

# Midkine in Advanced Prostate Cancer

Biological Impact and Biomarker Potential

Anna Nordin

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**Cover illustration:** Immunofluorescent staining of steroid deprived prostate cancer cells, labeled for MDK (green) and TUBB3 (red).

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- Biological Impact and Biomarker Potential

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# Midkine in Advanced Prostate Cancer

## - Biological Impact and Biomarker Potential

Anna Nordin

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University of Gothenburg, Gothenburg, Sweden

### ABSTRACT

Prostate cancer (PC) is generally an androgen-driven disease, why androgen deprivation therapy (ADT) is the cornerstone for treatment of advanced and metastatic hormone-naïve PC (HNPC). ADT generally offers a good initial response, but normally fails with time, and the disease relapses into lethal castration-resistant PC (CRPC). Neuroendocrine differentiation (NED) is a transdifferentiation process that results in the accumulation of neuroendocrine (NE)-like tumor cells. NED is increased in CRPC and in response to ADT, and may represent a therapy-driven escape mechanism. Midkine (MDK) is a pleiotropic growth factor that is highly expressed during human embryogenesis, but is also induced in many pathological conditions, as in most human carcinomas, including PC. In recent years, MDK has received increased attention as a tumor biomarker in different human carcinomas.

In addition to a lack of curative treatments for advanced PC, there is a lack of reliable prognostic and predictive biomarkers. There is a need to find new biomarkers and to better understand the mechanisms behind castration-induced transformation into CRPC, including NED and acquired resistance.

The purpose of this thesis was to evaluate the role and impact of MDK in PC, with a focus on the CR stage and castration induced transformations, including NED. In this work we found MDK to be highly expressed both in advanced HNPC and in progressed CRPC and that MDK is associated with NED in CRPC. MDK was found to be influenced by castration and is presumed to be functionally associated with the androgen receptor. MDK was associated with a profound biological role in androgen-sensitive PC cells *in vitro* and was found to promote PC cell survival during the initial phase of steroid deprivation. Lastly, MDK was demonstrated to represent a powerful prognostic biomarker in both advanced HNPC and at relapse into CRPC. NED, in response to steroid deprivation, was observed as a transient phase of adaptation before transition into castration resistance, and was furthermore inducible also in the CR-state in response to AR-targeting.

In conclusion, this thesis identifies MDK as an important tumor biomarker in PC, with the potential to improve clinical decisions in treatment of patients with both advanced HNPC and CRPC. Furthermore, the functional importance of MDK in tumor evolution was partly elucidated.

**Keywords:** Castration resistant prostate cancer, neuroendocrine differentiation, Midkine, steroid deprivation

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# POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostata cancer (PC) är den vanligaste cancerformen hos män i Sverige, med årligen ca 10 000 nyupptäckta fall. PC är även en den cancerform som orsakar flest cancerrelaterade dödsfall hos svenska män, ca 2350 varje år. PC växer ofta långsamt och är begränsad till prostatan och är utan symptom, men kan i vissa fall utveckla ett aggressivt tillväxtmönster där tumörceller sprider sig utanför prostatan och vidare till andra vävnader och organ som dottertumörer, metastaser.

Patienter med metastaserad PC kan inte botas, utan får i dagsläget bromsande och symptomlindrande behandling i form av kastration, oftast medicinsk så kallad androgen deprivationsterapi (ADT). Denna behandling blockerar produktionen av manligt könshormon, testosteron, från testiklarna, och därmed tumörtillväxten eftersom cancerceller i prostata är beroende av detta hormon. ADT ger ofta god effekt hos de allra flesta patienter, men tyvärr bara en kortare tid eftersom tumörerna normalt återupptar sin tillväxt efter några år, då som aggressiv så kallad kastrationsresistent PC (CRPC), för vilken det idag finns flera behandlingsalternativ som kan förlänga livet för vissa patienter upp till några år.

Övergången till CRPC förknippas ofta med en återupptagen aktivering av testosteron, trots att kastrationsbehandlingen pågår. Detta beror på att cellerna ökar sin känslighet för hormonet, eller att de börjat producera testosteron själva och därför inte längre är beroende av den normala produktionen. Det finns dock en grupp av CRPC patienter vars tumörtillväxt styrs av helt andra signaler. Dessa tumörer har ofta en ackumulering av cancerceller som har likheter med neuroendokrina celler, inklusive en produktion och utsöndring av diverse neuronala proteiner vilka stimulerar tillväxt och överlevnad, så kallade neuroendokrin (NE)-lika tumörceller.

Midkine (MDK) är ett protein som under fosterutvecklingen bidrar till bildningen av nervsystemet. Hos vuxna människor finns proteinet i princip endast vid sjukdomstillstånd som vid inflammation och olika tumörsjukdomar. MDK har i olika cancerformer förknippats med både tillväxt och ökad överlevnadsförmåga hos cancerceller, men hur detta sker och vad det har för betydelse i PC är delvis oklart.

För att kunna utveckla effektiva behandlingar mot CRPC, även mot de former som har NE-lika tumörceller, behöver vi bättre förstå hur ADT driver övergången till CRPC och hur detta leder till förekomst av NE-lika tumörceller. Vi behöver även bättre sätt att upptäcka och karaktärisera de farliga formerna av PC, och även förbättra metoder att utvärdera effekten av olika behandlingar.

Det övergripande målet med denna avhandling har varit att förstå funktionen och betydelse av MDK i PC, både före ADT, under utveckling av CRPC, och i CRPC, särskilt i samband med en ackumulering av NE-lik tumörceller. Dessutom studerades hur PC-celler förändrades av att inte ha tillgång till testosteron, och hur de över tid utvecklades till CRPC celler.

I dessa studier fann vi mycket MDK i vävnad från metastaserad CRPC jämfört med vävnad från tidigare, snällare, former av PC. I CRPC fanns MDK ofta i områden med mycket NE-lik tumörceller. Detta kunde också ses i cellodlingsförsök, där PC cellerna bildade mycket MDK då de utvecklades till NE-lik tumörceller efter att ha berövats testosteron. Genom att blockera cellernas produktion av MDK kunde vi även påvisa att MDK är viktigt för många grundläggande funktioner i en PC cell, så som tillväxt och reparation av skadade gener. Vi kunde också se att testosteron påverkade bildningen av MDK.

Dessa resultat tyder på att MDK har en viktig roll för PC. Därför undersöktes om analys av mängden av MDK i blodet från patienter med PC kan säga något om hur allvarlig PC man har. Studien visar att patienter med höga nivåer av MDK redan innan ADT påbörjas resulterar i en kortare livslängd. Dessutom visar resultaten att stora mängder av MDK i blodet när CRPC utvecklats tyder på att man har kortare tid kvar att leva.

Genom studier i cellodlingssystem kunde vi även se att utvecklingen av NE-lik tumörceller är ett övergående steg i processen att utveckla CRPC. Detta sker troligtvis som en anpassning till ADT och leder till utveckling av egenskaper hos PC cellerna för att överleva trots låga nivåer av manligt könshormon.

Sammanfattningsvis visar denna avhandling att MDK är viktig i metastaserad PC särskilt i relation till utveckling av CRPC och associerad ackumulering av NE-lik tumörceller, och att analys av MDK skulle kunna förbättra kunskapen om patienters PC sjukdom och prognos.



# LIST OF PAPERS

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

- I. **Nordin, A.**, Wang, W., Welén, K., Damber, JE. Midkine is associated with neuroendocrine differentiation in castration-resistant prostate cancer. *Prostate*. 2013; 73 (6): 657-67.
- II. **Nordin, A.**, Damber, JE., Welén, K. The role of Midkine in high-grade prostate cancer cells before and after steroid deprivation. Manuscript.
- III. **Nordin, A.**, Wang, W., Ahlgren, G., Josefsson, A., Welén, K., Damber, JE. Midkine is a progression biomarker in advanced prostate cancer. Manuscript
- IV. **Nordin, A.**, Welén, K., Damber, JE. Long-term steroid deprivation transforms prostate cancer cells to anaplastic castration resistant cells. Manuscript.



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# ABBREVIATIONS

|              |   |
|--------------|---|
| ADT          | Androgen deprivation therapy                                      |
| ALP          | Alkaline phosphatase  |
| AR           | Androgen receptor   |
| BPH          | Benign prostate hyperplasia                                       |
| CHGA         | Chromogranin A  |
| CR           | Castration resistant  |
| CRPC         | Castration resistant PC   |
| DCC-FBS      | Dextran charcoal treated - fetal bovine serum (DCC)               |
| DHT          | Dihydrotestosterone   |
| ELISA        | Emzyme-linked immunosorbent assay                                 |
| EMT          | Epithelial to mesenchymal transition                              |
| ENZ          | Enzalutamide  |
| FBS          | Fetal bovine serum  |
| GnRH         | Gonadotropin releasing hormone                                    |
| GS           | Gleason score   |
| HNPC         | Hormone naive PC  |
| ICC          | Immunocytochemistry   |
| IHC          | Immunohistochemistry  |
| iPS          | Induced pluripotency  |
| LNstrav.1, 2 | LNCaP starvation (LNCaP subline 1 and 2)                          |
| LNCaP        | Lymph node Carcinoma of the Prostate                              |
| MDK          | Midkine   |
| NE           | Neuroendocrine  |
| NED          | Neuroendocrine differentiation                                    |
| NES          | Nestin  |
| NEPC         | Neuroendocrine PC   |
| NSC          | Neuronal stem cell  |
| NSE          | Neuron specific enolase   |
| NT           | Neurotensin   |
| PC           | Prostate caner  |
| PIA          | Prostatic inflammatory atrophy                                    |
| PIN          | Prostatic intraepithelial neoplasia                               |
| PSA          | Prostate specific antigen   |
| PTN          | Pleiotrophin  |
| siRNA        | Small interfering RNA   |
| siMDK        | Cell transfected with siRNA directed towards MDK                  |
| siNEG        | Cell transfected with siRNA directed towards off target sequences |
| CSS          | Cancer specific survival  |
| SCPC         | Small cell prostate carcinoma                                     |
| SRD5A        | 5 alpha reductase   |
| SYP          | synaptophysin   |
| TUBB3        | Tubulin-beta III  |
| TURP         | Transurethral resection of the prostate                           |





# 1 INTRODUCTION

## 1.1 CANCER

Cancer is one of the leading causes of death worldwide and represents a collection of genetic diseases, all defined by uncontrolled cell proliferation. The array of human cancers is made up of around 200 different diseases, each generated from different cell types in the human body (1).

Most cancers, approximately 90% in humans, have an epithelial origin (endo- or ectodermally derived) and are known as carcinomas. A subtype of carcinoma, adenocarcinoma, arises from epithelial cells of glandular origin, for example, breast and prostate glandular tissue. The remaining non-epithelial cancers are mainly represented by neuroectodermal tumors and sarcomas. Neuroectodermal tumors are derived from cells in the central and peripheral nervous system, while sarcomas are of mesenchymal origin (e.g. cartilage, bone, or fibrous tissue). Furthermore, cancers of the blood, leukemia and lymphomas, represent subclasses of sarcoma that are of hematopoietic origin, arising from blood-forming cells and from cells of the immune system, respectively. While most cancers grow as solid masses of cells, i.e. tumors, cancer of the blood grows as individual cells (2, 3).

Primary cancer is a tumor mass present at the site of initial transformation and is normally of little clinical obstacle, excised by surgery or cured by localized treatment. Secondary cancer, i.e. metastasized cancer, represents a colonization and regrowth of primary tumor cells at distant sites and in contrast to primary cancer, metastasized cancer represents a great clinical challenge and accounts for the vast majority of cancer related deaths (4, 5).

### 1.1.1 The initiation and progression of cancer

Tumorigenesis refers to the process of tumor formation, including both benign (indolent) and malignant (invasive, aggressive) tumors, while carcinogenesis refers to the formation of malignant tumors, i.e. the development of cancer. Malignant tumors are characterized by their ability to invade nearby tissue and to spread to distant secondary sites via the circulatory or lymphatic systems through the process of metastasis (2, 3).

The initiation of cancer is a multistep process whereby normal cells are gradually transformed into cancer cells through a sequential accumulation of epigenetic alterations and mutations within the genetic material of the cell, the DNA. These acquired genetic changes eventually lead to uncontrolled clonal growth. Cancer cells tend to adhere poorly to each other, and invasive cancer cells possess the ability to

transform into a migratory phenotype through the process of epithelial to mesenchymal transition (EMT). During EMT, many cancer cells also upregulate the expression of different secretory proteases that will assist in the digestion of surrounding tissues. Without growth regulation, continuous cell expansion eventually leads to cell dissociation, EMT, and migration, and tumor cells that finally leave the primary tumor site through invasion and metastasis. The progression of cancer is characterized by continuous clonal selection of tumor cells with progressively increasing capacity for proliferation, survival, invasion, and metastasis (2, 3).

Similar to tumor initiation, metastasis is a multistep process. The initial steps preceding metastasis, i.e. the growth of the primary tumor, require the initiation of angiogenesis. The metastatic process then begins with tumor cell intravasation into the circulatory system (in blood vessels or lymphatics). Of the circulating tumor cells it is estimated that less than 0,1% (6) will survive the hostile environment, and many of the surviving cancer cells will exist in an arrested state in the circulation for longer periods before extravasation. At extravasation, the tumor cells leave the circulation and invade their new organs or tissues (2, 3). Common sites for metastatic spread include lymph nodes, lungs, the liver and the skeleton (National cancer institute; <https://www.cancer.gov/types/metastatic-cancer>). In this new environment, many disseminated tumor cells die or become dormant, sometimes for many years. Eventually, individual tumor cells might give rise to micrometastases through initiated proliferation. If the proper conditions are met, continuous growth will thereafter result in clinical metastases for which the initiation of angiogenesis is required. With induced angiogenesis, seeding of new metastases is further enabled (2, 3)

### 1.1.2 Hallmarks of cancer

Genetic susceptibility to cancer is determined by genetic variations that are inherited as well as by variations and alternations that are acquired during life. Cancer is therefore a disease associated with aging due to the time-dependent accumulation of genetic mutations, epigenetic alterations and cellular damage, and many key features of cancer cells and aging cells are thus shared, for example, genomic instability and epigenetic modifications. Cancer and aging differ however in that cancer cells have a 'gain of function and fitness' whereas aging cells are characterized by a 'loss of function and fitness' (7).

Genome instability and mutations are considered to be enabling characteristics of cancer, as described by Hanahan and Weinberg (8). Loss of function mutations in tumor suppressors and gain of function mutations in growth promoting proto-oncogenes are essential for the development of cancer. Also, mutations and modifications leading to loss of functions of genes involved in the DNA damage response are of central importance for cancer initiation and progression (3, 8, 9).

In addition to genomic instability and mutations, tumor-promoting inflammation is also considered an enabling characteristic, which highlights the close relationship between inflammation and cancer (10-13). The original six hallmarks as described by Hanahan and Weinberg in (9) are activation of invasion/metastasis, induction of angiogenesis, apoptotic escape, replicative immortality, sustained proliferation, and the ability to evade growth suppressors. In addition, two emerging hallmarks have been added to the six original features of cancer – the deregulation of cellular energetics and the ability to avoid immune destruction (8).

## 1.2 THE PROSTATE GLAND

### 1.2.1 Anatomy and physiology

The prostate, a walnut-shaped exocrine gland, is located in the pelvis below the urinary bladder (14) and is of endodermal origin. It arises from the proximal part of the urethra through epithelial budding into the surrounding mesenchyme. Under the influence of androgens and EMT interactions, epithelial budding is followed by glandular differentiation and branching morphogenesis to generate an epithelial-lined glandular and ductal system, while the mesenchyme differentiates into a dense fibromuscular stroma. Prostate organogenesis and maturation continue until the gland reaches its full size during puberty (15).

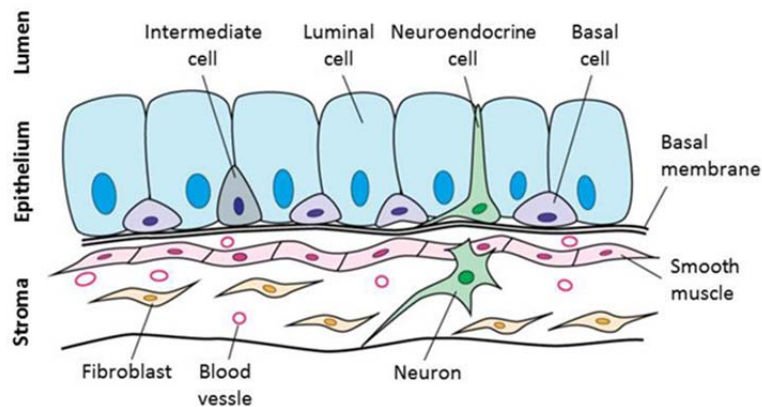
The human prostate, which is enclosed by a capsule-like structure, can be divided into three glandular zones – the peripheral zone, the transitional zone and the central zone, as well as one non-glandular zone termed the anterior fibromuscular stroma. The transition zone represents the exclusive site of benign prostatic hyperplasia (BPH) initiation, a common nonmalignant condition found in older men. The peripheral zone is the largest zone, containing the majority of the glandular tissue (about 70%), and representing the most common origin of prostate cancer (PC) and premalignant lesions such as prostatic intraepithelial neoplasia (PIN) (14, 16).

The prostate is part of the male reproductive system and produces a weakly alkaline secretion that drains into the urethra together with spermatozoa and secretions from the seminal vesicles at ejaculation (17). The prostatic secretion, which contains numerous proteins, ions, and electrolytes, functions as a liquefying agent that enhances sperm motility and survival, hence aiding in fertilization. The alkaline property of the secretion furthermore protects the sperm from the acidic environment of the vagina (18, 19). As a liquefying agent, one of the main functions of the prostatic secretion is proteolysis, and prostatic secretions are therefore rich in proteolytic enzymes of which one of the major constituents is the serine protease prostate specific antigen (PSA), also known as kallikrein III (KLK3).

### 1.2.2 Morphology and cell function

The glandular structures of the prostate are composed of an epithelial bilayer containing three distinct types of cells – secretory luminal cells, basal cells and a minor population of neuroendocrine (NE) cells. In addition, a small fraction of stem cells with basal cell characteristics is also believed to be scattered within the epithelium. An underlying basement membrane separates the epithelium from the surrounding stroma (**Figure 1**) (20, 21).

Prostatic epithelial cells are identified by their morphology, location, and distinct patterns of marker expression.



**Figure 1.** Schematic illustration of the human prostate epithelial compartment, the lining glandular ducts and acini, and the underlying stromal compartment. Adapted from (15) with permission.

The inner epithelial layer, facing the lumen of the duct, is made up of polarized columnar-shaped luminal cells (**Figure 1**), and these represent the predominant prostatic epithelial cell type. They produce and secrete the components of the prostatic fluid, including PSA, directly into the prostatic lumen. They are terminally differentiated and post-mitotic, have a high androgen receptor (AR) expression and are androgen dependent. They are further characterized by the expression of luminal cytokeratin (CK) 8 and CK18 (20, 21).

The outer epithelial layer, resting on the basal membrane, is made up of cuboidal basal cells, which represent the second major population of prostatic epithelial cells (**Figure 1**). The function of the basal cells is not fully understood, but in contrast to luminal cells, they lack a secretory function and are relatively undifferentiated, express low or undetectable levels of AR, have no PSA expression, and are independent on androgens for survival and growth. They are further characterized by the expression of P63 and basal CK5 and CK14. A fraction of the basal cells scattered within the basal cell layer are believed to represent a prostatic stem cell

population, and as such these cells are able to differentiate into the other epithelial cell types if needed. This is believed to occur via a basal-luminal intermediate (transit amplifying) cell type that expresses both basal and luminal CK, CK5 and CK18, as well as additional markers such as CK19. This undifferentiated population is AR negative, hence androgen independent (15, 22) and have been demonstrated to possess tumor initiating capabilities (23).

The third cell type, the NE cells, are scattered within the basal cell layer, and like the luminal cells, are terminally differentiated and post-mitotic (**Figure 1**). The NE cells are however AR negative and androgen independent. Furthermore, they produce numerous secretory products, including many neuropeptides, and are believed to support and regulate the growth, survival, and differentiation of surrounding epithelial cells in a mainly paracrine fashion (21). They can be of opened or closed morphology, where the opened NE cells extend dendritic protrusions toward the lumen. The NE cells are mainly characterized by the expression of different NE markers for example chromogranin A (CHGA) and neurons specific enolase (NSE) (24). They are furthermore characterized by express of a mixed basal – luminal CK pattern of CK5 and CK8 (22).

The prostatic stroma is composed of a heterogeneous mixture of cells, including mesoderm-derived smooth muscle cells and fibroblasts as well as infiltrating immune cells. The cells are embedded in a collagenous matrix together with blood vessels, lymphatics, and nerves (15). Both the stromal smooth muscle cells and the fibroblasts are to AR positive and produce andromedins – which are growth and survival-promoting peptides – in response androgen/AR signaling. The andromedins diffuse into the epithelial compartment where they control the growth and survival of both the luminal and basal cells (15, 25, 26).

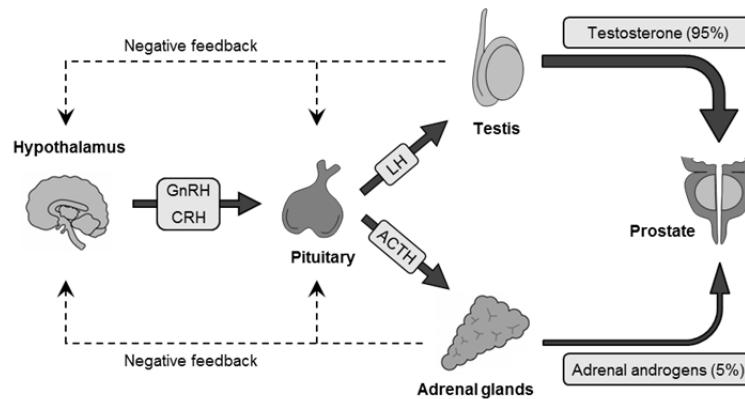
### 1.2.3 Androgen biosynthesis and AR activation

Androgens belong to a group of steroid hormones that promote the development and maintenance of the male sex characteristics. The prostate gland, as part of the male reproductive system, is highly dependent on androgens for normal development, growth, survival and function (15). The cellular response to androgens is mediated by the AR, which regulates a complex genetic program, affecting fundamental processes such as proliferation and differentiation as well as the DNA damage response (27, 28)

#### 1.2.3.1 *Androgen biosynthesis*

Circulating androgens are produced, in the testes and in the adrenal glands, under the regulation by the hypothalamic–pituitary axis (**Figure 2**). Endocrine secretion of the hypothalamic hormones, gonadotropin releasing hormone (GnRH) and corticotropin releasing hormone (CRH) regulate the secretion of the pituitary gland hormones

luteinizing hormone (LH) and adrenocorticotrophic hormone (ACTH), respectively. Testicular androgen in the form of testosterone is produced by the Leydig cells of the testes in response to LH, while the production of adrenal androgens, mainly made up of the weak androgen precursors dehydroepiandrosterone sulphate (DHEA-S, predominantly), DHEA, and androstenedione (AD), is regulated by ACTH (**Figure 3A**). Testicular androgen in the form of testosterone accounts for 95 % of the circulating androgens while the adrenal androgens account for the remaining 5% (29).



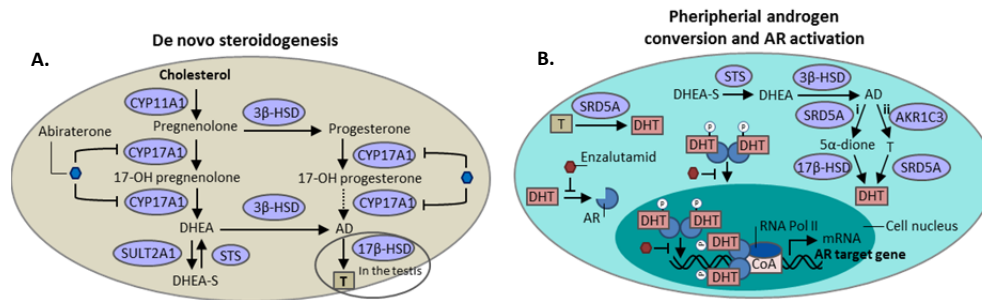
**Figure 2.** Androgen synthesis and regulation of the prostate gland. The illustration is reused from (30), with permission.

#### 1.2.3.2 Androgen receptor activation

Testosterone as well as adrenal androgens enter the stromal and epithelial cells of the prostate by passive diffusion. Within the prostatic cells, testosterone is converted into dihydrotestosterone (DHT) by one of two isoenzymes, steroid 5 $\alpha$  reductase 1 (SRD5A1) or SRD5A2, of which SRD5A2 predominates within the normal prostate. Both testosterone and DHT can bind and activate the AR, but DHT has a higher binding affinity and is a more potent androgen. Also the adrenal androgens can be converted into testosterone or DHT within peripheral tissues, including the prostate, for which the central enzymes include, 3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta$ 5-4-isomerase type 1 (3 $\beta$ -HSD), 17 $\beta$ -hydroxysteroid dehydrogenases (17 $\beta$ -HSD), aldo-keto reductase family 1 member (AKR1C3), and SRD5A (**Figure 3B**) (31).

In the prostate, the AR is present in both the stromal compartment (fibroblasts and smooth muscle cells) and in the secretory luminal cells of the epithelial compartment, while the basal cells and the NE cells are AR negative. Inactivated, ligand-free AR is present in the cytosol of AR-positive cells, where it is bound by heat shock proteins (Hsp-70 and Hsp-90) that stabilize the receptor and protect it from degradation. Androgen binding to the AR induces a conformational change that results in the dissociation of bound heat shock proteins (29). The ligand-receptor complex becomes stabilized and activated through subsequent homodimerization and

phosphorylation. The activated complex is thereafter translocated to the nucleus where, together with RNA polymerase II and diverse co-factors (co-activators or co-repressors), it binds to DNA target sequences known as androgen response elements (AREs) in order to activate or repress gene expression (**Figure 3B**) (31).



**Figure 3.** **A.** Androgen biosynthesis in the adrenal gland results in three main androgen precursors; DHEA-S (the predominant precursor), DHEA, and AD. De novo steroidogenesis from cholesterol also occurs in the Leydig cells of the testes, where the biosynthesis proceeds all the way to the potent androgen, testosterone. **B.** Conversion of testosterone and adrenal androgens into the potent androgen, DHT occurs within prostatic stromal and epithelial cells. AD conversion into DHT occurs through two alternative pathways (i versus ii), where the 5 $\alpha$ -dione pathway (i) represents a DHT bypass pathway that predominates in CRPC, while the testosterone pathway (ii) represents the normal route. Abbreviations: T, testosterone; CRPC; castration resistant PC, RNA pol II, RNA polymerase II; CoA, cofactors. The illustration is redrawn, inspired of PA Watson et al (31). Regarding abiraterone acetate (androgen biosynthesis targeting drug) and enzalutamide (AR-targeting drug), see section 2.3.2; Prostate Carcinogenesis.

## 1.3 PROSTATE CANCER

### 1.3.1 General background

In the industrialized Western world, PC is the most commonly diagnosed cancer in men and is the third leading cause of cancer-related deaths (32), this despite many therapeutic advances in recent year. In Swedish men, PC represents both the most commonly diagnosed cancer form and the most common cancer-related cause of death, with approximately 10,000 new cases diagnosed and 2.350 reported deaths annually in recent years (Cancer statistics in Sweden; <http://www.socialstyrelsen.se/statistik/statistikdatabas/cancer>).

Over the past decades, the incidence of PC has increased dramatically in most countries, especially in the Western world, while the PC mortality rate has remained fairly constant. The steady increase in PC incidence is primarily due to widespread PSA testing, which was introduced as a clinical option in the late 1980s (33) but is

also likely influenced by an aging population, due to the close association between PC and increased age (34).

In recent years, however, an ongoing decrease in mortality rate can be observed in many countries, for example in Sweden, the US and the UK (35). The decrease in PC mortality is likely due to recent advances in PC treatment options as well as to the earlier detection of PC due to PSA testing. The majority of the PC cases detected are thus represented by organ-confined primary tumors that are potentially curable through radiation or surgery, and to a less extent by lethal metastatic disease (36, 37).

A substantial portion of the primary tumors detected are however low-risk PC, i.e. tumors that will never progress or will only very slowly progress from a localized tumor to a locally advanced or a metastatic stage (37). Today there is no reliable diagnostic tool to differentiate between indolent and aggressive forms of PC and to predict clinical progression, and this remains one of the major challenges within the PC field (37, 38). To prevent overtreatment of indolent tumors, active surveillance is today the first choice for this patient group (39) (<http://www.socialstyrelsen.se>).

In addition to cancer, the prostate is subject to pathologies of prostatitis (inflammation) and BPH, both of which are more common in elderly men, as well as associated with increased PSA levels (40, 41). BPH presents as an enlargement of the prostate, including both the stromal and the epithelial compartments, and can, when sufficiently enlarged result in compression of the urethral canal and further complications (41). Prostatitis refers sometimes to a histological inflammation, sometimes chronic, that might be associated with an increased risk for PC (11).

#### 1.3.1.1 *Risk factors*

PC incidence, like the incidence of many other cancer forms, is strongly related to age, with clinical PC only being frequent in men over 60–65 years (42). The strong association between PC and age is at least partly due to the accumulation of genetic and epigenetic alterations that occur over time. Examples of genes that are commonly modified during the course of PC progression are the tumor suppressors *NKX3.1*, *CDKN1B*, *TP53* and *PTEN* and the oncogenes *AR*, *MYC* and *ERG* (11). In addition, as is also true for cancer in general, genetic inheritance can influence the predisposition for PC initiation and progression, and several susceptible genes have been identified, including *BRAC2*, *RNASEL*, *MSR1* and *CHEK2*, however all with low frequencies of genetic variation in the risk population (34). Although several of these genetic alterations represent strong risk factor within families (43), most PC cases appear to be sporadic with less than 10% estimated to be accounted for by heritable forms (40). PC incidence is much higher in men of Afro-american compared to other populations.

In addition, environmental factors, diet, and lifestyle are considered emerging risk factors for PC, as indicated by higher PC incidences in Europe and the US compared to in Asia. In support of an association between environmental and lifestyle factors



and PC, the risk of developing PC increases markedly in native Asians who migrate to the US (11, 34). As mentioned above, inflammation of the prostate, including lesions such as proliferative inflammatory atrophy (PIA) is considered to be associated with the development of PC (10-12).

#### 1.3.1.2 *Diagnosis and pathology*

PC is diagnosed as localized, locally advanced, or metastatic disease. Localized PC refers to invasive growth within the prostate gland, while locally advanced PC is characterized by invasive growth and spread outside the prostate capsule. Metastatic disease refers to the spread of tumor cells to distant sites through the lymphatic system or the circulation (34, 40). The most common site for PC metastasis is in lymph nodes (44) and in bone, with estimates of about 90% of men with metastatic castration-resistant (CR) PC (CRPC) developing bone metastases. Furthermore, as estimated from autopsy studies, bone metastases are found in around 70% of men who die from their PC (45, 46), and thus appear to be responsible for the vast majority of the morbidity and mortality in PC (20, 45).

Early-stage PC is often asymptomatic while locally advanced or metastatic disease is more often associated with severe symptoms like hematuria, lower urinary tract symptoms, bone pain, and fractures. Early-stage PC is thus commonly detected by a routine PSA blood test that is followed by further examination due to the low specificity and sensitivity of PSA. Examinations that follow an elevated PSA measurement include multiparametric magnetic resonance imaging (MRI) and transrectal ultrasound-directed biopsy sampling, where the ultrasound gives information about the size and the shape of the prostate and the MRI might detect areas in the prostate suspicious for cancer (34, 40, 47).

The collected biopsies are histologically examined by a pathologist for assessment concerning differentiation (Gleason Score (GS)) as a measurement of aggressiveness. After PC has been confirmed, additional examinations might be performed, for example, if metastatic spread is suspected as is the case in patients with a high PSA level in combination with a high GS. Examinations to determine metastatic spread include bone scintigraphy, MRI, computerized tomography (CT), and positron emission tomography-CT (PET-CT). The combined information is used for classification into different risk categories (34, 40, 47).

#### 1.3.1.3 *PSA and other biomarkers for PC*

Normal prostate architecture prevents PSA from reaching the circulation; however, during prostate pathologies, including BPH, prostatitis and PC, the epithelial layer and the underlying basement membrane become leaky, this in combination with an increased production of PSA, resulting in increased PSA levels that are detectable in the blood (48, 49). An increased PSA blood level might thus indicate PC, but is not sufficient for cancer diagnosis. Furthermore PSA cannot differentiate between indolent and aggressive forms of PC (38). A PSA level of 0–3 ng/mL is considered

normal, a level above 10ng/mL is considered indicative of local tumor growth, and a PSA value above 100ng/mL is considered indicative of metastatic disease. However, the majority of men with a PSA value of 3–10 ng/mL does not have PC but the benign condition BPH (40). In addition to the high proportion of false positives, PSA measurement is also associated with false negative results, i.e. patients with aggressive PC but without an elevation in PSA (38).

In addition to PSA, other biomarkers are sometimes evaluated at diagnosis, for example, alkaline phosphatase (ALP). ALP is primarily used to evaluate the degree of bone remodeling and hence the degree of metastatic burden (34, 40, 50). Furthermore, NE marker(s), for example chromogranin A (CHGA), neuron specific enolase (NSE), CD56 (also known as NCAM1), and/or synaptophysin (SYP) can be used when an evaluation of the degree of neuroendocrine differentiation (NED) is of interest (24, 51). An accurate estimate of the amount of NE-like tumor cells can, however, be difficult to achieve due to the heterogeneous nature of NE-like tumor cells and the lack of a consistent marker (47, 52, 53).

#### 1.3.1.4 *Gleason score*

The GS is a histological grading system to assign PC aggressiveness according to the degree of differentiation. First, the growth patterns of the tumor is assigned with Gleason grades, 1 to 5, with 1 being the least aggressive and 5 the most aggressive (the most dedifferentiated) (54). The GS is the sum of the most common and the most aggressive Gleason grades found in the tumor.

According to the new WHO guideline from 2016 (55), Gleason grade 3 should be the lowest grade to assign PC, and therefore a modified grading system now range from GS 6 to 10. The GSs are further divided into different grade groups, 1 to 5, with 5 being of highest risk for progression. The GS is generally considered a good prognostic factor (56, 57) where patients with GS 6 are classified as having a low risk for progression (grade 1), while patients with GS 8 (grade 4) and GS 9-10 (grade 5) are classified as high risk. GS 7, the most common score at diagnosis, has however a history of low prognostic value and should according to the new guideline from 2016 be separated into two different risk groups according to the Gleason pattern; gleason 3+4 (grade 2) versus 4+3 (grade 3), with significantly worse prognosis for the latter (55).

#### 1.3.1.5 *TNM staging*

The TNM staging system is a clinical classification that takes into account the tumor (T) and the presence of regional lymph node metastases (N) and distant metastases (M). Tumor stages T1 (non- palpable) and T2 (palpable) comprise tumors that are still confined within the prostate, while T3 and T4 stages comprise tumors that have penetrated the prostate capsule and spread into the surrounding tissue. In all T-stages, there is a possibility of metastatic spread, but the risk is increased in T3 and T4 stages. In addition, the tumor stages TX and T0 refer, respectively, to a primary

tumor that cannot be assessed and to no evidence of primary tumor. The M stages, MX, M0, and M1 refer, respectively, to distant metastases not assessed, not present, and present, while the N stages NX, N0, and N1 refer, respectively, to regional lymph node metastases not being assessed, not present, and present (National Cancer Institute; <https://www.cancer.gov/about-cancer/diagnosis-staging/staging>). For M1 patients the prognosis is poor, with an average survival of 24–48 months, while the prognosis for N1 patients is much better with a median cancer-specific survival (CSS) of 8 years (34).

#### 1.3.1.6 *Prognostic risk groups PC stages*

Prognostic risk groups for patients with localized PC, refers to the estimated risk – low, intermediate or high for progression into invasive growth and metastatic disease – and are based on the pre-treatment evaluation of T-stage, GS and PSA level (34). The overall PC stage, I to IV, refers to the progression state of the disease, with stage IV being the most aggressive stage represented by advanced disease (N1 and/or M1) – based on the PSA level, TNM staging and GS at diagnosis. Stage 4 is further divided in N1 versus M1 disease (American Cancer Society; <https://www.cancer.org/cancer/prostate-cancer/detection-diagnosis-staging/staging.html>).

### 1.3.2 Prostate carcinogenesis

#### 1.3.2.1 *Androgen dependence*

PC originates in the prostatic epithelium and is generally believed to arise from transformed luminal cells due to the androgen dependence of most PC tumors as well as to the expression of luminal markers in PC cells. The PC origin is, however, still a matter of some debate, with theories of a basal cell origin as well as a cancer stem cell or intermediate cell origin. Because PC stem cells do not express AR and therefore are truly androgen independent (20, 58, 59), their implication in PC initiation and/or progression is of central importance, especially concerning the transformation to a castration-resistant (CR) disease.

The normal prostate – as well as PC initiation and progression – is androgen dependent for growth, survival, and function (60). In the normal prostate, growth and maintenance of the prostate epithelium depends on paracrine signaling of andromedins (including growth factors and survival factors) produced by supporting stromal cells (smooth muscle and fibroblasts) in response to AR signaling (25). Androgen-induced AR signaling within the luminal cells is however required for the production of secretory proteins such as PSA and human kallikrein-2 and is furthermore growth suppressive, resulting in G0 growth arrest and terminal differentiation into secretory cells (26). During transformation into PC, the paracrine regulatory function mediated by stromal AR is lost and replaced by AR autocrine

regulation in the epithelial tumor cells. Furthermore, in these epithelial tumor cells, AR signaling goes from being growth suppressive to growth stimulatory (25, 26), usually measurable as an elevated level of PSA in the circulation due to a leaky epithelial compartment (REF). The transformed epithelial cells, now producing their own growth and survival factors become less dependent on the stromal cells. The oncogenic transformation of the AR, going from growth suppressive to growth promoting, is associated with an AR-regulated increase in *MYC* expression, an oncogene commonly upregulated in different human carcinomas (61).

#### 1.3.2.2 *PC initiation and progression*

PC is a multifocal and heterogeneous disease and is therefore believed to arise from multiple neoplastic foci that emerge and evolve independently, while metastases are believed to be clonally related and derived through selective advantages of individual clones (20). The progression of PC as well as cancer progression in general is a multistep process, that in PC involves – PIN, localized PC, locally advanced PC, metastatic disease and CR advanced and/or metastatic disease (**Figure 4**).

#### **PIN and PIA**

In PC, malignant tumors are believed to proceed from premalignant lesions known as PIN. PIN is characterized by luminal epithelial hyperplasia (increased proliferation) in combination with a reduction of basal cells, but with an intact basal membrane, resulting in intraglandular cell growth (**Figure.4**).

The premalignant cells of PIN are further characterized by cell depolarization and nuclear atypia and enlargement (20). (PIA) is a state of epithelial atrophy in combination with increased proliferation. Commonly, there is also increased stromal infiltration of inflammatory cells (11). PIA is a proposed precursor state of PIN as well as of PC (10, 11). The incidence and extent of both PIN (62) and PIA (11) are increased with age.

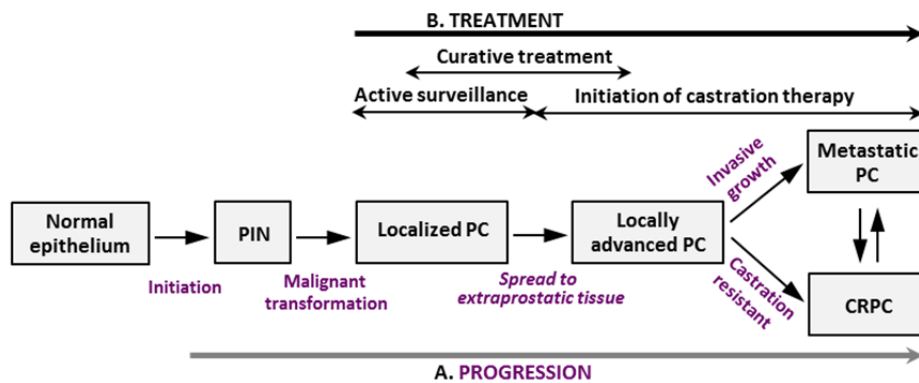
#### **Localized and locally advanced PC**

The progression of PIN to malignant lesions is characterized by the loss of the basal cells, disruption of the underlying basal membrane, and further invasion of tumor cells into the surrounding stromal compartment (**Figure 4**) (20). The primary tumor development goes from localized to locally advanced PC. Localized PC is confined within the prostate gland, while locally advanced PC is characterized by the penetration of the prostatic capsule and invasion of surrounding tissues, but without lymph node involvement or distant metastases (Cancer Research UK; <http://www.cancerresearchuk.org/about-cancer/prostate-cancer>).

#### **Metastasis**

Metastasis refers to the spread of tumor cells to distant locations, colonization, and regrowth (**Figure 4**). Metastatic PC almost invariably metastasizes to bone, and furthermore presents as characteristic osteoblastic rather than osteolytic lesions (20,

45) In addition to bone, PC also commonly forms metastases in the lymph nodes as well as in the lung and liver (63, 64). Extensive bone metastases are primarily responsible for PC patient morbidity and mortality (20, 45).



**Figure 4.** The progression of PC is a multistep process, and a potential curative treatment is only available before the transition to CR growth and/or metastatic disease. **A. PC progression:** Tumor cell transformation is normally initiated in premalignant lesions i.e. PIN. PIN lesions potentially can progress into prostate carcinoma, from localized to locally advanced and further to metastatic disease. Disease recurrence after primary castration treatment is referred to as CRPC, and normally occur as a metastatic disease but, however, sometimes within the prostate. **B. Treatment:** Localized PC is followed by active surveillance or is treated with curative treatment depending on the estimated progression risk. Locally advanced PC is treated with curative treatment under some conditions, but normally with castration (surgically or, more commonly, chemically) in combination with radiation. For CRPC, there is currently no curative treatment, but only palliative options.

### Castration-resistant PC (CRPC)

Disease recurrence after castration normally occurs after a couple of years, when the cancer has transformed into CRPC. Normally, disease recurrence occurs as metastatic disease, but it can sometimes occur locally in the prostate (65), in which case metastatic spread follows after CRPC transition (**Figure 4**). Relapse into CRPC is identified by a rising PSA level or from radiological and/or symptomatic progression despite ongoing castration therapy(34).

### 1.3.3 Treatment of localized PC

Treatment options for localized PC include active surveillance, radical prostatectomy, and radiation therapy. Radical prostatectomy and radiation therapy aim to remove the tumor, while active surveillance is carried out through regular controls followed by treatment in case of disease progression. Active surveillance is the choice for patients assessed with clinically low-risk PC, commonly in combination with a short life expectancy, while curative prostatectomy or radiotherapy commonly is the choice for patients with longer life expectancy and/or

with more poorly differentiated tumors (34). Active surveillance is, however, becoming a more common first option due to a history of over treatment of low-risk PC tumors (39).

In Sweden, removal of the prostate gland with radical prostatectomy is the most common curative treatment (34). Of patients that have undergone curative treatment in the form of prostatectomy or radiotherapy, about 30% will experience cancer relapse (66).

#### 1.3.4 Treatment of advanced PC

Advanced PC, including high-risk localized PC, locally advanced PC, and metastatic and/or CR disease, is characterized by invasive growth. Locally advanced PC has already penetrated the prostate capsule and spread to the surrounding extraprostatic tissue, while localized PC still grows within the prostate but is assessed as aggressive and rapidly progressive. Advanced localized PC should fulfill at least one of the following criteria: GS of 8 to 10,  $\geq$ T2c, or PSA > 20 ng/ml (34).

##### *Androgen deprivation therapy*

Castration, i.e. removal of circulating testosterone can be achieved by surgical castration (removal of the testes) or more commonly through chemical castration i.e. androgen deprivation therapy (ADT). ADT represents the cornerstone for primary treatment of locally advanced and metastatic HNPC. The beneficial effect of ADT in PC treatment was described already by Huggins and coworker in 1941 for which they were later awarded the Nobel Prize (67, 68).

During ADT, the production of testicular testosterone is suppressed by the administration of GnRH agonists or antagonists that disrupt the hypothalamic-pituitary-gonadal axis and hence the production of testosterone by the Leydig cells of the testes (**Figure 2**). The effect of androgens can be further blocked by AR antagonists, for example, bicalutamide, which are sometimes administrated together with ADT in what is known as total androgen blockade (34). The efficiency of ADT is referred to as the achieved testosterone level, where a castrated level is defined as < 0.5 nmol/l in comparison to the normal level of about 10 nmol/l (40).

ADT initially displays good clinical effect with tumor regression (decreased proliferation and increased apoptosis) and symptomatic ease, this in approximately 80% of the patients (34), although with considerable variation in response durations between patients (69). Tumor regression results in an approximately 30% reduction in prostate volume after a couple of months of treatment (70), a progression that normally can be followed by a decrease in circulating PSA (65), both due to the reduced tumor burden but likely also due impaired secretory function in persisting cells. Unfortunately, however, ADT fails, normally within a few years, and the disease relapses into lethal CRPC (65), this commonly as a metastatic disease.

ADT is therefore only palliative, and advanced and metastatic disease considered incurable.

### ***Locally advanced and metastatic PC***

For patients with locally advanced PC, local treatments like surgery and radiation therapy are less likely to eliminate the cancer by themselves but can sometimes be offered in the form of radiation therapy. The first-line option for locally advanced PC is, however, normally a combination of radiotherapy and ADT (71), due to the androgen dependence of most prostatic tumors.

Hormonal therapy also represents the first-line option for metastatic disease (34), this commonly in combination with other treatments, due to observed survival benefits compared to the administration of ADT alone. The combination therapy of the cytotoxic drug docetaxel together with ADT is since recently the new recommended primary treatment for patients with HNPC with a high metastatic burden. A combination therapy of abiraterone acetate (CYP17A1 inhibitor, **Figure 3A**) and ADT is also a promising new primary treatment for patients with metastatic HNPC, this regardless of tumor volume (38, 69, 72). Although these combination therapies have improved overall survival in recent years, most patients will relapse with time.

### ***Treatment of CRPC***

Recurrent CRPC, commonly metastatic, represents an aggressive and highly resistant disease that despite recent therapeutic advances still has an average survival of only approximately 3–4 years (69).

With some exceptions, there is a reactivation of AR signaling during castrate conditions, a reactivation that is considered crucial both for CRPC transition and for its continued growth (31, 38), due to which hormonal therapy in the form of ADT continues to represent the backbone therapy also at this stage of the disease. The continued reliance on AR signaling in CRPC has in recent years also been confirmed from the survival advantages seen for androgen-targeted and AR-targeted therapies using abiraterone acetate and enzalutamide (**Figure 3**), respectively, both approved for chemotherapy-naïve and post-chemotherapy metastatic CRPC treatments (31, 73). In addition to the novel androgen/AR-targeting therapies abiraterone acetate and enzalutamide, several other potent drugs are now available for combination therapy with ADT, including – chemotherapy with cabazitaxel and docetaxel and radiation therapy with radium-223 (69). These new targeted therapies offer no cure, but increase overall survival by a number of months before relapse and acquired resistance (69).

Furthermore, for treatment of bone metastases, radium-223, zoledronic acid (inhibition of osteoclasts) and denosumab (targeting of RANK ligand) could be considered, this to prevent and delay cancer-related skeletal events in CRPC (74).

Neuroendocrine PC (NEPC) represents an aggressive subtype of CRPC that has low or no dependence on the AR, and it believed to develop as a resistance mechanism to

castration and AR-targeted therapies (75), supported by recent observations that acquired resistance to abiraterone acetate and enzalutamide is associated with increased NED (76, 77).

### 1.3.5 Resistance mechanisms in CRCP

Disease progression leading to CRPC transition as well as the progression of CR disease is most commonly AR-driven, at least during the earlier stages, and involves the acquisition of different resistance mechanisms related to AR signaling (31, 38). There is, however, a subset of CRPC tumors that progress with little or no dependence on classical AR signaling, and this is often in association with lineage plasticity, the loss of luminal markers, and the acquisition of diverse NE features, hence the formation of NE-like tumor cells and further development into NEPC (31, 78). Dedifferentiated, AR-independent tumors without typical NE features can also evolve, commonly referred to as anaplastic PC (75).

Both AR-dependent and AR-independent resistance mechanisms are believed to evolve through adaptation to castration and AR inhibition. Resistance mechanisms to ADT and AR-targeted therapies can be divided into three general categories – restored AR signaling, AR bypass, and complete AR independence (31). Concerning AR signaling in CRPC, it has furthermore been shown that the AR regulates a distinct but overlapping transcriptional program compared to in HNCP (28).

#### 1.3.5.1 Restored AR signaling

Restored AR signaling can be achieved through several mechanisms, including AR amplification, AR-activating mutations, AR splice variants (ARVs), and intratumoral steroidogenesis. Restored AR signaling through any or a combination of several of these mechanisms is relatively common in CRPC (31).

#### **AR amplification**

AR amplification (**Figure 5.1**), which results in elevated AR levels, enhances the response to the limited androgens, and this is a common mechanism in CRPC and is estimated to occur in up to 30% of all patients. AR amplification is, however not common in HNCP and the mechanism hence considered to be treatment dependent (31).

#### **AR-activating mutations**

AR-activating mutations in PC are mainly represented by four different point mutations (L702H, W742C, H875Y, and T878A), all located in the ligand binding domain of the AR. These mutations share the property of promiscuous AR activation by non-canonical steroid ligands such as adrenal androgens, estrogen, and progesterone or, in the case of L702H, by glucocorticoids (**Figure 5.2**). These mutations can also convert anti-androgens into AR agonists. AR mutations are rarely



found in HNPC but are estimated to be present in about 15–20% of patients with CRPC (31).

#### **Ligand independent AR activation**

The expression of ARVs is increased in response to ADT and in resistant tumors, but can also be found in normal tissue. In these variants, truncation and exon skipping of AR mRNA results in a substantial or complete loss of the ligand binding domain, rendering some variants constitutively activated and androgen independent (**Figure 5.3A**), for example, ARV7, the most common ARV found in PC. The expression of constitutively active ARVs is believed to be involved in CR transformation and in acquired resistance mechanisms to CR-targeted therapies such as the AR-targeting drugs abiraterone acetate and enzalutamide (**Figure 3**) (31). ARV7, the best studied ARV, has been shown to regulate both AR-regulated genes and a unique set of AR-independent genes, an indication of an overlapping but distinct role compared to full-length AR in PC cells (79).

Ligand independent AR activation in CRPC can also be achieved through crosstalk phosphorylation by downstream tyrosine kinases (**Figure 5.3B**), for example from signal transduction pathways from several growth factors, including insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), epidermal growth factor (EGF) and HER-2/neu (80, 81). Furthermore, signal transduction by the neuropeptide neurotensin (NT) and gastrin releasing peptide (GRP) has also been reported to phosphorylate and activate the AR in the absence of androgens (82, 83).

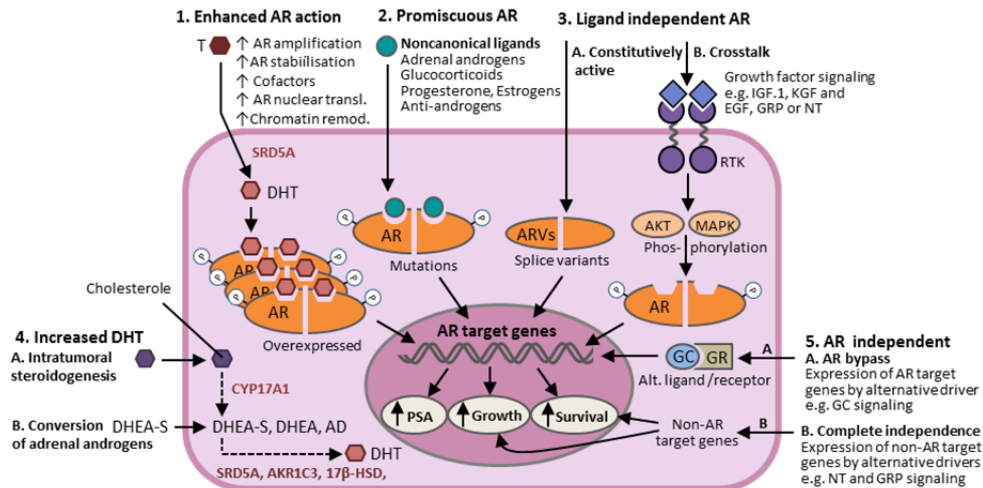
#### ***Intratumoral steroidogenesis and the utilization of adrenal androgens***

Medical or surgical castration reduces circulating levels of serum testosterone by >90%; however, physiologically significant amounts of intratumoral androgens remain after ADT in the prostate and in metastatic CRPC (79). A primary source of these residual (prostatic) androgens is the adrenal androgens DHEA-S, DHEA, and AD, which are not affected by ADT using GnRH antagonists and/or agonists (**Figure 2 and 3A**). These precursors can be further converted into testosterone and DHT in peripheral tissues (**Figure 3B and 5.4B**). In addition, intratumoral de novo biosynthesis from cholesterol might also be initiated to increase the pool of DHT. For this, the tumor cells upregulate enzymes for steroidogenesis, for example CYP17A1, ACKR1C3, SRD5A, and 17 $\beta$ -HSD, a mechanism that is commonly observed in CRPC (**Figure 3 and 5.4A**) (79).

#### **1.3.5.2 AR bypass**

AR bypass refers to the mechanism in which AR-targeted genes remain regulated, but without the involvement of the AR, and are instead regulated by a different hormone receptor. One example, observed in both cell culture and in clinical samples, is glucocorticoid receptor stimulation by glucocorticoid, which regulates a subset of AR-targeted genes in PC (**Figure 5.5A**) (31).

The glucocorticoid receptor and the AR share substantial homology within the DNA-binding domain, which could explain the appearance of this mechanism. Glucocorticoids are normally administered together with abiraterone acetate during AR-targeted therapy, this in the form of prednisone, to decrease symptoms of mineralocorticoid excess, which might have an impact on this resistance mechanism. The progesterone receptor and the mineralocorticoid receptor are two other nuclear steroid hormone receptor family members with structural resemblance to the AR, and therefore are also potential candidates for AR bypass mechanisms in PC. The expression of progesterone receptor has been demonstrated in prostate tumor cells in some, but not all, studies, and a high level of progesterone receptor staining in primary PC tumors has been reported to be associated with clinical recurrence (31).



**Figure 5.** Resistance mechanisms associated with the transition to CR growth and with acquired resistance to CR-targeted therapies. Most of these mechanisms are associated with restored and enhanced AR signaling, for example, through AR amplification (1), AR mutations (2), ligand-independent AR activation (3), or through intratumoral steroidogenesis (4A). The AR also commonly becomes hypersensitive due to, for example, increased stabilization and nuclear transfer, while its transcriptional activity is enhanced and altered by the expression of various cofactors as well as through chromatin remodeling. Ligand-independent AR activation can be achieved through the expression of constitutively active ARVs (3A) or through cross-activation by other signaling pathways (3B). Furthermore, AR-targeted genes can be regulated through bypass mechanisms (5A) where AR-response genes are alternatively activated by a different driver, for example, via glucocorticoid signaling. In addition, completely AR-independent mechanisms (5B) sometimes dominate, commonly in neuroendocrine PC (NEPC) and in PC stem cells, where other genes for growth and survival are transcribed and expressed than those that are under AR regulation. The illustration is reproduced and redrawn with inspiration from BJ Feldman et al (84).

### 1.3.5.3 *AR independent mechanisms*

In these AR-independent tumors, growth and survival is stimulated by non-AR-targeted genes and are activated by alternative drivers (**Figure 5.5B**). NE-like tumor cells, like normal NE cells, are known to express various growth and survival stimulating factors, for example, the neuropeptides NT and GRP (82, 83).

## 1.4 NEUROENDOCRINE CELLS

### 1.4.1 The normal NE cell

NE cells can be found in epithelial linings throughout the body, including the gastrointestinal tract, respiratory tract, the urogenital system, and the skin. Furthermore, they are found in endocrine glands, such as the hypothalamus and the thyroid, and in exocrine glands, such as the prostate and the mammary glands (24).

The origin of prostatic NE cells is still a matter of debate, but they are likely derived together with the basal and the luminal epithelial cells from a common endodermal pluripotent stem cell. Another suggestion is that the basal and the luminal cells have a common endodermal origin, while the NE cells instead are of neurogenic origin and are derived from migratory neural ectoderm (15, 24). Despite this uncertainty, it is believed that the prostate epithelium comprises a small number of tissue stem cells with basal-cell characteristics that are capable of tissue regeneration. Furthermore, it is clear that NE cells can transdifferentiate from luminal epithelial cells, giving rise to an NE-like cell type (15, 21, 52), thus mirroring the close relationship between the cells of the prostatic epithelium.

The normal NE cell of the prostate is terminally differentiated, postmitotic, and AR negative, and hence androgen independent (21, 52). These cells express a mixed CK pattern of basal CK5 and luminal CK8 (22) and are further characterized by high resistance to apoptosis, partly due to their low rate of proliferation (21, 52).

#### 1.4.1.1 *Characterization and function*

The NE cells are a normal component of both the glandular and ductal epithelium of the prostate, representing a minor cell population estimated to account for less than 1% of the epithelial cells (85), and they are found at the highest density within the periurethral and the ductal regions (86). The normal NE cell displays a combination of neuronal and endocrine features, having a partly neuron-like morphology and an endocrine-like secretory mechanism. In the epithelium, the prostatic NE cells are scattered within the basal cell layer and exist in either an open or a closed form (24). The open cell type extends cytoplasmic processes toward the lumen thus sensing the prostatic secretions and the environment, while both the opened but mostly the closed form extends dendritic processes between adjacent cells (52) that are responsible for cell-cell communication both with surrounding epithelial cells and

with each other. In addition, both afferent and efferent nerves innervate the prostatic NE cell population (24).

The NE cells are further characterized by the presence of cytoplasmic dense core granules of marked heterogeneity in size and form containing hormone peptides for storage and secretion, secreted either constitutively or in response to an incoming stimulus. Due to the wide diversity of these granules, as well as the diversity in secretory products produced by the NE cells, the NE cell population is believed to represent a number of subpopulations, each with its own set of secretory products and characteristic mode of regulation. Examples of NE secretory products include CHGA, NT, GRP, serotonin, parathyroid hormone-related protein (PTHrP), calcitonin, human chorionic gonadotropin (hCG), somatostatin, vascular endothelial growth factor, and glucagon (52).

Although morphologically different from the other epithelial cells of the prostate, the NE cells can normally not be recognized only with histological examination, and they need to be identified through immunohistochemical staining for specific NE markers (85, 86).

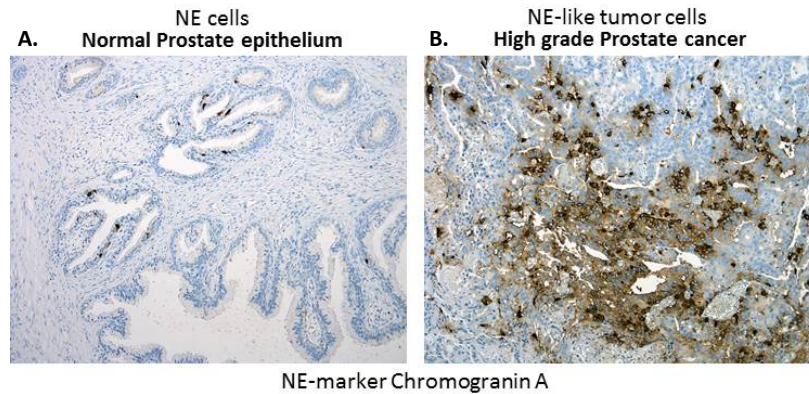
NE markers that are commonly used for identification include NSE (an isoenzyme), CHGA (a secretory product), neural cell adhesion molecule 1 (NCAM1 or CD56; a surface glycoprotein), and synaptophysin (SYP, a synaptic vesicle glycoprotein), which are expressed in most but not all NE cells (24, 51, 78). These markers are commonly also used to identify NE-like tumor cells both *in vitro* and in clinical samples, this with varying degree of correlation between focal NED and PC progression (24, 52).

In clinical settings, synaptophysin is considered the most sensitive and CHGA the most specific NE marker, while neuronal NSE in recent years has been proven to be rather unspecific for NE and NE-like tumor cells (24, 51).

The NE cells are involved in regulating the growth, survival, and differentiation of surrounding epithelial cells mainly through paracrine stimulation (21). In addition, the NE cells are involved in autocrine, endocrine, and neurocrine signaling mechanisms and are themselves under neural control (24). The function of NE cells is thus primarily to maintain normal prostatic differentiation and function, including secretory processes from the luminal cells (52), and might therefore during normal circumstances have a protective role against prostatic carcinogenesis. African-American males are known to have a lower number of prostatic NE cells compared to the general population (24) and are also more prone to the development of PC (11, 34).

## 1.4.2 Neuroendocrine-like tumor cells

During the progression of PC, there is commonly an accumulation of NE-like tumor cells within the prostate adenocarcinoma, especially in association with the transition to CR growth (87-90) and during the progression of CR disease (**Figure 6**), an increase that is accompanied by treatment resistance and poor prognosis (21, 87, 88).



**Figure 6.** Morphological presentation of normal prostate epithelium (A) and high grade adenocarcinoma with accumulated NE-like tumor cells (B), stained for CHGA. During PC progression, there is a general de-differentiation with gradual loss of glandular structures. In parallel, there is increased NED as identified by the expression of NE markers such as CHGA. NED is generally considered to be correlated with tumor grade and aggressiveness/de-differentiation. Immunohistochemical staining of CHGA in primary prostate tissue (TURP specimen), photographed with a light microscope.

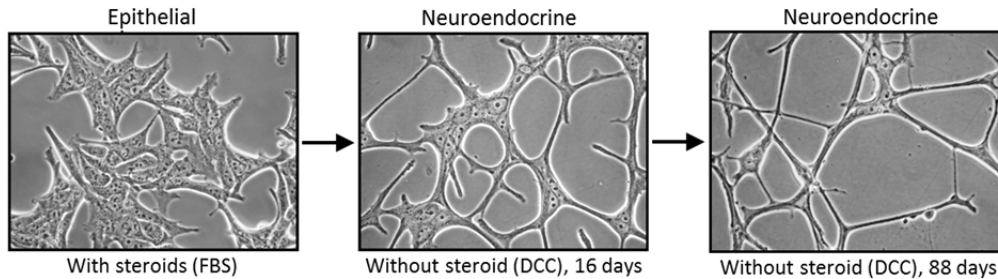
### 1.4.2.1 Neuroendocrine differentiation (NED)

Prostatic NE-like tumor cells are believed to arise through NED, which is a transdifferentiation from malignant luminal epithelial cells (**Figure 6**). The transdifferentiation pathway is supported by the fact that NED can be induced *in vitro* through steroid deprivation of PC cell lines with luminal characteristics as well as by the genetic resemblance between the NE-like tumor cells and luminal-derived cancer cells (21, 75).

NE-like tumor cells thus display a mixture of luminal markers (e.g. luminal CK8 and CK18) and NE/neuronal markers and are different from, and should be distinguished from, the normal NE cell population that presents with basal or intermediate characteristics (21, 22, 52). Furthermore, the NE-like tumor cells express several proteins not found in normal NE cells, for example, BCL2 (an anti-apoptosis marker) and AMACR (a marker associated with PC, including NE-like tumor cells) (21). Although NE-like tumor cells express secretory neuropeptides and NE/neuronal markers, in clinical samples they sometimes lack typical cellular processes that are characteristic of normal NE cells, and instead have a morphology that is similar to

the luminal-derived cancer cells (52). *In vitro*, however, steroid deprivation induced NED in androgen-sensitive PC cells, induces a morphological transformation toward a neuronal-like phenotype of tapering cell bodies and dendrite protrusions (**Figure 7**) (91, 92).

The NE-like tumor cells are further characterized by a ceased proliferation and of androgen independence; no or low expression of the AR and of AR targeted genes, such as PSA (21, 52).



**Figure 7.** *In vitro* steroid deprivation of the androgen sensitive PC cell line LNCaP is associated with NED, as represented by decreased proliferation, the upregulation of diverse NE/neuronal markers, and a neuron-like morphological transformation, i.e. tapering cell bodies and dendrite protrusions. Phase contrast imaging of LNCaP cell during basal conditions (FBS) and during steroid deprived conditions (DCC).

### Escape mechanism

NED in PC is generally believed to represent an escape mechanism driven by the castration therapy and by AR inhibition (21, 75), and this is supported by observations of induced NED in response to androgen depletion in many preclinical studies (92-94) as well as increased NED after ADT in clinical studies (89, 95). Accordingly, accumulated NED within PC tumors has been correlated with CRPC, both its acquisition (87-90) and progression, including a poor clinical prognosis (87, 96). However, there is little evidence for an independent impact of NED on progression (53). Accumulated NED has also recently been observed to be associated with AR-targeting therapies with enzalutamide (an AR antagonist) and abiraterone acetate (an androgen biosynthesis inhibitor) in CRPC (**Figure. 3**). The increased NED following abiraterone acetate or enzalutamide treatment is correlated with poor survival (76, 77), likely due to acquired resistance mechanisms. With the growing use of highly potent AR-targeting drugs like abiraterone acetate and enzalutamide, there is an expectation of increased treatment-related NEPC in the future, and this concern has resulted in an increased focus on the pathogenesis of NED (97).

### Cancer promotion

NED is believed to play an active role in CRPC transition and growth, partly due to the secretion of different growth-promoting neuropeptides from the NE-like tumor cells, for example NT and GRP (98-100), both of which also have been found to

activate the AR in the absence of steroids(82, 83).The NE-like tumor cells are also highly resistant to apoptosis and to most chemotherapeutic and radiation therapies, partially due to their low rate of proliferation (97, 101, 102). Furthermore, the adaptive state of highly resistant and slowly proliferating NE-like tumor cells is generally believed to represent a temporary state, and NED is a reversible process (103), i.e. the NE-like tumor cells can differentiate back to a proliferating state at the end of treatment or at the time of recurrence or, as suggested by Abrahamsson et al (52), they can gain selected traits from the NE cells such as the secretion of different neuropeptides but still remain proliferative.

### **Reprogramming**

It is evident that the process of NED relies on a network of transcriptional reprogramming, including epigenetic modifications (75, 104, 105), for example, the downregulation of repressor element-1 silencing transcription factor (*REST*, involved in chromatin remodeling) that is essential for the acquisition and maintenance of neuronal features (106-108), and the upregulation of the polycomb histone methyltransferase enhancer of zeste 2 (*EZH2*), which is associated with both CRPC (109, 110) and NED/NEPC (104, 105, 111). In recent years, it has also become evident that the NE-like tumor cells upregulate different stem cell markers, suggesting stem cell-like functions of these cells (75, 86, 106, 112). The process of NED might therefore be closely associated with dedifferentiation and reprogramming.

Other alterations frequently associated with NEPC include the loss of tumor suppressors *RBI*, *PTEN*, and *TP53*, mutations and amplification of *MYCN* (involved in the regulation of neural differentiation) and *AURKA* (involved in cell cycle regulation), upregulation of *ASCL1* (a pro-neural transcription factor), activation of mitotic programs, and genomic instability (75, 78, 86, 113, 114). In addition, NE-like tumor cells are generally considered to be AR low/negative, although several recent studies have confirmed AR expression in a substantial fraction of NEPC as well as AR expression in mixed tumors of NEPC and conventional adenocarcinoma (75, 86). Although many interesting candidate drivers for NED and the development of NEPC have been proposed in recent years, for example, *AURKA* and *MYCN*, a targetable driver resulting in clinical survival benefits has so far not been identified (113, 115). A better understanding of the underlying biology of NED and NEPC is still required in order to efficiently identify further therapeutic targets.

#### **1.4.2.2 Neuroendocrine PC (NEPC)**

principal, all adenocarcinomas of the prostate, as well as the benign prostatic epithelium, have some degree of NE-marker positive cells, normally presented in a sparse and scattered appearance. In advanced HNPC, an accumulation of focal NE-like tumors cells is a relatively common event, considered to occur in about 5–10%,

(85, 86, 116), while *de novo* pure NE tumors, like small cell prostate carcinomas (SCPC), are very rare at primary diagnosis (0.5-2%) (116-118). The incidence of both focal NED (88-90, 95) and SCPC (117, 119) are however more frequent in the CR stage of the disease. NEPC therefore mainly develops from adenocarcinoma and furthermore consists of a spectrum of subtypes ranging from conventional adenocarcinoma with accumulated NED to pure forms of NE tumors, of which SCPC represents the most aggressive form and is characterized by poorly differentiated, highly proliferative tumor cells with neuronal characteristics (53, 86, 117, 118). Furthermore, in the advanced stages, mixed tumors of conventional adenocarcinoma and SCPC can be observed (86) as well as clinical features of SCPC or “anaplastic” PC in morphologically heterogeneous CRPC (53).

NEPC is characterized by aggressive and rapid disease progression, often with an atypical pattern of metastatic disease, including visceral metastases, and a low PSA serum level that is out of proportion in relation to the tumor burden (120). These tumors commonly display clear features of NED, including neuronal characteristics, but might sometimes display an aggressive AR-independent phenotype without features of NED, sometimes referred to as anaplastic PC and sometimes included in the term NEPC (75, 117, 120).

Furthermore, according to autopsy studies, aggressive forms of NEPC have been considerably underestimated until rather recently, and NEPC is today estimated to be relatively common with up to 25% of lethal PC cases considered to be driven by NED(120).

#### **NEPC – diagnosis and treatment**

In the identification of NED, CHGA is the most commonly used and the most specific NE marker (85). However, CHGA is likely not optimal in defining NED due to the heterogeneous nature of NE tumor cells. There is also a lack of clear relationship between CHGA expression and tumor aggressiveness. Serum markers such as CHGA and/or NSE can be used for an initial screening but are not reliably high in all patients with increased NED or transformed NEPC (75, 120, 121).

Optimally, several serum markers should be followed simultaneously, and metastatic tumor biopsies are of great value as well as the evaluation of circulation tumor cells. Furthermore, the NED process should be followed in response to novel treatments to better be able to predict treatment responses and for increased knowledge concerning ongoing resistance mechanisms. Lastly, new and better biomarkers are needed.

NEPC has poor response to androgen modulation and AR-targeting therapies, with the most aggressive form, SCPC, being almost completely unresponsive. SCPC does, however, display frequent but short-lived responses to platinum-based chemotherapies, e.g. cisplatin (53, 120), but no more treatment options are available for this highly resistant patient group. There are, however, many ongoing preclinical



studies on different candidate targets, including targeted therapies against *ARUKA*, *MYCN* and *PARP* (113, 115, 122).

Because NED is primarily considered to be a therapy-driven adaptive process to ADT and AR-targeting therapies, and because NE-like tumor cells are characterized by high resistance to most therapies (53, 97, 119, 120), a biomarker that could differentiate for the predisposition of NED before primary treatment would be highly valuable.

## 1.5 MIDKINE

### 1.5.1 General background

Midkine (MDK) is a heparin-binding growth factor and cytokine that is involved in diverse biologic functions such as promoting survival, growth, migration, differentiation, and gene expression (123).

MDK was discovered in 1988 as a retinoic acid response gene in embryonic carcinoma (124), and was further confirmed to be highly expressed in response to retinoic acid during embryogenesis, especially during the mid-gestation period, hence its name (**mid**-gestation – **cytokine**). MDK is downregulated around birth and is thus developmentally regulated. During embryogenesis MDK is involved in neurogenesis, EMT, mesoderm remodeling and stem cell renewal. The involvement of MDK in neurogenesis is integral to proper development, and MDK is a neurotrophic factor involved in neurite outgrowth and in the survival of neurons and neural progenitors (123, 125, 126). Pleiotrophin (PTN) is a structural relative of MDK, and together they comprise a small family of heparin-binding proteins. MDK and PTN share about 50% sequence homology and have very similar three-dimensional protein structures. Furthermore MDK and PTN share receptors and are involved in similar activities, including fibrinolytic, anti-apoptotic, mitogenic, transforming, angiogenic, and chemotactic activities (127). MDK and PTN both display specific, but overlapping, expression patterns during neuronal development where PTN peaks after MDK, around birth (128).

The importance of MDK and PTN for development has been confirmed through deficiency studies in mice where homozygous deletion of either *Mdk* or *Ptn* results in slight developmental abnormalities mainly associated with the neurological tissues. However, homozygous deletion of both *Mdk* and *Ptn* results in severe developmental abnormalities, including frequent prenatal death, reduced sized, a shorter life span, and female infertility due to impaired follicular maturation(123, 129, 130). MDK deficiency during development is therefore largely considered to be compensated for by PTN.

Both MDK and PTN are downregulated after birth. In the adult, MDK is expressed at negligible levels, and the expression is restricted to specific sites such as in the kidneys where very low levels are found. Because MDK is a secreted protein, low levels can also be found in body fluids such as serum, plasma, and urine (131). MDK is, however, induced and highly expressed during tissue repair as well as in many pathological conditions such as in inflammatory diseases and in most human carcinomas (123, 125, 127, 131). PTN also has very restricted expression in the healthy adult but sometimes is upregulated in association with human carcinoma. In general, however, MDK is expressed more intensely and in a wider range of human carcinomas than PTN (123, 127, 128).

### 1.5.2 MDK regulation and protein structure

The human MDK gene (*MDK*), located on chromosome 11 (site p11.2), is composed of four coding exons (130). Several splice variants have been reported at the mRNA level, of which at least two have also been detected as protein products – VA-MDK and tMDK. VA-MDK has two extra amino acids (valine-alanine) at the N-terminus, while tMDK lacks exon 3, which corresponds to the N-terminal domain (131). VA-MDK is suggested to be naturally expressed simultaneously with conventional MDK *in vivo*, but a potential biological difference concerning activity and function compared to conventional MDK has so far not been found. It is, however, suggested that VA-MDK might represent a more stable product due to the importance of the N-terminus in MDK stabilization and thus might have a longer half-life. tMDK has been reported to be tumor specific and to be associated with lymph node metastasis in clinical samples and with invasion *in vitro* (130).

As mentioned above, MDK is induced by retinoic acid, and accordingly a retinoic acid response element (RARE) can be found in the *MDK* gene promoter region. Furthermore a hypoxia response element as well as an NF- $\kappa$ B binding site can be found within the promoters responsible for hypoxia (mediated by HIF1 $\alpha$ ) and NF- $\kappa$ B-induced MDK expression, respectively (123, 130, 132). The promoter region also contains two binding sites for the Wilms tumor suppressor gene product (WT1), which is responsible for suppressing MDK expression (123) and loss function of this gene product in Wilms tumors is reported to account for the overexpression of MDK in these tumors (133, 134). NF- $\kappa$ B has been reported to induce MDK expression in PC, and the induction of MDK has been confirmed to support survival (135). Furthermore, the expression and activity of NF- $\kappa$ B has been associated with PC stem cells (136) as well as been described as a transcription factor associated with NED (137).

Translation of MDK mRNA results in a low molecular-weight protein of 13-kDa that is rich in basic amino acids and cysteines. The MDK protein is composed of two structural domains, the N-domain (which is close to the N-terminus) and the C-

domain (which is close to the C-terminus), and the C-domain contains two heparin binding clusters that are important for the biological activity of MDK. Spontaneous dimerization of two MDK proteins sometimes occurs and is considered essential for some MDK activities. The N-domain appears to be important for the stability of MDK and is also involved in the dimerization process (123, 130).

### 1.5.3 Cell signaling and biological activity

The biological activity of MDK is mainly executed through intracellular signaling, where MDK acts by binding to complexes of multiple transmembrane receptors (127, 128). Due to the promiscuous nature of MDK and the great number of available receptors, as well as due to alternative downstream signaling pathways, MDK has been implicated in a diverse range of biological processes. In addition to receptor-mediated signaling, MDK also exerts some of its effects directly within the nucleus, and these activities are associated with the promotion of survival and with an increase in ribosomal RNA synthesis (130). However, exactly how these mechanisms are executed is still unknown. The MDK receptor, LDL receptor-related protein 1 (LRP1), is however involved in the MDK nuclear translocation, through endocytosis of bound MDK. In the cytoplasm, internalized MDK interacts with the cytoplasm-nucleus shuttle proteins nucleolin and laminin that translocate MDK to the nucleus (138, 139).

**Table 1 Receptors of MDK**

| Receptors   | Gene symbol                | Activity  |
|---|----------------------------|---|
| Syndecan 1, 3 and 4                                   | <i>SDC-1, -3, -4</i>       | Neurite outgrowth (130, 140, 141)   |
| Glypican 2  | <i>GLC2</i>                | Adhesion and neurite outgrowth (138, 142)   |
| Neuroglycan C   | <i>NGC</i>                 | Neurite outgrowth (138, 142)<br>Migration (138, 142, 143)   |
| Protein tyrosine phosphatase $\zeta$                  | <i>PTPZ</i>                | Abolished dephosphorylation of e.g $\beta$ -catenin (138)<br>Survival (130)                                       |
| LDL lipoprotein receptor-related protein 1            | <i>LRP1</i>                | Survival of neurons (130, 138, 144),<br>Anchorage independent growth (138, 145)<br>Nuclear targeting of MDK (139) |
| Anaplastic lymphoma kinase                            | <i>ALK</i>                 | Cell growth (130, 146)  |
| Integrins;<br>$\alpha 4\beta 1$ and $\alpha 6\beta 1$ | <i>ITGA4, ITGA6, ITGB1</i> | Neurite outgrowth, migration (142, 147)   |
| Notch 2   | <i>NOTCH2</i>              | EMT and drug resistance (130, 142, 148)   |

The PTP $\zeta$ , the syndecans, glypican 2 and neuroglycan C are proteoglycans, the others are not.

MDK has many identified receptors, but none so far are specific for the MDK/PTN family and many have a relatively low MDK binding affinity. It is therefore likely that MDK frequently binds to more than one component in the receptor complex in order to exert its function (130). MDK has, however, been confirmed to have a high

binding affinity towards both heparan sulfate and chondroitin sulfate chains in addition to heparin (a highly sulfated form of heparan sulfate). Several of the MDK receptors are therefore represented by proteoglycans in which the glycosaminoglycan portion is responsible for recognition and high-affinity binding of MDK (130, 142). For some of the receptors and proposed MDK-mediated activities see **Table 1**.

Downstream signaling pathways that are important for MDK signal transduction include PI3K/AKT, MAPK, ERK1/2, NCID (the activated intracellular part of Notch 2), Src, PKC, and Paxilin, and transcription factors that are affected by MDK signaling include NF- $\kappa$ B, Hes-1, and JAK/STATs (130). For the promotion of survival, MDK-dependent suppression of caspase-3 and the activation of Bcl-2 have been observed, while activities associated with differentiation, neurite outgrowth, and migration involve cytoskeletal remodeling (130, 138). Signaling via ALK is involved in increased proliferation, while signaling via Notch 2 is involved in EMT and in drug resistance mechanisms (130).

#### 1.5.4 MDK in cancer

As mentioned above, MDK is induced and upregulated in a wide array of pathologies, including inflammatory diseases such as rheumatoid arthritis and multiple sclerosis as well as in most human carcinomas. MDK is also upregulated during tissue generation and tissue repair as well as during hypoxia and in association with high blood pressure (123, 125, 130, 142).

The association with MDK and cancer is constantly getting more support, and the role of MDK in cancer and the applicability of MDK as a therapeutic target has gained increased attention in recent years. At present, MDK is reported to be overexpressed in over 20 different human carcinomas (127, 131), including PC (135, 149, 150), and an elevated MDK level in the circulation has been correlated with poor clinical outcome in various human carcinomas (131, 151-155). Collectively, MDK is considered to be a potential tumor biomarker (131, 151-155) and diagnostic tests, measuring circulating MDK is currently entering the clinic for several different carcinomas (131). Furthermore, MDK is also suggested to be a potential treatment target in some human carcinomas (154, 156-162).

In some human carcinoma, including PC, MDK is considered to be an early biomarker, detected already in premalignant lesions (127). As a progression biomarker, the early induction of MDK during cancer progression is an important feature because it allows for the early detection of cancer development and can potentially be used to discriminate between aggressive and non-aggressive cancers.

#### 1.5.4.1 *Biological function and impact*

MDK, being a pleiotropic protein, has been associated with many cancer-promoting features in different human carcinomas, including proliferation (163, 164), survival (165), transformation (166), angiogenesis (167, 168), and metastasis (169, 170), and thus it is considered to play an active part in tumor development and progression and to be associated with aggressiveness. In PC, MDK has been associated with cell survival (135) and proliferation (171). MDK has also been associated in numerous studies with resistance to chemotherapy (154, 156-161). For example, it possess a cytoprotective role against doxorubicin (interferes with DNA replication) in neuroblastoma (159), while it has varying effects with platinum-based chemotherapies cisplatin (interferes with DNA repair) in different cancer forms. In most carcinomas studied so far, MDK expression has been associated with a cytoprotective role against cisplatin (154, 156, 160, 161), but in some studies MDK expression has instead been associated with enhanced cytotoxicity of cisplatin (157, 158). The possible suppressing or enhancing effect of MDK on platinum-based chemotherapy in PC remains to be determined, but it opens up an interesting area for exploration because platinum-based chemotherapies are the treatment of choice for aggressive NEPC (53, 122). Collectively, however, MDK seems to have a strong value as a therapeutic predictor, at least regarding cisplatin, as well as representing a possible target in combination with cisplatin therapy for certain patient groups.

## 2 AIMS

The overall aim of this thesis was to evaluate the role and impact of MDK in PC, with a specific focus on the CR state of the disease and on castration-induced transformations, including NED.

*The specific aims are outlined as follows:*

- ❖ To evaluate the expression of MDK in relation to PC progression and NED
- ❖ To investigate the function of MDK and its dependence on androgens and/or AR-signaling
- ❖ To evaluate the clinical value of MDK as a biomarker in advanced prostate cancer
- ❖ To study MDK in relation to castration-induced transformation in PC as well as its relation to the CR-state

## 3 MATERIAL & METHODS

In this thesis, the effects of castration on PC pathogenesis and progression, ultimately leading to CRPC transformation, including NED and other resistance mechanisms, have been studied through *in vitro* experiments and through the analysis of diverse clinical materials. In the studies conducted in this thesis, the primary focus has been on the expression and function of MDK and its relation to AR signaling, dedifferentiation, and NED.

### 3.1 CLINICAL SAMPLES

For our studies of PC in humans, clinical samples in the form of primary prostatic tissue and plasma were evaluated. Prostatic tissue included transurethral resection of the prostate (TURP) specimens, needle biopsies, and tissue from prostatectomies. The use of patient material was approved by the local ethics committee.

#### 3.1.1 Transurethral resection of the prostate (TURP) (Paper I)

Archival TURP specimens were used to evaluate the expression of MDK in early compared to advanced primary PC as well as to evaluate any association of MDK with NED. The TURP specimens were collected from patients with early, low-risk HNPC (T1b, n = 29) and patients with progressed CRPC (n = 24). For the evaluation, MDK, the NE marker CHGA, and the neuronal marker tubulin-beta III (TUBB3) were stained on sequential sections with immunohistochemistry (IHC) techniques, and the expression pattern was compared between groups and between markers. Triple immunofluorescence (IF) was performed on a selection of specimens. It should be noted that the CRPC TURP specimens represent tumors with local progression, a rather uncommon event in PC where tumor relapse normally occurs at metastatic sites.

#### 3.1.2 Prostatectomies (Paper III)

Archival prostatectomy specimens were used to evaluate the expression of MDK and the NE marker CHGA in primary PC undergoing radical prostatectomy and randomized to 3 months of neoadjuvant ADT or not (172). Forty specimens from the treatment arm and 52 specimens from the control arm were used in the present study (cohort 3, paper III). For the evaluation, MDK and CHGA were stained on sequential sections with IHC, and the expressions were compared between treatment groups.

The patients had localized, intermediate-risk PC (T1b-T3a, N0, M0, G1-3) and were sampled prior to treatment initiation. For clinicopathological characteristics of the patients, see paper III, cohort.3.

### 3.1.3 Prostate needle biopsies (Paper III)

Archival needle core biopsies were used in paper III to evaluate the expression of MDK in primary PC tumors and over the course of PC progression after ADT. The patients had locally advanced or metastatic PC with a PSA level > 80 ng/ml prior to the initiation of ADT. For clinicopathological characteristics of the patients, see paper III, cohort.2.

The primary tumor biopsies were sampled from patients at baseline (n = 45), approximately 3 months post-ADT (n = 26), and at relapse into CRPC (n = 11). The specified n represents the specimen numbers used in the study. For evaluation, the biopsies were stained for MDK with IHC, and the expression was evaluated between the groups.

It should be noted that the time point for CRPC relapse was defined based on clinical progression as metastases or failure in PSA control, making the CR status of the prostate tissue a bit uncertain. However, the biopsies were evaluated by a pathologist, and viable cancer cells with only weak signs of regression were detected in the majority of the 11 cases.

### 3.1.4 Plasma (Paper III)

Plasma samples from two independent patient cohorts were used to evaluate the level of circulating MDK during PC progression after ADT. The inclusion of patients in cohort 1 (n = 40) and cohort 2 (n = 51) occurred prior to the initiation of ADT and included patients with locally advanced or metastatic PC with a PSA level > 80 ng/ml. For the clinicopathological characteristics of the patients, see paper III.

The plasma was collected at baseline, prior to the initiation of ADT, and at different progression time points post-ADT. For evaluation, MDK plasma levels were assessed with enzyme-linked immunosorbent assay (ELISA). In addition, plasma from a small control group (n = 6) of patients with low-risk tumors (T1c, NX, M0, GS < 7, and PSA level < 10 ng/ml) were also analyzed. The resulting MDK concentrations were compared within patient cohorts (over the course of progression) and against the controls (baseline levels; pre-ADT).

## 3.2 *IN VITRO* STUDIES

For *in vitro* studies on PC biology and progression, the androgen-sensitive human PC cell line **LNCaP** (Lymph Node Carcinoma of the Prostate), **FGC** (fast-growing clone) was used as a model in studies at basal conditions (with steroids) and under



castrated conditions (without steroids). In addition, two LNCaP-derived, castration resistant (CR) sub-lines, **LNstarv.1** (LNCaP starvation 1) and **LNstarv.2** were used to characterize the process of CRPC transition and for studies on the CR state.

The LNCaP cell line was originally established from a lymph node metastasis from a CRPC patient (173) and was obtained from the American Type Culture Collection (Rockville, MD, US).

LNCaP is one of the most common androgen-sensitive cell lines used in PC research (31), and the cells express both AR and PSA. However, these cells possess a mutated AR in which the ligand binding domain harbors a T877A mutation making the AR responsive to other steroid hormones than androgens, for example, progesterone and estradiol (174, 175). AR mutations are common in CRPC, and the T877A mutation found in LNCaP cells is one of the most common AR mutations encountered in clinical samples (31). However, due to this mutation, the LNCaP model can only represent PC that harbors this or a similar mutations resulting in a promiscuous AR, and this is a limitation in the present studies.

In addition to LNCaP, a few other androgen-sensitive PC cell lines have been established and are used in the field of PC research, for example, VCaP and LAPC4 cells, both of which, unlike LNCaP, possess a wild-type AR (31, 174). Comparative studies to the present LNCaP-based studies, using one or both of these wild-type AR cell lines, would be valuable.

For all experiments, including basal conditions and short- and long-term steroid deprivation, LNCaP cells between passages 11 and 13 were used, and cells were tested regularly and found to be free of mycoplasma.

### 3.2.1 Cell culturing

#### 3.2.1.1 *Baseline studies of LNCaP (Papers II and IV)*

For baseline studies (with steroids) of LNCaP the cells were maintained in phenol red-free RPMI 1640 culture media, modified according to the manufacturer's recommendation and supplemented with 10% fetal bovine serum. LNCaP cells were also exposed to the AR-targeting drug enzalutamide and to DHT administration under both basal (FBS) and steroid-deprived conditions

#### 3.2.1.2 *Short- and long-term steroid deprivation of LNCaP (Papers II and IV)*

For studies of LNCaP under steroid-deprived conditions (without steroids), the cells were exposed to both short-term (4, 8, 10, 16, and 20 days) and long-term (over 1 year) steroid deprivation. In steroid-deprived conditions, the cells were maintained in LNCaP basal medium supplemented with 10% steroid-depleted serum (DCC; dextran-charcoal-treated (DCC)-FBS) instead of complete serum (FBS).

LNCaP grown in DCC were also exposed to the AR-targeting drug enzalutamide and to steroids in the form of DHT.

In experiments of short-term steroid deprivation, the effect of castration during the initial phase of adaptation and NED was studied. In long-term steroid-deprivation experiments, the stepwise progression of LNCaP into a CR) including the NE phase was studied.

### ***FBS versus DCC***

The method of using FBS versus DCC-containing media for simulating basal versus steroid-deprivation conditions is a standard procedure in the field of PC research, but this entails a source of uncertainty due to the “unspecific” content of serum as well as a possible difference between FBS and DCC in addition to the depleted steroids. A preferable method would have been a well-controlled complete serum-free complement with and without the addition of androgens in the form of DHT.

### ***3.2.1.3 Generation of LNStarv.1 and LNStarv.2 (Paper IV)***

In two independent experiments, long-term steroid deprivation generated two LNCaP-derived “castration-resistant” sublines, LNstarv.1 and LNstarv.2. The characterization of the terminal CR state of LNstarv.1 and 2, as well as the characterization of the whole progression course, including the preceding NE phase and colony-forming phase is an ongoing project.

LNCaP cells from passage 11 were used when initiating LNstarv.1, and LNCaP cells from passage 12 were used when initiating LNstarv.2. Split (SP) 0 represents the initial cell seed, and SP1 represents the first de-attachment during the steroid deprivation timeline. LNstarv.1 was terminated in association with SP22 after 541 days of steroid deprivation, and LNstarv.2 was terminated in association with SP17 after 362 days of steroid deprivation.

In both experiments, the morphological transformation was followed and documented in each passage and throughout the entire steroid deprivation timeline. Cells for mRNA and protein evaluation as well as for freeze sampling were collected at each cell split. In addition, DNA and conditioned media were collected at each passage in the second experimental setup (LNstarv.2), but these have not yet been analyzed.

The morphological phenotypes that were associated with the different phases were similar, but not completely consistent, between LNstarv.1 and LNstarv.2, and furthermore LNstarv.1 needed a longer time within both the NE phase and the colony-forming phase before transition. These differences are likely due to different DCC products used in LNstarv.1 versus in LNstarv.2 (the DCC used for LNstarv.1 was discontinued), where LNstarv.1 most likely been grown under a more stringent steroid deprivation, which is supported by observed differences in gene expression levels.

### 3.2.2 Cell experiments on LNstarv.1 and LNstarv.2 (Paper IV)

Separate experiments along the transformation timeline were mainly performed with LNstarv.2 cells, which is a limitation in the present study. However, cells from both sublines from each cell passage have been frozen, and for the continued characterization of these cells we are planning to use these samples to compare morphologies and properties. Furthermore, experiments might be repeated with both sublines in parallel.

#### 3.2.2.1 *AR-targeting (Paper IV)*

For AR-inhibition experiments, 1–10  $\mu\text{M}$  enzalutamide was included in the experimental media in the different experimental setups. For experiments including enzalutamide, the corresponding concentration of DMSO was used as the vehicle control.

#### 3.2.2.2 *Reversibility experiments (Papers II and IV)*

Reversibility of the transformation process was analyzed through the re-introduction of steroids at different time points along the transformation timeline. Androgens were administrated in the form of steroid-containing media (FBS), DHT supplement, and conditioned media from LNCaP cells grown in the presence of steroids.

LNstarv.1 cells were analyzed in the NE phase and the colony-forming phase with FBS administration, while LNstarv.2 cells were analyzed in the NE phase and the colony-forming phase as well as in the CR state with FBS, DHT, or condition media administrations.

For experiments with DHT, the corresponding concentration of ethanol was used in parallel as the vehicle control.

Furthermore, the cells were exposed to the AR antagonist enzalutamide during steroid re-introduction in order to assess the influence of the AR under these conditions.

#### 3.2.2.3 *De-differentiation experiment (Paper IV)*

To further analyze the de-differentiation process and stem cell properties of long-term steroid-deprived LNCaP cells, LNstarv.2 cells from both the colony-forming phase and from the early CR state were exposed to growth in neuronal stem cell media with well-controlled serum-free supplements (DMEM F12 supplemented with B27 without retinoic acid, and bFGF), which also represented conditions of stringent steroid deprivation.

After passaging, cells were re-seeded on both standard and poly-L-lysine-coated culture plates in order to analyze their adhesive properties and morphology. The cells were also subjected to the AR antagonist enzalutamide on order to assess the influence of the AR under this condition.

### 3.2.3 Cell transfection – midkine silencing (Papers II and IV)

To determine the function of MDK in PC, we silenced MDK in LNCaP cells under both basal and steroid-deprived conditions (5 days of propagation). The cells were thereafter terminated 6 days post-nucleofection.

The MDK knock-down experiments were performed through electroporation using a Nucleofector 2b device and the Nucleofector Kit R (Lonza) according to the manufacturer's recommendation for LNCaP cells. The siRNA targeting MDK (siMDK, s8625) and a non-targeting control siRNA (siNEG, 4390846) were both from Ambion Silencer Select. The siRNA was chosen based on its performance among three different siRNAs targeting MDK and two non-targeting control siRNAs. Performance testing included a verified minimal MDK silencing effect on pleiotrophin (PTN, a structural relative to MDK) expression.

Transfected cells were seeded on poly-L-lysine-coated plates according to the manufacturer's recommendation, and the transfection efficiency was evaluated on both the mRNA (Q-PCR) and protein level (western blot).

It should be noted that there was no obvious morphological change in siMDK compared to siNEG cells under steroid-deprived or basal conditions; however, slight morphological differences were seen in both the siMDK and siNEG cells compared to parallel untransfected control cells (e.g. increased cytoplasmic vacuoles). Furthermore, all steroid-deprived cells, siMDKs, siNEGs, and control cells grown on poly-L-lysine-coated plates had a somewhat less pronounced NE morphology compared to cells cultured on uncoated culture plates. The difference in MDK expression between siNEG and control cells was, however, in a similar range. Taken together, the results imply that the poly-L-lysine coating might affect the morphological NE transformation and that the invasive nature of the transfection process itself has an impact on the cell phenotype.

To further evaluate the function of MDK in PC, it would be highly valuable to analyze the effect of MDK loss with the use of stable MDK-silenced cells, in contrast transient MDK-silencing using siRNA, as well as through the administration of an MDK inhibitor, for example, a neutralizing peptide. Furthermore, the effect should be analyzed in additional models of androgen-responsive PC, for example, in VCaP and LAPC4 cells, due to the heterogeneity in PC.

## 3.3 FUNCTIONAL ANALYSIS

To assess the performance and the functionality of MDK in LNCaP and LNstarv.2 cells, functional analyses in the form of proliferation and survival measurements was performed during both basal and different experimental conditions. More specifically, proliferation and survival were assessed in response to AR-targeting (LNCaP and LNstarv.2 cells), during steroid re-introduction (LNstarv.2 cells) and

during de-differentiation with neural stem cell media (LNstrav.2 cells) as well as during MDK silencing experiments (LNCaP cells).

### 3.3.1 Proliferation (Papers II and IV)

Proliferation was evaluated with a BrdU incorporation assay (Cell Proliferation ELISA; Roche Applied Science).

### 3.3.2 Survival (Papers II and IV)

Survival was assessed from fluorescent measurements generated from a specific protease that is restricted to intact and viable cells (Apo-Tox-Glow Triple Assay; Promega).

## 3.4 RNA ANALYSIS

### 3.4.1 RNA and cDNA preparation (Papers II and IV)

Total RNA from cultured cells, pelleted or directly lysed when possible, was purified with an RNeasy Mini Plus Kit (Qiagen) in accordance with the manufacturer's instructions. RNA concentrations and purity were determined spectrophotometrically (NanoDrop), and RNA intended for microarray analysis was quality checked using a Bioanalyzer (Agilent 2100).

cDNA was prepared by reverse transcription of total RNA using VILO Superscript III (Invitrogen) according to the manufacturer's instructions.

### 3.4.2 Quantitative Real Time Polymerase Chain Reaction (Paper II, IV)

Quantitative Real Time Polymerase Chain Reaction (Q-PCR) was performed with an ABI Prism 7500 Fast Sequence Detector (Applied Biosystems) for the analysis of individual TaqMan Gene Expression Assays and for TaqMan Array Gene Signature plates, and the analysis of microfluidics cards was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems).

#### 3.4.2.1 *Gene expression assays and gene signature plates (Paper II and IV)*

For mRNA expression evaluation and verification, individual TaqMan probes were used (gene expression assays, Applied Biosystems). For paper II, a panel of TaqMan probes was used, including probes directed toward the AR, different NE/neuronal and dedifferentiation markers, and MDK. For paper IV, verification of candidate genes is ongoing, and of these many major targets have already been validated, including MDK, PSA, AR, and many NE/neuronal markers in both LNstarv.1 and LNstarv.2 cells. The data were analyzed according to the  $\Delta\Delta C_t$  method for relative mRNA quantification and were normalized to *18S* or the average expression of three housekeeping genes – *TBP*, *POP4*, and *CASC3*.

TaqMan array gene signature plates, including Human Androgens (#4418741) and Human Stem Cell Pluripotency (#4418722), were used for initial screening after candidate genes in LNstarv.1 and LNstarv.2 split samples. The 96-well gene signature plates were pre-loaded with 96 TaqMan probes, including 4 endogenous controls.

### 3.4.3 Microfluidics (Paper IV)

Customized 384-well Microfluidic Cards for TaqMan Q-PCR (Applied Biosystems) were pre-treated with 96 specially selected TaqMan probes (TaqMan Gene Expression Assay) in duplicate, and these were run with selected samples from LNstarv.1 (SP1, SP2, SP3, SP4, SP5, SP6, SP7, SP8, SP10, and SP13) and LNstarv.2 (SP1, SP4, SP7, SP10, SP13, and SP17), with one sample per microfluidic card. In addition, as a reference, LNCaP cells grown under basal conditions (FBS: 2 and 2+12 days) and subject to short-term steroid-deprivation (DCC, 12 days) were also analyzed by microfluidics assay.

The microfluidics cards were run at the Genomics Core Facility at Gothenburg University, Sweden. For all samples in run 1 (LNstarv.1, including references) and for all samples in run 2 (LNstarv.2, including references), a common threshold for all genes was set. Data were thereafter analyzed according to the  $\Delta\Delta C_t$  method for relative mRNA quantification and were normalized to the average expression of two housekeeping genes – *18S* and *CASC3*.

#### 3.4.3.1 *cdNA microarray and analysis (Paper II)*

Alterations in gene expression in LNCaP cells in response to MDK silencing were evaluated through whole-genome expression microarray analysis (Human Gene 2.0 ST, Affymetrix) under both basal conditions (FBS) and during the early phase of steroid deprivation (DCC).

RNA processing, GeneChip hybridization, scanning, and data pre-processing (normalization) were performed at the Genomics Facility SCIBLU, Lund, Sweden. The data were filtered to delete probe sets displaying signal intensities below 50 in all samples and then were log 2 transformed. The gene expression for siMDK was compared to the expression for siNEG under basal conditions and in steroid-deprived conditions (see the statistical section for further information).

## 3.5 PROTEIN ANALYSIS

### 3.5.1 Protein preparation (paper II and IV)

Total protein from cultured cells, were prepared through cell lysis (direct when possible or of pre-pelleted cells), using Cell LyticM lysis buffer (C2978-50, Sigma-Aldrich) supplemented with protease (Complete Mini) and phosphatase (PhosStop)

inhibitors from Roche Diagnostics. Protein concentrations were determined by BCA Protein Assay Kit (Pierce Chemical).

### 3.5.2 Western blotting (paper II)

Protein samples (15–20 µg) were separated according to size on 4–12% Bis-Tris gradient gels (Invitrogen) under reducing conditions and subsequently transferred to polyvinylidene difluoride (PVDF) membranes using an i-Blot gel transfer system (Invitrogen). The membranes were incubated in 2% blocking solution (GE Healthcare) followed by incubation with primary antibody and subsequent incubation with horseradish peroxidase (HRP)-labeled secondary antibody. The immunoreactions were detected using the ECL Advanced Western Blotting Detection System (GE Healthcare) and visualized with a LAS4000 CCD camera. Primary antibodies used in paper II were directed against AR, PSA, NE markers, and MDK. As a loading control, an antibody against beta-actin was used (Sigma).

### 3.5.3 Enzyme-linked immunosorbent assay (ELISA) (Paper III)

The plasma level of MDK was evaluated with ELISA for human MDK (Cellmid Ltd.) in patient samples during the course of PC progression, before and after ADT (cohort 1 and cohort 2, paper III). As a reference, plasma MDK was also evaluated in a patient group with low-grade localized PC.

The ELISA procedures and calculations were performed according to the manufacturer's instructions.

### 3.5.4 Immunocytochemistry (ICC) (Paper II and IV)

ICC for AR localization studies was performed on fixated LNCaP cells from basal and castrated conditions as well as on LNCaP-derived CR-subline LNstarv.2 cells. ICC was performed with the Vectastain Elite ABC kit (Vector Laboratories), starting from the endogenous peroxidase blocking step and proceeding according to the manufacturer's protocol. Primary antibody labeling was performed with an anti-AR antibody (sc-7305, Santa Cruz Biotechnology), and as a negative control ICC was performed in the absence of primary antibody and with a corresponding isotype control.

### 3.5.5 Immunohistochemistry (IHC) (Paper I and III)

IHC was used in paper I to evaluate MDK, CHGA, and TUBB3 expression in TURP specimens of low-risk HNPC and progressed CRPC. In paper III, IHC was used to evaluate the expression of MDK and CHGA in prostatectomies with or without 3 months of ADT (cohort 3, paper III) and for the evaluation of MDK expression in primary biopsies during PC progression before and after ADT (cohort 2, paper III).

IHC of the TURP specimens and the prostatectomies was performed using the Vectastain Elite ABC kit (Vector Laboratories, Inc.), while the IHC of the biopsies was performed using the EnVision FLEX System (Dako) according to the manufacturer's protocols. Primary antibodies were anti-MDK (5479-100, BioVision, Inc.), anti-CHGA (KL2H10, Neomarkers), and anti-TUBB3 (TUJ1, Convance). For information concerning antibody concentrations and incubations, see papers I and III. As negative controls, IHC was performed in the absence of primary antibodies.

### 3.5.6 Immunofluorescence (IF) and confocal imaging (Paper I and II)

In paper I, triple IF was performed on a selection of TURP specimens to determine if the observed co-expression patterns of MDK, CHGA, and TUBB3 in CRPC tumors were co-localized in the cells. In paper II, MDK and TUBB3 double IF was performed of fixated LNCaP cells cultured under basal and steroid-deprived conditions to evaluate their expression pattern and co-localization.

Double IF ICC and triple IF IHC procedures were based on the Vectastain Elite ABC kit (Vector Laboratories, Inc.) using protocols for ICC and IHC, respectively (for details, see paper I and II), and they were performed by simultaneous labeling of primary antibodies followed by simultaneous detection with fluorophore-conjugated secondary antibodies. After primary and secondary labeling, the coverslips (fixated cells) or sections were washed, air dried, and mounted with Prolong Gold anti-fade with DAPI (Invitrogen). Negative and cross-reactivity controls were performed, respectively, by omitting primary antibodies and by staining with mismatched antibody couples in single IF labeling.

Primary antibodies were directed against MDK, CHGA, and TUBB3-targeted, and the primary antibodies targeted by isotype-specific fluorophore-conjugated secondary antibodies (Molecular Probes, Invitrogen). For further information concerning antibodies, concentrations, and incubations, see papers I and II.

Immunofluorescent confocal imaging was performed through sequential scanning using an inverted Zeiss LSM 510 META microscope (Carl Zeiss) equipped with a 63/1.4 oil NA objective at the Centre for Cellular Imaging, Sahlgrenska Academy, University of Gothenburg. Merged images were obtained and processed using ImageJ software (<http://rsb.info.nih.gov/ij>) (for further details, see papers I and II).

## 3.6 EVALUATION OF IMMUNOSTAINING

### 3.6.1 Quantitative IHC evaluation (Paper I and III)

Quantitative IHC evaluation was performed for MDK, CHGA, and TUBB3 staining of TURP specimens in paper I, for MDK and CHGA staining of prostatectomies in paper III, and for MDK staining of biopsies in paper III.



The staining was evaluated using a semi-quantitative scoring system combining the proportion of positive tumor cells and staining intensity in the malignant areas of each specimen. The proportion of positive cancer cells was scored as 0 = <5%, 1 = ≤33%, 2 = ≤66%, and 3 = > 66%, and the intensity was scored as 0 = no detectable signal, 1 = weak staining, 2 = moderate staining, and 3 = strong staining. For the biopsy scoring, a fourth grade was used for both proportion (>90%) and intensity (very strong staining).

### 3.6.2 Qualitative comparative IHC evaluation (Paper 1)

In the CRPC TURP specimens (paper I), there was a strong association of MDK with both CHGA and TUBB3 staining in terms of both the staining appearance and the amount of co-expression. Thus we performed a comparative evaluation of MDK, CHGA, and TUBB3 in the TURP specimens. In this evaluation, we compared the association of double and triple co-expression between HNPC and CRPC tumors and between the staining appearances of general and scattered.

For this analysis, individual hotspot areas were analyzed on serial sections stained for all three markers, and these were scored as positive or negative in the corresponding area/structure. The positive staining was furthermore characterized as general or scattered, and the distribution of co-expressing hotspots (single, double, and triple expressions) were compared between CRPC and HNPC tumors as well as between the general and scattered hotspot categories (see paper I for further information).

## 3.7 STATISTICAL ANALYSIS

Statistical analysis was carried out using the SPSS version 19.0 (paper II) and version 23.0 (papers II and III) for Windows software. For all tests,  $p \leq 0.05$ ,  $p \leq 0.01$  and  $p \leq 0.001$  were considered significant, if not otherwise stated.

Statistics in **paper I** concerned the evaluations of the immunostainings. The Mann–Whitney U-test was used to analyze differences in semiquantitative scores between two independent groups (HNPC vs. CRPC). Co-expressing hotspots from the comparative evaluations were analyzed as distributions of categorical variables using Fisher's exact chi-squared test, and comparisons were made between marker expressions (single, double, or triple expression) and group (CRPC vs. HNPC or general vs. scattered staining). Bonferroni correction ( $\alpha' = \alpha/m$ , where  $m$  = the number of performed tests) was used for multiple testing.

Statistics in **paper II** concerned calculations of microarray data, comparing functionality and the gene expression for siMDK with that for siNEG under basal conditions (with steroids, FBS) and under castrated conditions (DCC) in LNCaP

cells. The difference in gene expression was analyzed with Significance Analysis of Microarrays using the TMEV v4.0 software. The false discovery rate (q-value = adjusted p-value) was set at  $q = 0\%$ , and the genes were ranked according to fold change. The fold change cut-off was set at 1.4 for changes in FBS, while all altered genes are shown for castrated conditions in DCC.

Gene ontology analysis was performed on differently expressed genes in FBS using the Database for Annotation, Visualization and Integrated Discovery (DAVID) tools (<http://david.abcc.ncifcrf.gov/>).

Statistical calculations of differences in proliferation and in viability between siMDK and siNEG in FBS and in DCC were analyzed with the unpaired t-test.

Statistics in **paper III** concerned calculations on patient samples, group comparisons, correlations, and prognostic calculations.

The Mann–Whiney U-test was used to compare protein expression between non-related groups, and Wilcoxon’s signed-rank test was used to compare protein expression between related groups. Spearman’s rho correlation was used to analyze bivariate correlations of prognostic factors.

The Kaplan–Meier method with log rank statistics and univariable Cox (proportional hazards) regression analysis were used to estimate the unadjusted association of MDK and of all prognostic factors with cancer-specific survival (CSS) respectively. Multivariable Cox regression analysis of selected variables was performed to assess adjusted associations with CSS. The influence of plasma MDK in different models was evaluated using likelihood ratio tests of nested models comparing the overall p-value of the full vs. the reduced model. The best prognostic parameter/model for CSS was assessed through stepwise backward and forward multivariable procedures. The prognostic value of plasma MDK was further evaluated and confirmed with nonparametric receiver operating characteristics curve analysis, analyzing the discriminative power of MDK levels for a short vs. a long CSS time (mean months).

No statistical analysis has yet been performed in **paper IV**.

## 4 RESULTS AND COMMENTS

### 4.1 Midkine is associated with neuroendocrine differentiation in castration resistant prostate cancer (PAPER I)

The study for Paper I was designed in light of the evidence for MDK's association with cancer and the lack of information concerning its involvement and expression pattern in PC. Furthermore, we took into consideration earlier results from our group showing that MDK is upregulated in the CR cell line LNCaP-19 compared to its androgen-responsive parental cell line LNCaP (176).

The aim of the study for Paper I was thus primarily to evaluate the expression of MDK in PC by comparing the expression of MDK protein in low-risk (localized, T1b, stage II) HNPC) to the expression in CRPC. The evaluation of MDK expression was performed in an archival material of transurethral resection of the prostate (TURP) specimens, including 29 low-risk HNPC samples and 24 CRPC samples, that contained adenocarcinoma with adjacent benign prostate tissue.

The MDK expression was found to be highly upregulated in CRPC compared to HNPC. The MDK staining was mainly observed in the cytoplasm, but occasionally in the nucleus, and included both general staining and areas of a distinct scattered or nested cells appearance. The scattered MDK appearance was mainly observed in CRPC specimens, and the occurrence and amount of MDK increased with tumor aggressiveness (dedifferentiation). Due to the characteristic appearance of the scattered MDK staining, it was presumed to belong to NE-like cells, which led us to investigate a possible relation between MDK and NED and therefore to analyze the NE-marker CHGA and the early neuronal marker TUBB3.

In addition to the expression of MDK in cancer, we could observe the initiation of MDK expression in premalignant lesions such as PIN, usually as a general cytoplasmic staining but sometimes as a solely MDK-positive NE-like cell. MDK could sometimes also be observed in other deregulated glands such as solely MDK-positive NE-like cells in PIA. Furthermore, in these areas of deregulated glands, MDK could occasionally also be observed in infiltrating immune cells such as macrophages or in the endothelial cells of blood vessels. Collectively, these results demonstrate that MDK is induced early during the initiation and progression of PC.

As observed for MDK, both CHGA and TUBB3 were found to be highly upregulated in CRPC compared to HNPC tumors. All three markers, but especially TUBB3, also had very low expression in the HNPC specimens. The distinct difference in

expression between low-risk HNPC tumors and CRPC tumors links the upregulation of all three markers to an advanced and/or CR state.

MDK and TUBB3 were found to have both general and scattered appearances, while CHGA was mainly found to have a scattered staining appearance, typical of NE/NE-like cells. The scattered MDK appearance was confirmed to be expression in NE-like tumor cells because CHGA was found to have a similar expression pattern as MDK and because MDK-positive NE-like cells were found to co-express CHGA or, more commonly, CHGA together with TUBB3. MDK hotspots of sparse scattered staining appearance were found in both HNPC and CRPC tumors, while large amounts of scattered staining were found exclusively in CRPC specimens. MDK-expressing NE-like tumor cells therefore increased with the progression of PC and were associated with the CR state.

MDK was furthermore found to be highly expressed in both CHGA and in TUBB3 hotspots, and expression of MDK in these hotspots was associated with CHGA together with TUBB3 as triple expressions or with either marker alone as double expressions. CHGA and TUBB3 were rarely found to be co-expressed unless MDK was also present. Collectively these results suggest MDK to represent an over-bridging marker between different populations of NE-like PC cells in CRPC.

As a generic NE marker, CHGA was expressed in both NE cells from normal glands and in NE-like tumor cells in PC, in contrast to both MDK and TUBB3, which were only expressed in NE-like tumor cells in cancerous lesions.

In cancer lesions, CHGA was mainly found with a scattered appearance, and this in areas of varying levels of differentiation, while TUBB3 was found with both general and scattered staining, but an intense general staining for TUBB3 was only seen in poorly differentiated areas and thus appears to be strongly associated with aggressiveness. Although widely expressed, it should be emphasized that the expression of CHGA was also increased along with dedifferentiation in CRPC, but not to the same extent as TUBB3.

Furthermore, while MDK expression generally displayed a good accordance with TUBB3, including both general and scattered staining, CHGA was primarily associated with TUBB3 scattered staining. TUBB3 and CHGA were rarely seen to be co-expressed unless MDK was also present. Collectively, these results suggest that CHGA and TUBB3 expression can differentiate between tumors, where CHGA is expressed in a broader differentiation range, including both HNPC and CRPC tumors, while TUBB3 is predominantly expressed almost exclusively in more de-differentiated CRPC tumors. There is however a large overlap between CHGA and TUBB3 expressing cells and MDK expression is seen in both populations. Taken together, these results suggest that TUBB3 might be a possible marker for high grade (poorly differentiated) NE-transformed cells in PC, similar to the previously suggested role for TUBB3 as a marker for pulmonary cancer [46].

In conclusion, MDK was found to be significantly upregulated in CRPC tumors and was further found to be co-expressed with both CHGA and TUBB3 in CRPC tumors. The results of this study suggest that MDK is expressed in overlapping populations of NE-like tumor cells with different characteristics and/or differentiation status, possibly as part of the NED process. In these transformed tumor cells, MDK might also be involved in inducing lineage plasticity and in the acquisition of different resistance mechanism or in survival signaling. In addition, MDK was detected as a weak/spares staining in premalignant lesions and thus represents an early tumor marker in PC. Collectively, MDK represents a promising candidate biomarker in PC.

## 4.2 The role of Midkine in high-grade prostate cancer cells before and after steroid deprivation (PAPER II)

Based on the previous results showing upregulation of MDK in CRPC and its association with NED, we sought to evaluate the biological function and impact of MDK in PC, both in an androgen-sensitive state and in relation to NED in response to castration (*in vitro* steroid deprivation). For this study, the PC cell line LNCaP was used as a model and was cultured with steroids (FBS) – representing the basal state, and without steroids (DCC) – representing the steroid-deprived state, this for up to 20 days. The cells were also administered androgens in the form of DHT.

In Paper II, MDK was confirmed to be expressed in LNCaP cells under basal conditions and was shown to play a fundamental biological role at this stage, by promoting proliferation and affecting essential gene expression. More specifically, MDK silencing by siRNA resulted in the downregulation of a large number of genes involved in essential biological processes such as DNA replication and repair, in the cell cycle, including the G1/S checkpoint, and telomere maintenance. MDK silencing also resulted in a large number of upregulated genes related to processes such as cell migration, the inhibition of cell differentiation, and TGF-beta signaling.

Interestingly, many of the biological processes found to be associated with MDK silencing in the presence of FBS are processes known also to be associated with AR signaling, including DNA replication and repair, telomere maintenance, and proliferation (27). Furthermore, many of the signaling pathways and biological processes that were affected in the presence of FBS in response to MDK silencing, including both upregulated and downregulated genes, are similar to the effects observed in response to castration (DCC-treated cells vs. FBS-treated cells) *in vitro*. For example, steroid deprivation of LNCaP cells is associated with the downregulation of genes associated with the cell cycle and with, DNA replication and repair, as well as with the upregulation of genes associated with adhesion, synapse formation, axon guidance, pluripotency signaling, and TGF-beta signaling, which was similar to the effect of MDK silencing in FBS-treated cells (results not shown).

Collectively, these results suggest a strong relationship between MDK and androgen signaling in the steroid-supplemented basal state based on both the number of genes affected by MDK silencing and on the many similarities with AR signaling. To further support the association between MDK and AR signaling, several of the genes that were upregulated in response to MDK silencing in FBS-treated cells are known AR-regulated genes, for example, *AGR2*, *AZGP1*, *CRYM*, *EAF2*, *IGFBP3*, and *LPXN*, which is also an AR co-activator associated with PC progression.

The profound biological importance of MDK in androgen-sensitive PC presented in the paper II suggests that MDK represents a possible target in advanced HNPC, and the potential for this needs to be thoroughly evaluated. In support of the importance of MDK for PC progression, several of the genes originally suppressed by MDK in FBS-treated cells were inversely associated with PC (for example, *AZGP1*, *CRYM*, *EA2*, and *IGFBP3*), while MDK silencing downregulated several PC-promoting genes (for example, *OR51E1* and *TM4SF1*).

In response to steroid deprivation, MDK displayed a profound but transient upregulation (with a peak at around 8 days of steroid deprivation), and this was associated with the initial phase of castration-induced NED as demonstrated by the concomitant upregulation of several NE/neuronal markers as well as an obvious morphological NE transformation, including tapering cell bodies and neurite protrusions. The initial phase of castration was also associated with the apparent upregulation of stem cell/dedifferentiation markers, and this provided supporting evidence for simultaneous NE/neuronal transformation and acquisition of stem cell features, as recently suggested by others (75, 106, 112).

Steroid deprivation in combination with DHT administration inhibited most of the effects of castration-induced NED, in terms of both marker expression and cell morphology, and this supports a repressive role for androgens in NED (92).

However, in contrast to the other markers that were analyzed, castration-induced upregulation of the neuronal marker TUBB3 and the PC stem cell marker CD133 was not inhibited by DHT supplement, but instead was stimulated. Both TUBB3 and CD133 therefore seem to be stimulated by androgens, and thus some repressive factor might be present in normal FBS that is not found in DCC.

TUBB3, a tubulin beta isoform and hence a component of microtubules, is upregulated in various human carcinomas (177), including CRPC, as observed by us (paper I) and by others (178, 179). In CRPC, TUBB3 is associated with poorly differentiated tumors and NED (paper I).

TUBB3 is characterized by enhanced tubulin depolymerization activity, and in various human carcinomas TUBB3 expression is associated with poor prognosis and with resistance to tubulin-targeting drugs such as docetaxel (a microtubule stabilizing drug) (177). Because the study for paper 2 indicated that TUBB3 is induced by DHT, the efficiency of docetaxel in CRPC might be improved by combining docetaxel with different AR-targeting therapeutic options. In support of this, a combination therapy of docetaxel and ADT for patients with metastatic HNPC has recently been shown to substantially increase survival times (38, 69, 73).

In contrast to the major impact of MDK in the presence of steroids, during the initial phase of castration MDK silencing resulted in limited alterations in gene expression. A functional role of MDK at this stage could, however, be demonstrated by an MDK-dependent increase in survival. Furthermore, it was demonstrated that MDK

depletion downregulated the expression of a subset of genes associated with PC progression. Of specific interest was androgen-regulated *AGR2*, which has been associated with metastasis and an aggressive NE subgroup in CRPC (180), which suggests an association of MDK with both aggressiveness and the NE phenotype during the initial phase of castration.

In comparison to clinical data from our study in paper I, where MDK was significantly upregulated in the CR state, the steroid deprived state in paper II represent the initial phase of castration with cells still dependent on classical androgen signaling and in which MDK expression was clearly affected by the androgen status. We therefore hypothesized that the observed upregulation of MDK in the early phase of steroid deprivation is an attempt by the cells to maintain the many MDK-regulated cellular processes, and maybe also to compensate for the loss of AR signaling. However, the obvious similarity between the expression profile of MDK and several NE markers during this phase also suggested an association between MDK and NED. Furthermore, due to the high expression of MDK in CRPC(149, 150), in which the AR signaling axis is normally restored (31), we hypothesized that MDK regains, at least partially, the impact on cellular functions that it has in the androgen-sensitive state.

A functional role for MDK under both basal conditions and in response to steroid deprivation was further supported by the expression of MDK receptors in the LNCaP basal state, several of which were also upregulated by steroid deprivation. Potential receptors responsible for promoting MDK-dependent survival, as observed during castration, include ALK and LRP1, of which LRP1 also displayed castration-induced upregulation. PTP $\xi$ , another MDK receptor associated with survival, was, however, not found to be expressed in LNCaP cells. Potential receptors for the possible involvement of MDK in castration-induced neurite outgrowth include members of the syndecan family (SDC1, SDC3, and SDC4), GPC2, and NGC, of which, SDC1, SDC3, and SDC4 were found to be expressed in LNCaP cells in the study for paper II. Furthermore, the involvement of MDK in mechanisms of lineage plasticity and EMT and in diverse resistance mechanisms might be derived from Notch2 activity under both basal and steroid-deprived conditions in LNCaP cells. Finally, the observed MDK-dependent proliferation seen under basal conditions might be derived from ALK activation, which in addition to promoting survival is also involved in growth promotion and is expressed in both the basal and the steroid-deprived state. The potential involvement of LRP1 and ALK in the MDK-dependent promotion of survival and proliferation, respectively, would be interesting to evaluate, for example, through the use of receptor inhibitors or neutralizing antibodies under basal and steroid-deprived culture conditions.

In conclusion, the study for paper II demonstrated that MDK has a significant impact in PC cells in the presence of steroids and that it affects fundamental biological



processes, including DNA replication and cell proliferation, probably in close association with androgen signaling, and these results might be translatable to advanced HNPC. In addition, MDK affects the biology of PC cells during early steroid deprivation, thus connecting MDK to the process of castration-related progression in terms of aggressiveness and increased survival. Furthermore, we hypothesized that when MDK is upregulated in CRPC it regains, at least partially, the impact on cellular functions that it has in the androgen-sensitive state together with the restoration of AR signaling. In addition, it is possible that truly androgen-independent mechanisms of MDK signaling might emerge due to the many receptor and signaling pathways involved in MDK signaling.

### 4.3 Midkine as a biomarker in advanced prostate cancer (PAPER III)

There is currently a lack of reliable biomarkers for PC diagnosis, prognosis, and treatment prediction as well as for monitoring disease progression (38). One of the major challenges within the PC field is the discrimination between indolent and aggressive forms of low-risk localized PC, which is why new biomarkers strongly associated with aggressiveness and clinical progress are urgently needed. Likewise, biomarkers that can predict NED and the development of NEPC are also needed for the prediction of treatment outcomes. Based on our previous and parallel findings of MDK in PC concerning its expression pattern, responsiveness to steroid deprivation, and functional impact, we sought to evaluate the applicability of MDK as a clinical progression biomarker in advanced PC before and during ADT.

The prognostic value of MDK was assessed both in plasma and in primary tissue both at baseline (pre-ADT) and at the time of relapse into CR growth. This was done in relation to established prognostic factors in current clinical use, including clinical (TNM staging) and pathological (GS) factors and serum biomarkers (PSA and ALP). Furthermore, the NE markers CHGA and NSE were included in the analysis because we wanted to evaluate the association of MDK with increased NED as well as to assess the prognostic performance of MDK in relation to these NE markers.

In several recent studies on different human carcinomas, circulating MDK has been evaluated and proven to be closely associated with cancer progression (131, 151-155). In this study we showed that plasma MDK is also a powerful progression biomarker of advanced PC. More specifically, plasma MDK, both at baseline and even more so at relapse, was shown to be a negative prognostic marker for cancer-specific survival (CSS) and was found to be an independent marker. Furthermore MDK outperformed all other prognostic factors commonly used in PC clinical settings, especially at relapse.

Unlike plasma MDK, tissue (primary tumor) MDK was only found to be a weak biomarker for CSS at baseline and showed no association with CSS at the time of relapse.

The observed discrepancy between plasma and tissue MDK as progression biomarkers was supported by their complete lack of correlation, both at baseline and at relapse, and this is likely explained by the contribution of MDK from circulating tumors cells and from metastatic and pre-metastatic sites, which are accounted for in plasma measurements but not in the evaluation of primary tissue. Plasma MDK thus represents an estimate of the total tumor burden and also reflects a more progressed disease.

The lack of translation between plasma and tissue MDK seen in this study concerns advanced stages of PC (pre-ADT and at relapse), and it is likely that there is some translation at an earlier progression time point before metastatic dissemination.

In this study, MDK was confirmed to be increased in advanced PC compared to low-risk localized PC (paper I)(150) and to be affected by castration (paper II). Like PSA, MDK displayed a temporary decrease after castration but began to recover around the time of relapse. In comparison to PSA and ALP, MDK displayed a robust and positive correlation with both markers. Furthermore, MDK displayed a robust and positive correlation with pathological grade (GS) and with M-stage (distant metastasis), but not with T-stage (tumor).

Concerning the NE markers CHGA and NSE, plasma MDK showed a positive correlation with the plasma levels of both markers at baseline but not at relapse. The observed loss of correlation between MDK and NE markers after castration is probably, at least partly, explained by the fact that CHGA and NSE, unlike MDK, are unaffected by castration. It should also be noted that CHGA and NSE did not have any correlation with each other at baseline or at relapse, confirming the heterogeneous nature of NE and NE-like tumor cells.

None of the NE markers had any prognostic value at baseline, while CHGA had a weak prognostic value at relapse that was enhanced by NSE, but this prognostic value was completely confounded by plasma MDK. This suggests that the power of CHGA as a prognostic marker increases with CRPC progression as well as in combination with other NE markers as previously suggested (53). Furthermore, these results indicate that MDK might identify pre-treatment patients who are predisposed to the development of increased NED, and might account for at least some of the aggressiveness associated with NEPC, and these hypotheses need to be tested in future studies. The results also indicate that MDK, in addition to NEPC, identifies aggressive variants of anaplastic CRPC without typical NE features such as CHGA and NSE expression, thus offering a more widely applicable biomarker for aggressiveness.

In this study, circulating MDK was confirmed as being a powerful negative prognostic biomarker, but these results need to be further tested in well-controlled prospective studies. However, based on the findings in this work, it is tempting to speculate that patients with a high MDK level at baseline might benefit more from some alternative primary treatment than from ADT alone, such as upfront chemotherapy and radiation. Furthermore, for prognostic evaluation of locally advanced and metastatic HNPC, our results suggest that circulating MDK alone or in combination with T-stage might be the best prognostic option, while circulating MDK alone, being superior, is the best prognostic option for evaluation at relapse post-ADT.

We speculate that MDK could be used together with PSA to screen for aggressive PC at an earlier stage than in this study. We also speculate that MDK can aid in the identification of patients who are predisposed for NED and the development of NEPC and that systemic MDK might therefore assist in the selection of treatment. Furthermore, the possibility of using MDK as a therapeutic target should be evaluated.

#### 4.4 Long-term steroid deprivation transforms prostate cancer cells to anaplastic castration resistant cells (PAPER IV)

In order to be able to prevent or at least prolong the time until relapse into CRPC, as well as to be able to efficiently target CRPC growth, we need to better understand the biology behind castration-induced transformation, including the NED process. This study therefore aimed to evaluate the process of long-term castration-induced transformation, including the NED process and its relation to AR signaling. Furthermore, based on previous findings for MDK, including its profound upregulation in CRPC (paper I), its association with NED (papers I and II), its responsiveness to castration (papers II and III), and its biological impact in PC (paper II), as well as its functional association with both survival and proliferation (paper II), we sought to evaluate the expression and effect of MDK throughout the process of transformation and ultimately in CR growth.

For the evaluation of long-term steroid deprivation in PC, we used the LNCaP cell line as a model, which was the same androgen-sensitive PC cell line that was used for the short-time steroid deprivation study in paper II. In this study, LNCaP cells were subjected to continuous steroid deprivation in two independent experiments – LNstarv.1 and LNstarv.2. Furthermore, steroid-deprived cells at different time-points after the initiation of steroid deprivation were subjected to the administration of DHT, to the reintroduction of FBS-containing media, to the AR-targeting drug enzalutamide (for studies of AR inhibition), and to stringent steroid deprivation in the form of neural stem cell media, this mainly in the second experiment (LNstarv.2).

In this study, continuous steroid deprivation was observed to sequentially transform the androgen-sensitive PC cell line LNCaP into highly proliferative CR cells via a stem cell-enriched NE phase and a subsequent colony-forming phase. This transformational process was observed in both LNstarv.1 and in LNstarv.2 cells and suggested that classical NED only represents a transient and adaptive phase during the progression towards CRPC.

The different morphological phases were overlapping, and the transformations were gradual, especially between the NE phase and the colony-forming phase. The morphological phenotypes that were associated with the different phases were similar but not completely consistent between the LNstarv.1 and LNstarv.2 experiments, and LNstarv.1 required a longer time within both the NE and the colony-forming phases before transition.

The NE phase was characterized by growth cessation, a typical NE morphological switch (tapering cell bodies and neurite protrusions), and the upregulation of diverse NE and neuronal markers such as *NSE*, *MAP2*, and *NCAMI (CD56)*.

The observed NE phase was furthermore associated with dedifferentiation and stemness based on the observed upregulation of induced pluripotency markers such

as *POU5F1*, *SOX2*, and *NANOG*. The NE-like tumor cells also displayed an overlapping EMT with the upregulations of, for example, *SNAIL* and *VIM*, and the NE-like tumor cells also displayed a modest AR downregulation in association with ceased expression of AR target genes such as *PSA*, *TMPRSS2*, and *NXK3.1*. Finally, confirming our earlier study (paper II), MDK was observed to be transiently upregulated in the NE phase and to have a similar expression pattern as many NE/neuronal markers and dedifferentiation markers. The association of NED with both dedifferentiation and EMT was more pronounced in LNstarv.1 compared to LNstarv.2 cells, where LNstarv.1 represented the first experiment and cells that were subjected to a presumably more stringent castration treatment due to the efficiency of DCC complementation. A comparison between the two DCC products is planned for the future.

The subsequent colony-forming phase was characterized by small cells that were tightly packed, with or without neurite protrusions at the colony borders and showing gradually increasing proliferation. The colony-forming cells were further characterized by a reduction in NE/neuronal markers and a reduction in induced pluripotency markers, but they showed sustained expression of EMT markers and increased expression of PC stem cell markers (upregulation of *CD133* and recovery of *CD44*) and the neural stemness-associated transcription factor *HES1*. Furthermore there was altered expression of genes involved in reprogramming (upregulation of *XIST* and downregulation of *REST*), which could indicate overall epigenetic instability and transformational reprogramming.

The colony-forming phase thereafter gradually transformed into a scattered cell type with a small cell morphology (high nucleus to cytoplasm ratio, round to oval shape) in combination with a continued increase in proliferation. The colony-forming phase represents a relatively short transition phase between the adapting phase of NED and the CR state of growth-modified and proliferative CR progeny.

In addition to the morphological switch at the recovery in proliferation described above, the CR terminal state was characterized by an upregulation of the AR. In association with AR upregulation, there was a massive alteration in gene expression. Several NE/neuronal and pluripotency markers were recovered from the NE phase, for example, *NSE*, *AMACR*, *NR5A2*, *NANOG*, and *KLF4*, while others mainly had a peak and pronounced upregulation during the CR state, for example, the stemness marker *GABRB3*, the primitive neuronal markers *NES* (nestin) and *EOMES*, and the early neuronal marker *TUBB3*. Furthermore, markers associated with EMT were found to have continued upregulation, for example *VIM*, *SNAIL*, and *BMP4*. These data are in line with recent studies and suggest an association between NED and stem cell-like phenomena, i.e. the acquisition of stemness by NE-like tumor cells during the progression of PC (18, 24, 26), a causal relationship that could explain many aggressive features in CRPC. The results also suggest that AR recovery is associated with dedifferentiation in CRPC.

Furthermore, *MDK*, which was downregulated in the colony-forming phase, was recovered and upregulated at CR transition, and this was associated with the upregulation of many of its receptors (including *LRP1*, *SDC3*, and *NOTCH2*) as well as with upregulation of *TUBB3*. These results confirm our earlier theories of regained MDK expression and activity in the CR state of PC. Furthermore, these results also support theories of androgen regulation of both *MDK* and *TUBB3* expression in PC. In addition to the upregulation of genes associated with dedifferentiation, neuronal characteristics, and EMT, there was upregulation of genes associated with general aggressiveness and cancer progression, for example, *SHH*, *CD24*, and *CTNNB1* and the transcription factors *JUN*, *FOS*, and *LEF-1*.

The AR was confirmed to have a nuclear localization and to be growth promoting in the CR state, but without any recovery in AR-regulated genes such as *PSA* and *TMPRSS2*, suggesting an altered signaling axis in these cells.

In addition to the observed *AR* upregulation, altered steroidogenesis (upregulation of *AKR1C3* and *SRD5A1*) and/or potential bypass mechanisms (upregulation of steroid hormone receptors *ESR1* and *PGR*) were indicated to be involved in the acquired AR activity. Furthermore, the AR might be activated through crosstalk mechanisms, for example, by the neuropeptide NT (neurotensin), which is known for this activity (83) and to have a high mitogenic activity in an androgen deprived condition (83, 98, 100), in addition to its observed pronounced upregulation in the CR state. The mechanism of AR activation in the CR state should be further evaluated, including an analysis of ARVs, of which ARV7 is of greatest interest.

In this study, in addition to the confirmed nuclear localization, the AR was confirmed to be growth promoting, even after prolonged steroid deprivation, and was shown to be essential for proliferation and survival and likely also for the differentiation status.

Inhibition of AR signaling by the administration of enzalutamide resulted in a pronounced effect on morphology, survival, and proliferation in steroid-deprived LNstarv cells. Inhibition of growth and survival by enzalutamide was, however, more pronounced and of longer duration in the colony-forming phase compared to the CR state, in which rapid recovery was observed. Resistance mechanisms in the colony-forming phase further involved short-lived NE features, supporting NED as a resistance mechanism as well as suggesting that NED is inducible multiple times during acquired resistance. In contrast, the morphological impact of enzalutamide observed in the colony-forming phase in cells of the CR state was relatively short-lived and mild, and the associated alteration needs to be further evaluated at the mRNA and protein level. Collectively, the results suggest that AR-targeting drugs such as enzalutamide might be efficient on the reactivated AR in CRPC, although therapeutic efficacy might not be detected by effects on PSA levels. However, AR-targeting drugs is likely more efficient before transformation to a fully CR state has been achieved.

The capacity of LNstarv cells, both from the colony-forming phase and the CR state, to survive and proliferate in neural stem cell media, i.e. during stringent castration, was confirmed to be enhanced and completely AR dependent. Spontaneous spheroid formation and dedifferentiation to a presumed primitive state was induced in cells from both the colony-forming phase and the CR state. AR targeting resulted in the inhibition of spheroid formation, ceased proliferation, and massive cell death, and there was no recovery with time. Collectively, these results suggest that the AR might be efficiently targetable in the CR state if a state of complete castration is achieved.

Reintroduction of androgen to the steroid-deprived cells demonstrated the reversibility of the transformation process to encounter more resistance with prolonged starvation time and with the progression state of the cells. In the NE phase and early colony-forming phase, which are associated with the expression of NE markers and stem cell markers, the reversion was relatively rapid and complete, while in the fully CR state the cells did not respond to steroids with reversion but instead with increased proliferation. Furthermore, androgens in the form of DHT had a toxic effect in the early colony-forming phase, and slightly so also in the late colony-forming phase, indicating that the cells were sensitized to DHT or that they did not use this androgen for activation at this stage.

These results identify different effects of androgens during the progression into CRPC, and these might be important to consider in relation to the reintroduction of steroids in the clinic.

Major findings in this study include the observed gradual process of castration-induced transformation and the sequential transformation of LNCaP cells into a terminal CR state via a phase of classical NED and a subsequent colony-forming phase. Furthermore, we made the observation that MDK was associated with both the NE phase and with CR growth, that MDK was recovered in association with the AR, and that the AR was confirmed to be growth promoting and associated with proliferation, survival, and differentiation status in the CR cells.

From this and previous studies, we hypothesize that MDK has a functional role both during NED induction and in CR growth and that MDK and the AR are functionally associated. Furthermore we propose that classical NED represents a transient phase that is closely associated with dedifferentiation and is inducible multiple times as a mechanism of adaptation and associated transformation.

Interpretations of the results from this study must be made with caution, however, because they were obtained from only one cell line, and they need to be confirmed in other studies as well as in other PC cell lines, for example, in VCaP and/or LAPC4 cells. In addition, LNCaP cells, which were used as the PC model in this study, carry a T877A mutation in the ligand binding domain of the AR, making it, in addition to androgens, also responsive to progesterone and estrogen (52), and this might have

influenced the final results. Furthermore, the expression data presented in this paper need to be verified at the protein level as well as through regular Q-PCR, which is an ongoing project.



## 5 GENERAL DISCUSSION

Metastasized HNPC and CRPC both represent incurable stages of PC pathogenesis despite several therapeutic advances in recent years. Treatment advantages for patients with metastasized HNPC involve combination treatments on a backbone of ADT, and ADT in combination with the cytotoxic drug docetaxel has resulted in a substantial survival benefit in comparison to the administration of ADT alone (38, 69, 72, 74). However although an increased survival rate is obtained, most patients ultimately relapse into lethal castration resistant prostate cancer (CRPC) as therapy fail.

### 5.1 LETHAL PC

CRPC is lethal and normally results in cancer-related death within 3–4 years due to complications associated with metastatic disease (69). Despite recent advances also at this stage, including the use of the potent AR/androgen-targeting drugs enzalutamide (targets the AR) and abiraterone acetate (targets androgen biosynthesis), the treatment is only palliative and the response durations are relatively short due to different mechanisms of acquired resistance. Eventually, highly resistant variants of PC emerge that no longer respond to any available treatment (69, 74, 75, 97, 122).

In addition to the lack of curative treatments for patients with metastasized HNPC and CRPC, there is an urgent need for reliable biomarkers for disease monitoring and prognostics.

PSA represents the primary biomarker in PC but has limitations concerning both sensitivity and specificity, and thus it cannot discriminate between indolent and aggressive forms of low-risk PC and is insufficient in disease and therapy monitoring, especially in late stages of the disease (38). We therefore need new biomarkers to use in addition to or alongside PSA to be able to make better diagnostic and treatment decisions.

To be able to develop curative treatments, or at least long-lasting palliative treatments, for patients with advanced HNPC and CRPC, we need to better understand the process of castration-induced transformation in terms of acquired resistance, including AR-related mechanisms, stem cell-related dedifferentiation, and NED. We also need to find targetable drivers responsible for these transformations that can be used in combination with or instead of AR targeting.

## 5.2 CRPC TRANSFORMATION

PC is generally an AR-driven disease, and current therapies therefore largely focus on androgen ablation and AR-targeting therapies. Resistance mechanisms to these therapies largely involve AR-related mechanisms that sensitize and restore AR activity and/or signaling (31). Before transformation into a CR state, the tumor undergoes massive cell apoptosis. The surviving cells comprise those cells that can survive despite castrate levels of testosterone, including cells already androgen independent (e.g. prostate stem cells and NE cells) and/or androgen-dependent cells that are capable of a rapid adaptation (21, 24, 181), such as the commitment to NED, which is the transdifferentiation process of NE-like tumor cells from luminal epithelial cancer cells (21). These NE-like tumor cells are characterized by androgen independence, ceased proliferation, and high resistance to apoptosis as well as by a general dedifferentiation, including the upregulation of pluripotency markers (21, 52, 86) as demonstrated in the present thesis (papers II and IV). The NE-like state is furthermore both reversible (182) and temporary, representing a transition phase as seen in paper IV. This window of dedifferentiation and transformational reprogramming, before the emergence of CR disease, might be efficiently targetable if focusing on the right driver. The AR is possibly also targetable at this stage, as seen in paper IV where the growth and survival of colony-forming steroid-deprived cells was temporarily inhibited by enzalutamide. However, targeting the AR at this stage is likely not enough. Possibly, however, targeting a driver associated with a primitive state or with associated reprogramming, for example, *EZH2*, might be more efficient. It is likely, however, that a combination therapy targeting several drivers will be needed.

### 5.2.1 Targets in NED and in CRPC

In recent years many candidate drivers for NED and the development of NEPC have been suggested, for example, *AURKA* and *MYCN*; however, none so far have been proven to be associated with a clinical survival benefit (113, 115, 122). Interestingly, in paper IV, *AURKA* was downregulated during the early stages of steroid deprivation-induced transformation (in the NE phase and the colony-forming phase), and its expression was recovered at the transition to the CR state in association with the upregulation of AR. These results might support the upregulation of *AURKA* in NEPC, but they contradict *AURKA* as a driver of NED. A driver for NED and presumably also for NEPC should optimally represent an early marker or event during the transformation process and thus be strongly associated with the initiating phase of NED.

*REST* is another gene with a reported strong association with NED (108) that is downregulated during NED, but in paper IV it was shown to be upregulated in association with CR transition and AR recovery. *REST* has been reported to have a strong association with the AR (108), and in addition to its known repressive

functions in neuronal differentiation it has recently been assigned a tumor-promoting role in neural cells and shown to be involved in EMT and in stem cell self-renewal (106). Furthermore, *REST* is highly expressed in stem cells, including neuronal progenitors (107). Collectively, the upregulation of *REST* in LNstarv.1 cells supports an active AR in these cells as well as a primitive neuronal state, because several primitive neuronal markers, including *NANOG*, *EOMES*, *TUBB3*, and *NES* (nestin), were upregulated in parallel with *REST* and *AR*.

Finally, an efficient target for NED, as part of the transformation process, might be targetable within the transformation window for PC in general regardless of a predisposition for NEPC or not.

### 5.2.2 The AR in CRPC

In the CRPC, at least in therapy-naïve CRPC, the AR generally represents the main driver for disease initiation and progression; however, it is difficult to efficiently target due to mechanisms of acquired resistance (31). The AR might, however, be more efficiently targetable if a completely castrated context could be achieved, as seen in paper IV concerning the devastating effect of enzalutamide administration during stringent steroid deprivation. *In vivo*, however, such a stringent castration is hard to achieve due to the presence of background adrenal androgens and acquired intratumoral steroidogenesis.

An absolutely essential role of the AR in CR growth after prolonged steroid deprivation was therefore observed in paper IV, and this involved survival, proliferation, and differentiation status. These results were obtained in LNstrav.2 cells and should be verified in LNstrav.1 cells. The CR state in LNstrav.1 cells was however associated with marked AR upregulation, which was in association with massive gene alterations, but without any PSA expression, which might be indicative of an altered androgen/AR signaling axis. The characterization of LNstrav.1 and LNstarv.2 is an ongoing project and might reveal the acquisition of different resistance mechanisms in these sublines.

### 5.2.3 NED as a transient phase of adaptation

In paper IV, continuous steroid deprivation was observed to sequentially transform LNCaP cells into a highly proliferative CR state, and classical NED was shown to represent a stem cell-enriched phase during this progression. The NE phase later merged into a colony-forming phase that gradually transformed further into the CR progeny. NED was also observed as a transient adaptive response in colony-forming cells in response to enzalutamide (paper IV). NED has also been reported clinically to be part of the acquired resistance in CRPC (31, 76, 77).

Additionally, before reaching the fully transformed state of CR growth, the process of castration-induced transformation was shown to be reversible by the re-introduction of androgens. In the terminal CR state, however, the transformation process was not reversible but the androgens were instead shown to be growth

promoting. Collectively, these results suggest that NED represents a transient adaptive phase that is associated with dedifferentiation and reprogramming and is part of a mechanism of acquired resistance. Thus NED in CRPC might be targetable with the same procedure as primary NED during CRPC transition if an efficient target can be identified.

## 5.3 MDK IN PC

### 5.3.1 MDK expression in PC

In paper II, MDK was shown to promote proliferation and to alter gene expression associated with essential biological processes in androgen-sensitive LNCaP cells under basal conditions, and it was suggested that there is a close association between MDK and AR signaling and/or AR-regulated genes. The close relationship between MDK and AR was further confirmed in paper IV, where a concomitant recovery of expression and upregulation of MDK and AR was observed at the transition to CR growth. The responsiveness of MDK to steroid deprivation observed *in vitro* (papers II and IV) was further verified clinically (paper III), where MDK showed a similar, but not as pronounced, expression profile as PSA.

In paper I, MDK was shown to be upregulated in CRPC tumors and to be associated with NED in these tumors. MDK upregulation in CRPC was confirmed in paper III where MDK expression started to increase in association with CRPC relapse and to continue to increase with disease progression. An association of MDK with NED was also seen *in vitro*, where MDK displayed transient upregulation in response to the initial phase of steroid deprivation-induced NED and associated dedifferentiation (papers II and IV). MDK was thereafter downregulated during the succeeding colony-forming phase but recovered its expression at CR relapse (paper IV). Furthermore, MDK had an impact on early steroid deprivation, as shown by MDK-dependent survival as well as transcriptional association with the pro-metastatic *AGR2*, which is known to be associated with an aggressive NE phenotype in CRPC as well as to represent a new promising biomarker for characterization, monitoring, and directing therapies for patients with metastatic NEPC (122, 180). Collectively these results suggest that MDK and AR are in a close functional relationship and that MDK is associated with the NE phase and with CR growth.

### 5.3.2 MDK as a prognostic biomarker

The clinical findings of MDK in paper I suggested a prognostic value for MDK, and this was later confirmed in paper III in which circulating MDK was demonstrated to be a significant negative prognostic biomarker for CSS in advanced HNPC, pre-ADT, and at relapse into CRPC.

Circulating MDK furthermore outperformed all other analyzed factors, including clinicopathological factors (TNM staging and GS) and biomarkers (PSA, ALP, NSE, and CHGA) that are currently in clinical use. In the same study, however, tissue MDK levels evaluated from primary tumors were of no prognostic value and were therefore not translational to MDK levels measured in plasma. This discrepancy is most likely due to the contribution of MDK from pre-metastatic and metastatic sites accounted for in plasma levels but not to the levels in primary tumors.

A high MDK level is thus associated with a short survival time both in advanced HNPC and at transition to CRPC. Circulating MDK might therefore represent a marker for aggressiveness and as such be able to differentiate between slow and fast-progressing tumors at an earlier stage in disease progression, for example, in localized PC. MDK levels in plasma might, however, also be a reflection of total tumor burden. To further evaluate the applicability of MDK as a biomarker, it would be of specific interest to evaluate the prognostic value of MDK in patients with low-risk localized tumors with an uncertain clinical progression course, for example, GS 7 tumors. MDK could potentially differentiate between indolent and aggressive variants from an even earlier time point, which should be evaluated separately. It should be noted that the CRPC tumors analyzed in paper I (TURP specimens) represent advanced CRPC and tumors with a local relapse post-ADT, while the tumors analyzed in paper III (patient cohort 2) were prostatic biopsies taken from patients at the time of CRPC diagnosis as defined by distant tumor relapse or increasing PSA levels, and this is important when considering the non-prognostic value of MDK in tumor biopsies in paper III.

## 5.4 FUTURE PERSPECTIVES

### 5.4.1 MDK as a predictive biomarker

Due to great variations in patient response duration for different treatments, reliable biomarkers for treatment predictions are highly valuable.

The findings of MDK as a significant negative prognostic biomarker in advanced HNPC, both for progression-free survival (results not shown) and for CSS (paper III), indicate that patients with a high MDK level will not respond as well to standard therapy with ADT as those with lower MDK levels. Instead, they might benefit more from some alternative therapy, for example, chemotherapy, but this needs to be evaluated separately. Likewise, patients with a high degree of focal NED generally respond more poorly to ADT (24, 87, 97), while patients with SCPC (an aggressive form of NEPC) are considered unresponsive to ADT and AR-modulating therapies. An association between MDK and NED was observed in all papers in this thesis work, as indicated by the observed correlation between MDK and the NE markers CHGA and NSE in advanced HNPC (paper III), the co-expression of MDK with both CHGA and TUBB3 in CRPC tumors (paper I), and the observed upregulation of

MDK during the initial phase of NED *in vitro* (papers I and IV). Furthermore, MDK was observed to be upregulated in association with primitive neuronal markers at the transition to CR growth (paper IV).

Collectively, these results suggest that MDK might offer a therapeutic predictive value in HNPC, for the evaluation of which, the MDK pre-treatment level should be analyzed along different treatment arms and in relation to disease progression.

Due to the strong association of MDK levels at relapse with time to death, the predictive value of MDK should also be evaluated at this time point in the treatment-naïve CR state. For this, the MDK levels at relapse into CRPC should be analyzed along different treatment arms and in relation to acquired resistance and survival.

An evaluation of the predictive value of MDK in relation to therapy with platinum-based cytotoxic drugs, e.g. cisplatin (interferes with DNA repair), would be of specific interest due to its association with NED and NEPC (a patient group that might receive this therapy) (53, 122) as well as due to the reported impact of MDK on therapy efficiency to this category of drugs (154, 156-158, 160, 161).

The possible impact of MDK on platinum-based chemotherapy in PC should be evaluated separately; however, published data in other cancer forms imply that MDK has high value as a therapeutic predictor at least regarding cisplatin, and might represent a possible target in combination with cisplatin therapy for certain patient groups.

#### 5.4.2 MDK as a therapeutic target

Due to the pronounced biological impact assessed *in vitro* for MDK under basal condition (paper II) as well as the observed strong association of MDK with CSS in patients pre-ADT (paper III), it might be hypothesized that MDK is targetable in patients with advanced HNPC, perhaps in combination with AR targeting.

The data from this thesis imply that MDK in CRPC might regain its biological impact and many of its functions from the basal state (paper II) in association with AR re-activation and the restoration of an androgen-influenced context. Furthermore, because MDK was identified as an excellent negative biomarker at relapse (paper III) as well as being confirmed to be upregulated in CRPC, both *in vitro* (paper IV) and in clinical samples (paper I and III), it is possible that MDK is targetable at this stage as well. In CRPC, it would be of great interest to target MDK in a small clinical trial composed of patients with multi-resistant and highly aggressive PC variants that no longer respond to any available therapies.

Furthermore, as mentioned earlier, MDK might also represent a target in combination with cisplatin therapy as well as a possible target during NED induction.

#### 5.4.3 Concluding remarks

Treatment of patients with metastatic PC is rapidly changing with the introduction of new drugs, as well as the earlier introduction of both cytotoxic drugs and the new

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generation of hormonal agents. In this changing landscape, good biomarkers are necessary and, MDK represents a cancer biomarker that is of great potential in several stages of the disease.

The results from paper III demonstrate MDK to be a powerful prognostic biomarker in PC, both in the advanced HNPC stage and at relapse. However, these results need to be further verified in well-controlled prospective studies. Furthermore a prognostic value of MDK should be evaluated retrospectively in localized PC, especially in those with an uncertain clinical progress/risk. MDK as a therapeutic predictor should also be evaluated both in HNPC and in therapy-naive CRPC, including if possible cisplatin patients.

MDK as a future therapeutic target in both HNPC and in CRPC should also be evaluated, primarily by a thorough analysis in preclinical settings. There is a humanized neutralizing MDK antibody available (CAB102, Cellmid Ltd.), which in a variety of xenograft models been associated with the inhibition of tumor growth and metastasis and of angiogenesis and to reduce chemoresistance ([http://www.cellmid.com.au/content\\_common/pg-lyramid.seo](http://www.cellmid.com.au/content_common/pg-lyramid.seo)). In future studies, a combined MDK and AR targeting should also be evaluated, based on their mutual dependency suggested by the work in this thesis. In addition, several interesting genes associated with MDK and NED should be evaluated further as prognostic tools and possible drug targets, for example *AGR2* identified in the present study.

## 6 CONCLUSIONS

*Based on the results of the present thesis, the following conclusions could be made:*

- ❖ MDK is upregulated along with disease progression in HNPC and after relapse in CRPC, resulting in high expression in progressed CRPC
- ❖ MDK is associated with dedifferentiation and NED in CRPC.
  
- ❖ MDK appears to be involved in fundamental biological processes in androgen-sensitive PC.
- ❖ MDK and the AR seem to be functionally connected.
- ❖ MDK is associated with increased survival during early steroid deprivation, thereby influencing the effect of castration.
  
- ❖ MDK represents a potentially powerful negative prognostic biomarker, in terms of CSS, for both advanced HNPC and at CRPC relapse.
  
- ❖ Steroid deprivation-induced transformation into a CR state is a gradual process where classical NED represents a transient phase associated with stemness.
- ❖ NED is also associated with an adaptive state preceding acquired resistance in the CR state.



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