Prognostic markers in prostate cancer

-Studies of a watchful waiting cohort with long follow up

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Till Cecilia och Emil

"Science may be described as the art of systematic over-simplification"

Karl Popper

Abstract

Background: Prostate Cancer (PC) is a common and highly variable disease. Using current diagnostic methods, the prostate specific antigen (PSA) blood test and histological grading of prostate tissue needle biopsies, it is often difficult to evaluate whether the patient has a PC that requires active treatment or not. The absolute majority of all 10,000 cases of PCs diagnosed annually in Sweden have tumours graded as Gleason score (GS) 6-7 and a PSA value in blood below 10. Many of these are harmless and can be left without active treatment and hence spared problematic post-therapy side-effects, others are highly malignant and require early diagnosis and treatment. Better prognostic markers are needed and the aim of this study was to evaluate prognostic markers and to test if these markers could identify patients with indolent tumours.

Methods: We have studied tumour material from 419 men consecutively diagnosed with PC at transurethral resection (1975-1990). The majority of these patients (295) had no metastasis at diagnosis and was not given any curative treatment and only hormonal treatment upon symptoms from metastatic progression. Standard histological sections and tissue microarrays (TMA) from these tumours and surrounding normal prostate tissue were stained and evaluated for cell proliferation (Ki67), blood vessels (endoglin and von Willebrand factor, vWf) and the extracellular matrix component hyaluronan (HA). An orthotopic rat PC model was used to explore hyaluronan staining, hyaluronic acid synthase (HAS)-1 mRNA levels and the effect of local HA treatment on tumour growth.

Results: Tumour cell proliferation (Ki67) and the density of intra-tumoural endoglin stained blood vessels were independent prognostic markers (i.e. they added prognostic information to the conventional prognostic markers; clinical stage and GS). None of the GS 6 patients with low staining for both Ki67 and endoglin died of PC within 15 years of follow-up. High HA staining in the tumour epithelium and stroma was a negative prognostic marker of cancer specific survival but they were not independent of GS. High HA staining and high vascular density in the stroma of the surrounding morphologically normal prostate were prognostic for short cancer specific survival. Implantation of tumour cells in the normal rat prostate resulted in an increase in HA and HAS-1 mRNA levels in the prostate tissue surrounding prostate tumours. Concurrently intra-prostatic injection of HA also stimulated tumour growth.

Conclusions: By evaluating both tumour cell proliferation (Ki67) and vascular density, it is possible to identify patients with very low risk of cancer specific death in the absence of active treatment. Prostate tumours influence the surrounding non-malignant prostate tissue, for example they cause an increased angiogenesis and synthesis of hyaluronan. Such responses can possibly be used to diagnose PC and to evaluate PC aggressiveness.

Svensk sammanfattning

Varje år diagnostiseras ca 10000 män i Sverige med prostatacancer. Majoriteten av dessa har en lokaliserad sjukdom där bot är möjlig (ca 80 %). De allra flesta små cancrar skulle, om de lämnades obehandlade, aldrig ge symptom under personens livslängd. Med dagens metoder kan man i de flesta fallen inte skilja på de tumörer som faktiskt behöver behandlas och de som skulle fortsätta att vara ”snälla” även utan behandling. Att behandla prostatacancer med strålning eller kirurgi innebär en stor risk för biverkningar i form av impotens, urinläckage och ibland även besvär med avföringstömning. Det skulle därför vara till nytta om man kunde veta vilka patienter som behöver behandling direkt, och vilka som utan risk kan vänta med behandling och på så sätt bespara patienter behandlingsrelaterade biverkningar.

Denna avhandling bygger på de undersökningar som vi har gjort av olika proteiners uttryck i prostatacancervävnad från patienter med lång uppföljning, som inte fått någon botande behandling utan endast hormonell behandling vid sjukdomsprogress.
Det första delarbetet i avhandlingen visade att det med hjälp av markörer för celldelning och blodkärl, tillsammans med en uppskattning av tumörens storlek, var möjligt att identifiera patienter med låg risk att dö av sin prostatacancer. I ett uppföljande arbete undersöktes 419 patienter (med hjälp av s.k. ”tissue micro arrays”; vävnadsstansar i ett matrissystem) för samma markörer. Vi bekräftade att det med dessa markörer var möjligt att identifiera patienter som inte dog av sin prostatacancer inom 15 år efter sin diagnos.

Den till synes omgivande friska vävnaden omkring en prostatacancer förändras och bland annat ökar mängden blodkärl och mängden av en molekyl som kallas hyaluronan (tidigare kallad hyaluronsyra). En ökad mängd blodkärl och hyaluronan visade sig vara kopplat till dålig prognos (kort överlevnad). Stöd för att hyaluronan påverkar cancerutvecklingen fick vi även av ett djurförsök där cancerceller växte bättre om hyaluronsyra sprutades in i cancern. Våra studier visade dessutom att proteinet som producerar hyaluronan ökar i omkringliggande vävnad om man planterar in prostatacancerceller i prostatan på försöksdjur.

Dessa tre arbeten och ytterligare andra arbeten som jag deltagit i talar för att man med relativt enkla metoder kan undersöka tumörvävnad samt vävnaden runt omkring tumören och på så vis identifiera patienter som trots att de inte får någon botande behandling har en mycket låg sannolikhet att dö av sin prostatacancer.

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Abbreviations

AR	Androgen receptor
CI	Confidence interval
CpG	regions of the DNA where cytosine and guanine are separated by only one phosphate
ECM	Extra cellular matrix
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
GnRH	Gonadotropin-releasing hormone
GS	Gleason score
GSTP1	Glutathione-S-transferase π
HA	Hyaluronan
HABP	Hyaluronan-binding protein
HAS	Hyaluronan synthetases
HGPIN	High grade prostate intraepithelial neoplasi
HIF	Hypoxia-inducible factor
HMW	High molecular weight
HR	Hazard ratio
HYAL	Hyarluronidases
IGF	Insulin growth factor
ISUP	International Society of Urological Pathology
LMW	Low molecular weight
PAI	Plasminogen activator inhibitor
PC	PC
PDGFβ	Platelet derived factor beta
PIA	proliferative inflammatory atrophy
ROC	Reciver operating characteristic
r_s	Correlation coefficient according to Spearman rank test
SNP	Singel nucelotid polymorphism
TGF	Transforming growth factor
TINT	Tumour indicating/instructed normal tissue
TMA	Tissue micro array
TUR-P	Transurethral resection of the prostate
TRUS	Transrectal ultrasonography
VEGF	Vascular endothelium growth factor
vWf	von Willebrandt factor (Factor VIII related antigen)

Original Papers

This thesis is based on the following Papers, which are referred to in the text by their Roman numerals

I. Josefsson A, Wikström P, Granfors T, Egevad L, Karlberg L, Stattin P, Bergh A

Tumour size, vascular density and proliferation as prognostic markers in GS 6 and GS 7 Prostate tumours in patients with long follow-up and non-curative treatment.
Eur Urol, 2005. **48**(4): p. 577-83

II. **Josefsson A**, Wikström P, Egevad L, Granfors T, Stattin P, Bergh A

Low vascular count and Ki67 staining scores in Gleason score 6 tumours may identify men with low risk of PC specific death when managed by watchful waiting.
Submitted

III. Josefsson A, Adamo H, Hammarsten P, Granfors T, Stattin P, Egevad L, Engström Laurent A, Wikström P, Bergh A

PC increases hyaluronan in surrounding non-malignant stroma, and this response is associated with tumour growth and an unfavorable outcome.
Am J Pathol, 2011. **179**(4): p. 1961-68.

During the course of this project I have also participated in following publications not included in the thesis

Chung SC, Hammarsten P, Josefsson A, Stattin P, Granfors T, Egevad L, Mancini G, Lutz B, Bergh A, Fowler CJ
A high cannabinoid CB(1) receptor immunoreactivity is associated with disease severity and outcome in PC.
Eur J Cancer, 2009. **45**(1): p. 174-82

Hägglöf C, Hammarsten P, Josefsson A, Stattin P, Paulsson J, Bergh A, Östman A
Stromal PDGFRbeta expression in prostate tumours and non-malignant prostate tissue predicts PC survival
PLoS One, 2010. **5**(5) e10747

Hammarsten P, Karalija A, Josefsson A, Rudolfsson SH, Wikström P, Egevad L, Granfors T, Stattin P, Bergh A
Low levels of phosphorylated epidermal growth factor receptor in nonmalignant and malignant prostate tissue predict favourable outcome in PC patients
Eur J Cancer, 2009. **45**(1): p. 174-82.

Thomasson M, Wang B, Hammarsten P, Dahlman A, Persson JL, Josefsson A, Stattin P, Granfors T, Egevad L, Henriksson R, Bergh A, Hedman H
LRIG1 and the liar paradox in PC: A study of the expression and clinical significance of LRIG1 in PC
Int J Cancer, 2011. **128**(12): p. 2843-52.

The prostate gland

The prostate is one of the male accessory sex glands, producing approximately 60% of the ejaculate and is believed to be involved in fertility by enhancing motility and surivival of the sperms.

The prostate measures approximately 20-30 cubic cm (chestnut) in young men and surrounds the urethra at the neck of the bladder. The main prostatic ducts, including two ejaculatory ducts connect to the urethra in a region of the prostatic urethra called the verumontanum. The prostate which is composed of three histologically different zones referred to as the central, transition and peripheral zones is surrounded by a fibrous and loose capsule (Figure 1).

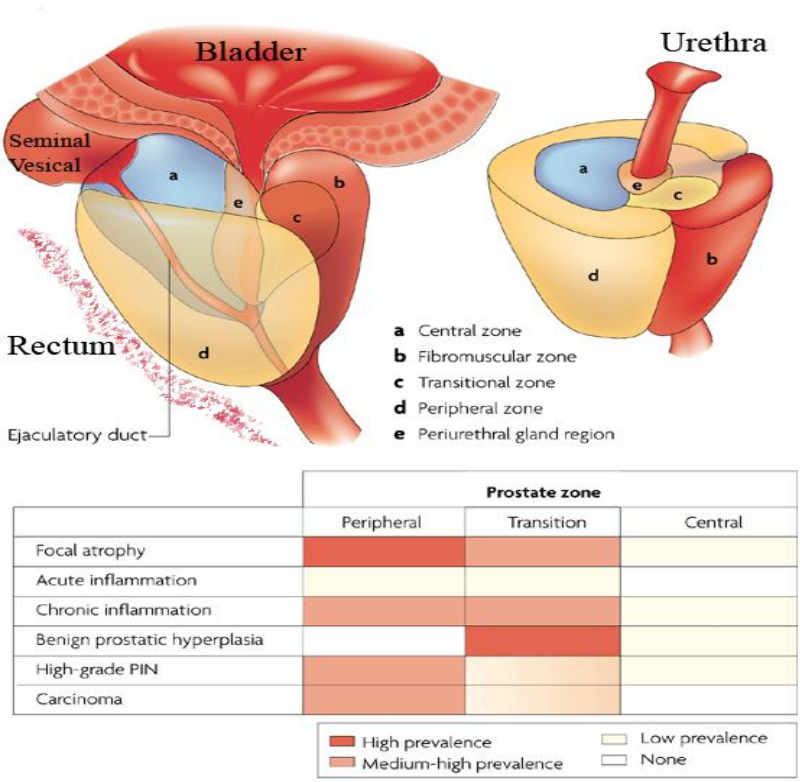

Figure 1 Schematic drawing to see the 3D localizations of the prostate zones and the relationship to different pathology in the different zones (the figure is adapted from De Marzo et al [1] with permission from De Marzo and Nature Publishing Group)

Posterior of the prostate, a small space called Denonvilliers fascia separates the capsule around the posterior peripheral zone from the rectum. The neurovascular bundle is attached to the posterior lateral aspects of the prostate capsule and contains nerves controlling erectile potency and incontinence. For this reason it is almost impossible to surgically remove the prostate or beam it with radiotherapy without interference to the neurovascular bundle. This interference can negatively effect erectile potency and urinary and stool continence. The proximity of the prostate to the rectum however makes it possible to take core biopsies (tissue samples) from the prostate guided by ultrasonography (TRUS; transrectal ultrasonography guided biopsies).

Androgen regulates normal prostate growth

The formation, differentiation and maintenance of all organs requires carefully choreographed programs of cell proliferation, apoptosis, adhesion, polarity, migration and differentiation. In the prostate these processes are dependent on reciprocal epithelial and stromal interactions [2], excretion of androgens, presence of androgen receptors (AR) and the protein 5-alpha reductase.

Testosterone is produced by the leydig cells in the testes and to a lesser extent in the adrenal glands. In the prostate, 5-alpha-reductase converts testosterone to dihydrotestosterone which binds more strongly to the AR. At binding, the AR is phosphorylated, which facilitates homodimer complex formation. The homodimer can then bind androgen response elements in the nucleus and subsequently induce changes in the transcriptional profile of the cell.

The prostate epithelium consists of two cell layers, the excretory luminal cells and the basal cells resting on the basement membrane. The luminal cells produce serine protease PSA, which cleaves the semenogelins in the ejaculate and thereby liquefies the ejaculate. In the AR negative basal cell layer, stem cells can be found which constitute 0.5 % of all basal cells in an adult prostate. These stem cells give rise to two distinct cell lineages. One possible lineage involves cells proliferating and differentiating into a non-proliferative and AR negative neuroendocrine cell which produce and secrete various neuropeptides and growth factors [3] such as synaptophysin and chromogranin A. The second lineage involves differentiation into another androgen receptor negative cell that is called a transit-amplifying cell. These transit-amplifying cells constitute the main bulk of cells in the epithelial

basal cell layer and eventually terminally differentiate to form the androgen receptor positive luminal-secretory layer.
All of these steps are dependent on epithelial cell interactions with stromal cells; smooth muscle cells and fibroblasts. Smooth muscle cells express both AR and 5-alpha reductace and stimulate the epithelial differentiation through paracrin interactions of produced growth factors called andromedins (e.g. IGF-1, FGF-7 and -10 and VEGF) [4]. For review see Issacs *et al* [5]). Androgens control differentiation and survival of the epithelial cells whilst andromedin activated proliferation of the transit-amplifying cells is inhibited in the luminal cells due to expression of the AR mediated up regulation of p21 and p27.

Prostate cancer incidence and mortality

Recently the incidence of prostate cancer (PC) has increased dramatically, with an age-standardized annual increase of 2.1% over the last 10 years in Sweden. The mean PC incidence per year between 2005 and 2009 was 9503, which constituted 36.3% of all cancers diagnosed. In 2010 over 10 000 new PC cases were diagnosed in Sweden.

PC is the leading cause of cancer related deaths in Swedish men contributing to 2460 deaths each year (average between 2005 and 2009). Indeed in 2010 the lifetime risk for a man to die of PC in Sweden was 5.5%. The number of men to die of PC in 2010 of different age groups is illustrated in Figure 2 [6].

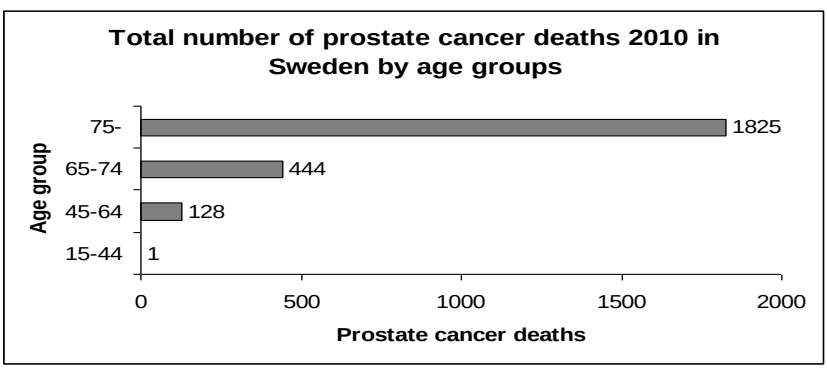

Figure 2 Number of PC deaths 2010 in Sweden by age groups [6].

Incidence and mortality rates of prostate cancer in Sweden spanning 24 years are illustrated in Figure 3 and 4. The estimated annual age-standardized decrease in mortality rate in Sweden over the latest 10 years has been 1.7% and predominantly among men below 74 years old [7] Of the men in Sweden diagnosed with PC, most are within the ages 65 and 69 years, whilst the majority of PC deaths occur in men over 79 years [6]. Between 1999 and 2003 the 1- and 5-year relative survival was 97 and 84 % respectively [7].

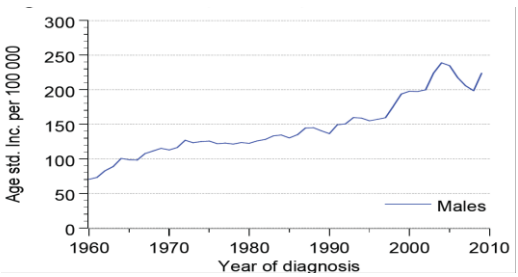

Figure 3 Age standardized incidence per 100 000 men in Sweden between the years 1960 and 2010 [6].

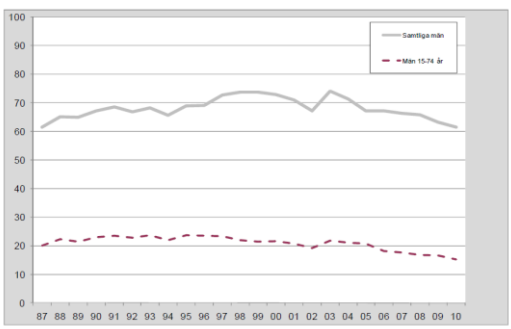

Figure 4 Prostate cancer mortality rates between the years 1987 and 2010, in all swedish men (the solid line) and between 15 to 74 years old (dashed line). Figures are age-standardized death rates per 100 000[6].

The decline in mortality has also been accompanied by a decline in the proportion of men with distant metastases at diagnosis. From 1998 to 2009 the proportion of men with metastasis at diagnosis have declined from >20% to approximately 11%. The proportion of men with low risk PC

at diagnosis has increased from approximately 13 % to 28% and in 2007 30% of all PC patients were diagnosed without symptoms [8]. It is conceivable that the decline in metastasized PC at diagnosis and the decrease in PC mortality is due to increased awareness of the disease followed by access to curative treatments.

Prostate cancer prevalence

By the year 2009 there were 75647 men living with a PC diagnosis (1627/100 000 men; 1.6% diagnosed prevalence) [7], however prevalence of PC has been examined in various autopsy studies with PC indicated as the most prevalent cancer of all cancer forms [8] with a prevalence of 40-50% in 50 year old men [9-11]. In addition an autopsy study of accidental deaths of 249 men with an age between 20-69 showed that cancer could be found as early as in the 3rd decade in some cases (2%) and was found in 55% of men in the 5th decade of life [12]. Similar prevalence was found by Sanchez-Chapado *et al* with 64% PC in men between 50-69 years of age [13], while lower numbers were found by Yin *et al* in an autopsy study of previously healthy donors, showing a prevalence of 30% in men between 60-69 and 46% in men above 70 years of age [14].

More than 70% of all cancers in the prostate arise in the peripheral zone and probably in the apex region [15], whilst less than 20% and 10% of the PCs are located primarily in the transition zone and central zone, respectively [15-16].

Prostate cancer etiology

Cancer develops through the accumulation of genetic and epigenetic changes leading to the inactivation of tumour suppressor and caretaker genes, and the activation of oncogenes. These changes are due to environmental exposure and acquired or inherited genetic predisposition. The etiology of PC is unknown, but the three most established risk factors are age, race and family history of PC which indicates a genetic component.

The incidence and mortality of PC is differently distributed in the world and not only due to race and variant expectancy. The prevalence of small cancers in autopsy studies is similar in different geographical regions, and correlates with age but clinically significant tumours are more common in countries with western lifestyles [11]. This implies that environmental factors in addition to genetic factors may facilitate the progression of cancer.

Etiology -Environmental factors

Epidemiological studies have demonstrated that Asian countries have much lower cancer incidence compared to the USA and Europe [17]. Asian men moving to the USA will experience a much higher PC prevalence. [18-19]. Diet

differs greatly between Asian and Western countries. The Asian diet is rich in soy and vegetables (cruciferous), garlic and green tea but also red wine and tomatoes are believed to decrease PC risk [20-22]. The more Western diet, that is rich in fat, meat and dairy products may instead increase the PC risk [23].

Etiology -Genetic factors

The higher risks of PC incidence in African-American men compared to Caucasian men implicate a genetic component in the etiology of PC. Twin studies have indicated that as many as 42% of all PC cases could be explained by heritable factors [24]. With one first-degree relative with PC the relative risk to be diagnosed with prostate cancer is 1.65 in a Swedish register based study [25].

Recently, the largest genome-wide association study performed in PC has reported the identification of 40 loci that could explain approximately 25% of the familial risk of PC. This indicates a huge number of different low risk genetic changes that in combination could lead to a higher risk of PC [26]. Many important genes are located in the 40 loci identified as being involved in PC etiology, for example those genes involved in androgen synthesis (e.g. the gene for 5α-rducatse-1 and the gene CYP17 [27], where a commercial inhibitor of the gene product for CYP17 (abiraterone acetate) is available of treatment of castrations resistant prostate cancer) and the immune response (e.g. RNASEL, involved in reducing antiviral activity [28]). Indeed alterations in inflammatory genes such as cyclooxygenase, interleukin-1 and 8, tumour necrosis factor alpha and toll like recetor-4 have also been found to be associated with prostate cancer risk [29].

Studies of single-nucleotide polymorphisms (SNPs) in a genome-wide association study in Sweden found a small group consisting of 1.4% of the studied population with 5 SNPs which had 9.46 times increased PC risk (a group with four SNPs (8.2% of Swedish men) had 4.7 times increased risk of PC) [30]. Proteins coded in regions with found polymorphisms include many different receptors and enzymes including angiotensin converting enzyme, methyltransferases, lipases, nitrix oxide synthesis, PSA , vitamin D- and human epidermal growh factor receptor 2, interleukins, TGF-β1, VEGF, E-cadherin, HIF-1α and PAI-2 and enzymes involved in androgen metabolism [31].

Etiology -Inflammation

Chronic infection and inflammation have been shown to cause cancer in several different organs including the stomach, liver, large intestine and penis. The inflammatory process is characterized by sustained tissue damage, recruitment of inflammatory cells, production of different paracrin growth factors, damage-induced cellular proliferation and eventually induced signals of tissue repair, including angiogenesis. There is also accumulating evidence that inflammatory cascades are involved both during PC initiation, progression, and metastasis [1].

Many cytokines, chemokines and inflammatory cells involved in inflammation have been linked to different aspects of PC progression and have been shown to facilitate angiogenesis, tumour growth, tumour cell invasion, tumour cell escape, and the resettlement of distant metastasis (see Lu *et al* and De Marzo for review [1, 32]).

From normal epithelium to prostate cancer

PC is thought to develop from normal prostate epithelium through a histologically defined stage called proliferative inflammatory atrophy (PIA) or from PIA via high grade prostate intraepithelial neoplasia (HGPIN) to PC [1, 33]. It has been suggested that HGPIN can form without the pre-cancerous lesion of PIA. The previously mentioned autopsy study by Sakr *et al* showed that approximately 3/4 of all men with HGPIN also had a PC and 24% of all men with PCs also harboured HGPIN [12]. The same chromosomal abnormalities that are seen in PIA are also seen in PIN and PC cells. Another association between PIA/HGPIN and PC is the zonal co-localization; chronic inflammation and focal PIA are seen in the peripheral zone, where more than 70% of all PC is localized. In addition both HGPIN and PC are absent in the central zone [34] (see also Figure 1).

Many different stimuli can trigger PIA lesions, such as bacteria, viruses or fungi. Other mechanisms for PIA response can be the exposure of estrogens, physical irritation by prostate stones (corpora amylacea), even the presence of urine can trigger lesion formation. Dietary carcinogens such as charred meat have also been shown to trigger DNA damage and mutations and result in inflammatory response in the prostate epithelium [1].

The regenerative response after tissue injury leads to the PIA lesions which histologically can be seen as atrophic cells intermixed with highly

proliferating cells. Glutathione-S-transferase π (Gstp 1) is up regulated in these proliferative cells and function as a genome-protective protein. Hyper-methylation of CpGs in the gene promoter of GSTP1 results in down-regulation of this protein and is widely seen in PC [35]. This methylation is one of the earliest molecular abnormalities characteristic of PC cells. Other early changes seen in pre-stages of PC are the shortening of the telomeres, up regulation of hepsin and down regulation of the protein products of Nkx3.1, CDKN1B and PTEN [36-39].

Collectively, these changes increase the risk of accumulating genetic damage, decrease the caretaker phenotype and may lead to genetic instability and thereby acceleration of genetic changes. In this vulnerable state, gene rearrangements can easily occur. The most common type of gene fusions involve fusion of the androgen regulated chromosomal region TMPRSS2 to the oncogenic ETS transcription factor family members ERG (in 30-70% of all PC:s) [40-42]. This androgen mediated activation of the ETS gene products mediates MYC activation, modulation of prostaglandins and regulation of various matrix proteinases and angiogenesis-related genes [41-43]. Recently, Shaikhibrahim *et al* have identified many connections between the ETS gene family and immunity-related genes showing another link between PC and inflammation [44].

Clinical manifestation of prostate cancer

Localised PC does not cause any condition-specific symptoms, but rather shares symptoms with other non-malignant prostatic diseases such as benign prostate hyperplasia. These symptoms include voiding problems, poor stream and high micturation frequency and/or urgency. The majority of men diagnosed with PC in Sweden are without symptoms. As the disease progresses, symptoms can manifest, localised advanced PC can obstruct the ureters and give rise to uraemia, metastasised PC can give rise to pain and pathological fractures due to metastases to bone. In advanced and disseminated PC fatigue and weight loss called cachexia is due to mostly non known effects, but can also be the effect of anaemia due to bone marrow suppression and/or uraemia.

Prostate cancer diagnosis

The diagnosis of PC is based on histological evaluation of resected prostate tissue. Men who present with elevated prostate specific antigen (PSA) in blood or if PC is suspected upon digital rectal examination of the prostate, undergo further investigation with core biopsies from the prostate. This is performed with ultrasound (TRUS, see below) guided multiple punctuations with an 18-gauge needle. The histological evaluation is graded according to Gleason score (GS).

Staging

Non palpable tumours are referred to as clinical t-stage 1 (cT1), palpable tumours are cT2, palpable tumours that seem to penetrate the prostate capsule are cT3, and if the tumours extend to the surrounding organs they are called cT4. It should be noted that pathological staging is a histological staging performed after surgical removal of the prostate (pT1-4).

Historically it was common to carry out explorative surgical removal of the obturatorius lymph nodes and to assess possible lymph node metastasis before making a therapeutic decision. Today, magnetic resonance imaging (MRI) or computer tomography X-ray could be of some help if lymph node metasasis are suspected. In order to detect bone metastasis, a nucleotide based bone scan is used.

Grading according to Gleason

Today the grading of PC is carried out according to Donald F Gleason (1920-2008) and is based on the histological patterns/organisation of the PC cells [45]. In 2005, the International Society of Urological Pathology (ISUP) drew up recommendations of how to use the Gleason score (GS) system and also further defined the grades within the system. Different approaches are also used to construct the GS depending on the tissue samples to be evaluated.

As part of these recommendations each cancer pattern in biopsy material is graded between 3-5 (1-2 should be rarely, if ever used [46]) and the GS is composed of the most common grade and the highest grade, which mean that GS can be 6 to 10 in biopsy materials.

In radical prostatectomy specimens each PC biopsy is graded between 2 to 5 (pattern 1 should be used rarely, if ever) and the GS is composed of the most common and the second most common grade. If any smaller foci are composed of a higher grade this should be mentioned together with an estimated percentage of the extent of this grade.

In Sweden, tissue samples from trans-urethral resections of the prostate (TUR-P) are evaluated in concordance with the grading used in radical prostatectomy tissue, but it could be argued that the biopsy approach should be used instead.

Prostate specific antigen

Normally PSA leaks out into the serum in very small proportions. PSA is increased in the serum for various reasons; elevated levels are seen during infection/inflammation of the prostate, injury to the prostate (e.g. punctuation of the prostate during biopsy), benign prostatic hyperplasia (BPH) and PC. Disruption of the basal membrane and altered intra-luminal cleavage could also account for the increased serum PSA seen in PC [47]. It is also probable that increased PSA during injury and inflammation is caused due to interference of the barrier between the epithelium cells and the capillaries. In BPH the PSA leakage is proportional to the size of the prostate.

Higher PSA density (PSA value/prostate volume) is more common in men with PC and a PSA density less than 0.1 ng/ml*cm-3 with negative core biopsies makes PC less likely.

As discussed PSA is not cancer specific and PC cells actually produce less prostate specific antigen (PSA) per cell than normal prostate epithelium.

Serum PSA-values can be up to many thousands in metastatic PC and with PSA-values above 100 ng/ml a metastatic disease is indicated. PSA value is fluctuating over time intra-individual and a man with elevated PSA value one years (above 3.0 ng/ml) could be below the cut off the next two following years in as many as 20% of the cases in a screened population [48].

Due to the natural occurrence of PSA in serum and the increase during benign prostate hyperplasia there are no absolute cut off between normal and abnormal PSA values. In Sweden 3 ng/ml is used as cut off and the specificity is then reported to be 89% in a screened population [49]. The proportion of men with levels above 3 ng/ml and actually diagnosed with PC on biopsy is approximately 24% [49]. In the largest study of PC incidence after biopsy, as much as 15.2% of patients with PSA-values less than 4 ng/ml were found to have PC [50].

Treatments of prostate cancer - Curative treatment

Patients with clinically localized PC and with a life expectancy of more than 10-15 years may either be offered direct treatment with curative intention or monitored by active surveillance. Active surveillance is recommended as an alternative for patients with low grade cancers and with PSA values less than 10 and includes close monitoring with PSA tests, DRE and recurrent core biopsies. Upon disease progression therapy with curative intention can be performed. The rational of this approach is to avoid over treatment of indolent tumours. In a recent active surveillance study approximately 30-40% of all men were offered treatment within 4 years due to either risen PSA-value, GS progression or other reasons for active therapy. Of those patients that ended active surveillance and were treated, 9 of 70 had PSA-relapses with a median follow up of 37 months [51] the other study with much older patients had 59/117 (50%) PSA-relapses after median follow up after 48 months [52]. These figures raise the question of wether the right patients were chosen for active surveillance, or rather if we today have predictive markers good enough to find those patients with indolent tumours.

Therapy with curative intention is either surgery or radiotherapy. Surgery can be done by open retro-pubic radical prostatectomy, laparoscopic prostatectomy or robotic assisted laparoscopic prostatectomy (RALP). Radiation therapy can be seed implantation, external beam radiotherapy and/or internal brachytherapy.

Improvements in surgical procedures with robotic assisted laparoscopic prostatectomy have been shown non-inferior with oncological results and

are associated with less pre-operative blood loss, post-operatively less pain and fewer in–hospital days. Side-effects of curative therapies are still significant however with 80-90% of pre-operatively potent men classified as impotent 18 months post-operative and 14-20% considered post-operatively incontinent [53]. There is also a small risk of urethral stricture. Estimations show that screening for PC makes approximately 4 extra men impotent and 1 extra man incontinent for each life saved from PC death [54]. In the Swedish SPCG-4 study they found that abrupt onset of the erectile dysfunction in those men (84% of all) after radical prostatectomy was a psychological problem for the men more than a decade after the procedure (median age at questions was 77 years old) [55].

Radiotherapy techniques have also improved and with adjustments to the external radiotherapy with movement of the target higher doses can be given without higher frequencies of side effects. With a dose escalating regime, 78 gray can be given and with a combination of external and brachy radiotherapy more than 100 gray can be given. Long follow ups have shown that radiotherapy for localized disease is non-inferior to surgery in disease free survival [56]. About 64% have erectile dysfunction after 5 years and the corresponding figure for urinary leakage is 13%. Proctitis with haemorrhage and problem at passing stool after radiotherapy has been seen in approximately 18% of cases [57].

Treatments - Non-curative treatment

Watchful waiting, also called deferred treatment or delayed hormonal treatment was previously the most used approach. This means that hormonal treatment is given upon symptoms of the disease, compared to active surveillance where primary treatment with surgery or radiotherapy is given before any symptoms arise. Watchful waiting is suitable for patients with a life expectancy of less than 10-15 years and where primary treatment with curative intention is not suitable (because of co-morbidity and/or aggressive disease). Locally advanced disease can also be treated with radiation therapy with neo-adjuvant, concomitant and adjuvant endocrine therapy with good results [58] Also surgery in locally advanced disease states has been show to have more favourable outcomes than non treatment approaches [59].

Treatments - Metastatic disease

In a case of disseminated metastasised PC with symptoms direct hormonal treatment such as androgen deprivation is offered. It can be accomplished by surgical castration, oestrogen injections or by stopping the testosterone production by either gonadotropin-releasing hormone (GnRH) agonist or GnRH antagonist (the agonist functions as a antagonist after approximately one month through down regulation of the receptor). Treatments with oral estrogens have been shown to have cardio-vascular side effects and are now less commonly used. Parenteral injection of estrogen gave fewer side effects, however less than 1% of hormonal treatments in Sweden today are estrogen injection.

Side effects of androgen deprivation therapy are hot flushes (similar to those in post menopausal women), mood changes, and accumulation of abdominal fat, cardio vascular diseases and loss of libido.

Prostate cancer screening

The lack of symptoms, high incidence and increasing awareness of the disease have initiated the discussion of screening of all men above a certain age for the presence of PC. This question is beyond the scope of this background; however some recent publications can be mentioned.

The Gothenburg randomised population based PC screening trial (the longest follow up of the European randomised study of screening for PC, ERSPC) have shown that PC mortality was reduced almost by half over 14 years (the numbers of PC deaths were small with an absolute risk in the control group and the screening group of 0.9 to 0.4 %, respectively). It should be noted that at least 12 patients needed to be diagnosed and 293 needed to be screened to prevent one PC death [60].

The Prostate, Lung, Colorectal and Ovarian cancer screening trial (PLCO) study conducted in the US could not confirm similar findings with decreased PC mortality in the screening arm. The differences between the two trials have been thoroughly reported by Schröder and Roobol in 2010 [61]. One of the suggested reasons for the lack of difference in PC-related deaths between study arms of the US study was the high prevalence of non organised screening in the control arm; 52% had been PSA tested during the study and both the control and study arm had been pre-screened with PSA tests before

the study. The men that had conducted one or two PSA tests before the study entry had a 25% risk reduction of getting PC during the study.

With a prevalence of at least 30% PC in men above the age of 50, screening of all men in Sweden would lead to the diagnosis of even more small, indolent cancers. This would hence make curative treatment possible for some men, but with the risk of overtreatment for many others.

The prostate cancer dilemma

The prostate is as previously described an organ with very high frequency of cancer. Most cancers are however not clinically significant (indolent) and if undiagnosed, would be symptom free and would not influence the affected man within his lifetime. There is today no way of telling if a low grade tumour will progress to lethal disease or not. GS, PSA, extent of cancer on core biopsies and clinical stages are used and can roughly be simplified to discriminate four groups. The worst group with very advanced disease with only palliative treatment available (GS 8-10 with PSA above 200 with metastasis), the second worst group with some available treatment that can prolong life but where the possibility of curation is minimal (GS 7-8 with large tumours, PSA above 30 and with suspected metastasis) and the 3rd group of quite good prognosis with men where curation may be possible and where primary treatment is suggested to be done quickly (GS 6-7 with large tumour and with PSA above 10) and the 4th group with the most favourable prognosis where curation is possible but may not be needed (non palpable, small GS 6 tumours with PSA<10). In the 4th group there are however some tumours behaving more aggressively and those should not be considered as indolent. Screening has shown reduced PC deaths compared to control groups but at the cost of side effects for those patients treated. If all men with a prevalent PC could be found, by the introduction of more effective screening, an even larger fraction of men would be diagnosed with a low grade, low volume, low PSA and non-palpable tumour, and many men would be recommended for surgery or active surveillance, with either reduced quality of life due to post operative side effects or the risk of missing the window of opportunity to be cured from the disease. In contrast, if no man was offered PSA tests and surgical removal of the prostate, men would die of PC who would have been saved in a screening scenario. As mentioned before the increasing use of PSA-testing leads to both earlier detection of prostate cancers that would become symptomatic with time, but also of an increasing number of small tumours that probably never would progress during lifetime. In the European screening study ERSPC over diagnosis has been estimated to be as high as 48%[62].

A prostate cancer diagnosis is made through PSA measurement, digital rectal examination and by transrectal ultrasound guided biopsies. As prognostic markers these are neither sensitive nor specific enough to distinguish significant from in-significant prostate cancers. PSA testing in a screened population had a 24% positive predictive value to find prostate cancer at sextant core biopsies [63]. A positive finding of digital examination is considered a very blunt tool and as much as 70% of a positive rectal palpation is negative the next year [48]. Transrectal ultrasound (TRUS) examination enhanced by Doppler could be used as a tool for detecting suspected PC areas but is again neither specific nor sensitive. The positive predictive value of hypo-ecogenic area in the peripheral zone is 25-30% [64]. The grading of the tumour found at core biopsy are a very good prognostic marker for tumors with GS below 6 or ≥8, but the absolute majority of newly diagnosed PCs today have GS 6 and for those patients GS has no prognostic value. Novel prognostic markers are urgently needed for identification of patients where management with active surveillance can be a safe alternative to direct curative treatment and thereby avoid or postpone side effects of treatments.

"Always remember that it is impossible to speak in such a way that you cannot be misunderstood: there will always be some who misunderstand you."

Karl Popper

Novel prognostic markers in prostate cancer

Prognostic markers for PC are most often searched for in radical prostatectomy material using PSA-relapse as an outcome. Pound et al has shown that only about 50% progress from PSA-relapse to metastatic disease within 10 years [65]. Pre-operative core biopsies could also be used and with PSA-relpase as outcome. These approaches are suitable when looking for markers of poor outcome in patients who have been actively treated, that is cases where extra-prostatic growth and metastases are present prior to the starting of treatment. If markers are found that are able to prognosticate PSA-relapse or PC death after radical prostatectomy these could be useful in decision of additional neo-adjuvant, adjuvant or extended therapy but not to find those that should not have needed therapy, that is they can not help us to find the indolent tumours.

The material used in this study (see Material and methods) is from patients diagnosed with PC after transurethral resection of the prostate (TUR-P). These patients were diagnosed between 1970 and 1990, and most did not receive any curative treatment but were followed with watchful waiting, according to the therapy tradition in Sweden. This TUR-P material is suitable for the search of potential markers for indolent disease, due to its long follow up and the fact that the majority of the patients were not given any curative treatment.

Prognostic markers - where to look?

PC growth and spread is dependent on the tumour cell phenotype but also on reciprocal interactions with stroma cells. The cancer stroma supports cancer development by supplying the tumours with cytokines, growth factors, and tissue remodelling enzymes, all of which may facilitate tumour cell growth, invasion and angiogenesis [66].

The progression from normal epithelium to invasive cancer have been investigated and reviewed by Weinberg and co-workers and the prerequisites for cancer development and progression, identified so far, are called “the hallmarks of cancer” and include [67-68]:

- Self-sufficiency in growth signals
- Insensitivity to anti-growth signals
- Evasion of apoptosis
- Limitless reproductive potential
- Angiogenesis
- Tissue invasion and metastasis
- Deregulated metabolism
- Evasion of the immune system
- Unstable DNA

In this thesis, markers of proliferation, angiogenesis and extracellular matrix have been investigated. They may reflect some of the hallmarks of cancer. Proliferation may reflect both self-sufficiency in growth signals and insensitivity to growth inhibition, but also deregulated metabolism. Vessel markers should reflect angiogenesis and changes in the extra cellular matrix may reflect and have effects on diverse hallmarks such as tissue invasion, inflammation, angiogenesis, and the evasion of the immune system.

Angiogenesis

Already in 1945 Algire *et al* suggested that the development of a rich vascular supply was essential for the rapid growth of tumours [69]. Folkman *et al* suggested that tumours are needed to recruit blood vessels to be able to grow more than a few mm [70]. During cancer progression tumours must therefore at some point switch to an angiogenic phenotype to grow. The blood vessels are needed to give good supply of oxygen and nutrients but also enable tumour cells to metastasise. The angiogenic processes is induced and sustained by different paracrine signals, where the production of vascular endothelial growth factor (VEGF) is the crucial regulator. Hypoxia in the cells may lead to reduced growth in tumours and lead to the production of VEGF, but some degree of hypoxia may also be potentially favourable for tumour progression [71]. The tumour vasculature is different from normal vasculature and is both irregular in shape and has a physiologically dysfunctional organization (arterio-venous shunts and blind ends). Tumour vessels have discontinuous lining of the basement membrane, peri-endothelial cells and are therefore more leaky [72].

New blood vessels are formed by various mechanisms, all of which are known mechanisms in cancer angiogenesis. Sprouting angiogenesis (often referred to as angiogenesis) is the most investigated, and considered the central method of vascular recruitment to hypoxic tissue, although other

mechanisms probably go on simultaneously. After the initial breakdown of the basal membrane and extra cellular matrix the “leading” endothelial cells, tip cells, will migrate towards the hypoxic area [72].

Other mechanisms by which tumours recruit new blood vessels are through recruitment of endothelial precursor cells from the circulation. These cells become angioblasts and differentiate into endothelial cells. They are then further organized into mature vessels by attracting and interacting with mural cells (Vasculogenesis). These vessels can then expand through sprouting angiogenesis. Vascular mimicry can also be employed in the development of new vessel. This involves dedifferentiated tumour cells forming vessels either dependent or independently of other angiogenic processes [72].

The maturation phase of angiogenesis includes the formation of the basement membrane and the recruitment of peri-endothelial cells that enclose and stabilise the vessels. Platelet derived factor beta (PDGFβ) and TGFβ are important in the maturation process and are required for the recruitment and longitudinal spreading of vascular smooth muscle cells and pericytes that will cover the blood vessels [73-74]. Vessels without peri-endothelial cells or basement membrane are more easily penetrated by escaping PC cells, recruited stem cells or inflammatory cells. Wikström *et al* have shown that endoglin (CD105) is expressed in the endothelium of immature blood vessels, i.e. vessels without stabilizing pericytes [75]. Von Willebrand factor (vWf) or factor VIII related antigen, CD34 and CD31 are expressed in most endothelial cells but not always in newly formed vessels.

Endoglin is a trans-membrane protein, considered as an accessory TGF-β super family receptor subtype, which is predominantly expressed in vascular endothelial and smooth muscle cells. Endoglin plays an important role in homeostasis of the vessel wall by modulating TGF-β response [76-77]. Endoglin has also been seen in cancer epithelium, but with conflicting results. Cell line experiments have suggested an inhibitory effect on tumour cell motility [76, 78-79]. Serum and urine endoglin levels is higher in patients with more aggressive PCs and have been associated with higher risk of PSA relapse after prostatectomy [80-82]. Cell line experiments have proposed KLF6 to control endoglin expression which could be induced by both hypoxia and TGF-β [83]. Blood vessels expressing endoglin have been seen primarily in tissues that have sustained injury or inflammation and have also been found in thyroid disorders, psoriasis, scleroderma, ischemic stroke and also different tumours [76].

Vessel density in prostate cancer

In line with the hypothesis that cancers need to shift to an angiogenic phenotype in order to be aggressive, micro vessel density has been associated with worse prognosis in many different cancers. Endoglin is a promising marker of newly formed vessels and has been shown to be a stronger prognostic marker in breast cancer patients than CD34 micro vessel density [84]. Vascular density has been shown to prognosticate outcome after radical prostatectomy and also after TUR-P in watchful waiting cohorts [85-89].

High micro-vessel density has also been associated with metastatic disease, highlighting the proposed escape route of PC cells through vessels into the circulation [86, 88, 90].

Wikström *et al* have previously examined endoglin and vWf vascular count in a sample of PC patients from the same material as used in this project. The hot spot vascular count of endoglin was associated to TGFβ expression and proliferation index. Approximately 34% of the blood vessels in prostate cancer diagnosed at TUR-P expressed both endoglin and vWf. About 18% expressed only endoglin and in line with the hypothesis that endoglin is expressed in newly formed vessels; only 19% of these vessels had pericyte coverage. Higher tumour grades had less coverage of endoglin stained vessels with pericytes. vWf was expressed alone in approximately 48% of the vessels and almost half of these had pericyte coverage. Both increased endoglin and vWf vessel staining score were associated with shorter cancer specific survival however endoglin was also prognostic in the subgroup of GS 5-7.[75]. El-Gohary *et al* have counted hot spots of endoglin in PC patients who underwent prostatectomy and showed that high endoglin v.d. was associated with survival [91]. High vascular density measured with vWf in 66 radical prostatectomy patients was associated with biochemical recurrence [92]. Borre *et al* have shown that maximum vWf vascular count was an independent prognostic marker for cancer specific survival in a TUR-P cohort of patients managed with watchful waiting, however the mean vascular count could not be used as a prognostic marker [87, 93].

Proliferation

Proliferation is regulated by mitogenic and anti-mitogenic signals controlling the cell cycle. The cell cycle is divided into different phases; G_1, S, G_2, and M-phase. G_0 is the non proliferating phase. The proliferation marker protein Ki67 is not present in G_0 phase and early G1 phase but is present and maintained through all subsequent steps of the cell cycle. The monoclonal

antibody against human Ki67 was first available for cryostat sections 30-years ago [94]. In 1992 Cattoretti *et al* made a mouse monoclonal antibody (MIB-1) against recombinant parts of the Ki67 which made it applicable to immunohistochemistry on paraffin wax sections [95]. Very little is known about the function of Ki67 in cells but it is thought to play a role in the organisation of chromatin structure [96]. Ki67 is suggested as a promising prognostic marker in PC and has been shown to give independent prognostic information in many cohorts. In radical prostatectomy tissue Ki67 has been shown to independently predict both PSA-relapse and cancer-specific outcome [97-117]. In biopsy studies it has shown to prognosticate post-operative PSA-relapse and cancer-specific survival [118-124]. Moreover, Ki67 could, in evaluated biopsies, predict radiotherapy failure [125-128].In TUR-P material it has also been shown to be a independent prognostic marker of prostate specific death [129-133]. Other studies have shown that ki67 measured in biopsy could be associated with hormonal treatment failure [134-135].

There are however some conflicting results with some studies failing to show association between Ki67 and cancer specific outcome in both TUR-P material [136], radical prostatectomy material [137-140] and core biopsies materials [141].

Changes in the extra cellular matrix

Tumour stroma has been shown to undergo alteration during cancer progression which includes both changes in the cell composition and the extracellular matrix (ECM). The loss of smooth muscle cells and the accumulation of fibroblasts, myofibroblasts, tumour associated macrophages, mast cells and other inflammatory cells together with angiogenesis and changes in collagen, hyaluronan, versican composition all characterise the tumour stroma and many of these changes have been linked to prognosis [93, 142, 143 , 144]

These recognizable changes of the extra cellular milieu of tumours can also be called the “reactive stroma” which reflects the theory that these changes are the effect of signals from the tumour. Other changes seen in areas surrounding the tumours are referred to as the “field effect”. The “field effect” is referred to the supposed pre-cancerous milieu that enhances the initiation of cancer.

Hyaluronan in prostate cancer

The extra cellular matrix (ECM) is re-constructed around PC including accumulation of collagen I, collagen III, different fibronectin isoformes, hyaluronan and versican [145]. These changes have all been associated to PC

outcome [142, 146 , 147-149]. ECM changes are either a consequence of cancer progression or are self facilitating the cancer progression in different ways. These changes have both been shown to facilitate the recruitment of cells from the circulation, the modification of stroma cells, and also to facilitate the migration of tumour cells into the circulation.

Hyaluronan (HA) is a sugar-chain macromolecule in which N-acetyl-glucosamine (GlcNAc) and Glucuronic acid(GlcA) are linked together by alternating beta-1,3 and beta-1,4 linkages. HA-binding molecules can link with high molecular weight (HMW) HA into aggregates which form part of the ECM. The three isoforms of the proteins that synthesize HA are referred to as hyaluronan synthesis 1, 2 and 3 (HAS1, 2 and 3) . As discussed in Paper III there are many PC associated growth factors and mechanisms that also activate the production of HA (e.g. TGF-β, IGF-1 and hypoxia).

Degradation of HA is by hyaluronidases called HYAL-1,2,3 and PH-20. Depending on which HYAL that is present, HA seems to be degraded into different sizes which gives rise to different biological effects (see Discussion).

HA is a versatile molecule that can regulate cell behaviour both through mechanically changing the surrounding and by acting on the intra cellular signalling pathways through interaction with cell surface receptors. It has also been associated with the regulation of cell adhesion, cell polarity, motility, growth, angiogenesis, differentiation during embryogenesis, inflammation, wound healing, metastatic process, cancer progression and chemo resistance [150-158].

HA has been shown to accumulate in both epithelium and tumour stroma and is also associated with poor prognosis in both prostate, breast, ovarian and thyroid cancers [159]. In PC HYAL-1 seems to promote tumour growth, tumour cell invasion and metastases [160-162]. HYAL-1 has also been identified as an independent predictor of metastasis in PC patients [149, 163].

Changes in the normal prostate tissue surrounding tumours

Our group has previously suggested that adaptive changes in the surrounding morphologically normal prostate tissue outside the tumour should be called “Tumour Indicating/Instructed Normal Tissue” (TINT, see discussion) and that the magnitude of TINT changes are related to tumour aggressiveness. TINT changes can be seen in either the stroma or the glandular epithelium in the surrounding morphologically normal (non-malignant) prostate [143, 164-166].

AIMS

The overall aim of this thesis was to evaluate prognostic markers for PC in patients followed with watchful waiting and with a long follow-up, and in particular to ascertain if these markers could be used to identify patients with a good prognosis also without curative treatment.

Specific aim of Paper I

To explore if tumour vascular density (assessed by endoglin and vWf stained vascular count), tumour cell proliferation (Ki67) and tumour size was prognostic for cancer-specific survival in patients with GS 6 and 7 tumours.

Specific aims of Paper II

To study the same markers evaluated in Paper I (angiogenesis and proliferation), in additional tumour samples (GS 4 to 10) through the use of tissue-micro arrays (TMA) including both tumor and non-malignant areas.

To evaluate the use of TMAs in the search for prognostic markers in a cancer as heterogeneous as PC.

Specific aim of Paper III

To study the distribution of hyaluronan in malignant and non-malignant tissue areas adjacent to PC in patients followed with watchful waiting and to evaluate its correlation to cancer specific death.

To assess hyaluronan levels in normal prostate tissue of an orthotopic rat model for PC, and moreover to investigate tumour effects of hyaluronan injection in this tumour model.

Materials and methods

All studies were approved by the research Ethics Committee in Umeå, Sweden

Population study (Paper I-III)

The population study was based on retrospectively collected material of all men (n=419) diagnosed with PC at transurethral resection of the prostate (TUR-P) between 1975 and 1991 at the county hospital in Västerås (Sweden). The surgical procedure was performed due to voiding problems. No information of Prostate specific antigen (PSA) was available because the test was not used in Sweden at that time. Information of clinical stage, metastatic lesion on radio nuclide bone scan and cause of death were collected by examination of patient records. Estimated tumour volume was evaluated as the percentage PC of the total resected prostate tissue area (see also Egevad *et al* [167]). All PCs were retrospectively re-assessed and graded in line with the recommendations presented by ISUP 2005 by one experienced pathologist [167].

At last follow up (2003) 37 (8%) were still alive, 221 (53%) had died of other causes, 156 (37%) had died due to PC and 5(1%) had an unknown cause of death. Median age at diagnosis was 73.6 (range 51-95). Of those patients available for survival analysis, without known metastasis at diagnosis and followed with watchful waiting (n=295), 30 (10%) were still alive 197 (37%) had died of other causes and 68 (23%) had died due to PC. Median age in this group was 74.3(range 53-95). The 10-year PC–specific mortality was 28% which is in concordance with a nation wide study of cancer mortality of all men diagnosed with PC at TUR-P in the pre PSA-era showing a 28.2% PC specific mortality [168]. For more clinical characteristics please see Table 1 in Paper II.

According to the therapeutic tradition in Sweden at that time, the majority of men were not given any curative treatment but were managed with watchful waiting with only 27 % receiving primary treatment. In Sweden watchful waiting management included either oestrogen or GnRH-agonists therapy upon symptoms from metastasis.

Transurethral resection of the prostate give rise to many small tissue chips that are assembled, formalin fixed, paraffin-embedded and stored. Whole sections from those embedded tissue chips are from now on referred to as standard tissue sections. On these standard tissue sections there are a mixture of chips containing PC, normal prostate epithelium and sometimes only stroma. Paper I is based on immunohistochemical evaluation of standard tissue sections and included the sub-population of patients with GS 6 or 7 tumours and no detectable bone metastases who were primarily managed with watchful waiting. From the paraffin embedded blocks, tissue micro arrays (TMA) were then constructed using a Beecher instrument number I (Sun Praire, WI, USA). Cancer areas were marked and tissue samples/cores were punched out from the paraffin blocks and re-embedded into a new paraffin embedded block containing 5-8 tissue samples of PC and 4 samples of non-malignant tissue from each patient. Each core had a diameter of 0.6 mm, approximately 0.28 mm^2. Paper II and III are based on immunohistochemical evaluation of sections from these TMAs and included all patients with available tissue for analysis in the constructed TMAs. In survival analysis, only those patients managed with watchful waiting were used. See Figure 5 below for an example of what a TMA section looks like.

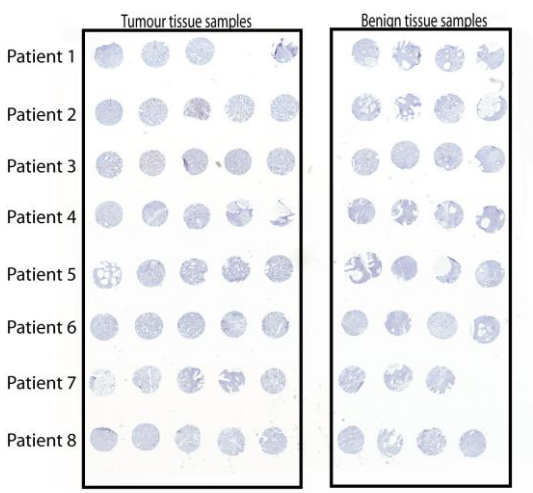

Figure 5 Example of Tissue-micro array stained for Ki67 including 8 patients with 5 tumour samples and 4 benign samples each.

Animal experiments

All the work was approved by the local ethical committee for animal research.

Orthotopic implantation of Dunning tumour cells into Copenhagen rat ventral prostate (Paper III)

A spontaneous tumour from a 22-month old Copenhagen rat was identified in the 1960s by Dr. W.F. Dunning, who named it Dunning R3327 [169]. This tumour cell line has given rise to many different cell lines with different characteristics which are all transplantable to immunocompetent and syngenic Copenhagen rats. In this thesis, the AT-1 and MatLyLu cell lines were used. Both these cell lines are androgen insensitive with low (AT-1) and respectively high (MatLyLu) metastatic capacity. In vitro they were grown according to the manufacturer's instructions (In RPMI 1640 with 10% fetal calf serum, 0.2% N-Bic, 50 μg/ml gentamycin and 250 nM dexamethasone in 37°C and 5% Co_2). Before orthotopicly implantation, the cells were grown to approximately 75% confluence, trypsinised, counted in a Burker chamber and diluted in RPMI to the appropriate concentration.

The orthotopic implantation of the cells was carried out after anaesthesia. Briefly, a lower abdomen incision was made and the ventral prostate lobes exposed. Carefully a total of 2000 AT-1 or MatLyLu cells in 50 μL saline was then injected into one of the lobes. Controls were injected with 50 μL saline. Animals with AT-1-injected tumours were sacrificed at days 7, 10 and 14.MatLyLu-injected animals were sacrificed at day 7.

In a parallel experiment, animals with AT-1 injected cells were injected at day 8 with either 400 μg of Hyaluronan (Hyalgan, Nycomed Stockholm, Sweden) in 40 μL saline with Hyaluronan-free saline as control and sacrificed 4 days later.

Evaluation of tumour size after hyaluronan or saline injection was determined by measuring the estimated percentage of tumour in the tissue section of the removed prostate lobe. The tumour weight was then estimated by multiplying estimated percentage of tumour by the weight of the sample (see also Halin *et al* [170]

Methods for RNA preparation and quantitative RT-PCR analysis can be found in the "material and method"-section of Paper III.

Immunohistochemisty

Ki67, endoglin and von Willebrand factor

The paraffin embedded tissue were deparaffinised and rehydrated according to standard procedures.

All staining of Ki67, endoglin and factor VIII related antigen (vWf) were performed using the Ventana ES automatic staining machine according to the manufacturers instructions.

Sections examined for endoglin and vWf were pre-treated with protease-1 for 4 min and sections for Ki67 were boiled in citrate (pH6.0) before applying primary antibodies for endoglin (SN6h, DAKO Corporation, Carpinteria, CA), vWf (A0082, DAKO Corporation) and Ki67 (MIB-1, DAKO Corporation) diluted 1:10, 1:3000 and 1:50, respectively.

The secondary system was slightly different between Paper I and II. For standard whole slides the Basic AEC iVIEW detection kit (Ventana Medical systems Inc., Tucson, AZ) system was used and for the TMA staining the DAB iVIEW detection Kit (Ventana Medical systems Inc) was used.

Hyaluronan

For localization of hyaluronan (HA), a hyaluronan-binding protein (HABP) was used as a probe. For isolation and biotin-labelling procedures of the HABP together with the procedure of pre-incubation and testing with negative control see Hellström *et al* [171]. Incubation with HABP diluted 1:40 in PBS was performed at 4 C overnight. Vectastain-Elite Avidin-Biotin complex reagent (Vector Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine (DAB; Vector Laboratories) as chromogen was used for visualization.

Quantification of immunohistochemical reactivity

Ki67 (Paper I and II)

In Paper I, hot spots were indentified at low magnification. A lattice pattern in the eye-piece of a light microscope with 121 crossings (11 horizontal lines and 11 vertical lines) was used at 200 x magnification. All cancer nuclei crossing the 11 horizontal lines were counted in a total of 5 hot spots with a maximum of 100 cells per hot spot. The total number of stained cells of the 5 hot spots divided by the 500 counted cells was used as the Ki67 proliferation index in Paper I.

In Paper II, each tissue sample with representative tissue in the TMA was evaluated once at 200x magnification. All cell nuclei crossing the horizontal lines were counted without upper limit (range 0-363 per tumour core, 75 % of all tumour cores had less than 146 cells). The Ki67 proliferation index was expressed as the fraction of stained cells divided by the total number of cells crossing the lines. In normal tissue samples the prostate epithelium Ki67 proliferation index was evaluated in a similar fashion.

Endoglin and vWf (Paper I and II)

In Paper I hot spots were identified at low magnification and three hot spots were evaluated for each patient. All vessels were counted within the lattice and a visual vessel lumen was not required for counting. In total 0.75 mm^2 (3x0.25mm^2) was evaluated and vessel density was expressed as mean vascular count per mm^2.

In Paper II each tissue sample in the TMA, with enough tissue to be evaluated, were examined and total number of stained vessels were counted (without the lattice) per the approximated total core area of 0.28mm2. A visual lumen was not required for included as a countable vessel. In normal tissue samples the same method was used.

Hyaluronan (Paper III)

Three parameters were evaluated for each cancer tissue sample in the TMA whilst four parameters were evaluated for each normal tissue sample. The three common parameters were:

-Staining intensity from 0-4 (none, faint, moderate or very strong) in the cancer and normal epithelium respectively.
-Staining intensity from 0-4 (none, faint, moderate or very strong) in the cancer and normal stroma respectively
-Staining distribution from 0-4 (none, <10%, 10-50%, 50-90% and >90%) in the cancer and normal stroma respectively

The product of stroma staining and stroma distribution were used as the HA stroma variable in analysis and consequently the HA score values ranged from 0 to 16.
In normal tissue samples, the presence of staining in basal cell layer was also noted as 1 or 0.

Statistics

Please see the respective Papers´ statistical paragraphs. Certified statisticians have approved of methods and re-calculated the results for each Paper. SPSS statistical package was used in almost all tests but hazard proportional assumption in Paper II was tested in Stata. A p-value less than 0.05 was considered statistically significant.

In the thesis paragraph "Tumours effect the normal adjacent tissue-and the magnitude of these changes is associated with prognosis", linear regression analysis was used to investigate if the markers were more dependent on grade than on estimated tumour volume.

In Table 1 uni- and multivariate Cox proportional hazard analysis and ROC-curve analysis were used with PC death as event and death from other causes at follow up as censored events.

Summary of the results and conclusions

The results of paper I-III regarding uni-variate Cox proportional hazard regression analysis in different Gleason score groups (GS 4-10, GS 6 and GS 7) are summarized in Table 1-4, together with analysis of the markers potential to discriminate prostate cancer death in ROC analysis. The key results of each paper are furthermore summarized as below.

Paper I

Estimated tumour size, vWf and endoglin vascular counts in hot spots were prognostic for survival in GS 6-7, but not GS 7 patients.

Low estimated tumour volume and low vWf vascular density identified a favourable group of men with GS6 tumours where 2.5% died of PC within 15 years.

Paper II

Ki67 proliferation index, endoglin vascular density and vWf vascular density were all prognostic for survival in univariate Cox analysis of patients with GS 4-10 tumours followed by watchful waiting.

vWf vascular density in stroma adjacent to GS6-7 tumour areas was a prognostic marker for cancer-specific survival.

Tumour Ki67 index and endoglin vascular density could, in combination, identify 35% of all GS 6 tumours with no PC death within 15 years.

Paper III

Hyaluronan staining score in normal stroma adjacent to tumour was associated with clinical characteristics and cancer specific survival but could not prognosticate the outcome in GS 6 tumours.

Hyaluronan staining score in tumour cells and in the tumour stroma were associated with clinical characteristics and cancer specific survival.

Animal experiments using a transplantable rat tumour model confirmed that hyaluronan levels are up-regulated in cancer-adjacent stroma , and indicated that hyaluronan may promote PC progression.

Table 1 Univariate Cox analysis and ROC-curve analysis of patients with GS 4-10 tumours, without metastasis at diagnosis and managed by watchful waiting with PC death as event. The table on top treat the variables as continuous and the table below use the specified cut offs for each variable in the analysis.

	GS 4-10	Range of the variables	Area under the curve	n	HR	p-value	95% CI
Continuous variables	Hyaluronan in tumor stroma	0.4-15.5	0.66	277	1.19	<0.001	1.09-1.29
	Hyaluronan in tumor epitheilium	0-4	NS	277	1.40	<0.001	1.11-1.76
	Hyaluronan in normal stroma	0.5-14.0	0.61	282	1.14	<0.01	1.04-1.25
	Ki67 proliferation index in PC	0-35	0.73	274	1.14	<0.001	1.10-1.19
	Ki67 proliferation index, normal epithelium	0-31	NS			NS	
	Endoglin vascular density in PC	0-123	0.66	270	1.06	<0.001	1.03-1.09
	Endoglin vascular density in normal areas	0-60	NS			NS	
	vWf vascular density in PC	7-201	0.61	273	1.02	<0.001	1.01-1.03
	vWf vascular density in normal areas	3-118	NS			NS	
	Gleason score	4-10	0.83	295	2.12	<0.001	1.82-2.46
	Estimated tumor size	5-100	0.76	295	1.03	<0.001	1.02-1.04
	GS 4-10	**Cut off**		**n**	**HR**	**p-value**	**95% CI**
Dichotomoused variables	Hyaluronan in tumor stroma	median		277	1.95	<0.01	1.18-3.22
	Hyaluronan in tumor epitheilium	median		277	1.67	<0.05	1.02-2.72
	Hyaluronan in normal stroma	median		282	2.08	<0.01	1.24-3.50
	Ki67 proliferation index in PC	median		274	3.98	<0.001	2.34-6.78
	Ki67 proliferation index, normal epithelium	median				NS	
	Endoglin vascular density in PC	median		270	3.31	<0.001	1.92-5.70
	Endoglin vascular density in normal areas	median				NS	
	vWf vascular density in PC	median		273	1.74	<0.05	1.05-2.87
	vWf vascular density in normal areas	median				NS	
	Gleason score	4-7 vs GS 8-10		295	8.655	<0.001	5.31-14.12
	Estimated tumor size	20%		295	5.90	<0.001	3.40-10.25

Abbreviation: NS= not significant, HR= Hazard ratio, CI= Confidence interval

Table 2 Univariate Cox analysis and ROC-curve analysis of patients with GS 6 tumours, without metastasis at diagnosis and managed by watchful waiting with PC death as event. The table on top treat the variables as continuous and the table below use the specified cut offs for each variable in the analysis.

	GS 6	Range of the variables	Area under the curve	n	HR	p-value	95% CI
Continuouse variables	Hyaluronan in tumor stroma	0.4-13.6	0.67			NS	
	Hyaluronan in tumor epitheilium	0-4	NS			NS	
	Hyaluronan in normal stroma	0.5-14	NS			NS	
	Ki67 proliferation index in PC	0-10	0.76	89	1.32	<0.01	1.12-1.55
	Ki67 proliferation index, normal epithelium	0-31	NS			NS	
	Endoglin vascular density in PC	0-123	0.69	88	1.06	<0.05	1.01-1.11
	Endoglin vascular density in normal areas	0-60	NS			NS	
	vWf vascular density in PC	13-100	NS			NS	
	vWf vascular density in normal areas	3-118	NS			NS	
	Gleason score	6	-			-	
	Estimated tumor size	5-95	0.75	97	1.03	<0.001	1.01-1.05
	Endoglin counted in hot spots whole slides	0-14		79	1.01	=0.5	0.99-1.02
	Ki67 counted in hot spots whole slides	0-136		79	1.16	=0.15	0.95-1.43
	vWf counted in hot spots whole slides	12-235		79	1.01	=0.07	1.00-1.02
	GS 6	**Cut off**		**n**	**HR**	**p-value**	**95% CI**
Dichotomoused variables	Hyaluronan in tumor stroma	median				NS	
	Hyaluronan in tumor epitheilium	median				NS	
	Hyaluronan in normal stroma	median				NS	
	Ki67 proliferation index in PC	median		89	5.93	<0.01	1.97-17.82
	Ki67 proliferation index, normal epithelium	median				NS	
	Endoglin vascular density in PC	median		88	9.65	<0.01	2.09-44.63
	Endoglin vascular density in normal areas	median				NS	
	vWf vascular density in PC	median				NS	
	vWf vascular density in normal areas	median				NS	
	Gleason score	-				-	
	Estimated tumor size	20%		97	6.16	<0.01	2.06-18.37
	vWf counted in hot spot whole slides	median				NS	
	Endoglin counted in hot spots whole slides	1st quartile				NS	
	Ki67 Counted in hot spots whole slides	1st quartile		79	4,18	<0.5	1,28-13,66

Abbreviation: NS= not significant, HR= Hazard ratio, CI= Confidence interval

Table 3 Univariate Cox analysis and ROC-curve analysis of patients with GS 7 tumours, without metastasis at diagnosis and managed by watchful waiting with PC death as event. The table on top treat the variables as continuous and the table below use the specified cut offs for each variable in the analysis.

	GS 7	Range of the variables	Area under the curve	n	HR	p-value	95% CI
Continuous variables	Hyaluronan in tumor stroma	2.3-14.4	NS			NS	
	Hyaluronan in tumor epitheilium	0-3	NS			NS	
	Hyaluronan in normal stroma	1.3-12.3	NS			NS	
	Ki67 proliferation index in PC	0-35.5	NS			NS	
	Ki67 proliferation index, normal epithelium	0-20	NS			NS	
	Endoglin vascular density in PC	0-84	NS			NS	
	Endoglin vascular density in normal areas	0-39	NS			NS	
	vWf vascular density in PC	19-134	NS			NS	
	vWf vascular density in normal areas	16-105	NS	47	1.03	<0.05	1.00-1.06
	Gleason score	7	-			-	
	Estimated tumor size	5-100	NS			NS	
	vWf counted in hot spot whole slides	37-411		49		NS	
	Endoglin counted in hot spots whole slides	0-235		49		NS	
	Ki67 Counted in hot spots whole slides	0-15		49		NS	
	GS 7	**Cut off**		**n**	**HR**	**p-value**	**95% CI**
Dichotomoused variables	Hyaluronan in tumor stroma	median				NS	
	Hyaluronan in tumor epitheilium	median				NS	
	Hyaluronan in normal stroma	median				NS	
	Ki67 proliferation index in PC	median				NS	
	Ki67 proliferation index, normal epithelium	median				NS	
	Endoglin vascular density in PC	median				NS	
	Endoglin vascular density in normal areas	median				NS	
	vWf vascular density in PC	median				NS	
	vWf vascular density in normal areas	median				NS	
	Gleason score	-				-	
	Estimated tumor size	20%				NS	
	vWf counted in hot spot whole slides	median				NS	
	Endoglin counted in hot spots whole slides	1st quartile				NS	
	Ki67 Counted in hot spots whole slides	1st quartile				NS	

Abbreviation: NS= not significant, HR= Hazard ratio, CI= Confidence interval

Table 4 Maximum value for each patients different variables were used in the Univariate Cox analysis and ROC-curve analysis of patients with GS 7 tumours, without metastasis at diagnosis and managed by watchful waiting with PC death as event. The table on top treat the variables as continuous and the table below use the specified cut offs for each variable in the analysis.

	GS 7	Range of the variables	Area under the curve	n	HR	p-value	95% CI
Continuous variables	Maximum Ki67 in tumour stroma	0-35				NS	
	Maximum Ki67 in normal stroma	0-25				NS	
	Maximum Endoglin in tumour stroma	0-46				NS	
	Maximum Endoglin in normal stroma	0-27				NS	
	Maximum vWf in tumour stroma	7-77				NS	
	Maximum vWf in normal stroma	7-43	0.58	47	1.09	<0.05	1.01-1.18
	GS 7	**cut off**		**n**	**HR**	**p-value**	**95% CI**
Continouse variables	Maximum Ki67 in tumour stroma	median				NS	
	Maximum Ki67 in normal stroma	median				NS	
	Maximum Endoglin in tumour stroma	75^{th} percentile		50	3.74	<0.05	1.37-10.20
	Maximum Endoglin in normal stroma	median				NS	
	Maximum vWf in tumour stroma	median				NS	
	Maximum vWf in normal stroma	median		47	4.10	<0.05	1.37-12.32

Abbreviation: NS= not significant, HR= Hazard ratio, CI= Confidence interval

Conclusions

From Paper I-III we conclude that it is possible to find potential prognostic markers for GS 6 and 7 PC patients, in TUR-P material from patients with long follow up and no curative treatment.

- High tumour cell proliferation indicates a bad prognosis for PC patients
- Angiogenesis both in the tumour stroma and adjacent TINT (tumour indicating/instructed normal tissue) stroma seem important for PC aggressiveness
- Hyaluronan is present in tumour epithelium, tumour stroma and adjacent TINT stroma and is indicative of a bad prognosis.
- GS 7 tumours can be prognosticated by the vascular density in the tumour stroma and in the adjacent TINT stroma
- A combination of tumour proliferation and vascular markers can identify GS 6 patients not suitable for active surveillance.

In-conclusive results

We reported that Ki67 proliferation index was a promising prognostic marker for cancer specific survival in Paper II, however in Paper I it did not reach statistical significance. Identified differences are listed below:

- The staining technique used was slightly different. The secondary system used in Paper I was AEC whilst in Paper II DAB was used. Staining with AEC is often weaker than DAB and the staining is more affected by time than DAB [172].

- The quantification methods used were similar in the two Papers but the cores in the TMAs were randomly chosen from cancer areas in Paper II while in Paper I hot spots of the entire slide were quantified. The hot spot approach generated a slightly higher proliferation index than the mean TMA values. The staining scores of the hot spots in the standard whole sections compared to the maximum staining scores in the randomly choosen TMA cores are listed in Table 5 below.

Table 5 Ki67 score and vWf and endoglin vascular counts in GS 6-7 tumours of patients analyzed in both paper I and paper II.

Table 5	Hot spots paper I	Maximum values paper II
vWf	101 vessels per mm^2	68 vessels per mm^2
Endoglin	40 vessels per mm^2	41 vessels per mm^2
Ki67	2.0%	5.2%

The median value of the hot spots for ki67 staining in Paper I thus seems to be too low to be comparable with Paper II. It is also non-comparable with a previous Paper using the same material[130] and also another study using radical prostatectomy material by Ali *et al* [117].We believe that the staining was not sensitive enough in Paper I and that the results in Paper II are more valid for proliferation index. Whether this difference is due to the secondary system used or other factors influencing the staining is unknown. Indeed others have shown that there are higher inter-laboratory differences than in inter-observer reliability [173].

"Whenever a theory appears to you as the only possible one, take this as a sign that you have neither understood the theory nor the problem wich it was intended to solve"

Karl Popper

General discussion

Strengths with the patient material used

The long follow up time of the patients included in this thesis made it possible to measure putative markers association with definitive outcome. This is important in PC research, due to the sometimes slow progression of the disease. The cause of death was defined by experienced specialists in Urology by retrospective viewing of the patients´ records, which is of importance due to the fact that old age and co-morbidity may obscure accurate determination of death. Fall *et al* has recently shown a 14 % discordance between a chart review and the Cause of Death Register [174] but in a recent publication the same figure was 4%, this study however included PC deaths of men at younger age [175].

Another strength was the low proportion of patients surviving the observation period (8%) and the fact that we had a relatively high proportion of PC deaths (37%) which made the identification of insignificant tumours even more interesting. All tumours were re-graded by one pathologist, in order to eliminate time and inter-individual shift of grading. Also the fact that TUR-P was not a curative surgical procedure but left the tumour tissue in the prostate is a strength when looking for markers that define tumours with an indolent natural history.

Limitations of the patient material

Markers should be evaluated in the context of already established prognostic markers to see if the markers give any extra clinical information and if they are relevant in a clinical context. This material was collected prior to the PSA-era which limits the possibility to interpret the result in a contemporary clinical situation. The median age at diagnosis was 74 years and today the majority of diagnosed patients are approximately 5 years younger. Time until additional symptomatic disease that triggered hormonal treatment was unfortunately lacking.

TUR-P material is composed predominantly of tissue from the central parts of the prostate and thus finds transition zone cancers and large peripheral cancers. The absolute majority of cancers in the prostate are located in the peripheral zone. If transition zone cancers have a more favourable prognosis than peripheral zone cancers have been discussed but there are studies

showing a similar cancer specific mortality and time to biochemical relapse [168, 176-179]. There are however differences between contemporary diagnosed PC and the material presently used. In our material 41 % of the patients managed by watchful waiting had an estimated tumour size greater than 20% and 36% had a palpable tumour. These are larger tumours than those present in the majority of the men diagnosed today without symptoms and at core biopsies due to elevated PSA-value. Still prognostic markers found in TUR-P material with similar tumour characteristics have been shown to be able to be verified in radical prostatectomy materials [180].

The results in context

No other cancer is as common or has such a great need for prognostic markers as PC. GS, stage, PSA and tumour extent on core biopsies can give prognostic information, and the majority of men diagnosed today have low to intermediate risk for progression according to these parameters. If all men with prevalent cancer, perhaps as many as 30-50% of all men aged 50 years old, could be found and undergo treatment with surgery or radiation therapy, there would be many men with symptoms from the side-effect of treatment who if not detected with PC never would have experienced any PC symptoms during there lifetime.

We have found potential markers which may add prognostic information to the conventional markers used today for identification of patients with potential indolent PCs and longer time to clinical presentation than expected life-time left. The patient material used in this thesis is too different from the contemporary clinical situation (with small, PSA-detected and core biopsy-verified PCs) to suggest a direct application of these markers to be used in clinical practise of core biopsy diagnosed patients. However if verified with valid and reliable cut offs, using contemporary core biopsy material, a combination of the suggested markers could be of great clinical value, especially for patients that potentially could be managed by active surveillance.

Stage and grade as prognostic markers

GS was a very strong prognostic marker for the patients included in this thesis, with 10 years disease-specific median survival times of 20, 14 and 7 years for GS group 4-6, 7 and 8-10, respectively, and with a cumulative disease specific survival at 10 years of 93%, 62% and 32% respectively. This was not only verifying existing data, but was also indicating the importance

of finding additional markers for differentiating prognosis within the corresponding GS groups.

Clinical stage by digital rectal examination is also a well known prognostic marker, but a T2 tumour could in 70% of the cases be re-staged as a T1 tumour the next year (see background) making this marker some-what unreliable. The disease-specific median survival in the currently used material was 20, 11 and 6 years for stage T1, T2 and T3-4, respectively, with a cumulative disease specific survival at 10 years of 85%, 53% and 19%. This makes clinical stage an important variable to be included in multivariable Cox analysis when searching for markers that give additional prognostic information.

Estimated tumour size as prognostic marker

Estimated tumour size was a robust prognostic marker for survival for the patient material used with long follow up and without primary treatment. It was also independent of GS and clinical stage. Tumours with pT1a (non palpable tumour with less than 5% of the resected TUR-P tissue) was in a recently published study also followed expectantly, and shown to have a 8% risk of PC deaths at 10 years [181]. In our material, the group of patients with pT1a tumours (n=72) had no PC death within 15 years. To find a tumour size cut off with similar risk of PC we used a 20% estimated tumour size (which gave a 9% cumulative PC risk within 10 years). The material of the compared study had younger men (5 years lower median age) and if this reflected men with less co-morbidity it could be a reason for the difference between the studies. Rajab *et al* has also shown increasing HRs with increasing tumour size, with a HR of 1 with ≤10% estimated tumour size, HR of 2.5 in 10%-25%, HR of 5.4 in 25%-75%, and HR of 9.4 in >75% [181]. Similar results could be seen in our material with HR of 1, 2.1, 4.4, and 11.5 in the corresponding groups.

In core biopsies the total length of the biopsies and the number of biopsies with cancer have been shown be strong predictors of PSA-relapse after radical prostatectomy [182-184] but also to prognosticate cancer specific death [185]. Tumour size probably reflects a tumour that have acquired many aspects of the hallmarks of cancers in order to be able to grow and not only the angiogenic phenotype, HA rich stroma or higher proliferative index as our results indicates.

A palpable tumour (T2-T4) with a similar tumour size as a non palpable tumour (T1) has higher HA tumour stroma staining. Hyaluronan may be the

extra cellular component that enables palpation (T2-T4) as HA accumulates water which probably increases the tissue pressure .

Proliferation, vascular density, and hyaluronan are associated with worse prognosis.

As described in the background increasing angiogenesis and proliferation and accumulation of HA have previously been found in PC and also to be associated to some aspects of worse prognosis. We confirm that Ki67, endoglin, vWF and hyaluronan staining scores are higher in tumour areas as compared to normal areas. They are also associated with clinical characteristics and cancer specific survival in PC also in this material with very long follow up.

A markers independence to grade could be investigated by multivariate Cox analysis with GS as one of the predictive variables, but this assumes that the markers can give prognostic information in both low and high grade disease. The growth limiting factors for low grade tumours do not need to be the same as for high grade tumours and sub analysis of the variables within patient groups of the different Gleason grades was therefore also performed.

Univariate Cox analyses of the evaluated markers in relation to outcome are listed in Table 1-4 and show that higher endoglin, vWf, Ki67 and hyaluronan staining scores (tumour stroma and tumour epithelium) were associated with shorter disease specific survival. The prognostic potential of Hyaluronan and vWf staining scores were correlated to grade and estimated tumour size and did not provide independent prognostic information. The Ki67 proliferation index of the tumour cells and the endoglin vascular density in the tumour stroma, however, were independent prognostic markers of PC specific death, and provided information complementary to estimated tumour size, stage and grade (Table 3; Paper II).

The maximum vascular density is theoretically the best measurement for defining an aggressive tumour and this is strengthened by our results from Paper II. The maximum vascular count in the TMAs for endoglin and vWf are better prognostic markers as compared to the mean of the cores.

It was also found that the maximum of vWf vascular count in normal tissue and the maximum of endoglin staining score in tumour tissues could give prognostic information in the GS 7 tumours. In Paper I hot spots (another way of finding the maximum) on the whole slides were evaluated and both vWf and endoglin were prognostic for cancer specific survival in GS 6-7 patients.

Theoretically, the maximum Ki67 index should also be the best measurement for defining an aggressive tumour; but our results do not support this. Maximum ki67 staining index was not a better prognostic marker compared to the mean of the tissue cores in the TMAs. If a tumour has multiple cores with high values (not top values) they seem to be more aggressive than if they have some cores with top notations. Indeed previous studies have indicated that non proliferative cells also could have Ki67 staining and this could be an explanation for our findings [186-188].

In breast cancer, HA accumulation has been shown in the invasive front which suggests a role in invasion/migration and this is also supported in PC where HA accumulation has been associated with seminal vesicle invasion [189-190]. In our material, high epithelial HA staining was associated with metastasis further supporting the idea that HA is involved in invasion and migration of tumour cells. It is clear that in this material, HA score indicates a bad prognosis but may also play some role in the cancer progression. This is supported both of the finding that the cancer cells had HA staining in 66% of cases but was not found in the normal luminal epithelial cells. This was further supported by our animal experiments. Injected HA in the orthotopically injected AT-1 tumours gave larger tumours.

Another interesting connection between hyaluronan and PC is the finding that HA can deactivate macrophages and make them incapable of defeating the cancer cells [191]. Also that HA may protect the cancer cells from direct attachment of leukocytes on the cancer cell and thereby prevent proper immune reaction [159].

Hyaluronidases have been linked to worse prognosis in PC [144], also, the products of hyaluronan degradation, low molecular weight (LMW) HA, has been linked to many of the factors important in tumour progression such as increased angiogenesis, cell proliferation, lymph angiogenesis, tumour cell invasion, recruitments of inflammatory cells and epithelial to mesenchymal transition, and also to activation of a general inflammation response. [144, 159, 192]. LMW HA has been shown to be pro-angiogenic but HMW HA has been shown to be anti angiogenic. Indeed a number of other contradictory effects of the different lengths of HA have been reported [144]. The hyaluronan binding protein (HABP) used to stain HA in our studies is known to bind different lengths of HA molecules and we could therefore not determine the relation between HA lengths and the staining score. To determine the molecular size of HA, a new sensitive method for small samples has recently been tested with good results and could be of interest in future studies [193]. HA staining score is, in our material, associated to vWf vascular count (r_s=0.20) but not to endoglin vascular count. Whether this non association

between our HA staining and endoglin is due to the lack of specificity to HA lengths is not known.

In our material, tumour cores with high PDGFRβ staining scores in tumour cells [165] also had high HA staining scores (r_s 0.31 $p<0.001$), which could be in line with findings by others showing that PDGF-BB (a ligand to PDGFRβ) stimulates cardiomyocytes to produce hyaluronan [194]. There are many other potential mediators of HA production in PC including TGF-β which could be one of the paracrine mediators up regulating the HAS-1 RNA production seen in our animal experiment .

Tumours effect the normal adjacent tissue
- and the magnitude of these changes is associated with prognosis

PC is almost always multifocal, which means that PIN/HGPIN/cancer lesions develop concurrently at multiple sites. This suggests that a milieu of processes facilitating cancer initiation precedes the PC development. This phenomenon is called the cancer field effect and was named by Slaughter *et al* 1953 [195]. One of the first studies to show changes in the surrounding morphologically normal tissue was Ayala *et al* showing that phosphorylated AKT-1 in the non-malignant tissue was an independent marker of PC survival [196]. Merseburger *et al* showed in 2006 that that the PKB/Akt pathway was activated in the non malignant tissue [197]. Nonn *et al* have shown multiple biomarkers that support the existence of a field effect in prostate carcinogenesis [198]. Other studies have found hyper-methylation in the surroundings of PC [199-200]. The detectable/measurable effects (markers) in normal areas which correlate to already known prognostic markers are very interesting and even though they may not give independent prognostic information they may reflect the presence of the tumour elsewhere in the prostate.

In Paper III we showed that HA was up regulated in the normal tissue surrounding tumours and that these changes were associated with GS, tumour volume and cancer specific survival. This up-regulation in the surrounding tissue was supported by our animal experiments. An orthotopically injected AT-1 or MatLyLu tumour at one place up regulated the Hyaluronan synthase-1 mRNA and showed an increased hyaluronan staining score in the surrounding normal tissue. In Paper II we found that vWf vascular density was prognostic for cancer specific survival in normal TINT stroma. We thus claim that these two markers, should be added to our list of previously reported potential TINT markers all of which indicate aggressive tumours; phosphorylated-EGFR, increased levels of PDGFRβ and mast cells and decreased levels of androgen receptors in the normal stroma [143, 164-166].

The potential clinical applications of the suggested TINT markers are very interesting. Upon suspicion of prostate cancer, a tissue examination of the prostate often includes 8-12 biopsies, where each biopsy samples about 1/1000 of the whole prostate. Most men are diagnosed with only one or two tumour-positive core biopsies and seem to have small low grade tumours judged from the core biopsies, but after surgical removal of the prostate 30% of the tumours have a higher grade than anticipated [117]. If the normal looking tissue could prognosticate the presence of a high grade tumour, as we suggest it could, the pre-operative "negative" biopsies would contain information useful in pre treatment decision.

In breast cancer, changes in the tumour surroundings are seen at least 1 cm from the tumour [201]. How far from the tumour that these TINT markers can be seen in PC has not yet been investigated. Findings from a magnetic resonance (MR) study have also shown that there are measurable changes of metabolites in the TINT which reflect the grade of the tumour of the histologically verified PC [202-203]. If either TINT markers in the normal looking core biopsies or MR will be used in the future it is possible that they can indicate the presence of an aggressive tumour elsewhere in the prostate and thereby could help in safely selecting patients for active surveillance.

PC can become aggressive in different ways

Our results show that better prognostication could be accomplished with a combination of markers. This is consistent with the idea that multiple markers are needed to correctly identify aggressive tumours. Penney *et al* recently showed that a signature made of a panel of 157 mRNAs could prognosticate outcome for patients with GS 7 tumours [180].

Of the markers investigated in this thesis, proliferation and tumour stroma angiogenesis seem to be important factors determining the behaviour of GS 6 tumours. In Paper II we demonstrated that endoglin and Ki67 staining scores were only weakly correlated (r_s=0.18) and that men with GS 6 tumours with neither high Ki67 nor endoglin (34/97 =35%) experienced no PC death within 15 years compared to a 65% cumulative risk of PC deaths over 15 years in the other group. There were 16 cancer deaths among GS <7 tumours (Table 4, Paper II) and only one of these were classified as being in the low risk group and he died after 18 years.

Other studies that have found prognostic markers in GS 6 tumours include for example Zellweger *et al* who found high Ki67 index in preoperative core biopsies to be associated with shorter PSA free survival [124]. From a Swedish

group that has investigated a similar group of TUR-P diagnosed PC with long follow up Andren *et al*, Mucci *et al* and Cuzick *et al* have reported that MUC-1, an multigene signature and another RNA expression signature of proliferation could prognosticate the outcome in GS 6 tumours [204-206].

In GS 7 tumours, angiogenesis seems to be a limiting factor for progression, as vascular density in tumour stroma (maximum endoglin) or the normal TINT stroma (maximum vWf) could significantly prognosticate outcome. Penney *et al* have recently shown that a 157 gene signature built on the ability to discriminate GS 4-6 from 8-10 tumours could also prognosticate in GS 7 tumours of the TUR-P and this was also verified in radical prostatectomy material [180].

Our group has previously shown that mast cells, cannabinoid receptor, pEGFR, LRIG1 and PDGFRβ are prognostic markers of PC survival independent of GS in the whole material of GS 4-10.

Many different markers can prognosticate the outcome in PC and by evaluating different aspects of the tumour the ability to discriminate a favourable group seems to be more accurate. If the markers should be used in a clinical setting the method needs to be easy and reproducible and also inexpensive. Our immunohistochemical analysis could be done in any clinical laboratory in Sweden and were neither expensive nor complicated to score. If validated and reliably reproduced in contemporary settings they may become available for clinical use.

Future aspects

In the future PSA testing in men will probably increase and with this the of the diagnosis of small low grade PC with moderately increased PSA-values Many of the men diagnosed with small tumors will be suggested for active surveillance whilst some will be recommended for direct primary treatment. Side effects of primary treatment are well characterized and will impact the quality of life for the rest of their lifes. The psychological distress and the risk of missing the window of opportunity in those managed by active surveillance is currently under investigation in a large nation wide study.

Results presented here and in similar studies, which show that markers in normal tissue areas could indicate tumour characteristics, should be tested in a Swedish contemporary biopsy material. Our results together with other findings show that a panel of markers probably is needed in order to reach satisfying prognostic specificity. New –omics methods are available that can be used in the seach for prognostic markers now also in paraffin embedded tissue.

Recent findings indicate that protein-, DNA- or RNA-based biomarkers quantified in urine could be used to identify patients with higher probability of positive core biopsy whilst some are also predictive of extra capsular extent. The problem with assessment of newly collected materials is the question of a gold standard for outcome. The gold standard of significant or indolent tumours is cancer specific death. Time however is a factor as the outcome is assessed 15 to 25 years after radical prostatectomy which is a long time to wait for results (see Roobol *et al* for review [207]).

An increasing number of studies show the importance of micro RNA, hyper- and hypo-methylation, and other epigenetic changes in PC. (recent reviewed by Jeronimo *et al* [208]). These studies are very interesting and reveal another layer of complexity to the understanding (or lack of understanding) of the processes involved in different stages of PC.

Currently castration is the primary treatment of metastatic PC, new therapies however have become available and offer interesting results with secondary docetaxel treatment being more and more common in castration resistant PC. Also abirateron acetate has now been introduced in Sweden and proven to improve survival of patients with castration resistant PC. Pharmacological treatments attacking angiogenesis are theoretically interesting but have not yet shown any convincing results. For all of these new therapies biomarkers for selecting the right patients for different

treatments and also for predicting therapy response are of great importance. This is due to the fact that therapies have serious side-effects and also due to economy, they can be cost prohibitive. An interesting potential biomarker is circulating tumour cells, which has been shown to give prognostic information of castration resistant PC, and could hopefully also be useful in the identification of patients suitable for new treatments and to predict and follow therapy response. (see also review by Danila *et al.* [209])

I hope that the future will give us tools to find those men with PC that will benefit from treatment at the right time (not too late but also not too early) so that we safely can save men from PC death. Of equal importance are tools to distinguish those men with indolent tumours that do not need to worry about PC and let them die without the knowledge of their small PC.

"Emperor Meiji: Tell me how he died.
Algren: I will tell you how he lived. "
Quotes from the film "The last Samurai"

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