB had a significantly reduced risk for recurrence after radical prostatectomy (hazard ratio (HR)=0.710; 95% confidence interval (CI) 0.394-0.556; P<0.001). MSMB expression remained a significant independent predictor in multivariate analysis adjusted for clinicopathological parameters. We did not find any correlation between CRISP3 and recurrence.

Expression levels were quantitatively assessed by the automated image analysis tool, the *IHC-MARK* algorithm. The *IHC-MARK* algorithm is learning-based meaning that it must be trained to recognize and differ between the morphology of a tumour cell and any other cell types that are present in the tissue. The algorithm then quantifies percentage of stained tumour cells (0-100%) and staining intensity (0-255). We demonstrated a high correlation between manual and automated analysis in this study, although the impact of heterogeneous morphology often seen among prostate cancer cells remains to be fully evaluated.

Automated annotations are becoming more prevalent as a tool for histopathological assessments since they offer a sensitive and reliable system and remove inherent inter- and intraobserver variability associated with manual assessment [192]. Furthermore, automated image analysis may be a key feature of systems pathology, enabling more personalised prediction tools to better match disease grade and therapy [136, 193].

To find the most suitable cut-off levels for defining high and low expression levels, we used classification regression tree (CRT) analysis. This analysis is recognized as a robust and accurate way to predict outcome in that it is not sensitive to background noise, such as missing cases, and readily illustrates the analysis.

Surprisingly, it appears that an MSMB-positive tumour cell fraction as small as 8-10% greatly reduces the risk of recurrence. In normal prostate and benign prostatic hyperpla-

sia, virtually all epithelial cells express MSMB, suggesting that there is a redundancy in protein expression. Perhaps a fraction of MSMB-expressing cells sufficiently maintains any potential tumour suppressing effect(s) that MSMB have. This does not explain the fact that surrounding benign epithelial cells express high levels of MSMB, which may also act in a paracrine manner on tumour cells. Furthermore, MSMB intensity appears to be of less significance compared to fraction of positive tumour cells. Interestingly, the cut-off values we find to optimally define MSMB high and low expression in the current cohort is very similar to the cut-off values found in our previous study of an independent cohort [171].

In the current study we found no significant correlation between CRISP3 expression and biochemical recurrence, neither regarding intensity nor regarding fraction of CRISP3 positive tumour cells. However, similar to our previous findings, there was a trend suggesting that patients with high CRISP3 expression had increased risk for recurrence. Additional studies on long term survival are required to evaluate whether MSMB and/or CRISP3 will be of use in the clinic as prognostic tissue biomarkers for prostate cancer.

Despite the risk of overtreatment of a large number of patients with relatively indolent prostate cancer, the number of clinically applicable predictive and prognostic biomarkers is disappointingly low. Currently only serum PSA levels are included in clinical assessments, despite the low specificity of this test in localized prostate cancer [194]. Here, we emphasize the role of MSMB as a prognostic biomarker for prostate cancer outcome after radical prostatectomy.

Androgen regulation of MSMB and CRISP3 expression in prostate cancer tissue and cell lines (paper II and III)

The androgen signalling pathway is critical to the development and progression of prostate cancer, and with ADT being the first line treatment for patients with advanced prostate cancer, we wanted to examine the impact of short and longterm ADT on prostate cancer outcome predictors MSMB and the MSMB-binding protein CRISP3.

In **paper II**, we used an Affymetrix cDNA array to investigate the expression of *MSMB* and *CRISP3* genes in a small set of tumour specimens from patients that had received ADT prior to radical prostataectomy (n=17) or no neoadjuvant therapy (n=23). Included was also a small collection of metastases (n=9). For reference, we used the *KLK3* and *AR* genes, encoding PSA and the androgen receptor, respectively, and we found compelling similarities between *MSMB* and *KLK3* expression. Firstly, *MSMB* and *KLK3* are expressed at similar expression levels, which may be expected of two highly secreted proteins. Among those patients not receiving neoadjuvant ADT, more

inter-patient variation was seen for MSMB expression, compared to *KLK3*. *MSMB* and *KLK3* levels were reduced by ADT, however, *MSMB* levels decreased more than *KLK3* levels. In contrast to previous studies, this indicates an androgen dependent expression [195].

In line with previous studies [168], we find that *MSMB* expression was low or absent in metastatic prostate cancer, whereas *KLK3* was expressed at moderate-high levels. Rising levels of PSA is considered a hallmark for biochemical recurrence. This indicates that despite similarities in androgen effect on *KLK3* och *MSMB* expression in the normal prostate and primary prostate cancer, it is obvious that they are regulated in different ways in progressive disease. Apparently, the overexpression of *AR* often associated with aggressive prostate cancer will readily induce rising *KLK3* levels but not *MSMB* levels. This could be due to *MSMB* promotor methylation performed by *EZH2*, which we find to be up-regulated in metastases.

For *CRISP3* and *AR*, the cDNA array revealed no expression changes upon ADT. *AR* expression was expressed 10-fold more than *CRISP3*. In general, metastatic lesions had higher expression of both *CRISP3* and *AR* compared to primary prostate cancer.

To verify these findings, in **paper III** we performed *in vitro* studies where the androgen sensitive cell line LNCaP was stimulated with synthetic androgen. In line with cDNA array data, we find that *MSMB* expression is up-regulated in the presence of androgen, but so is *CRISP3*. It has been reported that the *CRISP3* gene has AREs in its promotor region [190], thus androgen driven expression was not surprising. One may speculate that the reason why *CRISP3* expression is not decreased upon neoadjuvant ADT in the patient material, is that other factor(-s) are driving *CRISP3* expression there. Furthermore, since *MSMB* and *CRISP3* response to androgen was not as rapid as *KLK3* induction, it may be that these genes are not direct targets of the activated androgen receptor.

In line with androgen regulated expression, we found that in a panel of prostate cancer cell lines, *MSMB* and *CRISP3* were primarily expressed in those with androgen receptor. Interestingly, although LNCaP cells had high expression of both *MSMB* and *CRISP3*, the LNCaP-derived cell lines C4-2 and LNCaP-IL6+ had decreased expression (C4-2), or no expression (LNCaP-IL6+). The C4-2 cell line was derived from serially xenografted LNCaP tumours in castrate conditions, and is an androgen-responsive cell line with high *AR* expression [196]. The LNCaP-IL6+ cell line is a long-term IL-6 stimulated cell line grown in presence of IL-6 for more than 50 passages. This cell line lacks expression of *AR* and produces IL-6 for autocrine stimulation [197]. Both C4-2 and LNCaP-IL6+ have a radically different morphology compared to parental LNCaP.

In addition to our cDNA array in **paper II**, we also had access to tissue from 16 patients undergoing repeated transurethral recestion of the prostate (TURP), before and during

long-term ADT. In general, CRISP3 and androgen receptor expression are expressed in a majority of tumour cells, and CRISP3 expression is up-regulated during disease progression in 12 out of 16 patients. The high *CRISP3* expression in metastatic and recurrent tumours may be indicative of a role for CRISP3 in the progression of prostate cancer. In this cohort, MSMB expression is difficult to interpret, since it is very low already at the time of the first TURP, with a majority of patients having less than 25% of all tumour cells staining positive for MSMB. In all patients with MSMB expression in more than 25% of all tumour cells, MSMB was decreased during ADT and disease progression. Two patients out of 16 were carriers of the high-risk allele rs10993994, and had very low levels of MSMB.

The close connection between high androgen receptor and CRISP3 expression seen in both cell lines and tissue may explain the increased CRISP3 levels detected in a subgroup of prostate cancer tumours [171]. In these patients, CRISP3 is connected with aggressive disease and increased risk for recurrence, and in such patient groups, the androgen receptor is frequently highly expressed.

Although very small, the serial TURP tissue material is unique. It must be interpreted with caution, however, because of its size, but also since it reflects patients not only undergoing ADT, but also with recurrent CRPC.

In conclusion, *MSMB* expression appears to be androgen driven, and levels are readily decreased upon hormonal treatment. Since MSMB is a prompted tumour suppressor, it is most thought-provoking to note that according to this study, *MSMB* is downregulated and perhaps subsequently silenced by standard treatment. CRISP3 expression is induced by synthetic androgen *in vitro*, and is highly up-regulated in CRPC.

Regulation of CRISP3 and MSMB genes (paper III)

Since previous studies show that *MSMB* and *CRISP3* expression can not only be explained by androgen, we wanted to further study the regulation of their expression in **paper III**. Therefore, a promoter assay was performed to detect putative transcription factor binding sites in a region 1000 base pairs upstream of the transcription start site in *CRISP3* and *MSMB* promoter regions.

Interestingly, in the *CRISP3* promoter, we found putative binding sites for several transcription factors normally associated with stem cells, such as Oct, nanog and Sox, leading us to hypothesize that perhaps *CRISP3* is expressed in CSC or transiently amplifying cells. However, we discovered that CRISP3, along with MSMB, is a feature of well-differentiated cells, and neither protein is expressed in benign prostate stem cells or prostate CSC (data not shown, and personal communication with Prof Norman Maitland, York Cancer Research Unit, York, UK).

Interestingly, the *CRISP3* promoter also contains binding elements for transcription factors linked to inflammation and carcinogenesis. Other putative transcription factor binding sites were for factors connected to androgen receptor, such as Oct-1, a proposed androgen receptor co-factor [198], inflammation, such as STAT and NF-kappaB, or both, such as PPAR.

Both *CRISP3* and *MSMB* gene promoters contained several putative binding sites for the PPARγ-RXR complex. Interestingly, the PPARγ transcription factor is able to induce growth arrest and terminal differentiation in a variety of cancers [199-202], and expression is correlated to lower pT stage [203]. PPARγ has connections to both androgen receptor and inflammatory cytokine signalling. The PPARγ coactivator-1α (PGC-1α) interacts with the androgen receptor, and enhances its DNA-binding ability to AREs [204]. In PC-3 cells, IL-6 normally induce proliferation, but when IL-6 was added in combination with the ligand for PPARγ, the IL-6 induced proliferation was inhibited, and levels of STAT3 was decreased [205]. Future experiments will aim at eluding the role of PPAR in regulation *CRISP3* and *MSMB* genes.

Inflammatory stimuli affects MSMB expression (paper III)

We found the presence of NF-kappaB and STAT binding sites in the *CRISP3* promoter region most interesting since we had previously hypothesised that inflammatory stimuli may regulate expression of these genes. This hypothesis was based on observations of high expression of both MSMB and CRISP3 in PIN lesions, and due to their localisation in exocrine secretions and mucosa, both proteins have been implicated to function in immune responses. To investigate whether inflammatory stimuli could affect expression of our genes, we used the pro-inflammatory cytokine IL-6 which has been shown to have a crucial role in prostate cancer progression (reviewed in [206, 207]). Interleukin-6 is frequently elevated in prostate cancer patients, and correlate to poor prognosis [208-210]. Intriguingly, prostate cancer cells have been shown to produce and secrete IL-6 in a paracrine and autocrine manner [211].

Again, we used LNCaP cells to study the effect of IL-6 stimulation. In addition, we used a long-term stimulated cell line derived from LNCaP, but grown in presence of IL-6 for more than 50 passages (kindly provided by Dr Zoran Culig, Innsbruck, Austria). This cell line has a radically different morphology compared to parental LNCaP, and produces IL-6 for autocrine stimulation. This cell line is therefore denoted LNCaP-IL6+.

When LNCaP cells were subjected to IL-6 stimulation, they responded by a dramatic 3.75-fold increase of *MSMB* expression. Surprisingly, although the *CRISP3* promoter has putative binding sites for both STAT and NF-kappaB, there was no induction of gene expression.

There are two splice variants of the *MSMB* gene, depicted in Fig 6A. Both transcripts have been deteced in organs of both the male and female urogenital tract [212, 213]. Interestingly, the short isoform was found to constitute 98% of the total MSMB transcript levels in BPH, whereas there was a complete splice variant switch in cancer, where 96% of the total MSMB transcript levels was full-length MSMB [214]. Although it remains to be validated, this is an interesting finding. Also, the impact on protein level remains to be elucidated since the only study so far attempting to determine protein expression of the short splice-variant was unable to detect the protein [213].

Throughout these studies, we have used isoform-specific primers for full-length MSMB. In **paper III**, we did also investigate the expression of the short MSMB splice variant, and found that levels changed in manners very similar to full-length MSMB upon androgen and IL-6 stimulation (data not shown).

A

```
MSMBa mnvllgsvvi fatfvtlcna scyfipnegv pgdstrkcmd lkgnkhpins
MSMBb mnvllgsvvi fatfvtlcna scyfipnegv pgdstrmflh lwvmtkttak

MSMBa ewqtdncetc tcyeteiscc tlvstpvgyd kdncqrifkk edckyivvek
MSMBb essrrrtasi swwrrrtqkr pvlsvng

MSMBa kdpkktcsvs ewii
MSMBb
```

B

```
Human mnvllgsvvi fatfvtlcna scyfipnegv pgdstrkcmd lkgnkhpins
Rat   mkarlgsllv latlvtasna acsiqrlkrl pneksdectd vdggkhvlnt

Human ewqtdncetc tcyeteiscc tlvstpvgyd kdncqrifkk edckyivvek
Rat   ywqkncewcf cektaitcct ktlipvsydk krcqrqfhse nctysvvert

Human kdpktcsvs ewii
Rat   npgktcpvng wti
```

FIGURE 6. Human MSMB exists in two isoforms. The full-length isoform (MSMBa; accession number NP_002434) is comprised of 94 amino acids, whereas the short isoform (MSMBb; NP_619540) has 57 amino acids. The truncation is due to frame shift mutation in and loss of part of exon 3 (A). Comparison of the primary structure of human and rat (NP_062061) MSMB. Location of the MSMB-derived peptides (MSMB1125 and MSMB3145) used in paper IV, and previously attributed anti-tumour effect are highlighted in grey (B). Conserved amino acids are in bold; and signalling sequences are underlined.

It has been shown that IL-6 can bind and activate the androgen receptor in absence of androgen [215], and that IL-6 may regulate the expression of genes responsible for *de novo* synthesis of androgens in the prostate [216]. However, we did not detect any IL-6 induced up-regulation of either *KLK3* or *AR* expression in stimulated LNCaP cells,

and we do not consider the up-regulation of *MSMB* to be due to androgen receptor activation.

Since IL-6 was able to induce expression of *MSMB* in LNCaP cells, we were surprised to find that LNCaP-IL6+ cells completely lack *MSMB* expression. It has previously been reported that the *MSMB* promoter was silenced by methylation in PC-3 cells [161], and treating LNCaP-IL6+ cells with DNA methyltransferase inhibitor MSMB was re-expressed.

Although one may only speculate, the finding that IL-6 induces increased MSMB expression could perhaps be explained by a role for MSMB in the innate immune response. The finding that *MSMB* is epigenetically silenced in long-term IL-6 stimulated cells, on the other hand, may be due to other, non-immune response functions of MSMB, such as the level of cellular differentiation. That would be in line with previous findings from Birnie et al, who showed that MSMB is expressed in differentiated prostate epithelial cells and not in prostate CSC [115].

In this light, our findings indicate that chronic exposure to inflammatory stimuli may somehow allow silencing of the *MSMB* gene, either directly, or indirectly as an effect of cells undergoing EMT. Furthermore, if MSMB does have tumour suppressing functions, silencing the expression of this gene could allow the cell to progress into a more aggressive state.

To conclude, we show for the first time that *MSMB* is regulated by inflammatory stimuli, and that this gene is epigenetically silenced in a cell line that was long-term stimulated with IL-6. Studies of the gene promoters revealed transcription factors known to be involved in a variety of cellular responses. Taken together, we believe that MSMB and CRISP3 may be involved in inflammatory response, and/or in differentiation. Further studies are warranted to better understand the role of these proteins in both cancer and benign tissues.

MSMB re-expression induces decreased proliferation (paper IV)

Despite the large interest the *MSMB* gene has generated as a marker for prostate cancer detection, recurrence, and as a genetic factor predisposing for increased prostate cancer risk, very little is known about its function in the human body. Several reports have implicated that MSMB may have anti-tumour effects (reviewed in [217]), but many of the proposed anti-tumour effects that have been attributed to MSMB have been discovered in experimental settings with two major flaws: they lack proper controls, and the majority of experiments are performed on non-human prostate cancer cell lines. In

paper IV, we wanted to investigate the proposed anti-tumour function of MSMB in human prostate cancer cell lines.

To summarize previous studies, it was reported a decade ago that apoptosis was induced in the PC-3 prostate cancer cell line upon treatment with isolated human MSMB protein [218]. In addition, colony forming capacity and tumour initiating capacity were both reduced [218]. Contrary to this finding, a recent study showed the contrary, that MSMB over-expression in PC-3 cells did not lead to reduced colony forming capacity, as it did in LNCaP cells [219].

In the Mat Ly Lu rat cell line studied *in vitro* and *in vivo* as xenografts, isolated MSMB protein appears to have omnipotent effects, including reduced experimental skeletal metastasis, reduced tumour volume, decreased serum calcium levels, and induction of apoptosis *in vivo*, as well as reduced proliferation *in vitro* [220, 221].

A number of studies have also investigated the effect of a synthetic peptide corresponding to amino acids 31 to 45 of the mature MSMB protein (Fig 6B). Again using the Mat Ly Lu rat cell line *in vitro* and *in vivo*, this synthetic peptide was able to reduce experimental skeletal metastasis in a manner similar to that of isolated full-length MSMB, albeit requiring a 10-fold higher concentration [222]. Furthermore, this peptide was able to reduce expression of pro-MMP9 in the human fibrosarcoma cell line HT-1080 [223], and inhibit tumour associated vascularisation [224].

One may consider that human MSMB has limited sequence similarity to rat MSMB, and binding partners of receptors in the human cell may therefore differ greatly compared to the murine setting. The MSMB gene is rapidly evolving [225], and when we compared the amino acid sequence, human MSMB had only 46% sequence similarity to rat MSMB (Fig 6B) [182]. Furthermore, when we compared the 15 amino acid peptide sequence to the rat proteomic catalogue [182] we found no matches in the rat proteome. One may consider the risk that since the human protein or peptide is alien to the rat cell, potential binding partners may not recognize the human protein sequence, and thus a different response may be elicited. In addition, few studies aiming at understanding the function of MSMB has been performed, using human prostate cancer cell lines, and the results generated from these studies are not completely clear.

In a recent study, a benign prostate cell line aquired anchorage-independent growth capacity when MSMB expression was silenced [226]. In normal cells, apoptosis is induced if attachment to ECM and surrounding cells would be lost, a process called anoikis. Cancer cells are able to avoid this limitation, and anoikis has been proposed to be an additional hallmark of cancer [227]

We used the MSMB-derived peptide corresponding to amino acids 31-34 (MSMB3145), previously attributed anti-tumour properties, and an additional peptide corresponding

to amino acids 11-25 (MSMB1125; Fig 6B). Treating PC-3 cells with these peptides, or scrambled control peptides, we did not detect decreased viability. Since viability assays may be flawed by low sensitivity to detect apoptosis when highly proliferative cell lines are used, we also performed Western blots to detect cleaved caspase-3, a hallmark of apoptosis. Again, the MSMB3145 peptide did not generate caspase-3 activation. Interestingly, the MSMB1125 peptide did generate caspase-3 activation. This finding is puzzling and must be further examined. Potentially, this finding could be interesting for drug discovery.

In order to actually understand MSMB function, we abandoned the peptides and used a transient transfection vector to induce MSMB expression in two cell lines lacking endogenous MSMB expression. PC-3 and LNCaP-IL6+ cells over-expressing MSMB were visibly reduced in cell number, and this corresponded to decreased cyclin D1 levels.

Interestingly, the LNCaP-IL6+ response to MSMB over-expression was more dramatic compared to PC-3, in terms of decreased proliferation. In these cells, MSMB expression caused a reduction in cell number by 33% compared to control cells. Since we have not investigated transfection efficiency, one may speculate that this result could be even more pronounced using a stable transfection vector ensuring complete transfection efficiency.

One other study suggests a link between MSMB and cyclin D1. In a cDNA array based on the CWR22 cell line as it progressed into a castration-resistant state (the 22Rv1 cell line), *MSMB* was the most down-regulated gene, whereas the most up-regulated genes were hepatocyte growth factor (HGF) and cyclin D1 [163]. Interestingly, cyclin D1 may be a selective androgen receptor modulator, with effect on classic androgen receptor target genes [228, 229].

The clinical significance of this finding remains to be clarified, since cyclin D1 over-expression is rare in prostate cancer [230], whereas decreased or lost MSMB expression is more frequent. Potentially, MSMB may have targets that directly or indirectly affects cyclin D1 and prevent proliferation, but it remains to understand how these events are connected.

To conclude, we have investigated the cellular effects of MSMB in prostate cancer cell lines, and we demonstrate that MSMB expression is associated with decreased cyclin D1 levels and reduced proliferation. From a biological perspective, it will be interesting to understand whether the frequent loss of MSMB expression in prostate cancer has a role in cancer development and progression, or whether it is bystander event.

Conclusions

In this thesis, we have aimed to gain further insight into the role of MSMB and CRISP3 in prostate cancer.

We conclude that:

- Preserved expression of MSMB in tumour cells is a marker for favourable outcome after radical prostatectomy.
- Tissue expression of MSMB was decreased by ADT in primary prostate cancer.
- CRISP3 expression is highly up-regulated in a subset of aggressive prostate cancers but its prognostic value as an independent tissue biomarker is unclear.
- CRISP3 expression is elevated in CRPC and in metastases.
- Androgen affects *MSMB* and *CRISP3* gene expression *in vitro*, but time to response points towards implication of different regulatory pathways.
- Inflammatory stimuli up-regulates *MSMB* expression, but it is silenced by methylation in long-term IL-6 stimulated cells.
- Re-expression of MSMB *in vitro* leads to reduced cyclin D1 expression and subsequent decreased proliferation.

Populärvetenskaplig sammanfattning

Prostatacancer är en mycket vanlig form av cancer, och det har visats att cirka hälften av alla män över 70 år har tumör i prostata. Prostatacancer är ofta en latent form av cancer, vilket innebär att den sällan ger kliniska symtom förrän i ett framskridet stadium. Sedan PSA testet började användas på 90-talet har antalet diagnostiserade fall av prostatacancer blivit allt fler. Men PSA testet är inte specifikt för cancer, utan nivåerna kan påverkas av andra prostatasjukdomar såsom godartad prostataförstoring eller inflammation i prostatan. För att bekräfta att förhöjt PSA beror på cancer, måste man ta vävnadsprover. Allt fler fall av prostatacancer upptäcks i ett tidigt stadium, och det är svårt att avgöra om tumören är aggressiv eller latent. Aggressiva tumörer kan opereras eller bestrålas, medan för latenta tumörer (även kallade indolenta eller icke-signifikanta), kan aktiv monitorering vara tillräckligt. Aktiv monitorering innebär att nivåerna av PSA mäts med korta intervall, och först när tumören visar tecken på tillväxt påbörjas aktiv behandling.

Pågående forskning försöker hitta nya markörer som kan hjälpa det kliniska beslutstagandet, genom att på ett tidigt stadium kunna avgöra huruvida tumören är aggressiv eller indolent. För behandlingskrävande tumörer är man även i behov av markörer som kan förutse om sjukdomen sannolikt är återkommande. Målet med de studier som presenteras i den här doktorsavhandlingen har varit att studera två proteiner vars uttryck är förändrat vid prostatacancer jämfört med normal prostatavävnad. microseminoprotein-β (MSMB), uttrycks i mycket höga nivåer i normal prostatavävnad, men mycket lägre, eller inte alls, i många prostatatumörer. Cysteine-rich secretory protein-3 (CRISP3) uttrycks i låga nivåer i den normala prostatakörteln, men är mycket högt uttryckt i vissa fall av prostatacancer.

I **artikel I** använder vi en ny automatiserad metod för att kvantifiera uttrycksnivåer i en stor prostatacancervävnadssamling från 3268 patienter. Vi finner att höga nivåer av MSMB i tumören är en markör för minskad risk för återfall efter att prostata bortopererats. I motsats finner vi att höga nivåer av CRISP3 tycks vara kopplat till högre risk för återfall, men detta samband är inte lika starkt som för MSMB. I **artikel II** använder vi en mindre vävnadssamling för att undersöka om hormonell behandling av prostatacancer påverkar uttrycket av MSMB och CRISP3, och vi finner att *MSMB* uttrycket minskar vid kort hormonell behandling före operation (ca 3 månader), medan *CRISP3*

produktionen inte tycks påverkas. Vi visade också att CRISP3 kan bildas i stor mängd i metastaserad prostatacancer.

I **manuskript III** och **IV** använder vi prostatacancercellinjer för att närmare studerade de molekylära mekanismerna bakom produktionen av MSMB och CRISP3 i tumörceller. Utöver att *MSMB* och *CRISP3* regleras av manligt könshormon (androgen) i odlade tumörceller, så fann vi även att inflammatoriska faktorer ökar produktionen av MSMB i tumörceller. Däremot kan MSMB nedregleras genom så kallad promotormetylering i långtidsstimulerade prostatacancer celler. Detta är intressant eftersom inflammation i prostatan är ett mycket vanligt tillstånd, och har föreslagits vara ett sätt på vilket prostatacancer kan uppkomma. Om inflammatoriska stimuli kan tysta *MSMB* genen så kan detta vara kopplat till utveckling och tillväxt av prostatacancer. Genom att experimentellt inducera produktion av MSMB i prostatacancercellinjer som saknar eget MSMB-uttryck, påvisade vi minskad celldelning i dessa tumörceller. Detta påvisar indirekt en länk mellan nedsatt produktion av MSMB och progression av prostatacancer.

Sammanfattningsvis visar resultaten att MSMB är en godartad prognostisk markör för minskad återfallsrisk, och förlusten av MSMB-uttryck kan vara länkat till uppkomsten eller progressionen av prostatacancer.

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