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DUCTAL ADENOCARCINOMA OF THE PROSTATE

A MORPHOLOGICAL, IMMUNOHISTOCHEMICAL AND GENETIC STUDY

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Cover photo: Ductal adenocarcinoma of the prostate, hematoxylin and eosin, 20x lens magnification. The case with the most striking features in the series according to the author.
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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Till mamma och pappa

"Success isn't a result of spontaneous combustion. You must set yourself on fire"
—Arnold H Glasgow

ABSTRACT

Ductal adenocarcinoma of the prostate (DAC) is the second most common prostate cancer subtype. Patients with DAC often present at an advanced clinicopathological stage, with or without metastases. Patients have a high rate of extraprostatic extension, seminal vesicle invasion, a shortened time to biochemical recurrence as well as regional and distant metastases. The diagnosis of DAC continues to challenge pathologists half a century after its discovery. Histologically, DAC is an adenocarcinoma with papillary, cribriform, glandular or mixed architecture. The epithelium is tall, columnar, pseudostratified and the nuclei elongated with high-grade features. Our aim was to characterize the entity of DAC by morphology, immunohistochemistry and genetic analyses to provide better understanding and tools for diagnosis.

The prognostic significance of histopathological features of DAC were analyzed. Classic DAC shows tall, columnar, pseudostratified epithelium, elongated nuclei and papillary, glandular and/or cribriform architecture. The tumors may lack elongated nuclei or classical architecture and still have similar prognosis, which justifies their classification as DAC. However, cases with only stratified, high grade nuclei had better prognosis and should not be considered DAC. The reproducibility of the DAC diagnosis was evaluated among international experts on prostate pathology. The most useful diagnostic feature of DAC was papillary architecture while nuclear and cellular features were less important. The most common differential diagnoses to DAC included intraductal prostate cancer, acinar adenocarcinoma and high-grade PIN. The immunohistochemical profile of DAC showed overlap with staining patterns of acinar adenocarcinomas, but differences consistent with the more aggressive phenotype of DAC were noted, such as a higher expression of Ki-67, p53 and p16. The immunohistochemical profile of DAC was also compared with that of adenocarcinomas of non-prostatic origin. To diagnose the site of origin of metastases may be challenging as DAC resembles some other adenocarcinomas morphologically. The expression of cytokeratins and intestinal markers in DAC were not specific and may lead to diagnostic errors if not combined with prostate specific markers. In men with adenocarcinoma of unknown primary, the threshold for applying prostate specific immunomarkers should be low even when the morphology suggests a non-prostatic origin. The genetic profile of DAC was analyzed by sequencing and was found to be similar to that of advanced and/or metastatic acinar adenocarcinoma of the prostate. This can explain the aggressive behavior of this prostate cancer subtype and may also offer targets for future tailored therapies.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Prostatacancer är den vanligaste cancerformen i Sverige, under 2011 diagnostiserades 9 663 män i Sverige med sjukdomen. I dagsläget har cirka 20 olika typer av prostatacancer beskrivits. Den vanligaste varianten kallas för acinärt adenocarcinom och står för dryga 90 % av alla fall, medan den näst vanligaste varianten kallas för duktalt adenocarcinom och beskrevs för första gången på 1950-talet. När man tittar på tumörerna i mikroskop skiljer sig den duktala cancern jämfört med den acinära. Duktal cancer har har avlånga celler som ser ut att vara ordnade i flera lager, arkitekturen är oftast papillär eller kribriform men man kan se en blandning av båda dessa typer. Patienter som drabbas av duktalt adenocarcinom har en sämre prognos än de som drabbas av acinärt adenocarcinom men man har inte kunnat förklara varför. Trots att prostatan ofta opereras bort hos dessa patienter har man sett att duktal cancer ofta växer utanför prostatan, att all tumörvävnad inte är borttagen vid operationen och att de ofta har spridning av tumören till andra vävnader i kroppen. Vårt mål var att kartlägga duktal cancer och definiera diagnoskriterier, kartlägga dess mikroskopiska utseende och jämföra såväl proteinuttryck som genetiska skillnader mellan duktalt och acinärt adenocarcinom.

Vi kom fram till att den duktala cancern oftast växer tillsammans med en acinär cancer som i de flesta fall är elakartad. Tumören återfinns oftast i de perifera delarna av prostatan och hos de patienter där man opererar bort prostatan ser man i större utsträckning att tumören växer utanför prostatan, in i sädesblåsorna och att det ofta finns cancerceller kvar i området efter operationen. Det drag som är mest användbart för att ställa diagnosen duktal cancer är den papillära arkitekturen. Brist på typisk arkitektur är oftast anledningen till att patologer inte ställer diagnosen. Det finns flera olika typer av prostatacancer som kan misstas för duktalt adenocarcinom.

När vi undersökte proteinuttrycket visade det sig att duktal cancer oftare uttrycker proteiner som talar för en aggressiv cancer. Duktal cancer kan likna cancer från andra organ såsom bukspottkörtel, lunga, magsäck, tjocktarm eller urinblåsa. Detta gör att det kan vara svårt att avgöra om cancerceller man finner i andra delar av kroppen, så kallade metastaser, kommer från prostatan eller något annat organ. För att säkerställa att det rör sig om ett duktalt adenocarcinom bör man därför titta på proteiner som är specifika för prostatan då detta ökar chansen att ställa rätt diagnos. På DNA-nivå kunde vi se att duktalt adenocarcinom uppvisar förändringar som förekommer i avancerad och/eller metastaserad prostatacancer. Sammanfattningsvis är duktal cancer en aggressiv subtyp med förändringar i såväl proteinuttryck som på gennivå vilka kan förklara dess aggressiva beteende.

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I **Seipel AH**, Wiklund F, Wiklund NP, Egevad L.
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II **Seipel AH,** Delahunt B, Samaratunga H, Amin M, Barton J, Berney DM, Billis A, Cheng L, Compérat E, Evans A, Fine SW, Grignon D, Humphrey PA, Magi-Galluzzi C, Montironi R, Sesterhenn I, Srigley JR, Trpkov K, van der Kwast T, Varma M, Zhou M, Ahmad A, Moss S, Egevad L.
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V **Seipel AH**, Whitington T, Delahunt B, Samaratunga H, Wiklund P, Grönberg H, Lindberg J, Egevad L.
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Ductal adenocarcinoma of the prostate: histogenesis, biology and clinicopathological features
Pathology. August 2016, volume 48 (5), pages 398-405

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AMACR	Alpha-methylacyl-coenzyme A racemase
AR	Androgen receptor
CEA	Carcinoembryonic antigen
DAC	Ductal adenocarcinoma of the prostate
EGFR	Epithelial growth factor receptor
EPE	Extra-prostatic extension
GS	Gleason score
HGPIN	High-grade prostatic intraepithelial neoplasia
HMWCK	High molecular weight cytokeratin
IDC-P	Intraductal carcinoma of the prostate
IHC	Immunohistochemistry
ISUP	International Society of Urologic Pathology
PSA	Prostate-specific antigen
PSAP	Prostate-specific acid phosphatase
s-PSA	Serum prostate-specific antigen
PZ	Peripheral zone of the prostate
RP	Radical prostatectomy
SVI	Seminal vesicle invasion
TMA	Tissue microarray
TUR-P	Transurethral resection of the prostate

1 INTRODUCTION

1.1 PROSTATE CANCER IN SWEDEN

"Declare the past, diagnose the present, foretell the future"

—Hippocrates

Prostate cancer is the most common cancer in Sweden where it accounts for one third of all cancers in men. In 2011, 9 663 men were diagnosed with prostate cancer and together they accounted for nearly a third (32.2 %) of all newly diagnosed cancers. Uncommon before 50 years of age, only 85 men under the age of 50 were diagnosed with the disease in 2011. The 5-year survival is 91.6 % and a majority of patients eventually die of unrelated causes such as acute myocardial infarction or stroke. Nevertheless, prostate cancer is a heterogeneous disease with some patients living with indolent disease for decades while others present with metastases and a short overall survival (1).

The diagnosis is based on analysis of serum prostate-specific antigen (s-PSA), digital rectal examination and histopathologic examination of core biopsies acquired through transrectal ultrasound. In some patients magnetic resonance imaging (MRI) is performed. s-PSA levels may be elevated due to other causes than prostate cancer such as a urinary infection, prostatitis or benign prostatic hyperplasia. There is no national s-PSA screening program in Sweden but many men ask for the test at primary health care centers and s-PSA testing has increased during the last decade. However, the false-positive rate of s-PSA is high, which leads to unnecessary prostate biopsies and ultimately over-diagnosis of low-risk prostate cancers. Nevertheless, efforts are being made to improve the sensitivity and specificity of the diagnosis of prostate cancer. A recent study described a new model to reduce unnecessary biopsies without diagnosing less prostate cancers with a Gleason score (GS) of at least 7. This was achieved by combining plasma protein biomarkers and genetic polymorphisms. Clinical variables included age, family history, previous prostate biopsy and/or exam. s-PSA concentration was tested in all participants (2). Hence, in the near future, evaluation of a combination of variables may be the gold standard in daily clinical work.

Approximately 50 % of the patients diagnosed with prostate cancer are treated with radiation therapy, surgery or a combination. Radiation therapy may also be combined with hormonal treatment to reduce tumor burden prior to treatment. Patients with tumors of low GS may be subjected to active surveillance, where s-PSA and core biopsies are analyzed at certain time

intervals. Patients with metastasized disease at time of diagnosis may be subjected to surgical or chemical castration as well as chemotherapy. An estimated 25 % of patients suffer biochemical recurrence, i.e. a rising s-PSA after surgical treatment or radiation therapy (1).

1.2 PROSTATE CANCER GLOBALLY

An autopsy study by Franks in 1954 (3) revealed prostate cancer to be more common than previously thought. Franks identified 69 prostate carcinomas at autopsies of 220 patients, none of the cancers had been diagnosed before death and in none of the men were they the cause of death. Franks also demonstrated that prostate cancer is correlated to age: in the age group 60-79 years prostate cancer was found in approximately one third of patients, in the age group 80-89 years in almost 50 %, 90-99 years 75 % and in patients over 100 years old, all patients had prostate cancer. One could assume that these were all indolent, well-defined tumors, however, 20 % showed large, anaplastic areas.

Prostate cancer is the second most common cancer among men globally and the fourth most common cancer in both sexes combined (4). One point one million men were diagnosed with prostate cancer in 2012, accounting for 15 % of all cancers diagnosed in men. Of these, 70 % occurred in countries such as Australia, New Zealand and North America as well as Western and Northern parts of Europe where the incidence is high *Figure 1*. The incidence is high also in less developed regions including the Caribbean, Southern parts of Africa and South America while it is less common in Asia. In 2012, 307 000 men died of their prostate cancer globally, making it the fifth most common cause of cancer related death. The mortality rates are highest in black populations (4). The high incidence of prostate cancer is partly because s-PSA testing and core biopsies are common diagnostic tools in developed regions. The use of s-PSA dates back 30 years in high-income countries and has led to a higher incidence of prostate cancer as the detection rate increases. In 2001, 75 % of American males over 50 years of age had been subjected to s-PSA testing (5). In summary, the incidence of prostate cancer is largely dependent on the efforts to detect the disease. Some risk factors have been identified and include age, black race, family history of prostate cancer and certain genetic traits (6).

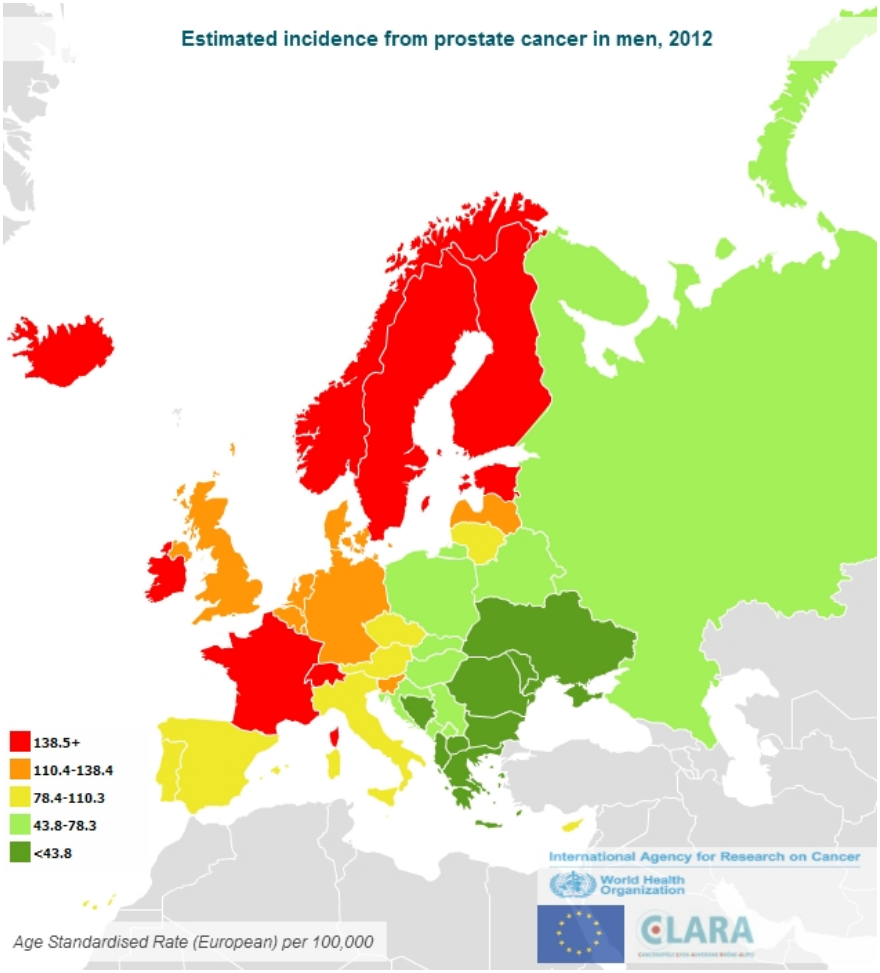


Figure 1 The incidence of prostate cancer in Europe. As can be seen, the incidence is high in the Nordic countries. Reprinted from http://eco.iarc.fr/eucan

1.3 PERSONALIZED PROSTATE CANCER MEDICINE?

"It is more important to know what sort of person has a disease than to know what sort of disease a person has" —*Hippocrates*

It is known that there are several molecular subtypes of cancer and that we are entering an era where treatment for each individual patient may be based on the unique tumor signature and not only tissue origin or anatomical site. A normal cell develops into a cancer cell by accumulating genetic changes, which may be acquired sporadically or through inheritance. Inherited genetic changes allow clinicians to identify patients at risk of developing a certain cancer type and provide treatment and recommend life style changes (7). For example, the BRCA1 and BRCA2 genes can be identified and patients subjected to early treatment to prevent death from breast cancer. Prostate cancer is the most common cancer among men in the Western world but better predictors of individual treatment response are needed to provide customized treatment. There is currently a risk of over treatment of patients with

indolent and low-risk disease where patients are put at unnecessary risk of post-radiation or post-surgical complications.

Prostate cancer treatment today is generally not based on the histological subtype or genetic signature but the TNM system where T-category, s-PSA and GS stratifies the patients into low, intermediate and high risk groups, predicting the risk of biochemical recurrence (8) *Figure 2*. For patients with low-risk disease, active surveillance may be sufficient. However, the patient may wish to be treated with radiation therapy or radical prostatectomy (RP), treatment decision thus being a combination of tumor biology and the patient's wish. If histological review shows positive surgical margins or extraprostatic extension (EPE), patients may benefit from radiotherapy (9). Those with high-risk prostate cancer benefit from RP or radiation therapy followed by androgen deprivation therapy. Despite treatment some patients develop metastatic disease and palliative disease is then managed by chemotherapy, androgen deprivation, radiation or secondary hormonal treatment. There are currently no tests to tell who will benefit from single or combination therapy. The lack of predictors of treatment outcome and prognosis makes personalized prostate cancer medicine challenging. Multifocality and interpatient as well as intrapatient heterogeneity in prostate cancer biopsies contribute to potential sampling bias. Despite the prognostic factors s-PSA, GS and T-stage, we cannot estimate a patients' risk of developing aggressive disease and a deeper understanding of the tumor heterogeneity is needed before we can offer personalized treatment. Prostate cancer is a heterogeneous disease and several foci are typically seen in one patient together with several clonal subpopulations (10). Aggressive prostate cancers seeding metastases early or harboring local resistance to therapy would benefit from early detection such as genetic signatures predicting which patients to select for aggressive and immediate treatment. Presence of subtypes such as ductal adenocarcinoma of the prostate (DAC) may affect clinical outcome. It is already known that the subtype intraductal prostate cancer (IDC-P) is a risk factor for aggressive disease (11).

Although GS allows for the identification and grading of separate foci, further subgrouping by genomic alterations could be of benefit in clinical practice, such as TMPRSS2ERG gene fusions, c-myc, PTEN and/or NKX3.1 status (12, 13). Genetic signatures in core needle or liquid biopsies may tell us if the patient will develop aggressive disease, before we send our patient to the operating theater or radiation therapy. In RP specimens with several tumor foci we must also be sure of which clone decides the outcome and why. Recent studies have aimed to evaluate expression signatures with estimated overall survival time and results could

hopefully be used in clinical diagnostics for optimal treatment decisions based on the survival benefit (14).

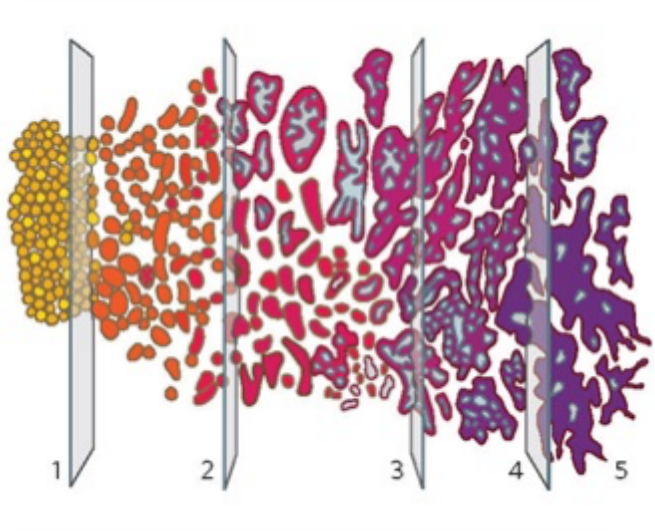


Figure 2 The Gleason grading scale from least aggressive (1) to most aggressive (5). Adapted and reprinted with permission from *The Prostate Cancer Foundation Australia*

1.4 HISTOLOGICAL SUBTYPES

Today, 9 out of 10 patients are diagnosed with acinar adenocarcinoma while DAC is the second most common subtype. Other histological variants can be grouped into acinar and non-acinar carcinoma variants. Variants of acinar adenocarcinoma include atrophic, foamy, signet-ring, colloid, oncocytic, pseudohyperplastic and lymphoepithelioma-like adenocarcinomas. Non-acinar carcinomas include DAC, basal cell carcinoma, neuroendocrine tumors, squamous and adenosquamous adenocarcinoma, urothelial carcinoma and sarcomatoid carcinoma. Since the WHO 2004 classification several subtypes have emerged, such as pleomorphic giant cell carcinoma, large-cell neuroendocrine carcinoma, microcystic carcinoma and prostatic intraepithelial neoplasia-like adenocarcinoma (15). Some of them are extremely uncommon and rarely encountered in daily clinical work while others, such as DAC, are more common. The subtypes differ in clinical course as well as in histopathologic features and may be assigned different GS, hence correct identification and classification is important for patient management and outcome.

1.5 THE STORY OF DAC

1.5.1 The first case

A 66-year-old man of previously good health is admitted to Parkway Hospital, New York in March 1966 due to a delay in onset of urination, nocturia and dribbling. Physical examination shows no abnormalities and the digital rectal examination reveals a firm but not nodular

prostate. The clinical impression is benign prostatic hyperplasia. Dr George Golding performs a suprapubic prostatectomy on March 8 1966, but what was thought to be a benign hyperplastic prostate turns out to be a prostate malignancy never seen before. The microacini are not arranged in a back-to-back fashion, in cribriform or solid areas but instead masses of columnar cells are seen, with the greatest concentration near the urethra. This is currently viewed as the first description of what would later be known as ductal adenocarcinoma of the prostate (16).

DAC is known to have an adverse prognosis with shortened time to biochemical recurrence, increased mortality rate, short term failure rate after radical prostatectomy, positive surgical margins, EPE, seminal vesicle invasion (SVI) and positive pelvic lymph nodes (17-19). At the time of its discovery, DAC was thought to stem from a Müllerian duct remnant, hence a female tissue origin, due to its resemblance to endometrial adenocarcinoma (16). Although 50 years have passed since the first description there are still controversies regarding diagnosis, patient management and outcome.

1.6 EARLY DESCRIPTIONS OF DAC

Ever since the first description DAC has been a source of conflict and debate. The tumor type has been referred to as endometrial carcinoma of the prostatic utricle, endometrial carcinoma of the prostate, adenocarcinoma with endometrioid features, primary prostatic duct adenocarcinoma as well as papillary carcinoma of the prostate, but is today commonly referred to as ductal adenocarcinoma of the prostate.

The patient described by Melicow and Pachter is commonly considered to be the first reported patient with DAC (16). The microscopic appearance with a large volume of columnar cells arranged in bands with a pseudopapillary architecture led the authors to believe that the malignancy stemmed from the “uterus masculinus”. The rationale behind this was that the malignant cells were concentrated around the verumontanum, which is a structure in the dorsal wall of the urethra forming a conical elevation that protrudes into the urethral lumen. Located at the apex is a structure known as the utricle, described 400 years ago (20) *Figure 3*. This was earlier viewed as a Müllerian duct remnant, hence a tissue of female origin. It was even viewed by some as the male correspondence to the female vagina, hence the term “vagina masculina”. The thought that some of these female glands would persist and become neoplastically changed did not seem inconceivable (21).

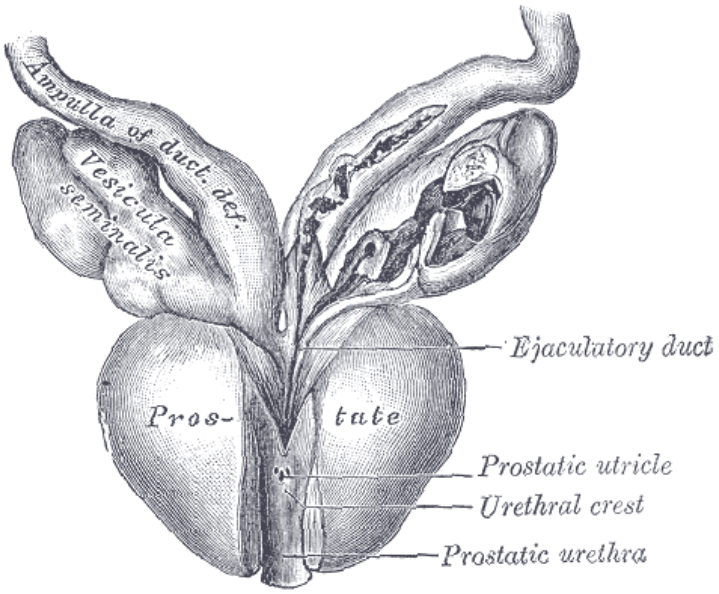


Figure 3 The supposed origin of ductal adenocarcinoma of the prostate, the prostatic utricle. Reprinted from *Anatomy of the Human Body, Henry Gray, 1918*

The Müllerian origin was to great extent based on an article by Glenister whom in 1962 described the prostatic utricle arising at the site of merging of the urogenital sinus, mesonephric and paramesonephric ducts on the Müllerian tubercle, which later becomes the colliculus seminalis (22). The epithelium lining the cranial portion of the utricle is derived from the paramesonephric ducts while the caudal portion from the mixed epithelium, namely endodermal urogenital sinus cells, mesodermal Wolffian cells and the paramesonephric Müllerian cells, which also covers the colliculus seminalis or the verumontanum (22). An earlier study by Zondek *et al*. (23) also confirmed the utricle to be formed from the fused caudal ends of the Müllerian ducts, hence a supposed female tissue rest.

More recent studies, however, support a resemblance to prostatic tissue derived from the urogenital sinus and not the Müllerian derived endometrial tissue (24). Wernert *et al*. (25) were the first to perform an immunohistochemical investigation of the utricle. They demonstrated a prostate-specific antigen (PSA) and prostate-specific acid phosphatase (PSAP) positivity of the utricle from the 5th month of life, as well as keratins, carcinoembryonic antigen (CEA) and peanut agglutinin. The positivity was similar to that of normal prostate glands, suggesting that the epithelium of the urogenital sinus participated in the lining of the utricle during embryogenesis. The debate was settled in 2004 when Shapiro *et al.* (26) proved the utricle to have its origin in the urogenital sinus. The authors used immunohistochemical biomarkers p63, uroplakins, vimentin, PAX-2 and Ki-67 to show that

male fetuses demonstrate a weak expression of p63 in the basal layer of the urogenital sinus around 9 weeks of gestation. At 11 weeks of gestation, the Müllerian ducts express PAX-2 and p63 to some extent. Meanwhile, the p63 staining of the urogenital sinus increases. By 14-15 weeks of gestation, the Müllerian ducts are undergoing apoptosis and uroplakin-expressing epithelium grows into the periurethral stroma, forming a sheet of p63 positive cells beneath the urogenital sinus, which is also Ki-67 positive. As for the remaining Müllerian duct epithelium, it is at the time p63 negative, vimentin and PAX-2 positive. By 17 weeks of gestation, the sheet of p63 positive cells is elongating and forms the utricle, which is PAX-2 and vimentin negative. Hence, the utricle is formed by an ingrowth of cells from the dorsal wall of the urogenital sinus while the Müllerian ducts regress.

Melicow published a second article in the early 1970s describing six cases of endometrial carcinoma of the prostatic utricle (27). All cases showed histopathological features similar to those described in the first article and none of the patients received hormonal therapy nor orchiectomy due to the possible adverse effects on a tumor of Müllerian origin. Instead, they were treated with transurethral resection of the prostate (TUR-P), RP or radiotherapy. During the time that followed, several cases were described, with features such as shaggy-appearing tissue close to the verumontanum, elongated glandular structures arranged in a back-to-back fashion and areas of papillary architecture complete with true fibrovascular stalks (28). Nuclei were described as oval with a nucleolus. Mitotic figures could be seen and the luminal aspect of the cytoplasm sometimes showed a frayed appearance with tongues of cytoplasm projecting.

Carney and Kelalis (28) discussed the similar appearance of Melicow and Pachter's endometrioid adenocarcinoma to an already known entity called ductal adenocarcinoma, both demonstrating tall, columnar epithelium and a papillary architecture. The most convincing feature for the presumed utricular origin was thought to be ciliated tumor cells, which can be seen in endometrial adenocarcinoma but never in prostate cancer. However, these were later confirmed to be microvilli and not ciliae (29).

1.7 TWO TUMOR TYPES -ONE DIAGNOSIS?

Reviewing the literature around the time of the discovery of DAC reveals two similar tumor types; one originating from the prostatic utricle, described as endometrial carcinoma, and a second group of tumors arising from the prostatic ducts referred to as ductal adenocarcinoma. But were they actually members of the same group? Shortly before Melicow and Pachter's discovery, several groups wrote of a tumor type arising from the prostatic ducts, the earliest

description by Foot *et al*. in 1950 (30). The histopathologic features were described as tall, columnar epithelium, papillary or cribriform growth, elongated, crowded, hyperchromatic nuclei and abundant cytoplasm (31-33). Tumors of the primary prostatic ducts were rarely reported at the time (34).

A study on prostatic adenocarcinomas of ductal origin was published in 1973 describing 55 cases of ductal type. The cases were divided in two subgroups, adenocarcinomas of the primary prostatic ducts and the secondary prostatic ducts (32). Adenocarcinomas of the primary ducts were described as composed of papillary fronds with fibrovascular cores, the epithelium consisted of a single layer of tall, columnar epithelium with elongated nuclei and pale eosinophilic cytoplasm. Adenocarcinomas of the secondary prostatic ducts were described as consisting of tall, columnar epithelium, with eosinophilic or clear cytoplasm, containing a large, pleomorphic hyperchromatic nucleus. A papillary pattern was observed but also areas of cellular outfoldings bridging the cell lumen, creating a papillary-cribriform architectural pattern. The lumen was sometimes filled with eosinophilic-like debris, such as comedonecrosis, which has later been described in a majority of cases by Christensen *et al*. (17). As many as 95 % of patients had obstructive symptoms.

Despite the similarities to the endometrial adenocarcinoma of the prostatic utricle described by Melicow *et al*. (16), the authors claimed that the primary duct carcinomas should be separated from this entity due to more glandular features in the tumor described by Melicow compared to the papillary architecture described by Dube *et al*. (32). Moreover, the endometrial adenocarcinomas did not show osteoblastic metastases nor an elevation in serum acid phosphatase as did the primary duct carcinomas. Also, Dube *et al*. (32) described immunohistochemical expression confirming a prostatic origin, with positive acid phosphatase reactions and negative aminopeptidase expression.

Bostwick *et al*. (35) later suggested that since endometrioid carcinomas and primary or large duct adenocarcinomas shared similar clinicopathological features these should be grouped together and named prostatic adenocarcinomas with endometrioid features. Due to microscopic, ultrastructural and immunohistochemical findings, the term endometrial adenocarcinoma was later discarded and the tumors were regarded as adenocarcinomas arising from primary prostatic ducts. Due to the similar clinicopathological features of endometrioid carcinomas and primary or large duct adenocarcinomas, a distinction between the two types did not seem justified but these were grouped together and treated like conventional prostate cancers (25).

Given this information, it is possible that Melicow and Pachter were actually not the first to describe what would later be known as DAC, but merely one of several groups at the time describing the same tumor type. Hence, the first description of what would later be known as DAC may have been that of Foot *et al*. (30).

1.8 CLINICAL FEATURES

Studies show that patients diagnosed with DAC are slightly older than those with acinar adenocarcinoma, the mean age at time of diagnosis ranging from 60-80 years (17, 19, 24, 36, 37). The urologist may see a patient referred because of macroscopic or microscopic hematuria, urinary obstruction, diminished urinary stream or urinary retention (38-41). Less common symptoms are anal pain (42) and hemospermia, the latter due to tumor fragmentation into the urethra (43). The symptoms described are also seen in patients with acinar adenocarcinoma but they are more common in patients with DAC due to the sometimes periurethral location (37). Investigation with cystoscopy may show a villous lesion protruding from the urethra (24, 33, 42, 44-47). Due to the peripheral as well as periurethral location, digital rectal examination may be normal but could also reveal a palpable nodularity or induration (17, 19, 35). The patients often present with a clinical stage of T2b or higher, classifying the tumors as advanced even at the time of discovery. The s-PSA range is wide and does not predict findings at RP. Even in metastatic disease, s-PSA may be widely scattered and has been reported at 0.3-698 ng/mL with a median of 26 ng/mL, not correlating with neither site nor size of the metastasis. Authors have described s-PSA as both lower and higher than in patients with acinar adenocarcinomas and the tumor to be less likely to be detected by s-PSA screening (17, 48). The conclusion is that s-PSA cannot be reliably used to risk stratify patients and that s-PSA does not seem to correlate with findings at RP (17). Of interest is that many patients are diagnosed with DAC in RP or TUR-P specimens rather than the initial core biopsy of the prostate (49). Lee *et al*. (50) noted that five of six patients with ductal adenocarcinoma had blood group O, but this has not been further studied.

1.9 COEXISTENCE WITH ACINAR ADENOCARCINOMA

DAC is often seen in combination with an acinar adenocarcinoma and is rare in its pure form. Tannenbaum reviewed the pathological files at Columbia-Presbyterian center in 1975 and concluded the prevalence of pure DAC to be a mere 0.2 %, the majority being associated with an acinar adenocarcinoma (51). Other authors have reported 0.4 % (50) and 0.8 % (35), however, it was not specified whether these were pure DAC or mixed tumors. Dube separated pure DACs (1.3 %) from mixed (6.3 %) (32). The acinar component is often

advanced with a GS of 4 or 5 (35). No difference has been observed between pure DAC and mixed cases regarding s-PSA, age, GS, clinical or pathologic stage (49). In summary, DAC is most often found together with an acinar component while pure DACs are extremely rare.

1.10 HISTOPATHOLOGY

Today, a DAC diagnosis is based on morphology alone. DAC is composed of tall, pseudostratified, columnar epithelium with abundant cytoplasm, which is usually amphophilic but may be pale or clear (24, 29, 38) *Figure 4*. Cytoplasmic vacuoles and apical snouting are commonly seen (38, 52). The nuclei are basally located and often show a prominent nucleolus and a clumped chromatin pattern (38, 53). Mitotic figures (51, 52, 54) and luminal necrosis may be seen (35). DAC shows a variety of architectural patterns; papillary, cribriform, solid or glandular. Although several patterns are commonly seen in a specimen one is usually predominant (32, 52). The papillary architecture is characterized by true fibrovascular stalks and the cribriform pattern by back-to-back large glands and intraepithelial bridging, forming slit-like lumina (24, 50) *Figure 5*.

The location of DAC was initially described as central, often involving the verumontanum and the large periurethral prostatic ducts. The peripheral zone (PZ) was not thought to allow papillary formations or complex glands due to limited space and stromal formation (35). Nevertheless, it has since been shown that DAC involves the PZ preferentially (17, 19, 55).

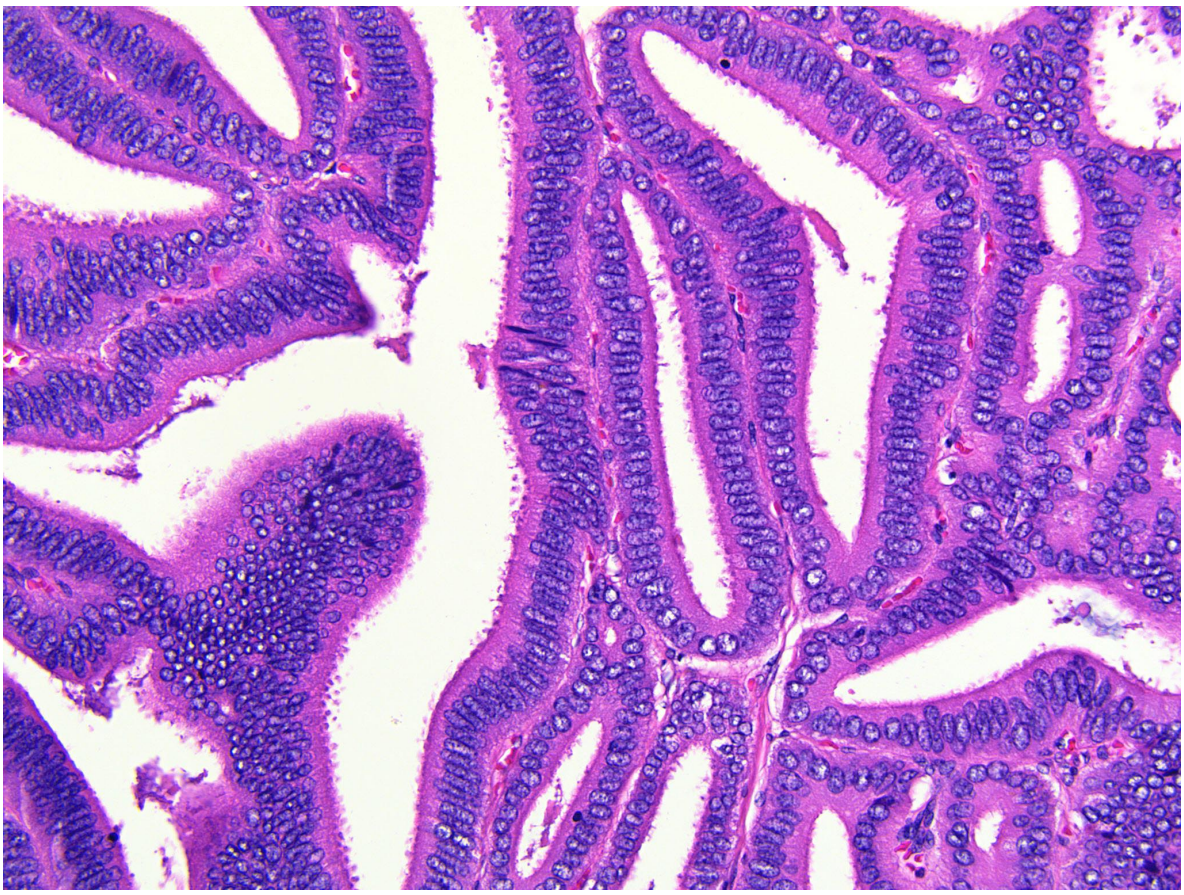


Figure 4 Ductal adenocarcinoma of the prostate (DAC) with typical tall, columnar epithelium and elongated nuclei with high-grade features. Hematoxylin and eosin, 20x lens magnification.

To this day the Gleason grading system remains one of the most powerful predicting factors in prostate cancer (56). In the 2006 upgrade of the Gleason grading system, is was concluded that a pure DAC should be assigned a GS of 4+4=8 unless comedonecrosis is present, which would warrant a GS of 9-10. However, patients with pure DAC have survived for long periods despite a high GS (8-10), which suggests that GS may not be useful a prognostic factor in this group (49).

Electron microscopy shows two types of cells arranged in well-defined glandular structures. Cells are divided in two types: light and dark. The light cells are composed of secretory droplets, lipid filled vacuoles and vesicles, occasional lysosomes and free ribosomes. Dark cells have an electron dense cytoplasm with smooth and rough endoplasmic reticuli, ribosomes, polyribosomes and numerous mitochondria. In both cell types, a well developed Golgi apparatus is found (35, 38, 57). Microvilli are seen on the luminal surface (29). The light cells bear resemblance to a well differentiated prostatic adenocarcinoma while the dark cells are found in less differentiated parts of the tumor (38).

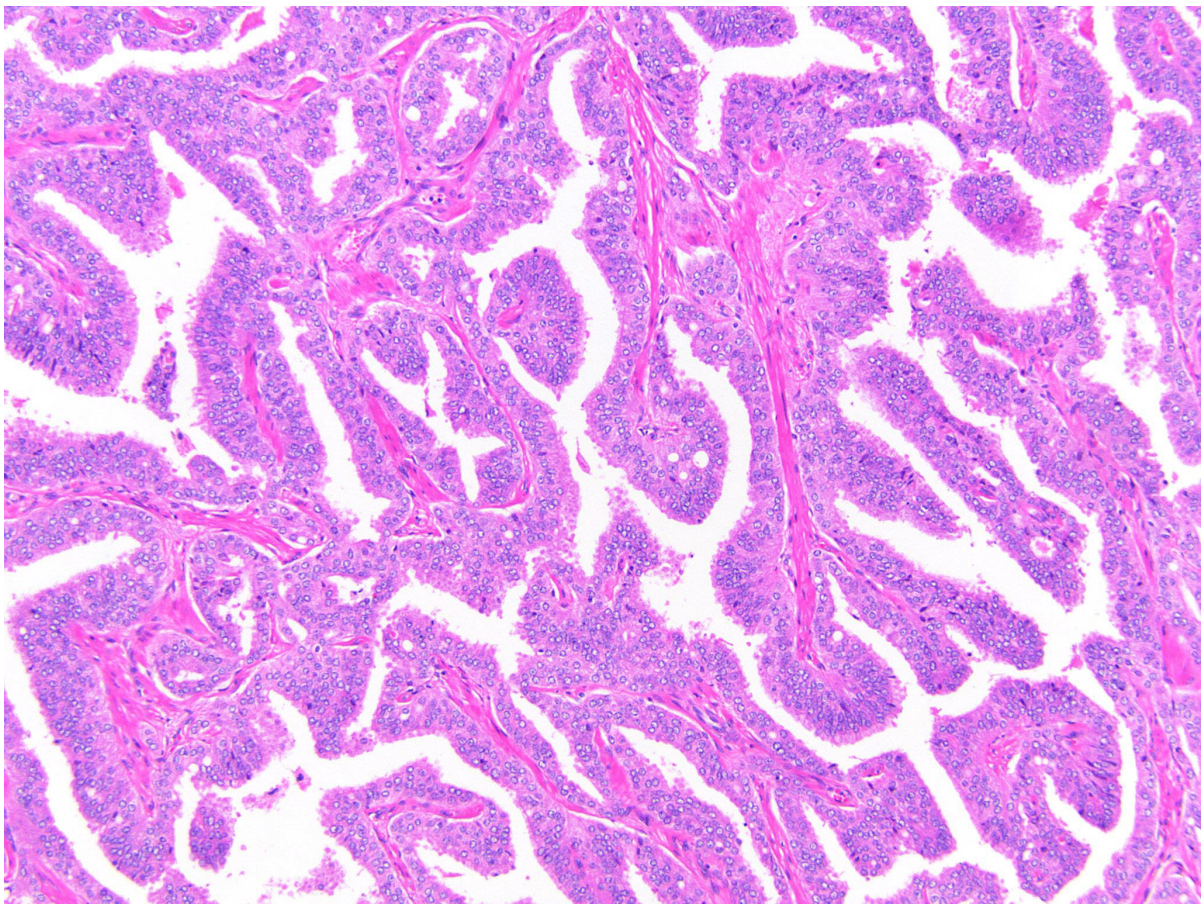


Figure 5 Ductal adenocarcinoma of the prostate (DAC) with tall, columnar, pseudostratified epithelium. Nuclei are elongated with high-grade features and the architecture is papillary with true fibrovascular cores. Hematoxylin and eosin, 10x lens magnification.

1.11 IMMUNOHISTOCHEMISTRY

The immunohistochemical profile of acinar adenocarcinomas is well characterized but for DAC it is not. Conventional acinar adenocarcinomas express PSA and PSAP and are negative for CK7 and CK20 although expression increases with increasing GS. Alpha-methylacyl-coenzyme A racemase (AMACR) is over-expressed and basal cell markers such as p63 and high molecular weight cytokeratin (HMWCK) are negative (58). Although DAC tumors stain positively for PSA and PSAP, which confirms the prostatic origin (24, 44, 47, 52, 59) the staining may be of variable intensity (57). Two completely PSA and PSAP negative DAC tumors have been reported (21, 54), as well as PSA negative metastases (60). Nevertheless, it is also known that PSA and PSAP are less expressed in non-DAC tumors of GS 8 and above (61).

DAC may show a focal CK7 and a patchy CK20 staining (62) and a remnant of basal cells as demonstrated by HMWCK and p63 staining (58). These are not detected in classical adenocarcinomas of the prostate (63).

CDX2 and villin are antibodies used to separate prostate cancer from adenocarcinomas of the gastrointestinal tract as these are negative in acinar prostate cancer. However, both CEA and CDX2 positivity has been demonstrated in DAC (35, 64).

1.12 GENETIC PROFILE

During the last decade efforts have been made to reveal the genomic landscapes of not only prostate cancers but all common forms of human cancers. A small number of genes are altered in most of the tumors while a large number of genes are infrequently altered. A decade ago, sequencing one tumor would set researchers back more than 100 000 USD, however, the prices have dropped and sequencing is now the norm, thus facilitating the understanding of tumor biology and the development towards personalized cancer medicine.

Most tumors show different genetic signatures. This is also seen in the daily clinical work of urologists and oncologists: not two patients follow an identical clinical course. Possible explanations are non-genetic and host factors, including vascular permeability to drugs or germline variants deciding the drug half time (65). Another explanation is somatic mutations in the tumors. A DAC tumor may have several different mutations but if you compare tumors from two different patients they may only share a few mutations, often located in driver genes (66). Nevertheless, even if the mutations occur in the same driver genes, the mutations themselves may differ. As different domains of the protein will be altered, the cellular properties may differ (67).

Frequently mutated genes in prostate cancer include PTEN, TP53, FOXA1 and SPOP (68, 69) while the TMPRSS2-ERG fusion is the most common fusion gene in prostate cancers, occurring in 40-60 % of all cases (70). The gene fusion occurs through a deletion on chromosome 21 or through a translocation, the deletion being more common. TMPRSS2 is an androgen regulated gene while ERG is a member of the ETS transcription family. The fusion results in ERG being brought under the control of an androgen regulated promotor, hence there is an over expression of protein (70). In reports, the fusion has not been found to correlate with prognosis (71). Other genetic alterations include AR amplification and loss of NKX3.1. Together or separately, these alterations account for the development of prostate cancer and for some patients the development to metastatic, castration resistant disease (72, 73).

Although DAC has been known for 50 years and recent advances have been made in the technique of genome sequencing, few studies have investigated the genetic profile of DAC. One study showed a high degree of molecular relatedness to acinar adenocarcinomas but a

lower frequency of the TMPRSS2ERG gene fusion (74). Although the TMPRSS2ERG fusion does occur in DAC, it is less commonly seen than in pure acinar adenocarcinomas. The fusion gene was found in 45 % of acinar adenocarcinomas but only in 11 % of DAC and 5 % of the acinar components of mixed tumors (74, 75). As the expression was low also in the acinar component, this suggests that different genetic events may lead to a mixed ductal-acinar cancer than to a pure acinar adenocarcinoma. It has been hypothesized that as DAC sometimes presents with a low s-PSA, the level of circulating androgens may be lower, which could lead to less frequent rearrangements (75).

AR is a ligand-dependent nuclear transcription factor and the main therapeutic target in prostate cancer. The AR signaling pathway is disrupted in many prostate cancers, often through AR itself but also through its pathway members such as FOXA1, SPOP or NCOR1/2 (76). Together with ETS fusions, SPOP is one of the driver events in tumorigenesis (68). PIK3C may be altered in metastatic, castration resistant prostate cancer through amplifications, fusion and/or hot spot mutations (76). FOXA1, when mutated, represses androgen signaling and promotes tumor growth through promotion of cell cycle progression (77, 78). It is unknown whether any of these genetic changes occur in DAC.

Sanati *et al*. (79) showed that DAC and acinar adenocarcinomas are highly molecularly related. Among the differentially expressed gene transcripts were CD24 and cadherin-like 23, involved in cell adhesion and the prolactin receptor, which was over expressed. Prolactin has been shown to promote ductal morphogenesis and increase the ductal epithelium in rodent prostates (80, 81).

Subsequent genetic events occurring after cancer initiation includes PTEN homozygous deletions. In a recent study, 37 DAC and 18 matched acinar adenocarcinomas were examined by immunohistochemistry with genetic validation (75). PTEN loss was often seen in acinar adenocarcinomas but was infrequent in both pure and mixed DAC. Also, PTEN loss was not enriched in ERG positive tumors, a common observation in acinar adenocarcinoma (75). As PTEN and ERG status are similar in DAC and the acinar component it is possible that the two types are clonally related.

DAC may grow in an environment low of sex hormones, shown as a decreased expression of SQLE, 5AR2 and aromatase. SQLE is the rate limiting enzyme of cholesterol synthesis expressed in prostate cancer cells, especially in high grade prostate cancer (82). An up-regulation of BCAR1 and Src is also seen, both molecules are associated with aggressive prostate cancer. Src belongs to the family of Src kinases that is responsible for signal

transduction from certain receptors on the cell surface. The downstream cytoplasmic effector is BCAR1/p130 CAS, which is involved in proliferation, migration and survival (83). These have also been shown to be over-expressed in castration resistant prostate cancer.

Metastatic castration resistant prostate cancer shows copy number alterations and amplifications in PIK3CA, PIK3CB and CCDN1 as well as deletion peaks in CHD1, PTEN, RB1 and TP53, the latter being the most commonly mutated gene in all human cancers (84). CHD1 negative tumors may be ETS negative and show mutations in SPOP and this may be viewed as a separate class of prostate cancer (77). Patients with DAC may present with metastases but it is unknown which genetic modifications they may harbor.

In summary, the few studies made on the genetic profile of DAC show that the ETS fusion is less common, PTEN loss is less frequently seen in both DAC and the associated acinar component in mixed cases and PTEN loss is not enriched in ERG positive tumors, which is seen in acinar adenocarcinomas (75).

1.13 PROGNOSIS

The prognosis of DAC is generally considered poor with patients presenting at an advanced clinicopathological stage, with or without metastases (17, 19). To this day, it is not known whether the poor prognosis is the result of the high grade features or the sometimes central location allowing occult growth and metastasizing (59). Moreover, it is not fully known how the size of the DAC component influences outcome in a mixed ductal-acinar tumor. Amin *et al*. (85) found that the pathologic stage of GS 7 cancers with less than 10 % DAC did not differ from that of acinar adenocarcinomas of GS 7. Moreover, the combination of DAC and acinar cancer makes it difficult to tell whether the acinar or the ductal component accounts for disease progression.

DAC was initially thought to have a favorable prognosis as no patients died from their disease in early follow-ups ranging from 0-60 months (16, 28, 39, 45, 86, 87). The hypothesis was that the intraurethral location led to an early discovery when patients sought health care because of hematuria, obstructive or irritative symptoms. Hence, the tumors were found at a low grade (16, 28, 39, 45, 86, 87). Some authors even thought of DAC as a tumor with no metastatic potential (51). Mixed ductal-acinar cases were thought to have a worse prognosis, the acinar component being viewed as the outcome-determining factor (31, 50, 52).

More recent studies have confirmed DAC to be a subtype with poor prognosis. A study by Bostwick *et al*. demonstrated the 5-year survival to be a mere 15 % with more than 50 % of

patients dying of metastatic disease within 9-70 months after diagnosis (35). Oxley *et al*. showed that DAC patients with a high proliferative index as demonstrated by Ki-67 were dead of disease one, three and four years, respectively (88). Ki-67 labeling index is known to be an independent prognostic factor in prostate cancers treated by RP (89). DAC has a high short-time failure rate after RP compared to acinar adenocarcinomas (17). Patients with DAC may progress without biochemical recurrence, illustrating that DAC may not produce PSA to the same extent as acinar cancers (90). This suggests that even a patient showing a complete response in s-PSA after treatment may progress in his disease.

Christensen *et al*. (17) examined DAC tumors in RP specimens and compared them against acinar adenocarcinomas of similar stage. DAC cases had a large tumor volume and the pathologic stage was advanced, 93 % of patients had EPE, 47 % positive surgical margins, 40 % SVI and 27 % had tumor infiltration of pelvic lymph nodes. The DAC component in mixed ductal-acinar cases often contributed to the EPE. EPE and SVI are more frequently observed as tumor volume increases together with perineural invasion. The progression rate of DAC has been compared to that of GS 7-9 acinar tumors (19). Pure DAC tends to pursue an indolent course but has an increased risk of local recurrence (49). This suggests that local treatment and control may improve the clinical course for patients with pure DAC.

1.14 METASTATIC SITES

Metastases are three times more common at time of diagnosis in patients with DAC than in patients with acinar adenocarcinoma (18). The metastatic sites were initially thought to be similar to those of acinar adenocarcinoma, namely bone and lymph nodes. However, DAC has later been shown to have a propensity to spread to visceral organs such as lungs and brain (35, 49, 91) but also to the penis and testes, which are unusual metastatic sites for acinar adenocarcinoma (92, 93). Metastases to the penis from any site are rare and until recent date only about 400 cases have been reported in the literature, the first one already in 1870 (94). It may be difficult to recognize a DAC metastatic to the penis because of the unusual anatomic site for prostate cancer, varying morphology, no history of prior prostate cancer or a long interval from the primary prostatic lesion (93). DAC has also been found to metastasize to the anterior urethra, liver and skin, prostate cancer metastatic to the skin being very rare with an incidence of 0.36 % (60, 90, 95, 96). Metastases may be pure DAC, acinar or mixed (24, 57, 60).

It is crucial to diagnose the site of origin of a metastatic adenocarcinoma as cancer therapies are increasingly organ specific. DAC is over-represented at unusual metastatic sites and may

resemble other adenocarcinomas histologically, meaning that the tissue origin may be difficult to establish. Immunohistochemical stains may be useful for the identification of tumor origin. Looking at morphological features, it is not difficult to understand why the first authors thought that DAC was related to endometrial adenocarcinoma *Figure 7*. Gastrointestinal tumors such as rectal and colon adenocarcinomas may show overlapping features with tall, columnar epithelium and complex glands (97). However, staining for intestinal markers such as CDX2 and CEA in combination with prostate specific markers PSA, PSAP or prostein can be used to confirm the intestinal origin. Copeland and al. (91) have described the possibility of misdiagnosing DAC metastases in the lung as a primary pulmonary or colorectal adenocarcinoma, demonstrating the need for immunohistochemical panels.

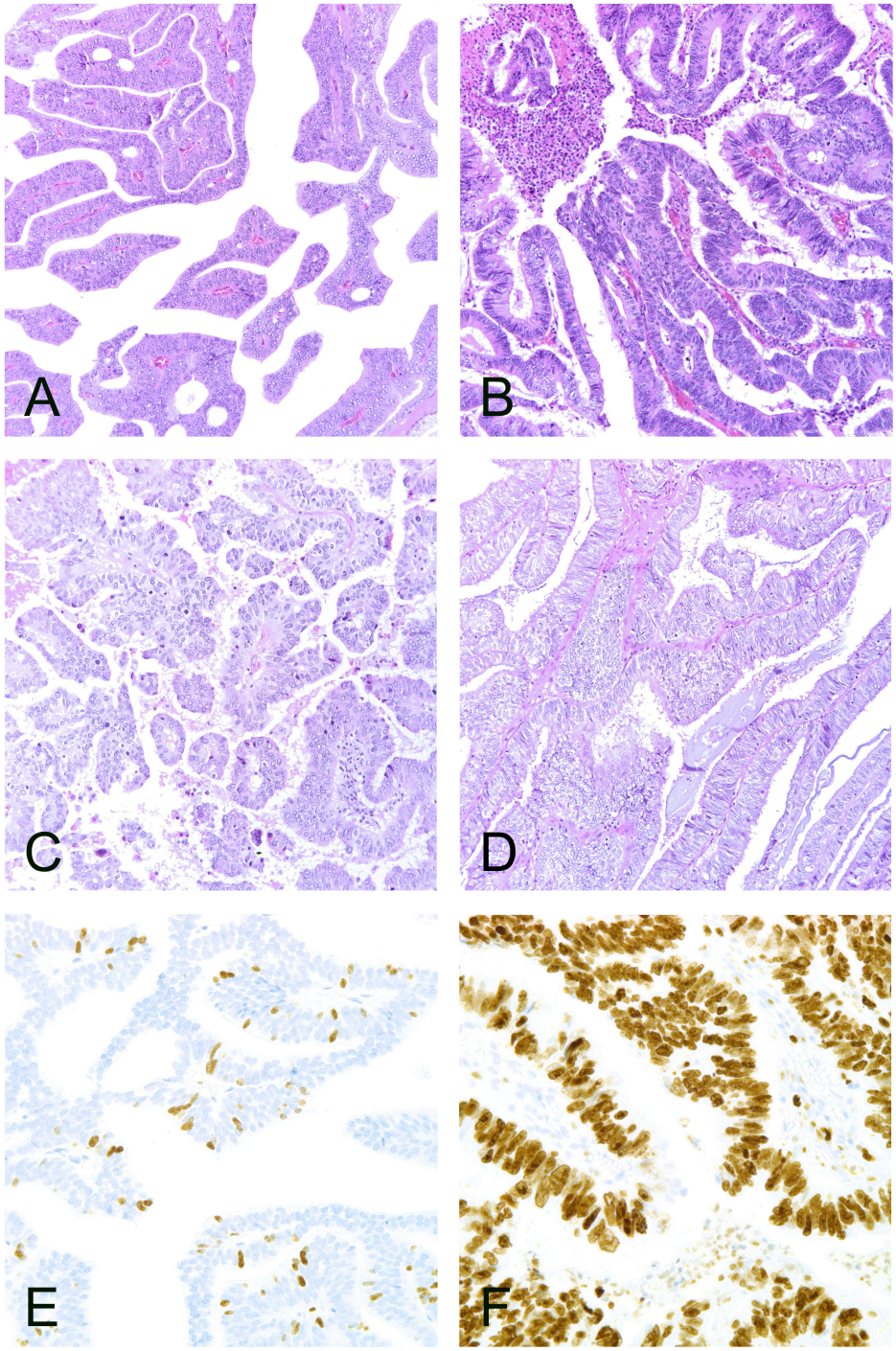


Figure 7 A) Ductal adenocarcinoma of the prostate (DAC) with papillary architecture and fibrovascular stalks. Tall, columnar epithelium with high-grade nuclei and prominent nucleoli. Hematoxylin and eosin (H&E), 20x lens magnification. **B)** Colon adenocarcinoma with columnar epithelium, elongated high-grade nuclei and prominent nucleoli. H&E 20x. **C)** Lung adenocarcinoma with papillary architecture. Cells are columnar with elongated high-grade nuclei and prominent nucleoli. H&E, 20x. **D)** Endometrial adenocarcinoma with papillary architecture and fibrovascular stalks. Cells are columnar with prominent nucleoli. H&E, 20x. **E)** DAC stained with Ki-67. H&E, 40x. **F)** Colon adenocarcinoma stained with Ki-67, demonstrating a higher labeling index than seen in DAC (E). H&E, 40x. *Seipel et al. 2015*

1.15 TREATMENT

Because of the supposed female tissue origin, Melicow and Pachter believed that treatment with orchiectomy or estrogen was contraindicated (16). They were supported by a clinician who wrote a letter to the editor of Archives of Pathology & Laboratory Medicine in 1973, stating that castration and other forms of hormonal therapy were probably worthless as treatment for DAC (98).

Nevertheless, the tumors were later found to respond well to hormonal treatment as well as orchiectomy (24, 29, 99). As further studies proved a prostatic origin of these tumors DAC was treated in the same manner as conventional adenocarcinomas. Modern treatment modalities for DAC include RP, TUR-P, radiation therapy in form of external beams or brachytherapy, hormonal therapy or a combination of the above (100). Patients with DAC are more likely to undergo RP than patients with an acinar adenocarcinoma of GS 8-10 (101). The response to hormonal therapy was initially described as poor (32, 102) but is now considered similar for DAC and acinar adenocarcinomas, with similar relapse and regression rates (103). Orihuela and Green suggested that a combination of radiation and hormonal therapy might be the best treatment option for patients with localized DAC (90). Radiotherapy has been shown to be of benefit for local control of local recurrence but not as curative treatment (104). Staging with whole body scan and computer tomography (CT) of chest, abdomen and pelvis should be performed in all patients, possibly even of the brain due to the distribution of metastases of this tumor subtype. Due to the aggressive nature of DAC tumors, it is possible that adjuvant treatment should be added (105), nevertheless conflicting results have been shown (35).

The large proportion of EPE, positive surgical margins, SVI and pelvic lymph node metastases in patients who were initially thought to have resectable disease suggests a clinical underestimation of the extent of DAC tumors (17, 19). RP may even be a poor choice of treatment strategy for these patients. A high risk of local recurrence has also been demonstrated after RP (106). However, it should be noted that some studies were conducted before the PSA era, thus it is not possible to determine whether poor results post-RP are due to a delayed diagnosis or a poor choice of treatment.

In summary, studies show variable efficacy of RP, hormonal treatment, radiotherapy and chemotherapy, which is likely the result of small studies, a timeline of 50 years and variable inclusion criteria. A prostate cancer with a DAC component of any size should be treated as high-risk disease due to its potential to spread locally and the likelihood of metastasizing (36).

1.16 DAC -A TRUE SUBTYPE?

Since the discovery of DAC it has been debated whether it should be considered a separate clinical entity (107). About 40 years ago, Rotterdam and Melicow described a mixed ductal-acinar case, stating that the two components should be considered as neoplasms with different cell origin, biologic potential and histopathologic features (46).

In 1999, Bock and Bostwick questioned the existence of DAC, challenging its status as a unique subtype due to the considerable overlap with acinar adenocarcinoma, particularly in small tissue specimens. Reviewing a series of 338 consecutive RP specimens, the authors identified cases with clinicopathological findings of acinar adenocarcinomas. The series included typical PZ cancers that apparently did not involve the large periurethral prostatic ducts or verumontanum. Among the 338 cases the authors searched for ductal features in terms of papillary or cribriform architecture, which were at least 5 mm in diameter. In 5 % of the cases, ductal features were identified. They concluded that adenocarcinomas arising in the PZ of the prostate may display papillary or cribriform growth, features associated with DAC. Together with the almost constant association of DAC and acinar adenocarcinoma this led them to the conclusion that DAC is the result of the spread of typical acinar cancer to the large periurethral ducts and stroma. The only unique histologic feature of DAC would be the site of growth (107).

Supporting the view of DAC as a distinct subtype are current immunohistochemical findings, clinical behavior, genetic profile and morphology. The morphology with tall, columnar, pseudostratified epithelium arranged in papillary, cribriform and/or glandular structures is different from a typical acinar adenocarcinoma and the clinical outcome with a large extent of EPE, SVI, positive surgical margins and rate of metastasis suggest that it should, in fact, be viewed as a separate entity and treated accordingly. Despite evidence suggesting that DAC is a separate subtype, no immunohistochemical markers or diagnostic features can with certainty be used to diagnose DAC.

1.17 DIFFERENTIAL DIAGNOSES

Several benign and malignant lesions may be confused with DAC. Lesions protruding into the urethra, such as papillary transitional cell carcinoma of the prostatic urethra and inverted urothelial papilloma may mimic the appearance of DAC (28). A benign fibroepithelial or prostatic polyp in a young male may cause macroscopic hematuria, a symptom sometimes seen in DAC (108). Other lesions occurring in the urethra are proliferative papillary urethritis, caruncle, urothelial papilloma, polypoid adenocarcinomas and nephrogenic adenomas. As

DAC may arise from villous polyps, a centrally located DAC can mimic benign urethral polyps, clinically and histopathologically (62, 86).

High grade prostatic intraepithelial neoplasia (HGPIN) consists of glands lined by atypical cells and may mimic DAC in a RP or core biopsy specimen. The architectural pattern is tufting, micropapillary, cribriform or flat. The micropapillary pattern is characterized by papillary projections into a glandular lumen (109). As opposed to DAC, HGPIN does not contain true fibrovascular stalks, the glands show the same size and distribution as benign glands and not the large, back-to-back oriented glands found in DAC. Features seen in DAC but not in HGPIN also include perineural invasion, hemosiderin deposition and stromal fibrosis (19). Separating DAC from HGPIN on core needle biopsies may be difficult due to the limited tissue available. As an adjunct to histopathological characteristics, Ki-67 staining can be used, the expression being higher in DAC (110).

Pseudohyperplastic adenocarcinoma has a columnar appearance, papillary infoldings and abundant cytoplasm, features similar to those of DAC (111). The nuclear atypia is generally less pronounced than in DAC. Distinction is important as pseudohyperplastic adenocarcinoma is considered a Gleason pattern 3 while DAC usually is assigned a Gleason pattern 4. The PIN-like ductal adenocarcinoma of the prostate may be seen as long strips of PIN-like epithelium lining the edge of a biopsy specimen, however, the marked pleomorphism, cribriform, papillary or solid architecture seen in DAC is missing (112). Intraductal carcinoma (IDC) is also known for its resemblance to DAC, however, the cells form dense cribriform patterns with rounded lumina or micropapillary tufts without fibrovascular stalks (85). The nuclei are more rounded and the epithelium is not as columnar but the distinction is not always obvious (85). IDC represents intraductal growth of acinar adenocarcinoma and may co-exist with DAC in as many as 16 % of RP specimens. More than 30 % of DAC has been show to grow intraductal, which further complicates the distinction (58) *Figure 8*.

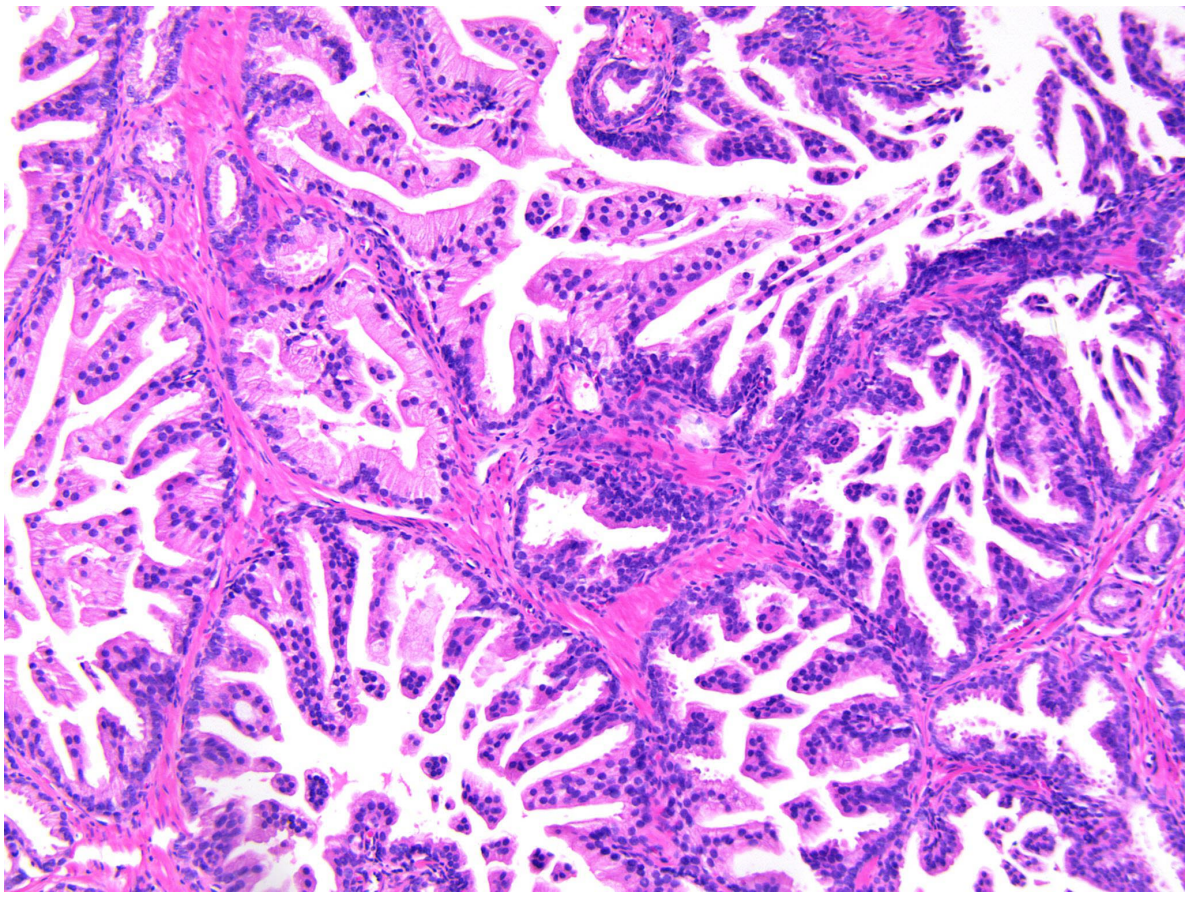


Figure 8 Intraductal prostate cancer, a common differential diagnosis to ductal adenocarcinoma of the prostate (DAC). The typical tall, columnar epithelium and elongated nuclei seen in DAC are missing. Hematoxylin and eosin, 10x lens magnification.

DAC may also be found together with other rare histological patterns such as mucinous, goblet cell, foamy gland, Paneth cell, neuroendocrine, micropapillary or cystic features although these account for only ten known cases in the literature to date (113).

1.18 FUTURE CHALLENGES

Although half a century has passed since the first descriptions of DAC, the clinical and prognostic implications of DAC remain somewhat controversial. The major limitation in studies is that the tumor is rare. A majority of studies include only a handful of cases as large series are difficult to collect at single institutions. Recent studies including a large number of cases have used database searches, which is an unreliable method as the diagnosis of DAC is known to be challenging even for experienced uropathologists (18, 101). Hence, DAC cases may have been overlooked or differential diagnoses included. The collection of DAC cases should therefore be done by central review by an experienced pathologist. Multicenter studies with central review are needed for verification of results from smaller single-center studies.

The lack of diagnostic criteria for DAC may explain some of the differences observed between studies. There is a need for standardized diagnostic criteria as well as further studies on the genetic as well as the immunohistochemical profile of DAC. As we move towards personalized cancer medicine, acquiring knowledge about aggressive subtypes such as DAC may be the key to optimize treatment and clinical outcome.

2 AIMS

The aim of this thesis was to investigate and better characterize the prostate cancer subtype ductal adenocarcinoma (DAC), which continues to challenge pathologists half a century after its first discovery. Briefly, our aims were to examine;

- Which cancers should be classified as DAC and which criteria should be used for diagnosis?
- Do any of the histopathologic characteristics of DAC show a correlation with poor prognosis?
- Does DAC have a different immunohistochemical profile than acinar adenocarcinomas?
- How do we distinguish DAC from adenocarcinomas of other origin if sampled at a metastatic deposit?
- Are there genetic differences between DAC and acinar adenocarcinomas?
- Is DAC a separate entity or merely a variant of acinar adenocarcinoma?

3 MATERIAL AND METHODS

"It always seems impossible until it's done"

—Nelson Mandela

3.1 TISSUE COLLECTION AND PREPARATION

Cases for Study I-IV (114-117) were identified after the review of a consecutive series of 1'156 specimens from patients who underwent RP at Karolinska University Hospital between May 1998 and December 2005. Patients were excluded if they had received neoadjuvant treatment (hormonal treatment or radiotherapy), gone through TUR-P or if there was no clinical follow-up available. A total of 1 051 cases remained for study. For Study III and IV additional cases were acquisitioned from Aquesta Pathology, Queensland, Australia and for Study V (118) by searching records at Karolinska University Hospital for DAC cases with fresh-frozen tissue available.

The RP specimens were formalin-fixed overnight in 4 % buffered circulating formal saline, inked and totally embedded, sections were then cut horizontally at 4 mm thickness. Slices were either mounted or cut into 2-6 segments. The apex and base were handled using the shave technique 1998-2003 and from 2004 by coning with sagittal slicing of the apex and shaving of the base. The RP specimens were then dehydrated, paraffin embedded and cut at 4 μm. The sections were stained with hematoxylin and eosin (H&E) for histopathologic evaluation.

Fresh-frozen tissue for Study V was collected from RP specimens by cutting a horizontal slice with a double-bladed knife. The slice was cut into smaller blocks and put in cryomolds filled with OCT gel, snap frozen in liquid nitrogen and stored at -80°.

3.1.1 Study I

All slides from the series of 1 051 RP specimens were reviewed by the author for any presence of DAC, which was later confirmed together with a senior pathologist (Lars Egevad, LE). Ductal cancers were classified into three subgroups according to histopathological features.

DACC

A case was classified a ductal adenocarcinoma of classical type (DACC) if the tumor occupied more than one visual field, had tall, columnar, pseudostratified epithelium, prominent nucleoli, elongated or oval nuclei, papillary/cribriform/glandular architecture or a mixture of several types. The nuclei were crowded and had high-grade features with open chromatin and rough chromatin texture. The cytoplasm was amphophilic or clear. *Table 1.*

DACB

A ductal adenocarcinoma with borderline features (DACB) showed several of the characteristics described for DACC but would lack elongated nuclear shape or typical architectural features. A case was also classified as DACB if the extension was less than focal, or less than one medium power field. Two different types were often seen, one where the nuclei would be located basally but show stratification and a complex glandular architecture with papillary infoldings. A second type showed elongated, stratified nuclei as seen in DACC, but the architecture was simple and glandular *Table 1, Figure 9.*

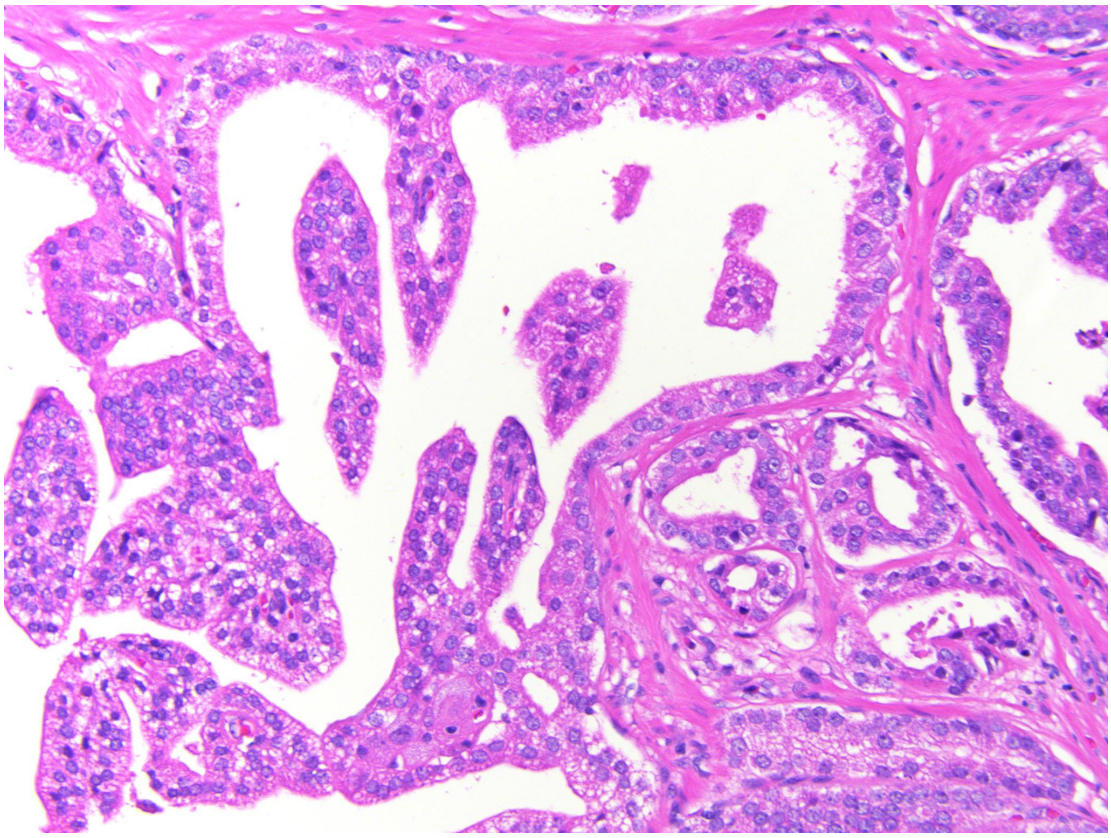


Figure 9 Ductal adenocarcinoma with borderline features. The tall, columnar epithelium can be seen, however, the typical nuclear features are missing.

PCDF

A prostate cancer with ductal features (PCDF) had stratified, high-grade nuclei but lacked the other characteristics of DACC. The nuclei were not elongated but had other high-grade features *Table 1, Figure 10.*

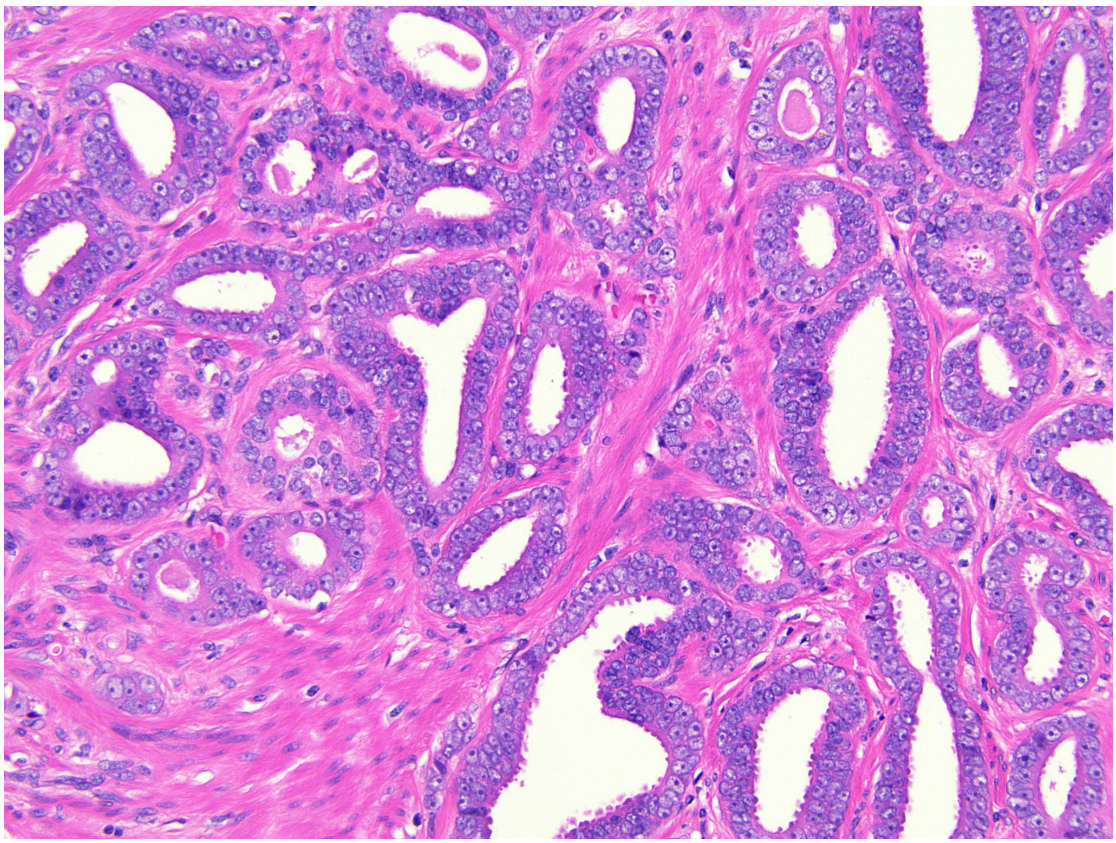


Figure 10 Prostate cancer with ductal features. The nuclei are stratified and of high-grade but the typical architecture and the columnar, pseudostratified epithelium seen in ductal adenocarcinoma of the prostate is missing. Hematoxylin and eosin, 20x lens magnification.

	DACC	**DACB**	**PCDF**
Feature			
Columnar, pseudostratified epithelium	Yes	Yes	No
Elongated nuclei	Yes	Y/N	No
Stratified, high grade nuclei	Yes	Yes	Yes
Architecture, one or more:			
Cribriform	Yes	Y/N	No
Papillary	Yes	Y/N	No
Glandular	Yes	Y/N	No

Table 1 Describing the histopathological features of the three subgroups of ductal adenocarcinoma of the prostate

Cases identified

A total of 86 cases were identified of which 27 were classified as DACC, 42 as DACB and 17 as PCDF.

3.1.2 Study II

For Study II, 21 cases from the DACC and DACB group of Study I were included; 14 DACC and 7 DACB. Cases with a range of histopathological features were selected.

3.1.3 Study III and IV

A total number of 35 DAC cases from the DACC and DACB group of Study I were chosen based on sufficient tumor tissue volume. Acinar adenocarcinomas matched for GS were chosen as controls and a total number of 46 cases were included.

Cases from Aquesta Pathology, Brisbane, Australia were added to increase the total number of DAC cases. For Study III, records were searched for RP or TUR-P specimens containing DAC tissue and a total of 25 cases were identified; 23 RP and 2 TUR-P specimens. Blocks were sent to Karolinska University Hospital and all cases were included in the tissue microarray constructed for Study III and IV.

For Study IV, records were searched at Karolinska University Hospital for adenocarcinomas with morphological features similar to those of DAC. A total number of six colonic adenocarcinomas, seven endometrial adenocarcinomas, five gastric adenocarcinomas, seven lung adenocarcinomas, five pancreatic adenocarcinomas and seven urinary bladder adenocarcinomas were included.

3.1.4 Study V

Records were searched at Karolinska University Hospital for prostate cancers with a DAC component and available fresh frozen-tissue. Formalin-fixed paraffin embedded sections were reviewed for presence of DAC and the corresponding fresh-frozen tissue was then cryosectioned, stained and reviewed for any presence of DAC. Benign tissue from all cases was used as control. Eleven cases were included in the study.

3.2 PHOTOMICROGRAPHS

For Study II photomicrographs were taken and distributed together with a questionnaire, using a web based survey tool (www.surveymonkey.net). A set of six photomicrographs were taken, three at 10x and three at 20x lens magnification. Pictures of several areas were taken and critically examined in order to ensure an accurate, objective and valid description of each case (119). Photographs were reviewed together with a senior pathologist (LE) before the final series was chosen.

3.3 TISSUE MICROARRAYS

For Study III and IV a tissue microarray (TMA) was constructed consisting of two paraffin blocks. One core of tumor tissue of 1 mm size was harvested from each case, resulting in two slides with 145 cases in total *Figure 11*.

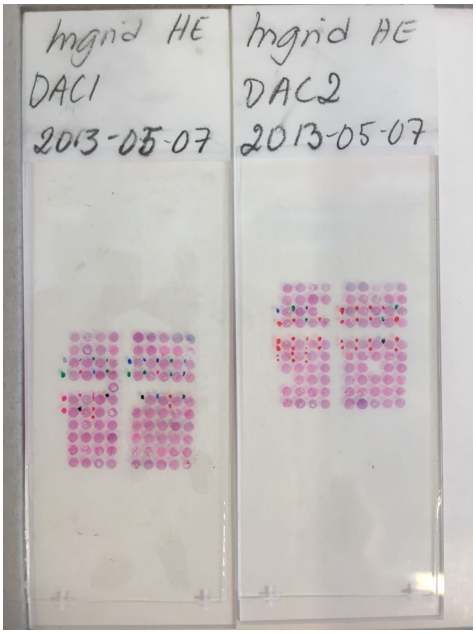


Figure 11 One section from each final paraffin block stained with hematoxylin and eosin. Cores are arranged in asymmetrical patterns to allow easy identification of each separate case.

A TMA is a method, which is used to collect a large number of cases in a single paraffin block. The method was described by Battifora in 1986 as a method for immunohistological testing of a large number of samples on a single slide with only one drop of antibody (120). The method was modified in 1990 when the tissue was distributed in a checkerboard pattern (121). Kononen et al. (122) subsequently refined the method and enabled in-situ detection of DNA, RNA and protein targets, allowing up to 1 000 cores in one 45 x 20 mm block.

The first step in the construction of a TMA is to use an H&E section from the donor block to identify areas of interest and verify that enough tissue is still available in the block. One or more tissue cores are punched out from the donor block and transferred to pre-made holes in the recipient block. Cores are arranged in precise but asymmetrical patterns to allow identification of separate cases while maintaining orientation. Sections are then cut from the recipient block using a microtome and these sections are later stained and analyzed. TMAs are used for histochemical, immunohistochemical and immunofluorescent staining as well as DNA and RNA hybridization. Depending on the core length, one block may theoretically provide 100 to 200 sections (123), although the number of sections that can be harvested is usually lower in practice.

3.3.1 Number of cores

In our studies we used one 1 mm core of tumor tissue from each case. It has been debated how many cores should be sampled from each tumor and what the core size should be.

Kononen *et al*. (122) stated that as many tumors are heterogeneous, a single sample is insufficient for representation of the biological properties. Instead, several samples should be taken from the most representative areas of each tumor. Core size may vary, common sizes are 0.6, 1.0, 1.5 and 2.0 mm. Large cores of 2.0 mm size may damage the donor as well as the recipient block. Rubin *et al*. (124) concluded that no less than three 0.6 mm cores should be taken to assess tumor protein expression while more than 4 cores of 0.6 mm did not add significant information. However, Tennstedt (125) showed that using multiple 0.6 mm cores does not necessarily increase the ability to associate biomarkers with prognosis or tumor phenotype but may introduce statistical errors because of unequal amounts analyzed per tumor. The authors also debated the use of only one section of an RP specimen as the golden standard to find relevant biomarkers for prostate cancer. The mean volume of prostate cancer in 1 657 RP specimens was 4.4 cm^3, meaning that one 4 μm section containing 2x1 cm of tumor would only analyze 0.00008 cm^3 of the tumor, hence 0.0018 % ! Including two more cores does not add a substantial amount of information. Also, the addition of multiple positively stained cores may not reflect tumor heterogeneity but rather false positive or non-specific staining (125). The use of several cores is expensive, time consuming and depletes the paraffin blocks of tumor tissue. Hence, the use of 1 mm cores in our studies is supported by the literature and as DAC tissue is sparse, the choice was also made not to exhaust the blocks by using more than one core.

3.4 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is used to analyze protein expression in tissue and the method was used in Study III and IV. A summary of antibodies used is seen in *Table 2.* Antibodies to estrogen, progesterone and androgen receptor, prolactin, PSA, prostein, PSMA, PSAP, CDX2, lysozyme, villin, monoclonal CEA, CK7, CK20, HMWCK, p63, p504s, c-myc, epithelial growth factor receptor (EGFR), Ki-67, p16, p21, p27, p53, PTEN, ERG, PAX-2 and PAX-8 were used. Sections were stained at the routine lab of the Department of Pathology, Karolinska University Hospital using a Leica Bond robotic immunostainer (Leica Microsystems, Wetzlar, Germany), or a Ventana automated immunohistochemistry system (Ventana Medical Systems, Tucson, AZ, USA).

Antibody/clone	*Species/Type*	*Dilution*	*Antigen retrieval*	*Vendor*
Androgen receptor	Rabbit/monoclonal	Prediluted	CC1 64 min	Ventana 760-4605
CDX2	Rabbit/monoclonal	1:50	H2	Cell-Marque 23R-16
CEA monoclonal	Mouse/monoclonal	1:400	H1	Dako M7072
CK7	Mouse/monoclonal	Prediluted	H2	Novocastra PA0942
CK20	Mouse/monoclonal	1:100	E1 (10)	Dako MR019
C-myc	Mouse/monoclonal	1:24	CCI 64 min Amp+u-w	Neomarker M5139
EGFR	Mouse/monoclonal	1:600	E1 (5)	Dako M7239
ER	Rabbit/monoclonal	Prediluted	CC1 64 min Amp+u-b	Ventana 790-4324
HMWCK	Mouse/monoclonal	Prediluted	Protease 1 8 min	Ventana 790-4373
Ki-67	Rabbit/monoclonal	Prediluted	CC1 36 min + u-b	Ventana 790-4286
Lysozyme	Rabbit/polyclonal	1:2500	E1 (5)	Dako A099
p16	Mouse/monoclonal	1:400	H1	Santa Cruz sc5660
p21/WAF-1	Mouse/monoclonal	1:50	H1	Calbiochem OP64
p27	Mouse/monoclonal	1:40	H1	Novocastra NCL-P27
p504S/AMACR	Rabbit/monoclonal	1:100	CC1 36 min + Amp	Dako M3616
p53	Mouse/monoclonal	Prediluted	CC1 64 min +Amp u-b	Ventana 800-2912
p63	Mouse/monoclonal	Prediluted	CC1 52 min + Amp u-w	Ventana 760-4509
PAX-2	Rabbit/polyclonal	1:100	H2	Abcam ab23799
PAX-8	Mouse/polyclonal	1:200	H2	Proteintech Europa 10336-1-AP
PgR	Rabbit/monoclonal	Prediluted	CC1 64 min + u-b	Ventana 790-2223
Prostein/P501S	Mouse/monoclonal	1:100	H1	Dako M3615
PSA	Mouse/monoclonal	1:100	H2	Novocastra NCL-PSA-431
PSMA	Mouse/monoclonal	1:50	H1	Dako M3620
PSAP	Mouse/monoclonal	1:200	H1	Novocastra NCL-L-PAP
PTEN	Mouse/monoclonal	1:400	H2	Novocastra NCL-PTEN
Villin	Mouse/monoclonal	Prediluted	H1	Novocastra PA0106
ERG	Rabbit/monoclonal	Prediluted	CC1 36 min	Ventana 790-4324
Prolactin	Rabbit/polyclonal	Prediluted	CC1 36 min + u-w	Ventana 760-2803

Table 2. Antibodies used in Study III and IV. CCI= citrate buffer pH 6.0, heated 36/52/64 minutes, u-b= ultra block, u-w= ultra wash, E1= 1 drop of conc. enzyme+ 7 ml Bond enzyme solution, Protease 1= Proteolytic enzyme, Amp= amplification of DAB antigen, H1= pretreatment with Bond Epitope Retrieval Solution 1, H2= pretreatment with Bond Epitope Retrieval Solution 2. *Seipel et al. 2014*

The principles of IHC date back to the 1930s but it was not until 1942 that Coons *et al*. (126) used labeled antibodies to identify Pneumococcal antigens in infected tissue. Tissue samples must be rapidly preserved to prevent breakdown of cellular proteins and architecture. Formaldehyde is commonly used for fixation, creating a semi-reversible covalent crosslinking. The tissues are then embedded in paraffin to preserve the natural shape and architecture for histopathologic examination. Sections are cut and mounted on glass slides.

In order for the antibodies to recognize and reach the target antigens, samples must be de-paraffinized. Removal of the paraffin is done by washes with xylene followed by graded washes with ethanol to remove the xylene and washes with ethanol and water to rehydrate the specimen. The formaldehyde creates methylene bridges between proteins, thus masking epitope recognition by primary antibodies. These bridges are removed either using heat-induced epitope retrieval or proteolytic-induced epitope retrieval.

Endogenous target activity must then be blocked to avoid recognition of endogenous biotin with the targeted antigen. Biotin is a co-enzyme in many reactions and is conjugated to antibodies and enzymes because it has a strong binding affinity with avidin and thus facilitates visual recognition of the complex. Our cells, however, may contain high levels of biotin, which causes the avidin to bind, thus creating background staining. To prevent this, free avidin is administrated to the sample and biotin is then added, making the biotin fill all available biotin-binding sites on the avidin molecule.

Antibodies have an affinity for specific epitopes but may nevertheless bind to non-specific sites similar to their binding site on a specific epitope. This also causes background staining. To reduce this, samples are incubated with a buffer that blocks the reactive sites.

Primary and secondary antibodies are diluted to promote homogeneous distribution, stabilize the antibody and to reduce non-specific binding. The sample is rinsed between the application of the primary and secondary antibody to wash off any excess antibody that may have bound to a non-specific site. Antigen detection methods are indirect or direct *Figure 12*. In the direct method, a primary antibody conjugated to an enzyme recognizes its epitope and binds. The enzyme is activated by adding a substrate, which allows visual detection of the complex. The indirect method uses a secondary antibody specific for the primary unlabeled antibody, which is added first. As multiple secondary antibodies may bind to the primary antibodies this allows for amplification of the signal, thus facilitating visual recognition of the detectable product.

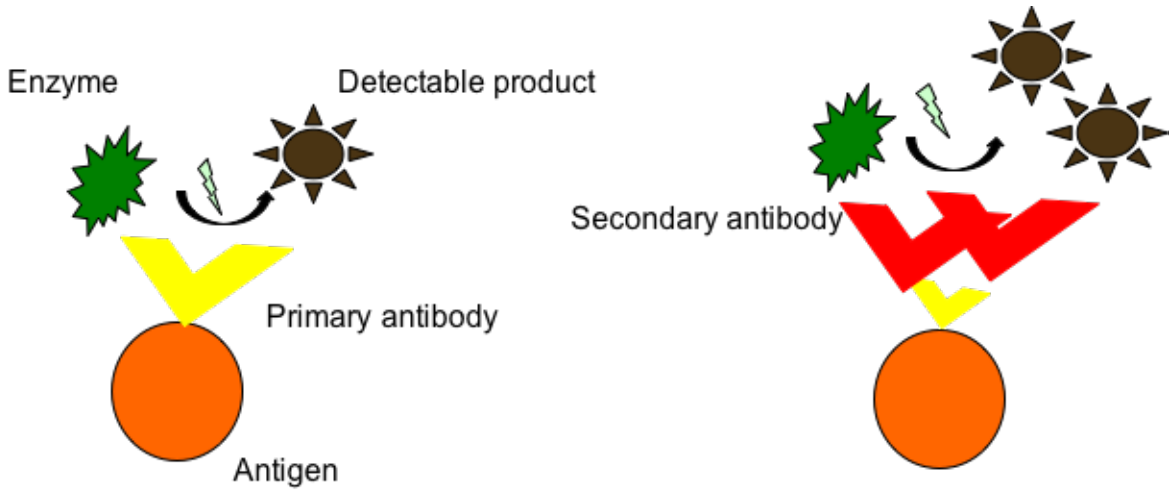


Figure 12 Describing the two principles of antigen detection, direct (left) and indirect (right).

3.4.1 Evaluation

Immunohistochemical staining in Study III and IV was evaluated by the author. Cases with interpretation difficulties were re-assessed in open discussion together with a senior author (LE).

3.5 LASER CAPTURE MICRODISSECTION

All tissues in the human body are composed of different cell types. Cells in complex tissues are affected by their surroundings and the microenvironment. The analysis of gene expression patterns requires pure cell populations without contamination of other cell types. If the DNA or RNA acquired are not from a homogeneous population but from several cell types, no conclusions about the specific disease morphology can be made. Laser capture microdissection (LCM) allows the isolation of a pure group of targeted cells in a microscopic region for further analysis and has been modified since its introduction in the early 21th century.

Different methods have been used to isolate pure cell populations; one method was to destroy the unwanted regions using an ultraviolet laser beam and then collect the remaining wanted cells mechanically. This was described already in 1976 by Meier-Ruge *et al*. (127) and used by several authors at the time (128, 129). Another option was to remove the desired region manually using a tool such as a needle or a blade (128, 130).

The modern day LCM was invented at the National Cancer Institute of the National Institutes of Health in Bethesda, USA (131). The authors described a method in which a thin, transparent film was placed over a tissue section. The cells of interest were adhered to the film using a pulse from an infrared laser. The film together with the area of cells of interest could then be transferred directly into, for example, a buffer for downstream analysis. The advantages were time, preservation of original morphology, avoiding manual manipulation and contamination of the material and above all, it was a simple method fulfilling the needs of clinicians and researchers in daily work (131). The method is an example of an infrared

laser capture microdissection system and the method was quickly commercialized by Arcturus Engineering.

Another platform is the ultraviolet laser microbeam microdissection system, which was described by Schütze and Lahr in 1998 (132). The sectioned tissue is mounted on a membrane slide and a laser beam is used to cut the region of interest on the slide under direct visualization, creating a gap between the region of interest and the surrounding cells. The power of the laser is then increased and the cut region is catapulted up into a collecting tube. This method was commercialized by PALM Zeiss Microlaser Technologies and used in our studies.

The greatest advantages of LCM are the speed and the precision, allowing even the dissection of single-cells without destroying the adjacent tissue. A range of tissue can be used, from formalin-fixed paraffin embedded material to live cells and cell cultures, fresh-frozen tissue, stained or unstained tissues *Figure 13*. Limitations include the lack of a coverslip, which may cause difficulties in the recognition of histological features of some organs. The user may also experience difficulties when catapulting the area of interest into the collecting tube. This could be caused by too low laser power, too thick a section or incomplete tissue dehydration.

In Study V, snap frozen tissue was sectioned using a cryostat, stained and reviewed for presence of DAC. If sufficient tumor volume, sections were cut and placed on membrane slides and underwent standard staining protocol with H&E. The tissue was then dehydrated before using the PALM Microbeam (Carl Zeiss MicroImaging, Bernried, Germany) for LCM and collection of tumor cells.

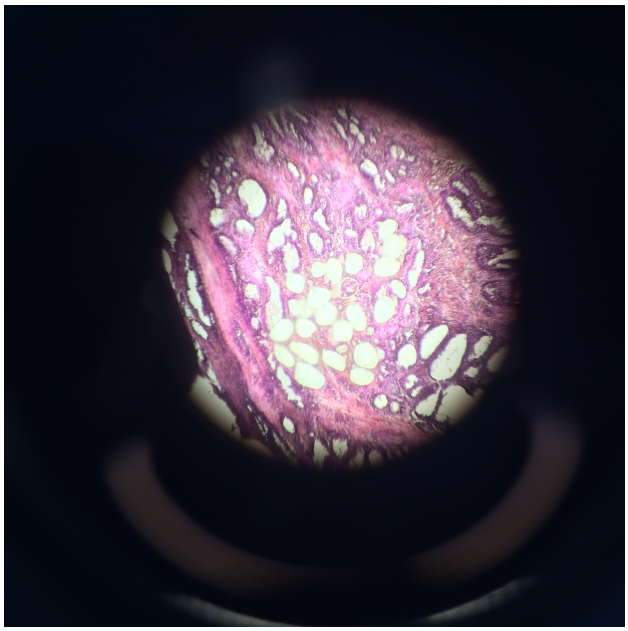


Figure 13 Demonstrating a DAC case during laser capture microdissection, the holes seen in the central part of the picture shows the appearance of the tissue after the areas of interest have been dissected and catapulted into the collecting tube.

3.6 GENETIC STUDIES

In Study V after the collection of tumor cells, DNA was extracted using the AllPrep DNA/RNA Mini Kit (Qiagen, Venlo, Netherlands), the kit allowing high-quality DNA to be used for downstream analysis. DNA was used for library preparation with ThruPlex FD kit (Rubicon Genomics, Ann Arbor, USA) creating indexed libraries through end repair, adapter ligation and high-fidelity library amplification.

Recent advances make it possible to catalog genetic variations, thus creating a foundation for the understanding of prostate cancer. Sanger sequencing was described in 1977 and has since become the gold standard for DNA sequencing (133). Whole-genome sequencing is a comprehensive method for analysis of the genome and allows further downstream analysis when needed. Low-pass whole genome sequencing was used to determine copy number alterations in all cases using the Illumina platform (Illumina, San Diego, USA). Reads were aligned to a reference genome using Burrows-Wheeler Aligner (134) and quality controlled using the Picard software (Broad Institute, Cambridge, USA) (135). Copy number alterations were called from low-pass wgs data using Vardict (136) and variants annotated through the use of SndEff (137).

3.7 STATISTICAL ANALYSIS

Statistical analysis was performed using R statistics software (The R Project for Statistical Computing, Vienna, Austria) or SPSS (IBM SPSS Statistics for Macintosh, Version 22.0, Armonk, NY: IBM Corp). Univariate and multivariate Cox regression models were used for analyses of time to biochemical recurrence and Kaplan-Meier curves and log-rank test to compare biochemical recurrence-free survival between groups. The *Chi-square test* was used in Study I to IV for comparison of categorical variables between different groups while the *unpaired t-test* was used in Study III and IV to compare numerical variables between different groups. *Fisher's exact test* was used in Study IV to compare differences in groups of small sample size. The predictive performance of explored markers in Study III was assessed by the area under the receiver operating characteristic (ROC) curve (AUC). In Study II interobserver variability was estimated by the use of pairwise unweighted kappa statistics. In all studies, *p*-values of less than 0.05 were considered significant.

3.8 ETHICAL CONSIDERATIONS

Study I-V were approved by the Regional Ethic Review Board, Stockholm (2006/1014-31, 2013/1451-32, 2010/710-31/2) and the Aquesta Ethics Committee, Brisbane, Australia (AQ376421).

4 RESULTS AND DISCUSSION

"A problem is a chance for you to do your best"

—Duke Ellington

4.1 PAPER I: DAC IS MORE AGGRESSIVE THAN ACINAR ADENOCARCINOMA

The aim of our first study was to retrospectively review RP specimens from Karolinska University Hospital to identify cases with a DAC component. Histological variants of DAC and their histopathological features were to be correlated with biochemical recurrence as little is known about the contributing factors to the poor prognosis of DAC. Hence, the aggressive clinical behavior of DAC makes it important to accurately define and diagnose this subtype.

Of 1,051 sections reviewed, 86 (8.2 %) had a ductal component, two of these were pure DAC (2.3 %). Hence, pure DAC accounted for 0.2 % (2/1051) of all specimens reviewed. All other cases were mixed with acinar adenocarcinoma, which supports earlier reports that a majority of DAC are associated with acinar adenocarcinoma. The acinar component is often of high GS, which was true also in our study (35). A reason for the high prevalence of DAC cases is that these were identified by central review and not by database search, which is commonly used by other authors. Although studies have shown that DAC patients present at an older age (17, 36, 37), the mean age in the DAC and the acinar group at time of diagnosis was similar (64 and 62.3 years) as well as the preoperative s-PSA, which was 9.8 ng/ml and 8.2 ng/ml, respectively. s-PSA is not reliable for risk stratification of patients with DAC and has not been shown to correlate with clinical outcome (17).

DAC tumors originated mainly from the PZ, a fact that seemed improbable at the time of discovery of DAC as it was thought that the tumors only arose in the periurethral ducts and the prostatic utricle (16). However, some DAC tumors are identified on cystoscopy as an exophytic growth into the urethra (24, 33). A majority of our cases (56.9 %) were diagnosed with palpable disease (T2), which is more than expected in a series of RP specimens and consistent with previous studies (35). Similar to the study of Christensen *et al.* (17), positive surgical margins, EPE and SVI were more common in DAC than in acinar adenocarcinomas. The most common architecture was a mixture of papillary and cribriform growth patterns.

DAC is diagnosed on morphology alone and we attempted to classify potential DAC cases into subgroups and run statistical analyses to investigate the prognostic significance of histopathological features. The cases were divided into three groups as described in the material and methods section based on their resemblance to a classic DAC; DACC, DACB and PCDF *Figure 14.* The outcome data showed that DACC and DACB had similar hazard ratio and relapse rate while PCDF, hence cases with only stratified high-grade nuclei should not be classified as DAC. This is the first study attempting to describe a group with vague ductal features.

In line with other studies our results suggest that DAC has a worse prognosis than acinar adenocarcinoma although statistical significance was not reached, likely due to a low number of cases *Figure 15*. However, when biochemical recurrence was defined as a PSA of equal to or >0.5 ng/ml instead of 0.2 ng/ml, statistical significance was reached. A possible explanation for the results seen at this value is poor surgical techniques at the time of case collection when prostatic tissue may have been left in the resection area, thus secreting PSA and contributing to false-positive biochemical recurrence.

In summary, DAC should be defined as a cancer with tall, columnar, pseudostratified epithelium, elongated or oval nuclei and/or papillary, glandular or cribriform architecture. Patients are more likely to present with EPE, SVI and positive surgical margins. We could not show that necrosis, stromal invasion versus intraductal spread, percentage of DAC component or localization within the prostate have any clinical significance. DAC is likely to be more aggressive than acinar adenocarcinoma, which is seen when biochemical recurrence is defined as a s-PSA of >0.5 ng/ml.

The combination of DAC and acinar adenocarcinoma is commonly reported in the literature, likely because of underrepresenting as well as unclear diagnostic criteria. As the diagnosis of DAC is complicated by a wide spectrum of differential diagnoses such as HGPIN, IDC-P, pseudohyperplastic adenocarcinoma, urothelial carcinoma, colorectal adenocarcinoma and benign prostatic polyps, establishment of diagnostic criteria would facilitate diagnosis.

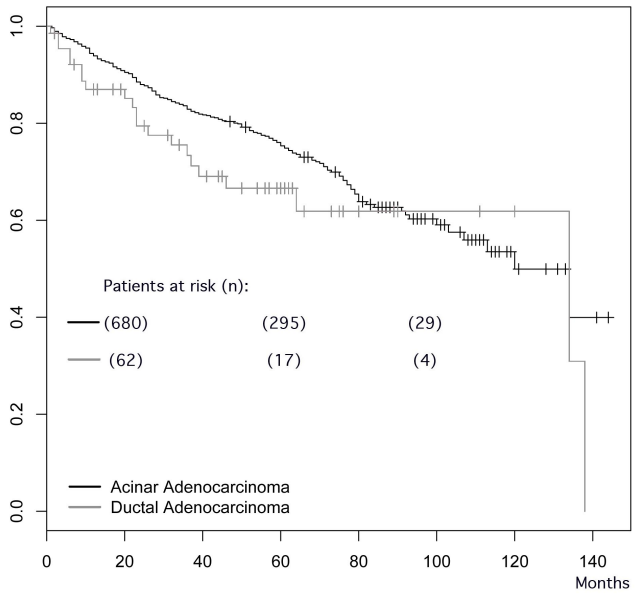


Figure 14 Kaplan-Meier curve showing the survival probability for ductal versus acinar adenocarcinoma. *Seipel et al. 2013*

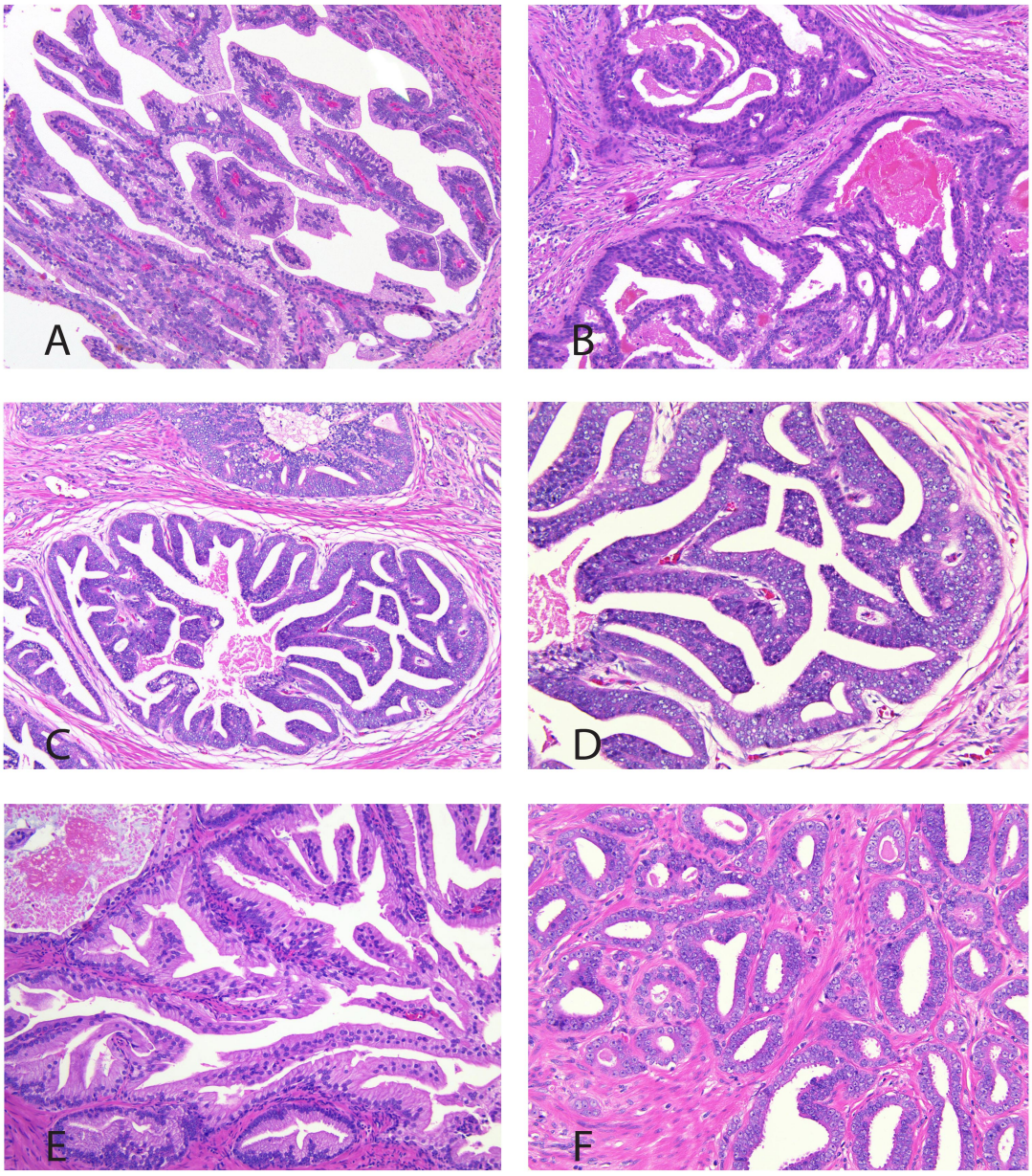


Figure 15 A) Ductal adenocarcinoma, classical type (DACC) with papillary architecture, tall, columnar epithelium with stratified nuclei with high-grade features. Hematoxylin and eosin, H&E, 10x lens magnification **B)** DACC with cribriform, invasive glands also showing a tall, columnar epithelium with stratified, high-grade nuclei. H&E, 10x **C)** DACC with papillary architecture, tall, columnar epithelium with crowded, stratified nuclei. H&E, 10x **D)** Same DACC case as in c showing papillary fronds with stromal cores, crowded, stratified nuclei with high-grade features. H&E, 20x **E)** Ductal adenocarcinoma, borderline type (DACB). The lining epithelium is columnar and branching but the nuclear features are more bland than in DACC and nuclei are rounded. H&E, 10x **F)** Prostatic carcinoma with ductal features (PCDF). The glands are lined with epithelium with stratified high-grade nuclei but the typical architecture of DACC is missing and the nuclei are not elongated. H&E, 20x. *Seipel et al. 2013*

4.2 PAPER II: PAPILLARY ARCHITECTURE IS THE MOST USEFUL DIAGNOSTIC FEATURE OF DAC WHILE NUCLEAR AND CELLULAR FEATURES ARE CONSIDERED LESS IMPORTANT

Twenty expert uropathologists from over the world were asked to participate in our second study aiming to define diagnostic criteria for DAC. Two of these did not consider DAC to be a histological subtype but merely a variant of acinar adenocarcinoma and were therefore excluded from analyses.

A set of six photomicrographs; three at 10x and three at 20x lens magnification were distributed using a web-based survey tool. These were all DACC and DACB cases from Study I. There was a 2/3 consensus for a DAC diagnosis in 11/21 (52 %) cases and consensus against DAC in 5/21 (24 %) cases. For the eleven consensus cases of DAC, papillary architecture was reported as the most important histopathological feature for diagnosing DAC followed by elongated nuclei, cribriform architecture, stratification of nuclei, tall columnar epithelium and high-grade nuclear features *Figure 16*. For the five cases with consensus against DAC, suggested diagnoses were acinar adenocarcinoma, IDC-P, HGPIN, pseudohyperplastic adenocarcinoma and PIN-like cancer. Some of the cases are displayed in *Figure 17*.

IDC-P was the most frequently reported differential diagnosis and is notoriously difficult to separate from DAC. IDC-P, however, is an intraductal extension of high-grade acinar adenocarcinoma, hence cuboidal or low columnar cells with round nuclei and not the tall columnar epithelium seen in DAC. Nevertheless, the distinction is not always obvious. A cribriform DAC is composed of columnar epithelium with intraepithelial bridging and slit-like lumina, while the lumina in IDC-P are typically rounded with a “punched out” appearance (85). To make matters even more confusing, we distinguish between intraductal spread of ductal adenocarcinoma and IDC-P, which represents intraductal spread of acinar adenocarcinoma. There is obviously still a need for better distinction between these two entities as morphological features may be shared between them. Two helpful features to distinguish between the two types are the papillary fronds with true fibrovascular stalks and the elongated nuclei that are seen in DAC but not in IDC-P.

The second most common differential diagnosis was HGPIN, however, this is not as difficult to separate from DAC as IDC-P but distinction is critical for patient management and outcome. Micropapillary formations may be seen in HGPIN but they lack the true fibrovascular stalks seen in DAC. The size of the glands in HGPIN is similar to that of benign glands. Their spatial distribution is almost normal while the glands in DAC are often arranged back-to-back and enlarged. Comedonecrosis may also be seen in DAC (19). One can also look for mitotic activity, perineural invasion and hemosiderin deposition to separate DAC from HGPIN (99). In summary, HGPIN shows a less complex architecture than DAC, the nuclear atypia is not as high grade and papillary fronds with true fibrovascular stalks are not seen.

The distinction between DAC and acinar adenocarcinomas may be complicated as they typically co-exist and the histopathological features may overlap. However, small gland patterns are commonly seen in acinar adenocarcinomas while DAC is a large-gland adenocarcinoma. Acinar adenocarcinoma may have columnar epithelium and nuclear stratification, while DAC may present with rounded nuclei. They can both show intraluminal necrosis, cribriform patterns and high-grade nuclear features. As the acinar component is often of high GS in mixed tumors, the clinical implication of separating a GS 8 tumor with or without a DAC component does not affect patient management today, but it may very well be that patients with DAC should be treated aggressively considering the poor prognosis compared to acinar adenocarcinomas. However, it has been suggested that a DAC component of less than 10 % does not affect outcome (85).

It is, however, important to separate pseudohyperplastic carcinoma and PIN-like carcinoma from DAC as they are considered Gleason pattern 3, reflecting their less aggressive behavior. Pseudohyperplastic carcinoma and DAC share the tall, columnar epithelium but the nuclear atypia and stratification are less conspicuous in pseudohyperplastic carcinoma. PIN-like carcinoma shows less nuclear atypia than DAC and has a less complex architecture.

Lack of typical architecture was the most common reason to reject a DAC diagnosis. Other reasons were round nuclei, lack of nuclear stratification, only a limited amount of DAC present and the absence of true papillae. Or as one participant wrote, the tumor simply did not look nasty enough. The architecture was also the most helpful feature for establishing a DAC diagnosis.

The multirater kappa value was a mere 0.23, pointing to the difficulties in diagnosing DAC and also the need for standardized criteria. Our study shows that the subtype of DAC is not as well defined as previously thought. The diagnosis of DAC is important for patient management as it has a poor prognosis but also for research purposes as the tumor is rare and there are few studies on larger materials. As histological subtypes other than acinar adenocarcinomas are unusual and account for less than 10 % of all prostate carcinomas, diagnosis is a clinical challenge. The disease specific mortality for DAC has been reported at 12 % compared to 4 % for patients with acinar adenocarcinomas (18). This implies that a prostate cancer showing any component of DAC should be treated as high-risk disease owing to its potential to progress and metastasize (49).

One could argue that five cases defined and analyzed as DACC and DACB in Study I were not diagnosed as DAC by the group of international pathologists in Study II. Hence, this may have affected the results of Study I as some of the cases should not be grouped and analyzed as DAC. Nevertheless, DAC has proved to be a challenging diagnosis with poor reproducibility even among some of the most experienced uropathologists worldwide and for all cases in this study, there were always one one more participants diagnosing the case as DAC even though the majority classified the case as something else. The results were expected for a tumor with poorly defined morphology and emphasize the need for standardized diagnostic criteria.

In summary, the finding of papillary architecture in combination with stratified nuclei supports a DAC diagnosis but needs to be correlated with outcome data in larger datasets.

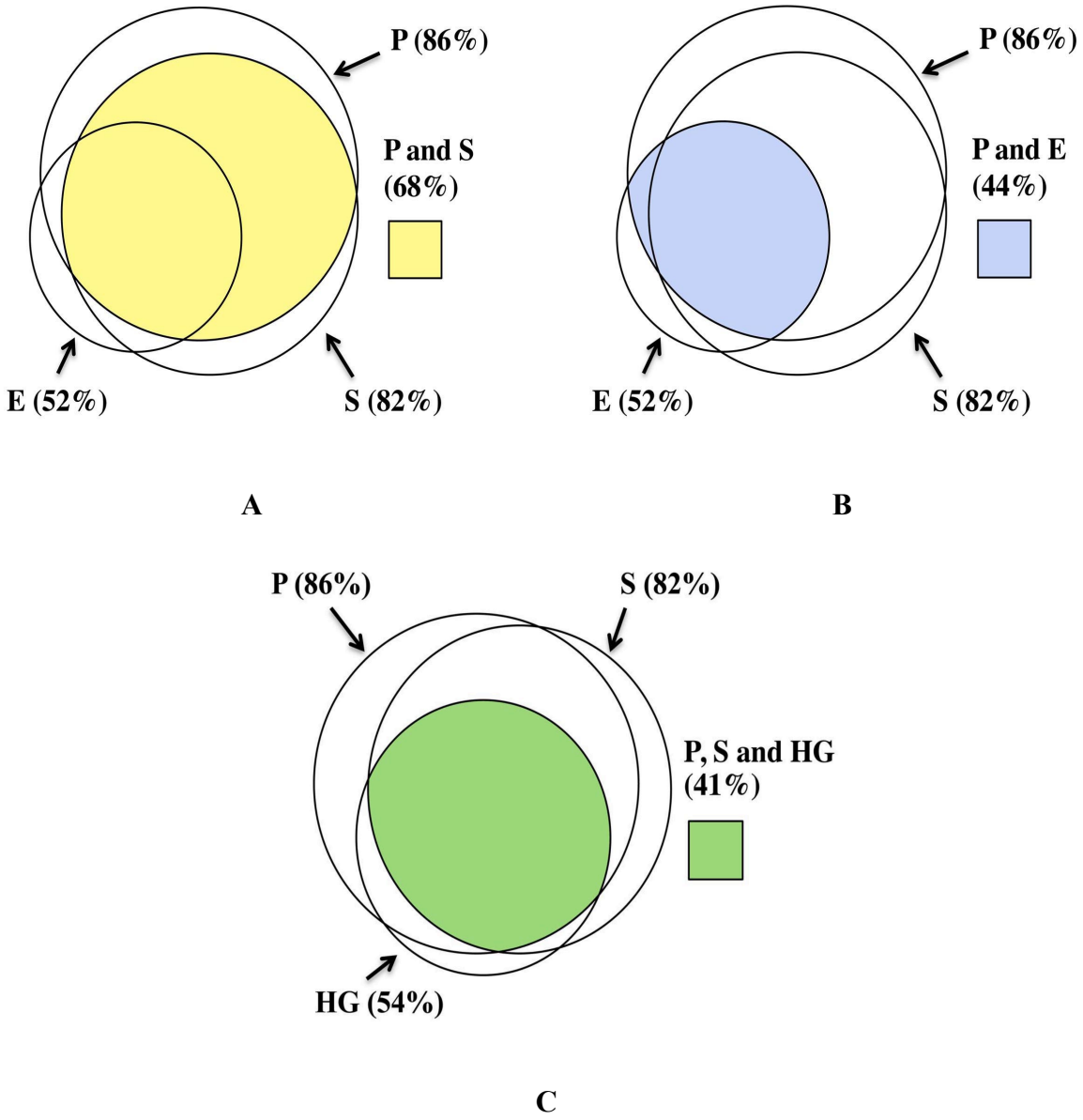


Figure 16 Venn diagrams illustrating combinations of features in ductal adenocarcinomas. **A)** In 99 % papillary architecture (P), stratification of nuclei (S) or elongated nuclei was seen. P and S were combined in 68 % of cases. **B)** In 44 % P was combined with elongated nuclei **C)** In 41 % a combination of P, S and high-grade features (HG) was seen. *Seipel et al. 2014*

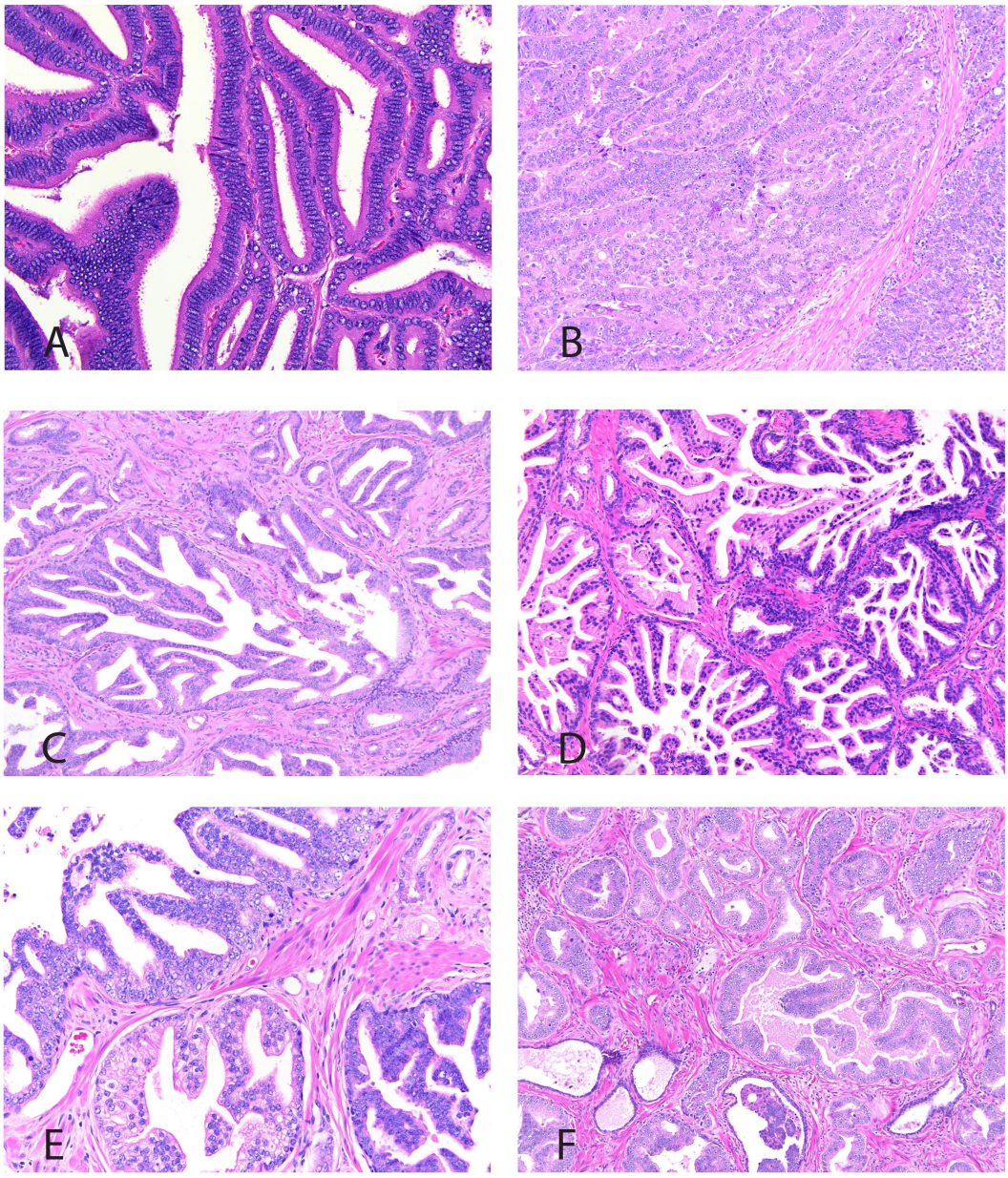


Figure 17 A) Consensus for ductal adenocarcinoma (DAC). Glandular pattern, pseudostratified epithelium with tall columnar cells and abundant cytoplasm. Nuclei are elongated with high-grade features. Hematoxylin and eosin, H&E, 20x lens magnification **B)** Consensus for DAC. Solid pattern and high-grade nuclear features. H&E, 10x **C)** Consensus against DAC. IDC-P lacking the tall, columnar epithelium seen in DAC. Rounded nuclei are seen H&E, 10x **D)** Consensus against DAC. IDC-P lacking the tall, columnar epithelium seen in DAC. Rounded nuclei are seen. H&E, 20x **E)** Consensus against DAC. HGPIN with less complex architecture than typically seen in DAC. Nuclear features are not as high grade. H&E, 20x **F)** Consensus against DAC. Acinar adenocarcinoma with small gland pattern. H&E, 10x. *Seipel et al. 2014*

4.3 PAPER III: DAC SHOWS A MORE AGGRESSIVE PHENOTYPE THAN ACINAR ADENOCARCINOMA

Few studies have examined the immunohistochemical features of DAC compared to acinar adenocarcinoma, even though DAC is considered more aggressive and the diagnosis is based on morphology alone (58, 138, 139). IHC is routinely used for the diagnosis of acinar adenocarcinoma and efforts have been made to identify antibodies of prognostic significance (140). We aimed to examine several types of markers including prostate-specific markers, cell-cycle related markers, oncogenes, hormones and hormonal receptors, cytokeratins and intestinal markers. To correctly identify subtypes of prostate cancer is important as clinical behavior varies according to morphotype.

Prostate specific markers were chosen as a patchy PSA positivity is sometimes seen in DAC and there have even been reports on PSA negative cases (21, 54). Hormone receptors were added because of the presumed origin of DAC, a Müllerian tissue rest, hence a female tissue, which should stain positive for estrogen receptor. Intestinal markers were included as DAC may mimic a gastrointestinal adenocarcinoma. Ki-67 was included as DAC seem to behave more aggressively than matched acinar adenocarcinoma and we wanted to investigate whether this was also reflected in the proliferation index. For the same reason several cell-cycle specific markers were also added. We hypothesized that cytokeratin expression may differ in DAC tumors located close to the urethra due to the gradual change from the CK7/CK20 negative acinar adenocarcinoma of the prostate to the CK7/CK20 positive urothelium.

In a TMA 60 DAC cases were matched by GS against 46 acinar adenocarcinomas and stained with immunomarkers. Five out of 28 markers were differentially expressed in DAC vs. acinar adenocarcinoma; HMWCK, p16, Ki-67, CK20 and p53 *Figure 18*. Of the intestinal markers, CEA was positive in one DAC case and villin in two.

Acinar adenocarcinoma is typically PSA and PSAP positive, CK7/CK20 negative and does not stain for basal cell markers such as p63 or HMWCK (58). PSA staining may be weak or patchy in specimens of high GS (61, 141) and may be weak, focal or completely negative in DAC. A distinct focal CK20 staining was observed more often in DAC than in acinar adenocarcinomas. There are descriptions of CK20 staining in acinar adenocarcinomas of high GS but more than 50 % staining is rarely seen (61). The hormone receptor status of DAC was similar to that of acinar adenocarcinoma. Three markers support the more aggressive nature of DAC compared to acinar adenocarcinomas; Ki-67, p16 and p53. Several papers have suggested Ki-67 as an independent prognostic marker (142, 143). We found that DAC had a higher labeling index than grade matched acinar adenocarcinomas with a mean labeling index of 9.2 % and 2.6 %, respectively ($p=<0.001$). This supports findings in previous studies and the view that DAC is an aggressive subtype. p16 is a tumor suppressor protein inhibiting the interaction between cyclin D1 and CDK 4 or 6, thus leaving the cell in G1 arrest. It is thought to play a role in cancer progression, poor outcome and tumor recurrence (144, 145).

The debate of whether to call DAC a distinct subtype of prostate cancer or merely a variant of acinar adenocarcinoma is ongoing, however, our study supports the view of DAC as an aggressive subtype, supported by the expression of Ki-67, p53 and p16 and its unique morphological features.

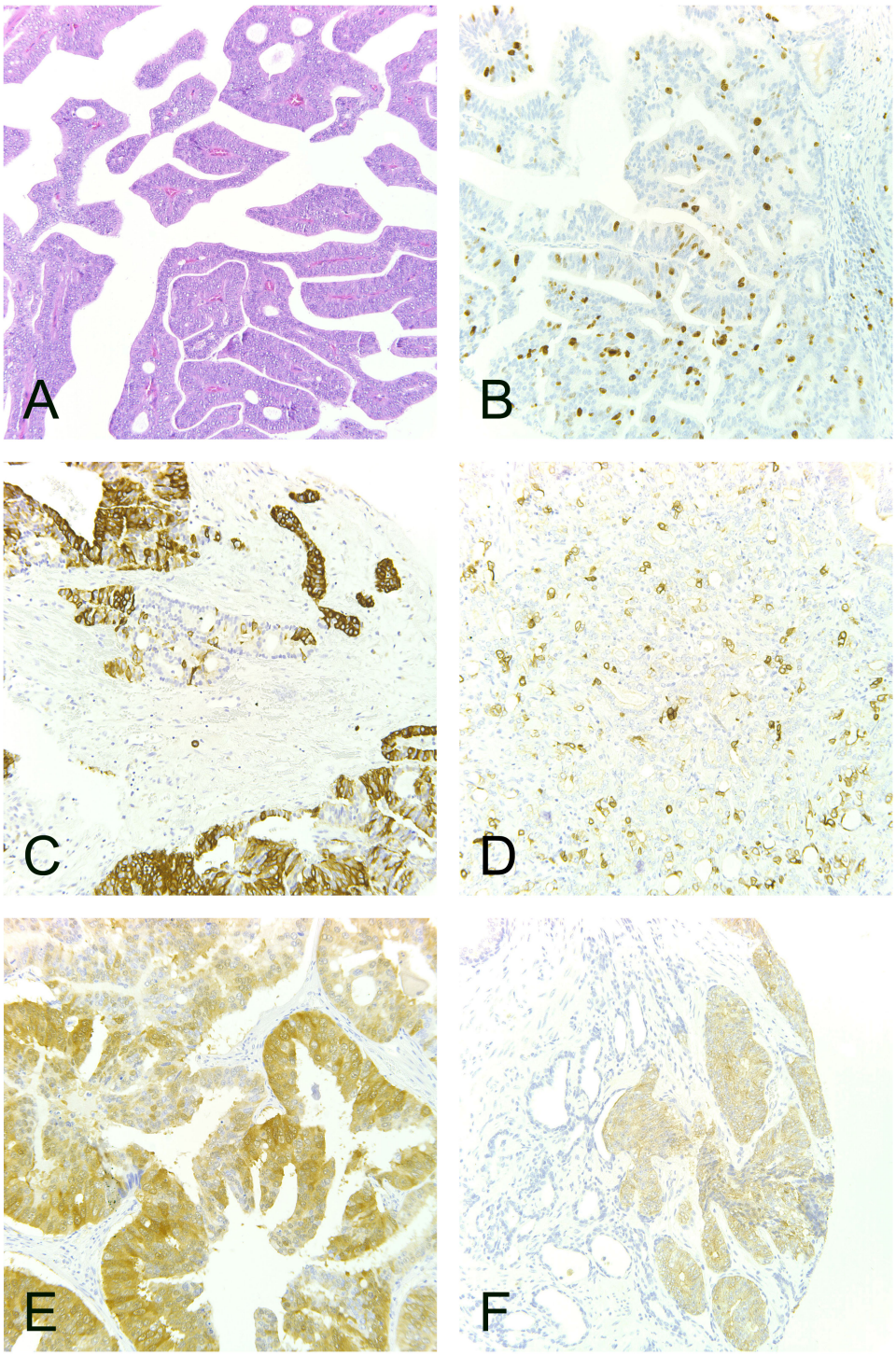


Figure 18 A) Ductal adenocarcinoma of the prostate with papillary architecture and tall, columnar epithelium. Hematoxylin and eosin, 10x lens magnification B) DAC stained with Ki-67 showing high labeling index. H&E, 20x C) DAC stained with CK20 showing a patchy positive staining pattern. H&E 20x D) Acinar adenocarcinoma stained with CK20 also showing a patchy positive staining. H&E 20x E) DAC stained with p16. H&E 20x F) DAC stained with villin, one of two positive cases. H&E 20x. *Seipel et al. 2014*

4.4 PAPER IV: PROSTATE SPECIFIC MARKERS SHOULD BE ADDED IN MEN WITH ADENOCARCINOMAS OF UNKNOWN ORIGIN EVEN IF THE TUMOR MORPHOLOGY SUGGESTS A NON-PROSTATIC ORIGIN

To diagnose the site of origin of a metastatic adenocarcinoma has clinical implications as today's cancer therapies are increasingly organ specific. IHC is a cheap diagnostic tool compared to radiology, surgical procedures or endoscopy as these modalities are not only expensive but time-consuming, inconvenient and may be unsuccessful (146). IHC and histopathologic examination are the key to optimal patient management as they shorten time to diagnosis while at the same time being cost-effective (147). Our aim was to compare DAC to adenocarcinomas of other origin and suggest a use of IHC to establish the tissue origin at a metastatic site. Although endometrial adenocarcinoma is not a differential diagnosis to prostate cancer it was included because of the historical presumed origin of DAC from Müllerian duct remnants (16).

While metastases from prostate cancer are typically seen in lymph nodes, bone, lung, liver, pleura and adrenals, DAC also metastasizes to unusual locations such as the testis and penis (92, 93, 148, 149). When encountered at a metastatic site, DAC tumors may resemble adenocarcinomas of non-prostatic origin, thus making diagnosis challenging. Colorectal cancers may also extend directly into the prostate, mimicking DAC by also displaying tall, columnar epithelium and complex glands (150, 151). A combination of prostate specific markers and intestinal markers is preferably used for diagnosis, including CDX2, villin and β-catenin (97). We found all colorectal adenocarcinomas and nine DAC to be CDX2 positive and two DAC cases also stained positively for villin. However, all colonic adenocarcinomas were negative for all prostate specific markers. Separating DAC from colorectal adenocarcinoma may not only be difficult in the prostate but also in the lung, DAC in fact being the second most common type of prostate cancer metastasizing to the lung (91). A plot showing the proportion of staining for each individual organ is seen in *Figure 19*.

Adenocarcinomas of the urinary bladder are best identified using a combination of markers such as GATA3, CK20, p63 and HMWCK (152). We noted overlap in CK20 staining while p63 was negative in all DAC cases but positive in a majority of urothelial adenocarcinomas. HMWCK was less often expressed in DAC than in urothelial cancer. Nevertheless, there is an overlap between the two types and prostate-specific markers as well as GATA3 should be added to determine the site of origin.

Our study supports previous studies claiming DAC to be a tumor of prostatic origin, staining negatively for ER, PAX-8 and progesterone receptor and positively for prostate specific markers PSA, PSAP and prostein (24, 52). The International Society of Urologic Pathology (ISUP) recommend combinations of markers to establish tissue origin and recommend a combination of PSA, PSAP, prostein and NKX3.1 to establish a prostatic origin (97).

Although we have shown that DAC has a higher Ki-67 labeling index than grade matched acinar adenocarcinomas, it was still lower than in other adenocarcinomas included in our study. There were overlap between tumor types but a high proliferation rate may be useful to distinguish DAC from adenocarcinomas of non-prostatic origin.

A limitation of our study is that few cases of each tissue type were included, which may have affected the power to detect differences between the organs. Biopsies from metastatic DAC are rare so all tissues were harvested from primary tumors. As we used primary tumor tissue from DAC, we also used primary tissue from the other organs.

In summary, the combination of prostate specific markers PSA, PSAP and prostein likely confirms the prostatic origin. In men with cancer of unknown origin the threshold for applying prostate specific stains to verify a possible prostatic origin should be low, even when the morphology suggests that the tumor is non-prostatic.

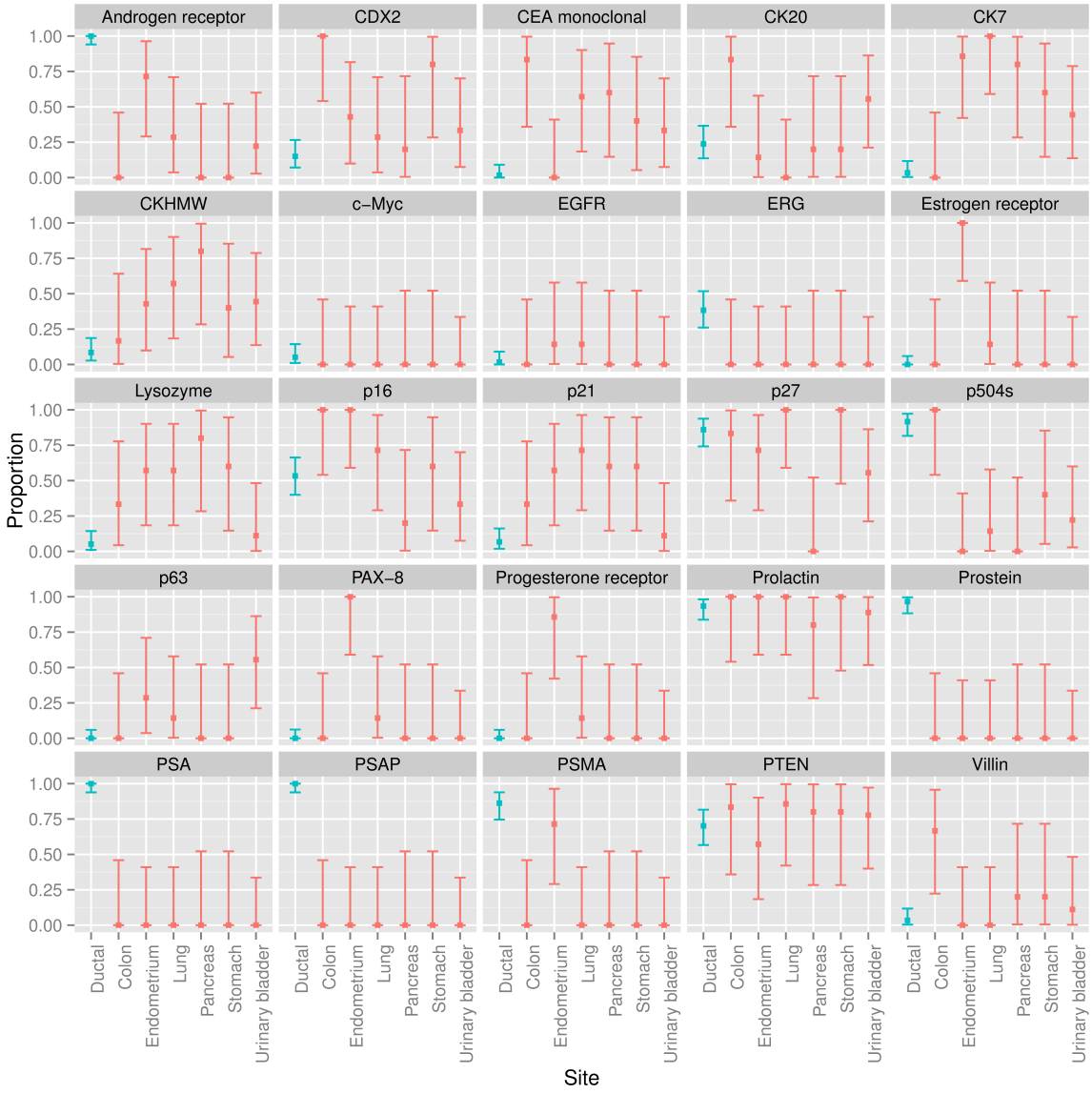


Figure 19 Plot showing the proportion of positive staining presented by marker and individual organ. *Seipel et al. 2016*

4.5 PAPER V: THE GENETIC PROFILE OF DAC IS SIMILAR TO THAT OF ADVANCED AND METASTATIC PROSTATE CANCER

Despite the recent progress and diminishing cost of whole genome sequencing, little is known about the genetic profile of DAC compared to acinar adenocarcinomas. Understanding the different geno- and phenotypes of prostate cancer is crucial as we move towards personalized cancer medicine.

We aimed to examine the genetic profile of DAC in search for genetic properties accounting for its aggressive biological behavior. DNA was sequenced using low-pass sequencing for copy-number alterations (CNA), mutations and indels. We also compared the fraction of genome affected by copy-number alterations in DAC to primary acinar adenocarcinomas, as the fraction of affected genome is associated with poor prognosis (153, 154). We found that the fraction of genome affected by CNA in DAC was similar to that of GS 8-9 tumors *Figure 20*. The genetic alterations varied in the eleven cases, some showing several genetic alterations while others showed alterations in single genes only *Figure 21*.

Two DAC cases had frameshift indels in APC, two had TP53 mutations and two had FOXA1 mutations. FOXA1 is a member of the AR signaling pathway, which is often disrupted in prostate cancer and a main therapeutic target. The signaling pathway may be disrupted in AR itself but commonly through members of the pathway such as FOXA1 or SPOP. SPOP mutations are possible driver events in prostate cancer and are seen in both localized and metastasized prostate cancer (68). If mutated, FOXA1 represses androgen signaling, thus promoting tumor growth (77).

Prostate cancers may be classified into genetically different subgroups defined by mutations in SPOP, FOXA1, IDH1 and ETS fusions to name a few (155). The ETS transcription factor family is well known in prostate cancer, primarily through the well known TMPRSS2-ERG fusion (70). Other members include ETV1 and ETV5, both associated with metastatic disease and described as clonal events in prostate cancer (156). We identified two cases with ETV1 and/or ETV5 amplifications. Interestingly, ETS rearranged tumors may also show lesions in the PI3K and TP53 signaling pathways, which was observed in four of our cases.

TP53 is the most commonly mutated gene in all human cancers (84). One case showed a germline mutation in BRCA2, which is associated with the highest risk of developing prostate cancer known to date (157). Copy-number altered genes included deletions in APC, CHD1 and HDAC2, results that may be associated with treatment response to PARP inhibitors (158).

ERG negativity is more common in DAC than in acinar adenocarcinomas (75, 159) and is linked to MAP3K deletions, MAP3K acting as a tumor suppressor in prostate cancer (160). Deletions are associated with early biochemical recurrence, advanced tumor stage, lymph node metastases and a high GS, all are features seen in DAC (114). Also common in metastatic tumors are CHD1 deletions, which were also seen in two of our DAC cases (77, 84).

Other alterations supporting the aggressive behavior of DAC tumors are BDH1 amplifications associated with a high GS and androgen independent prostate cancers (161), knock-down of SMYD3 attenuating the malignant phenotype of prostate cancer (162), expression of genes associated with epithelial to mesenchymal transition such as PTPRN2, facilitating spread of cancer cells (163) and alterations in tumor suppressor APC, altering the Wnt pathway (164).

In summary, our study shows that DAC has a genetic profile similar to that of advanced and/or metastatic prostate cancer and gives a possible genetic explanation for the aggressive biological behavior.

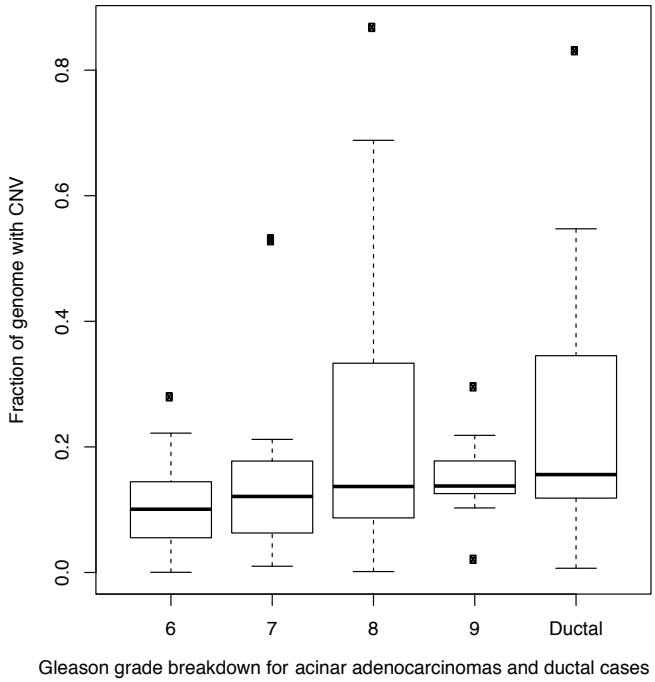
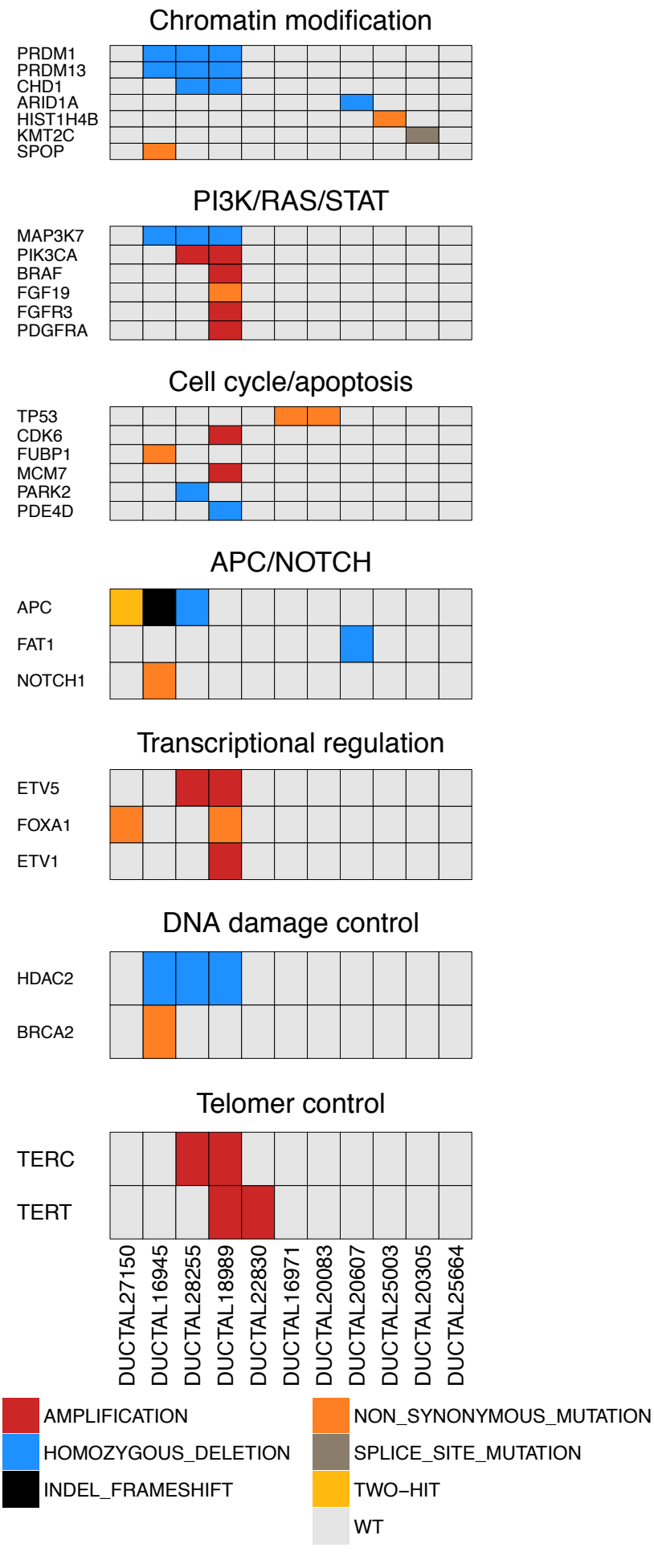


Figure 20 Fraction of the genome altered by copy-number alterations. Y-axis; fraction of the genome altered. X-axis; Gleason score 5-9 acinar adenocarcinomas and the eleven ductal adenocarcinomas. *Seipel et al. 2016*

Figure 21 (as seen on next page): Genes affected by mutations and copy-number alterations. Genes were grouped according to the core pathway affected. In instances with few genes assigned to individual pathways, data were aggregated into larger groups. Y-axis; gene names. X-axis; individual tumor IDs. Mutational data is missing from DUCTAL22830, DUCTAL28255, DUCTAL25664, DUCTAL20607 due to low library complexity. *Seipel et al. 2016*


Chromatin modification
PRDM1
PRDM13
CHD1
ARID1A
HIST1H4B
KMT2C
SPOP
PI3K/RAS/STAT
MAP3K7
PIK3CA
BRAF
FGF19
FGFR3
PDGFRA
Cell cycle/apoptosis
TP53
CDK6
FUBP1
MCM7
PARK2
PDE4D
APC/NOTCH
APC
FAT1
NOTCH1
Transcriptional regulation
ETV5
FOXA1
ETV1
DNA damage control
HDAC2
BRCA2
Telomer control
TERC
TERT
DUCTAL27150
DUCTAL16945
DUCTAL28255
DUCTAL18989
DUCTAL22830
DUCTAL16971
DUCTAL20083
DUCTAL20607
DUCTAL25003
DUCTAL20305
DUCTAL25664
AMPLIFICATION
HOMOZYGOUS_DELETION
INDEL_FRAMESHIFT
NON_SYNONYMOUS_MUTATION
SPLICE_SITE_MUTATION
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5 CONCLUDING REMARKS

DAC is more aggressive than acinar adenocarcinoma when defined as a tumor with tall, columnar epithelium with stratified, elongated or oval, high-grade nuclei and a papillary, cribriform and/or glandular architecture. Cancers with only stratified, high-grade nuclei should not be considered DAC. Patients with DAC are more likely to present with extraprostatic extension, positive surgical margins and seminal vesicle invasion. Necrosis, stromal invasion versus intraductal spread, percentage of DAC tumor or localization does not seem to have clinical significance.

The diagnosis of DAC is not as well-defined as previously thought. Papillary architecture in combination with nuclear stratification support the diagnosis, while nuclear and cellular features are less important. The most common differential diagnoses to DAC are IDC-P and HGPIN.

There is some overlap in the immunohistochemical expression of DAC and acinar adenocarcinoma. However, DAC shows greater expression of Ki-67, p53 and p16 than acinar adenocarcinoma and the differences between these subtypes are consistent with DAC being biologically more aggressive.

The combination of prostate specific markers PSA, PSAP and prostein likely confirms the prostatic origin of a DAC tumor if encountered in a metastatic deposit. In men with cancer of unknown origin, the threshold for applying prostate specific stains to verify a prostatic origin should be low, even when the morphology suggests that the tumor is in fact non-prostatic.

The genetic profile of DAC is similar to that of advanced and/or metastasized prostate cancer, which may explain its aggressive biological behavior.

Immunohistochemistry, morphological features, clinical course and genetic profile all support the current view of DAC as a distinct clinical entity.

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Key contributors and co-authors

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