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TISSUE BIOMARKERS IN PROSTATE CANCER

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Till mamma och pappa

ABSTRACT

Prostate cancer (PC) is the most common male cancer in the western world. Better biomarkers are needed to support diagnosis, prediction of prognosis and treatment decision.

Radical prostatectomy (RP) specimens are routinely immersed in formalin overnight. Formalin may also be injected into the prostate for improved fixation. We report that formalin injection does not alter tissue volumes compared to conventional fixation. Formalin may affect epitopes for immunohistochemistry (IHC). Immunoreactivity was compared between fixation methods with no significant difference for the majority of 15 antibodies.

We investigated the transcription factor pancreatic duodenal homeobox-1 (PDX-1) and heat shock proteins (HSP) 27, 60 and 70 as prognostic markers in PC. A tissue microarray (TMA) of 289 PCs was constructed and immunostained. HSP 27 and 60, but not HSP 70 and PDX-1 correlated with biochemical recurrence. In multivariate analysis including histopathological prognostic factors, only HSP 60 was an independent predictor of prognosis. PDX-1 was overexpressed in cancer vs. benign tissue but also in atrophy and high-grade prostatic intraepithelial neoplasia (PIN) vs. cancer.

The role of GAD1 (glutamate decarboxylase 1) as prostate-specific biomarker was investigated. A TMA of benign and malignant tissues from prostate, rectum, lung and bladder was stained for GAD1, prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA, glutamate carboxypeptidase). Presence of GAD1 protein was validated by Western blot and real-Time PCR (RT-PCR). By IHC, the expression of GAD1 and PSA was stronger in prostatic tissues than in controls. PSMA was stronger in prostate cancer than in urothelial and rectal cancer but had lower specificity than GAD1 and PSA.

The intra- and interobserver reproducibility of IHC evaluation in TMA were assessed. Intensity and extent of PDX-1 immunostains of 50 PCs were scored twice by 4 independent observers. Mean weighted kappa for intra- and interobserver agreement was 0.85 and 0.80 for intensity and 0.43 and 0.21 for extent with similar results for 2 pathologists and 2 non-pathologists. Thus, subjective assessment of intensity is highly reproducible while estimation of staining extent is less reliable.

In conclusion, we find that TMA is a valuable tool for tissue-based biomarker research. We have attempted to optimize the procedures from tissue handling to evaluation. We also present HSP 27 and HSP 60 as potential predictors of prognosis in PC and GAD1 as a new prostate-specific biomarker.

LIST OF PUBLICATIONS

I. **Jonmarker S**, Valdman A, Lindberg A, Hellström M, Egevad L.
Tissue shrinkage after fixation with formalin injection of prostatectomy specimens.
Virchows Archiv 2006, 449: 297-301

II. **Jonmarker Jaraj S**, Egevad L.
Formalin fixation and immunoreactivity in prostate cancer and benign prostatic tissue.
Manuscript

III. **Jonmarker S**, Glaessgen A, Culp WD, Pisa P, Lewensohn R, Ekman P, Valdman A, Egevad L.
Expression of PDX-1 in prostate cancer, prostatic intraepithelial neoplasia and benign prostatic tissue.
APMIS 2008, 116: 491-8

IV. Glaessgen A, **Jonmarker S**, Lindberg A, Nilsson B, Lewensohn R, Ekman P, Valdman A, Egevad L.
Heat shock proteins 27, 60 and 70 as prognostic markers of prostate cancer.
APMIS 2008, 116: 888-94

V. **Jonmarker Jaraj S**, Augsten M, Häggarth L, Wester K, Pontén F, Östman A, Egevad L.
GAD1 is a novel prostate–specific tissue biomarker.
Manuscript

VI. **Jonmarker Jaraj S**, Camparo P, Boyle H, Germain F, Nilsson B, Petersson F, Egevad L.
Intra- and interobserver reproducibility of interpretation of immunohistochemical stains of prostate cancer.
Virchows Archiv *In press*

CONTENTS

LIST OF ABBREVIATIONS

AR	Androgen receptor
BPH	Benign prostatic hyperplasia
CZ	Central zone
DRE	Digital rectal exam
ED	Erectile dysfunction
FNAC	Fine-needle aspiration cytology
GAD	Glutamate decarboxylase
GS	Gleason score
HGPIN	High grade PIN
HSP	Heat shock proteins
IHC	Immunohistochemistry
IRP	Immunoreactivity product
LGPIN	Low grade PIN
PC	Prostatecancer
PDX-1	Pancreatic duodenal homeobox-1
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate-specific antigen
PSMA	Prostate-specific membrane antigen
PZ	Peripheral zone
RP	Radical prostatectomy
RT-RNA	Reverse transcriptase RNA
TMA	Tissue microarray
TRUS	Transrectal ultrasound
TZ	Transition zone

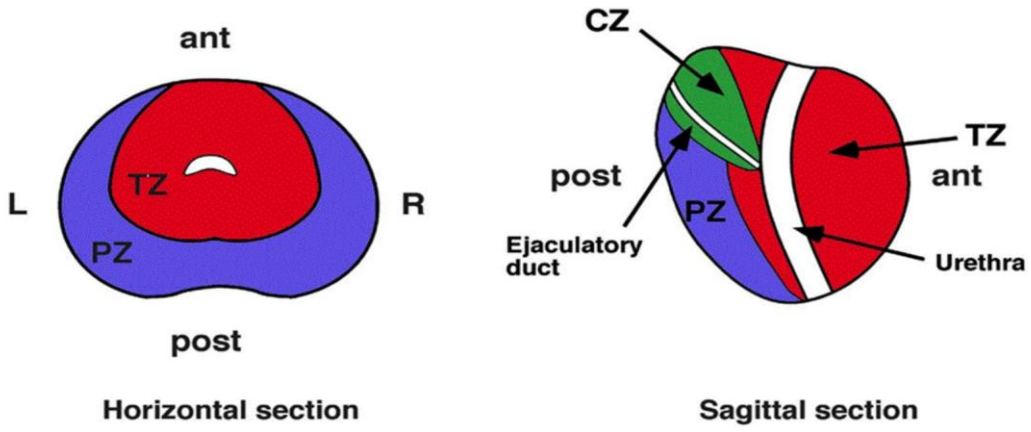
1 BACKGROUND

1.1 PROSTATE

1.1.1 Anatomy

The prostate is a walnut sized gland that is part of the male reproductive system. It consists of fibromuscular stroma and glands built of acinar cells surrounded by basal cells. The prostate is surrounded by a so called capsule, which in fact is a transition between the condensed intraprostatic stroma and the loose extraprostatic connective tissue, rather than a true capsule. It weighs a little more than 20 g in the young man and is slightly cone-shaped with the base facing the urinary bladder and the apex pointing downwards. The posterior part borders to the rectum, from which the prostate can be palpated. The urethra passes through the gland. The seminal vesicle enters the upper posterior part of the prostate and merges with vas deferens that opens into the ejaculatory duct. The prostate has three major anatomical regions: the peripheral zone (PZ), the central zone (CZ) and the transition zone (TZ), occupying 65%, 25% and 10%, respectively, of the prostate volume [123] (**Figure 1**). The biology of these regions differs, which is important for the development of cancer and other histological lesions.

Figure 1*: The anatomical zones of the prostate*



1.1.2 Physiology

The prostate stores and secretes a slightly alkaline fluid that is added to the semen upon ejaculation. A large part of the ejaculate consists of prostatic fluid, which serves to nourish and protect the semen. The prostatic fluid contains numerous enzymes including proteases such as prostate-specific antigen (PSA), acid phophatase, zinc,

potassium, citric acid and prostaglandins. Testosterone, which is secreted by the testis, is metabolized to dihydrotestosterone by 5-alpha-reductase in the prostate.

1.2 PROSTATE CANCER

1.2.1 Cancer development

The development of prostate cancer (PC) occurs through the accumulation of genetic and epigenetic changes, leading to an inactivation of tumor suppressor genes and activation of oncogenes [54]. These alterations most likely take several decades and cancer development can be considered a continuous transformation from benign cells, cancer precursors and malignant cells [134]. In PC there are two major putative premalignant lesions: prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA). Of these, PIN has the most convincing association with cancer [9,21,22,132]. This lesion is an intraductal, or intra-acinar, epithelial proliferation of luminal cells with pronounced nuclear atypia [21,124]. PIN is a histopathological diagnosis, only visual through the microscope. Four architectural patterns have been described: tufting (i.e. undulating epithelium), micropapillary, cribriform and flat (i.e. thin, attenuated epithelium). These are seen in 87%, 85%, 32% and 28% of the cases, respectively [23]. Prominent nucleoli are vital for the diagnosis. The morphology of PIN is similar to that of invasive cancer, however, in PIN the basal cells layer remains intact [121,126]. PIN is classified into low-grade and high-grade PIN (LGPIN and HGPIN) [9,24].

The points of association between HGPIN and cancer are several. HGPIN is more frequent in populations with a high incidence of PC [156]. The incidence of HGPIN is higher in prostate glands containing cancer, and more pronounced in cases with extensive multifocal carcinomas [80,126,179]. The pattern of distribution for PC and HGPIN within the prostate is similar, and both lesions are most frequently found in the peripheral zone [54,127,179]. Areas of HGPIN can be seen close to or as a continuation of invasive cancer ducts, indicating that invasive cancer stems from HGPIN [129]. Several biomarkers are over or underexpressed in both PIN and cancer, compared to benign prostate tissue, further indicating that HGPIN is a likely premalignant lesion [28,80].

PIA consists of proliferative epithelial cells without the ability to differentiate to the common secretory cells [123,134]. In 1999, DeMarzo et al first proposed that PIA is a precursor to cancer [53]. The association between PIA and cancer is still not understood, although some association between PIA and cancer can be seen. Similar to both PIN

and cancer, atrophy is seen in multiple sites in the prostate, the incidence of PIA lesions increases with age and PIA is most common in the peripheral zone [53,123,154]. PIA is often seen close to PIN or cancerous lesions [53,154,160]. PIA is associated with chronic inflammation, which is known to have a role in cancer development [55]. However, whether PIA is a premalignant lesion or not remains to be further elucidated, as there are conflicting reports [12,19,122].

Prostatic epithelial cells with a phenotype intermediate, between basal and secretory cells (intermediate cells), have been postulated to be the cellular origin of PC [183]. In a stem cell model proposed by Isaacs and Coffey, the stem cell population gives rise to amplifying cells that by transient proliferation modulate development of the entire epithelium [91]. This cell group undergoes translocation from basal toward luminal cell compartment while gradually shifting its gene expression profile from stem cells toward terminally differentiated cells [88,183].

1.2.2 Epidemiology

PC is the most common cancer and the most common cause of cancer related death in western males [77]. This is the elderly man´s disease with a mean age at diagnosis around 72 to 74 years, and very few reported cases in patients below the age of 50. Autopsy material has shown that 2/3 of men in their seventies have PC [155] which indicates that although this is the most common cause of cancer death in western males, most men die with the tumor, not from it, thus, PC has a diverse biology, where some tumors are indolent and slow growing, and others aggressive with high metastatic potential [26,35,42].

Incidence of PC has increased drastically during the past 30 years [77]. In Europe, around 2.6 million new cases are diagnosed each year [83]. The increase in incidence is in large parts due to better diagnostics, mainly an increase in PSA testing, and a longer life expectancy [1,32,176]. However, an increase in cancer incidence has also been seen in developing countries, where better diagnostics can not be attributable, indicating an actual incidence increase [138]. As an effect of earlier diagnosis, a shift from higher to lower tumor stage has been seen [1,32,37].

1.2.3 Histology

The most common histological type of PC is adenocarcinoma, which is an epithelial neoplasia with infiltrative growth pattern. However, the morphology is heterogeneous and several subtypes exist [150]. Most of these are rare, and the prognostic importance is unknown, but others are known to be aggressive. Cancer development is most common in the peripheral zone, but also seen in the transition zone, whereas PC less often arises primarily in the central zone [128].

1.2.4 Risk factors

The risk of developing PC is known to be hereditary, with a reported estimated risk of 27-42 % in studies on monozygotic twins [78,114,140]. The hereditary pattern most likely follows an autosomal dominant inheritance [77]. It has been suggested that patients with familial PC have a worse prognosis than sporadic cases. These trends seem to have disappeared after 1992 when PSA testing became widely available, which may at least in part be due to a raised awareness in high risk groups [104]. To date, no genetic test is available for evaluating PC risk.

Dietary factors are postulated to have an effect on cancer development, as an increase in PC incidence is seen in populations with low PC frequency, one generation after moving to a "high prevalence" area. Notably, the new incidence does not reach the levels seen in the new host country [77].

1.2.5 Diagnosis

1.2.5.1 Digital rectal exam, biopsy, ultrasound

The first examination of the prostate, in patients with urogenital symptoms, is often digital rectal examination (DRE). This is a simple diagnostic method with minimal complications [11]. DRE can detect prostate malignancy regardless of PSA elevation and also diagnose non-malignant causes of elevated PSA, such as prostate hyperplasia or infection [30,36,104]. However, many clinically significant tumors are missed at DRE as only the posterior part of the prostate is palpable. Although the detection rate of tumors using DRE is not as high as for serum PSA measurements, a combination of the two methods has higher detection rate than each alone [30]. Tumors discovered through DRE are often more advanced than PSA-detected cancers [30].

As with most suspected malignancies, the histological diagnosis of PC is essential before treatment. Biopsies from the rectum have been used to diagnose PC for more than half a century [15]. Today, ultrasound scanning (TRUS) with sector biopsies is the clinical standard for retrieving material for diagnosis [83,166]. Tissue biopsy is the only way to obtain a Gleason score (GS), which is important for the choice of treatment. Biopsy is performed on the basis of abnormal serum PSA or DRE [104]. The recommended number of biopsies varies. Not surprisingly, most studies show a higher detection rate with a greater number of biopsies [83]. In Sweden, the number of biopsies is commonly eight to ten. Often the procedure is repeated at intervals if PSA levels indicate that cancer may be present although biopsies are negative.

Known complications of rectal biopsies include infection, bleeding, haematuria, haemospermia and urinary difficulties [145,163]. Minor complications are common, but severe complications are rare and hospitalization rates low [145,166]. The most common cause of hospitalization is prostatitis and/or urosepsis [145]. Although major complications are rare, it is important to consider that elderly and sickly patients are at higher risk. Many patients find the procedure painful [50,58].

1.2.5.2 Fine needle aspiration cytology vs. core biopsies

Fine-needle aspiration cytology (FNAC) of the prostate was introduced in 1960 [70]. Cytological grading of PC has been shown to correlate with survival [67]. It is a simple, quick and cheap method and early studies showed that the sensitivity of FNAC for detection of PC was comparable to that of core biopsies [2,5,10,135], however, these studies compared the results of FNAC with less extensive core biopsy protocols and less advance ultrasound guidance than those used today. In recent years, TRUS-guided core biopsies have become the routine method for PC diagnosis. Core biopsies can be combined with immunohistochemistry which increases the diagnostic accuracy and adds clinically important information on extent of disease and GS. Thus, there has been a decline in the use of FNAC. Because of low complication rates and the minimal invasiveness, FNAC could still be considered in elderly patients with high co-morbidity, where transrectal core biopsies could become an additional health risk.

1.2.6 Treatment

Treatment choices for PC include watchful waiting, radical prostatectomy (RP), radiotherapy, hormonal or combination treatment. Recommended treatment varies

depending on tumor characteristics, including GS, serum PSA levels, clinical and pathological stage, as well as co-morbidity and life expectancy of the individual patient [83]. RP and radiotherapy are the only two curable treatments. Of these, RP is the most common, especially for younger and healthier patients [47,83,139].

Unlike most other types of malignancies, not all PCs are considered lethal, which is the reason why non-curable treatment is sometimes recommended. Studies indicate that patients with an estimated life expectancy exceeding 15 years, benefit from radical treatment, but patients with shorter life expectancy and early stage tumors can be subject to watchful waiting or active monitoring [94,171]. Once PC has metastasized, no curative treatment is available [17,38,49,57].

There is a substantial overtreatment of PC. Side effects of treatment hugely affect quality of life, and choice of treatment should therefore be carefully considered. The most commonly reported side effects of radical RP and radiotherapy are erectile dysfunction (ED), urinary incontinence and bowel problems, of which the first two are more common after RP [102,117]. Korfage et al reported ED in 88% of the patients after RP vs. 64% after radiotherapy [102]. However, as ED was seen in both groups prior to treatment, the actual increase after surgery or radiotherapy was 57% and 24%, respectively. Likewise, urinary leakage was more commonly reported in the RP group compared to radiotherapy (31% vs. 13% with 19% vs. 5% increase compared to before treatment). Bowel function problems were more common in the radiotherapy group, but the largest prevalence of symptoms, seen 52 months after treatment, was only 4%. Although percentages differ slightly, this is in line with previous studies [117,143]. Differences in disease-specific survival after RP and radiotherapy need to be further investigated.

1.2.7 Prognostic factors

Predicting prognosis is of importance for all types of disease. In the case of PC, where the biology is very diverse, better prognostic markers would revolutionize choice treatment. In the area of research, better prognostic factors would allow a more accurate stratification of patients for clinical trials and enable better evaluation of therapeutic changes [167]. Prognostic factors are also important in the struggle to understand the complex biology behind cancer development.

Prognostic factors for tumors have been classified into three different categories by The College of American Pathologists (CAP) [26,81].

- Category I factors are well supported by literature and generally used in patient management. For PC these include TNM stage grouping, GS, preoperative PSA levels and surgical margin status. Notable is that surgical margin, full gland GS and certain cancer staging parameters are only available after RP.
- Category II are factors that have been extensively studied biologically and/or clinically but with few clinical outcome studies. In the 1999 CAP guidelines these include tumor volume, histological type and DNA ploidy.
- Category III are factors that currently do not meet criteria for I or II. This is understandably a large group, and include factors such as perineural invasion, neuroendocrine differentiation, microvessel density, nuclear roundness, chromatin texture, other karyometric factors, proliferation markers and PSA derivates.

1.2.7.1 Tumor volume

Tumor volume is known to correlate to tumor progression and disease outcome [25,65,127,169]. Epstein et al reported 26% 5 year recurrence after RP in men with tumors smaller than 4 ml, as opposed to 49% of men with larger tumors [65]. Stamey et al reported recurrence rate after RP of 14%, 39%, 67%, and 97% with tumor volumes of 0.5–2, 2–6, 6–12, and >12 ml, respectively [169]. The percentage of cancer and linear length of cancer in core biopsies have been shown to predict cancer volume [63,79,146]. Tumor volume is a reported predictor of other prognostic factors, including extra-prostatic extension, seminal vesicle invasion and positive surgical margins [125], and in studies based on large tumors, volume has been reported an independent prognostic factor [90,137]. However, the role of tumor volume is not fully established, as some authors have reported that tumor volume does not provide additional prognostic information beyond other predictors of prognosis in multivariate analysis, as reviewed by Epstein et al [64,97]. Due to the evolvement of PSA testing, a shift towards lower stage and a decrease in tumor volume at diagnosis has been observed. As tumors are now substantially smaller [55], tumor volume as a prognostic factor is less important now, than before the PSA era.

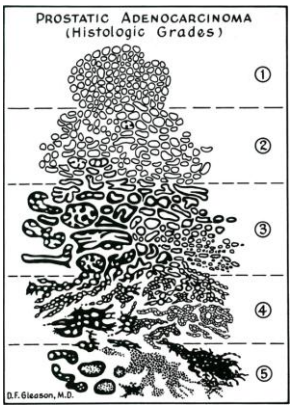
1.2.7.2 Stage, surgical margins and seminal vesicle invasion

The TNM system for staging of PC is the predominant method for defining disease extent [150]. Staging takes into account local extent, lymph node metastasis and evidence of distant metastasis. Positive resection margins and vascular invasion are well known prognostic factors [150]. Seminal vesicle invasion in RP specimens predicts a poor prognosis with a 5-year biochemical progression-free survival between 5 and 60% (mean 34%) as reviewed by Epstein et al [64].

1.2.7.3 Gleason Score

The Gleason grading was developed by Donald F. Gleason [74]. This 5-tier grading system is based on the architecture of the cancerous glands and more de-differentiated carcinomas receive higher grades (**Figure 2**). The two predominant patterns are graded and added to obtain the GS [73]. The Gleason system was accepted by the WHO as the international standard of PC grading in 1993 [133]. GS is one of the most important prognostic factors in PC, both for prediction disease-specific survival in non-treated patient [8,62], and for predicting biochemical recurrence after RP [66,89] or radiotherapy [76]. As a predictor of prognosis GS 7 shows a significant difference in survival length compared to GS 6 or less and 8 or more, indicating a grouping of GS separating GS 7 from higher, and lower scores, in prognostic studies [175]. A limitation of the Gleason grading is that a majority of newly diagnosed tumors are in the mid range scores, somewhat undermining the utility as a predictor of prognosis [62,108].

***Figure 2**: Gleason grading system*



1.2.8 Biomarkers

A tumor biomarker has been defined as an ”antigen or protein which is secreted by the tumor itself or by the surrounding tissues in response to the tumor and that can be

determined in the serum samples of the patient" [33]. The concept of biomarker may also include proteins measured in the tumor tissue itself. Biomarkers can be used for diagnosing cancer, for prediction of prognosis, and for choosing and monitoring treatment. In clinical cancer care, all these categories of biomarkers are of great interest. Although this is a research field that has received considerable attention, the number of tumor biomarkers of clinical relevance is still very small [130].

1.2.8.1 Prostate-specific antigen

Prostate-specific antigen (PSA) was purified in 1979 by Wang et al [184]. It is a protease that belongs to the kallikrein family and is produced by the columnar epithelial cells of the prostatic ducts [170,184]. It is a secretory protein involved in liquefaction of semen, but a minor part leaks into the bloodstream [193]. Although malignant cells produce lower levels of PSA, serum levels of PSA increase in most cases of PC, due to a higher proportion of leakage [166]. The clinical use of serum PSA for detection of PC was first reported by Stamey et al [168], and PSA has since then become the most well known cancer biomarker [157]. PSA is of importance both for diagnosing PC and for monitoring treatment response and tumor recurrence. As PSA is prostate specific, rather than cancer specific, a moderate increase is also commonly seen in benign conditions such as prostatitis and benign prostatic hyperplasia (BPH) [115,157].

Although PSA is mostly used as a serum biomarker, it can also be detected in tissue by IHC. PSA is therefore useful for diagnosing PC in cases of tumors of unknown origin. Studies of IHC of PSA have shown a loss of immunoreactivity in higher GS [75,187] but no correlation to prognosis [27]. Sporadic weak PSA staining has been reported outside the prostate in both benign and malignant tissue [39,186].

The role of PSA for detecting tumor recurrence is well-established. PSA screening, in which raised PSA levels are used for detection of PC in asymptomatic men, is still controversial. A screening method for cancer should be safe, affordable and offer a high sensitivity and specificity. The screened cancer type must have a high prevalence, be responsible for substantial morbidity and mortality, and treatment must be available for the majority of detected tumors [104]. The sensitivity and specificity of serum PSA depends on cut-off levels. Thompson et al showed that a PSA cut-off at 4.1 ng/mL had a sensitivity and specificity of 20.5% and 93.8%, respectively [178]. Thus, 80% of tumors would remain undetected. Lowering the PSA threshold would results in increased

sensitivity, but decreased specificity, leading to a large group of patients undergoing unnecessary biopsy procedures.

Although PSA is not the ultimate diagnostic marker, it still remains the best tumor biomarker available. The cost for a screening program, including PSA measurement, DRE and TRUS-guided core biopsies, is comparable to that of breast cancer screening [104]. The high incidence of PC, and its impact on health economics, makes this cancer suitable for screening. The major argument against PSA screening is that a majority of PCs will never cause clinical symptoms if left untreated [155]. Detection of these tumors would instead result in a large number of patients undergoing unnecessary treatment.

PSA density, defined as the serum PSA level related to prostate volume, is a predictor of advanced disease, and preoperative serum PSA is a predictor of prognosis for PC [170]. Newly reported data from two large, case control studies showed contradictory results regarding the effect of PSA screening. Andriole et al showed that PSA screening did not affect mortality after 7 to 10 years [11], whereas Schröder et al found a 20% reduction of death from PC [159]. According to Schröder et al, to prevent one death in PC, 1410 men would have to be screened, and 48 men would have to undergo surgery. Thus, the risk of overdiagnosis, i.e. the risk of diagnosing PC in patients that would not experience clinical symptoms during their lifetime, was high. Overdiagnosis is defined somewhat differently in different studies [60] and consequently the risk of overdiagnosis using PSA screening varies widely between 25% and 84 % [59,68,120,180]. A recent study by Draisma et al showed overdiagnosis ranging from 23 % to 42 % [60].

1.2.8.2 Prostate-specific membrane antigen

Prostate-specific membrane antigen (PSMA) is an integral membrane glycoprotein produced by epithelial cells in the prostate [157]. It was first discovered in prostate epithelium in 1987 [87]. PSMA is expressed in all types of prostatic tissue and an increased expression is associated with aggressive cancer [27,106,118,157]. Some groups have found PSMA to be an independent predictor of prognosis in multivariate analysis [118,151]. Low levels of PSMA expression has also been reported in non-prostatic tissue [98,105].

1.2.8.3 Pancreatic duodenal box 1

Pancreatic duodenal homeobox 1 (PDX-1) is a Hox-type transcription factor expressed during early embryonic stages of pancreatic development [14]. In the adult pancreas,

PDX-1 activity has been detected in 90% of beta cells and 15% of delta cells of Langerhans islets [3]. PDX-1 is also found in endocrine cells of the duodenum [131] and gastric antrum [107]. PDX-1 activates gene transcription of insulin, somatostatin, islet amyloid polypeptide, glucose transporter type 2 and glucokinase [14]. PDX-1 gene mutations are associated with early onset of diabetes type 2 [172]. Elevated levels of PDX-1 have been reported in both benign and malignant tissues from patients with pancreas, breast, colon, prostate, gastric carcinomas and kidney cancers [113,185] and PDX-1 has been reported to be an independent prognostic factor in pancreatic cancer [99].

1.2.8.4 Heat shock proteins

Heat shock proteins (HSPs) are considered useful as diagnostic and/or prognostic predictive factors in a variety of tumors [45]. HSPs are ubiquitous molecules required for proper folding, localization and stability of cellular proteins, as well as degradation of senescent proteins. They also act as molecular chaperones, protecting cells against stress-related injury [71]. Altered expression of HSP has been seen in a wide range of human neoplasms, including carcinomas of the prostate, breast, female genital tract, gastrointestinal tract, liver, pancreas, lung, skin and urinary tract, hematological malignancies, sarcomas and endocrine adenomas [45].

HSPs have been suggested as prognostic markers for PC [48,103,109]. Proteomic studies by our group have shown that HSP 60 and HSP 70 are overexpressed in PC compared to benign prostatic tissue [6,7,111], and overexpression correlated with prognostic factors such as GS and DNA ploidy [112]. According to several reports, HSP 60 is upregulated in PC [34,48,111], while HSP 27 and 70 have been reported both to be downregulated [48,173,177] and upregulated in PC [103,111].

1.2.8.5 Glutamate decarboxylase 1

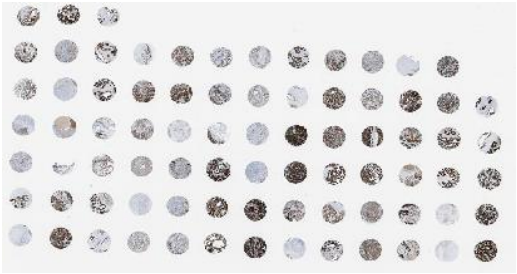
Glutamate decarboxylase 1 (GAD1) is derived from a gene with the same name, located on chromosome 2 [29]. Similar to PSMA, GAD1 is involved in the regulation of glutamate [40]. Strong expression of GAD1 is seen in the brain and small amounts have also been reported in the testis [40]. In the brain, GAD1 catalyzes the synthesis of inhibitory neurotransmitter gamma-amino butyric acid (GABA) from glutamate [4,40]. Altered GAD1 expression in postmortem brain tissue has been associated with psychiatric diagnosis including schizophrenia, bipolar disorder and autism [4]. Autoantibodies directed towards GAD1 have been seen in individuals who later

develop type 1 diabetes [96,194], and are also known to be associated with other autoimmune diseases including autoimmune polyendocrine syndrome (APS) and stiff person syndrome, a rare progressive disease of the nervous system [20,61,144]. Studies on mice have shown that loss of GAD1 leads to craniofacial development defects and is not compatible with postnatal life [13]. No association to cancer, or to the prostate, has previously been described.

1.3 TISSUE MICROARRAY

Tissue microarray (TMA) is a method for collecting large numbers (up to several hundreds) of tissue samples in one paraffin block. This enables large tumor series to be investigated on a few slides. The method of collecting small tissue samples from multiples tumors was introduced by Battifora in 1986 [16]. The early multitumor tissue blocks differed in several ways from those used today, the most important difference being that individual tumor samples could not be identified. In 1998 Kononen introduced TMA, a method that enables collection of identified specimens, thereby connecting tissue-derived data to other factors, such as clinical follow-up [101]. Since then, TMA has become a major research tool, both for the validation process and for identifying new tissue biomarkers (**Figure 3**).

***Figure 3**: TMA*



1.3.1 Construction

For construction of TMA, the histological slides are reviewed, and areas of interest are marked. A core biopsy is taken from the corresponding areas of the paraffin block, the so called donor block, and transferred into pre-made holes in a recipient block. This procedure is repeated until the desired number of cores has been collected. Both the cores and the holes in the recipient block are made by the same instrument, and the cores can therefore be arranged in any desired pattern. Once the recipient block is completed, it can be sectioned and used for any type of analyses performed on formalin-fixed, paraffin-embedded material.

1.3.2 Categories of TMA

TMAs are mainly used for research, mostly within the cancer field, and TMA blocks are commonly used for more than one study. The purpose of the TMA block is important for the selection of material. Although several types of TMAs are used for research, the terminology of TMA classification varies between groups. We separate our TMAs into the following categories: Prognostic, diagnostic and progression TMA. Other forms include predictive and control tissue TMA. Several of these terms are used by other groups [95,142].

1.3.2.1 Prognostic TMA

Prognostic TMAs are used to investigate biomarkers of prognostic importance [95,142]. For construction of this type of array, it is importance to know which cases develop advanced disease. For PC, where death usually is a late event, it is wise to include other clinical endpoints in addition to survival time, for example pre- and postoperative PSA, Gleason grade, surgical margins and TNM stage.

1.3.2.2 Diagnostic TMA

Diagnostic TMAs are used to investigate possible tissue- or tumor-specific markers. These could consist of an array of tumor and benign material from the same tissue, or include different tissue types, in a multitissue array, depending on the specific purpose. For studies on tissue-specific markers, malignant and benign material should be included from tissues that are possible challenges as differential diagnoses.

1.3.2.3 Progression TMA

In a progression array, expression of biomarkers of importance for tumor development, from benign material to metastatic disease, are investigated. This type of TMA includes a spectrum of tissues from benign and premalignant changes, high and low-grade malignant tumors and metastases [95,142]. For PC, a progression TMA would ideally include benign prostate tissue from different anatomical zones, atrophy, HGPIN and PC cases of different GSs.

1.3.2.4 Predictive and control TMA

Predictive arrays are used to investigate biomarker expression for prediction of therapy response [142]. Cases can for example be stratified according to hormone therapy response. Control TMAs can be designed for positive and negative controls in routine

IHC staining. Control TMAs are also important for establishing experimental protocols [142].

1.3.3 Representativity

TMA cores represent a very small sample of the tumor. It has therefore been questioned whether the cores are representative of the tumor. However, studies show that the size of cores may be compensated for by increasing the number of cores taken from each case. A higher number of cores may be reasonable in very heterogeneous material, whereas homeogenous tissue require a smaller number of cores. Three to five cores per tumor have been shown to be representative for PC [152,165]. Most commonly, one [100], two [141,188], or three cores [69] are used per case. It can be assumed that the diameter of the cores also affects representativity. In the first report of TMA, 0.6 mm samples were used [101]. This is still the case in a majority of TMA studies [86,152,164], although diameters up to 2 mm are possible with TMA instruments available today [31].

1.3.4 Fixation of material

1.3.4.1 Formalin fixation

The conventional fixation method for RP specimens consists of overnight immersion in 10% buffered formalin [85,153]. This is the standard fixation for most surgical specimens in the routine laboratory. Fixation is important for immunoreactivity, which may be affected both by over- and underfixation [52,110,153,174,189]. Optimal fixation is also of great importance for preservation of morphology [189]. Tissue shrinkage is a well known side effect of specimen fixation [136,158].

1.3.4.2 Formalin injection

Formalin injection of RP specimens prior to formalin immersion has emerged during the past decade, and is now routine procedure in some laboratories [52,85,153]. Injection of formalin leads to a faster and more homogeneous fixation [85], which is known to have a positive effect on immunoreactivity [52,153].

1.3.5 Evaluation of immunohistochemistry

The most commonly used laboratory technique applied to TMA material is IHC. The interpretation of IHC staining is critical for the results of the analysis. Most important, the correct material needs to be present and evaluated. In PC, premalignant and benign glands are often located adjacent to malignant lesions, and it is important that the

observer is familiar with the histopathology of prostatic tissue. The same core may include several different histopathological entities, and only those tissue components that are of interest should be evaluated. The scales used for IHC evaluation are usually not thoroughly discussed, although the evaluation scales are evidently important when comparing results between research groups. In some studies, immunoreactivity is described as "negative" "weak" or "strong" [182], whereas other groups have a more complex evaluation system. The system used should be based on the type of protein studied, for example, a nuclear protein is usually evaluated by cell count [46,116], whereas a cytoplasmic antibody often is evaluated using a scale for both intensity and extent [51,100,148,149,181,191].

Several different scales for intensity and extent have been presented. We used a scale where the strongest intensity, and the extent of this intensity, are graded, and presented as a product from 0-9. Chung et al graded all intensities present in the sample core on a scale of 0-3, and multiplied each of them by the percentage of extent. All products for the same sample were then added together, to obtain a final sum from 0 to 3 [44]. Depending on the type of scale for intensity and extent, this type of scoring system, where several intensities and their extent are added together, is often referred to as the Histon score [119]. In the Allred score, percentage of positive cells are graded from 0 to 5 (0 = no staining, 1 < 1 %; 2 = 1-10%; 3 = 10-33%; 4 = 33-66%; 5 > 66%) and added to the estimated average intensity, graded from 0 to 3, resulting in a total score of 0 to 8 [82]. This is similar to the scoring system used by Reiner et al, where the average intensity (0-3), and the extent of positive cells (1 < 10%; 2 = 10-30%; 3 = 30-70%; 4 > 70%) are scored, giving a total sum of 0-7 [147]. Detre et al introduced the so called quickscore, in which the average intensity, graded from 0 to 3, and extent of positive staining cells, scored from 1 to 6 (1 = 0-4%; 2 = 5-19%; 3 = 20-39%; 4 = 40-59%; 5 = 60-79%; 6 = 80-100%), are evaluated and presented both as a sum and as a product [56]. Both the "additive" and "multiplicative" quickscore obtained similar results when compared to the Histon score [56].

Instead of scoring intensity and extent separately, Volante et al used a system where subcellular localization and extent of staining is scored simultaneously on a 4-tier scale signifying 0 = absence of immunoreactivity; 1 = pure cytoplasmic immunoreactivity, either focal or diffuse; 2 = membranous reactivity in less than 50% of tumor cells, irrespective of the presence of cytoplasmic staining; 3 = circumferential membranous

reactivity in more than 50% of tumor cells, irrespective of the presence of cytoplasmic staining) [192].

Often, more than one observer participate in the evaluation [72,82,191]. The slides may be read by several observers in an open discussion at a multiheaded microscope [72], or the observers may do individual and independent observations, followed either by calculation of an average score, or by an open discussion about cases with conflicting results [141,192].

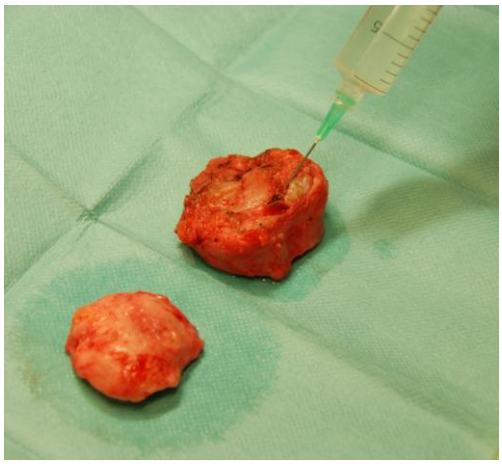
2 MATERIAL AND METHODS

2.1 MATERIAL

2.1.1 Study 1: Tissue shrinkage after formalin injection

142 consecutive RP specimens were collected from 2003 to 2004 at the Karolinska Hospital. The unfixed prostate was weighed, and the transverse, sagittal, and vertical diameters measured. Of the 142 RP specimens, 84 (59%) were formalin-injected at multiple sites with a total of 20 ml 10% buffered formalin solution (4% formaldehyde) (**Figure 4**). This was done using a 20-ml syringe and a 23-G (0.6mm) needle. For homogeneous fixation, the syringe was inserted deep into the prostate, and then slowly retracted while injecting the solution. The specimens were weighed after injection. After pinning of the prostatic capsule to a cork plate, all 142 specimens were immersed in 10% buffered formalin for overnight fixation. The weight was measured again after fixation. The microscopic sections were measured on glass slides with a ruler at 1 mm precision. On whole-mount slides, the maximum transverse diameter of the prostate was measured. On quadrant sections, the widths of two adjacent quadrants were added.

Figure 4: *The prostate was injected at multiple sites with a total of 20 ml 10% buffered formalin solution*



2.1.2 Study 2: Formalin fixation and immunoreactivity

42 RP specimens were collected during 2003-2004 at the Karolinska Hospital. None of the patients had received hormonal therapy prior to surgery. In the pathology laboratory, 21 (50%) prostates were injected with formalin according to the procedure of Study 1 (**Figure 4**). All 42 cases were then immersed in 10% buffered formalin for overnight fixation. A TMA consisting of one benign and one cancer core from each

case, hence, a total of 84 cores, was constructed using a Beecher Manual Arrayer 1 (Beecher Instruments, Silver Spring, Maryland).

IHC staining was performed using 15 antibodies, including nuclear and cytoplasmic markers known to be positive in prostate tissue: pan cytokeratin, P504S, high-molecular weight (HMW) keratin, PSA, vimentin, actin HHF35, thioredoxin-1, peroxiredoxin-2 (PRDX-2), PDX-1, BAX, p27, androgen receptor (AR), HSP 27, 60 and 70. Differences in staining intensity in cancer and benign tissue were compared separately, except for HMW keratin (which should be negative in cancer).

2.1.3 Study 3: Pancreatic duodenal box 1

Prognostic TMA

333 consecutive RP specimens were collected from 1998 to 2002. After exclusion due to lack of follow-up data, and in one case due to lack of cancer in the TMA, 289 cases remained. None of the patients had received hormonal therapy or radiotherapy prior to surgery. Mean age of patients at surgery was 61.1 years (median 61.3, range 46–74 years). Mean preoperative serum PSA was 9.0 ng/ml (median 7.7, range 0.5–58 ng/ml). Clinical stage was T1c in 191 (66.1%), T2 in 94 (32.5%), and T3 in 4 (1.4%) cases. Mean follow-up time was 44.1 months (median 48.9, range 1–101 months) after RP. Biochemical recurrence was defined as two consecutive serum PSA values $\geq$0.2 ng/ml.

The main tumor originated from the PZ, TZ and CZ in 244 (84.4%), 40 (13.8%), and 1 (0.3%) specimen, respectively. The zonal origin was unclear in four specimens (1.4%), with large cancers growing in both the PZ and TZ. Extraprostatic extension was found in 135 (46.7%), seminal vesicle invasion in 36 (12.5%), and positive surgical margins in 133 (46%) of the specimens. All cases with seminal vesicle invasion also had extraprostatic extension. GS of the main tumors were 5 in 10 (3.5%), 6 in 127 (43.9%), 7 in 118 (40.8%), 8 in 14 (4.8%), 9 in 19 (6.6%), and 10 in 1 (0.3%) case. The Gleason patterns of individual TMA cores were 2 in 16 (1.8%), 3 in 597 (68.9%), 4 in 224 (25.8%), and 5 in 30 (3.5%) cores. To obtain adequate representation of the tumor, two cores were obtained from the primary Gleason pattern, and one from the secondary pattern. A total of 14 blocks were constructed. After evaluating hematoxylin and eosin stained sections of the blocks, an additional block was made from five tumors that were poorly represented in the original TMA blocks. Each block also contained 3 cores of benign prostatic tissue, serving as benign controls.

Progression TMA

A TMA from 40 consecutive RP specimens collected during 2005 was designed to contain a pathogenetic sequence of prostatic tissues, including benign tissue, precursor lesions and invasive carcinoma from the same cases. From each case, seven cores were taken: two from cancer, two from HGPIN, and one each from atrophy, non-atrophic benign tissue (without morphologic signs of hyperplasia) and BPH. The BPH sample was taken from TZ and the others from PZ. For analysis purposes, non-atrophic glands of PZ and hyperplastic glands of TZ were also combined as benign non-atrophic glands by calculating mean values. The GS of the cancers were 5 in 1 (2.5%), 6 in 18 (45%), 7 in 20 (50%), and 9 in 1 (2.5%) case.

Metastasis array

20 lymph nodes with PC metastases from 2002 to 2005 were collected. Two cores were taken from each lymph node. In three cases, no representative material was obtained in the immunostained sections, leaving 17 cases for analysis.

All TMAs were constructed using a Beecher Manual Arrayer I with a punch diameter of 1 mm.

2.1.4 Study 4: Heat shock proteins 27, 60 and 70

The prognostic TMA used for Study 3 was used for this study as well.

2.1.5 Study 5: GAD1

Multitissue array

A multitissue TMA was constructed from 15 high-grade urothelial cancers, 4 lung adenocarcinomas, 5 rectal adenocarcinomas and 36 PCs. Specimens from bladder, lung and rectum were collected from 2001-2002. Prostatic tissue was taken from RPs performed 2005. None of the patients had received radiotherapy or chemotherapy prior to surgery. All cases were fixed in formalin overnight at arrival to the pathology department, and thereafter paraffin embedded. The Beecher Manual Arrayer I was used for TMA construction. Each tumor was represented by two cores, while benign cases were represented by one core. Benign tissue was taken from 8 prostate, 4 lung, 5 bladder and 5 rectum samples. IHC for GAD1, PSA and PSMA was performed. The GAD1 antibody was validated by Western blot. Real-Time PCR (RT-PCR) for GAD1,

PSA and PSMA was performed on malignant and benign samples from each tissue type.

2.1.6 Study 6: Reproducibility of evaluation

From our prognostic TMA (Study 3 and 4) 50 cores were selected from consecutive cases, with one core from each case. Only cores containing a large amount of cancer were selected. Reference cores for standardization of intensity were selected from the same blocks, but not included in the dataset.

2.2 IMMUNOHISTOCHEMISTRY

Antibodies, dilutions, pretreatment conditions and manufacturers for all antibodies used in Study 2-6 are listed in Table 1.

IHC staining (Study 2 – 4 and 6) of TMA sections was done using commercially available antibodies. Sections were cut at 4 µm. After deparaffinization in xylene and rehydration through graded ethanol, the sections were microwave treated for antigen retrieval (Vector H 3300) for 15 min. Non-specific binding sites were blocked with 5% skimmed milk in PBS with 0.1% BSA. The slides were incubated with a primary antibody at 4°C overnight. The secondary antibody was applied for 30 min at room temperature. The signal was increased with the ABC kit (20 ml A+20 ml B/ml PBS-0.1% BSA) at 37°C for 45 min and detected with DAB (Vector kit SK 4100). The slides were counterstained with hematoxylin. Positive and negative controls were used according to the manufacturers' instructions. HMW keratin, PSA, actin HHF35 and AR (Study 2) were stained using a Leica Bond robotic immunostainer (Leica Microsystems, Wetzlar, Germany) (**Table 1**).

For Study 5, slides were incubated in 60°C for 45 min, deparaffinized in xylene (2x15 min) and hydrated through graded alcohols. Endogenous peroxidase was blocked with H_2O_2 in 95% alcohol. A Decloaking chamber (Biocare Medical, Walnut Creek, CA) where slides were immersed in Citrate buffer®, pH6 (Lab Vision, Freemont, CA) and boiled for 4 min at 125°C was used for antigen retrieval. Immunostaining was performed using an automated staining instrument, Autostainer 480® (Lab Vision). Primary antibodies against GAD1, PSMA and PSA and a dextran polymer visualization system (UltraVision LP HRP polymer®, Lab Vision) were incubated for 30 min each at room temperature. The slides were rinsed in wash buffer® (Lab Vision) between all

steps and Diaminobenzidine® (Lab Vision) was used as chromogen for 10 min. Slides were rinsed in tap water and counterstained in Mayers hematoxylin (Histolab, Gothenburg). The stained slides were scanned using an automated high-resolution scanner (Aperio Technologies, Vista, CA).

Table 1. *Antibodies/clone, species/type, dilutions, antigen retrieval (MW=microwave treatment) and vendor*

Antibody/clone	Species/type	Dilution	Retrieval	Vendor
Pan CK	Mouse/monoclonal	1:50	HH	Novocastra
P504S	Rabbit/polyclonal	1:200	MW	Dako
HMW Keratin	Mouse/monoclonal	1:400	Enzymatic	Novocastra/Leica
PSA	Mouse/monoclonal	Prediluted	None	Novocastra/Leica
Vimentin	Mouse/monoclonal	Prediluted	MW	Zymed
Actin HHF35	Mouse/monoclonal	1:1500	Enzymatic	Dako
Thioredoxin-1	Goat/polyclonal	1:350	Steam	R&D Systems
PRDX-2	Rabbit/polyclonal	1:400	MW	Proteintech
PDX-1	Rabbit/polyclonal	1:4000	MW	Abcam
BAX	Rabbit/polyclonal	1:800	MW	Santa-Cruz
p27	Mouse/monoclonal	1:1000	MW	Novocastra
AR	Mouse/monoclonal	1:100	Heat	Dako
HSP 27, 2B4	Mouse/monoclonal	1:20	MW	Novocastra
HSP 60, SPA-829	Mouse/monoclonal	1:100	MW	Biosite
HSP 70, 8B11	Mouse/monoclonal	1:100	MW	Novocastra
GAD1	Mouse/monoclonal	1:300	Heat	Santa-Cruz
PSMA	Rabbit/polyclonal	1:100	Heat	Atlas antibodies
PSA	Rabbit/polyclonal	1:150	Heat	Atlas antibodies

2.2.1 Evaluation of IHC

For Study 2-6, intensity of immunoreactivity of each TMA core was evaluated using a 4-tier scale. For cytoplasmic staining, negative, weak, medium and strong intensity was designated 0, 1, 2 and 3, respectively. When a core was heterogeneously stained, the strongest intensity was graded. For nuclear antibodies, a 4-tier scale was used, signifying negative, <5% positive, 5-25% positive and >25% positive nuclei, respectively.

For Study 3-6, the extent of staining was evaluated using a 4-tier scale and the immunoreactivity product (IRP) of intensity and extent was calculated, thus an IRP from 0 to 9 was obtained. When a case was represented by more than one core, the mean IRP was calculated. In Study 3 and 4, the scale from 0 to 3 evaluating extent of staining signified 0–4%, 5–25%, 26–50% and >50%, respectively. For Study 5 and 6, the scale was modified to facilitate evaluation, and scoring of extent of staining was graded from 1 to 3, signifying 1-33%, 34–66% and >66%, respectively.

All evaluations in Study 2 were done by one of the authors (SJJ). For Study 3 and 4, two independent observers (SJJ and AG) evaluated the staining. The mean score of the two observers was calculated. For Study 5, two observers (SJJ and LH) evaluated the staining and grading was the result of a consensus decision.

In Study 6, four independent observers, including two senior pathologists specialized in urogenital pathology (LE, FP) and two medical doctors with no formal training in pathology (HB and SJJ), performed the evaluations. The material was evaluated at two occasions with a two-week interval.

Digital image analysis was used in Study 6. The color intensity (brown) was measured by digital image analysis (Noesis, Gif sur Yvette, France). No selection was made of areas involved by color intensity measurement, i.e. false positive areas in stroma were not excluded.

2.3 WESTERN BLOT

For Study 3, seven PC samples and three benign prostatic tissue samples from RP specimens, two PC cell lines (LNCaP, DU145) and a pancreatic cancer cell line (PaCa-2) for positive control were immunoblotted. The samples were homogenized and proteins were isolated and separated by 1D gel electrophoresis, then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Primary antibodies included rabbit anti-human PDX-1 (1:1000, Abcam, Cambridge, MA) and rabbit anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, Abcam) used as protein loading control. The blot was initially probed for PDX-1 and then stripped and probed for GAPDH. Horseradish peroxidase-conjugated anti-rabbit (1:3000, GE Healthcare, Piscataway, NJ) secondary antibody was used with GE Healthcare's

enhanced chemiluminescence (ECL) detection kit. Chemiluminescence was detected with a Nikon CCD camera.

In Study 5, Western blot was performed on 15 µg of total protein lysate from normal prostate tissue, on a precast 10% to 20% criterion sodium dodecyl sulfate-polyacrylamide gel electrophoresis-gradient gel (Bio-Rad Laboratories, Hercules, CA) under reducing conditions, followed by transfer to polyvinylidine fluoride membrane (Bio-Rad Laboratories). The membrane was soaked in methanol and blocked (5% dry milk, 0.5% Tween 20, 1xTris-buffered saline; 0.1 M Tris-HCl, 0.5 M NaCl) for 1 hour at room temperature during constant shaking. The membrane was incubated with primary antibody against GAD1 for 1 hour, followed by washing (1xTris-buffered saline Tween-20) and incubation with a secondary (Goat anti-mouse 1:7000) peroxidase-conjugated antibody. Detection was carried out using a CCD-camera (Bio-Rad Laboratories) with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL).

2.4 REVERSE TRANSCRIPTASE RNA

The gene expression analyses in Study 6 were performed according to the following procedure: Fresh frozen tumor and benign prostate tissue derived from ten matched patient samples, 4 samples each from urothelial, lung and rectal cancer. RNA isolation was performed using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was quantified with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE). For each tumor and benign tissue, a 20 µm thick section was used for RNA isolation. The average amount of extracted RNA was 1, 2 µg. cDNA was synthesized with the SuperScript III First-Strand Synthesis System (Invitrogen) using poly-dT primers, according to the manufacturers´ instructions. 1 µl of cDNA was used to run the real-time PCR analysis using the SYBR Green Universal PCR Master Mix (Applied Biosystems, Foster City). Primers were used at a concentration of 200 nM. The reaction was performed with the Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems). The primer sequences for GAPDH, GAD1, PSA and PSMA are listed in Table 2. The expression levels of GAD1, PSA and PSMA were normalized to the expression of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

***Table 2**: Primer sequences for GAD1, PSMA, PSA and GAPDH (control)*

	Forward primer	Reverse primer	Annealing temperature
GAD1	CGAGGACTCTGGACAGTAGAGG	GATCTTGAGCCCCAGTTTTCTG	60°C
PSMA	CAGCGTGGAAATATCCTAAATCTGA	TTGGATGAACAGGAATACTTGGAA	60°C
PSA	TTGGAAATGACCAGGCCAAG	AGCAACCCTGGACCTCACAC	60°C
GAPDH	ACCCACTCCTCCACCTTTGA	CATACCAGGAAATGAGCTTGACAA	60°C

2.5 STATISTICAL ANALYSES

Paired and unpaired student *t*-test were used to analyze differences in mean intensity (Study 3) and to compare time spent on analyses (Study 6). Unpaired *t*-test was used for calculations of weight differences, differences in percent of shrinkage between groups (Study 1), to compare the effect of fixation method on immunoreactivity (Study 2) and for comparison of intensity between different antibodies (Study 5).

Chi-2 test was used for comparison of distribution of GSs (Study 2).

Spearman rank correlations were used for correlation of immunoreactivity with GS and Gleason patterns (Study 3, 4, 5) and for comparison of results of image analysis and subjective assessments (Study 6). ANOVA test was used for overall comparison of immunoreactivity and diagnostic categories (Study 3).

Logistic regression analysis was used for correlation of the immunoreactivity with extraprostatic extension and seminal vesicle invasion (Study 4).

Univariate and multivariate Cox proportional hazards models were used to compare prognostic parameters (Study 3 and 4).

A *p*-value <0.05 was considered significant.

Weighted kappa was used for assessment of inter- and intraobserver variability of immunoreactivity scores (Study 3, 4 and 6). A kappa value of 0 to 0.2, 0.21 to 0.4, 0.41 to 0.6, 0.61 to 0.8 and 0.81 to 1 was considered as slight, fair, moderate, substantial and almost perfect agreement, respectively. Compared to unweighted kappa statistics, weighted kappa analysis also factors in the magnitude of the difference between assessments.

2.6 ETHICS COMMITTEE DECISION

All studies were approved by the Regional Ethics Committee, Stockholm according to the following decisions: Dnr 03-658 and 03-660 (Study 1); 2006/4:10 (Study 2-6); Drn 01-353 (Study 5). Analyses performed in France (Study 4) were approved by the Ethics Committee at IARC Lyon (06-08).

3 RESULTS AND DISCUSSION

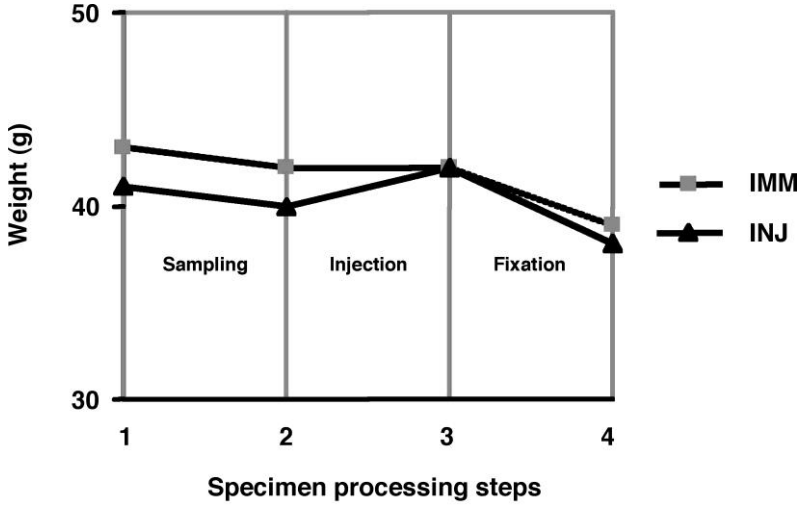
3.1 TISSUE SHRINKAGE AFTER FORMALIN INJECTION

3.1.1 Results

The average weights in the injection and standard fixation groups were 41.2 and 42.9 g, respectively ($p = 0.57$). The average weight loss after final fixation was 5.8% and 8.6% for formalin-injected and standard-fixed specimens, respectively ($p < 0.001$) (**Figure 5**). For all specimens, the average linear shrinkage (as determined by measurements on glass slides compared to unfixed specimens) was 4.5%, which translates into a linear correction factor of 1.047 (100/95.5). Because the volumetric shrinkage correction must take into account linear shrinkage in three dimensions, the volume correction factor obtained was $(1.047)^3 = 1.15$. There was no significant difference in shrinkage between formalin injected specimens and standard-fixed specimens (4.1% and 4.9%, $p = 0.59$), indicating that formalin injection does not have any effect on calculations of PC volume.

The theoretical tumor volumes of the processed specimens (i.e., based on microscopic sections) were derived by dividing the weights of unfixed prostates with the correction factors calculated for the formalin injection and standard fixation groups (1.13 and 1.16, respectively). The mean volumes obtained were almost identical (mean 36.3 and 36.9 ml, respectively; $p = 0.83$).

Figure 5: *Extrapolated average weight changes assuming formalin-injected (INJ) and immersion fixated (IMM) specimens had identical initial weight*



3.1.2 Discussion

Despite the dramatic decrease of tumor volume observed in RP specimens in recent years [55] there are still populations with relatively large PCs [79], indicating that determination of tumor volume remains clinically significant. The degree of tissue shrinkage due to formalin fixation depends on type of specimen and local circumstances, and a laboratory-specific shrinkage correction factors should be calculated. The volumetric tissue shrinkage correction factor obtained in the present study is almost identical to the one reported by Schned et al [158].

Formalin injection, as an addition to standard fixation procedure, does not affect tissue shrinkage as measured on the microscopic slides and, hence, does not have any effect on cancer volume calculations. This supports the routine usage of formalin injection fixation for RP specimens.

3.2 FORMALIN FIXATION AND IMMUNOREACTIVITY

3.2.1 Results

For 10 antibodies there was no significant difference in staining intensity between the fixation methods in benign and malignant tissue (**Table 3**). Five antibodies showed different staining intensity between groups, either in malignant, or benign tissue, or both, as listen in Table 3. The expression of HSP 27 in benign tissue was stronger in formalin injected cases (2.33 vs. 1.67, $p = 0.002$). The expression of AR was stronger in both benign and malignant tissue in formalin injected prostates (1.67 vs. 2.5, $p = 0.003$ and 2.25 vs. 2.72, $p = 0.03$ respectively). For HSP 60, 70 and PRDX-2, immunoreactivity was stronger in conventionally fixed prostates. For HSP 60, there was a significant difference in expression in both malignant and benign tissue (0.95 vs. 0.43, $p = 0.01$ and 1 vs. 0.38 $p = 0.02$, respectively). A significant difference in expression of HSP 70 and PRDX-2 was seen in cancer (1.43 vs. 0.76 $p = 0.005$) and benign tissue (2.62 vs. 1.95 $p = 0.003$), respectively.

***Table 3**: Average staining intensity for fixation method in benign and tumor material. Conv = conventional fixation, Inj = formalin injected material. p-value referring to differences in fixation method within tissue type.*

Antibody	Benign			Tumor		
	Conv	**Inj**	***p*-value**	**Conv**	**Inj**	***p*-value**
Pan CK	2.43	2.62	ns	2.9	2.67	Ns
P504S	0.05	0.05	ns	1.52	1.63	Ns
HMW Keratin	2.89	2.94	ns	N/A	N/A	N/A
PSA	2.9	2.75	ns	2.45	2.22	Ns
Vimentin	1	1.33	ns	0.33	0.38	Ns
Actin HHF35	2.89	2.83	ns	2.85	2.89	Ns
Thioredoxin-1	2.33	2.15	ns	2.14	1.9	Ns
PRDX-2	2.62	1.95	0.003	1.95	1.8	Ns
PDX-1	1.43	1.75	ns	2.05	2.42	Ns
BAX	1.81	1.7	ns	1.81	1.95	Ns
p27	1.29	1.45	ns	1.57	1.21	Ns
AR	1.67	2.5	0.003	2.25	2.72	0.032
HSP 27	1.67	2.33	0.002	1	1.1	Ns
HSP 60	0.95	0.43	0.021	1	0.38	0.012
HSP 70	1.24	1.24	ns	1.43	0.76	0.005

3.2.2 Discussion

Previous studies have reported a positive effect on immunoreactivity after formalin injection [52,153]. In this study, the choice of fixation method did not have a major effect on staining intensity. In 22 of 29 analyses performed, we were unable to demonstrate a significant difference between fixation methods. For the remaining 7 analysis, three showed a stronger immunoreactivity in formalin injected prostates, while 4 showed a stronger immunoreactivity in conventionally fixed cases. The combination of formalin injection and formalin immersion should minimize the risk of underfixation. Theoretically it may even result in overfixation in some cases, which has been reported to have a negative effect on immunoreactivity [110,174,189]. However, immunoreactivity of different antibodies varies, and it has been noticed that some epitopes benefit from one fixation method, whereas others are better preserved using another method [189].

Technical differences in fixation and TMA construction are important for the outcome of IHC. A critical point is storage. It has been reported that pre-cut tissue slides stored in room temperature show a loss of antigenicity [18,92,148,189,190], which may be due to oxidation [148,162]. However, it seems unlikely that a potential effect on immunoreactivity, due to differences in fixation, is counteracted by oxidation, as storage time is the same for the whole TMA, and if immunoreactivity becomes weaker, the differences between the fixation methods would still be expected to remain.

Most biological tissues are heterogeneous. In tumor tissue, protein levels and biological behavior are even less predictable. For this reason, it would be desirable to construct a TMA from organs where half the specimen is formalin injected and the other half is conventionally fixed. However, in PC considerable intratumor heterogeneity leads to variable protein expression within the same case. The diversity in protein expression may be of greater importance than the effect of fixation method.

This study indicates that for most antibodies, fixation method does not significantly affect antigen presentation. Until more research has been performed, formalin injection, prior to immersion, can therefore be recommended as other positive effects, such as decreased autolysis are desirable.

3.3 PDX-1

3.3.1 Results

PDX-1 was expressed in cytoplasm and occasional nuclei of epithelial cells of benign non-atrophic glands, PC, HGPIN and atrophic glands (**Figure 6-9**). In benign non-atrophic glands, basal and secretory cells expressed PDX-1, although generally at a low level. The mean values for PDX-1 IRP were as follows: benign non-atrophic glands 4.01 (normal PZ 4.67 and BPH 3.28), cancer 6.17, HGPIN 6.74, atrophy 7.33 (**Figure 10**). PDX-1 was overexpressed in cancer vs. benign tissue ($p < 0.001$), but also in atrophy and HGPIN compared to cancer ($p < 0.001$ and $p = 0.022$, respectively), and benign non-atrophic prostatic epithelium ($p < 0.001$). The expression was stronger in benign non-atrophic glands of PZ than in hyperplastic glands of TZ ($p < 0.001$) (**Table 4**). Mean IRP of primary cancers and lymph node metastases was 5.14 and 4.41, respectively ($p = 0.089$). None of the measures of PDX-1 staining (intensity, extent, IRP) correlated with biochemical recurrence ($p = 0.16$, 0.66 and 0.19, respectively) or GS ($p = 0.37$). However, there was a negative correlation with Gleason patterns of

individual TMA cores ($p < 0.001$). Mean IRP of Gleason patterns 2–3 and 4–5 was 5.26 and 4.84, respectively ($p = 0.032$). Weighted kappa for interobserver agreement of intensity, extent and IRP was 0.65, 0.13 and 0.54, respectively.

Western blot analysis validated the expression of PDX-1 in benign and tumor tissue. The band pattern of PC cell lines correlated with that of the positive control. Benign tissue samples displayed a weaker signal than cancer.

***Figure 6**: Weak staining for PDX-1 in benign non-atrophic glands*

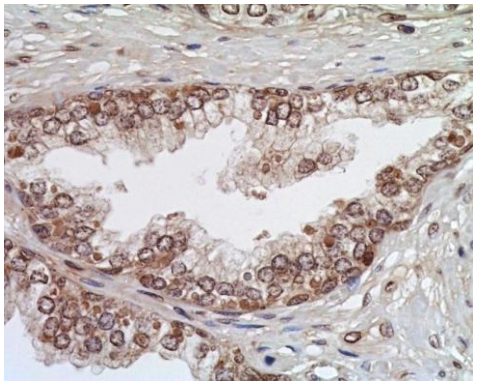


***Figure 7**: Strong staining for PDX-1 in PC, Gleason score 3 + 3 = 6*

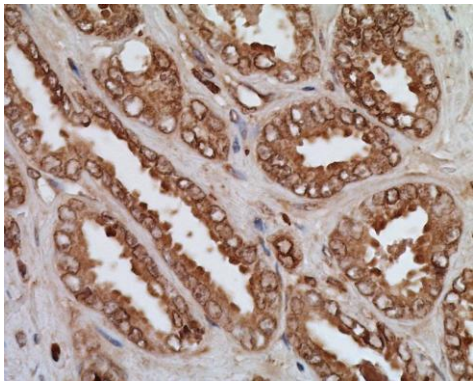


***Figure 8**: Strong staining for PDX-1 in HGPIN*

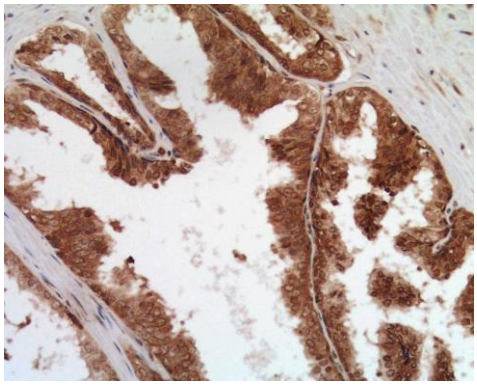


***Figure 9**: Strong staining for PDX-1 in atrophic glands*

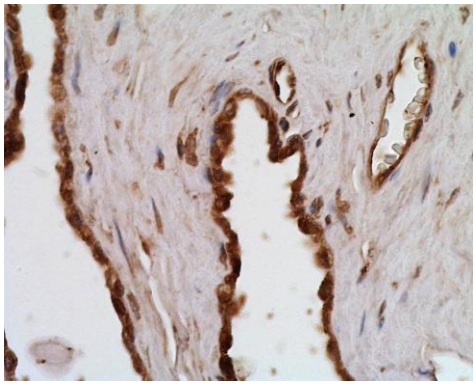


***Figure 10**: PDX-1 expression in benign prostatic tissue, atrophy, PIN, and primary and secondary PC*

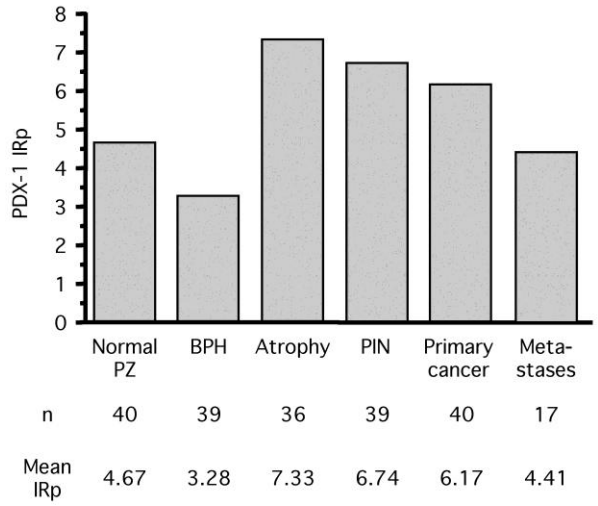


***Table 4**: Correlations of PDX-1 immunoexpression in prostatic tissues (p-values, paired t-test)*

	Benign non-atrophic	*Benign PZ*	*Benign TZ*	PC	HGPIN	Atrophy
Benign non-atrophic	-	-	-	<0.001	<0.001	<0.001
Benign PZ		-	<0.001	<0.001	<0.001	<0.001
Benign TZ			-	<0.001	<0.001	<0.001
PC				-	0.022	<0.001
HGPIN					-	0.102
Atrophy						-

3.3.2 Discussion

PDX-1 is suggested to play an important role in tumor cell proliferation and differentiation [99,185]. In a small study of 14 prostate samples, Wang et al. found a stronger PDX-1 expression in PC than in benign prostatic tissue using immunohistochemistry [185]. Similarly, we found that PDX-1 was overexpressed in PC specimens compared to benign tissue from the same cases. Our study also included HGPIN and prostatic atrophy, showing an even stronger PDX-1 expression. HGPIN is an established precursor of invasive cancer and several studies indicate that atrophy may be a precursor as well [53,55,154,160]. Thus, PDX-1 overexpression in these lesions may indicate the involvement of this protein in early carcinogenesis.

Despite the upregulation of PDX-1 in PC and its precursors, no correlation with prognosis was found and there was no overexpression in lymph node metastases compared to primary carcinomas. Thus, PDX-1 correlates better with pathogenetic development of PC than with its progression. In would be of interest to further investigate the role of PDX-1 in neoplastic transformation in the prostate.

3.4 HSP 27, 60 AND 70

3.4.1 Results

The three studied HSPs were expressed in the cytoplasm and occasionally in the nuclei of epithelial cells in benign and malignant prostate glands. Benign prostate tissue sometimes showed staining of basal cells. The cytoplasmic staining pattern varied from diffusely nongranular to finely granular, and in some cases showed a membranous pattern. The cytoplasmic staining for HSP 60 was more coarsely granular than that of the other HSPs.

Mean IRP of HSP 27, 60 and 70 was 2.62 (range 0–9), 4.13 (range 0–9) and 5.37 (range 1.3–9), respectively. IRP of HSP 27 and 60, but not of HSP 70, correlated positively with GS ($p < 0.001$, $p = 0.008$ and $p = 0.19$, respectively) (**Figure 11**). The IRP of HSP 27 correlated with seminal vesicle invasion ($p = 0.001$) and that of HSP 60 and HSP 70 correlated with extraprostatic extension ($p = 0.04$ and 0.02, respectively). The IRP of HSP 27 and 60, but not of HSP 70, predicted biochemical recurrence in univariate Cox analysis ($p = 0.014$, 0.034 and 0.160, respectively). Recurrence-free survival in patients with strong expression of HSP 27 and HSP 60 (IRP≥6) was shorter than in those with weak expression ($p = 0.019$ and 0.001, respectively) (**Figure 12**). In multivariate analysis, only HSP 60 remained an independent predictor of biochemical recurrence when extraprostatic extension, positive surgical margins, seminal vesicle invasion and GS were included in the model as explanatory variables. However, if preoperative clinical data (preoperative serum PSA and age at surgery) were added to the model, only surgical margins ($p < 0.001$), GS ($p = 0.004$), and serum PSA ($p < 0.001$) remained significant. Weighted kappa for interobserver agreement of the three HSPs was substantial to almost perfect for intensity (0.613-0.823), slight to moderate for extent (0.036-0.244) and moderate to substantial for IRP (0.584-0.719).

***Figure 11**: Distribution of IRP of HSP 27, HSP 60 and HSP 70 among Gleason score groups*

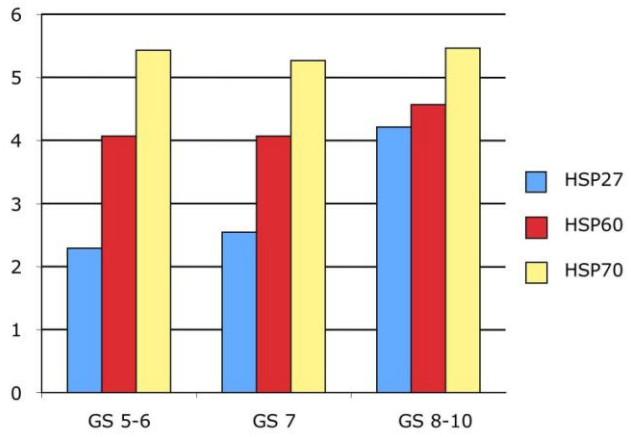
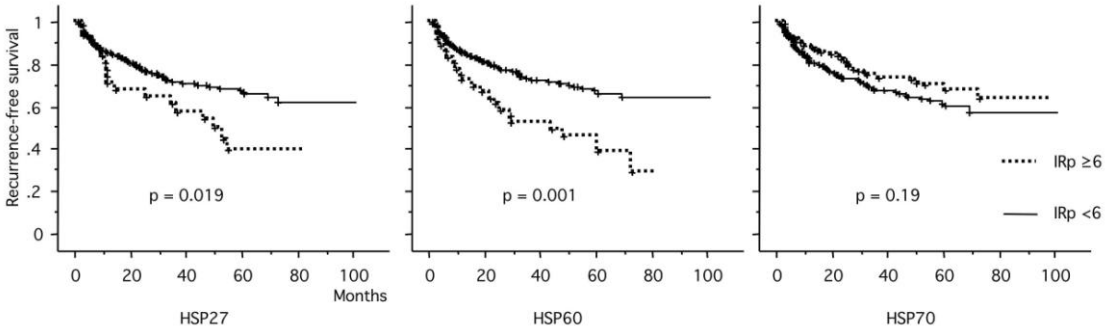


***Figure 12**: Recurrence-free survival after radical prostatectomy with strong and weak immunoreactivity*



3.4.2 Discussion

Using two-dimensional gel electrophoresis and mass spectrometry, we have previously shown that HSP 60 and 70 are overexpressed in PC [111], thus suggesting these HSPs to be potential prognostic markers of PC. HSP expression levels are known to be altered in a wide range of human neoplasms, and an association between HSP expression and prognosis has been seen for some cancers, although the prognostic impact varies between tumor types [45].

Thomas et al. showed that HSP 27 staining was reduced in PC and correlated with GS and tumor invasiveness. However, only a small set of frozen prostate samples was used, including 13 cancers and 15 benign cases [177]. In contrast to these results, and in line with our results, two larger studies have reported a correlation of high HSP 27 expression and biochemical recurrence and GS. Using a set of 120 cancers, Cornford et

al. saw a decreased HSP 27 expression in early PC [48]. Univariate analysis of 85 advanced cancers showed a correlation between high HSP 27 expression and poor clinical outcome. Using conventional sections from 172 cancers, Kurahashi et al. found an association between high HSP 27 expression in PC and pathological stage, GS, surgical margin status, lymph node metastasis, tumor volume and a shorter biochemical recurrence-free survival [103]. In line with our results, they did not find HSP 27 to be an independent predictor of biochemical recurrence in multivariate analysis.

Cornford et al. showed elevated HSP 60 expression in both high-grade PIN and PC compared to benign tissue, but no correlation to Gleason grade or clinical outcome [48]. Johansson et al. found an upregulation of HSP 60 in PC cell lines and in tumor tissue from 79 men but no correlation with GS [93]. We report a correlation of HSP 60 overexpression to both biochemical recurrence and GS. In multivariate analysis, HSP 60 was an independent predictor of biochemical recurrence when extraprostatic extension, positive surgical margins, seminal vesicle invasion and GS were included in the model. Thus, this is the first report showing a prognostic value of HSP 60 in PC.

HSP 70 stabilizes mutated p53, indicating that HSP 70 may be relevant for PC progression [84]. However, in the current study we saw no correlation between HSP 70 and biochemical recurrence or with other prognostic factors. These results are in line with other studies showing loss of HSP 70 expression in advanced PC compared with early cancer and benign tissue [48], but no correlation with Gleason grade or clinical outcome [48,103].

3.4.3 Reproducibility

For both Study 3 and 4, two independent observers (AG and SJJ) evaluated staining intensity and extent. Interobserver reproducibility of the staining intensity varied between substantial and almost perfect (weighted kappa 0.613 to 0.823), while that of extent was only slight to fair (weighted kappa 0.036 to 0.244). Estimating intensity and extent is a common method for assessment of immunostaining. However, our results indicate that the estimation of extent presents several difficulties and should be further investigated.

3.5 GAD1

3.5.1 Results

The staining results of the three antibodies are summarized in Table 5. GAD1 and PSA stains were stronger in benign prostate and PC than in benign and malignant tissues from other organs ($p < 0.001$). PSMA was significantly overexpressed in PC compared to urothelial and rectal cancer ($p < 0.001$), but not compared to lung cancer ($p = 0.14$). No significant overexpression of PSMA was seen in benign prostate ($p = 0.15$). The mean IRP of GAD1 in PC was slightly weaker than in benign prostate tissue ($p = 0.037$) while the opposite was true for PSMA ($p = 0.002$). PSA showed no significant difference in staining between cancer and benign prostate ($p = 0.22$). The GAD1 staining in PC correlated negatively with increasing GS ($r = -0.56$, $p = 0.001$). By contrast, PSMA staining was stronger in high GSs ($p = 0.015$). PSA expression did not correlate significantly with tumor differentiation ($r = 0.17$, $p = 0.32$). The sensitivity and specificity for different staining levels of GAD1, PSMA and PSA for recognizing cancers as PC are shown in Table 6. Stains of GAD1, PSA and PSMA in PC are shown in Figure 13.

Western blot on a lysate of normal prostate tissue showed a distinct single band at 47 kDa and no band on lysates of RT-4, human plasma and human liver (**Figure 14**).

The real-time PCR analysis demonstrated that GAD1, PSMA and PSA were expressed in all prostate tissue samples, with a higher abundance of PSA transcripts compared to PSMA and GAD1. The relative expression levels of PSMA, GAD1 and PSA showed large variations between the individual patients for both cancer and benign tissue. As opposed to PSA and PSMA, GAD1 mRNA was found in all types of control tissue.

***Table 5**: IRP in benign and cancer tissue from prostate and controls.*
p-value refers to difference between control and prostate tissue for each antibody

	GAD1		PSMA		PSA	
Cancer	IRP	*p*-value	IRP	*p*-value	IRP	*p*-value
Prostate	6.8	-	6.0	-	8.4	-
Urothelial	0.0	<0.001	0.3	<0.001	0.1	<0.001
Rectal	0.2	<0.001	0.7	<0.001	0.4	<0.001
Lung	0.0	<0.001	3.8	0.14	0.0	<0.001
All controls	0.0	<0.001	1.0	<0.001	0.2	<0.001
Benign						
Prostate	8.6	-	2.4	-	9.0	-
Urothelium	0.3	<0.001	2.7	0.8	1.7	<0.001
Rectum	0.0	<0.001	0.0	0.11	0.5	<0.001
Lung	0.0	<0.001	0.3	0.09	0.0	<0.001
All controls	0.1	<0.001	1.1	0.15	0.7	<0.001

***Figure 13**: A TMA core of PC stained for GAD1, PSMA and PSA*
(all with strong expression)

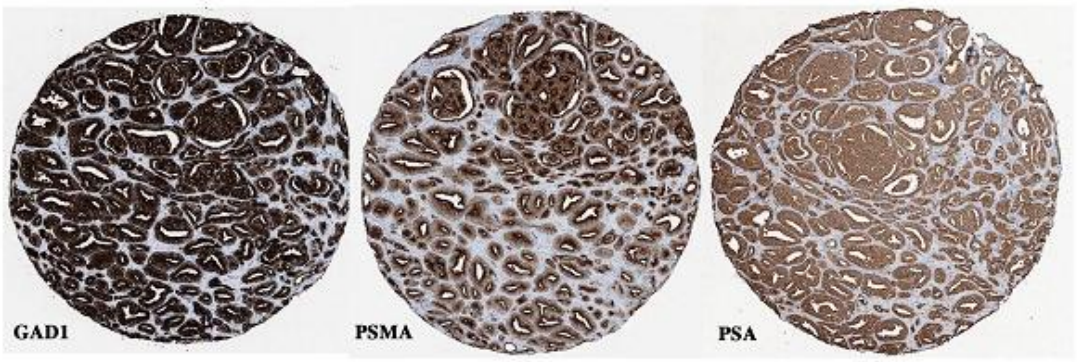
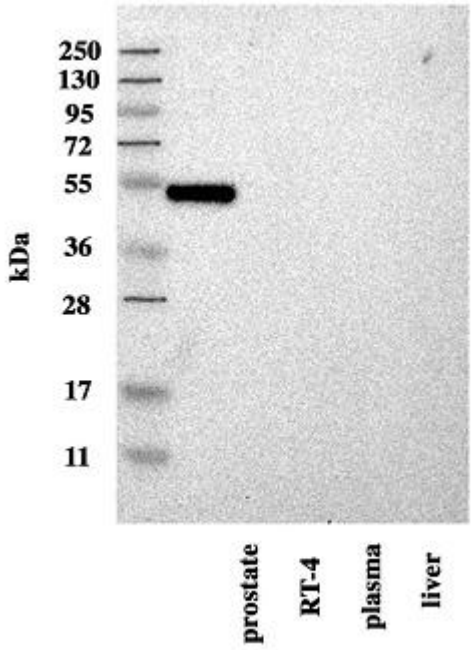


***Table 6**: Sensitivity and specificity of GAD1, PSMA and PSA according to IRP cut-off*
(sens = sensitivity, spec = specificity)

	GAD1		PSMA		PSA	
Cut-off	Sens	Spec	Sens	Spec	Sens	Spec
1	100.0	94.1	100.0	64.5	100.0	81.3
2	97.7	100.0	85.7	71.0	100.0	90.6
3	95.3	100.0	66.7	83.9	100.0	100.0
4	88.4	100.0	59.7	90.3	97.7	100.0

Figure 14: *Western blot on a lysate of normal prostate tissue showed a distinct single band at 47 kDa (first lane) and no band on lysates of RT-4, human plasma and human liver (right lanes)*



3.5.2 Discussion

In cases of advanced malignancies of unknown origin, tissue-specific antigens are of great importance for verifying diagnosis. Two of the most acknowledged tissue-specific markers for PC are PSA and PSMA [43,161]. However, there is still need of prostate-specific tissue markers in cases where PSA, PSMA and other stains show ambiguous results.

Interestingly, GAD1 is associated with the regulation of glutamate, similar to PSMA [40]. No association to cancer or prostate tissue has previously been described. In our IHC analysis, GAD1 showed a high specificity and sensitivity to prostate tissue. The expression of GAD1 was strong in both malignant and benign prostate tissue, while the expression in malignant and benign from the urinary bladder, rectum and lung was almost entirely negative. These results are clinically relevant, as these tumors are among the differential diagnoses to PC in cases with secondary cancer of unknown primary origin. Specificity and sensitivity was well comparable to PSA and slightly higher than PSMA. In contrast to the IHC analysis, RT-PCR indicated that mRNA for GAD1 was expressed in control tissue from lung, bladder and rectum. No mRNA expression of PSA and PSMA was seen in the control tissue.

It is well known that results from IHC and RT-PCR are not always concordant [40,41]. One plausible explanation is that mRNA is not translated into the same protein isoform in all tissues. GAD has three known isoforms in humans: GAD1, 25 and 65 (67, 25 and 65 kDa), all with slightly different expression profiles [29,40]. In our study, the detected mRNA in the control tissue may correspond to two or more of these isoforms while the antibody used for IHC only stains one of the isoforms, most likely GAD1, explaining the differences in results between IHC and RT-PCR. Further analysis of the GAD1 protein is necessary to clarify the role of this protein in prostate tissue and verify the data presented here.

3.6 REPRODUCIBILITY OF EVALUATION OF IMMUNOREACTIVITY

3.6.1 Results

Mean weighted kappa for intraobserver agreement of intensity, extent and IRP were 0.85 (range 0.81 – 0.89), 0.43 (range 0.38 – 0.51) and 0.83 (range 0.73-0.89), respectively (**Table 7**). Mean weighted kappa for interobserver agreement of intensity, extent and IRP were 0.80 (range 0.77 – 0.84), 0.21 (range 0.11 – 0.26) and 0.79 (range 0.67 – 0.88), respectively (**Table 8**). Pathologists and non-pathologists had similar intra- and interobserver agreement. The correlation coefficients between observers for evaluation of intensity, extent and IRP were 0.75-0.81, 0.17-0.48 and 0.72-0.82, respectively, in evaluation 1 and 0.77-0.86, 0.30-0.43 and 0.72-0.86, respectively, in evaluation 2. The subjective estimation of intensity by each observer correlated with results of automated image analysis ($r = 0.61 – 0.66$, $p < 0.001$). The results of the two automated measurements were not identical but correlated strongly with each other ($r = 1.0$, $p < 0.001$). The subjective estimation of the staining intensity correlated with the Gleason pattern of the core for 3 observers ($r = 0.31 – 0.39$, $p = 0.015 – 0.028$), but not for 1 of the non-pathologists ($r = 0.19$, $p = 0.19$). The automated analysis showed no correlation with Gleason pattern ($r = -0.001$, $p = 0.99$).

The pathologists needed less than half the time of that required by the non-pathologists to perform the first evaluation ($p = 0.001$). In the second evaluation, the non-pathologists improved their speed considerably and there was no significant difference in time needed ($p = 0.32$).

***Table 7**: Intraobserver agreement (weighted kappa) of the 4 observers.*
P = pathologists, NP = non-pathologists, IRP = immunoreactivity product

Observer	Training	Intensity	Extent	IRP
1	P	0.874	0.437	0.889
2	P	0.810	0.383	0.730
3	NP	0.828	0.506	0.872
4	NP	0.891	0.396	0.829
Average		0.851	0.431	0.830

***Table 8**: Interobserver agreement (weighted kappa) between the 4 observers.*
IRP = immunoreactivity product

Observer	Intensity	Extent	IRP
1 vs. 2	0.807	0.245	0.794
1 vs. 3	0.799	0.255	0.882
1 vs. 4	0.842	0.206	0.802
2 vs. 3	0.771	0.216	0.770
2 vs. 4	0.817	0.109	0.671
3 vs. 4	0.786	0.250	0.817
Average	0.804	0.214	0.789

3.6.2 Discussion

There is no gold standard for IHC annotation and perhaps there never will be, as each antibody must be considered individually. In this study, evaluation of staining intensity reached a strong inter- and intraobserver agreement. This indicates that subjective estimation of the staining intensity may be sufficient for reliable and robust measurement of IHC in TMA. By contrast, extent of staining showed a poor inter- and intraobserver agreement. This raises serious concern about extent as a reproducible measure in TMA assessment. There was no difference in the reproducibility achieved by trained pathologists and non-pathologists, indicating that IHC assessments can be performed with a high accuracy early in pathology training. Junior observers seem to compensate the lack of experience with more careful observation, as more time was required.

Objective assessment by image analysis is not necessarily better than subjective estimation and a standardization of this system is necessary to stabilize the analysis in the future. Whether subjective or automated measurements are the best predictors of patient outcome is not possible to evaluate based on the current series of 50 patients.

Although one scale of evaluation is likely not desirable or possible, we want to emphasize the importance of standardized scoring systems, in order to improve reproducibility within and between research groups.

3.7 CONCLUSION

Formalin injection as an addition to standard fixation procedures for RP specimens did not affect tissue shrinkage and, hence, did not have any effect on cancer volume calculations.

The volume correction factor for prostatic specimens after formalin fixation was 1.15.

Formalin injection did not affect immunoreactivity for the majority of antibodies studied.

The expression of HSP 27 and HSP 60, but not of HSP 70, significantly predicted biochemical recurrence of prostate cancer in univariate Cox analysis.

In multivariate analysis, HSP 60 expression remained an independent predictor of biochemical recurrence when extraprostatic extension, positive surgical margins, seminal vesicle invasion and GS were included in the model as explanatory variables.

PDX-1 was overexpressed in cancer vs. benign tissue, but no correlation to prognosis was found.

The expression profile of PDX-1 indicated an association to early carcinogenesis in prostate cancer.

GAD1 was expressed in benign and malignant prostatic tissue and may serve as a prostate-specific tissue biomarker.

A correlation of GAD1 expression to GS indicated that the protein may be of interest in prognostic studies.

Subjective assessment of intensity of immunoreactivity was highly reproducible while estimation of staining extent was less reliable.

The level of pathology training was not crucial for obtaining reproducible results in TMA evaluations.

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