DNA content based flow sorting combined with genomic high-resolution profiling in the context of the development of castration resistance in prostate cancer

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Statement

This work was performed at the Institute for Pathology in close collaboration with the Department of Urology of University Hospital Basel and the Translational Genomics Research Institute (TGen) in Scottsdale Arizona.

My thesis consists of an introduction covering several aspects related to my work followed by a result section which is composed of two peer-reviewed publications as a first author. Major findings and their implications are discussed in the following chapter. Literature References are provided after each chapter separately. Also, figures are numbered within chapters and a list of all figures is provided for each chapter. In the appendix a CV, as well as a list of publications and congress activities given.

Acknowledgments

I can not overstate the gratitude for all the people that continuously supported me during this admittedly long endeavor of writing this thesis.
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Abbreviations

ADT	androgen-deprivation therapy
FACS	fluorescence-activated flow sorting
FFPE	formalin-fixed and paraffin-embedded
FFE	fresh frozen
FISH	fluorescent in-situ hybridization
IHC	immunohistochemistry
PCa	prostate cancer
TMA	tissue microarray

Summary and Importance of This Work

Prostate cancer (PCa) is a common malignant disease in men. Due to increased screening efforts, PCa is often diagnosed in early stages and patients are therefore eligible for curative surgical approaches. Yet many patients, mostly due to age or extended disease, are treated primarily using androgen deprivation therapy (ADT). While this approach usually leads to disease stability or even reduction, unfortunately, this effect is usually not sustainable and tumors acquire resistance toward ADT commonly referred to as castration resistance. The resulting tumor progression usually leads to long term disease progression with increased morbidity and eventually the death of the patient.

Among other factors, changes in the tumor genome may confer resistance towards ADT, similar to what has previously been demonstrated for therapy resistance in other malignancies. This thesis focuses on different translational approaches aimed at tracking down the phenotypic and genomic changes occurring in PCa upon ADT.

In the first paper included in the thesis (*"ERG rearrangement and protein expression in the progression to castration-resistant prostate cancer"*), we explored one of the prototypic genomic alterations, namely the *TMPRSS2-ERG* gene fusion, and its role in the development of castration-resistant PCa. We used a tissue microarray (TMA) that comprised clinical samples of PCa prior to and after ADT using classical molecular analysis methods such as immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Interestingly we were able to identify a PCa subgroup that appeared to be associated with a partial shutdown of the androgen receptor (AR)-driven signaling axis.

Further, the notion of tumor evolution and intra-tumor heterogeneity has spawned an increased interest in recent years. The advent of high resolution, high throughput genomic analysis methods have provided the necessary tools to decipher the clonal complexity of tumors. This led to an improved understanding of the clonal evolution of malignant diseases. While most computational approaches focus on the bulk of the tumor, we developed and refined a combined approach relying on prior flow sorting of cytogenetically distinct tumor subpopulations and subsequent molecular and computational analyses to obtain a different perspective on intra-tumor heterogeneity.

The resulting findings are summarized in the second paper entitled *"Delineation of human prostate cancer evolution identifies chromothripsis as a polyclonal event selecting for FKBP4 driven castration resistance"*. To the best of our knowledge, this was the first paper describing tumor populations exhibiting features of chromothripsis, the catastrophic shattering, and the reconnection of chromosomes, that are related but distinct in the different subpopulations. This gains further clinical

significance as in the process the tumor acquired a new amplification of a chaperone protein, namely FKBP4, which is involved in AR signaling and highly correlates with patients' survival. Taken together, these findings, therefore, might open new doors in the development of prognostic and therapeutic tools.

Introduction

I. Cancer is a Disease of Genomic Instability

The second half of the last century brought significant advances in the understanding of the biology of cancer and tumorigenesis; cancer has therefore increasingly been understood as a disease of genomic instability and consequent altered behavior at a cellular and tissue level [1]. Especially in the last decade, the advent of high throughput genomic analysis (e.g. comparative genomic hybridization and next-generation sequencing) has now shed even more light on the nature of these drivers and importantly the dynamics of their acquisition.

Carcinogenesis involves the accumulation of changes in the underlying genetic code of the cell. Genome integrity constitutes a vital part of survival and over the course of evolution, the cellular apparatus has therefore been equipped with a multitude of molecular measures to ensure its integrity, such as DNA mismatch repair and DNA damage checkpoints [2]. Nevertheless, these changes can occur spontaneously, upon replication or provoked by mutagenic agents such as radiation or carcinogens like tobacco smoke. Their effect is often conferred through the introduction of double-strand breaks and failed DNA repair [3] resulting in small- or large- scale genomic alterations.

I.1 Small-scale Alterations

Common small-scale mutations are substitutions whereby a single nucleotide is replaced by an alternative base. Due to the chemical similarity, this usually results in the substitution of a purine for a purine (Adenine to Guanine or vice versa) or a pyrimidine for a pyrimidine (Cytosine to Thymine or vice versa). In addition, small scale nucleotide (1 - 50) deletions or insertions are found, so-called indels. The number of genomic small-scale mutations found in one tumor can vary from only a handful (e.g. 10-20) to a multitude (up to thousands) [4].

The majority of genomic changes found in cancer are so-called ‘passengers’, which do not exert a relevant modification of the biological behavior. But scientific research has also led to the discovery of multiple genomic ‘drivers’ that fuel the development of malignant disease [4]. As cancer therapy moves further into the direction of targeted therapies it is of utmost importance to correctly distinguish ‘driver’ from ‘passenger’ events.

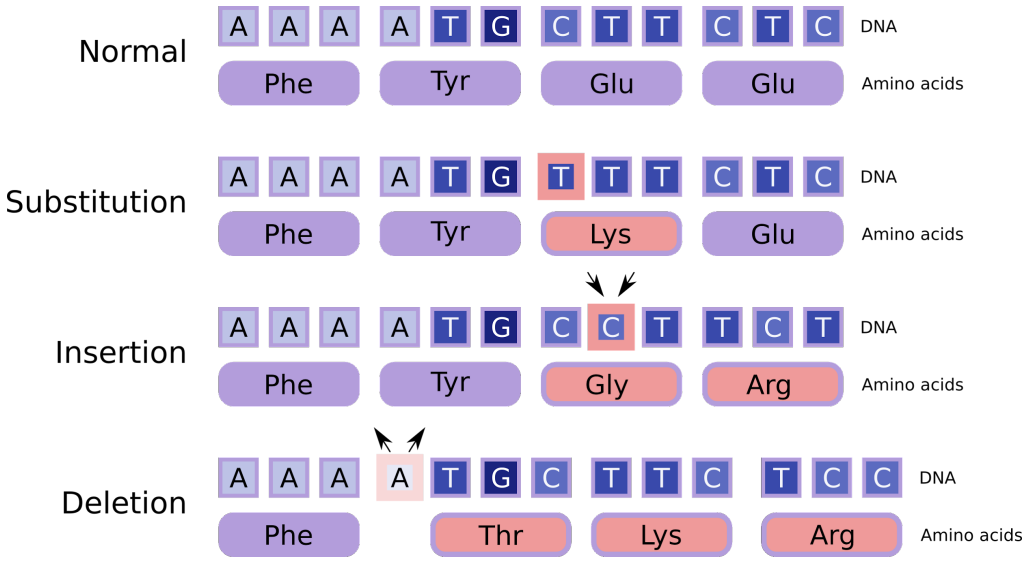


Figure 1: Types of genomic small scale alterations adapted from [5]

I.2 Large-scale Aberrations

Large-scale aberrations, on the other hand, encompass alterations of larger strips of DNA including the karyotype as a whole. Amplifications, defined by the overrepresentation of a genomic locus by an integer multiple. This might result in overexpression of genes in the affected genomic region, potentially harboring oncogenes such as *c-MYC* [6]. Deletions, on the other hand, refer to missing genomic loci. This might lead to the missing expression of affected genes and potentially also tumor suppressor genes such as *PTEN*[7]. Both are well known pro-oncogenic alterations. Additionally, the translocation of genomic regions within or between chromosomes can lead to the interruption of the function of the targeted regions where the translocated DNA is inserted or to the formation of novel fusion genes with potential different transcriptional activity or biological function. One example of such a phenomenon is the TMPRSS2-ERG fusion gene, which is commonly found in prostate cancer [8]. The extreme case of large-scale genomic aberrations whereby whole chromosomes are lost or present in non-exact multiples of copies greater than the physiological two copies are referred to as aneuploidy, while the multiplication of the whole set of chromosomes is referred to as polyploidy [9]. Aneuploidy itself is regarded as an essential hallmark of cancer as up to 90% of all solid tumors show signs of chromosome aberrations [10]. Defects in mitotic spindle assembly lead to chromosome missegregation during mitosis. Children, suffering from mosaic variegated aneuploidy (MVA), produce random aneuploidies in the body's cell. This, in turn, leads to the occurrence of leukemia and unusual solid tumor [11].

Research states that nearly all solid neoplasms are associated with large-scale genomic aberrations [12,13] and various studies support the notion of aneuploidy as the major driver of oncogenic transformation in contrast to small-scale mutations [12].

Aneuploidy also shows clinical significance as for example highly aberrant tumors are correlated with a worse outcome in melanoma patients treated with immunotherapy [14]

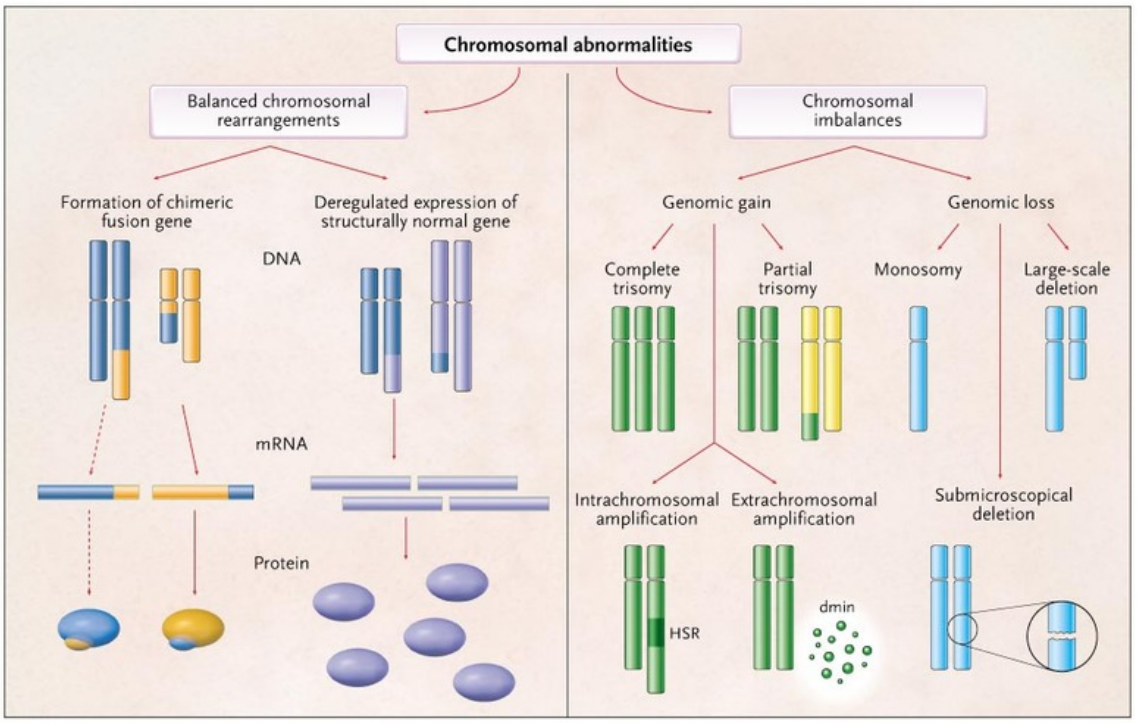


Figure 2: Large scale genomic alterations [13]

I.3 Genomic Instability

Mutation and chromosomal aberrations frequently occur as a consequence of environmental influences such as radiation or oxidative stress [7]. Under normal circumstances, the genomic integrity in every cell is protected and controlled for by built-in cellular machinery. We discriminate between caretakers and gatekeepers. Where caretakers are governing DNA repair and proofreading, implicating genomic stability and low mutation burden (eg. *ATM*, *BRCA2*, and *MLH*). While gatekeepers, on the other hand, orchestrate cell-cycle traverse and possible cell-death in case of chromosomal instability (e.g. *TP53*, *APC*, *CDH1*, *RB1*; or *PTEN*) [15]. Changes in these genes can lead to a higher acquisition rate of genomic aberrations [16]. Prostate cancer is no exception as up to 23% of all castration-resistant tumors exhibit defects in DNA-repair genes [7].

Impaired p53 function leads to sustained cell propagation even in the case of chromosomal instability [17,18]. Cyclin D1 overexpression attenuates the p53/p21 mediated cell cycle checkpoint allowing even tetraploid cells to proliferate [19,20].

While genomic instability appears to be a significant contributor to cancer development it does not only harbor beneficial effects for tumors. In several studies, tumors with an intermediate level of chromosomal instability showed the worst clinical outcome while low and high genomic instability was associated with a favorable outcome [21,22].

I.4 Tumor Evolution

In 1976, Nowell proposed "The clonal evolution of tumor cell populations", a concept mainly based on cytogenetic examination of tumors. He proposed the concept of a common ancestor with a stepwise accumulation of somatic mutations and clonal expansions [23]. This concept resembles the model of along with biologically non-relevant ('passenger') mutations [4]. Some authors even propose the establishment of a whole tumor ecosystem where predation, parasitism, and mutualism between the competing clones and the microenvironment is abundant [24,25]. The clinical importance lays in the fact that it is assumed that upon therapy additional evolutionary stress can be exerted on the tumor and therefore resistant clones can be selected which in turn leads to their outgrowth and the occurrence of relapsed disease. Also, it is thought that the ability for metastatic spreading is only acquired by a few clones and therefore additional insight could be gained comparing the genomic traits of competing clones [4].

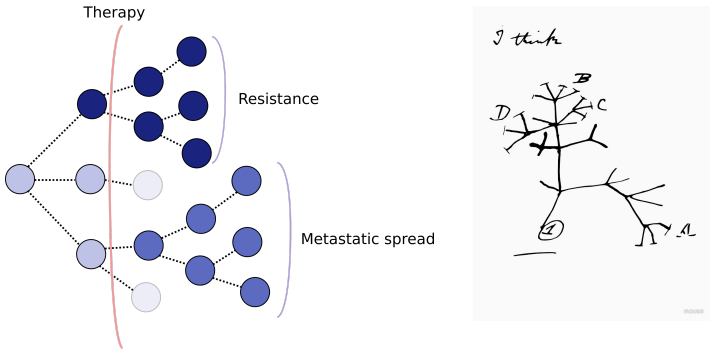


Figure 3: Illustrations of the 'evolutionary tree' (left) as a model of tumor evolution in comparison to the iconical Darwinian 'tree of life' (right) adapted from [4]. Circles represent tumor cell populations.

I.5 Tumor Heterogeneity

Clonal evolution may lead to tumor heterogeneity, a phenomenon that is frequently observed in many tumor types. Morphological studies of tumor cell lines established from murine mouse models have shown distinct tumor cell clones coexisting in the same solid tumor [26]. By leveraging the high throughput capabilities of next-generation sequencing, various studies have demonstrated the genomic tumor heterogeneity at a molecular level [27–31]. By whole-exome multi-region spatial sequencing of different

biopsies from different areas of a renal cell carcinoma, Gerlinger and colleagues were among the first to demonstrate that intratumoral heterogeneity on a genomic level. In this seminal work, 63 to 69% of all somatic mutations were non-congruent in the different locations which highlighted the importance of considering intratumoral heterogeneity in future oncological research [27].

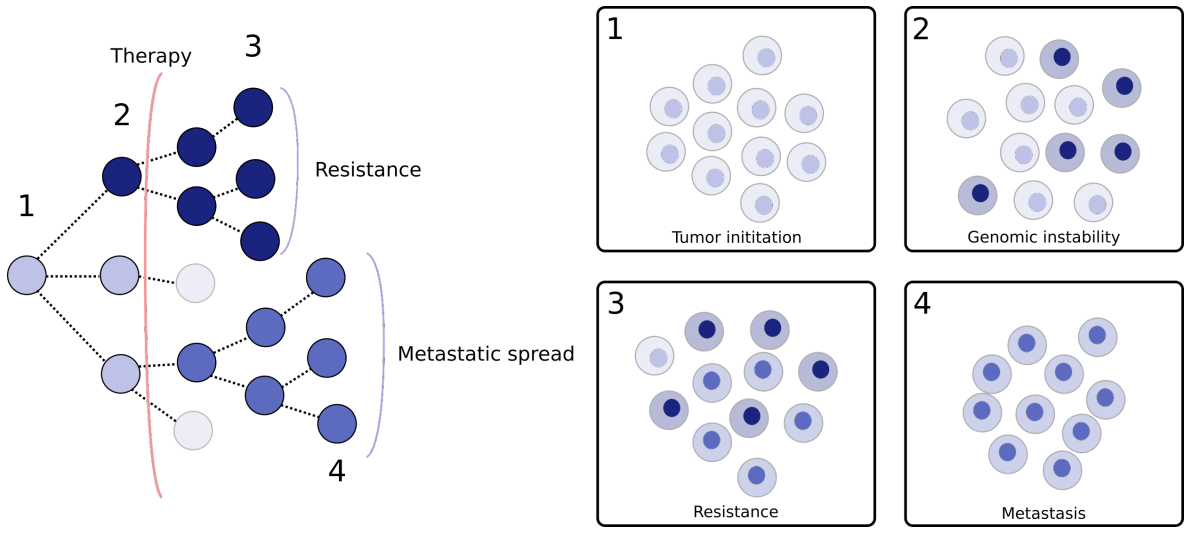


Figure 4: Illustration of intratumoral heterogeneity as it arises through the process of tumor evolution. After tumor initiation (1) novel subclones evolve due to genomic instability (2). Potential environmental factors such as therapies might lead to the selection of subclones (3). Only a small fraction of clones will eventually acquire the capability necessary for metastatic spread. Adapted from [32].

I.6 Genomic Instability, Tumor heterogeneity, and Evolution in Prostate Cancer

Heterogeneity is commonly observed in prostate cancer and has influenced pathological workup and interpretation more than in other solid malignancies. Given that the Gleason score is determined based on the 2 most prominent patterns of differentiation (see Figure 12) it may be regarded as a readout of intratumoral heterogeneity and can directly be linked to prognostic ratings [33,34]. Some authors even found more than 50% of all histological specimens analyzed showed more than three different grades at the same time [35].

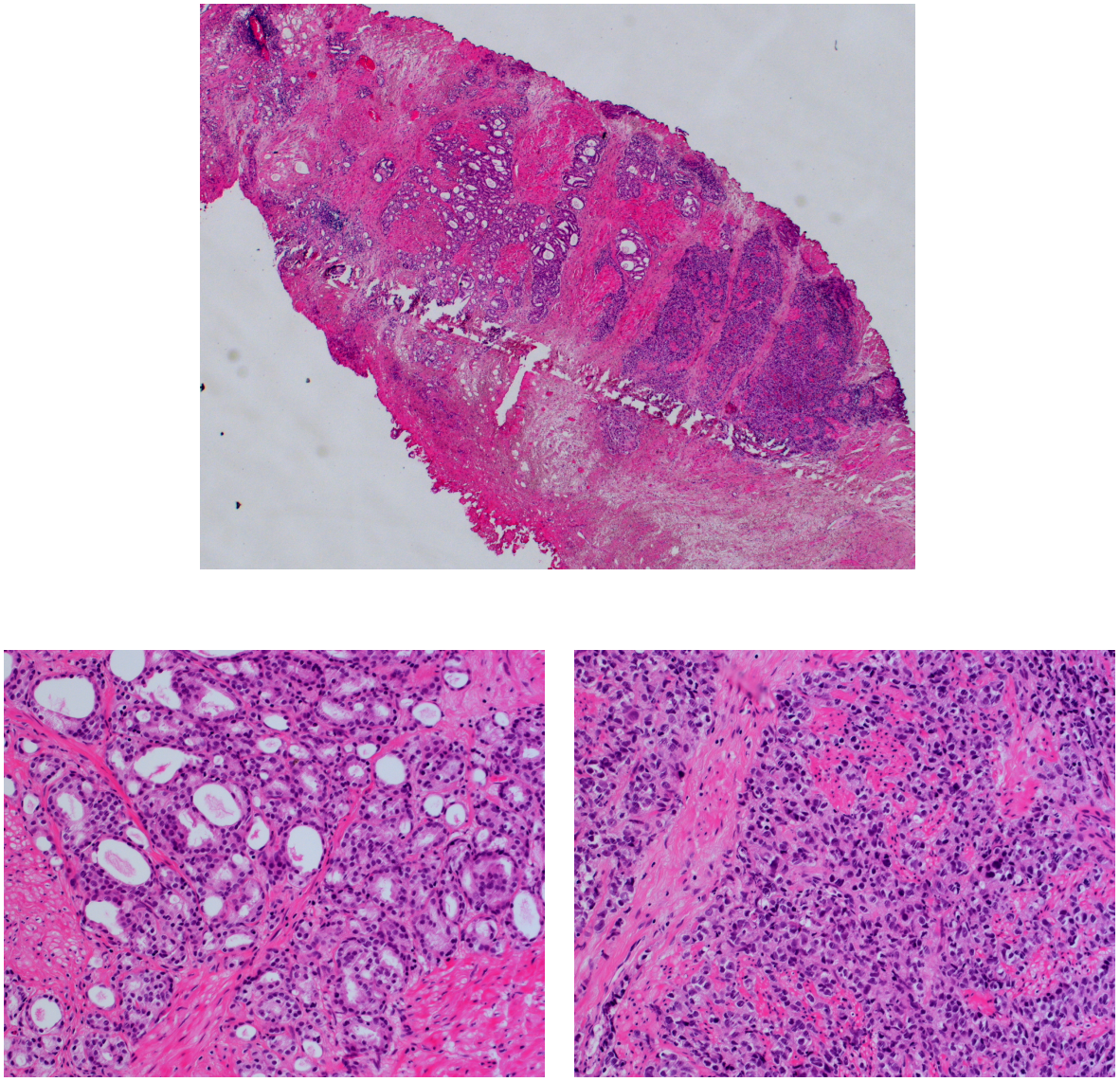


Figure 5: Histological specimen from a palliative transurethral resection showing easily distinguishable areas of differing morphology with distinct gleason patterns in the direct vicinity. (Overview above and close up below; Picture was taken at the Institute of Pathology Basel)

In addition, prostate cancer has been frequently shown to be multifocal as genetically different tumor foci can be found in the same patient's prostate [35,36]. Due to the spatial separation, it is assumed that these distant foci have arisen from independent cancer initiation events and are therefore not clonally related. Brocks and colleagues expanded the concept of intratumoral heterogeneity in prostate cancer and investigated epigenetic profiles of multiregional specimens. This way they were able to show that intratumoral heterogeneity not only exists on mutational but also on an epigenetic level[37].

Nevertheless, in the sense of evolutionary processes it seems that once PCa progresses into later stages, close clonal lines are responsible for metastatic spread [38,39], where selective advantages lead to the

outgrowth of the most aggressive/successful tumor clones, but can largely be influenced by therapeutic interventions changing the evolutionary pressure.

II. Prostate Cancer

As described in the previous section prostate cancer exhibits clear signs of clonal evolution and intratumoral heterogeneity and is also widely recognized as a major health burden in men. In the following section, we will discuss the physiological, pathological as well as the clinical aspects of prostate cancer.

II.1. Epidemiology of Prostate Cancer

Prostate cancer is one of the most prevalent malignancies affecting men worldwide and represents the most frequently diagnosed male solid neoplasm in western countries [40,41]. In 2012 8% of all cancers diagnosed were PCa resulting in 1.1 million people new patients worldwide. And newer numbers from 2018 estimate an age-standardized rate of 1,276.1 per 100'000 men. [42–44].

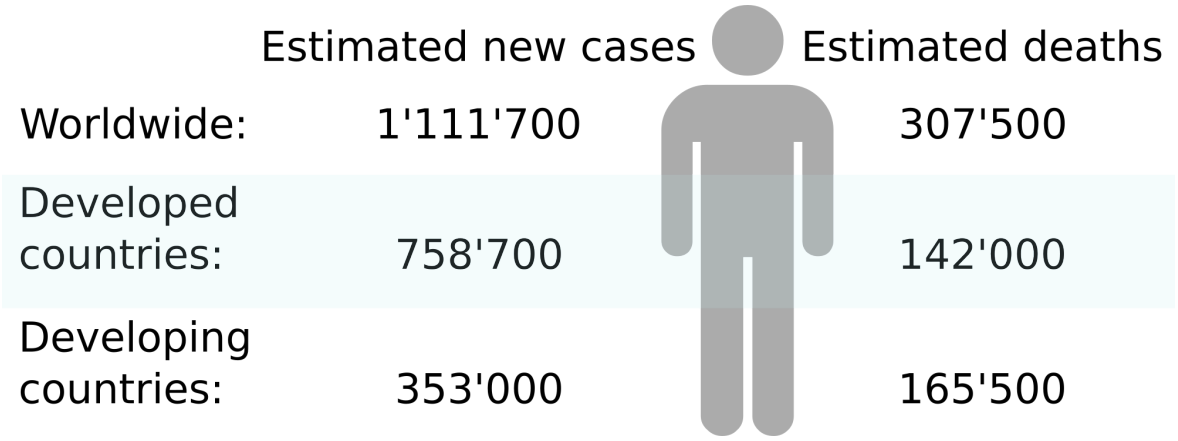


Figure 6: Estimated yearly incidence and mortality of PCa. Adapted from Torre et al. [41]

The incidence of PCa increases with age and is uncommon before the age of 50 years and still sparse before the age of 60 years (Figure 6). In concordance, the economic burden associated with PCa is increasing and accounts for major health care expenditures. Currently, it is estimated that the total economic costs of PCa in Europe exceed 8.43 billion euros [45].

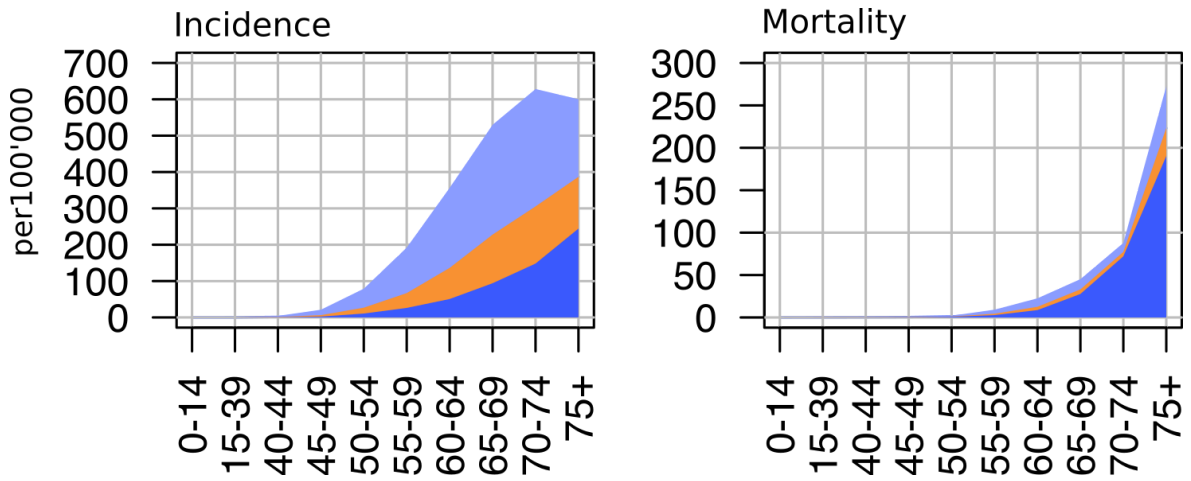


Figure 7: Age-adjusted incidence and mortality rates for PCa showing the correlation with age. Overall incidence rates are markedly lower in less developed countries. ■ *worldwide population;* ■ *high developed countries;* ■ *low developed countries. Adapted from GLOBOCAN data* [40,41,44].

While all risk factors for PCa are still poorly understood, factors such as increasing age, ethnicity, and heredity have been identified [46–48]. In addition, conditions like metabolic syndrome [49] or chronic inflammation has been proposed as a risk factor in promoting the development of PCa [50].

Also, the evidence for dietary influences is rather sparse. Several studies trying to establish a link between diet and PCa have yielded non-significant results. Probably most famous was the Selenium and Vitamin E Cancer Prevention Trial [SELECT] where 35,533 men were included in the study to test Vitamin E, Selenium or the combination to prevent PCa. The did not only fail to prove any preventive effect but surprisingly showed a trend, although not significant, towards higher PCa rates in the Vitamin E group [51]. But also dairy products [44] or lycopene, a member of the carotenoid family mainly present in red fruits, such as tomatoes[52], intake couldn't be associated with prostate cancer risk or prevention.

Based on these findings the European Association of Urology (EAU) currently does not promote any dietary measurements for the prevention of prostate cancer.

As a little side note, evidence exists, that coffee might reduce the risk of developing prostate cancer, even in a dose-dependent fashion. With a decrease in PCa risk of 2.5% (RR = 0.975; 95% CI: 0.957-0.995) for every two cups per day [53].

II.2 Physiology of the Prostate

II.2.1 Anatomy and Function

The Prostate is part of the male reproductive tract responsible to produce main parts of the seminal fluid and situated at the transition of the bladder to the urethra surrounding the later. With its walnut size, it can be divided into several anatomical parts namely the fibromuscular zone, the central zone, the transition zone and the peripheral zone (Figure 8). These anatomical zones are of clinical importance as they show different probabilities in developing the various kinds of prostate-specific diseases; It is indeed widely known and accepted, that benign prostate hyperplasia mainly develops in the transitional zone, while PCa usually starts in the peripheral zone [50].

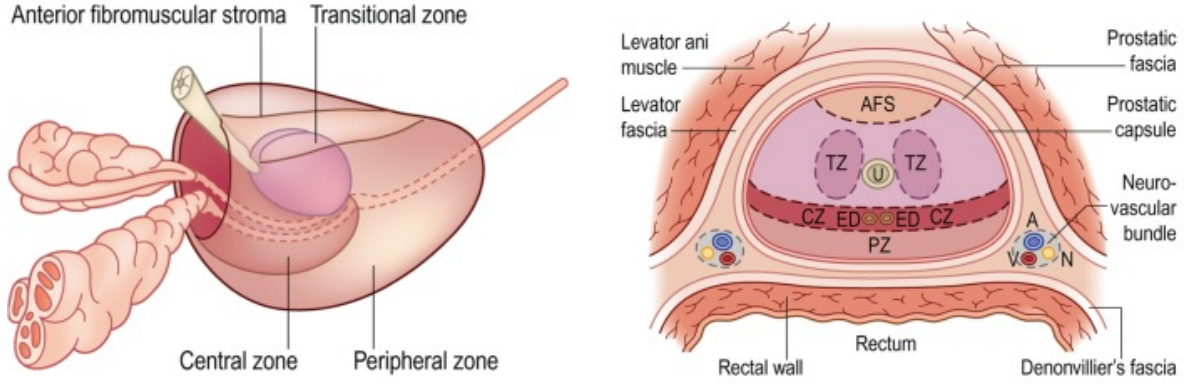
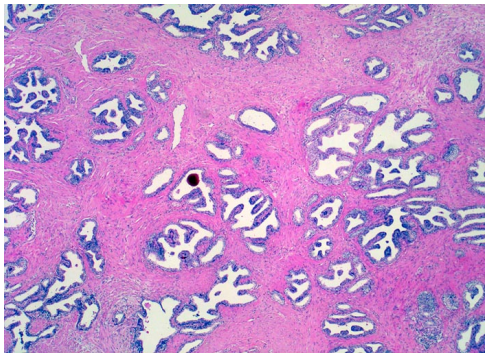


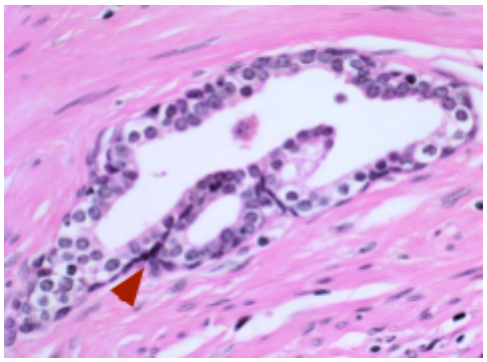
Figure 8: The Anatomical feature of the prostate. Axial and coronal views of the prostate gland and its close anatomical relationships. Zonal model of the prostate. Fascial planes around the prostate. A, artery; AFS, anterior fibromuscular stroma; CZ, central zone; ED, ejaculatory duct; N, nerve; PZ, peripheral zone; TZ, transition zone; U, urethra; V, vein [54].

II.2.2 Histology

The prostatic tissue can be divided into a stromal and an epithelial part, with the stromal compartment being mainly composed of smooth muscle cells, fibroblasts, endothelial cells, and nerve fibers. The epithelial compartment on the other side displays three distinct cell types referred to as basal (CK5+, CK14+, p63+) luminal (CK8+, CK18+) and neuroendocrine cells (ChromograninA+) which are organized in a pseudo stratification surrounded by a basal membrane. Especially the luminal cells are implicated in the prostate fluid production and have been shown to be androgen receptor (AR) positive and therefore sensitive to androgen stimulation [55–57].



Normal prostatic tissue with multiple glands regularly embedded in the stromal compartment.



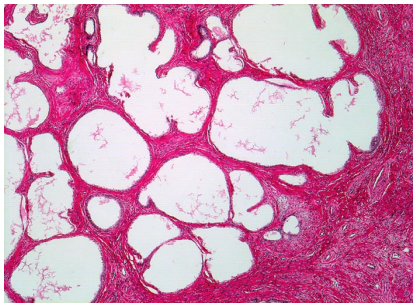
Prostatic gland with cuboidal epithelium and nicely visible basal cell layer (arrow)

Figure 9: Normal prostate

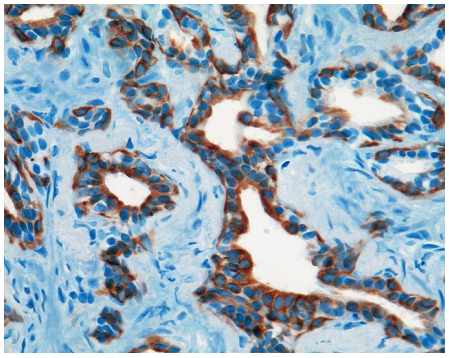
II.3 Histopathology of the Prostate

II.3.1 Benign Prostatic Hyperplasia

Benign prostatic hyperplasia originates from hyperplastic epithelial and stromal cells. It is characterized by a microscopic enlargement of the prostate with mostly myoepithelial characteristics in the histological evaluation. As it usually begins in the transitional zone it commonly leads to obstruction of the urethra and therefore symptoms associated with impairment of bladder voiding.



Hypertrophic fibromuscular stroma with enlarged prostatic glands.



Staining for cytokeratins (1, 5, 10, 14) indicating an intact basal layer (IHC, Antibody CK34betaE12)

Figure 10: Benign prostatic hyperplasia

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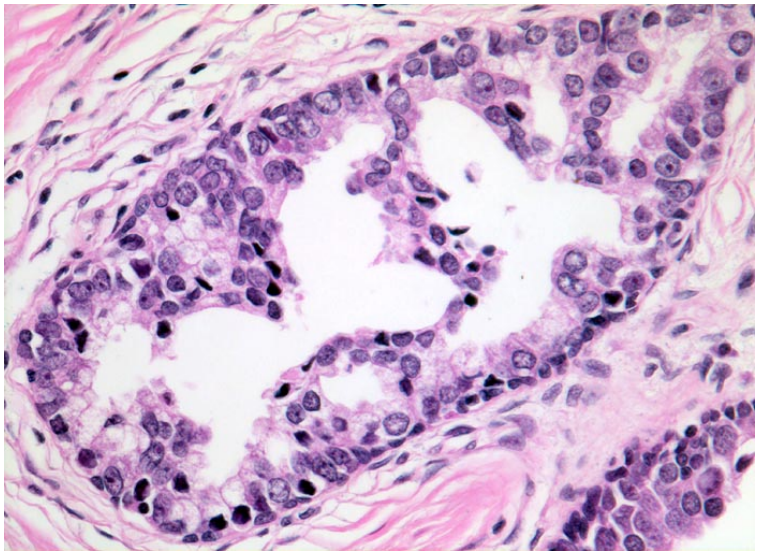
II.3.2 Prostatic Intraepithelial Neoplasia (PIN)

PIN is usually considered to be a pre-cancerous lesion of the prostatic epithelium, with the disruption of the basal layer [59]. This histological feature is of great importance in the pathological workup of PIN as the basal layer can be stained for cytokeratin 5 and 6 or p63 where its absence leads to the identification of PIN [60]. Additionally, PIN cells show changes in their chromatin structure similar to prostate cancer cells but overall lack invasiveness beyond the basal layer that defines PCa. Nevertheless, intraluminal growth can be observed [55]. As assessed by pathologists, PIN can be classified as either low-grade or high-grade PIN. This is of further clinical importance as low-grade, in contrast to high-grade, does not increase the risk for the development of PCa in general. Nevertheless, in the presence of only PIN in a needle biopsy, a further biopsy should be considered as up to 50% show cancerous lesions [61].



Larger glandular duct with epithelial proliferation and enlarged and atypical nuclei with prominent nucleoli but still visible basal cell layer.

Figure 11: Prostatic intraepithelial neoplasia (PIN)

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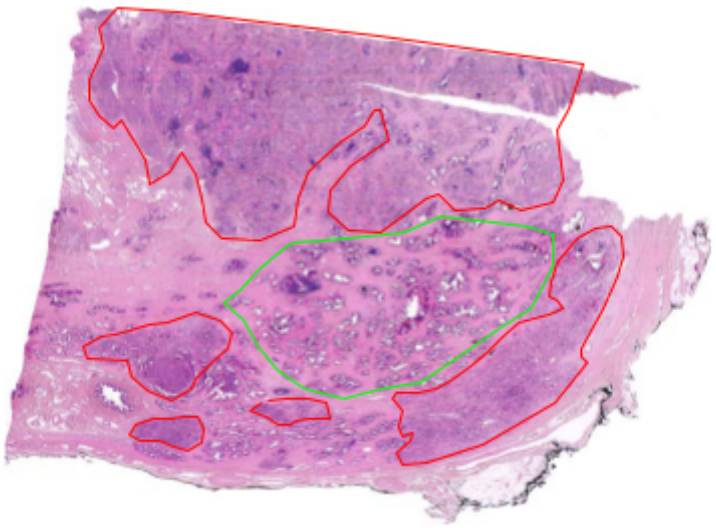
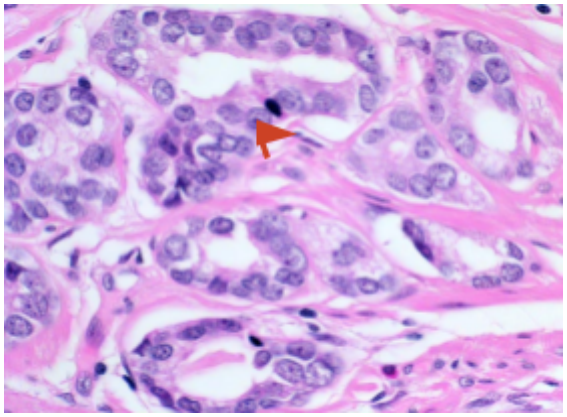
II.3.3 Prostate Cancer

Histopathology of PCa shares features with PIN, such as the irregular shape and chromatin irregularities. Glandular structures are usually denser and with a rather smaller volume. A typical finding is prominent nucleoli, which may also be present in PIN. Prostate adenocarcinoma is by far the most prevalent histological subtype with all the others combined (i.e.neuroendocrine, sarcomatoid carcinoma, squamous and adenosquamous carcinoma, basal cell carcinoma, large-cell neuroendocrine carcinoma, and pleomorphic giant cell carcinoma) constituting only around 5-10%[62].

In biopsy specimens, prostate adenocarcinoma diagnosis can be confirmed by the absence of immunostaining for p63 and cytokeratin 5/14 antibodies, both of which detect basal cells (Humphrey 2007; Grisanzio and Signoretti 2008). In addition, a diagnosis of prostate cancer may be supported by elevated immunostaining for a-methylacyl-CoA racemase (AMACR), a luminal marker that is overexpressed in carcinoma (Luo et al. 2002; Jiang et al. 2005; Humphrey 2007).



Multiple cancerous lesions with smaller glandular structures and sparser interspersed stromal tissue (red) as compared to the normal gland formations (green)



Cancerous gland with cuboidal epithelium but missing basal cell layer and atypical nuclei with prominent nucleoli (arrow).

Figure 12: Prostate cancer
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The most important histological grading of PCa is done using the Gleason's Pattern where different areas of the tumor are graded based on their differentiation (see also Figure 12). In contrast to most solid malignancies where only the highest grade (least differentiated) is reported the Gleason's Score consists of a sum of the two most abundant differentiation patterns. For example, a tumor with a major pattern of moderate differentiation (e.g. Gleason 3) and a minor pattern of poor differentiation (e.g. Gleason 5, in at least 5%) would be reported as Gleason score 3 + 5 = 8 [33,63].

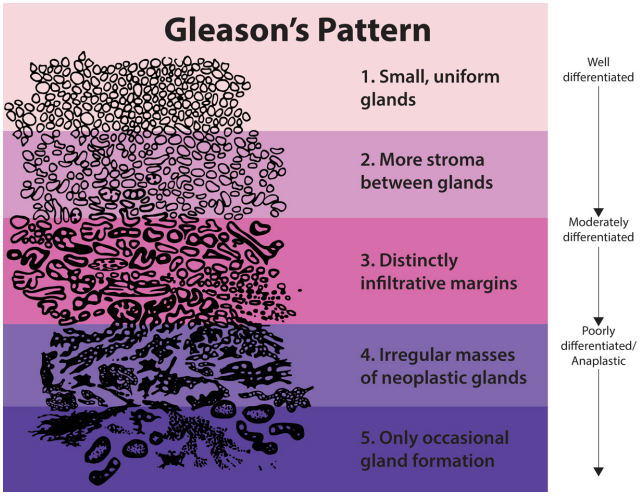

Figure 13: The Gleason grading scheme[64].

In 2013, a new grading system was proposed with five distinct grade groups (Grade Group 1 = Gleason score ≤ 6, Grade Group 2 = Gleason score 3 + 4 = 7, Grade Group 3 = Gleason score 4 + 3 = 7, Grade Group 4 = Gleason score 4 + 4 = 8, Grade Group 5 = Gleason scores 9 and 10) and is now commonly reported alongside the classical Gleason pattern (e.g. Gleason score 3 + 3 = 6 (Grade Group 1)) [65]. The Gleason

Score is of major importance as it has a direct and strong prognostic value (see Figure 13) with higher Gleason Scores being associated with worse prostate cancer-specific survival[66].

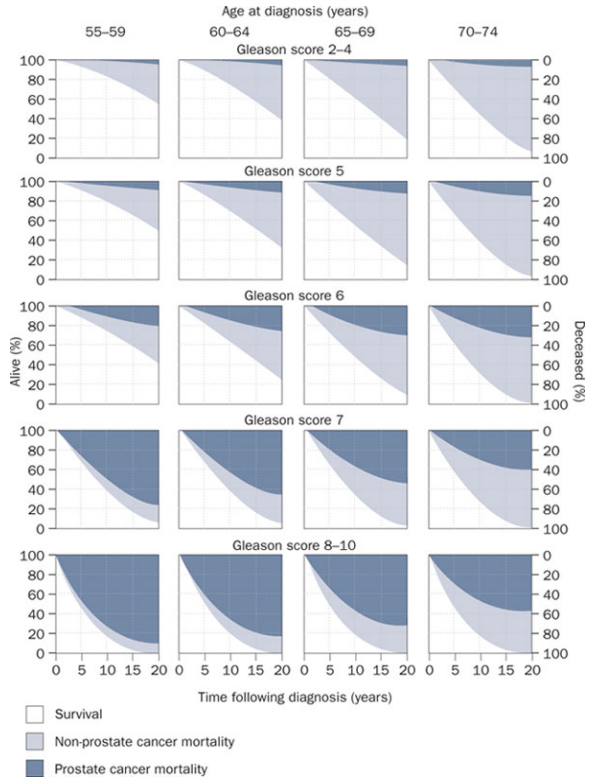

Figure 14: Prostate cancer and non-prostate cancer mortality in correlation with Gleason Score and age showing the prognostic implications of the Gleason Score[66].

II.4 Molecular Pathology of Prostate Cancer

II.4.1 Genetic Predisposition

A large body of evidence suggests a hereditary risk for PCa. So are for example sons of affected fathers at a higher risk (Hazard ratio 2.1), a risk which increases with additional affected family members [47]. Another study showed that brothers of patients with high grade, aggressive PCa tumors more susceptible to also developing PCa (standardized incidence ratio: 4.00 (95% CI, 2.63-5.82) for a Gleason score 8-10)[48]. Populations studies screening for genome-wide associations have identified around 100 susceptibility loci and are estimated to account for around 33% of all PCa cases [67] but with only around 9% true hereditary disease (defined as three or more affected relatives, or at least two relatives who have developed early-onset disease before the age of 55) [68].

Prostate cancer susceptibility loci have been mapped to 1q24-25 (*HPC1*), 17p11 (*HPC2*), Xq27-28 (*HPCX*), and 8q24. The latter directly affects the *MYC* oncogene which has been shown to be amplified in PCa in multiple studies and may be overexpressed in PIN lesions even in the absence of amplification [55].

Patients with hereditary risk profiles have a higher chance of developing PCa and seem to develop clinically relevant disease up to six to seven years earlier. But most studies fail to show a difference in terms of the aggressiveness of the disease. The only exception repeatedly reported up until now seems to be the *BRCA2* germline mutation, most famously implicated in breast and ovarian cancer, that also seems to predispose toward earlier onset and more aggressive PCa[68–70].

II.4.2 Acquired Genomic Aberrations

Several different genomic aberrations such as amplifications and mutations have been shown to play a role in prostate cancer carcinogenesis. Although several models of tumorigenesis have been proposed, one of them can be regarded as prototypical such as the model followed by colon cancer proposed by Vogelstein and colleagues [71].

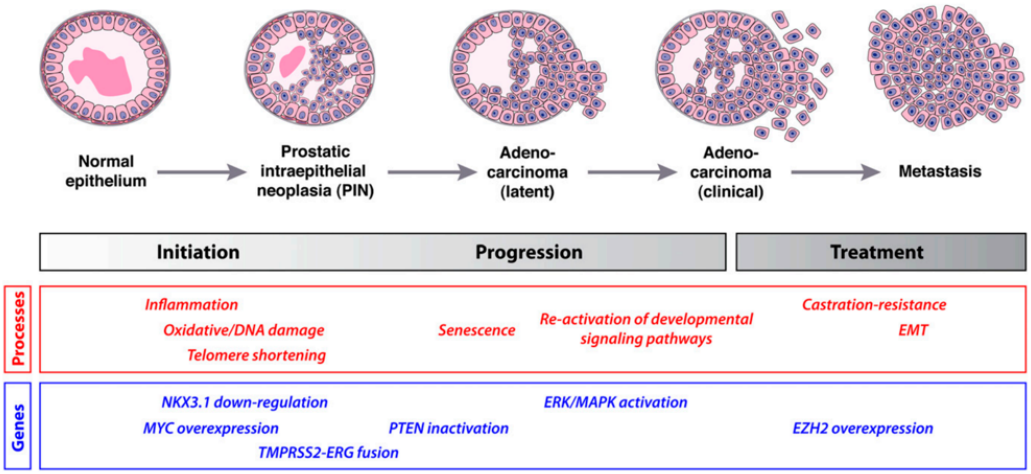

Figure 15: Common molecular cascade in prostate carcinogenesis with oncogenic processes (red) and frequently affected genes (blue).[55]

Nevertheless, several recurrent genomic events have been identified. including chromosomal aberrations (e.g. gains at 8q and losses at 3p, 8p, 10q, 13q, and 17p [28,72,73]). These genomic locations harbor several genes that are repeatedly implicated in prostate cancer, including *NKX3.1* at 8p21, *PTEN* at 10q23, *p27/Kip1* at 12p13, *KLF5* at 13q21 and *MYC* at 8q24 [72]. Importantly, several of these genetic alterations have also been identified in PIN which further emphasizes the pathological relationship between PIN and PCa. Some of the studies also highlighted the deletion of other known tumor suppressor genes such as *CDKN1B* (at 12p13) and *RB1* (at 13q14) [28].

Interestingly, in contrast to other solid malignancies, prostate cancer is characterized by structural genomic rearrangement and copy number alterations, while point mutations are rather infrequent [73]. Yet notable exceptions include *SPOP* (mutated in ~13% of tumors), *FOXA1*, *MED12*, *THSD7B*, *SCN11A* and

ZNF595 [74]. Among others, PTEN (Phosphatase and tensin homolog) deletion is one of the more frequent genomic alterations occurring during carcinogenesis [72]. *PTEN* is a known tumor suppressor gene that is implicated in a lot of malignant transformations as it directly negatively regulates the Akt/PKB signaling pathway. Consistent with its function in the human context, the inactivation of *PTEN* has been shown to promote PCa in murine models [75]. Further evidence exists that PTEN reduction or loss in prostate cancer predisposes to the emergence of castration-resistant prostate cancer [55].

The most prominent and prostate cancer-related genomic alterations are represented by chromosomal rearrangements involving the ETS family of transcription factors (*ERG*, *ETV1*, and *ETV4*). The most common of these rearrangements is the *TMPRSS2-ERG* fusion gene, resulting in the expression of a truncated ERG protein under the control of the androgen-responsive promoter of TMPRSS2 [8]. *ERG* and *TMRPSS2* are both located on chromosome 21q (3 Mb apart) and fusion either occurs through interstitial deletion or unbalanced inter-chromosomal translocation [55]. *ERG* itself can bind to AR targets and therefore affect prostate epithelium differentiation. About 15% of PIN lesions and 50% of PCa harbor the *TMPRSS2-ERG* fusions, indicating that its acquisition may be an early event in the development of PCa from pre-cancerous lesions. Growing evidence directly links androgen signaling with the occurrence of ETS family fusion events; in LNCaP cells (i.e. an androgen-sensitive prostate cancer cell line), the spatial proximity of the *TMPRRS2* and *ERG* loci upon stimulation with androgens has been shown [76].

Interestingly, ETS rearrangements and in particular *TMPRSS2-ERG* were found to be more prevalent in early-onset PCa (~90% of positive cases in patients < 60y) as compared to late-onset PCa (~40% of positive cases in patients > 60y). This may suggest a direct effect between higher androgen levels in younger patients and the development of gene fusion products [77]. Notably, *SPOP* mutations and ETS family gene rearrangements seem to be mutually exclusive and therefore may represent early and divergent driver events in prostate carcinogenesis [74]. In contrast, *PTEN* deletions appear to be positively correlated with ETS family fusions in most aggressive PCa tumors [78]. Yet despite the frequent occurrence of *TMPRSS2-ERG* in PCa, its clinical significance is still poorly understood as no clear association with survival has been shown [55,79].

II.5 Clinical Features of Prostate Cancer

II.5.1 Diagnostics

Clinical symptoms are rare in the early stages. Taking into account the high incidence due to age, screening programs for early detection are warranted. Their goal lies in the reduction of mortality due to

PCa and maintaining the quality of life especially through avoidance of the development of metastatic lesions.

One of the most elementary screening methods available is the digital rectal examination, which can be performed by a physician on routine examinations. Due to the relatively higher occurrence of PCa in the posterior lobe of the prostate, neoplastic foci, which usually present hard to the touch, can be detected. The diagnostic value has rather declined in the past years as the sensitivity (53.2%), specificity (83.6%) and positive predictive value (17.8%) can not compete with prostate-specific antigen (PSA) (sensitivity: 72.1%, specificity: 93.2% and positive predictive value: 25.1%)[80].

PSA, a kallikrein-related serine protease that is produced in normal prostate secretions, but is released into the blood as a consequence of disruption of normal prostate architecture (e.g. cancer, BPH, inflammation), has proven to be a very sensitive marker in the detection of PCa[81]. The use of PSA as a biochemical screening method leads to the increased detection (RR: 1.3; 95% CI: 1.02-1.65) of more localized (RR: 1.79; 95% CI: 1.19-2.70) and less advanced (T3-4, N1, M1 RR: 0.80; 95% CI: 0.73-0.87) lesions that are not detectable by palpation[82].

On the downside, the widespread adoption of PSA screening has also led to the detection of clinically localized low Gleason grade carcinomas that could be managed sufficiently with conservative approaches ("watchful waiting", ADT) sparing the surgical removal. False-positive results or clinically insignificant results (up to 50%[83]) lead to the unnecessary histological sampling of the prostate which also brings along the risk of complications, such as pain, bleeding or infection. Not to mention the anxiety that patients might experience being confronted with the potential diagnosis of a malignant disease [34,84,85].

A recent Cochrane meta-analysis of 5 studies with a total of 341,342 participants showed no statistically significant difference in prostate cancer-specific mortality between the screening and the control group (risk ratio (RR) 1.00, 95% confidence interval (CI) 0.86 to 1.17). Only one of the substudies (European Randomized Study of Screening for Prostate Cancer ERSPC) reported a 21% significant reduction of prostate cancer-specific mortality in a pre-specified subgroup of men aged 55 to 69 years [82].

The use of PSA screening is a matter of ongoing and harsh debate in the urological community at the moment and current European guidelines suggest an individualized risk-adapted strategy. Only well-informed men with at least 10-15 years of life expectancy should be subjected to PSA screening. However, the risks of over-treatment and -diagnosis have to be weighed against the potential benefits on a single patient basis, which requires a lot of clinical judgment of the treating urologist until further evidence has been gathered [61].

Several new markers or marker combinations such as the The Prostate Health Index (mathematical formula that combines total PSA, free PSA and proPSA[86]) or PCA3 (a prostate-specific, non-coding mRNA, measured in urine sediments obtained after prostatic massage[87]) have been proposed and seem to predict the result of subsequent biopsies more reliably than PSA screening alone. Nevertheless, they have not yet made into clinical routine.

Following the clinical diagnosis, either via palpation, biochemical or molecular analysis multiple biopsies, usually about 12, of the prostate are obtained under sonographic (transanal) guidance. These biopsies, in turn, are reviewed by the pathologist for signs of PCa (see also the section about Prostate Pathology).

Only a small portion of patients is not diagnosed until metastases are present. These patients usually present with complications, e.g. pathological bone fractures at the sites of osteoblastic metastases.

Additional radiological imaging techniques such as magnetic resonance tomography (MRI) [88] and the newer positron emission tomography (PET) using radioactively labeled antibodies against prostate prostate-specific membrane antigen (PSMA) in conjunction with computer tomography (CT) help to assess the tumor size and to identify present metastatic spread[89].

Complete histological and radiological workup are important pieces for the clinical decision of how aggressively a prostate tumor should be targeted. The usual habit is to report the TNM stage either form clinical/radiological assessment or if applicable after pathological workup of prostatectomy specimens and builds a cornerstone alongside the Gleason score for predicting disease outcome[89].

II.5.2 Treatment

Due to the, in most cases, prolonged progression of prostate cancer and the often advanced age of the patients at the primary diagnosis the main challenge in prostate cancer might not be to improve overall survival as it often is not the live limiting condition as it is the case in other malignancies. Up to 45% of all patients with localized PCa detected during screening will not profit from definitive treatment. Therefore conservative management schemes for the long disease course generally require a combination of different treatment modalities [90]. Current EAU guidelines suggest the classification of prostate cancer patients in low-, intermediate- and high-risk groups. Treatment modalities for prostate cancer are very diverse. In the following, I will restrict the description to the most commonly used ones.

Conservative Treatment Modalities

Low-risk patients with localized disease can be subjected to active surveillance or watchful waiting regimen. Active surveillance aims to prevent overtreatment of indolent prostate cancer by closely

monitoring disease progression (repeat biopsy, digital rectal examination, and PSA control). In contrast to watchful waiting, it still follows a curative intention and a switch to active treatment is always possible. Watchful waiting, on the other hand, has a palliative intention. Active treatment is therefore only initiated if the symptomatic disease occurs[90].

Surgery

If the disease stage and patient state allow radical prostatectomy, meaning the removal of the prostate, as well as the seminal vesicles and some of the surrounding tissue, is the optimal treatment regimen. If the tumor is completely removed, chances are the tumor is fully eradicated. It is the only treatment providing an improvement in overall survival compared to a more conservative initial approach [61]. But it also bears the risk of complications and side effects, namely incontinence and erectile dysfunction.

Radiotherapy

Dose-escalated intensity-modulated radiation therapy (IMRT) can be used alone for patients that do not qualify for extended surgery in order to achieve local tumor control or in addition to surgical removal, where just recently it could be shown that patients with nodal dissemination of the tumor could profit from adjuvant radiotherapy in terms of overall survival [91].

Chemotherapy

Advanced prostate cancer can be treated with chemotherapy. In 2004 two major clinical studies (TAX327 and SWOG 99-16) showed the taxane docetaxel to be superior to mitoxantrone in terms of overall survival [92,93]. Cabazitaxel is a newer generation taxane which can add additional benefit (prolonged survival (up to 15.1 months) and a reduced risk of death (up to 30%) even in docetaxel resistant patients [93].

PARP inhibitors

Recent genetic studies show that up to 23% of metastasized prostate cancer harbored somatic DNA repair pathway alterations (like *BRCA1/2* and *ATM*) [7]. Inhibition of Poly(ADP-ribose) polymerase (*PARP1*) leads to an accumulation of single-strand breaks which are commonly repaired through homologous recombination and repair. In the case of DNA repair-deficient tumors, this can lead to synthetic lethality of tumor cells [94]. The treatment of DNA repair-deficient patients suffering from metastasized prostate cancer with olaparib (a PARP inhibitor) showed a response rate of 88% [95] and therefore received FDA breakthrough therapy designation in 2016 [96]. Newer ongoing clinical studies now investigate the combination of PARP inhibitors with other systemic treatments such as enzalutamide [97]or even immunotherapy [98].

Besides surgery, chemotherapy, and radiotherapy, Antiandrogen therapy is one of the pillars of the treatments of prostate cancer. Usually, tumors show a reduction in size but after a median time of about 11.2 months [99] relapse resulting in progressive disease and castration resistance. Antiandrogen therapy, due to its seminal importance in this work is discussed separately in the following chapter.

III. Androgen Deprivation Therapy for Prostate Cancer

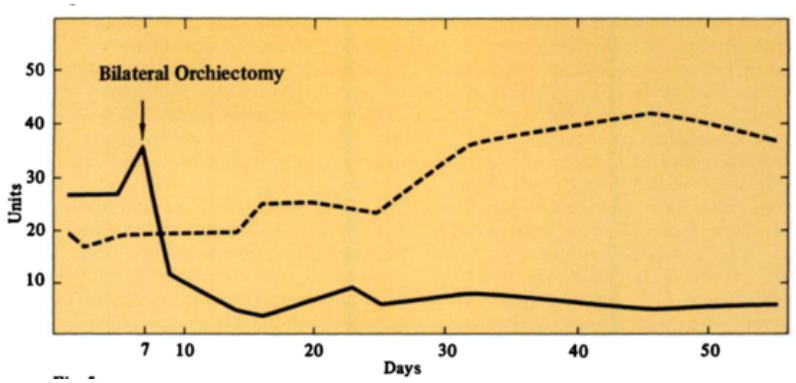

Figure 16: Charles Brenton Huggins received the 1966 Nobel Prize in medicine "for his discoveries concerning the hormonal treatment of prostatic cancer". Showing that PSA levels, as well as tumor volume, decreased upon bilateral orchiectomy (y-axis showing PSA levels) (Huggins and Hodges 1941).

Maturation of the male sexual organs, including the prostate, as well as the development of secondary sexual characteristics, depend on androgens, which is also their defining property. In the 1940s Huggins and colleagues showed that surgical orchiectomy and therefore the removal of testicular androgens leads to the regression of prostate tumors. In accordance, the administration of androgens promoted the growth of the tumors [100]. It is since a cornerstone in the treatment of prostate cancer.

III.1 Androgens and their Mechanism of action

Androgens are a diverse class of C19 steroids synthesized through the enzymatic oxidation of cholesterol (C21), of which testosterone can be considered the prototypic androgen. The adrenal cortex produces primarily dehydroepiandrosterone (DHEA), DHEA sulfate (DHEAS), androstenedione (A4), androstenediol and 11β-hydroxyandrostenedione (11OHA), with DHEA being the most abundant androgenic steroid in the human body. These adrenal androgens mainly serve as a metabolic intermediate in the biosynthesis of other androgens but also estrogens. Most importantly, in the given context, they are converted to more potent androgens in the prostate. Taken for themselves they display only weak androgenic potency as compared to testosterone [101,102]. DHEA for itself could be, under the concept of competitive binding, considered as an antiandrogen taking into account its low intrinsic activity on the AR (~10% of testosterone activity), but with its low affinity (Ki = 1μM) it does not seem to

be relevant in clinical relevant circumstances [103]. Nevertheless also minor quantities of testosterone itself can be synthesized by the adrenals. In men, this constitutes about 1% of circulating testosterone, while it accounts for about 30-50% in females [102].

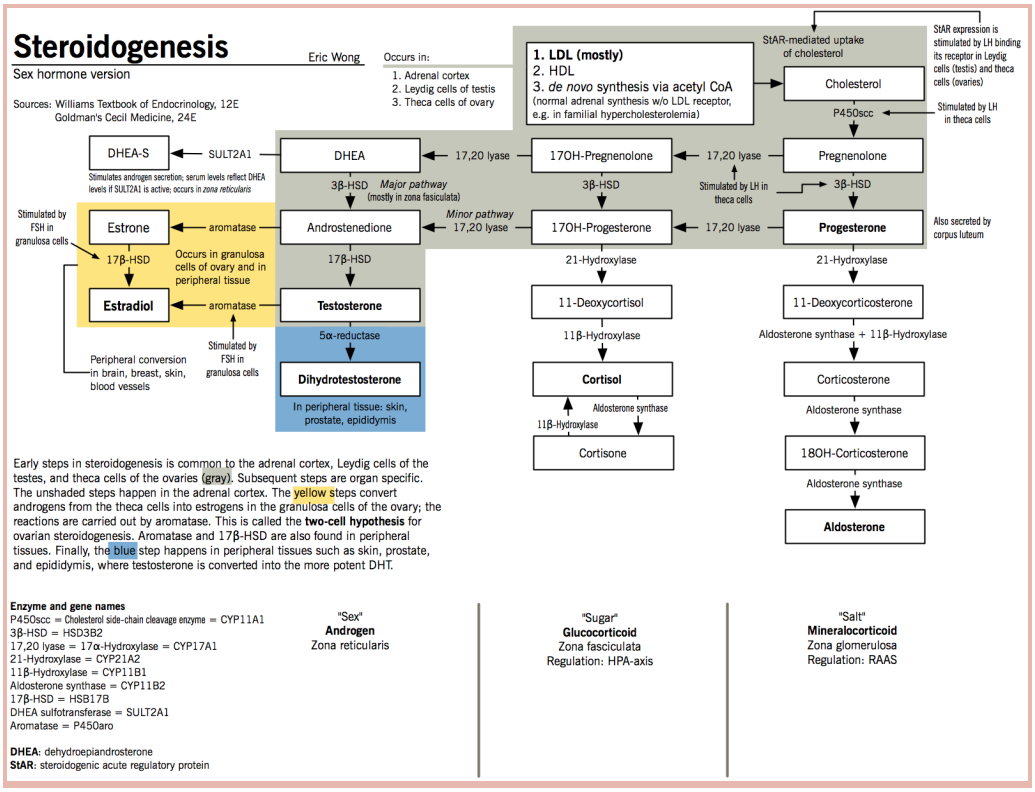

Figure 17: Steroidogenesis[104]

In males, the main androgens synthesis takes place in the testes, with the Leydig cells producing about 90% of the testosterone. Approximately 5% of testosterone undergoes enzymatic 5α-reduction into DHT, mainly in the skin and also the prostatic and seminal vesicle tissue. DHT is characterized by increased activity at the AR, partly due to its prolonged half-life (53 minutes) as compared to testosterone (34 minutes), and this may account for some of the difference in their potency [105]. Unlike testosterone, DHT cannot be aromatized into estrogen, and for this reason, has no potential estrogenic effects.

Production of sex hormones (androgens and estrogens) is mediated through the hypothalamic–pituitary–gonadal axis (HPG axis). Gonadotropin-releasing hormone (GnRH) is secreted from the hypothalamus. In turn, the anterior portion of the pituitary gland produces luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and the gonads and adrenals produce estrogen, testosterone, DHEA or similar respectively.

III.2 The Androgen Receptor Signaling Pathway

The androgen receptor (AR), a ligand-dependent transcription factor and member of the class I subgroup of the nuclear receptor superfamily, subsumed under the steroid receptors. Its downstream signaling plays a pivotal role in male sex differentiation, spermatogenesis, but also nerve and muscle development[106]. The AR consists of four regions, the N-terminal domain, a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD). It is encoded on the X-Chromosome (q11-12, 8 exons) and plays a major role in PCa carcinogenesis and progression. It is generally thought that the main AR-mediated androgen effect is mediated through direct binding of the AR on sequence-specific androgen response elements (AREs), acting as a transcriptional regulator of target genes [107].

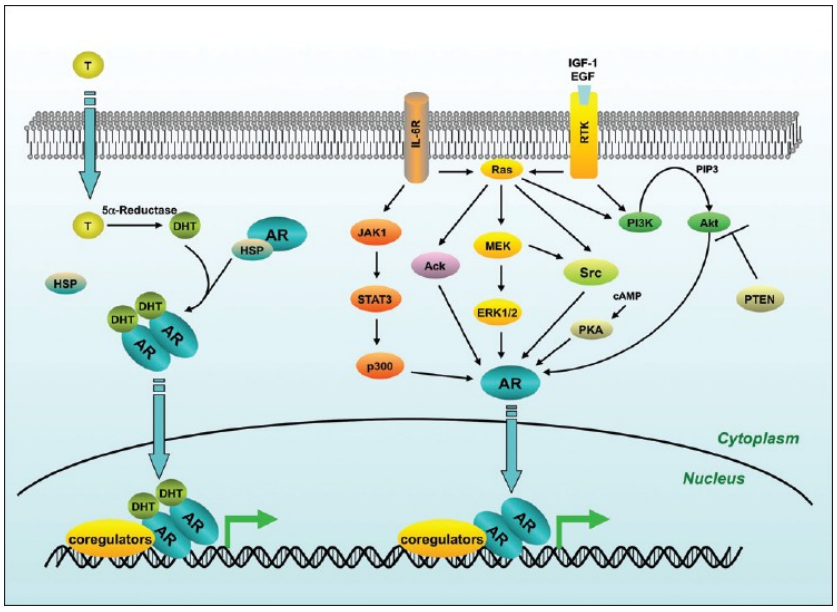

Figure 18: AR receptor activation in the prostate. On the left through direct ligand binding (T = testosterone, DHT = dihydrotestosterone) upon which AR dissociates from bound chaperones (HSP = heat shock protein) and dimerizes prior to translocation into to nucleus to modulate transcriptional activity. On the right alternative AR enhancing pathways are shown which increase AR translocations, such as ERK or even Interleukin 6 (through STAT3 signaling)[108].

The unbound and monomeric form of the AR is mainly located in the cytoplasm and associates with other proteins (e.g. heat shock protein (HSP) -90, -70, -56, cytoskeletal and FK506 binding proteins) [108]. Upon binding with androgens (mainly testosterone and DHT) the AR dimerizes and translocates into the nucleus. Although various androgen response elements have been identified through expression studies or chromatin immunoprecipitation (ChIP) analysis [109], the elucidation of their clinical significance requires further investigation. Probably the two most prominent proteins being translated upon AR activation, at least in the context of PCa, are PSA and TMPRSS2. Further roles of AR include regulation of

cellular growth signals (e.g. IGF1R, APP), cell cycle control signals (e.g. UBE2C, TACC2), of lipid and cholesterol metabolism, as well as some micro-RNAs (e.g. mi-21) [109–111].

III.3 Effects of Androgens on Prostate Cancer

II.3.1 Androgen Deprivation Therapy

Upon androgen deprivation the prostate tissue undergoes rapid cellular apoptosis and involution entering a regressed state [55]. Commonly androgen depletion or ADT is achieved through surgical (orchiectomy) or medical treatment.

As the testis are the main production site of androgens, their surgical removal logically leads to decreased androgen [112]. Although this leads to significantly decreased testosterone levels, there is residual androgen synthesis, mainly weak acting dehydroepiandrosterone (DHEA), in the zona fasciculata of the adrenals or the direct ectopic production and conversion of other steroid hormones in the tumor itself. To achieve further inhibitor of the AR axis orchiectomy is usually combined with an antiandrogenic drug resulting in a complete androgen blockade.

Antiandrogens or androgen antagonists are a diverse class of drugs that prevent the effects of testosterone or dihydrotestosterone in the body. They either act on the production (e.g. GnRH agonist), the conversion of weak androgens into more potent androgens (e.g. Abiraterone) or competitive binding of the androgen receptor (e.g. bicalutamide).

GnRH Agonists

Injectable gonadotropin-releasing hormone agonist (GnRH agonist, commonly also referred to as LHRH agonist), e.g. Goserelin, stimulate androgen synthesis in a non-physiological, because of non-pulsatile fashion. LHRH-Receptor binding leads to the continuous production of the luteinizing hormone in the pituitary gland. After an initial flare-up of androgen production, a disruption of the physiological feedback loop and long-term down-regulation LH-receptors in the testes sets in (14-21 Days). Androgen synthesis ceases in turn, reaching levels of castrates [113]. Although theoretically possible and also clinically available direct GnRH antagonists (e.g. Cetrorelix) are not commonly used in the treatment of prostate cancer. A high dose of estrogens leads to negative feedback on the hypothalamic-pituitary-gonadal axis (HPG axis) and in turn, achieves a lowering of testosterone levels similar to GnRH agonist. Because of its own hormonal activity and the associated side effect it is not used in the clinical treatment anymore [114]. Direct GnRH antagonists (e.g.e Degarelix) result in a quicker lowering of circulating testosterone without the flare-up observed with GnRH agonists [115] but are not commonly used in clinical routine.

Androgen Synthesis Inhibitors

Recently, Abiraterone (Abiraterone acetate) has had a marked impact on clinical practice. It is a specific inhibitor of 17α-hydroxylase/17,20-lyase(CYP17). Abiraterone inhibits both adrenal androgen and intratumoral androgen synthesis. Treatment with abiraterone is associated with marked declines in PSA levels, radiological tumor regression and relief of symptoms [116–118]. Importantly this holds also true for patients with castration-resistant disease that have failed prior antihormonal treatment [119].

Androgen Receptor Antagonist

Androgen receptor antagonists directly bind to the AR receptor and through competitive receptor binding to diminish the effect of androgens. They can be subdivided into steroidal and non-steroidal androgen receptor antagonists. Steroidal antiandrogens (e.g. spironolactone, cyproterone acetate) share a higher molecular similarity with androgens and can act as partial agonists of the AR receptor themselves in the absence of androgens. They are therefore of lesser use in the treatment of prostate cancer and are mostly replaced by non-steroidal androgen receptor antagonists (e.g. bicalutamide) which act as a silent antagonist. These show a significantly lower affinity to the AR than androgens, but in therapeutic concentration, they are able to competitively bind to the AR, thereby efficiently blocking androgen function. Enzalutamide (also known as MDV-3100) is a newer AR antagonist. It shows a higher affinity to the AR receptor as compared to bicalutamide and not only blocks AR signaling but also its translocation to the nucleus and its DNA binding capability [119]. It shows promising results in CRPC patients with or without chemotherapy (docetaxel) pretreatment[73,120]. Hussain and colleagues showed in phase III clinical trial including 1401 chemotherapy-naïve CRPC patients a significant increase in metastatic-free survival (36.6 vs 14.7 months) in Enzalutamide-treated vs. placebo-treated patients [120]. Similarly, Apalutamide (ARN-509) is a nonsteroidal antiandrogen with even greater efficacy than enzalutamide [121].

Current AR inhibitors all target the binding domain (LBD) of the AR; In contrast, N-terminal domain antagonists represent a new class of antiandrogens that block the N-terminal domain of the AR as opposed to more common AR-antagonists which target its ligand domain. The subsequent interaction of the AR with its downstream targets gets hampered. As an alternative approach, small molecule inhibitors and antibodies against point mutations and receptor truncations of the AR are being developed. They are currently tested in clinical trials [106].

5α-Reductase Inhibitors

5α-Reductase inhibitors inhibit the conversion of testosterone into its more potent form namely dihydrotestosterone (DHT). Although they are classified as anti-androgen drugs, they are routinely used

for the treatment of BPH but not prostate cancer. Debated evidence exists regarding their role in prostate cancer prevention with some studies suggesting that treatment with dutasteride leads to decreased prostate cancer incidence[122,123].

Androgen receptor degraders

Newer and still experimental approaches include the use of androgen receptor degraders. These drugs lead to continuous degradation of the androgen receptor in the cells leading to a decreased bioavailability [124,125].

While ADT is commonly used and followed by decreased tumor burden practically all patients relapse as the disease progresses towards castration resistance.

III.4 Castration Resistance

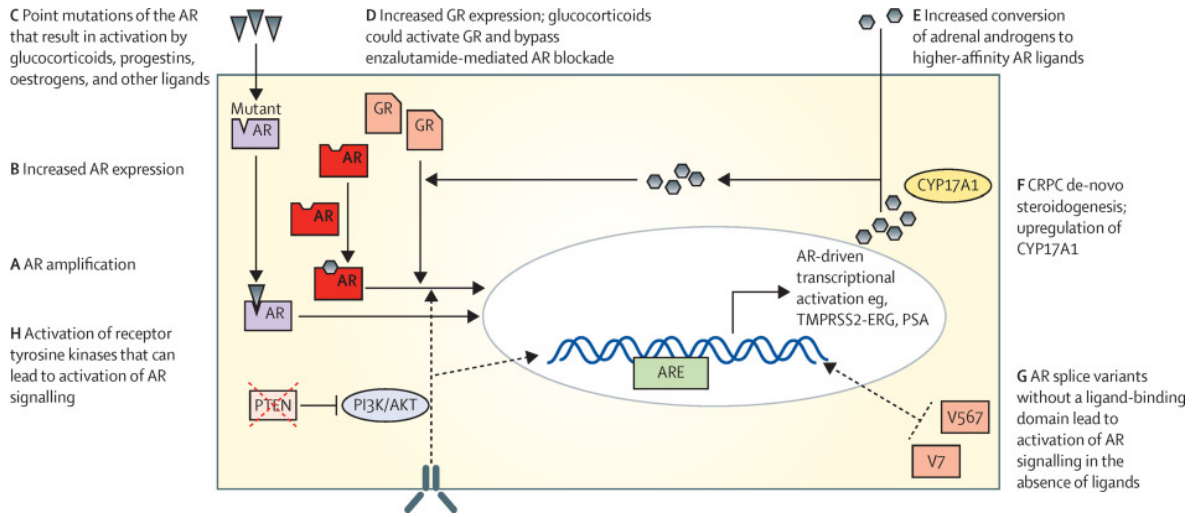

Figure 19: Possible modification leading to castration-resistance[126].

As stated previously, ADT usually leads to tumor regression but eventually, castration resistance develops. This development is usually diagnosed with a rise in detectable PSA levels and is not curable with current treatment options.

Several mechanisms of castration resistance have been proposed:

Sustained Androgen Effects

Autocrine androgen production, whereupon androgen blockade the PCa cells in collaboration with the adjacent stroma switch from a paracrine state into an autocrine one. They self-produce androgens for sustained androgen-stimulated growth [127]. Accordingly, upregulation of steroid synthesis-associated genes (*FASN*, *CYP17A1*, *HSD3B1*, *HSD17B3*, *CYP19A1*, and *UGT2B17*) can commonly be found to be upregulated in CRPC [128,129]. Also, weak adrenal androgens can be converted into more potent testosterone and DHT via enzymatic conversion in the prostate cancer cells. Accordingly, aldo-keto reductase family 1 member C3 (*AKR1C3*), one of these converting enzymes, overexpression can be found in castration-resistant prostate [130]. The gain of function mutations N367T leads to a stabilization of the 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1), which is considered to be one of the rate-limiting steps in the conversion of adrenal dehydroepiandrosterone into DHT 131.

AR Alterations

AR alterations include (1) Upregulation of AR synthesis. Interestingly this overexpression has also been reported to convert AR antagonists to weak AR agonists in an in vitro setting [132]. (2) AR amplification which occurs in up to one-third of all carcinoma and helps to sustain AR signaling activity even under castration levels of androgens [133].

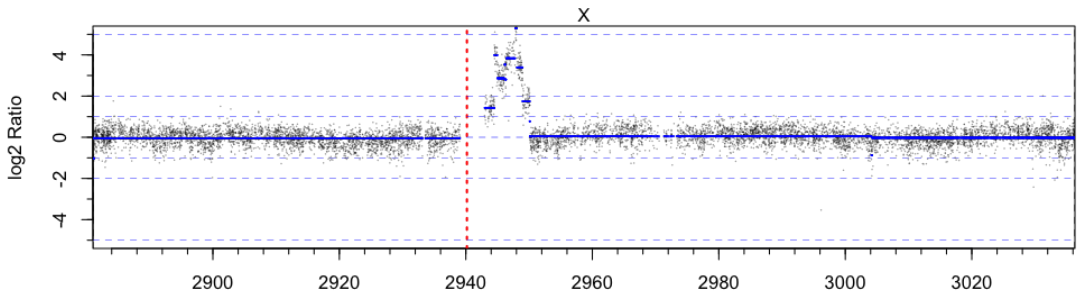

Figure 20: Androgen receptor as seen in with array comparative hybridization showing chromosome X. The hybridization signal (blue line) is shifted towards more positive log2 ratios indicating focal amplification where the AR is located. A patient with castration resistance after total androgen blockade (own data).

(3) *AR* mutations are found in 10% - 30% of tumors, conferring a gain of function either through increased stability/resistance towards degradation, increased sensitivity, higher promiscuity of the receptor toward other steroid hormones, constitutional activation without ligand binding or increased recruitment of AR coactivators [134–139]. The T878A mutation, for example, renders the AR-responsive to binding through progesterone, estrogen, flutamide, bicalutamide, and enzalutamide by lowering its specificity [135,140,141]. (4) *AR* splice variants, specifically the *AR-V7* splice variant has been described rather

recently and implicated with more aggressive, castration-resistant behavior as well as resistance towards treatment with enzalutamide and abiraterone [142].

Nuclear AR expression seems to persist in the majority of castration-resistant tumors even after progression toward castration resistance [143] persists. In conjunction with still measurable levels of circulating androgens even after castration and total androgen blockade, this leaves space for the postulate that at least a portion of PCa never fully dissociates from its androgen dependence but rather reacts through self-production or increased sensitivity. This is also supported by the clinical experience that further treatment with newer anti-androgens such as abiraterone still shows good clinical efficiency despite the fact that these tumors have traditionally been classified as hormone-independent. On the other hand, up to 30% show a primary resistance towards abiraterone and enzalutamide suggesting a true hormone independence[126].

Neuroendocrine Dedifferentiation

Neuroendocrine dedifferentiation is associated with an aberrant immunohistochemical expression as for example chromogranin A is more prevalent in PCa following ADT treatment. This histological subtype seems to confer castration resistance and AR expression usually lacks. It has to be assumed that alternative growth factor pathways play a role in its development[62,144,145]. Recent studies highlight the role of *RB1* and *TP53* deficiencies and attenuated AR signaling in the development of neuroendocrine prostate cancer [146,147]. Functional studies showed these losses to induce transdifferentiation of luminal cells into neuroendocrine cells mainly through epigenetic reprogramming mediated by EZH2 and SOX2 [148]. Also, *c-MYC* amplifications are found in up to 40% of all neuroendocrine tumors [149–151].

Additionally, to the above-mentioned ways of castration resistance, non-androgen dependent alterations have been found in a minority of cases and include for example activation of alternate growth factor pathways, e.g. activating mutations in the Akt/mTOR or MAPK signaling pathways [126].

I.V Aims

This work aims to contribute to the understanding of the development of castration resistance under androgen deprivation therapy. Specifically what phenotypic and genomic changes are newly developed during the treatment course in matched prostate cancer samples from our biobank prior to and after ADT. In order to achieve this, we applied several strategies that to our understanding build the strength of this project.

V. Experimental Strategies

In the following, I will describe the distinct experimental strategies that were pivotal for the results acquired in this thesis.

V.1 Tissue Microarrays

First, we made use of various clinically annotated patient cohorts that we have access to. Specifically, we used different tissue microarrays compromising a total of 557 patients. Importantly for one TMA with 114 patients, we had access to matched hormone naïve and castration-resistant samples. We were able to quickly screen candidate genes in a large clinical cohort.

Tissue microarrays are an invaluable tool for the study of large retrospective cohorts. Formalin-fixed and paraffin-embedded (FFPE) histological samples are punched wich results in cylindrical TMA cores. Up to a thousand of TMA cores with a typical diameter of about 6 mm can then be mounted on a glass slide. Allowing the immunohistochemical staining, FISH, and analysis of all samples simultaneously[152,153].

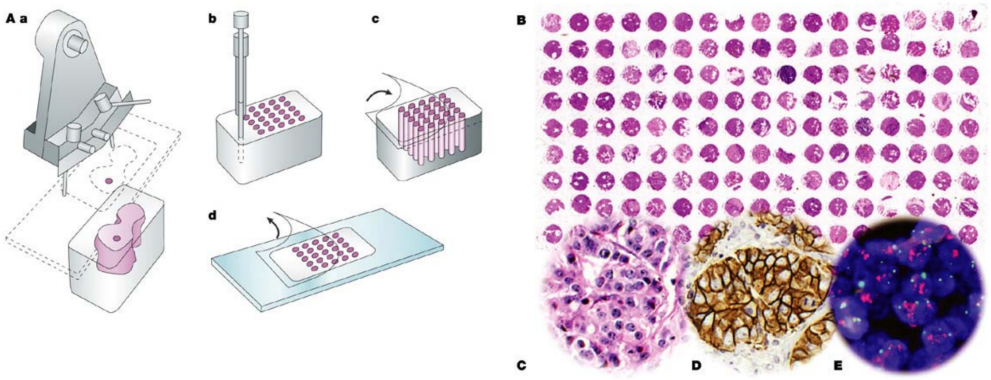

Figure 21: Process of TMA generation. A: a) cylindrical TMA cores are cut from a "donor" block. b) aggregation of TMA cores in a "receiver" block c) regular cutting of 4-6 um slides and d) mounting on a conventional glass slide. B: Simultaneous multiparametric analysis of hundreds of patient samples[152].

Their clinical value has been repeatedly demonstrated on a variety of tumor types [154]. We were, for instance, able to show a more unfavorable clinical outcome connected with the expression of the estrogen receptor β and the phosphorylation of the androgen receptor using the above specified matched prostate cancer TMA [155]

V.2 DNA Content Based Nuclei Sorting

Bulk genomic analysis of solid tumors commonly poses two major obstacles. i) selecting the samples with sufficient tumor content and ii) deconvoluting the underlying genomic heterogeneity. Advanced techniques like Laser capture microdissection do not fully solve these issues, as tumor populations often

are not easily distinguishable by morphology alone and purification methods are time-consuming and not readily feasible in larger screening studies. Additionally, the resulting admixture of different tumor clones, as well as benign cells, immune cells or tumoral stroma, dampers diagnostic resolution in most genomic approaches. Various computational approaches do in fact exist and show credible and promising results, but do all heavily rely on model assumptions[156].

Fluorescence activates flow sorting (FACS) ist capable of analyzing thousands of single cells per minute. Additionally, cells can be efficiently sorted based on their light scattering characteristics and optional fluorescent labeling[157]. Genomic aberrations might lead to changes in the overall DNA content of the cell nuclei. The DNA can easily be labeled using 4′,6-diamidino-2-phenylindole (DAPI) and DNA content can, therefore, be used as a parameter in the FACS analysis. Ruiz and Barrett pioneered the approach of DNA content bases nuclei sorting in 2011[158]. The approach enabled them to track the evolutionary history of one pancreatic cancer patient over multiple disease locations[158]. The separation of biologically relevant tumor clones with distinct aneuploidy status (DNA content) highly increased the diagnostic sensitivity as compared to bulk analysis.

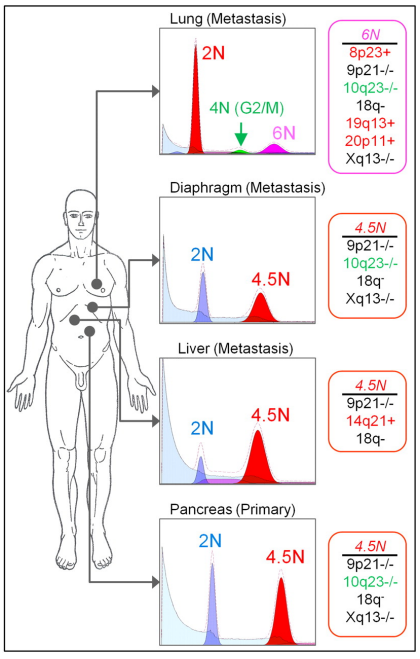

Figure 22: Analysis of a rapid autopsy patient with pancreatic cancer. Using DNA content bases nuclei sorting, Ruiz et. al were able to track the DNA content profile and evolution of chromosomal abberations across tumor sites[158].

The initial studies were all performed on fresh frozen (FF) tumor specimens. Briefly, clinical prostate cancer samples are minced in the presence of a lysing buffer (complete protocols not included but available upon request). The resulting nuclei suspension is then stained using DAPI and analyses using a BD Influx cytometer with ultraviolet excitation. Due to the proportional DAPI integration depending on DNA content this results in a histogram of the distribution of DNA content per nucleus. The distribution is then fitted to a polynomial model of the cell cycle as implemented in the MultiCycle (Phoenix Flow Systems, San Diego, CA) software, which also accounts for debris generated during the sample

preparation [159]. After the allocation of the appropriate gates, the distinct cell populations can be sorted and separately analyzed resulting in distinct genomic profiles.

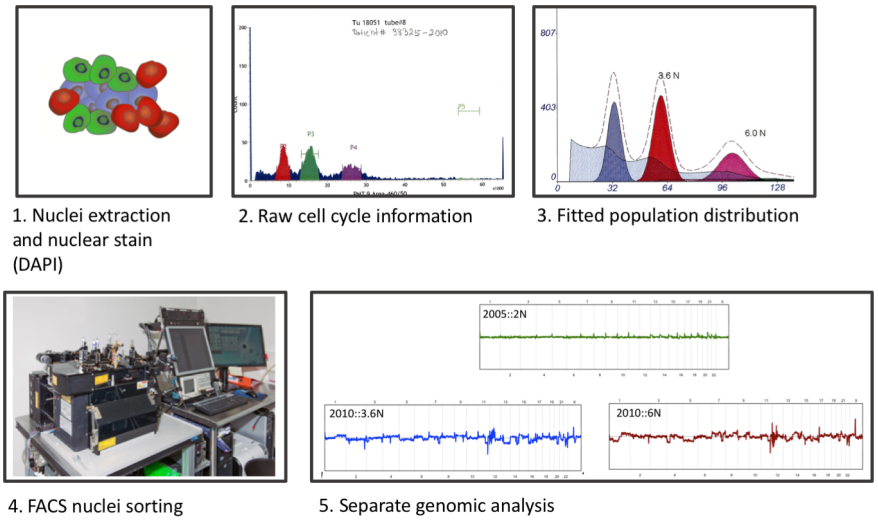

Figure 23: Workflow for DNA content based cell nuclei sorting.

During the time of this thesis, we also extended and the technique to be able to analyze archived FFPE samples in a collaboration between the Translational Genomics Research Institute (TGEN) in Scottsdale Arizona and our institute. Due to the paraffin embedding and the ubiquitous protein cross-linking in formalin-fixed specimens a completely adapted protocol for de-paraffinization and nuclei separation had to be developed[160] (protocols not included but available upon request).

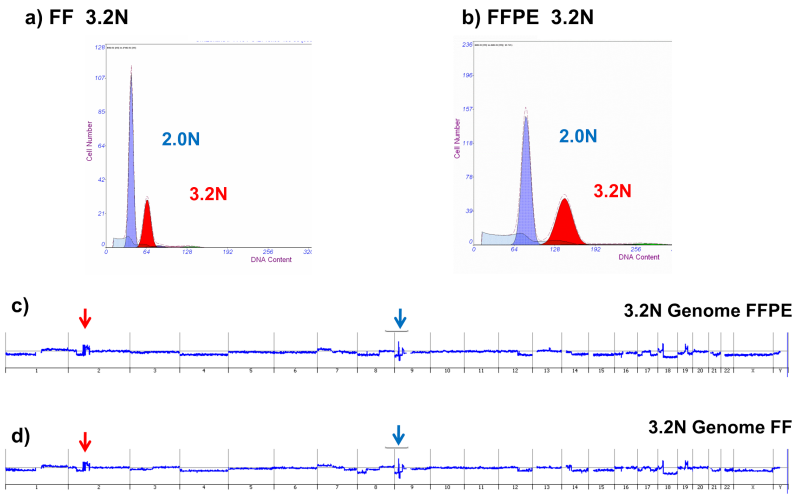

Figure 24: Comparison of copy number profiles between fresh frozen (FF) and paraffin embedded (FFPE) showing their concordance. Patient with ductal pancreatic cancer[160].

Just recently Lorber and colleagues from our institute were able to even further augment the technique by also incorporating a nuclear antibody staining for TTF-1 in order to more efficiently separate lung

cancer cells from adjacent tissue for genomic downstream analysis. This approach increased the mean tumor purity from 54% (range 7–89%) of unsorted material to 92% (range 79–99%) after sorting[161].

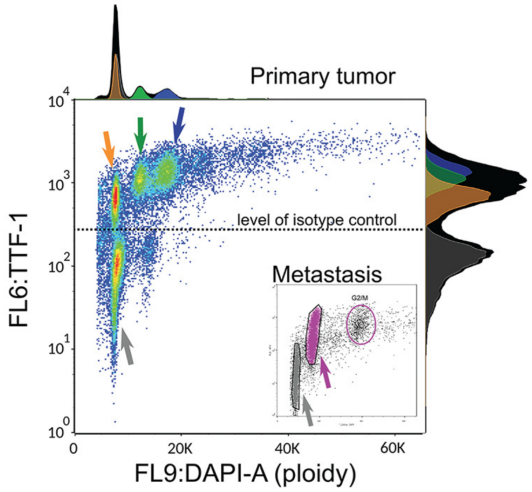

Figure 25: DNA content (DAPI on x-axes) vs. TTF-1 nuclear staining (y-axes) in a lung cancer patients. Primary tumor, metastatic site in the lower right corner[161].

Overall DNA content based nuclei sorting gives us a very flexible and valuable tool to i) improve tumor content in downstream analysis and ii) deconvolute intratumoral heterogeneity.

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Results

I. Publication 1

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ORIGINAL ARTICLE

ERG rearrangement and protein expression in the progression to castration-resistant prostate cancer

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BACKGROUND: Approximately half of the prostate carcinomas are characterized by a chromosomal rearrangement fusing the androgen-regulated gene *TMPRSS2* to the oncogenic *ETS* transcription factor *ERG*. Aim of this study was to comprehensively analyze the role and impact of the ERG rearrangement and protein expression on the progression to castration-resistant (CR) disease.
METHODS: We used a tissue microarray (TMA) constructed from 114 hormone naive (HN) and 117 CR PCs. We analyzed the *ERG* rearrangement status by fluorescence *in situ* hybridization and the expression profiles of ERG, androgen receptor (AR) and the proliferation marker Ki67 by immunohistochemistry.
RESULTS: Nearly half of the PC tissue specimens (HN: 38%, CR: 46%) harbored a *TMPRSS2-ERG* gene fusion. HN PCs with positive translocation status showed increased tumor cell proliferation ($P<0.05$). As expected, TMPRSS2-ERG gene fusion was strongly associated with increased ERG protein expression in HN and CR PCs (both $P<0.0001$). Remarkably, the study revealed a subgroup (26%) of CR PCs with ERG rearrangement but without any detectable ERG protein expression. This subgroup showed significantly lower levels of AR protein expression and androgen-regulated serum PSA (both $P<0.05$).
CONCLUSIONS: In this study, we identified a subgroup of *ERG*-rearranged CR PCs without detectable ERG protein expression. Our results suggest that this subgroup could represent CR PCs with a dispensed AR pathway. These tumors might represent a thus far unrecognized subset of patients with AR-independent CR PC who may not benefit from conventional therapy directed against the AR pathway.

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Keywords: castration resistance; TMPRSS2-ERG; ERG

I.2 Introduction

Prostate cancer (PC) is the most frequently diagnosed cancer among males in western countries and the second leading cause of cancer-related death.[1] Although the mortality of PC has decreased mainly due to earlier detection, this disease still accounts for 9% of the total cancer deaths. Most PCs are nowadays diagnosed at an early stage. They initially depend on androgens for their growth and are thus referred to as hormone naive (HN) PC. Based on this dependence, the standard treatment for patients harboring these tumors is androgen-deprivation therapy (ADT). Although this therapy is initially effective, most of the treated tumors recur after a few months or years as castration-resistant (CR) PC. Mechanisms responsible for this progression are not fully understood.

PC research was revolutionized by the discovery of the TMPRSS2-ERG gene fusion in 2005.[2] Later on, it was realized that this rearrangement was part of a whole family of gene fusions that connect the

promoter region of androgen-regulated genes, most frequently the TMPRSS2 (transmembrane protease inhibitor 2) with transcription factors of the ETS (erythroblastosis virus E26 transforming sequence) family of transcription factors.[3,4] Of these fusions, the rearrangement involving the genes TMPRSS2 and ERG is by far the most common (> 90%) and is present in approximately 50% of prostate tumors.[5] The two involved genes are <3 Mb apart on chromosome 21, and their fusion can occur through various rearrangement mechanisms, most frequently deletion of the intervening region on chromosome 21 (reviewed in Tomlins et al.[6] and Perner et al.[7]). This rearrangement results in androgen regulation of the ERG gene, leading to the overexpression of this gene in prostatic adenocarcinoma (reviewed in Sreenath et al.[8]). Despite the extensive studies about the role of the ERG rearrangement and expression, its clinical significance remains controversial.[9,10] Recently, Minner et al.[11] did not observe any prognostic impact in a larger cohort of radically operated PCs.

In CR PC, ERG rearrangement has been shown to prevail in 34– 45% of the tumors.[12,13] Very recently, we observed a higher frequency of ERG rearrangements (45%) in recurrent CR PC specimens and a lower frequency of 25% in metastatic CR PCs.[13] In contrast to the rearrangement, which is present on a genomic level, ERG protein expression is more dynamic, as it depends on the presence and activation of the androgen receptor (AR). In the CR disease state, the tumor may adapt to very low levels of androgens. Thus, it is not evident if these levels are sufficient for the activation of ERG transcription. Data from these investigations have provided controversial results: whereas in some CR PC xenograft experiments ERG mRNA expression was not detectable,[14] others have shown ERG protein expression in rearranged CR PC samples and xenografts.[15,16]

In the present study, we used a tissue microarray (TMA) consisting of 231 locally advanced PCs that were collected either before (HN) or after recurrence to ADT (CR). We used this TMA to comprehensively interrogate and characterize the ERG protein expression and rearrangement comparing HN and CR PCs. We included standard markers into our analyses known to be relevant in PC, such as AR protein expression and Ki67 labeling. Here, we show that a considerable fraction of ERG-rearranged CR PCs loses ERG protein expression. We hypothesize that this might be due to a dispensed AR pathway.

I.3 Materials and Methods

TMA and patients

The use of clinical specimens for the construction of the castration resistance TMA (crTMA) was approved by the ethical committee of the University and the University Hospital of Basel, Basel, Switzerland. The crTMA was manufactured as previously described.[17] Briefly, tissue cylinders with a diameter of 0.6 mm were punched from the 'donor' tissue blocks containing the TURP specimens using a

home-made, semi-automatic robotic precision instrument. Three cores from each specimen were arrayed. The composition of the crTMA has been previously described and is summarized in Supplementary Table S3.[18] Briefly, it is composed of 697 spots from 231 TURPs from a total of 202 patients treated with advanced, locally obstructive PC. In addition, it contains 12 specimens from BPH. Castration resistance was defined as locally obstructive recurrence and/or PSA-recurrence during ADT.

Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH)

IHC was performed according to the standard indirect immuno-peroxidase procedures. The primary antibody was omitted for negative controls. All slides were read manually by an experienced pathologist (LB). Data from AR and Ki67 were available from a previous study on the same TMA block.[18] Briefly, the antibodies M3562 and M7240 (both DAKO, Carpinteria, CA, USA) were used for AR and Ki67 staining, respectively. The anti-ERG mouse monoclonal antibody 9FY was from Biocare Medical (Concord, CA, USA).[19] FISH analysis for detection of ERG rearrangement was performed as previously reported.[13] Images were obtained by usage of the AXIO Imager.A1 microscope equipped with an AxioCam and the AxioVision 4.6 software (all from Zeiss, Jena, Germany).

Cutoffs, data analysis and statistics

For protein expression analysis of AR, Ki67 and ERG, the percentage of positive tumor cells was noted by an experienced pathologist (LB) and used as score.[18] For dichotomous stratification of ERG, samples with any specific positivity were considered as ERG positive (that is, cutoff >0) and were considered negative in reference to endothelial ERG-positive staining.[19,20] Cutoffs for definition of low or high for Ki67 labeling index were used as previously described.[18] For correlation studies between different markers, every evaluable spot was considered for the analysis, that is, the analyses were performed on a 'spot-by-spot' basis. All other analyses (that is, descriptive tables, association with clinical data, such as treatment status, cM, cT and survival data) were performed on a 'one-value-per-biopsy' basis, thereby considering only one value per biopsy/specimen. If more than one spot/value per biopsy/specimen was evaluable, the spot with the maximal score was included in the analysis.

Statistical analysis was performed with the R Framework Version 3.0.121 including the 'coin' package.[22] Differences between two groups were analyzed with the Wilcoxon's rank-sum test; differences between more than two groups were analyzed using the Kruskal–Wallis rank-sum test for metric variables, for example, expression score. $\chi 2$ and Fisher's exact test were used to analyze contingency tables. Survival curves were plotted by usage of the Kaplan–Meier method, and differences were assessed using the log-rank test. P-values < 0.05 were considered as statistically significant.

I.4 Results

ERG expression and TMPRSS2-ERG rearrangement in HN and CR PC and association with clinicopathological features

To interrogate ERG protein expression and rearrangement by IHC and FISH, respectively, in the context of progression to castration resistance, we used the recently described crTMA that was constructed for this purpose.[18] In addition, we included IHC data for AR and Ki67 expression from a previous study.[18]

For ERG expression analysis, 78 (68%) and 88 (77%) out of 114 HN and 117 CR TURPs, respectively, were evaluable (Figure 1). Of note, only cases with unequivocal nuclear staining for ERG in endothelial cells were considered as evaluable. ERG FISH analysis was successful in 94 (83%) and 94 (81%) of the 114 and 117 HN and CR PCs, respectively. ERG protein positivity, as well as the presence of ERG rearrangement, showed similar distributions between HN and CR PC (Table 1a). We found ERG protein positivity in 47% (37/78) and 40% (35/88) of the HN and CR PC samples. Similarly, 38% (36/94) and 47% (44/94) of the same samples showed ERG rearrangement. High-grade prostatic intraepithelial neoplasias were not present in this TMA and thus not analyzed in this study. We did not observe ERG positivity in the 10 evaluable BPH samples present on this TMA. In addition, the crTMA comprises a unique set of 36 matched PC samples from the same patients before (HN) and after hormonal ablation therapy (CR). The analysis of this subset revealed a change of ERG status in individual patients to be rare (1/21 and 2/30 for IHC and FISH, respectively; Supplementary Table S1).

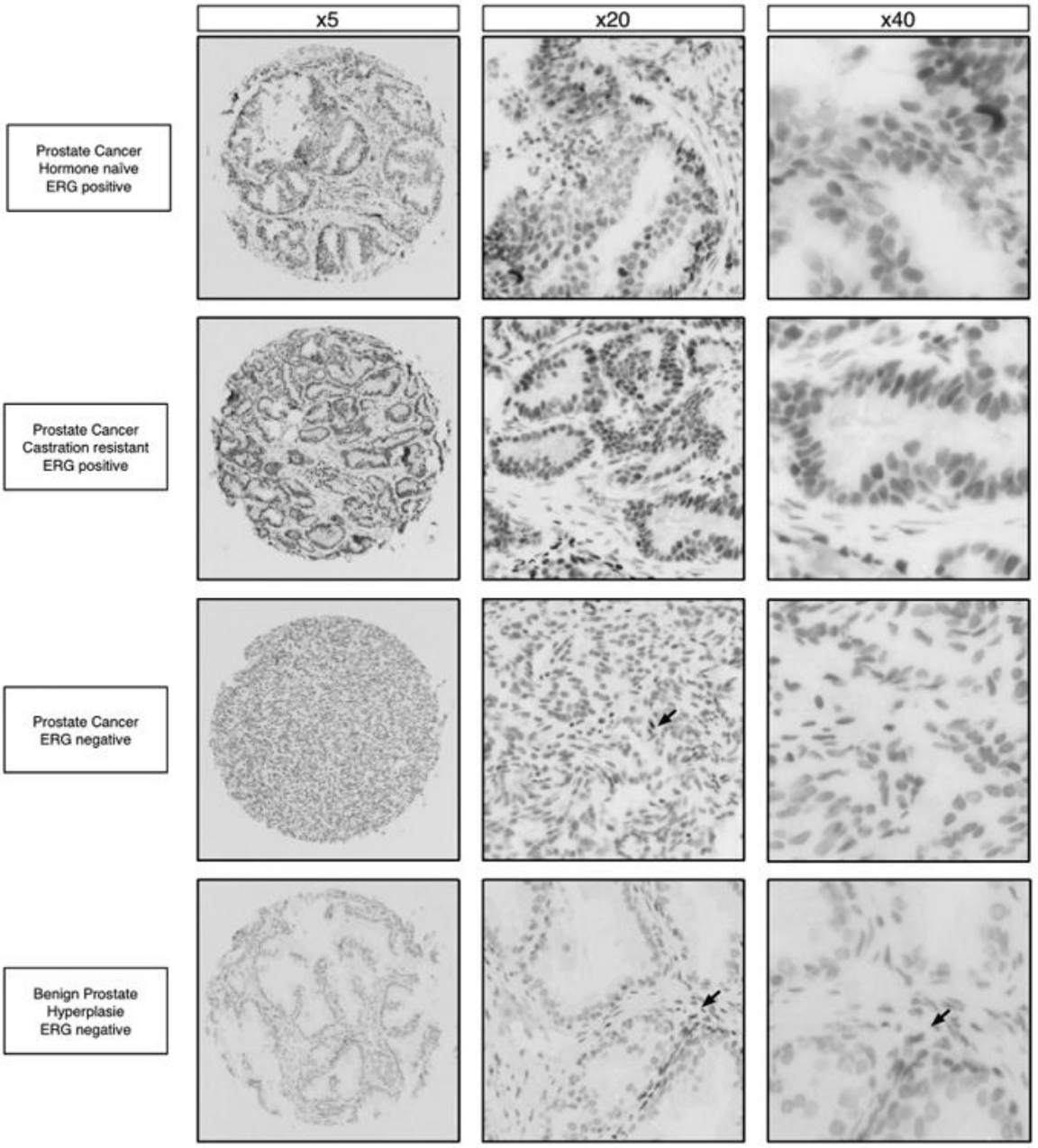


Figure 1: Representative images of ERG-stained prostate samples from the castration resistance tissue microarray (crTMA). Endothelial cells (black arrows) were used as positive control for the ERG staining.

(a)

					NS			
	BPH		All PC		HN		CR	
	n	%	n	%	n	%	n	%
FISH								
Not rearranged	10	100	108	57	58	62	50	53
Rearranged	0	0	80	43	36	38	44	47
Immunohistochemistry								
ERG negative	10	100	94	57	41	53	53	60
ERG positive	0	0	72	43	37	47	35	40

(b)

Gleason Pattern	*NS*				**P-value < 0.05*			
	FISH				Immunohistochemistry			
	Not rearranged		Rearranged		ERG negative		ERG positive	
	n	%	n	%	n	%	n	%
HN								
3	7	58	5	42	1	14	6	86
4	45	58	33	42	27	44	34	56
5	61	70	26	30	44	67	22	33
CR								
3	0	—	0	—	0	—	0	—
4	21	54	18	46	17	49	18	51
5	80	58	58	42	90	70	39	30

Table 1: Overview of the ERG status on the castration resistance tissue microarray (crTMA) (a) ERG status was significantly different between BPH and all PCs, but not between HN and CR. Fisher's exact tests were used for comparisons. (b) HN and CR prostate cancer samples without ERG protein expression are characterized by higher Gleason pattern. This association was not true for ERG rearrangement. The $\chi 2$ test was used for comparison between the groups: Not rearranged vs rearranged and ERG + vs ERG — in HN samples. Fisher's exact test was used for CR samples. Abbreviations: CR, castration resistant; FISH, fluorescence in situ hybridization; HN, hormone naive; NS, not significant; PC, prostate cancer.

We next investigated a potential association between ERG status and clinicopathological features, such as cM and cT stages, and Gleason pattern. ERG status was not differentially distributed across different cM and cT stages (data not shown). Interestingly, only ERG protein expression but not ERG rearrangement revealed a significant decrease of positivity toward higher Gleason pattern. This was true in HN (P=0.004) as well as in CR PCs (P = 0.019) (Table 1b). As PCs of higher Gleason pattern are characterized by higher tumor cell proliferation, we investigated a potential correlation between ERG status and Ki67 labeling index. We did not observe a correlation between ERG protein expression and increased tumor cell proliferation. This was also true for ERG rearrangement. However, stratification into HN and CR revealed that the proliferation index in ERG-rearranged HN was significantly higher than in those HN where ERG was not rearranged (55% vs 38%, P< 0.05, Supplementary Table S2).

No significant association of ERG status with overall survival of HN or CR PC patients

We analyzed the potential impact of ERG protein expression and rearrangement on overall survival. In both cohorts, HN as well as CR, neither ERG staining nor ERG rearrangement were related to patient prognosis in Kaplan–Meier survival analysis (Supplementary Figure S1).

Decreasing correlation of TMPRSS2-ERG translocation with protein expression of ERG in CR PC

It is well established that ERG protein expression is dependent on the presence of an ERG rearrangement in prostatic adenocarcinoma. Here we investigated the power of this correlation in the cohort of the crTMA, which is composed of highly advanced PCs before (HN) and after ADT (CR). As expected, a high correlation between ERG rearrangement and ERG protein expression was observed (P<0.0001). This was also true if PC samples were stratified according to their hormonal treatment status HN and CR (P<0.0001, Table 2). Intriguingly, whereas in HN PCs, the number of FISH-IHC discordant results were minimal (7% FISH positive, but ERG negative and 9% FISH negative, but ERG positive), in CR PCs, 26% (13 spots) of the ERG-rearranged samples did not show detectable ERG protein expression (Table 2). This surprisingly large group of ERG-rearrangement positive, but ERG-protein-negative PC samples in the CR, but not in the HN group, can hardly be explained by a technical phenomenon. These findings rather suggest that losing the high concordance between ERG FISH and ERG IHC toward more advanced PC samples may be due to the existence of a specific subset of CR PC patients whose tumors have lost the ability of expressing the ERG protein despite the presence of an ERG rearrangement. Of note, these 13 spots were from 11 different TURPs from 10 distinct patients.

Table 2. Correlation of *ERG* rearrangement and protein expression

	***P-value < 0.0001			
	FISH			
	Not rearranged		*Rearranged*	
	n	%	n	%
Immunohistochemistry				
HN				
ERG negative	59	91	3	7
ERG positive	6	9	42	93
CR				
ERG negative	63	93	13	26
ERG positive	5	7	37	74

Table 2: Correlation of ERG rearrangement and protein expression. Abbreviations: CR, castration resistant; FISH, fluorescence in situ hybridization; HN, hormone naive. A highly significant correlation was found between ERG rearrangement and ERG protein expression in each of the subgroups. ERG FISH-positive CR prostate cancers showed by far the highest discordant rate (26%). Analyses were performed on a spot level by usage of the Fisher's exact test.

TMPRSS2-ERG-positive CR PCs without detectable ERG protein expression

We next interrogated the association between the AR protein expression and the ERG status. As previously described,[18] AR protein expression was present in almost all analyzed PC samples and maximal (score = 100) in >90% of the specimens. Overall, we were not able to see a significant association between ERG rearrangement or positivity and AR expression (P> 0.05 both, data not shown). To analyze whether the ERG FISH vs IHC discrepancy in CR PC with ERG rearrangement but absent ERG protein is due to a loss of AR, we stratified the PCs into the different ERG subgroups according to the two treatment status. Although AR expression was present at high levels (score 90–100) in almost all PC samples, independent of the ERG status, only ERG-rearrangement-positive CR PCs with absent ERG protein were characterized by lower levels of AR protein (P = 0.002, Figure 2a). Further, we interrogated a correlation of ERG protein expression with serum protein levels of the AR target gene PSA in the subgroup of ERG-rearranged CR PCs. As expected, the group of ERG-rearrangement positive and ERG-protein-negative CR PC samples had lower PSA levels than ERG-rearranged- and ERG-protein-positive samples (P< 0.05, Figure 2b). However, it must be considered that PSA serum information was only available for four ERG-rearrangement-positive but ERG-protein-negative CR PC samples.

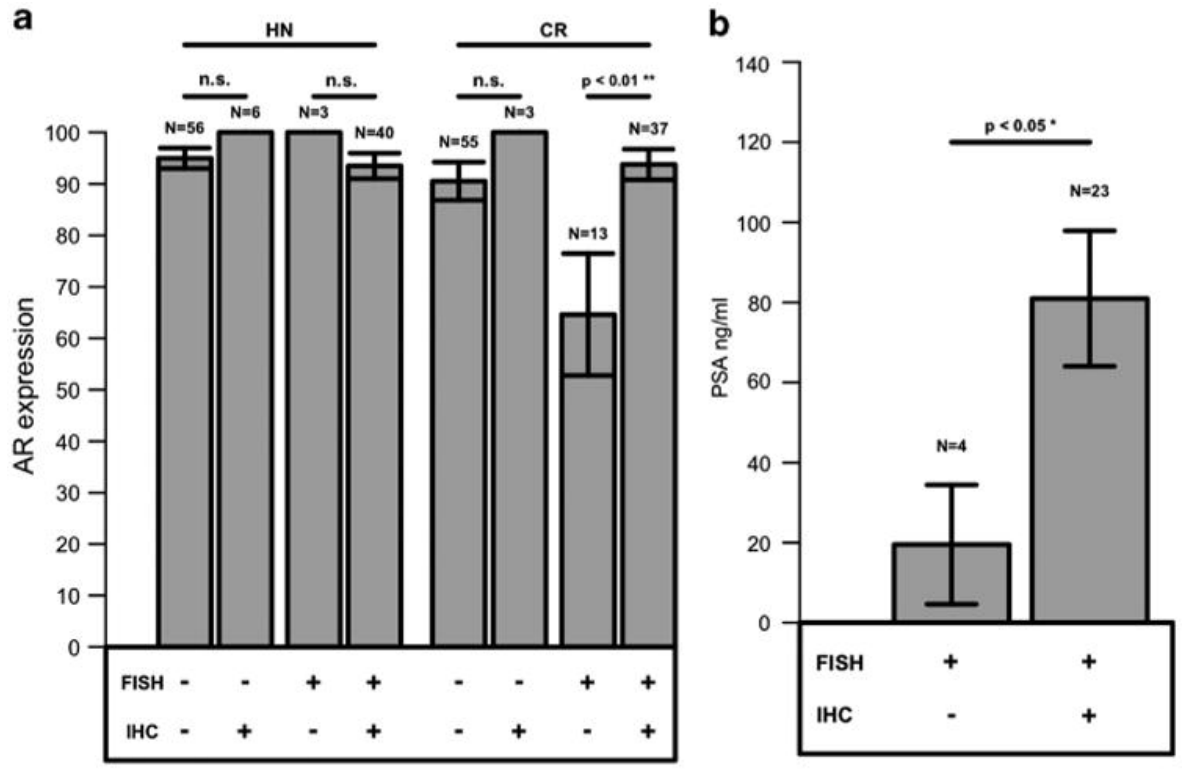


Figure 2: Differential androgen receptor (AR) and PSA protein expression in ERG-rearranged castration-resistant (CR) prostate cancer (PC). (a) AR protein expression in hormone naive (HN) and CR PC. Only ERG-rearranged CR PCs without ERG expression (discordant samples) showed significantly lower levels of AR protein expression. (b) Serum PSA levels in ERG-rearranged CR PC. The discordant samples (see Figure 2a) showed reduced levels of serum PSA. Statistical test used: Wilcoxon rank sum. n.s., not significant.

I.5 Discussion

The rearrangement of the ERG gene[2] and its associated expression in PC23 has been the subject of numerous studies. Depending on the cohort used, the prevalence of the rearrangement and protein expression varies extensively (15–80%).[13] Most of the studies have focused on the analysis of material from surgically resected prostates. In this study, we interrogated the ERG status on gene and protein levels in TURP specimens originating from HN and CR prostate tumors. For this purpose, we used a TMA specifically constructed for the analysis of disease progression.[18]

We observed an overall ERG positivity rate of 43% of both ERG rearrangement and IHC positivity across all PC samples. This is similar to recent reports that found ERG protein positivity in 47% and 52% of the PC samples[11,24,25] and ERG rearrangement in 47– 55%.[11,26,27] Stratification into HN and CR PC revealed a broader range (38–47%), but no significant differential positivity between these two groups could be detected. Concordantly, in the matched patient cohort, virtually all of the patients retained their ERG status after recurring under ADT. Although earlier reports that had focused on ERG RNA expression analysis or were based on tissues from xenografts had reported controversial prevalence rates in CR

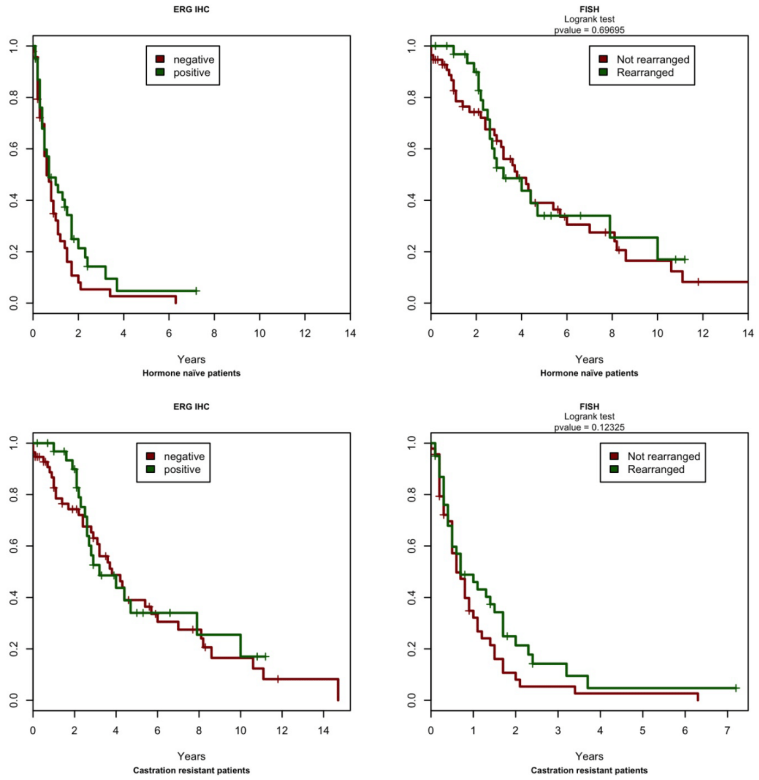
PC,[28] our findings are in line with a very recent study by Teng et al.[29] in which the authors observed the ERG expression in 37% of human CR PCs. These data strongly suggest that even lower levels of circulating androgens, as is the case under ADT therapy in patients with CR disease, are sufficient to sustain ERG expression in ERG-rearranged PC. Although no correlation of ERG status with clinico-pathological features, such as cM or cT stage, was found, we observed that at least for the protein expression, positive ERG status was associated with lower Gleason pattern (Table 1b). Of note, this TMA was not tailored for the analysis of the Gleason pattern, as most (97%) of the arrayed PCs show a high Gleason pattern (4 or 5) (Supplementary Table S3). In previous studies, TMPRSS2-ERG-negative PCs have already been associated to the highest Gleason category studied.[11,30] Similarly, we observed that a high fraction of tumors with Gleason Pattern 5 is ERG negative: 67% and 70% for HN and CR PCs by IHC, respectively, as well as 70% for HN PC by FISH (Table 1b). Interestingly, the lower number of ERG FISH-negative CR PCs with Gleason Pattern 5 (58%) might be explained by the higher number of ERG-discrepant CR PC samples in this study (see below).

As expected, we found a strong correlation between genomic rearrangement and protein expression in HN as well as in CR PCs, confirming that ERG expression depends on the presence of the ERG rearrangement, also in more advanced CR PCs. Stratification into the four different FISH (negative/positive) and disease (HN/CR) subgroups revealed that in the subgroup of ERG-rearranged CR PC the rate of discordant samples was surprisingly high (26%), suggesting that every fourth ERG-rearranged CR PC will no longer express the ERG protein. As the discordance rates in the other three groups were between 7% and 9%, the high discordance rate of 26% might be attributed to the defects of androgen signaling. Very recently, Teng et al.[29] had also reported a decrease in the consistency rate in the group of CR PC. However, their detected decrease was mainly due to ERG rearrangement negativity that needs to be further explored. Here, our findings suggest that up to 26% of the ERG-rearranged CR PC have lost their ability to express the ERG protein. These findings are consistent with a defective AR pathway.[31,32] Indeed, only the discrepant samples of this subgroup (CR PC, ERG FISH positive) had significant lower levels of AR protein expression. Concordantly, this minor group of samples also had lower serum PSA levels. One could hypothesize that such patients with PCs who do not express androgenresponsive genes any longer might not be good candidates for a continuing ADT. However, it must be considered that serum PSA level information was only available for four patients of the subgroup of ERG-rearranged but ERG-protein-negative CR PCs. In a recently published study, we reported a subgroup of advanced CR PC patients whose tumors were characterized by the lack of AR expression and had a worst overall survival.[18] Half of those tumors were classified as neuroendocrine prostate tumors, suggesting that they had circumvented AR dependency possibly by neuroendocrine-responsive trans-differentiation mechanisms.[33] In contrast, in the subgroup of ERG-discrepant samples (CR PC, ERG FISH positive but IHC negative), only four out of the 13 stained

positive for neuroendocrine markers, thus suggesting that neuroendocrine trans-differentiation alone cannot explain the characteristics of this subgroup. The four poorly differentiated neuroendocrine CR PCs included two small cell prostate carcinomas and two large cell neuroendocrine carcinomas. Further studies are needed to investigate the specific characteristics of this ERG FISH-positive but ERH IHC-negative subset of PCs on a molecular level and to define the role of ERG rearrangement and expression.

A limitation of our study is that our cohort comprises locally advanced and obstructive tumors from palliative TURPs. Materials from TURPs for TMA construction must be rigorously examined before construction to exclude areas with technical artifacts originating from the resection procedure (for example, heat/mechanical damage). However, PC specimens from these TURPs represent very valuable tissue samples, especially in the context of hormonal ablation. In this study, the stratification into different disease states (HN/CR) and FISH positivity groups limited the sample number in the different subgroups. Thus, studies with larger cohorts of HN and CR PC samples from TURPs are needed to further assess these findings and to evaluate the AR-downstream signaling pathways in the distinct HN/CR ERG subgroups. Despite these limitations, in this study we were able to show the prevalence of ERG positivity in HN and CR PC and that this positivity is not differentially distributed between these two disease groups. Importantly, we provide evidence for the existence of an ERG-rearranged PC subset of cases that has apparently lost the ability to express androgen-regulated genes.

I.6 Supplementary Material



Supplementary Figure 1: Overall survival of HN and CR patients. No significant association of ERG status with overall survival was for HN (upper panel) or CR (lower panel) was observed.

Technique	Status in		N (%)
	HN	CR	
IHC	Negative	Negative	11 (52%)
	Positive	Positive	9 (43%)
	Negative	Positive	0
	Positive	Negative	1 (5%)
FISH	Negative	Negative	15 (50%)
	Positive	Positive	13 (43%)
	Negative	Positive	1(3%)
	Positive	Negative	1 (3%)

Supplemental Table 1: ERG status in matched HN/CR patient cohort. Nearly all (IHC: 20/21, FISH 28/30) of the PC samples retained their ERG status after recurrence to castration resistance.
Abbreviations: IHC = immunohistochemistry, FISH = fluorescence in-situ hybridization.

	HN: * pvalue < 0.05; CR: n.s.					n.s.			
	FISH					Immunohistochemistry			
	Not Rearranged		Rearranged			ERG negative		ERG positive	
Ki67	n	%	n	%		n	%	n	%
HN:									
Low	64	62%	27	45%		41	59%	32	55%
High	39	38%	33	55%		28	41%	26	45%
CR:									
low	36	42%	31	38%		37	40%	26	43%
high	50	58%	50	62%		56	60%	35	57%

Supplemental Table 2: Correlation of ERG status with tumor cell proliferation. Significant association between ERG status and Ki67 (tumor cell proliferation) was only observed for FISH in HN PC. Fisher's exact tests were used for comparison.
Abbreviations: HN = hormone naïve, CR = castration resistance

	Surgical Specimens		Spots on TMA	
BPH	12		36	
Hormone naïve PC	114		340	
CRPC	117		357	
	Hormone naïve	Castration resistant	Hormone naïve	Castration resistant
Age at surgery	Mean = 77.5 Median = 78.4 Min = 43.7 Max = 99.46	Mean = 77.7 Median = 79.8 Min = 49.8 Max = 90.9		
Gleason pattern			3: 19 4: 140 5: 145 na: 36	3: 1 4: 73 5: 256 na: 27
cT	1: 9 2: 8 3: 53 4: 26 na: 18	1: 1 2: 3 3: 22 4: 82 na: 8		
cM	0: 69 1: 27 na: 18	0: 31 1: 67 na: 18		
Surgery to death	Median = 3.8 (± 2.9 -5.4) years Records = 109 Events = 68	Median = 0.9 (± 0.7 - 1.2) years Records = 110 Events = 88		

Supplemental Table 3: Overview of the cohort analyzed on this TMA. Summary of the HN and CR PC specimens on the crTMA used.

I.7 Acknowledgements

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II. Publication 2

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ORIGINAL PAPER

Delineation of human prostate cancer evolution identifies chromothripsis as a polyclonal event and FKBP4 as a potential driver of castration resistance

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Abstract

Understanding the evolutionary mechanisms and genomic events leading to castration-resistant (CR) prostate cancer (PC) is key to improve the outcome of this otherwise deadly disease. Here, we delineated the tumour history of seven patients progressing to castration resistance by analysing matched prostate cancer tissues before and after castration. We performed genomic profiling of DNA content-based flow-sorted populations in order to define the different evolutionary patterns. In one patient, we discovered that a catastrophic genomic event, known as chromothripsis, resulted in multiple CRPC tumour populations with distinct, potentially advantageous copy number aberrations, including an amplification of FK506 binding protein 4 (FKBP4, also known as FKBP52), a protein enhancing the transcriptional activity of androgen receptor signalling. Analysis of FKBP4 protein expression in more than 500 prostate cancer samples revealed increased expression in CRPC in comparison to hormone-naïve (HN) PC. Moreover, elevated FKBP4 expression was associated with poor survival of patients with HNPC. We propose FKBP4 amplification and overexpression as a selective advantage in the process of tumour evolution and as a potential mechanism associated with the development of CRPC. Furthermore, FKBP4 interaction with androgen receptor may provide a potential therapeutic target in PC.

Keywords: FKBP4; FKBP52; chromothripsis; punctualism; prostate cancer; castration resistance; evolution; hormone-naïve; survival

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No conflicts of interest were declared.

II.1 Introduction

Prostate cancer (PC) is the most frequently diagnosed cancer among males in developed countries and the third leading cause of cancer-related death in men[1, 2]. The long disease course requires different treatment modalities [3, 4]. Aside from surgery and radiotherapy, androgen deprivation therapy (ADT) is one of PC's treatment pillars. Despite an initial clinical response, patients relapse after a median time of about 11.2 months [5], with castration resistance (CR) and progressive disease [6, 7]. The resistance to ADT in PC exemplifies the selective pressure exerted by medical treatment on tumours and highlights their

ability for adaptive changes, referred to as tumour evolution [8-10]. According to conventional evolutionary theory, multiple lineages of tumour cell populations can lead to intratumoural heterogeneity, a phenomenon abundant in PC [11]. The deconvolution of the resulting clonal mixtures poses a major challenge even with high-definition genomic tools, including array comparative genomic hybridisation (aCGH) and next-generation sequencing (NGS) [12]. In previous studies, we demonstrated the feasibility of DNA content-based flow-sorting followed by genomic profiling of tumour cell populations to determine the clonal composition of different tumour types and the resulting intratumoural genomic heterogeneity [8, 13]. For this study, we analysed seven patients with multiple time points of PC biopsies, each with at least one biopsy under ADT. We combined DNA copy number analysis and whole-exome sequencing to investigate the patient-specific evolutionary patterns arising under the selective pressure of ADT.

II.2 Materials and Methods

Clinical Samples

This study was approved by the Ethics Committee of Northwestern and Central Switzerland (EKNZ, No EK13/11). Patient characteristics are given in Table 1. All samples used in this study were collected for clinical (diagnostic) purposes from patients who underwent transurethral resection or radical prostatectomy due to clinical symptoms such as urinary obstruction or in a curative attempt. Samples were collected in liquid nitrogen and stored at –80 °C. Surplus tumour tissue is routinely collected in liquid nitrogen at our institution if enough tissue from the same specimen is available for formalin fixation and diagnostics. Samples complying with the inclusion criteria – (i) histologically diagnosed PC, (ii) multiple biopsies and/or time points available, (iii) concurrent ADT, and (iv) sufficient quality and amount of material – were carefully selected by an experienced pathologist (LB). Both TMAs used in this study (crTMA and the prostate progression TMA) were manufactured as previously described [14, 15].

	At first diagnosis						Anti-hormonal treatment			Clinical signs of metastatic spread during disease course		
Patient	Year	Age	TNM	PSA (ng/ml)	Gleason score	Surgical treatment[a]	Start	Treatment	Further time points	Bone	Nodal	Distant
A	2000	78	pT2 Nx Mx	164	7 (3 + 4)	Radical prostatectomy	2000	Orchiectomy; non-steroidal anti-androgen	2007 2008	Yes	Yes	No
B	2011	68	–	–	10 (5 + 5)	TUR-P	2011	GnRH agonists	2012	No	Yes	No
C	2003	69	pT3 pN0 M0	12.8	7 (4 + 3)	Radical prostatectomy	2003	GnRH agonists; non-steroidal anti-androgen	2011 2011	No	No	Yes (liver)
D	2005	74	–	42.4	7 (4 + 3)	TUR-P	2005	Orchiectomy; non-steroidal anti-androgen	2010	No	Yes	No
E	1999	70	cT3 Nx Mx	–	10 (5 + 5)	TUR-P	1999	GnRH agonists	2000	No	No	No
F	2008	67	T4 N1 M1	13.9	9 (5 + 4)	Radical prostatectomy	2012	GnRH agonists; non-steroidal anti-androgen	2012	No	Yes	Yes (kidney)
G	2009	50	–	403	7 (4 + 3)	TUR-P	2009	GnRH agonists; non-steroidal anti-androgen	2012	Yes	Yes	No

All specimens were acquired through transurethral resection if not stated otherwise (radical prostatectomy as primary treatment).
GnRH = gonadotrophin-releasing hormone.
[a]TUR-P: transurethral resection of the prostate.

Table 1: Patient details

Flow Cytometry and aCGH Microarrays

Biopsies were minced in NST buffer containing 146 mm NaCl, 10 mm Tris–HCl (pH 7.5), 0.2% Nonidet P40, and DAPI according to published protocols [8, 13]. Nuclei were disaggregated and then filtered through a 40-μm mesh before flow sorting with an Influx cytometer (Becton-Dickinson, Allschwil, Switzerland) with UV excitation and DAPI emission collected at >450 nm. DNA content and cell cycle were analysed using the software program MultiCycle (Phoenix Flow Systems, San Diego, CA, USA) and R [16]. For aCGH analyses, the Agilent microarray platform (Agilent Technologies, Santa Clara, CA, USA) was used as previously described [8]. All microarray files and metadata have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE108203 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE108203). ACGH data analysis was performed using the Limma package [17] in the R environment [16]. Segment calling was performed with the circular binary segmentation (CBS) algorithm [18, 19]. Segments with a log2 ratio higher than 0.58 (upper boundary) or lower than –1.0 (lower boundary) were defined as genomic aberrations.

Genomic Analysis

DNA was extracted using Qiagen micro kits (Qiagen, Hilden, Germany). For each hybridisation, 100 ng of genomic DNA from each sample and of pooled commercial 46,XX or 46,XY reference (Promega, Madison, WI, USA) were amplified using the GenomiPhi amplification kit (GE Healthcare, Little

Chalfont, UK). One microgram of amplified sample and 1 μg of amplified reference template were digested with DNaseI and then labelled with Cy-5 dUTP and Cy-3 dUTP using a BioPrime labelling kit (Invitrogen, Carlsbad, CA, USA). All labelling reactions were assessed using Nanodrop before mixing and hybridisation to either 244 000 or 400 000 feature CGH arrays (Agilent Technologies). Whole-exome paired-end sequencing on a HiSeq 2000 (Illumina Inc, San Diego, CA, USA) was performed after exome capturing using the Agilent Sure Select enrichment system (Agilent Technologies). Resulting bam files were uploaded to Five3 Genomics (Santa Cruz, CA, USA) and processed according to their protocol (Five3 Genomics). In brief, the human genome reference build 37 (hg19) was used to align the data with BWA [20]. In turn, duplicates were marked by samblaster [21] and indel realignment and base quality recalibration were performed by GATK v2.3 [22]. Due to missing germline DNA as a reference, exome sequencing data were only used for determining structural variants. Structural variants were identified using methods described on structural variation in glioblastoma multiforme tumours using the BamBam algorithm [23, 24]. They were required to have a minimum support of at least six reads, with an average mapping quality greater than 30.

IHC and FISH of FKBP4

FKBP4 staining was performed with the mouse monoclonal antibody AT4D3 (LS-C103478; LifeSpan Biosciences, Seattle, WA, USA) in a 1:800 dilution, and p53 staining with the mouse monoclonal antibody DO-7 (800-2912; Ventana, Tucson, AZ, USA), both on a Ventana Benchmark XI instrument (Ventana), according to standard indirect immunoperoxidase procedures. FKBP4 custom FISH probes were created according to published protocols [25, 26] by use of the BAC clone RP11-958H19 (Empire Genomics, Buffalo, NY, USA). As a reference, a SpectrumGreen-labelled chromosome 12 centromeric probe was used (Vysis Abbott Molecular, Abbott Park, IL, USA).

Cell Culture Experiments

The BPH cell line was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA). Media were supplemented with 20% heat-inactivated fetal bovine serum (FBS), 2 mm glutamine, 100 U/ml penicillin/streptomycin, 20 ng/ml testosterone (Sigma Aldrich, St Louis, MO, USA), and 1× Insulin-Transferrin-Selenium Supplement Mix (Thermo Fisher Scientific, Waltham, MA, USA). Cells were transfected with Lipofectamine LTX and Plus reagent (Thermo Fisher Scientific) according to the manufacturer's specifications. Plasmids for FKPB4 overexpression and silencing were obtained from GeneCopoeia (Rockville, MD, USA). Cell growth was assessed using the xCELLigence system (ACEA Biosciences, San Diego, CA, USA). Twenty-four hours after transfection, cells were washed twice with PBS and resuspended in DMEM without Phenol Red supplemented with charcoal-stripped FBS (Thermo

Fisher Scientific) with or without testosterone (70 nm). Five thousand cells per well were plated in an 18-well plate and the growth rate was measured in real time at the indicated time points.

Statistics

Differences between two groups were analysed with the Wilcoxon's rank sum test or Student's t-test for in vitro experiments. Correlations were tested according to Spearman. Survival curves were plotted by the Kaplan–Meier method and differences were assessed using the log-rank test. P values less than 0.05 were considered significant. Pearson's correlation was used to assess array similarity and evolutionary distance.

II.3 Results

Heterogeneous nature of clonal cancer evolution under the selective pressure of androgen deprivation therapy

	At first diagnosis						Anti-hormonal treatment			Clinical signs of metastatic spread during disease course		
Patient	Year	Age	TNM	PSA (ng/ml)	Gleason score	Surgical treatment[a]	Start	Treatment	Further time points	Bone	Nodal	Distant
A	2000	78	pT2 Nx Mx	164	7 (3 + 4)	Radical prostatectomy	2000	Orchiectomy; non-steroidal anti-androgen	2007 2008	Yes	Yes	No
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E	1999	70	cT3 Nx Mx	–	10 (5 + 5)	TUR-P	1999	GnRH agonists	2000	No	No	No
F	2008	67	T4 N1 M1	13.9	9 (5 + 4)	Radical prostatectomy	2012	GnRH agonists; non-steroidal anti-androgen	2012	No	Yes	Yes (kidney)
G	2009	50	–	403	7 (4 + 3)	TUR-P	2009	GnRH agonists; non-steroidal anti-androgen	2012	Yes	Yes	No

All specimens were acquired through transurethral resection if not stated otherwise (radical prostatectomy as primary treatment).
GnRH = gonadotrophin-releasing hormone.
[a]TUR-P: transurethral resection of the prostate.

Table 1: Patient characteristics

The clinical data and pathological information of the patients and the corresponding biopsies are summarised in Table 1. The genomic data from patient A have been described in a previous study (see above and ref [8]): in the observed branched evolutionary pattern, one diploid tumour population represented the trunk of the tumour lineage with a gradual evolution of aneuploid branches under the selective pressure of ADT. This lineage gave rise to morphologically less differentiated and genomically more aberrant tumour populations that were eliminated by therapeutic escalations (Figure 1A). As in our previous study, we applied DAPI-based flow-sorting followed by genomic profiling by array CGH (aCGH) of all, and exome sequencing of selected, sorted populations [8, 13]. Tumour populations were

defined by DNA content (± 0.2 N) and by the presence of aberrant genomic intervals detected by aCGH. We identified three evolutionary patterns under ADT that were congruent with conventional evolutionary theory and NGS-based cancer models [9, 27, 28] (Table 1, Figure 1A–C, and supplementary material, Figure S1): (i) linear evolution with gradual acquisition of genomic aberrations (Figure 1B and supplementary material, Figure S1), as well as (ii) branched evolution with (iia) gradual (Figure 1A, C) or (iib) punctual (Figure 1D) acquisition of genomic aberrations. Whereas in gradual evolution few events (here: genomic aberrations) are slowly acquired over time, a punctual evolution (Figure 1D) represents the occurrence of multiple decisive events in a single or at least short time frame with a mostly quiescent evolution in between [27]. In contrast to the evolutionary pattern of the patient introduced in our previous study (Figure 1A), a linear rather than a branched evolution was found in most patients under ADT (four out of seven). In this predominant pattern, a stable but genomically aberrant tumour population acquired new genomic aberrations without evident generation of a stable parallel tumour population. The ploidy remained constant (up to 2 years), and the low number of newly acquired copy number aberrations (CNAs) after therapy initiation advocated for a gradual evolution. For example, the copy number variations within the 3.7 N population from patient B, which were acquired under ADT, affected only 82 genes (Figure 1B; see the Materials and methods section). This is in stark contrast to the two CRPC populations in patient D that resulted from a punctual evolution with 599 and 1554 genes affected by genomic CNAs (see below).

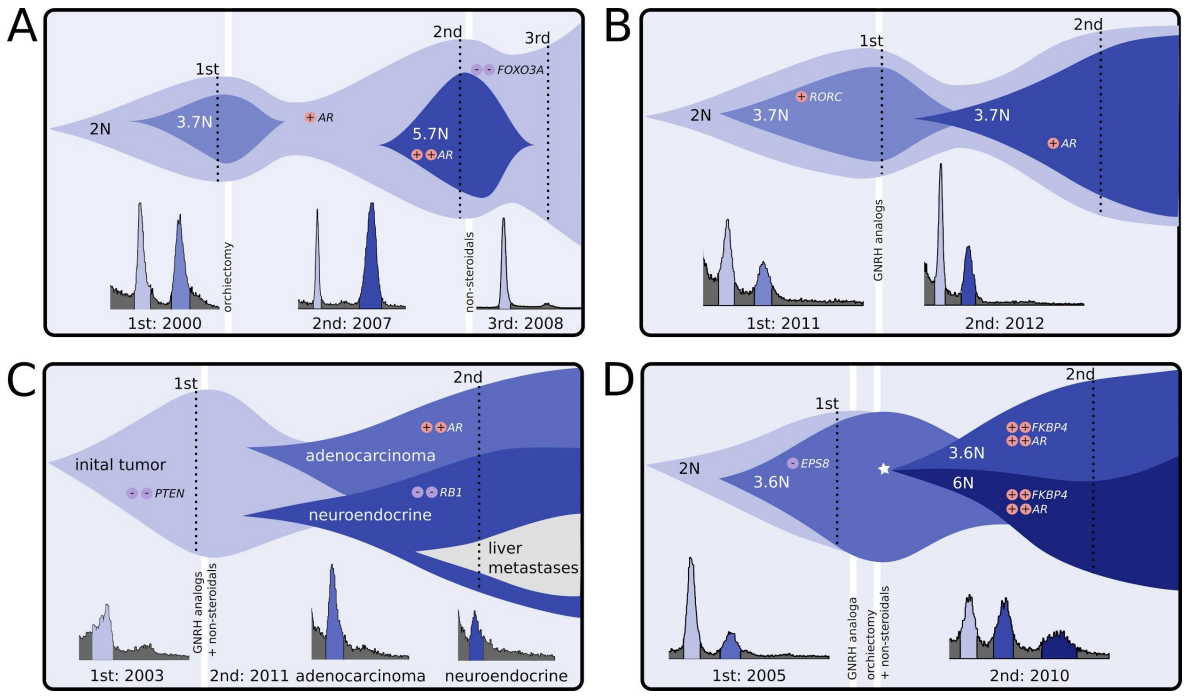


Figure 1: Different pathways for the development of castration resistance. (A) Branched development of two aneuploid tumour populations from a diploid tumour population with gradual acquisition of genomic aberrations. (B) Linear development with gradual acquisition of pathognomonic aberrations, such as the AR gene amplification after initiation of ADT. (C) Branched evolution is resulting in concomitant occurrence of diploid adenocarcinoma and neuroendocrine CRPC tumour populations. (D) Punctual development (chromothripsis denoted by a star) resulting in the parallel occurrence of two aneuploid populations. DNA content-based FACS histograms of biopsies are at the bottom of each box. The x-axes are not drawn to scale; the vertical dotted lines represent the time point of the biopsy. Vertical text on a white background describes the therapies that the patient received. GNRH = gonadotrophin-releasing hormone; + = amplification; ++ = high-level amplification; – = deletion; – – = homozygous deletion.

We next investigated androgen receptor (AR) gene amplification as a known mechanism for CRPC. The AR gene was amplified in five out of the seven patients: the copy number levels varied from duplication in the diploid population of patient A to multiple folds (log2 ratio = 2.1) in one of the aneuploid populations of patient C (Figure 1C). Analysis of this CRPC tissue specimen revealed the co-existence of an adenocarcinoma (AC) and a neuroendocrine (NE) cancer component. Genomic profiling based on aCGH of these two different compartments revealed a clonal relationship with shared as well as private genomic aberrations: The AC cells, but not the NE cells, showed AR gene amplification (supplementary material, Figure S2). This is in line with the current understanding that NE differentiation is a mechanism of castration resistance circumventing the AR signalling pathway [29, 30]. The liver metastasis of the same patient was genomically more closely related to the NE than to the AC compartment (0.58 versus 0.64 Pearson's rho), suggesting the NE compartment as the origin of the liver metastasis. This was further exemplified by specific genomic aberrations, such as the homozygous deletion of the RB1 gene, which was only shared between the liver metastasis and the NE compartment of the primary tumour (supplementary material, Figure S2). This is in line with recent findings showing that RB1 alterations are a hallmark of NE carcinomas in general [31].

We next focused on the rate of CNAs acquired under the selective pressure of ADT and the underlying evolutionary models. In line with conventional evolutionary theory, our observed patterns fitted the two concepts of gradualism and punctualism described in 1977 [27]. The evolution of virtually all tumours in this study (six out of seven; Table 1 and Figure 1A–C) can be attributed to the first model since CNAs were acquired in small and gradual steps. In contrast, in patient D, ADT resulted in two distinct aneuploid CR populations (3.6 N and 6.0 N), both having massive rearrangements of chromosome 12 that were not present in the preceding HN 3.6 N aneuploid population. This suggests that a punctual event consistent with chromothripsis must have occurred after initiation of ADT (star in Figure 1D), leading to massive rearrangements within this chromosome.

Analysis of the Different Populations Arising from Chromothripsis Identifies FKBP4 as a Potential Driver

To determine the potential role of chromothripsis as a selective event for progression to castration resistance, we next focused on the genomic profiles of the two tumour populations in the CRPC specimen of patient D. Chromothripsis involves the shattering of one or a few chromosomes in a single event, resulting in a large number of CNAs and massive intrachromosomal rearrangements [32]. In these two co-occurring aneuploid populations, chromothripsis affected only chromosome 12, in particular the whole p-arm and about 30 Mb of the q-arm (Figure 2A). CNAs in the remaining chromosome regions were mostly shared by the two populations, although not entirely. For example, the 6 N population contained a unique deletion spanning 35 Mb at the beginning of chromosome 4, which was not present in the 3.6 N population. A remarkable amount of differently sized amplifications and deletions was detected in the chromothriptic region. A total of 361 genes were affected by CNAs in this region (3.6 N: 212; 6 N: 134). As the chromothriptic regions show significant differences between the samples, only 30% (81) and 24% (23) of the 265 amplified and 96 deleted genes, respectively, were shared by both populations (Figure 2B and supplementary material, Figure S3). Whole-exome sequencing revealed 12 interchromosomal structural rearrangements (supplementary material, Figure S4) but also a total of 69 intrachromosomal rearrangements on the chromosome region affected by the chromothripsis: 27 for the 3.6 N population and 42 for the 6 N population (Figure 2C). None of these intrachromosomal structural variants, which are likely products of chromothripsis, were shared between the two aneuploid populations. These findings suggest that the two aneuploid CRPC populations did not originate from each other but evolved in parallel from a common ancestor that had undergone chromothripsis. The 3.6 N HN PC population from 2005 is likely to be this common ancestor since the CNAs in its genome were still present in the two 2010 aneuploid CRPC populations. Accordingly, both of the later CRPC tumour populations showed the same correlation coefficient as the putative common ancestor population (Pearson's rho = 0.58; supplementary material, Figure S5). Analysis of the AR amplicon shows different breakpoints and amplicon sizes for the 3.6 N and 6 N populations and therefore does not support synchronous development (supplementary material, Figure S6). It is debatable if chromothripsis may be a selective driver event and not just a bystander process [33]. We thus hypothesised that these two aneuploid CRPC populations were selected due to a common genomic aberration providing them with a selective advantage under the ADT environment. We interrogated the genome for aberrant segments with highly deviant log2 ratios (> 2 from baseline 0) in both of the aneuploid CRPC populations (2010) and with low absolute log2 ratio differences between the two populations (Figure 3A). Based on our hypothesis, we selected for genes with a reported function in CR or steroid pathway signalling: We identified FKBP4 as the most promising candidate and verified its genomic and expression status by FISH and IHC (Figures 3 and 4). Of note, FKBP4 (also known as FKBP52) is considered to be part of the AR complex and has been identified as an enhancer of AR function [34-37]. As

expected, FISH analysis with an FKBP4-specific probe revealed genomic amplification in the CRPC tissue biopsy from 2010 but normal gene status in the HNPC tissue from 2005 (Figure 4). The CRPC, but not the HNPC, was positive for FKBP4 by immunohistochemistry, suggesting that FKBP4 gene amplification emerging from chromothripsis might be the underlying mechanism for high protein expression.

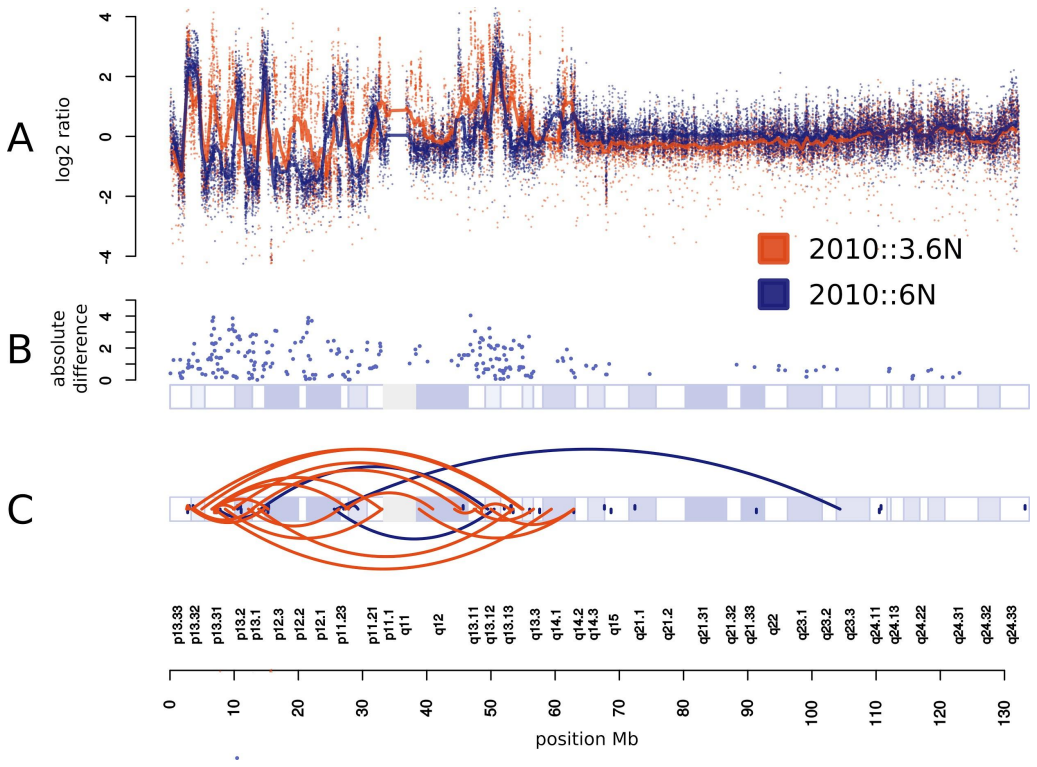
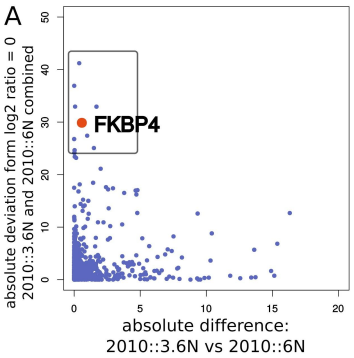


Figure 2: Chromothripsis results in two aneuploid tumour populations with private genomic aberrations. (A) Overlay of the copy number profiles (chromosome 12) of the two aneuploid populations from one biopsy taken from patient D. (B) Plot of genes (blue dots) with a difference in copy number status between the two aneuploid populations in chromosome 12. The blue dots represent the absolute difference of a gene between the respective log ratios (delta-squared). (C) Intrachromosomal structural rearrangements on chromosome 12 of the two aneuploid populations as determined by exome sequencing. Orange: 3.6N; blue: 6N.

A



B

	Gene symbol	Log 2 ratios	
		2010::3.6N	*2010::6N*
1	*EPS8*	-4.5239	-5.3468
2	*KRT77*	3.5191	2.9004
3	*NR4A1*	3.0707	3.0044
4	*ACVR1B*	2.7354	3.0044
5	*KRT74*	3.5191	2.2196
6	*KRT72*	3.5191	2.2196
7	*KRT73*	3.5191	2.2196
8	*KRT2*	3.5191	2.2196
9	*FKBP4*	3.114	2.3508
10	*ITFG2*	3.114	2.3508

Figure 3: Identification of FKBP4 as a potential driver of castration resistance. (A) Analysis of highly aberrant genes in the two aneuploid populations of patient D. Genes were selected with a high deviation of the log_2 ratio from 0 occurring in both populations (y-axis), but with a low absolute difference between the two populations (x-axis). (B) List of the top ten genes. Based on previous reports (function in CR or steroid pathway signalling), FKBP4 was selected for further analysis.

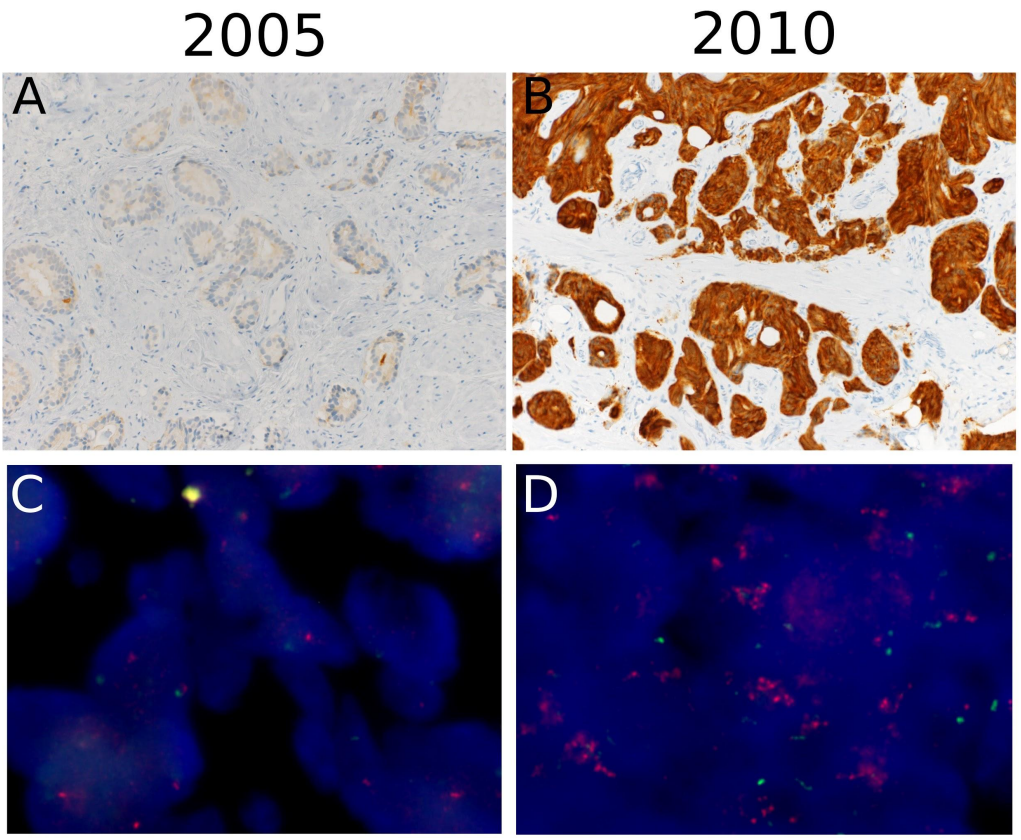


Figure 4: FKBP4 is amplified and overexpressed in the CRPC biopsy of patient D. (A, B) FKBP4 immunohistochemistry of the HNPC biopsy from 2005 (A) and the CRPC biopsy of the same patient from 2010 (B). (C, D) FISH analysis in these two samples. Red dots: FKBP4 gene signals; green dots: centromere 12.

FKBP4 is highly expressed in a subset of hormone-naïve prostate cancer and associated with poor overall survival

To further define a potential role for FKBP4 in progression to castration resistance and its association with androgen signalling, we performed in vitro cell growth experiments. We selected the benign prostate cell line BPH-1, due to its intact androgen axis, to study androgen-related effects in PC [38]. BPH-1 cells were grown in androgen-depleted medium and transfected with either an FKBP4 cDNA overexpressing plasmid under a CMV promoter or shRNA encoding plasmids aimed towards the silencing of FKBP4 (supplementary material, Figure S8). Both conditions were evaluated with and without exogenous addition of testosterone (70 nm) and assessed using real-time impedance measurements. Silencing and overexpression of FKBP4 in BPH-1 resulted in reduced and increased proliferation, respectively, of these cells in testosterone-supplemented medium (Figure 5A, B). We next investigated the role of FKBP4 amplification and protein expression in a larger cohort of human prostate cancers. For this purpose, we determined the gene copy number and protein level of FKBP4 by FISH and IHC, respectively, on the crTMA. This tissue microarray consists of 114 HNPC and 117 CRPC formalin-fixed and paraffin-embedded tumour specimens. It was specifically constructed for the analysis of markers in the progression of HN to CRPC [8, 15, 39, 40]. Copy number status was evaluable in 198 of 231 cases. We detected genomic amplification of FKBP4 in 4 of 98 HN (4%) and in 2 of 100 CRPC (2%) samples (supplementary material, Figure S9). Polysomy was found in another four HN (4%) and eight CRPC samples (8%). Immunohistochemistry revealed that 77/110 (71%) HN and 95/114 (84%) CRPC samples expressed FKBP4 (histoscore >0). Analysis of the matched HN/CRPC cohort showed an increase of at least ten histoscore units in FKBP4 protein in 50% (n = 11) of the 22 evaluable cases from HN to CR but a decrease in only 14% (n = 3). Although we observed a trend towards higher FKBP4 expression in PC samples with increased FKBP4 gene copy numbers (amplification or polysomy), tumours with normal FKBP4 gene copy number could also sustain high FKBP4 levels of up to 300 histoscore units, suggesting that other mechanisms besides amplification can lead to high FKBP4 protein expression. In contrast to the above-mentioned results of patient D, there was no general increase in FKBP4 protein in the CRPC versus HNPC samples on this TMA.

We speculated that this could be due to the advanced stage of the HNPC cohort of this crTMA since the samples were mostly from palliative transurethral resections of the prostate (TUR-P). Thus, we investigated FKBP4 protein levels on a second prostate TMA that was constructed for the general analysis of markers in the different PC stages, including 535 tumour and control samples ranging from incidentally detected localised PC to advanced metastatic CRPC [41]. Indeed, on this TMA, FKBP4 protein expression was increased in CRPC in comparison to HNPC (Figure 5C), with a mean FKBP4 histoscore of 14.4 for the HN and 87.1 for the CR samples (Wilcoxon rank-sum test: p = 2.301e-07). Since FKBP4 has

been described as a co-activator of the AR, we investigated a potential correlation of FKBP4 protein with AR and AR phosphorylation (serine 210-phosphorylated AR: pARSER210). We used pARSER210 data that had been acquired on the crTMA for a previous study [15]. While no association was observed with total levels of AR, we detected a positive association between FKBP4 expression and pARSER210 in HNPC (Spearman's rho = 0.385, p = 0.013) and in CRPC (Spearman's rho = 0.468, p = 0.0005), emphasising the potential role of FKBP4 under hormone withdrawal to sustain AR pathway signalling (Figure 5D, E). Since the AR is known to activate kallikrein-related peptidase 3 (KLK-3) gene expression, encoding prostate-specific antigen (PSA), we further investigated a correlation between PSA values in the blood and FKBP4 protein on the crTMA. Indeed, increased FKBP4 protein was associated with higher blood PSA levels in CRPC but not in HNPC (Spearman's rho = 0.33, p = 0.022) (supplementary material, Figure S10). We next investigated the potential impact of FKBP4 on overall survival. Survival analysis (median follow-up time for HN and CRPC was 36.6 and 8.4 months, respectively) revealed a strong association between high FKBP4 protein expression (grouped by median histoscore) and poor overall survival in HN patients ($\chi 2 = 22.4$, n = 80, p = 8.59e-07) (Figure 5F). The survival rate at median follow-up time (33 months) was 79% and 38% for patients with low and high FKBP4 protein expression, respectively. This association was not observed in the CR population ($\chi 2 = 0.1$, n = 73, p = 0.735).

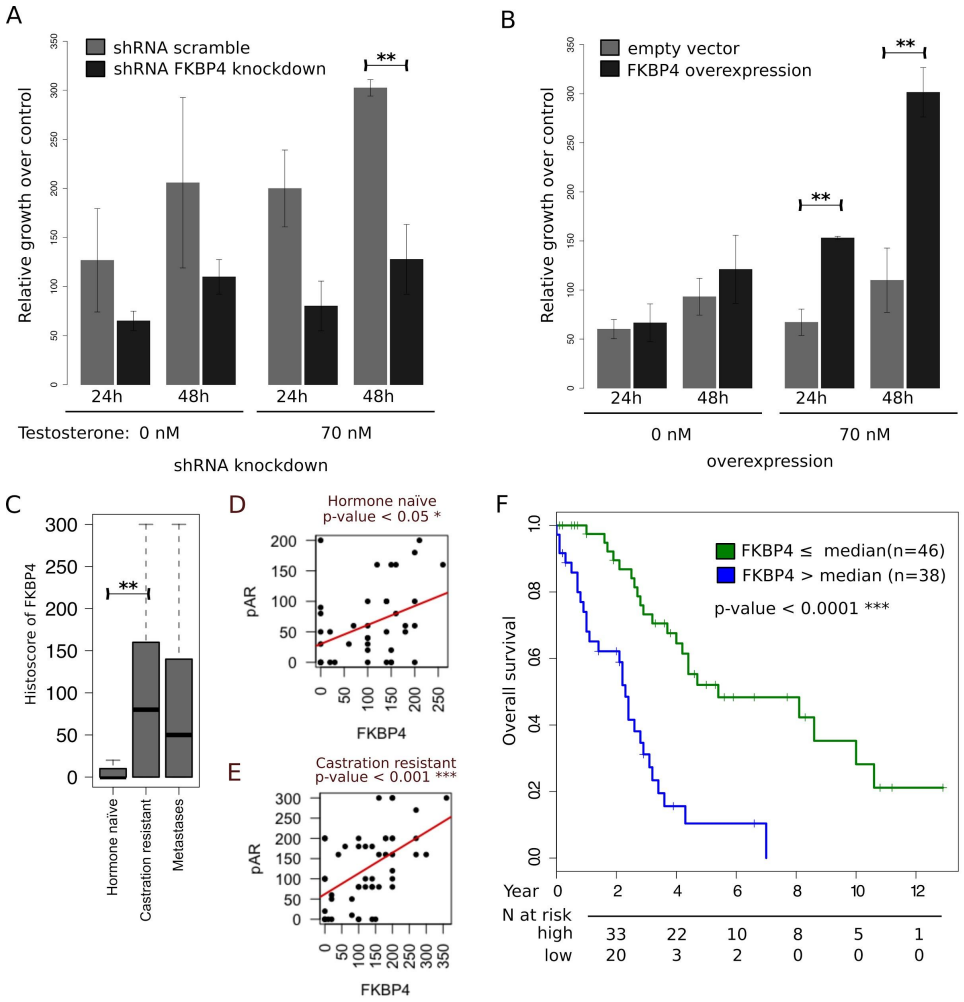


Figure 5: Clinical significance of FKBP4. (A, B) Growth of BPH-1 cells with (A) silencing of FKBP4 expression or (B) FKBP4 overexpression. (C) Analysis of FKBP4 levels by IHC in the progression TMA. (D, E) Correlation of FKBP4 expression with pARSER210 in HN (D) and CR (E) patients. (F) Overall survival analysis of patients with HNPC stratified according to FKBP4 level on the crTMA.

II.4 Discussion

In this study, we aimed at defining the different evolutionary patterns of PC under the selective pressure of ADT. Our approach is in contrast to previous ones describing the genomic landscape of PC [42] or applying mathematical modelling for determining clonality based on allelic frequencies [43]. Even though such computational approaches to capture tumour heterogeneity show impressive advances, they rely on model assumptions, which might not cover the whole complexity of human cancers and their evolution under treatment over time [44-46]. We selected patients with multiple longitudinal biopsies, including at least one obtained under ADT. Time intervals of up to 9 years and the availability of fresh-frozen material allowing DNA content-based flow-sorting before genomic profiling were

essential for this study. We identified different patterns of tumour evolution highlighting the heterogeneity of PC and the pressure of ADT during selection for CR tumour cell populations.

Comparisons between tumour evolution and evolution of species in the context of conventional evolution theory have been made previously [9]. Classically, evolution has been divided into punctual and gradual events [27], which have both been cited in the context of PC evolution [28]. We tracked the evolution of clinically relevant tumour populations over time and demonstrated a predominance of gradually developing, partly tree-shaped evolutionary patterns. In patient C, both concurrent CR populations emerged from the same adenocarcinoma HN population via different mechanisms: (i) androgen sensitisation through genomic amplification of the AR gene, and (ii) bypassing of the AR pathway via neuroendocrine differentiation. We have demonstrated that mechanisms for escaping ADT are not mutually exclusive, but can occur concurrently within the same tumour. Interestingly, one of our seven patients showed chromothripsis as an extreme case of punctuated evolution. Although it has been reported that 5–6% of PCs show signs of chromothripsis [47], [48], to the best of our knowledge, this is the first time that chromothripsis has been shown in the context of progression to CRPC. Of note, the biopsy affected by chromothripsis showed a normal p53 protein expression by immunohistochemistry, arguing against a p53 mutation (supplementary material, Figure S7). This is in contrast to reports in other tumour entities [49, 50], where chromothripsis has been linked to TP53 gene mutation. The co-existence of two distinct populations with chromothripsis escaping ADT towards CRPC in a single tumour specimen provides strong evidence for a selective advantage [33]. It is unlikely that the different copy number profiles in the different tumour populations could have been discovered by genomic analyses of bulk material, i.e. without prior purification by the use of flow sorting.

Several mechanisms have been proposed for the development of chromothripsis [33, 51]. Our results indicate two distinct genomics patterns arising from chromothripsis in one single tissue specimen and provide important new insights into the elucidation of this phenomenon. The sequential generation of one chromothriptic population from the other is rather unlikely and advocates that both populations must have resulted from a single catastrophic event. One might speculate that even more populations emerged under the premise of high genomic instability, but that only the ADT-resistant populations survived. Our data suggest that both populations have been selected independently from each other but that the shared genomic aberrations in the affected chromosome 12 region might have conferred resistance to ADT. The comparative evaluation led to the identification of FKBP4 as a shared amplified candidate gene.

The FKBP4 protein is known as a component of the AR complex that is required for AR's nuclear translocation after ligand binding. FKBP4 has been suggested as a potential therapeutic target in CRPC, and there is a synthetic inhibitor with anti-proliferative effects in androgen-stimulated PC cells in vitro

[37, 52]. This concept is in line with our findings showing decreased cell growth after shRNA-based knockdown of FKBP4, as opposed to increased cell proliferation after FKBP4 overexpression. Of note, overexpression was ineffective in stimulating proliferation in the total absence of androgens, suggesting that FKBP4 function depends on remaining low levels of androgens. In fact, FKBP4 might enhance androgen axis function through increased receptor affinity and nuclear translocation [35] and therefore might be able to sustain AR function under ADT. Recently, it has been reported that the interaction of beta-catenin with FKBP4 potentiates hormone-dependent, as well as hormone-independent, AR activity [53]. This further supports the model that FKBP4 amplification and/or overexpression may be a potential mechanism in the progression towards CRPC. Our finding of a significant association between FKBP4 and poor overall survival in advanced hormone-naïve PC patients emphasises a critical role for FKBP4 in advanced PC. Here, for the first time, we have demonstrated the significance of FKBP4 in the context of CR in a large cohort of clinical samples. FKBP4-positive advanced HNPC might be pre-selected and conditioned to resist subsequent ADT, which would explain the poor overall survival in this patient group. Once CR disease has been achieved, the specific mechanism of CR does not affect progression or prognosis.

In contrast to FKBP4, CR mechanisms such as AR amplification and AR mutation appear to be selected for after initiation of ADT, as they are almost never detectable in HNPC. Further studies are needed to clarify if FKBP4 amplification and/or expression can serve as a predictor of poor response to ADT. Interestingly, FKBP4 does not interact with the truncated AR protein resulting from the AR-V7 splice variant, which lacks the ligand binding domain, the site of interaction with FKBP4. In the presence of AR-V7, FKBP4 inhibitors lose their anti-proliferative effect in cell line experiments [36]. According to public databases, amplification of FKBP4 can be detected in up to 3.3% of PCs [54]. This is in line with our detected amplification rates of 2% and 4% in HN and CRPC. Importantly, we have shown high FKBP4 expression in a much larger proportion of our cohort, suggesting that other mechanisms besides gene amplification can lead to increased expression of FKBP4. Our study was not designed to fully elucidate the role of FKBP4 in the context of androgen-driven PC but based on the results of our copy number profiling, we identified FKBP4 as a promising candidate biomarker to better predict the response to ADT and as a potential therapeutic target to be tested in future studies.

II. 5 Supplementary Material

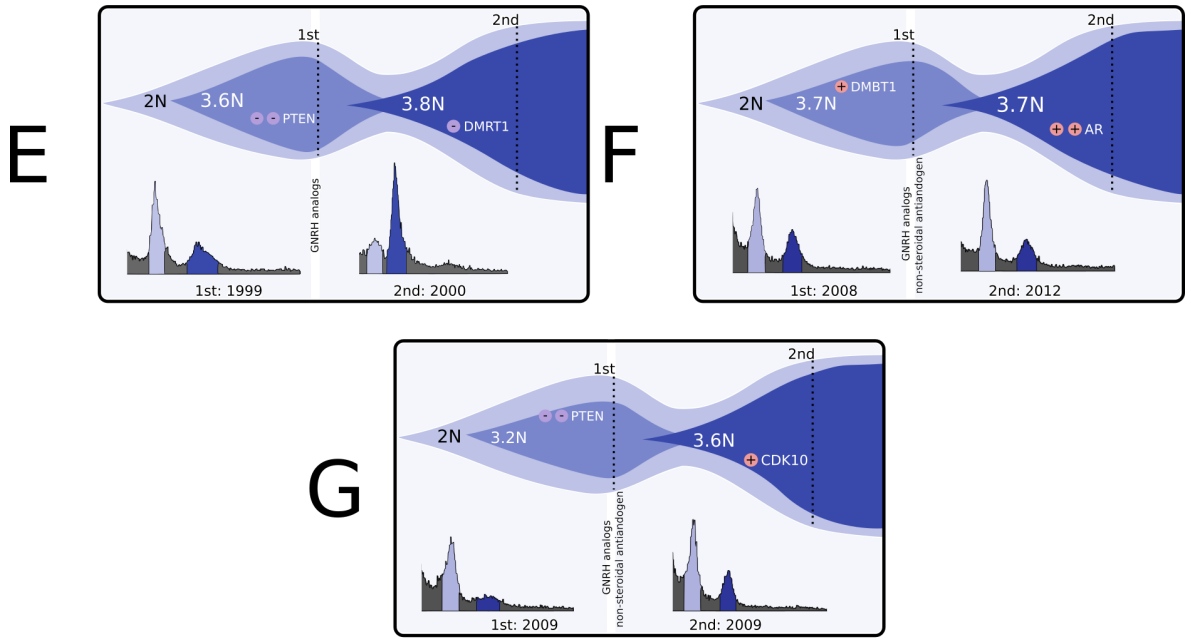


Figure S1. Illustration of patients E–G analogues of Figure 1. All patient samples following a gradual pattern of evolution

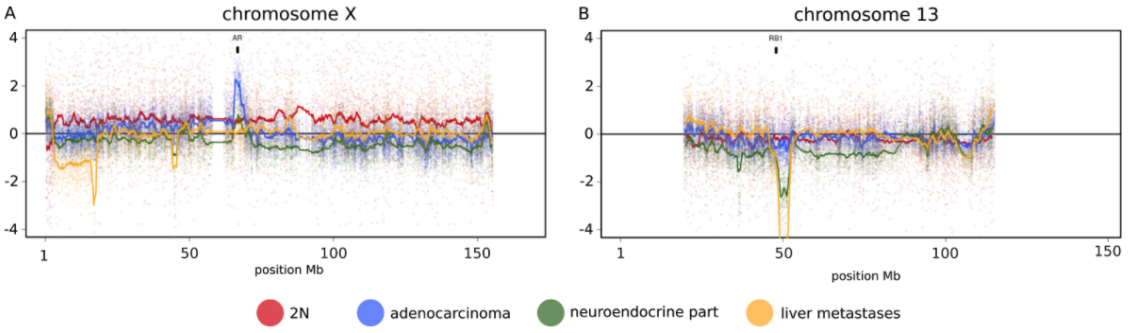


Figure S2. Array CGH profiles of chromosomes X (A) and 13 (B) of the CRPC specimen from patient C

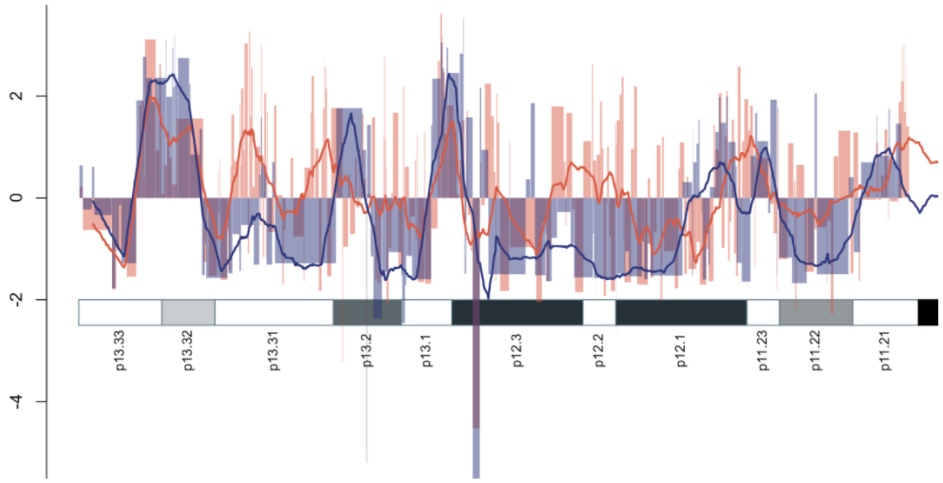


Figure S3. In-depth representation of the p-arm of chromosome 12 for patient D

Figure S4. Circos plots of whole-exome sequencing

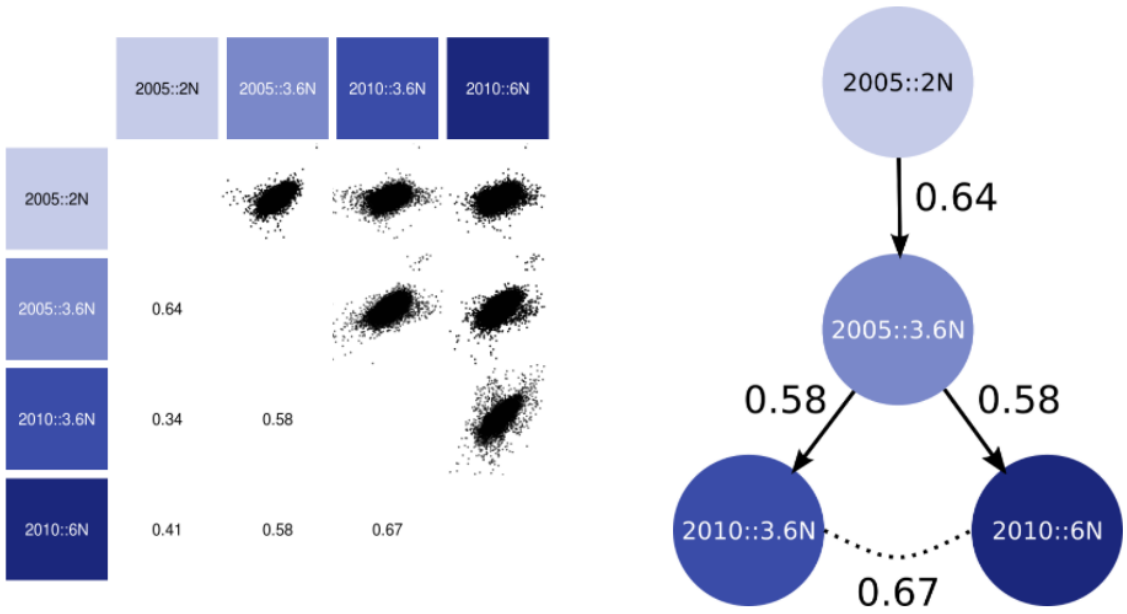


Figure S5. Correlation matrix of the tumour populations from patient D

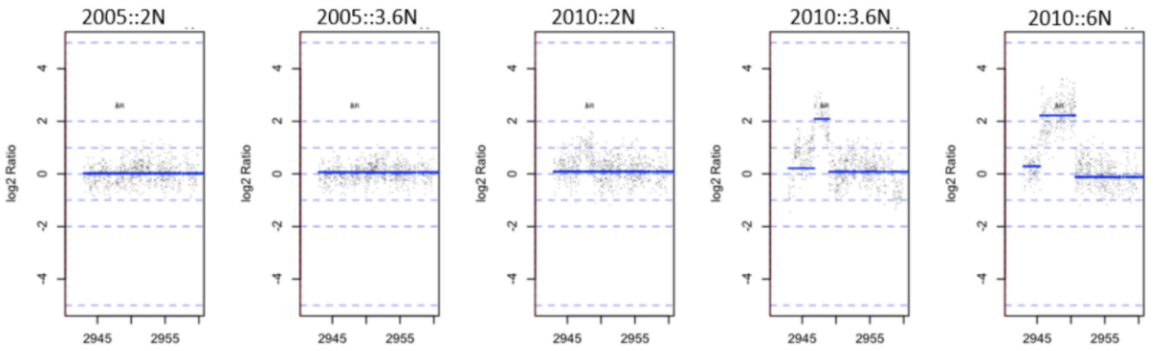


Figure S6. Evolution of the AR amplification illustrating different amplicon sizes for the 2010::3.6N and 2010::6N amplicons

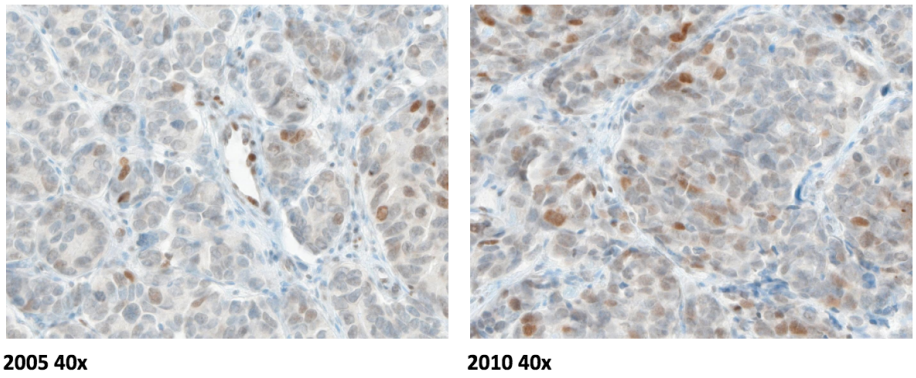


Figure S7. Immunohistochemistry for p53 protein expression

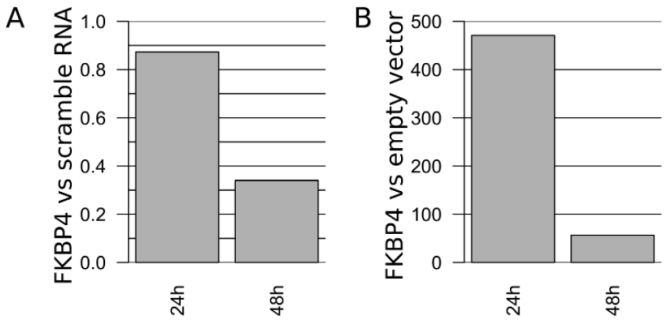


Figure S8. Validation of FKBP4 knockdown and overexpression

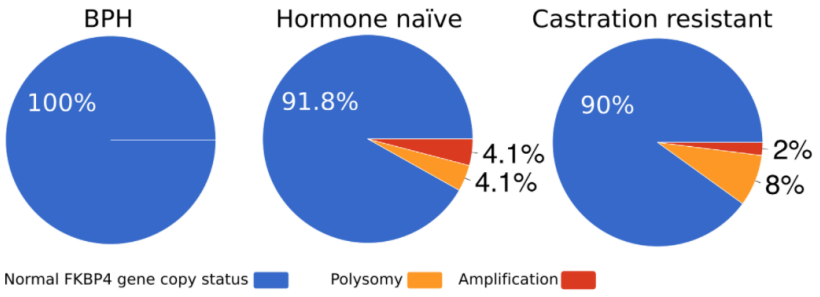


Figure S9. FKBP4 copy number assessment

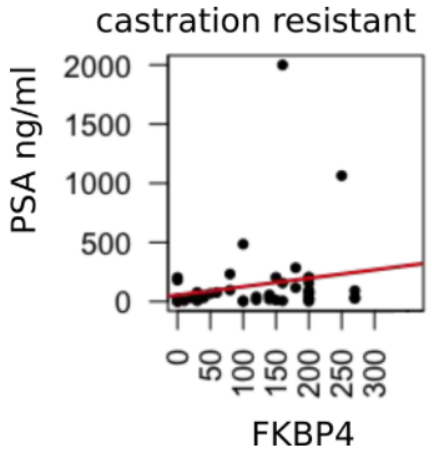


Figure S10. Correlation of FKBP4 protein expression with PSA serum levels

II. 6 Acknowledgements

We thank Sandra Schneider and Petra Hirschmann for their excellent support with FISH and IHC, respectively. Funding was provided by Krebsforschung Schweiz (Oncosuisse KFS-02780-02-2011).

II.7 Abbreviations

AC adenocarcinoma

aCGH array comparative genomic hybridisation

ADT androgen deprivation therapy

AR androgen receptor

BPH benign prostate hyperplasia

CAN copy number aberration

CR castration resistance

CRPC castration-resistant prostate cancer

FKBP4 FK506 binding protein 4

HN hormone-naïve

IHC immunohistochemistry

NE neuroendocrine

NGS next-generation sequencing

PC prostate cancer

RB1 retinoblastoma 1

shRNA short hairpin RNA

TMA tissue microarray

TUR-P transurethral resection of the prostate

II.8 References

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Discussion

In the first study [1], we investigated the correlation of the pathognomonic *TMPRSS2-ERG* rearrangement with the actual ERG protein translation. To assess so, *TMPRSS2-ERG* gene fusion was analyzed at the genomic (FISH) and post-translational (IHC) level using a "PCa progression" TMA. In agreement with previous studies, we did not find an association between *TMPRSS2-ERG* fusion and ERG expression. We showed that ERG expression was correlated with proliferation (as measured by Ki67 Expression) exclusively in hormone naïve-samples and without any measurable effect on overall survival. Here, we may hypothesize that in hormone-naïve tumors, the AR axis is of greater importance and ERG expression can just be used as a surrogate marker for its activation. Moreover, poorly differentiated tumors as defined by Gleason score seem to have lower ERG expression profiles and might depend less on AR signaling. Most interestingly, we found a subgroup of ADT-treated tumors that exhibit *TMPRSS2-ERG* rearrangement yet do not express ERG. Taking into account the high expression of AR in most of the tumor samples, it has to be assumed that these specific tumors are able to thrive even with a non-activated, although still present AR. In a more recent study, Udager and colleagues found discordant rearrangement status and *ERG* expression in up to 63% of all cases in various metastatic sites [2]. With more available large scale high throughput data we might be able to learn more about the pattern of *AR* and *ERG* expression in different subpopulations, which will eventually lead to the design of novel mechanistic experiments to understand their association in this special context.

In the second paper entitled "Delineation of human prostate cancer evolution identifies chromothripsis as a polyclonal event selecting for FKBP4 driven castration resistance", we genomically dissected a small patients' cohort with multiple time points before and after therapy. Using and refining our method of sorting multiple tumors prior to the genomic analysis, we were able to identify and confirm the presence of different evolutionary pathways in cancer with linear, parallel and branched development [3,4]. Additionally, we considered another major current dispute in cancer evolution namely the distinction between gradual vs. punctuated evolution. While most of our samples showed more of a gradual evolution pattern with the slow acquisition of new genomic aberrations one patient exemplified an extreme at the other end of the spectrum. In a catastrophic shattering and rearrangement of chromosome 12 the tumor further on took its path with branched clones originating from the same event, commonly described chromothripsis, and therefore acquired multiple new genomic aberrations, judged on their evolutionary distance from the last common ancestor at the same time and in parallel. Chromothripsis is a rather recently described phenomenon of cancer progression. But has since it's the first description by Stephens and colleagues [5] gained a massive interest by a diverse community of cell and molecular biologists as well as due to its inevitable associated genomic complexity by

bioinformatics. Under normal circumstances, any cell experiencing this amount of damage to the DNA should inevitably undergo apoptosis and the underlying mechanisms are not yet fully understood. Telomere dysfunction [6], loss of function mutations in DNA repair and replication control genes combined with flawed homologous repair of double-strand breaks [7] and especially the formation of micronuclei [8,9] have been implicated in the development of chromothripsis. While at the time being several studies did already tried to decipher intratumoral heterogeneity and evolutionary patterns[10–14] they all rely heavily on mathematical model assumptions. Nuclei sorting does not guarantee complete separation of tumor subclones but does show biologically true rather than mathematically estimated subpopulations. We think only due to this separation the detection of two distinct chromothripsis events has been made possible. In further work in our group, we extended the technique to also incorporate antibody staining of nuclei to further enhance its applications. Nowadays newer work in the field takes advantage of single-cell sequencing which has clear advantages in terms of resolution over nuclei sorting [15,16]. Probably it will, despite still being technically challenging, replace our technique in most cases. One exception might be the analysis of archived formalin-fixed samples where we achieved promising results, while whole-cell extraction is not feasible [17]. In any case, it becomes increasingly evident that intratumoral heterogeneity is not only restricted to a genomic but also an epigenetic, transcriptomic, phenotypic, and functional level [15,18]. It will be one of the major challenges in the field to integrate all these different aspects into meaningful insights.

By looking in-depth at the genes being affected by chromothripsis we found one gene, *FKBP4*, to be of major interest. *FKBP4* is a member of the androgen receptor complex and thought to not only stabilizing its dimerized form and therefore saving it from ubiquitination and [19]. Functional loss of FKBP4 ist known to induce male infertility and FKBP4-KO mouse models lack any development of a prostatic gland [20].

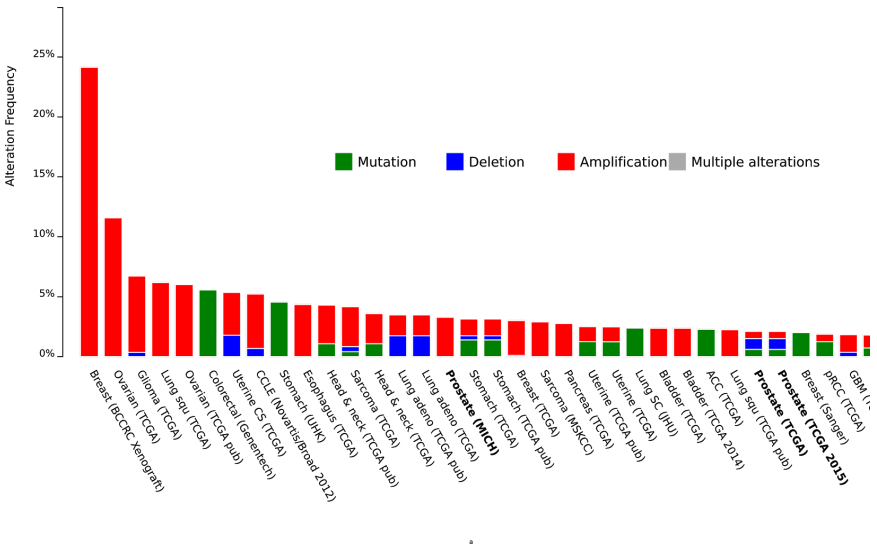

Figure 24: Distribution of genomic alterations of FKBP4 among various tumor types as provided by cBioportal. Amplifications of FKBP4 in prostate cancer can be observed at levels up to 4%.

Despite frequent high IHC expression, we identified *FKBP4* amplification in only 4 out of 98 HN (4%) and in 2 out of 100 CRPC (2%). This number corresponds to the ones reported in publicly available datasets (also see Figure 24). But other studies also confirm the overexpression of FKBP4 in castration-resistant prostate cancer reporting up to a 5-fold increase in the transcriptional and an over 2-fold increase in the protein level [21,22]. To this time point, it remains unclear what other mechanisms besides amplification lead to the increase in *FKBP4* expression we and others observed. One study suggested a direct link between the overexpression of *c-MYC* and in turn the upregulation of *FKBP4* [21]. In ADT treated patients *c-MYC* amplification has been reported in up to 57% [23]. Alternatively, epigenetic modifications could also be a possibility, but this hypothesis still lacks proof. Even if the modality of *FKBP4* overexpression was not fully explained in our work, we did see a clear clinical association with the development of castration resistance in prostate cancer. The subsequent functional studies were conducted in a transfected benign prostatic hyperplasia cell line (BPH-1) as commonly available prostate cancer cell lines are either castration-resistant (e.g. PC-3, DU145), harbouring AR mutations (e.g. AR-V7 in V-CaP), or are at least able to sustain growth even in androgen deprived environments (LN-CaP) [24]. Although we attempt to generate reliable gene knockdown in these cells we were not able to generate them in the quantities necessary for the planned experiments. We are currently trying to establish new primary prostate cancer cell lines, organoids, and xenografts. Once established it would definitely be a major goal to conduct further mechanistic studies on FKBP4 in these models. This is of clinical importance as in vitro models using the small molecule inhibitor MJC13, targeting the AR-Hsp90-FKBP52 complex, lead to suppressed prostate cancer cell growth in vitro [25]. Unfortunately, the effect disappears in cell lines harboring the AR-V7 splicing variant [26]. This indicates that although alterations in FKBP4 do confer castration resistance it is by no means the single way resistance can emerge. Only through an understanding of all the different modes of castration resistance can ultimately lead to a truly tailored treatment.

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Conclusion

Prostate cancer is a common disease among men and responsible for a lot of morbidities and economical burden. It is not the deadliest of all cancers due to its sheer abundance it still has considerable mortality. For clinical urologist one of the main questions is the identification of prostate cancer patients that are at risk of developing an aggressive treatment. Although this was not one of the main aims of the work in this thesis we identified, in this clinical context, new biomarker, *FKBP4*, that, given additional studies were conducted to proof its predictive value, could help clinicians to identify patient that are more susceptible toward ADT and less likely to develop castration resistance in a short time of therapy, castration resistance poses another hurdle in the management of PCA. Castration resistance is hard to overcome by current therapy and there too *FKBP4* could be an attractive target for attacking the AR from another angle [25]. Unfortunately, castration resistance is a heterogeneous phenomenon with different evolutionary pathways rendering tumor populations resistant and sometimes it seems that tumor cells are indeed able to shut down androgen-dependent signaling altogether as we hypnotized based on our TMPRSS2-ERG data. These tumors will need another concept of therapy altogether.

Most importantly I for myself had to come to the realization that in times of large scale genomic analysis, the cancer genome atlas and big data there still lays potential in the in-depth analysis of smaller cohorts and even single patients. It has traditionally always been a trait of researchers to come up with hypotheses from single observations and through logical reasoning that then can lead to a new insight after they are confirmed in a bigger context. As attractive the new approaches of more data equals more knowledge, as attractive these techniques are also very much to myself, we should not lose sight of the single case among all the thousands. Especially now due to my daily clinical work with cancer patients I can not evict the notion that true “personalized” medicine, as the name implies, has in first priority to take notice of the single patient.