# Molecular determinants of prostate cancer aggressiveness

## an analysis of the interconnection between biomarkers, disease drivers and resistance mechanisms towards novel diagnostic, theranostic and therapeutic approaches

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# Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Dissertation von mir selbst und ohne unerlaubte Hilfe angefertigt wurde. Es wurden keine anderen als die angegebenen Hilfsmittel benutzt. Ferner erkläre ich, dass die vorliegende Arbeit an keiner anderen Universität als Dissertation eingereicht wurde.

Teile dieser Arbeit wurden bereits in folgenden Publikationen veröffentlicht:

1. Where is the limit of prostate cancer biomarker research? Systematic investigation of potential prognostic and diagnostic biomarkers. **Kremer A**, Kremer T, Kristiansen G, Tolkach Y. *BMC Urol. 2019 Jun 6;19(1):46.* doi:10.1186/s12894-019-0479-z

2. Androgen Receptor Splice Variants Contribute to the Upregulation of DNA Repair in Prostate Cancer. Tolkach Y*, **Kremer A***, Lotz G, Schmid M, Mayr T, Förster S, Garbe S, Hosni S, Cronauer MV, Kocsmár I, Kocsmár É, Riesz P, Alajati A, Ritter M, Ellinger J, Ohlmann CH, Kristiansen G. *Cancers (Basel). 2022 Sep 13;14(18):4441.* doi:10.3390/cancers14184441
   *equal contributions

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# List of abbreviations

| | |
|---|---|
| **22Rv1$^{LT}$** | Long-term treated 22Rv1 |
| **22Rv1-Apa$^{LT}$** | Long-term APA treated LNCaP |
| **22Rv1-Bica$^{LT}$** | Long-term BICA treated LNCaP |
| **22Rv1-Daro$^{LT}$** | Long-term DARO treated LNCaP |
| **AA** | Antiandrogen |
| **ACTB** | Beta actin |
| **ADT** | Androgen deprivation therapy |
| **AF** | Activation-function motif |
| **APA** | Apalutamide |
| **AR** | Androgen receptor |
| **ARE** | Androgen response element |
| **AR-FL** | Full-length androgen receptor |
| **ARSi** | Androgen receptor signaling inhibitor |
| **AR-V** | Androgen receptor splice variant |
| **AR-V7** | Androgen receptor splice variant 7 |
| **BBB** | Blood brain barrier |
| **BCR** | Biochemical recurrence |
| **BICA** | Bicalutamide |
| **BPH** | Benign prostate hyperplasia |
| **BRCAness** | Lack of ability to repair double strand breaks |
| **BSA** | Bovine serum albumin |
| **cDNA** | Complementary DNA |
| **CE3** | Cryptic exon 3 |
| **CRPC** | Castration-resistant prostate cancer |
| **CT** | Computed tomography |
| **Ctrl** | Control |
| **DARO** | Darolutamide |

| | |
|---|---|
| **DBD** | DNA binding domain |
| **DDR** | DNA damage response |
| **DHT** | Dihydrotestosterone |
| **DNA** | Deoxyribonucleic acid |
| **DNA-R** | Homologous DNA repair |
| **DSB** | Double strand break |
| **e.g.** | For example |
| **ENZA** | Enzalutamide |
| **ER** | Estrogen receptor |
| **FBS** | Fetal bovine serum |
| **FBS-DCC** | Charcoal-stripped fetal bovine serum |
| **FDA** | U.S. Food and Drug Administration |
| **FDR** | False discovery rate |
| **FFPE** | Formalin-fixed paraffin-embedded |
| **FSH** | Follicle-stimulating hormone |
| **GC** | Glucocorticoid |
| **GG** | Grade group |
| **GnRH** | Gonadotropin-releasing hormone analog |
| **GR** | Glucocorticoid receptor |
| **GS** | Gleason score |
| **GSEA** | Gene set enrichment analysis |
| **H&E** | Hematoxylin and eosin |
| **HR** | Homologous recombination |
| **HRE** | Hormone-response element |
| **HSP** | Heat-shock protein |
| **$IC_{50}$** | Half maximal inhibitory concentration |
| **ISUP** | International Society of Urological Pathology |
| **LBD** | Ligand binding domain |
| **LH** | Luteinizing hormone |

| | |
|---|---|
| **LHRH** | Luteinizing hormone releasing hormone |
| **LNCaP$^{LT}$** | Long-term treated LNCaP |
| **LNCaP-Apa$^{LT}$** | Long-term APA treated LNCaP |
| **LNCaP-Bica$^{LT}$** | Long-term BICA treated LNCaP |
| **LNCaP-Daro$^{LT}$** | Long-term DARO treated LNCaP |
| **mCRPC** | Metastatic castration-resistant prostate cancer |
| **mPCa** | Metastatic prostate cancer |
| **MR** | Mineralocorticoid receptor |
| **MRI** | Magnetic resonance imaging |
| **mRNA** | Messenger RNA |
| **Myco** | Mycoplasm |
| **N.A.** | Not available |
| **NLS** | Nuclear localization signal |
| **nmPCa** | Non-metastatic prostate cancer |
| **NSE** | Neuron-specific enolase |
| **NTD** | N-terminal domain |
| **P/S** | Penicillin/ streptomycin |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **PARP** | Poly(ADP-ribose)-polymerase |
| **PARPi** | Poly(ADP-ribose)-polymerase inhibitor |
| **PCa** | Prostate carcinoma |
| **PCR** | Polymerase chain reaction |
| **PET** | Positron emission tomography |
| **PR** | Progesterone receptor |
| **PRAD** | Prostate adenocarcinoma |
| **PRIM** | Primary prostate cancer |
| **PSA** | Prostate-specific antigen |
| **PSMA** | Prostate-specific membrane antigen |
| **PVDF** | Polyvinylidene fluoride |

| | |
|---|---|
| **qRT-PCR** | Quantitative real-time polymerase chain reaction |
| **RNA** | Ribonucleic acid |
| **RT** | Ionizing irradiation therapy |
| **SDS** | Sodium dodecyl sulfate |
| **SR** | Steroid hormone receptor |
| **STAMPEDE** | Systemic Therapy in Advancing or Metastatic Prostate Cancer |
| **TAE** | Tris-acetat-EDTA |
| **TBS** | Tris-buffered saline |
| **TBS-T** | Tris-buffered saline with Tween-20 |
| **TCGA** | The Cancer Genome Atlas |
| **TNM** | Tumor-(Lymph)Node-Metastasis |
| **vs.** | Versus |
| **ZF** | Zinc finger |

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# Summary

Das Prostatakarzinom ist eine der am häufigsten vorkommenden Krebserkrankungen des Mannes weltweit, die bei frühzeitiger Diagnose oft gut behandelbar ist. Die Prognose ist im Einzelfall jedoch unzureichend zuverlässig abschätzbar. Der daraus resultierende Bedarf an Biomarkern, welche präzisere Informationen über den Krankheitsverlauf oder ein Ansprechen möglicher Therapieoptionen geben können, ist größer denn je. Im Laufe der vergangenen Jahre wurden diverse potenzielle Biomarker erforscht, welche jedoch nur selten die Anforderungen für eine Anwendung im klinischen Alltag erfüllen.

**Kapitel eins** dieser Dissertation beschäftigt sich mit der bioinformatischen Analyse des Prostata-Adenokarzinom-Datensatzes des „The Cancer Genome Atlas“ Programms. Ziel dieser Analysen war es, einen Überblick über die Möglichkeiten der Biomarkerforschung im Prostatakarzinoms zu erhalten und bislang unerforschte prognostische Gene zu identifizieren. Die durchgeführten Analysen ergaben eine Vielzahl an Genen, welche mit der Rezidiv-Rate der Tumore und somit direkt mit dem Krankheitsverlauf assoziiert waren. Etablierte Prostatakarzinom-Marker wie z.B. PSA oder PSMA fanden sich interessanterweise jedoch nicht unter den signifikantesten Genen. Die aktuelle Untersuchung der Ergebnisse zeigt, dass etwa 15% dieser Gene bisher nicht für Prostatakarzinome beschrieben wurden und sich demnach noch als Biomarker erweisen könnten. Die verbleibenden Möglichkeiten zur Erforschung neuer Biomarker erscheinen mannigfaltig und eine bioinformatische Herangehensweise zur Analyse großer Datensätze könnte den Weg zu einer präziseren und individualisierteren Therapie des Prostatakarzinoms ebnen.

Interessanterweise wurden etwa 40% der bereits erforschten Gene im Zusammenhang mit DNA-Reparaturmechanismen untersucht. Neben einer veränderten Transkription von DNA-Reparaturgenen sind Alterationen des Androgenrezeptors (AR) eng mit der Entstehung eines sog. kastrationsresistenten Prostatakarzinoms verknüpft. Unter Androgendeprivationstherapie kann es zur Expression ligandenunabhängiger AR-Spleißvarianten (AR-Vs) kommen, welche wiederum hormonunabhängig agieren und somit die Androgendeprivationstherapie unwirksam machen.

**Kapitel zwei** widmet sich dem Auftreten von AR-Vs im Laufe des Prostatakarzinoms und mit deren Beziehung zur DNA-Reparatur. Die retrospektive Analyse einer Patientenkohorte von primären und fortgeschrittenen Prostatatumoren zeigte das sukzessiv steigende Vorkommen von AR-Vs mit Voranschreiten des Tumorstadiums. Der zuvor bereits *in vitro* beschriebene Zusammenhang zwischen AR-Vs und einer verbesserten DNA-Reparatur konnte nun erstmals in einer Patientenkohorte nachgewiesen und mittels Bestrahlung eines adaptierten *in vitro* Tumormodells validiert werden. Der direkte Zusammenhang von AR-V7 und einer damit assoziierten vermehrten Reparatur von Doppelstrangbrüchen legt eine Verwendung von AR-V7 als theranostischen Biomarker nahe: Das Auftreten von AR-Vs, insbesondere von AR-V7, könnte Rückschlüsse auf die Effektivität diverser Therapieoptionen wie z.B. einer Tumorbestrahlung, Androgendeprivationstherapie oder des Konzepts der synthetischen Letalität zulassen. Die Expression von AR-Vs bietet somit einen vielversprechenden Ansatz zur Therapie von kastrationsresistenten Prostatakarzinomen und bedarf weiterer Untersuchungen.

Die Entstehung von Therapieresistenzen ist jedoch nicht ausschließlich dem Aufkommen von AR-Vs zuzuschreiben. Eine Vielzahl weiterer Resistenzmechanismen sind bereits bekannt (z.B. AR-Mutationen/Amplifikationen, Bypass-/outlaw pathways, Co-Faktoren) und können auf diversen, therapieabhängigen Faktoren beruhen.

**Kapitel drei** umfasst die Untersuchung von Resistenzmechanismen *in vitro* nach Langzeit-Antiandrogenbehandlung zweier Prostatakarzinomzelllinien mittels unterschiedlicher Antiandrogene. Die resultierenden Prostatakarzinomzelllinien wiesen therapiespezifische Adaptationen auf: Während eines der Antiandrogene (Bicalutamid) lediglich zu geringen Effekten in beiden Zellinien führte, bewirkte die Behandlung mit einem weiteren Antiandrogen (Apalutamid) die nahezu vollständige Androgenresistenz und Androgenrezeptor-Inaktivität beider Zelllinien. Im Fall der langzeitbehandelten LNCaP Zellen konnte ein kompensatorischer Mechanismus nachgewiesen werden. Im Gegensatz dazu wirkt der dem dritten Antiandrogen (Darolutamid) zugrundeliegende Mechanismus einzigartig. Diese Zellen wiesen noch immer eine signifikante Androgensensitivität auf, wohingegen sie nahezu vollständig resistent gegenüber anderen Antiandrogenen waren. Interessanterweise konnte dieses Antiandrogen zur Re-sensitivierung anderer langzeitbehandelter Zelllinien genutzt werden, ohne dabei einen der betrachteten kompensatorischen Mechanismen

zu nutzen. Schlussendlich legen diese Beobachtungen eine Reihenfolge für die Verwendung der einzelnen Antiandrogenen nahe. Aufgrund der Komplexität dieser Befunde erscheint die Auswahl der zu betrachtenden Resistenzmerkmale von großer Relevanz. Eine spezifische Auswahl an Kriterien könnte zu einer Art „Resistenz-Phänotypen“ führen, welche Rückschlüsse auf wirksame Behandlungsmöglichkeiten oder die Erschließung neuer Therapieoptionen zuließe.

# 1. Introduction

## Preface

Prostatic diseases are known to affect elderly men worldwide. A significant increase in incidence rates has been observed over the last decades in almost all industrialized countries due to a continuously increasing life expectancy. Since the number of pathological molecular alterations of the prostate dramatically increases with age, a successively growing disease burden must be expected in the population. To ensure the most accurate diagnosis for each patient, the identification of targets that allow for a comprehensive interpretation of tumor features and the associated clinical behavior is mandatory. An overall deeper understanding of the connections between mechanisms and pathways underlying prostatic diseases will allow for the identification of specific biomarkers with the aim of improving therapeutic strategies.

## 1.1 The prostate gland

The prostate is the largest accessory gland of the male reproductive system. Anatomically, it is located at the base of the male bladder, surrounding the upper part of the urethra. Histologically, the prostate is composed of glandular and fibromuscular stroma which are tightly fused within a capsule of connective tissue[1,2]. The prostate's primary function is to produce seminal fluid, which transports and nourishes sperm upon ejaculation[3,4].

Growth and development of the prostate are highly dependent on male sex hormones, so-called androgens. This dependence is conserved in the adult prostatic tissue and requires continuous androgenic stimuli for the maintenance of its functional and structural integrity[3,4]. However, with increasing age, the risk for prostatic disorders increases due to pathologic alterations of the prostate[5]. Primary symptoms like dysuria or urinary retention are usually difficult to allocate and can point toward several chronic illnesses such as prostatic inflammation (prostatitis) or proliferative disorders like benign prostate hyperplasia (BPH) or prostate cancer (PCa)[6].

## 1.2 Benign and malignant proliferative disorders

*BPH*

The benign prostate hyperplasia describes a non-cancerous enlargement of the prostate gland which blocks the urine flow due to an enlargement of prostatic tissue towards the urethra and bladder. Even though the cause of prostate enlargement is yet unknown, it is associated to a hormonal imbalance in ageing men. Treatment options for BPH include α-adrenergic receptor blockers, 5α-reductase inhibitors, or surgery[7]. However, the occurrence of BPH does not predispose for an increased risk of PCa.

*Prostate cancer*

In 2020, 1,414,259 new cases of prostate cancer (PCa) and 375,304 related deaths were reported worldwide, which makes PCa one of the most common forms of cancer (next to skin cancer) in elderly men worldwide. In Germany, a total of 67,959 new cancer cases has been reported in 2020[8,9].The incidence of PCa increases with age, resulting in an average onset of disease at 72 years[10]. Only 1% of PCa patients are at an age <50 years[11]. Known risk factors for PCa are a positive family history, chronic prostatitis, diet, obesity, cigarette smoking, or environmental influences[12–14].

The clinical course of PCa differs dramatically: typically, primary PCa progresses very slowly, resulting in indolent, asymptomatic tumors that are clinically innocuous and only found occasionally. In the last decades, radical prostatectomy or primary radiation therapy were the treatment options of choice for localized PCa. As these approaches almost invariably confer a considerable iatrogenic morbidity that negatively impacts the quality of life, a novel treatment strategy called active surveillance has emerged for patients with tumors, that are estimated as having a low risk of disease progression. In these patients active therapy is merely postponed and they are closely monitored for PCa progression, thereby preventing overtreatment[12]. In contrast, a minor fraction of patients suffers from rapidly metastasizing, aggressive tumors that significantly shorten survival times[15]. This dichotomy of indolent and aggressive courses is colloquially distinguished as “pet type” vs. “predator type” PCa[16].

### 1.2.1 Diagnosis

Periodically performed checkups including digital rectal examinations and prostate-specific antigen (PSA) testing allow for early recognition of prostate diseases. Noticeable findings such as abnormalities during rectal examination, elevated PSA blood serum levels or an abnormal PSA velocity are suspicious for prostate carcinoma[2,17]. Thus, 90% of prostate cancers are diagnosed at a timepoint prior to tumor dissemination, resulting in profound treatment success rates at early stages[2,18–22].

The primary diagnosis of PCa is performed via systematic random or targeted biopsies of the prostate following a multiparametric MRI and measurement of the PSA value in blood serum. As standard, 10 to 12 biopsy cores are sampled transrectal or transurethral under sonographic monitoring/ magnetic resonance imaging for histopathological assessment with diagnostic and therapeutic purposes. Consequently, this method allows for the exclusion of malignancy in case of BPH or for staging and grading in case of an assured PCa diagnosis[17].

#### 1.2.1.1 Tumor classification

The pathological examination of prostatic tissue comprises not only the directly affected tissue but also the expansion of the disease within the human body. A tumor classification system hence allows to identify patients with a comparable clinical outcome and to develop appropriate treatment recommendations.

*TNM (Tumor Node Metastasis) staging*

The TNM (Tumor Node Metastasis) classification is an established system to assess the stage of tumor spreading. It defines tumor size but also local spreading of a primary tumor (T) as well as the presence of affected lymph nodes (N) or remote metastases (M) (Table 1). T1-2 tumors are localized tumors within the prostatic capsule, whereas T3-4 tumors have already invaded the prostatic capsule and are defined as locally advanced. For a more specific tumor classification, categories T1-3 are further subdivided into subcategories a to c. Classification of metastases separates regional

(pelvic) lymph node metastases (N1), from non-regional, distant metastases of lymph nodes, bones and other organs (M1)[21–23].

***Table 1: Tumor Node Metastasis (TNM) classification of PCa*** *(modified from the EAU guidelines[21,22,24]).*

| | T - Primary Tumor (stage based on digital rectal examination) |
|---|---|
| **TX** | Primary tumor cannot be assessed |
| **T0** | No pathological evidence of primary tumor |
| **T1** | Clinically inapparent tumor that is not palpable |
| **T2** | Tumor that is palpable and confined within the prostate |
| **T3** | Tumor extends through the prostatic capsule |
| **T4** | Tumor is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall |

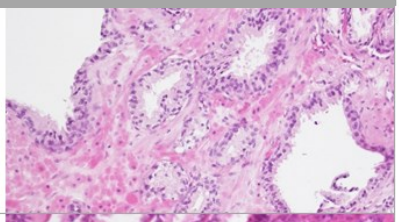
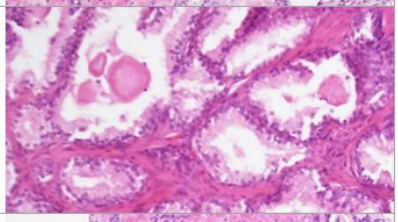
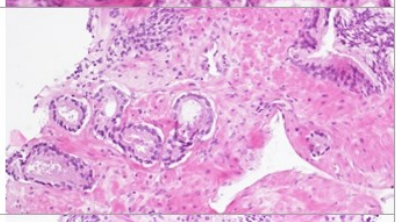
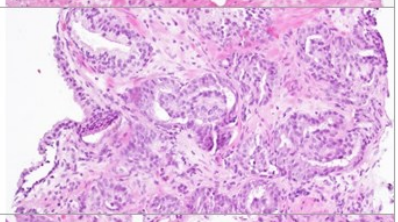
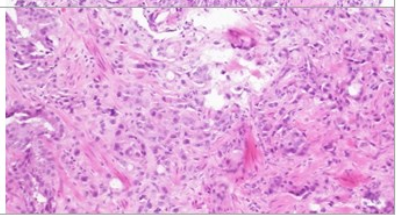
| | N - Regional (Pelvic) Lymph Nodes |
|---|---|
| **NX** | Regional lymph nodes cannot be assessed |
| **N0** | No regional lymph node metastasis |
| **N1** | Regional lymph node metastasis |

| | M - Distant Metastasis |
|---|---|
| **M0** | No distant metastasis |
| **M1** | Distant metastasis |

*Gleason/ ISUP grading*

Besides tumor staging, grading of cellular de-differentiation by inspection of the cellular tumor morphology is a good indicator of tumor aggressiveness. Prostate carcinoma is a paradigm for tumor heterogeneity, as it links clinical, spatial, and morphological heterogeneity to wide genetic divergence[16,25]. This suggests a high-level molecular genetic diversity which outlines the complex nature of this disease. Determining the grade of dedifferentiation of the tumor allows for biological classification of prognostic value (Table 2)[15,26].

***Table 2: Synoptic table of PCa tissue types and corresponding histologic images[22,24].***

| tissue type | H&E staining |
|---|---|
| Normal tissue |  |
| BPH |  |
| Gleason grades 1-3 |  |
| Gleason grade 4 |  |
| Gleason grade 5 |  |

Originally, the Gleason grading system classified tumor areas in 5 grades, ranging from 1 to 5, based on the histological tumor architecture. However, Gleason grades 1 and 2 were virtually eliminated in biopsies, which retains Gleason grades 3-5 only (Table 2). While pattern 1 tumor areas of well-formed glands, tumors of increasing grade show poorer differentiation. Subsequently, this grading system comprises the sum of the most extensive, primary pattern and the pattern with the highest grade of dedifferentiation (secondary pattern), irrespective of the extent of its contribution to a final Gleason Score[27]. In case of a single pattern, the duplication of its Gleason grade yields the final Gleason Score (GS). Hence, GSs can reach values from 6 to 10 (e.g.

3 (primary pattern) + 4 (secondary pattern) = 7a) (Table 2). In 2014, this grading system was updated by the International Society of Urological Pathology (ISUP). Besides amended histological criteria for the Gleason grading, the Gleason Score was migrated to a new system of ISUP grade groups (Table 2), summarizing GS 6 as Grade Group (GG) 1, GS 7a (3+4) as GG 2, GS 7b (4+3) as GG 3, GS 8 as GG 4 and GS 9-10 as GG5. These modifications aimed at refining tumor stratification, reduction of over-treatment of indolent tumors as well as to facilitate patient communication[22,24,27]. In general, a higher grading correlated with a worse prognosis for both grading systems.

#### 1.2.1.2 Molecular biomarkers

Histopathological diagnostics of tissue biopsies provide essential information to confirm a differential diagnosis and prognosis of PCa etiopathology, subsequently allowing for therapy recommendations. Besides the above-mentioned tumor characterization, pathological diagnostics are mainly supported by the detection and measurement of specific biomarkers that substantiate PCa diagnosis and treatment[28].

The detection of biomarkers in biological specimens like tissue samples or body fluids allows for the qualitative and quantitative assessment of aberrations that accompany tissue alterations and the pathogenesis of diseases. Those characteristic biological features play a central role in disease detection, stratification (indolent vs. aggressive), characterization, therapy selection and allow for a prognosis of treatment response when detected at an early stage of the disease[29 30–32].

Appropriate biomarkers are essential to drive and support important clinical decisions[26], particularly regarding the high occurrence and frequent morphological and molecular heterogeneity of PCa, Over the past two decades, cellular and molecular biomarker research has enabled the identification of new approaches to identify and target mechanisms that evolve during tumor progression[33]. The field of biomarkers can be divided into three main branches: diagnostic, prognostic and theranostic/ predictive markers[34] (Table 3).

*Diagnostic markers*

The diagnosis of PCa typically includes the pathological examination of histochemically stained prostate biopsies to identify a patient's risk of prostate cancer. Immunohistochemical staining, the most common technical platform used in biomarker studies[35], allows for the detection of various proteins such as PSA/KLK3, prostate-specific membrane antigen PSMA/FOLH1, NKX3.1 and others[36]. Different biomarkers can be used to further support a diagnosis, however, most are not FDA approved (Table 3).

***Table 3**: **Overview of a selection of potential biomarkers.** Classification according to the proposed field of application, analyte, target and FDA approval.*

| biomarker | analyte | target | FDA approval |
|---|---|---|---|
| **diagnostic** | tissue | FASN[37]<br>GOLPH2[38]<br>GST-pi[39]<br>NKX3.1[40]<br>p40[41]<br>p63/ racemase[42]<br>PSMA/ FOLH1[22,43]<br>TARP[44] | no |
| | blood | PSA/ KLK3[45] | yes |
| | urine | *PCA3*[45] | yes |
| **prognostic** | tissue | ESRP1[46]<br>KIAA1324[46]<br>MAP2[47]<br>PD-L1[48,49]<br>PTEN[22,50]<br>TMPRSS2:ERG[24,51] | no |
| | blood | PSA/ KLK3[45] | yes |
| **theranostic/ predictive** | tissue | PD-L1[48,49]<br>PSMA/ FOLH1[22,43] | no |
| | blood | PSA/ KLK3[45] | yes |
| Selected gene signatures | | | |
| **prognostic** | urine | SelectMDX[24]<br>*HOXC6, DLX1* | no |
| **diagnostic** | urine | ConfirmMDX[24]<br>*RASSF1, GSTP1, APC* | no |

*Prognostic markers*

Prognostic biomarkers intend to predict a specific clinical outcome, for example patient survival, biochemical recurrence or clinical relapse after radical prostatectomy. Only three established prognostic markers are currently recommended by the PCa Guidelines Panel[24]. In addition, only the serum-based PSA level is approved as reliable marker, prefiguring the possibility of PCa if the measured value is elevated (> 20 ng/dl)[22,24] (Table 3). Even after multiple years of research, no further prognostic markers were recommended for utilization in the clinical routine by the FDA, due to the lack of prospective validation and long-term data[22].

Theranostic/ predictive markers

The term “theranostics” is composed of the terms “therapy” and “diagnostics”. Theranostic markers aim to facilitate individualized therapy approaches by predicting a patient’s response to a specific, usually drug-based therapy. One example for a biomarker of this category is PSMA, which is used to predict response to radiopharmaceuticals (PET/CT)[52] (Table 3). In general, this species of biomarker exploits predetermining dispositions to ensure optimal patient support[35], thus granting the right treatment at the right time.

This broad field of biomarker research is equally crucial and challenging in research settings. It allows for determining a specific type of cancer and, moreover, helps verify diagnoses and prognoses of cancer and its progression. Currently, only few target genes are sufficiently specific for a given tumor entity to be used as reliable biomarkers. In consequence, the application of genes signature biomarkers which combine the specificities of multiple target genes gained increasing popularity. The identification of potential targets, such as single genes or gene combinations in a prognostic setting, further provides an opportunity to further prioritize research targets and will have an impact on research methodology. Considering the tremendous prospects left for biomarker research will help to overcome the yet unmet need to identify further strategies for implementing and controlling PCa treatment throughout its progression[34,53].

## 1.3 PCa progression and therapy

PCa treatment is highly dependent on the state of its progression (Figure 1). Radical prostatectomy or radiation therapy are typically used for the treatment of organ confined PCa**.** Within the last decade, active surveillance[54] has become more frequent in its application for early-stage and low-risk tumors. In consequence, overtreatment is prevented by periodic examination of blood PSA levels (chapter 1.2.1), supervision via MRI scans, and inspection of cancer biopsies until a therapy change is required due to disease progression**.** When tumor size increases and symptoms accumulate, usually androgen-deprivation therapy (ADT) is applied, exploiting the dependence of PCa growth on androgens[55].

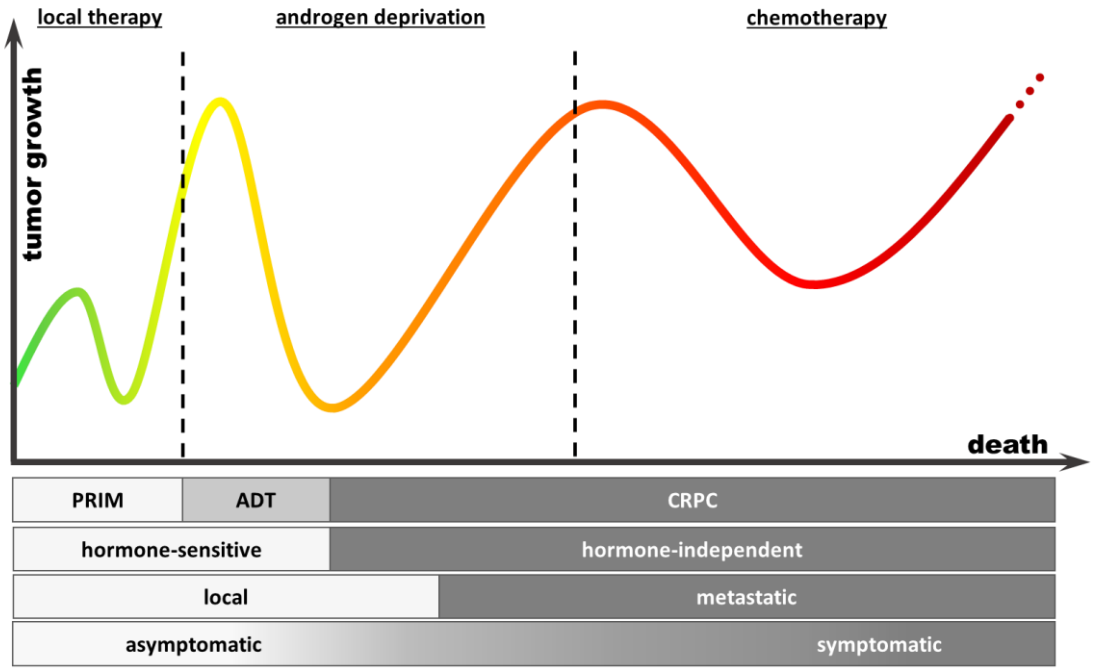



***Figure 1: Schematic diagram representing the natural history, characteristics (tumor classification, hormone status, localization, symptoms), and corresponding treatments of prostate cancer (PCa).*** *Localized tumors are removed via surgery. During the progression of the disease, PCa is treated by androgen deprivation therapy (ADT), while castration-resistant prostate cancer is treated by chemotherapy. Treatment groups are divided in primary tumors (PRIM), androgen-deprivation tumors (ADT), and castration-refractory tumors (CRPC) (modified from Wang, 2022[56]).*

ADT, a basic therapy concept for hormone-sensitive PCa, implements inhibition of androgen synthesis by either surgical or chemical castration. During chemical castration, gonadotropin-releasing hormone (GnRH) analogs[57], also referred to as

luteinizing-hormone-releasing hormone (LHRH) analogs, impede AR signaling[22] by blocking testicular testosterone synthesis. GnRH analogs act on the hypothalamus and pituitary, thus suppressing the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In turn, these act on the gonads by suppressing the production of testosterone and dihydrotestosterone (DHT) that prostate cancer growth relies on. In addition to ADT, androgen receptor signaling inhibitors (chapter 1.4.4.1) are used to block AR signaling by competitively inhibiting the binding of residual androgens to the AR[55,58].

However, recurrent growth and tumor progression typically emerges within one to three years of ADT[59]. Most PCas adapt to hormone depletion, thereby progressing to a clinical state termed castration-resistant prostate cancer (CRPC). Originally, tumors were considered to become independent of the androgen signaling pathway which resulted in the term castration-refractory PCa. However, recent evidence in PCa tumor biology demonstrate that those tumors are progressive despite ongoing ADT, but still retain AR signaling through a number of mechanisms[32,60] highlighting the AR as important target for PCa therapy (chapter 1.4.4)[61]. CRPC is considered established if PSA levels rise or radiographic tumor progression is detected despite ongoing ADT (testosterone levels < 20 ng/dl)[62]. Even in these advanced disease stages, androgen deprivation should be continued as basic therapy for the reasons mentioned above[22,63].

Especially throughout the development of metastatic PCa (mPCa), androgen deprivation is maintained by the continuous application of antiandrogens (chapter 1.4.4.1) to complement chemotherapy. Chemotherapeutics such as cabazitaxel and docetaxel, cytostatic drugs that inhibit cell division and growth[64], are the default standard therapies for both hormone-sensitive as well as castration-resistant mPCa[65]. In general, taxanes are well tolerated and improve overall survival after first-line long-term hormone therapy as shown in the STAMPEDE (Systemic Therapy in Advanced Metastatic Prostate Cancer) and other trials[66,67]. As already mentioned, the permanent deprivation of steroid hormones plays a major role in the treatment of multiple types of cancer throughout all stages of this disease[68,69]. Consequently, steroid hormone receptors (SR) came into research focus as relevant drug targets in cancer therapy.

## 1.4 Steroid hormone receptors

The AR, one of the main drug targets in PCa therapy, belongs to the family of SRs, which in turn belong to the superfamily of nuclear hormone receptors**.** Upon binding to steroid hormones, these predominantly ligand-induced transcription factors regulate the expression of a multitude of genes [70]. SRs are expressed in a broad range of tissues where they are involved in multiple physiological pathways to coordinate important biological processes like proper organ development and function, tissue differentiation, but also the promotion of oncogenic transformations[71–73].

### 1.4.1 Members and structure of steroid receptors

The best studied SRs are members of the intracellular nuclear receptor subfamily 3 (NR3) that include the estrogen receptor (ER, NR3A1), glucocorticoid receptor (GR, NR3C1; chapter 1.4.3), mineralocorticoid receptor (MR, NR3C2), progesterone receptor (PR, NR3C3) and the androgen receptor (AR, NR3C4; chapter 1.4.4)[74–76].

Apart from the ER, the above-mentioned SRs are structurally derived from the PR[77] and share common features and a structure of three domains that are functionally homologous[78] (Figure 2).

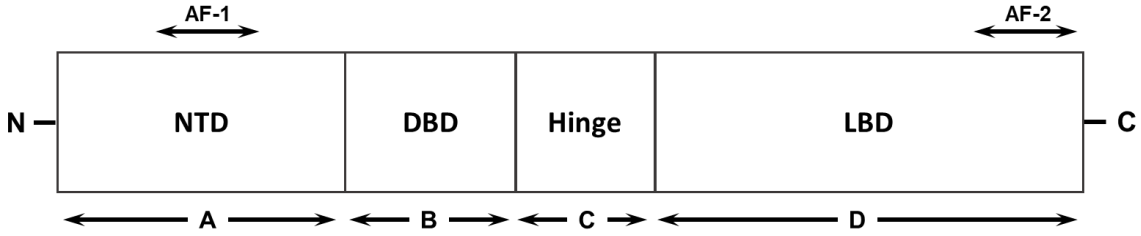



***Figure 2: Basic modular protein domain structure of steroid hormone receptors.*** *Structure including the N-terminal domain (NTD, A) with its activation-function 1 (AF-1, target for post-translational modifications), DNA binding domain (DBD, B), Hinge region (C), and ligand-binding domain (LBD, D) with its activation-function 2 (AF-1, induced by ligand-binding on the LBD) (modified from Saha et al.[79]).*

The **N-terminal domain** (**NTD**, Figure 3), also referred to as transactivation (**TA**) domain, is the most variable domain among the different receptors. It possesses at least one activation function-1 region (AF-1) that acts ligand-independentlyr[80,81] and is target for multiple posttranslational modifications with transcription modulation properties[82–84]. The **DNA binding domain** (**DBD**) is the most conserved, centrally located domain of the SR family[85]. It generally possesses two zinc finger (ZF) motifs. The first one specifically interacts and binds to the hormone-response element (HRE) in the promoter/enhancer region of a target gene while the second motif promotes receptor dimerization**.** The following region that interconnects the DBD to the ligand binding domain (LBD) is the hinge region. This short and less conserved sequence stretch facilitates receptor translocation to the nucleus, is associated with increased transcriptional activation by posttranslational modification and harbors a nuclear localization signal[78]. Both DBD and hinge region play essential roles in target gene selection[85]. C-terminally located is the **ligand-binding domain** (**LBD**), also called the hormone binding domain, which is rather structurally complex. Its tertiary structure, composed of twelve $\alpha$-helices and three $\beta$-strands, forms a hydrophobic ligand-binding cavity[72,79]. Receptor-specific properties of the amino-acid sequence of the LBD determine its ligand specificity. The LBS harbors a nuclear localization signal (NLS) that gets exposed upon ligand binding, thus leading to nuclear translocation of the AR. Besides, the LBD harbors a ligand-dependent activation-function motif (AF-2)[86–89] with coactivation properties[85,89–91].

### 1.4.2 Ligand binding

Unliganded, hormone-sensitive steroid receptors such as the AR are usually located in the cytoplasm where they form heat-shock protein (HSP) containing complexes. Upon ligand binding, the AR-HSP complex partly dissociates. As a result, the bipartite NLS located in the hinge region (Figure 2, C) gets exposed, allowing the receptor-hormone complex to translocate to the nucleus[92–94], where it accumulates and acts upon target gene transcription[70,85]. Most ligand-/ hormone-activated steroid receptors bind hormone-response elements (HREs) in chromatin as mono-, di- or tetramers[95,96] to modulate target gene transcription by recruiting co-activators or co-repressors[78].

Although the previously mentioned steroid receptors (chapter 1.4.1) resemble each other in structure, however, their diverse functionalities rely on their ligand specificity, tissue-specific expression, and distinct functions throughout transactivation processes[85,97,98]. To some extent, this results in complex interplays between the different receptors as they are known to interact and compete for HRE binding sites, as in the case of AR and GR in CRPC. While their physiological roles are reflected in their clinical application, AR and GR can therapeutically be targeted in PCa[85,97,98].

### 1.4.3 Glucocorticoid / glucocorticoid receptor signaling

Glucocorticoids (GCs) act via the glucocorticoid receptor (GR) to control various physiological processes such as reproduction, development, immune response, metabolic homeostasis, and many more[99–101] by up- or down-regulating the transcription of thousands of GC target genes[102]**.** Because of their extensive anti-inflammatory capacities[103], synthetic GCs are widely used to treat inflammatory and immunologic disorders[104] such as rheumatoid arthritis, asthma or as co-medication in the prevention of organ transplant rejection[85,105,106]**.** Consequently, synthetic GCs like prednisone, prednisolone or dexamethasone are used as co-medication for e.g. taxanes[107] to ease symptoms related to chemotherapy or cancer[108,109]**.** Besides their pain-relieving capacity, GCs are known to suppress androgens[110], thus offering a convenient therapeutic approach for androgen-dependent PCa, leading to a decreased proliferation of tumor cells, except for CRPC[111,112]. The application of GCs in PCa, however, encounters limitations sufficiently severe to affect the therapy outcome or cause other negative effects [113–115]:

On the one hand, GC treatment is known to be associated with adverse effects. Patients chronically treated with synthetic GCs showed a significantly increased risk for severe side effects such as hyperglycemia, obesity, cardiovascular diseases, hypertension, and bone fractures due to osteoporosis[113–115]**.** On the other hand, clinical studies indicate an increased risk for chemotherapy failure of solid tumors [108,116]. As shown by Montgomery et al.[117], GCs exhibit a pro-tumorigenic role in CRPC, contributing to poor prognosis due to increased proliferation and tumor progression[118]. It is known that ADT or antiandrogen treatment can result in the overexpression of GR (chapter 1.4.3), which was found to be associated with the loss of antiandrogen control

of proliferation. Thus, the GR can be upregulated and co-expressed with the AR[119]**.** It was postulated that the GR might take over the role of the ADT- and antiandrogen- (AA) inhibited AR, hence leading to CRPC[120], a further clinically relevant mechanism that induces resistance to therapeutics by targeting the AR signaling axis[121,122].

To dampen adverse inflammatory effects, treatment of CRPC patients during ADT is routinely supplemented with synthetic glucocorticoids[123]. However, the pro-tumorigenic interplay between the AR and GR brought the glucocorticoid signaling pathway into the focus of late-stage PCa treatment[113,118,124].

### 1.4.4 Androgen / androgen receptor signaling axis

A key element for the growth of normal and cancerous prostate tissue is the AR. The AR encodes a nuclear transcription factor that is directly activated by androgen hormones such as testosterone and dihydrotestosterone synthesized in the testis and adrenal gland[90,123]. The cellular effects of androgens are transduced by the AR, and regulate an extensive transcriptional program including cellular events like the development and maintenance of reproductive organs, proliferation, apoptosis, migration, DNA damage response (DDR), invasion and differentiation[121,125–127]**.**

Androgen signaling emerges to be one of the main effectors in the development and progression of PCa[63]. Already in 1941, Huggins and Hodge described their idea of treating advanced PCa by surgical castration[123,128]**.** In the 1970s, the concept of chemical castration became the mainstay of therapeutic hormone-naïve PCa treatment[129]. Nowadays, this concept is still widely applied[55] by combining GnRH analogs with antiandrogens, resulting in a decrease in tumor volume and serum PSA level in the majority of hormone-sensitive PCa patients[90,130].

#### 1.4.4.1 Androgen receptor signaling inhibitors

Androgen receptor signaling inhibitors (ARSi) are divided into two groups, according to their mechanism of inhibition. The group of androgen synthesis inhibitors, like abiraterone (Figure 3A) works by blocking *de novo* androgen biosynthesis[131,132].

Abiraterone is a steroidal, selective, irreversible CYP17A1 inhibitor that is used for the treatment of metastatic CRPC (mCRPC)[133,134]. Because of its steroidal structure, it shows hormonal side effects like the suppression of estrogen levels alongside with testosterone levels as well as the activation of AR[135–137].

In contrast, competitive AR antagonists, referred to as antiandrogens (AAs), do not exhibit steroidal structures. They constitute the second group of ARSi and impede AR signaling by competing with androgens for binding to the AR (Figure 3B). Inhibited AR activation through antiandrogens leads to diminished PCa growth[55,138] by increased apoptosis**.** However, the vast majority of tumors eventually relapse, resulting in androgen non-responsive tumor cell growth due to compensatory mechanisms which include the reactivation of the AR signaling axis[139]. Commonly within one to three years the disease would recur, resulting in tumors termed castration-refractory[140,141].

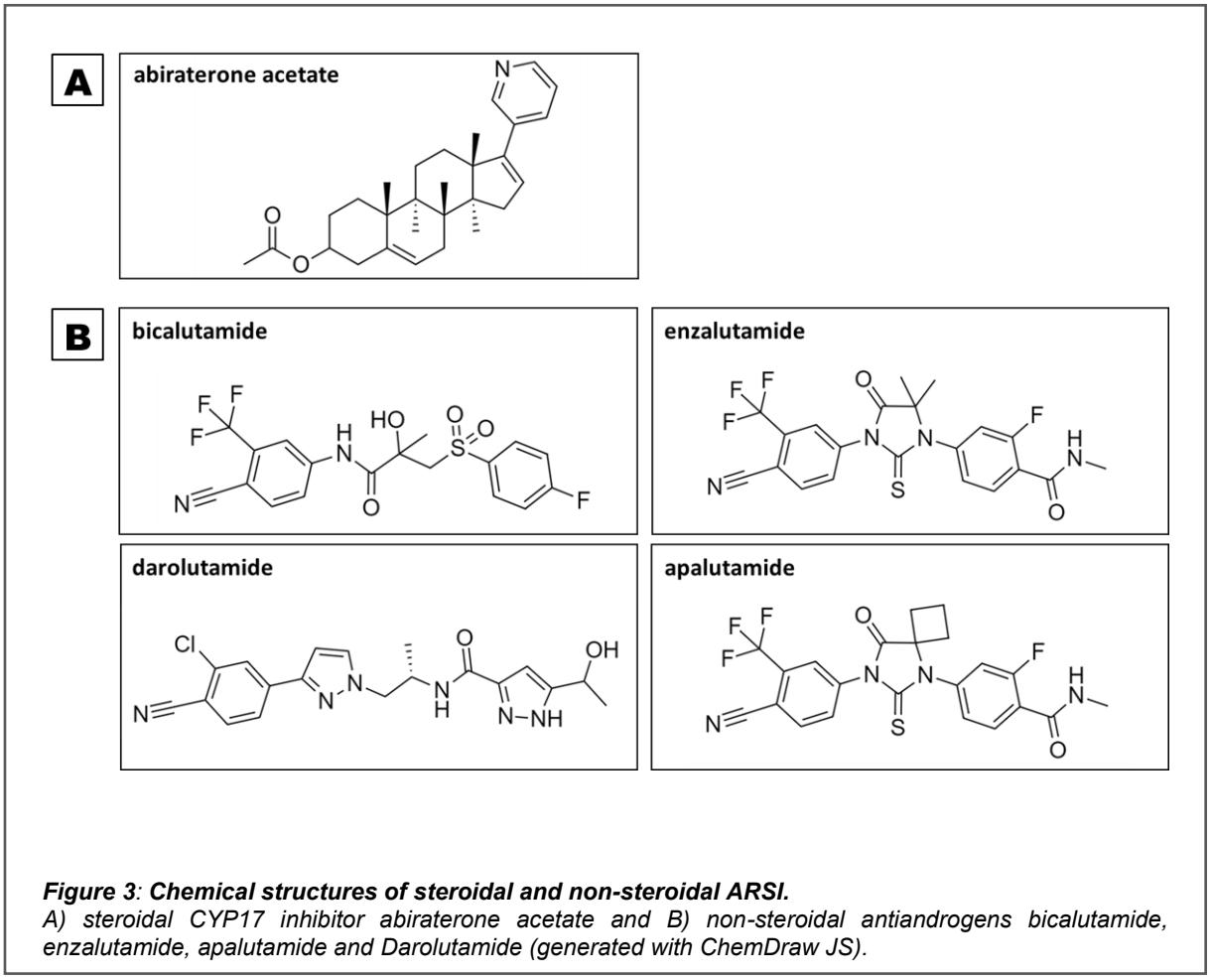



***Figure 3**: **Chemical structures of steroidal and non-steroidal ARSI.***
*A) steroidal CYP17 inhibitor abiraterone acetate and B) non-steroidal antiandrogens bicalutamide, enzalutamide, apalutamide and Darolutamide (generated with ChemDraw JS).*

In contrast to CYP17 inhibitors, non-steroidal AAs (Figure 3B) are used for the treatment of non-metastatic and metastatic CRPC[142]. Despite their effectiveness at the beginning of treatment, members of the first generation of AAs such as bicalutamide (Table 4), flutamide, etc. lead to resistance after 2-3 years, mostly because of AR point mutations that result in an antagonist-to-agonist conversion[143]. The second generation of AAs (Table 4, Enzalutamide, Apalutamide) was developed to overcome resistance to first-generation AAs, showing further improvements such as a higher affinity for AR and a broadened applicability[144]. The latest generation of AAs (Table 4, Darolutamide) has chemical structures unrelated to first- and second-generation AAs, that do not pass the blood-brain barrier (BBB), bind AR with an even higher affinity and show a unique antagonistic profile compared to other AR antagonists[145,146].

***Table 4: Synoptic table of non-steroidal antiandrogens bicalutamide, enzalutamide, apalutamide, and darolutamide on basis of different categories****: generation, application, structure, operating principle, affinity (ranging from low to high), penetrance of the blood-brain barrier (BBB), antagonist to agonist switch (due to AR point mutations).*

| | bicalutamide | enzalutamide | apalutamide | darolutamide |
|---|---|---|---|---|
| **drug name** | ICI-176334<br>Casodex®<br>BICA | MDV3100<br>Xtandi®<br>ENZA | ARN-509<br>Erleada®<br>APA | ODM-201<br>Nubeqa®<br>DARO |
| **generation** | first | second | second | latest |
| **indication** | nmCRPC | mCRPC | mCRPC | nmCRPC |
| **structure** | non-steroidal | non-steroidal | non-steroidal | non-steroidal |
| **operating principle** | competitive,<br>modulation of DNA binding, translocation, and binding of co-factors | | | |
| **affinity for the AR** | low | moderate | moderate | high |
| **penetrance of BBB** | yes | yes | yes | no |
| **antagonist to agonist switch** | frequent | occasional | occasional | not observed |
| **study** | EPC trial | PROSPER | SPARTAN | ARAMIS |
| **sources** | 138,143,147–150 | 148–155 | 148–150,156–158 | 142,148–151,156,157,159–164 |

The efficacy of these AA-based therapies highly expanded with the occurrence of second-generation AR antagonists, beginning with the approval of Enzalutamide

(Table 4) in 2012, followed by Apalutamide (Table 4) in 2018, and finally Darolutamide (Table 4) in 2019. Each of these agents improved the survival and life quality of PCa patients with applications for both androgen-dependent and/ or castration-resistant disease by delaying the development of metastases that are eventually associated with both morbidity and prostate cancer-specific mortality[142,157,165–167].

#### 1.4.4.2 Adaptive mechanisms leading to castration-resistance

Despite the advent of AR-targeted therapies, disease progression by maintained/ reactivated AR signaling is common[168]**.** A multitude of adaptive mechanisms can contribute to the progression of hormone naïve PCa towards castration resistance. Most adaptations are mediated by the AR that maintains its role as a key element in PCa. However, AR-independent adaptive mechanisms may also lead to treatment failure and affect the growth of CRPC[126,153]. Due to the variety of known resistance mechanisms, only a selection will be introduced in the following section including *AR* hypersensitization, AR outlaw pathways, *AR* mutations, AR bypass mechanisms and the expression of constitutively active *AR* splice variants[148–150].

*AR hypersensitization*

Hypersensitization of the AR can be achieved by different mechanisms that increase AR sensitivity, AR amplification or increased AR stability. Overexpression and increased stability of the AR ensure tumor cell growth even under minimal activation through androgens[169,170] by hypersensitizing CRPCs to low levels of androgens, a mechanism that eventually culminates in androgen resistance under ADT[171]. It was shown that AR protein overexpression does not occur in primary PCa but predominates in tumors under ADT. Eventually, 80% of CRPC tumors display aberrant ARexpression[55,172–174]. Several co-activators and co-repressors are known to affect AR gene activity and thereby promote tumor development. An imbalance between co-regulators like HOXB13, FOXA1, GATA2, EZH2, NCOA1/SRC1, etc. can positively affect AR transcription, resulting in reduced antagonistic effects of AAs[175]. AR hypersensitization achieved by intratumoral androgen-synthesis is blocked by the CYP17A1 inhibitor abiraterone (1.4.4.1 – Androgen receptor signaling inhibitor).

*AR mutations/ promiscuity*

The incidence of AR mutations increases with tumor progression, which results in an accumulation of point mutations in CRPCs[32]. By broadening the specificity for the accepted ligand, point mutations contribute to the acquisition of agonistic properties of antiandrogens or the acceptance of related steroid ligands by the mutant AR[121,125]. Most of these mutations occur in the LBD of the AR[121]. One of the most frequently occurring mutation in the promiscuous AR is T878A (former nomenclature: T877A), a gain of function mutation that converts the mutant AR to be activated by multiple hormones (progesterone, estrogen, glucocorticoids) as well as first- and second-generation antiandrogens such as bicalutamide, flutamide or enzalutamide. Further mutations confer treatment resistance such as F876L for enzalutamide, T878A or L702H for abiraterone, L701H and L701H/T878A that are highly responsive to both glucocorticoids cortisol and cortisone[122,149,176,177].

*AR outlaw pathways*

AR signaling can be affected by a number of mechanisms that allow for an alternative activation of this pathway: several growth factors like insulin-like growth factors, keratinocyte growth factors or epidermal growth factors can activate the AR[55]. A model example of the pathways known to affect tumor growth is the PI3K/AKT pathway, which is frequently upregulated in PCa. Loss or inhibition of tumor-suppressing genes like PTEN and others lead to a diminished suppression (i.e. activation) of the PI3K/AKT pathway, which consequently results in tumor growth. Interestingly, activation of AKT signaling due to downregulation of tumor suppressors was found upon inhibition of the AR by ARSIs[175]. However, further pathways (EGFR/HER-2, MAPK, PTEN, etc.) are described for their impact on tumor growth and resistance formation[55].

*AR bypass / AR substitution (GR)*

Activation of androgen-independent, alternative pathways can allow for bypassing the AR. For example, the overexpression of the pro-survival protein Bcl-2 is associated with androgen-independent tumor growth[55]. Another interesting resistance mechanism in PCa is the activation and upregulation of the GR (chapter 1.4.3). Under ADT, GR

substitutes the AR to activate a partly overlapping but distinguishable set of target genes that are necessary for the maintenance of the resistant phenotype[120]. Apart from GR overexpression, neuroendocrine differentiation and immune-mediated resistance are hallmarks in the development of treatment resistance in CRPC[153,178,179].

*AR splice variants*

One of the therapeutically relevant mechanism to overcome conventional ADT, especially in the state of CRPC, is the emergence of C-terminally truncated, ligand-independent AR splice variants (AR-Vs)[180], leading to a castration-resistant phenotype with reduced survival., castration-resistant phenotype[22,181]. The synthesis of truncated AR variants through structural rearrangements and alternative mRNA splicing are at least two mechanisms for the expression of AR-Vs in CRPC[182–184], initially described by Dehm et al.[185]. More than 30 distinct splice variants of the AR have been identified, with only a few known to be of clinical relevance. Interestingly, most of these AR-Vs are C-terminally truncated, thus lacking a functional LBD. These AR-Vs show constitutive activity, ligand independence, and insensitivity to traditional treatments including antiandrogens (Figure 4)[180,186–188].

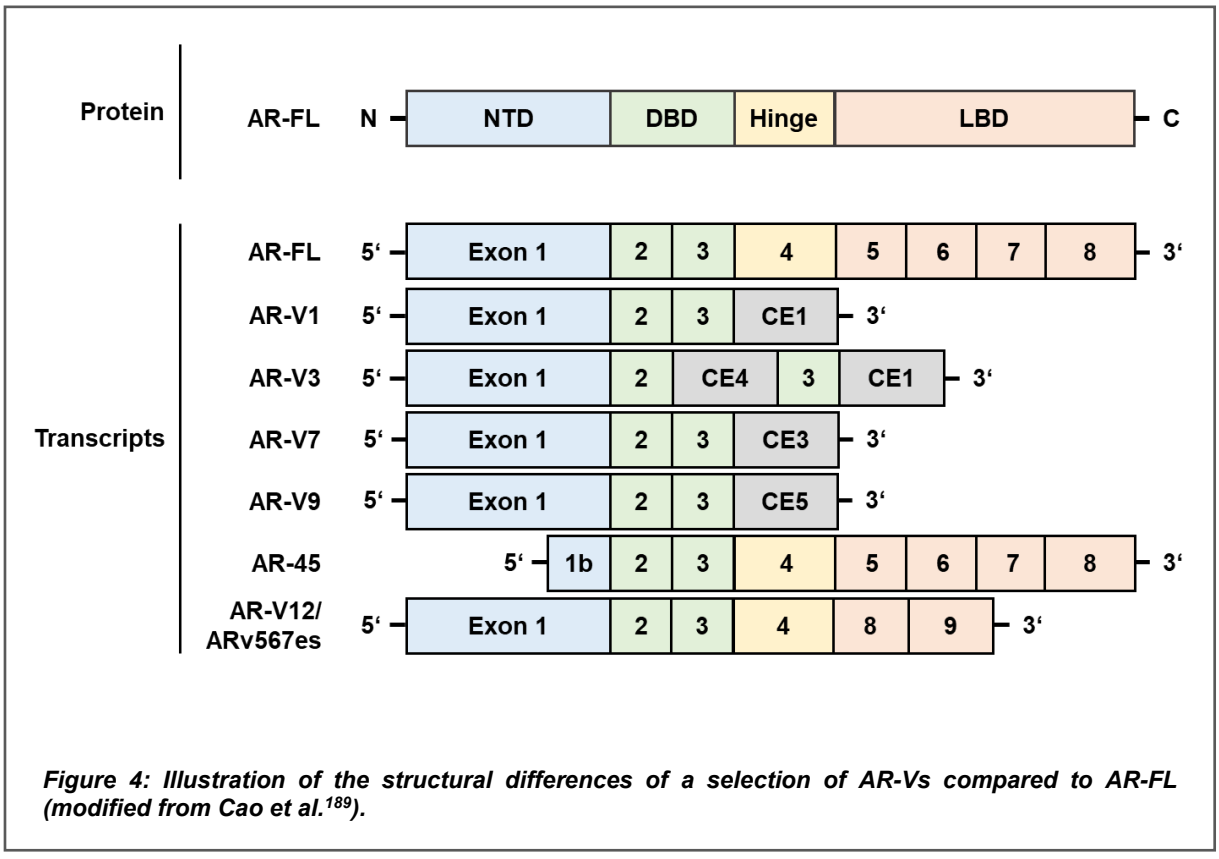



***Figure 4: Illustration of the structural differences of a selection of AR-Vs compared to AR-FL (modified from Cao et al.[189]).***

A characteristic pattern of six of these AR-Vs including AR-V1, AR-V2, AR-V3, AR-V5, AR-V7 and AR-V9 appear co-expressed in advanced PCa tissue. While AR-V3 appears to be the variant with the highest expression in CRPC, AR-V7 is the most important one identified so far due to its more abundant and frequent expression in CRPC[190]. Their presence is associated with shorter progression-free survival after second-line treatment[191]. It arises through aberrant splicing and cryptic exon inclusion, resulting in a conserved NTD and DBD, a partial hinge region, and a unique C-terminal 16-amino-acid sequence encoded by the so-called cryptic exon 3 (CE3). This constitutively active AR variant is rendered ligand-independent because of the absence of its LBD [192–194] (Figure 4). Its expression correlates with androgen-independent cell proliferation and disease progression, as indicated by increased AR protein levels in CRPC patients[195,196].

#### 1.4.4.3 DNA repair

As the AR controls multiple cellular effects including cell division, it is not surprising that there is a close interplay between AR signaling and the cellular DNA damage response (DDR) machinery[127]. When activated, the AR transcriptionally upregulates a large subset of DNA repair genes, thereby enhancing the DDR capacity by stimulating the expression of a number of DNA repair genes including BRCA1, BRCA2, CDK12, PALB2, ATM, ATR, RAD51, RAD51C, MRE11, CHEK2 and XRCC2/3[197–199]. This “AR-associated DNA repair gene signature”[198] preserves genome integrity and guarantees the survival of prostate cancer cells. In this context, the treatment of CRPC patients with ADT and ionizing irradiation therapy (RT) is suspected to lead to enhanced DNA damage. The concomitant induction of AR-Vs may trigger DDR and represent a potential mechanism of resistance to combined ADT and RT[200,201].

A biological concept named synthetic lethality exploits tumor cell vulnerabilities with defects in DDR. Two genomic events that are each separately harmless become lethal when occurring simultaneously[202]. Utilization of Poly(ADP-ribose)polymerases (PARP) inhibitors (PARPi) results in the progression of single-strand breaks to potentially toxic double-strand breaks (DSB). Cells exhibiting a status called “BRCAness” lack the ability to repair DSBs via HR. This inability for HR may be induced by the presence of pathogenic mutations, resulting in the defectiveness of genes involved in DSB repair

or by ADT. In consequence, combining PARPi and BRCAness leads to the accumulation of eventually lethal mutations[127,197,203]. The application of this concept becomes increasingly inappropriate as the disease advances, apparently due to AR alterations such as splice variants, amplifications and mutations[127,204,205]. In consequence, constitutively active AR-Vs may preserve DNA repair activity by mediating the expression of key genes essential for DNA repair[199,206,207].

It was shown that AR-Vs regulate a distinct set of DNA-damage response (DDR) genes compared to AR-FL[206]. The modulated DNA repair gene expression is linked to increased DNA repair activity in advanced PCa *in vitro*. Identification of a "gene signature" of involved DNA repair genes could offer molecular features that may be exploited as new treatment targets in CRPC[208], with the potency to serve as a novel biomarker for treatment selection[180,200,209–211].

## 1.5 Aims of this thesis

Specific biomarkers allow for the comprehensive interpretation of tumor features in a diagnostic or prognostic context. The identification of appropriate PCa biomarkers that allow for further disease stratification is crucial for the clinical treatment of highly heterogeneous PCa.

**Chapter I** aims at answering the following questions:

- Is it possible to outline the remaining opportunities left for PCa biomarker research using a holistic, bioinformatic approach?
- Does this method allow for the identification of diagnostically or prognostically promising target genes for PCa biomarker research?

Castration resistance following AA treatment is one of the main issues in PCa therapy and is significantly affected by AR-Vs. Since the AR combines disease driver, drug target and resistance effector characteristics, androgen-axis components like AR-Vs are a promising starting point for novel therapeutic approaches.

**Chapter II** aims at answering the following questions:

- When do AR-Vs emerge throughout PCa evolution in PCa patients*?*
- Do AR-Vs have an impact on DNA repair?
- Can AR-Vs be used as a novel biomarker for PCa?
- Is there a link between AR-Vs and the success of novel treatment strategies like PARPi?

Besides AR-Vs, various aspects can lead to castration-resistance following long-term androgen deprivation. Unravelling the interconnection of pathways and mechanisms that underly therapy resistance is complex but crucial to ensure specific therapeutic measures for PCa patients.

**Chapter III** aims at answering the following questions:

- What are the impacts of long-term AA treatment on cellular PCa features *in vitro?*
- Do long-term AA treated PCa cell lines develop resistance mechanisms?
- Is the long-term AA treatment of PCa cell lines linked to the emergence of cross-resistances towards other AAs?
- Can resistance-mechanisms be overcome using different AAs for re-sensitization?

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Buffer recipes

| Buffer | Recipe |
|---|---|
| **10% SDS gel (separation gel)** | 7.9 ml aqua bidest., 6.7 ml acrylamide/ bisacrylamide (30%), 5 ml Tris-HCl (pH 8.8), 200 µl SDS 10%, 200 µl APS 10 %, 8 µl TEMED |
| **10X running buffer** | 0.25 M Tris base, 1.92 M glycine, 0.1 % SDS ad 1 L aqua bidest.<br>(30.3 g Tris base, 144.0 g glycine, 50 ml 20% SDS) |
| **10X transfer buffer** | 390mM glycine, 480mM Tris base, 0,375% SDS ad 1L with aqua bidest.<br>(29.3g glycine, 58.2g Tris, 18.75 ml 20% SDS solution) |
| **1X TAE** | Diluted from 10X TAE |
| **1x TBS** | Diluted from 10X TBS |
| **1X transfer buffer** | 100ml transfer buffer 10x ad 800ml with bidest. water, add 200ml methanol, |
| **6 % SDS Gel (stacking gel)** | 4.1 ml aqua bidest., 1 ml acrylamide/ bisacrylamide (30%), 750 µl Tris-HCl (pH 8.8), 60 µl SDS 10%, 60 µl APS 10 %, 6 µl TEMED |
| **Blocking solution** | 5% milk powder in TBS or TBS-T |
| **RIPA lysis buffer (+Protease/ Phosphatase Inhibitor)** | Dissolve 1 cOmplete Protease inhibitor cocktail tablet per 50 ml RIPA lysis buffer, add 10 µl Halt Protease/ Phosphatase inhibitor (100X) per ml RIPA buffer |
| **Sample buffer** | 6X sample buffer + 10 % β-Mercaptoethanol |
| **TBS-T** | 0.05% Tween-20 in 1x TBS |

### 2.1.2 Cell lines

| Cell line | Article no. | Standard growth medium | Reference |
|---|---|---|---|
| **22Rv1** | ACC 438 | DMEM/F-12 GlutaMax (+ 10% FBS, 1% P/S (10,000 U/ml)) | ATCC, Manassas, USA |
| **DU145** | ACC 465 | DMEM/F-12 GlutaMax (+ 10% FBS, 1% P/S (10,000 U/ml)) | DSMZ, Braunschweig, Germany |
| **LNCaP** | CRL-1740 | DMEM/F-12 GlutaMax (+ 10% FBS, 1% P/S (10,000 U/ml)) | DSMZ, Braunschweig, Germany |
| **PC-3** | CRL-1435™ ACC 465 | DMEM/F-12 GlutaMax (+ 10% FBS, 1% P/S (10,000 U/ml)) | DSMZ, Braunschweig, Germany |
| **LNCaP/AR** | | RPMI1640 1% P/S (10,000 U/ml) 10% FBS/ 10% FBS-DCC | Kindly provided by Dr. A. Alajati, Clinic of Urology, University Hospital Bonn |
| **LNCaP/V7** | | RPMI1640 1% P/S (10,000 U/ml) 10% FBS/ 10% FBS-DCC | Kindly provided by Dr. A. Alajati, Clinic of Urology, University Hospital Bonn |

### 2.1.3 Cell culture supplies

| Name | Company |
|---|---|
| **Charcoal-stripped fetal bovine serum (FBS-DCC)** | Biowest, Nuaillé, France |
| **DMEM/F-12 GlutaMax** | Gibco Life Technologies, Paisley, UK |
| **DMSO** | Honeywell, Charlotte, USA |
| **DPBS** | Gibco Life Technologies, Paisley, UK |
| **Fetal bovine serum (FBS)** | Gibco Life Technologies, Paisley, UK |
| **Penicillin-Streptomycin (10.000 U/ml)** | Gibco Life Technologies, Paisley, UK |
| **RPMI1640** | Gibco Life Technologies, Paisley, UK |
| **Trypan blue Solution 0.4%** | Gibco Life Technologies, Paisley, UK |
| **Trypsin-EDTA 0.05%** | Gibco Life Technologies, Paisley, UK |
| **Bicalutamide Darolutamide Apalutamide Enzalutamide** | Selleck Chemicals GmbH, Planegg, Germany and MedChemExpress, Monmouth Junction, USA |

### 2.1.4 Chemicals and reagents

| Name | Company |
|---|---|
| **10% (w/v) ammonium persulfate - APS** | VWR, Darmstadt, Germany |
| **100bp GeneRuler** | ThermoFisher Scientific, Waltham, USA |
| **20% sodium dodecyl sulfate SDS solution** | Applichem, Darmstadt, Germany |
| **Acrylamide/ bisacrylamide solution, 30%** | CarlRoth, Karlsruhe, Germany |
| **Agarose** | Sigma-Aldrich, St. Louis, USA |
| **β-Mercaptoethanol** | CarlRoth, Karlsruhe, Germany |
| **cOmplete Protease Inhibitor Cocktail** | Roche, Basel, Switzerland |
| **Ethanol** | Merck Millipore, Darmstadt, Germany |
| **Glycine** | CarlRoth, Karlsruhe, Germany |
| **Halt Protease & Phosphatase Inhibitor** | ThermoFisher Scientific, Waltham, USA |
| **HDGreen plus** | Intas, Göttingen, Germany |
| **Isopropanol** | ThermoFisher Scientific, Waltham, USA |
| **KAPA2G Fast HotStart genotyping mix** | Sigma-Aldrich, St. Louis, USA |
| **Kapa2G Fast HS Genotyping Mix** | Sigma-Aldrich, St. Louis, USA |
| **Laemmli SDS sample buffer, reducing (6X)** | Alfa Aesar, Haverhill, USA |
| **Methanol** | Acros Organics |
| **Milk powder, skimmed** | CarlRoth, Karlsruhe, Germany |
| **Nuclease-free water** | Sigma-Aldrich, St. Louis, USA |
| **PageRuler Prestained Protein Ladder** | ThermoFisher Scientific, Waltham, USA |
| **Resolving gel buffer for PAGE** | Bio-Rad, Hercules, USA |
| **Restore Western blot stripping buffer** | ThermoFisher Scientific, Waltham, USA |
| **RIPA lysis buffer** | ThermoFisher Scientific, Waltham, USA |
| **RNase AWAY decontaminator** | ThermoFisher Scientific, Waltham, USA |
| **RNase-free water** | Qiagen, Hilden, Germany |
| **Stacking gel buffer for PAGE** | Bio-Rad, Hercules, USA |
| **TAE buffer, 10X** | ThermoFisher Scientific, Waltham, USA |
| **Tetramethylethylenediamine TEMED** | CarlRoth, Karlsruhe, Germany |

| | |
|---|---|
| **Tris base** | US Biological Life Sciences |
| **TRIS-buffered saline (TBS, 10X) pH 7.4** | Alfa Aesar, Haverhill, USA |
| **Tween-20** | AppliChem, Darmstadt, Germany |

### 2.1.5 Consumables

| Name | Company |
|---|---|
| **0.2 mL polypropylene PCR tube strips** | Axygen, California, USA |
| **100 µl PCR reaction tubes** | Axygen, California, USA |
| **15 mL PP centrifuge tubes** | Corning, New York, USA |
| **50 mL PP centrifuge tubes** | Corning, New York, USA |
| **6-well clear flat bottom plate, TC-treated** | Corning, New York, USA |
| **96-well clear flat bottom plate, non-treated** | Corning, New York, USA |
| **96-well clear flat bottom plate, TC-treated** | Corning, New York, USA |
| **Cell scraper L** | Sarstedt, Nümbrecht, Germany |
| **Combitips advanced, Biopur (1 mL, 5 mL)** | Eppendorf AG, Hamburg, Germany |
| **CryoPure cryotube, 1.8 mL** | Sarstedt, Nümbrecht, Germany |
| **Disposable scalpel no. 10, feather** | PFM Medical, Köln, Germany |
| **Filter tips (10 µl, 100 µl, 200 µl, 1000 µl)** | Nerbe Plus, Winsen/Luhe, Germany |
| **MicroAmp fast optical 96-well reaction plates, 0.1 mL** | AppliedBiosystems, Waltham, USA |
| **MicroAmp optical adhesive film** | AppliedBiosystems, Waltham, USA |
| **Parafilm M** | Pechiney Plastic Packaging, Chicago, USA |
| **Pipette tips (10 µl, 100 µl, 200 µl, 1000 µl)** | Greiner, Bio-One, Kremsmünster, Austria |
| **PVDF membrane low flourecence 0,2µm** | GE Healthcare/ Cytiva, Marlborough, USA |
| **Rectangular canted neck cell culture flask with vent cap (T-25, T-75, T-175)** | Corning, New York, USA |
| **Safe-lock tubes (0.5 mL, 1.5 mL, 2 mL, 5 mL)** | Eppendorf AG, Hamburg, Germany |
| **Serological pipets (5 mL, 10 mL, 25 mL)** | Corning, New York, USA |
| **Whatman botting paper 3 mm** | Merck Millipore, Darmstadt, Germany |

### 2.1.6 Equipment

| Name | Company |
|---|---|
| **7500 Fast Real-Time qPCR** | AppliedBiosystems, Waltham, USA |
| **Axiovert 40C inverted phase contrast microscope** | Carl Zeiss, Oberkochen, Germany |
| **Centrifuge 5417R** | Eppendorf AG, Hamburg, Germany |
| **CKX53 microscope** | Olympus, Tokyo, Japan |
| **Clean bench Herasafe** | Thermo Fisher Scientific, Waltham, USA |
| **$CO_2$ incubator BBD6220** | Thermo Fisher Scientific, Waltham, USA |
| **$CO_2$ incubator Cytoperm2 Heraeus** | Thermo Fisher Scientific, Waltham, USA |
| **Fusion S imaging system** | Vilber Lourmat, Radolfzell, Germany |
| **Infinite 200 PRO microplate reader** | Tecan, Männedorf, Swiss |
| **MIDI 2 elektrophoresis chamber** | CarlRoth, Karlsruhe, Germany |
| **Mini-PROTEAN Tetra cell casting module** | Bio-Rad, Hercules, USA |
| **Mini-PROTEAN Tetra vertical electrophoresis cell** | Bio-Rad, Hercules, USA |
| **MS1 Minishaker** | IKA, Staufen, Germany |
| **Multifuge 3 S-R Heraeus** | Thermo Fisher Scientific, Waltham, USA |
| **Multipette M4** | Eppendorf AG, Hamburg, Germany |
| **NanoDrop ND-1000 spectrophotometer** | Thermo Scientific, Waltham, USA |
| **nCounter Pro analysis system** | NanoString Technologies, Inc; Seattle, USA |
| **NucleoCounter NC-200** | Chemometec, Allerod, Denmark |
| **Olympus CKC53 inverted fluorescence microscope** | Olympus, Hamburg, Germany |
| **Pipette controller Accu-Jet** | BRAND, Wertheim, Germany |
| **Pipette controller Macro** | BRAND, Wertheim, Germany |
| **Pipette set research** | Eppendorf AG, Hamburg, Germany |
| **Power-Pac 200** | Bio-Rad, Hercules, USA |
| **Power-Pac Basic** | |
| **Power-Pac HC** | |
| **Sartorius Cubis laboratory balance** | Sartorius, Göttingen, Germany |

| | |
|---|---|
| **Sartorius Entris laboratory balance** | Sartorius, Göttingen, Germany |
| **Shaker RS-RR10** | Phoenix Instrument GmbH, Garbsen, Germany |
| **Tabletop centrifuge 2-7** | Sigma, Osterode, Germany |
| **Thermocycler MJ research PTC 200 peltier** | Bio-Rad, Hercules, USA |
| **Thermomixer comfort** | Eppendorf AG, Hamburg, Germany |
| **Thermomixer compact** | |
| **Thermomixer R** | |
| **Trans-Blot SD semi-dry transfer cell** | Bio-Rad, Hercules, USA |
| **Tube roller RS-TR10** | Phoenix Instrument GmbH, Garbsen, Germany |
| **VARIA 1 elektrophoresis chamber** | CarlRoth, Karlsruhe, Germany |
| **Vortex mixer RS-VA 10** | Phoenix Instrument GmbH, Garbsen, Germany |

### 2.1.7 Kits

| Name | Company |
|---|---|
| **CellTiter 96 AQueous One solution cell proliferation assay (MTS)** | Promega, Walldorf, Germany |
| **Maxima SYBR green/ ROX qPCR master mix** | ThermoFisher Scientific, Waltham, USA |
| **NucleoSpin gel and PCR clean-up, mini kit** | Macherey-Nagel, Düren, Germany |
| **Pierce BCA protein assay kit** | ThermoFisher Scientific, Waltham, USA |
| **Pierce ECL Western blotting-substrate** | ThermoFisher Scientific, Waltham, USA |
| **PureLink FFPE RNA isolation kit** | ThermoFisher Scientific, Waltham, USA |
| **RevertAid first strand cDNA synthesis kit** | ThermoFisher Scientific, Waltham, USA |
| **RNeasy Plus mini iit** | Qiagen, Hilden, Germany |
| **SuperSignal West Dura extended duration substrate** | ThermoFisher Scientific, Waltham, USA |

### 2.1.8 Antibodies

#### 2.1.8.1 Primary Antibodies

| Antibody | Application | Company | Cat # |
|---|---|---|---|
| **AR Clone AR441** | Western Blot | Agilent Technologies, Santa Clara, USA | #M356201-2 |
| **AR-V7 (RM7)** | Western Blot | RevMab Biosciences, San Francisco, USA | #31-1109-00 |
| **β Actin** | Western Blot | Abcam, Cambridge, UK | #ab6276 |
| **GR (D6H2L)** | Western Blot | Cell Signaling Technology, Danvers, USA | #12041 |
| **phospho-Histone H2A.X** | γH2A.X Assay | Millipore, Temecula, USA | #05-636 |

#### 2.1.8.2 Secondary Antibodies

| Antibody | Application | Company | Cat # |
|---|---|---|---|
| **Anti-mouse IgG/IgM Alexa Fluor 488-conjugated antibodies** | γH2A.X Assay | Dianova, Hamburg, Germany | #315-545-044 |
| **Goat anti-mouse, horseradish peroxidase-conjugated antibody (HRP)** | Western Blot | Abcam, Cambridge, UK | #ab6789 |
| **Goat anti-rabbit horseradish peroxidase-conjugated antibody (HRP)** | Western Blot | Cell Signaling Technology, Danvers, USA | #7074S |

### 2.1.9 Probes

| Gene | Accession | Position | Target Sequence |
|---|---|---|---|
| **ABCF2** | NM_007189.1 | 1541-1640 | TCTCACCTTTGGAGTACATGATGAAGTGCTACCC AGAGATCAAGGAGAAGGAAGAAATGAGGAAGATC ATTGGGCGATACGGTCTCACTGGGAAACAACA |
| **AGR2** | NM_006408.3 | 581-680 | GACCCATCTCTGACAGTTAGAGCCGATATCACTG GAAGATATTCAAATCGTCTCTATGCTTACGAACC TGCAGATACAGCTCTGTTGCTTGACAACATGA |
| **ALAS1** | NM_000688.4 | 396-495 | AGAAAGCAGGCAAATCTCTGTTGTTCTATGCCCA AAACTGCCCCAAGATGATGGAAGTTGGGGCCAAG CCAGCCCCTCGGGCATTGTCCACTGCAGCAGT |
| **AR-V9** | AR_V9.1 | 30-129 | AAATGTTATGAAGCAGGGATGACTCTGGGAGGAC AACTTACCTGAGCAAGCTGCTTTTTGGAGACATT TGCACATCTTTTGGGATCACGTT |

| | | | |
|---|---|---|---|
| **AR-V3** | ENST00000514029.1 | 2050-2149 | CTCACATGTGGAAGCTGCAAGGTCTTCTTCAAAA<br>GAGCCGCTGAAGGATTTTTCAGAATGAACAAATT<br>AAAAGAATCATAATCAGACACTAACCCCAAGC |
| **AR-V7** | ENST00000514029.1 | 2446-2545 | GTCCATCTTGTCGTCTTCGGAAATGTTATGAAGC<br>AGGGATGACTCTGGGAGAAAAATTCCGGGTTGGC<br>AATTGCAAGCATCTCAAAATGACCAGACCCTG |
| **AR-FL** | NM_000044.4 | 3081-3180 | TGCTCAAGACGCTTCTACCAGCTCACCAAGCTCC<br>TGGACTCCGTGCAGCCTATTGCGAGAGAGCTGCA<br>TCAGTTCACTTTTGACCTGCTAATCAAGTCAC |
| **AR45** | NM_001011645.3 | 2278-2377 | TGCTCCTGACATTGCCTGTCACTTTTTCCCATGA<br>TACTCTGGCTTCACAGTTTGGAGACTGCCAGGGA<br>CCATGTTTTGCCCATTGACTATTACTTTCCAC |
| **AR-V1** | NM_001348063.1 | 2368-2467 | GTCGTCTTCGGAAATGTTATGAAGCAGGGATGAC<br>TCTGGGAGCAGCTGTTGTTGTTTCTGAAAGAATC<br>TTGAGGGTGTTTGGAGTCTCAGAATGGCTTCC |
| **ARv567es** | GU208210.1 | 2139-2238 | ACTGGGAGAGAGACAGCTTGTACACGTGGTCAAG<br>TGGGCCAAGGCCTTGCCTGATTGCGAGAGAGCTG<br>CATCAGTTCACTTTTGACCTGCTAATCAAGTC |
| **ARF1** | NM_001024227.1 | 1371-1470 | CAATTCTGCATGGTCACAGTAGAGATCCCCGCAA<br>CTCGCTTGTCCTTGGGTCACCCTGCATTCCATAG<br>CCATGTGCTTGTCCCTGTGCTCCCACGGTTCC |
| **ATM** | NM_138292.3 | 1324-1423 | TGAAGATAAAGAACTTCAGTGGACCTTCATAATG<br>CTGACCTACCTGAATAACACACTGGTAGAAGATT<br>GTGTCAAAGTTCGATCAGCAGCTGTTACCTGT |
| **ATR** | NM_001184.2 | 566-665 | AAGACTTGGTTTACCTCCATAGAAGAAATGTGAT<br>GGGTCATGCTGTGGAATGGCCAGTGGTCATGAGC<br>CGATTTTTAAGTCAATTAGATGAACACATGGG |
| **BRCA1** | NM_007305.2 | 1276-1375 | CATTAGATGATAGGTGGTACATGCACAGTTGCTC<br>TGGGAGTCTTCAGAATAGAAACTACCCATCTCAA<br>GAGGAGCTCATTAAGGTTGTTGATGTGGAGGA |
| **BRCA2** | NM_000059.3 | 116-215 | GGGGACAGATTTGTGACCGGCGCGGTTTTTGTCA<br>GCTTACTCCGGCCAAAAAAGAACTGCACCTCTGG<br>AGCGGACTTATTTACCAAGCATTGGAGGAATA |
| **CHEK1** | NM_001114121.1 | 2226-2325 | AGGGTGATGGATTGGAGTTCAAGAGACACTTCCT<br>GAAGATTAAAGGGAAGCTGATTGATATTGTGAGC<br>AGCCAGAAGATTTGGCTTCCTGCCACATGATC |
| **CHEK2** | NM_001005735.1 | 895-994 | GGAGAGGTAAAGCTGGCTTTCGAGAGGAAAACAT<br>GTAAGAAAGTAGCCATAAAGATCATCAGCAAAAG<br>GAAGTTTGCTATTGGTTCAGCAAGAGAGGCAG |
| **DMC1** | NM_007068.2 | 901-1000 | GCAGCAAAAATTGGCCCAGATGTTGTCACGACTC<br>CAAAAAATCTCAGAAGAATATAACGTGGCTGTTT<br>TTGTGACCAATCAAATGACTGCCGATCCAGGA |
| **ECI2** | NM_006117.2 | 941-1040 | AGTTAACAGCGGGAGAGGCATGTGCTCAAGGACT<br>TGTTACTGAAGTTTTCCCTGATAGCACTTTTCAG<br>AAAGAAGTCTGGACCAGGCTGAAGGCATTTGC |
| **ERG** | NM_001136155.1 | 342-441 | GCGGTGAAAGAATATGGCCTTCCAGACGTCAACA<br>TCTTGTTATTCCAGAACATCGATGGGAAGGAACT<br>GTGCAAGATGACCAAGGACGACTTCCAGAGGC |
| **EXO1** | NM_003686.3 | 2716-2815 | GCCAGAGCCAGTGGGCTGAGCAAGAAGCCGGCAA<br>GCATCCAGAAGAGAAAGCATCATAATGCCGAGAA<br>CAAGCCGGGGTTACAGATCAAACTCAATGAGC |
| **FANCA** | NM_000135.2 | 799-898 | CTGAGAAGAACTGTGGAGCCTGAAAAAATGCCGC<br>AGGTCACGGTTGATGTACTGCAGAGAATGCTGAT<br>TTTTGCACTTGACGCTTTGGCTGCTGGAGTAC |
| **FASN** | NM_004104.4 | 5388-5487 | GAGGTGCTTGGCTACGCACGGTCGCTTCCTGGAA<br>ATTGGCAAATTCGACCTTTCTCAGAACCACCCGC<br>TCGGCATGGCTATCTTCCTGAAGAACGTGACA |
| **GDF15** | NM_004864.2 | 181-280 | ACTCCAGATTCCGAGAGTTGCGGAAACGCTACGA<br>GGACCTGCTAACCAGGCTGCGGGCCAACCAGAGC<br>TGGGAAGATTCGAACACCGACCTCGTCCCGGC |

| | | | |
|---|---|---|---|
| **GFM1** | NM_024996.5 | 1611-1710 | GAAATCTATGCTCAGAGGCTGGAAAGAGAGTATG<br>GCTGTCCTTGTATCACAGGAAAGCCAAAAGTTGC<br>CTTTCGAGAGACCATTACTGCCCCTGTCCCGT |
| **HPRT1** | NM_000194.1 | 241-340 | TGTGATGAAGGAGATGGGAGGCCATCACATTGTA<br>GCCCTCTGTGTGCTCAAGGGGGGCTATAAATTCT<br>TTGCTGACCTGCTGGATTACATCAAAGCACTG |
| **KLK3** | NM_001030049.1 | 434-533 | GTGTGTGGACCTCCATGTTATTTCCAATGACGTG<br>TGTGCGCAAGTTCACCCTCAGAAGGTGACCAAGT<br>TCATGCTGTGTGCTGGACGCTGGACAGGGGGC |
| **MCPH1** | NM_024596.2 | 643-742 | CCACCTCTTCCCAAATGATTCAGCAGTCTCATGA<br>TAATCCAAGTAACTCTCTGTGTGAAGCACCTTTG<br>AACATTTCACGTGATACTTTGTGTTCAGATGA |
| **MKI67** | NM_002417.2 | 4021-4120 | AGCAGATGTAGAGGGAGAACTCTTAGCGTGCAGG<br>AATCTAATGCCATCAGCAGGCAAAGCCATGCACA<br>CGCCTAAACCATCAGTAGGTGAAGAGAAAGAC |
| **MRE1** | NM_005591.3 | 506-605 | TTCCATGGGTGAACTATCAAGATGGCAACCTCAA<br>CATTTCAATTCCAGTGTTTAGTATTCATGGCAAT<br>CATGACGATCCCACAGGGGCAGATGCACTTTG |
| **NBN** | NM_002485.4 | 1061-1160 | GACTACAAAGAATTACTGTGATCCTCAGGGCCAT<br>CCCAGTACAGGATTAAAGACAACAACTCCAGGAC<br>CAAGCCTTTCACAAGGCGTGTCAGTTGATGAA |
| **PARP1** | NM_001618.3 | 3017-3116 | AAGGTTTGGGCAAAACTACCCCTGATCCTTCAGC<br>TAACATTAGTCTGGATGGTGTAGACGTTCCTCTT<br>GGGACCGGGATTTCATCTGGTGTGAATGACAC |
| **PARP2** | NM_005484.3 | 1155-1254 | GTTATGAGTTCAAAGTGATTTCCCAGTACCTACA<br>ATCTACCCATGCTCCCACACACAGCGACTATACC<br>ATGACCTTGCTGGATTTGTTTGAAGTGGAGAA |
| **PCNA** | NM_002592.2 | 281-380 | GGTGTTGGAGGCACTCAAGGACCTCATCAACGAG<br>GCCTGCTGGGATATTAGCTCCAGCGGTGTAAACC<br>TGCAGAGCATGGACTCGTCCCACGTCTCTTTG |
| **PGK1** | NM_000291.2 | 1031-1130 | GCAAGAAGTATGCTGAGGCTGTCACTCGGGCTAA<br>GCAGATTGTGTGGAATGGTCCTGTGGGGGTATTT<br>GAATGGGAAGCTTTTGCCCGGGGAACCAAAGC |
| **PTEN** | NM_000314.6 | 5345-5444 | CTTCAGATACTCTTGTGCTGTGCAGCAGTGGCTC<br>TGTGTGTAAATGCTATGCACTGAGGATACACAAA<br>AATACCAATATGATGTGTACAGGATAATGCCT |
| **RAD21** | NM_006265.2 | 1081-1180 | GATGAGGATGATAATGTATCAATGGGTGGGCCTG<br>ATAGTCCTGATTCAGTGGATCCCGTTGAACCAAT<br>GCCAACCATGACTGATCAAACAACACTTGTTC |
| **RAD51** | NM_133487.2 | 567-666 | AGACCACCAGACCCAGCTCCTTTATCAAGCATCA<br>GCCATGATGGTAGAATCTAGGTATGCACTGCTTA<br>TTGTAGACAGTGCCACCGCCCTTTACAGAACA |
| **RAD51AP1** | NM_001130862.1 | 1126-1225 | TCTGTGAAGTCTCCCAATCAGAGTCTCCGCCTTG<br>GCTTGTCCAGATTAGCACGAGTTAAACCTTTGCA<br>TCCAAATGCCACTAGCACCTGAGTGTGGTACA |
| **RAD51C** | NM_002876.2 | 301-400 | GCACTGGAACTTCTTGAGCAGGAGCATACCCAGG<br>GCTTCATAATCACCTTCTGTTCAGCACTAGATGA<br>TATTCTTGGGGGTGGAGTGCCCTTAATGAAAA |
| **RAD54B** | NM_012415.2 | 827-926 | TAGACAGAATGATTTCCAAAATTGCAAACCACGC<br>CATGACCCATATACGCCAAATTCCCTCGTTATGC<br>CACGACCAGATAAGAATCACCAGTGGGTATTC |
| **RAD54L** | NM_003579.2 | 1436-1535 | ATGAACCAGCGTGGAGCCAGGGTGTCTTCTCCCA<br>TCCTCATCATTTCCTATGAGACCTTCCGCCTTCA<br>TGTTGGAGTCCTCCAGAAAGGAAGTGTTGGTC |
| **RB1** | NM_000321.1 | 2111-2210 | CCTATCTCCGGCTAAATACACTTTGTGAACGCCT<br>TCTGTCTGAGCACCCAGAATTAGAACATATCATC<br>TGGACCCTTTTCCAGCACACCCTGCAGAATGA |
| **RMI1** | NM_024945.2 | 528-627 | TGTTAACTTGAGTCAGGCCCAAATGAATAAACAA<br>GTGTTTGAGCAGTGGCTCCTTACTGATCTGAGGG<br>ATTTGGAGCATCCTCTTTTACCCGATGGCATT |

| | | | |
|---|---|---|---|
| **RMI2** | NM_152308.1 | 891-990 | AAGACAGACTGTGTAAAAAAGGAATGACATCCTG<br>GCTCCTCATCTTCTTCATCAGCAACTACCATAAC<br>CAGTTTGCGAGTCAAATGGCATTTCCTAACGG |
| **SEC61A1** | NM_013336.3 | 2246-2345 | TCTGTGCACCTATTGGCTCTTCTAGCTGACTCTT<br>CTGGTTGGGCTTAGAGTCTGCCTGTTTCTGCTAG<br>CTCCGTGTTTAGTCCACTTGGGTCATCAGCTC |
| **STIL** | NM_001048166.1 | 2477-2576 | GGAAGCACAGTCTTCCCCTGGCTTGCACATGAGA<br>AAAGGTGTAAGCATTGCTGTGAGCACAGGTGCTA<br>GCTTGTTTTGGAATGCAGCAGGTGAGGATCAA |
| **TM4SF1** | NM_014220.2 | 96-195 | AATCGCAGTATTTAAGAGGTAGCAGGAATGGGCT<br>GAGAGTGGTGTTTGCTTTCTCCACCAGAAGGGCA<br>CACTTTCATCTAATTTGGGGTATCACTGAGCT |
| **TMPRSS2** | NM_005656.3 | 1080-1179 | AGACAATCTTTCATGTTCTATGGAGCCGGATACC<br>AAGTAGAAAAAGTGATTTCTCATCCAAATTATGA<br>CTCCAAGACCAAGAACAATGACATTGCGCTGA |
| **UBE2C** | NM_181803.1 | 270-369 | CTTTTAAGAAGTACCTGCAAGAAACCTACTCAAA<br>GCAGGTCACCAGCCAGGAGCCCTGACCCAGGCTG<br>CCCAGCCTGTCCTTGTGTCGTCTTTTTAATTT |
| **XRCC2** | NM_005431.1 | 537-636 | CTGTCAGCTTTTTACTGGATAGACCGCGTCAATG<br>GAGGAGAAAGTGTGAACTTACAGGAGTCTACTCT<br>GAGGAAATGTTCTCAGTGCTTAGAGAAGCTTG |

### 2.1.10 PCR primer sequences

| Target gene | Sequence | |
|---|---|---|
| **MycoC-FW** | GGGAGCAAACAGGATTAGATACCCT | synthesized by biomers.net GmbH, Ulm, Germany |
| **MycoD-RV** | TGCACCATCTGTCACTCTGTTAACCTC | |

### 2.1.11 qRT-PCR primer sequences

| Target gene | Forward (5'->3') | Reverse (3'->5') |
|---|---|---|
| **ATM** | TGGTGCTATTTACGGAGCTGA | AGCCTGAAGTACACAGAGAACA |
| **ATR** | AGGCCAAAGGCAGTTGTATTG | CAAATGACAGGAGGGAGTTGCT |
| **BRCA1** | TGCGGGAGGAAAATGGGTAG | CTGGGATTCTCTTGCTCGCT |
| **BRCA2** | GACTCTGCCGCTGTACCAAT | GTGGACAGGAAACATCATCTGC |
| **CHEK1** | CATGGCAGGGGTGGTTTATCT | CGAAATACTGTTGCCAAGCCA |
| **EXO1** | TGCGTGGGATTGGATTAGCA | TGGCCCGAATAAACCCGTTG |
| **FANCA** | GGACCTGAATGCCCTTTTGC | AGGCTTGATCCTGCAAAGCA |
| **MCPH1/ BRIT1** | AAATCTTTCCCCCACCTCTTCC | ATGAGTGTAAGCCACCAGCA |
| **MRE11** | TCCCAGAGGAGCTTGACTGA | CCTCTGACTGCATCTTTCTCCA |
| **NBN** | GCAGAAATTGGATTGGCGGT | AAGGCTTGGTCCTGGAGTTG |

| | | |
|---|---|---|
| **PPIA** | GCTGGACCCAACACAAATGG | GGCCTCCACAATATTCATGCCT |
| **RAD21** | CAAAGCCCATGTGTTCGAGTG | GTCCTGATGTCCGTAATGCC |
| **RAD51** | TCAACACAGACCACCAGACC | CTGAAAGCTCACCTCGACCC |
| **RAD51AP1** | TGCGGCCTGTGAGACATAAG | TCCTTTGGTGCTGTTCTGGA |
| **RAD51C** | AAACCCTCCGAGCTTAGCAA | TGTGACTCAGATGTACCAGCA |
| **RAD54B** | ACTGTTTCCCTCTTGTGGATGT | AGCTCCACATCTGCCATTCA |
| **RAD54L** | GCTGGCCAAGAGAAAACCTG | ATCTGGGTCTCACTGCTGGA |
| **RMI1** | TGTCATCAAATCCTGTGCTGCT | TGCAGCTAAAAGCCAAGTTTCA |
| **RMI2** | CGGCCCTGTCTAGTCCCA | CCTCCAGTTCCCACATACTTTC |
| **XRCC2** | GGGCGATGTGTAGTGCCTT | CTTCTACCTTCAAGTCGGGCA |

### 2.1.12 Software and databases

| Name | Purpose | Reference |
|---|---|---|
| **ChemDraw JS** | chemical structures of ARSi | https://chemdrawdirect.perkinelmer.cloud/js/sample/ |
| **Citavi 6 (v.6.14)** | literature and source nanagement software | https://www.citavi.com/ |
| **GIMP (v2.10.32)** | image editing | https://www.gimp.org/ |
| **ImageJ** | quantification of Immunoblots | http://imagej.nih.gov/ij/ |
| **Microsoft Excel** | database management | Microsoft, Redmond, USA |
| **NCBI** | collection of biomedical/ genomic information | http://www.ncbi.nml.nih.gov/ |
| **nSolver analysis software (v.4.0.70)** | datamining, normalization, analysis of expression data | Nanostring Technologies, Seattle, USA |
| **QuPath software (v.0.3.2)** | analysis of γH2A.X foci | https://qupath.github.io/ |
| **R (v.3.6.0)** | datamining, analysis, and graphics | https://www.r-project.org/ |
| **TCGA Database** | data collection (PRAD) | https://portal.gdc.cancer.gov/ |

## 2.2 Molecular and bioinformatical methods

The methods described in the following sections are part of publications as indicated by citations. Unless stated otherwise, all methods are described in brief below and were performed by Anika Kremer. A more detailed description is found in the corresponding publication (provided in section 6.2).

### 2.2.1 Chapter I

*Modified from Kremer et al., 2019;*
*"*Where is the limit of prostate cancer biomarker research?
Systematic investigation of potential prognostic and diagnostic biomarkers."[34]
*Published in BMC Urol. 2019 Jun 6;19(1):46.*

#### 2.2.1.1 Patient cohort

This bioinformatic transcriptome analysis involved 53 normal tissue and 499 PCa patients of the prostate adenocarcinoma (PRAD) cohort of The Cancer Genome Atlas (TCGA) program. A detailed description of clinicopathological and follow-up data is provided in Kremer et al., 2019[34] (section 6.2.1).

#### 2.2.1.2 mRNA expression data

mRNA expression was available for all above-mentioned samples. Merging of clinical and mRNA expression data was performed by barcode as identifier, resulting in mRNA expression data of 17,681 genes that met all criteria.

#### 2.2.1.3 Bioinformatical approach/statistics

A fully automated bioinformatical pipeline allowed for statistical analyses using R (R Foundation for Statistical Computing; version 3.6.0). For each gene, the correlation

between the mRNA expression and Gleason Score was assessed. Survival analyses of dichotomized data (median and best cut-off) were performed by univariate and multivariate Cox regression. Additionally, Kaplan-Meier estimates and curves were generated for all genes.

#### 2.2.1.4 Further methods

Quality control of clinical data, normalization of mRNA data and GSEA analysis were performed by Dr. Yuri Tolkach (formerly: Institute of Pathology, University Hospital Bonn; currently: Institute of Pathology, University Hospital Cologne) as published.

### 2.2.2 Chapter II

*Modified from Tolkach, Kremer, et al., 2022;*
*"Androgen Receptor Splice Variants Contribute to the Upregulation of DNA Repair in Prostate Cancer"*[207]
*Published in Cancers (Basel). 2022 Sep 13;14(18):4441.*

#### 2.2.2.1 Patient cohort

FFPE tumor tissue samples of 184 patients are included in this study: 167 of the patient samples showed different stages of PCa, further 17 patient samples constituted the control groups (normal and BPH). A detailed description of all samples, clinical characteristics and providers is available in Tolkach, Kremer et al., 2022[207] (section 6.2.2).

#### 2.2.2.2 RNA extraction from FFPE tissue

Macrodissection allowed for harvesting specific tissue areas in formalin-fixed paraffin-embedded (FFPE) material to investigate the heterogeneity of primary and metastatic PCa. Total RNA was extracted from and analyzed for 273 tumor samples.

#### 2.2.2.3 RNA expression analyses

RNA expression of a gene panel (including AR, AR-Vs, transcriptional targets of the AR, as well as DDR and proliferation-associated genes) was analyzed using the nCounter platform (NanoString Technologies).

#### 2.2.2.4 Statistical analyses

Raw RNA expression data were processed by nSolver analysis software v. 4.0.70 (Nanostring Technologies). Further analyses were carried out in R (v. 3.6.0, The R Foundation for Statistical Computing). AR-V expression was analyzed quantitatively

(correlation analyses, heatmap) and qualitatively (present/not present). An AR pathway score (AR score calculated from transcriptional AR target expression; n=10) and a HR DNA repair score (DNA-R score calculated from expression data associated with HR DNA repair; n=20) were calculated based on z-scores. Parametric, non-parametric and correlation tests allowed for the comparison of the respective groups and parameters.

#### 2.2.2.5 H2A.X assay – Foci analysis and evaluation

Foci formation following the H2A.X assay (at least 200 cells per condition; n = 3) was counted using the QuPath Software v0.3.2[212].

#### 2.2.2.6 Mycoplasma PCR

Cell lines were cultured in the absence of mycoplasma-active antibiotics for one week before submission and maintained in the same media without dilution for three days before testing. At 80-90% confluency, a sample of cell culture supernatant was heat-inactivated, taking 2 µl of cell supernatant and 23 µl of pre-mix (Table 3) per sample for mycoplasma PCR. PCR conditions are as follows: 94°C for 3 min, 35 cycles of 94°C for 30 sec., 55°C for 30 sec., 72°C for 45 sec. with a subsequent hold stage at 10°C.

***Table 5: Composition of the pre-mix for Mycoplasma PCR***

| reagent | 1 reaction |
|---|---|
| **Myco C (100µM)** | 1.0 µl |
| **Myco D (100µM)** | 1.0 µl |
| **$H_2O$** | 8.5 µl |
| **KAPA 2x MM** | 12.5 µl |
| **total** | 23.0 µl |

Agarose gel electrophoresis was conducted using a 2% TAE agarose gel and HDGreen plus (Intas, Göttingen, Germany). Gel documentation was done via Fusion S imaging system (Vilber Lourmat, Radolfzell, Germany).

#### 2.2.2.7 Quantitative qRT-PCR

mRNA expression analysis of DDR genes in LNCaP cells stably overexpressing AR-FL or AR-V7 was conducted 6h after irradiation (6 Gy). RNA was extracted and reverse-transcribed into complementary DNA (cDNA) for subsequent use in real-time polymerase chain reaction (qRT-PCR). PPIA (peptidyl prolyl isomerase A) served as housekeeping gene to assess the relative gene expression by the $\Delta\Delta C_t$ method.

#### 2.2.2.8 Further methods

Implementation of the linear regression analysis was performed by Prof. Dr. Matthias Schmid, (IMBIE, University Bonn). Expression heatmap clustering was performed by Dr. Yuri Tolkach (formerly: Institute of Pathology, University Hospital Bonn; currently: Institute of Pathology, University Hospital Cologne). H2A.X assay (including cell culture, irradiation, γH2A.X staining of cells and immunoblotting) was performed by Dr. Sarah Förster (Institute of Pathology, University Hospital Bonn). Sanger sequencing of the qRT-PCR amplification products was performed by Dr. Thomas Mayr (Institute of Pathology, University Hospital Bonn). as described in Tolkach, Kremer, et al., 2022.

### 2.2.3 Chapter III

The complexity of resistance mechanisms following long-term antiandrogen treatment in PCa tumor models *in vitro* (unpublished)

#### 2.2.3.1 Generation of antiandrogen resistant PCa cell lines

LNCaP cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). 22Rv1 cells were obtained from the American Tissue Culture Collection (ATCC, Manassas, USA). Cells were cultured in standard growth medium containing DMEM/F-12 / GlutaMax medium supplemented with 1% penicillin and streptomycin (P/S) (10,000 U/ml) and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% $CO_2$. Both cell lines were maintained without antiandrogens (control) or either bicalutamide, apalutamide or darolutamide (Selleck Chemicals GmbH) until resistance formation. Antiandrogen treatment of LNCaP and 22Rv1 cells was performed in standard growth media under a gradual increase in AA concentration (1 µM to 10 µM). Increasing the AA concentrations resulted in stagnation of proliferation or cell death with at least 10% of cell survival. As a cell line adapted to the applied AA concentration by restarting proliferation for one to two weeks, AA treatment concentration was again increased. For LNCaP sublines treated with APA or DARO, final treatment concentrations were reached within three to five steps. 22Rv1$^{LT}$ and LNCaP-Bica$^{LT}$ cells reached final AA concentrations after more than 10 steps. The final concentration was limited by occuring off-target effects determined in PC-3 cells. Upon reaching the final AA concentrations (Table 6), all cell lines were grown with continuous application of AAs for six to nine months to allow for continuous cell proliferation. For both LNCaP and 22Rv1 cells, a control cell line was cultured in parallel in the absence of AAs. Over a culturing period of two years, a stable growth of control and long-term treated sublines was achieved.

Upon passaging, the cells were initially washed with DPBS and incubated with 0.05% Trypsin-EDTA for 3-5 min at 37°C. Trypsin was inactivated by addition of standard growth medium. After centrifugation, the cells were resuspended in standard growth medium and transferred into a new cell culture flask containing fresh standard growth medium and the appropriate concentration of antiandrogen. Cells were routinely checked to exclude mycoplasma contamination.

#### 2.2.3.2 Proliferation assay (MTS)

For cell proliferation assay, 7500 cells (LNCaP) or 5000 cells (22Rv1) were seeded per 96-well 24h prior to treatment. Cells were cultured in either standard growth media or in androgen-deprived media with FBS being replaced by the same amount of charcoal-stripped fetal bovine serum (FBS-DCC) at 37°C in a humidified atmosphere containing 5% $CO_2$.

*DHT treatment:* Cell lines were treated with DHT in concentrations ranging from 0.001 nM to 1000 nM. In addition, a non-treated control, as well as control wells containing no cells (medium control) were carried along as reference. Proliferation assay times were assessed by a serial time course (24 h, 48 h, 72 h, 96 h) using the untreated control PCa subline vs. long-term AA treated cells in the presence or absence of the various AAs (Supplementary Figure 1). These pre-experiments were performed in full media or androgen-deprived media and retrieved an optimal proliferation assay time of 96 h that was used in all further experiments.

*Antiandrogen treatment:* Cell lines were treated separately with two sets of AAs (bicalutamide, enzalutamide, apalutamide, darolutamide) from different distributors: (A) Selleck Chemicals GmbH, Planegg, Germany or (B) MedChemExpress, Monmouth Junction, USA for 96 h. Chemicals were diluted similarly to ensure comparability. Treatment occurred either at (a) the initial antiandrogen concentration that led to resistance of the cell line (Table 6) or (b) in concentration gradients ranging from 0.01 µM to 1000 µM of AA concentration. A non-treated control, as well as a medium control were carried along as reference. Experiments were performed in full media or androgen-deprived media and were repeated three times (n=3).

*Measurement:* Proliferation was measured using the CellTiter 96 AQueous One Solution cell proliferation assay (MTS). 20 µl of CellTiter 96 AQueous One solution was added to each well and the absorbance was measured at 490nm after 4h on an Infinite 200 PRO microplate reader. As reference, the wavelength was measured at 650nm

for background subtraction. In the analysis, normalization and background subtraction was carried out via reference wavelength and medium control. Subsequently, the effect of DHT or AAs on the cell lines was calculated in % of untreated control reference. Each concentration was measured in quadruplicates per experiment.

*Statistics:* Significances ($p < 0.05$) were assessed using an independent two-sample t test. Androgen sensitivity was assessed for all long-term AA treated sublines ($Bica^{LT}$, $Apa^{LT}$, $Daro^{LT}$) vs. $Ctrl^{LT}$ for LNCaP and 22Rv1 sublines, respectively. Antiandrogen sensitivity was assessed for all long-term AA treated sublines ($Bica^{LT}$, $Apa^{LT}$, $Daro^{LT}$) vs. $Ctrl^{LT}$ for LNCaP and 22Rv1 sublines, respectively. Re-sensitization of the long-term treated LNCaP and 22Rv1 sublines was assessed by comparison of all $LNCaP^{LT}$ or $22Rv1^{LT}$ sublines with most effective challenge vs. treatment with the respective AA.

#### 2.2.3.3 Immunoblot

Cell lysates were prepared using RIPA lysis buffer supplemented with cOmplete protease and phosphatase inhibitor cocktail and Halt Protease & Phosphatase Inhibitor. Cells were lysed for 30min on ice. Lysates were centrifuged at 20,000 xg and 4°C for 15min. The supernatant was subsequently transferred into a new reaction tube for protein quantification using the BCA protein assay kit according to the manufacturer's instructions. Bovine serum albumin (BSA) served as standard. All samples and standards were measured in triplicates. For every sample, 30 µg of protein lysate was separated by SDS PAGE and transferred to PVDF membranes pre-treated with methanol. Membranes were blocked in 5% skimmed milk TBS-T and incubated with primary antibodies directed against AR-FL (AR-Clone AR441), AR-V7, GR (D6H2L) and beta actin (ACTB). As secondary antibodies, goat anti-mouse HRP-conjugated antibody and goat anti-rabbit HRP-conjugated antibodies were used. Signals were detected using ECL Western Blot Substrate or SuperSignal West Dura extended duration substrate on a Fusion S imaging system. For detection of AR-

V7/AR-FL, blots were initially incubated for AR-V7. After development, the blots were stripped for 15 min using Restore Western blot stripping buffer, re-blocked for 1h in 5% skimmed milk TBS (AR-FL) or 5% skimmed milk TBS-T (GR, AR-V7) and incubated with either AR-FL or GR primary antibody. Quantification of the immunoblots was performed using ImageJ. Samples were normalized to beta actin (loading control). The relative protein expression was assessed for GR, AR-FL and AR-V7 with either LNCaP-Ctrl$^{LT}$ or with LNCaP-Apa$^{LT}$ + APA as reference.

# 3. Research projects

## 3.1 Chapter I: Deciphering the limits of PCa biomarker research

**Kremer Anika**, Kremer Tobias, Kristiansen Glen, Tolkach Yuri

Where is the limit of prostate cancer biomarker research?
Systematic investigation of potential prognostic and diagnostic biomarkers.[34]

BMC Urol. 2019 Jun 6;19(1):46

(publication provided in section 6.2.1)

### 3.1.1 Introduction

Primary PCa diagnoses frequently may occur at advanced stages of the disease, thus significantly reducing the chances of curation. Disease treatment is further impeded by the high heterogeneity within and between PCa tumors. The identification of appropriate biomarkers is not only highly relevant to ensure early diagnosis, but also for stratification between indolent and aggressive tumors[213]. The use of biomarkers as clinical tools moreover allows for risk assessment, point-of-care diagnosis, elaborated prognosis, individualized treatment and improved treatment efficacy, thus increasing a patient's life span and quality of life. Expanding the clinical armamentarium by identifying new research targets that allow more precise and individualized therapy options for PCa patients is essential for today's clinical practice[35]. Over the last decades, a large variety of studies described new applicable targets for the three categories of biomarkers: diagnosic, prognostic and theranostic. Unfortunately, both prognostic and theranostic PCa biomarkers are only insufficiently or not at all established for clinical use. Few diagnostic biomarkers are considered reliable[214] and are hence recommended in professional guidelines[22,24]. However, the number of potential target genes and their combinations seem almost endless. Commercially available kits already combine the expression patterns of single genes into so-called gene signature biomarkers that allow for further stratification and identification of specific diseases. The following comprehensive, holistic bioinformatical approach

aimed at re-evaluating and roughly delineating the tremendous opportunities and applications for biomarker research that are still undiscovered by transcriptome analysis[34,47].

### 3.1.2 Results

This study cohort comprised 499 PCa and 53 normal tissue patients (TCGA PRAD cohort[15]) with available clinicopathological information. For 452 PCa patients, follow-up information including biochemical recurrence (BCR) was available. mRNA expression data for approx. 18,000 genes were available. Data curation was primarily performed by Dr. Yuri Tolkach. Bioinformatics were performed in close collaboration between Dr. Yuri Tolkach as supervisor and Anika Kremer with the help of Dr. Tobias Kremer. Data were analyzed by Anika Kremer with the help of Dr. Tobias Kremer and Dr. Yuri Tolkach. Visualization and writing of the original draft was performed by Anika Kremer and Dr. Yuri Tolkach. A specifically developed bioinformatical pipeline allowed to analyze gene expression differences in tumors with different aggressiveness (ISUP grading based on the Gleason score) vs. benign prostate tissue[34]. Analysis of the diagnostic potential yielded large amounts of prospective target genes that were significantly upregulated (n = 1553) or downregulated (n = 1754). Approx. 8% of these genes showed high levels of deregulation. In addition, Gene set enrichment analysis[215] (GSEA, performed by Dr. Yuri Tolkach) showed that 16 of 30 of the most enriched biological processes (53%) were related to alterations of epigenetic regulations like chromatin organization or gene silencing.

Correlation of mRNA expression with tumor aggressiveness yielded 8724 genes that were significantly correlated to the ISUP grading ($p<0.05$). Of these genes, one-third was found to be negatively correlated, while the other two-thirds were positively correlated to tumor aggressiveness.

Examining the prognostic role of all genes for patient survival identified 3571 genes significantly associated with BCR-free survival dichotomized by the median in the univariate analysis. In the multivariate analysis, 571 genes (16%) were independently associated with BCR-free survival after radical prostatectomy according to the stringent criteria of REMARK[216]. Under optimized conditions, using the best cut-off instead of the median for dichotomization, even 9273 genes in univariate analysis and

2435 genes in multivariate analysis (26%) were significantly associated with BCR-free survival. Lowering the stringency of the analysis criteria inevitably leads to an increased error susceptibility. Nevertheless, the additionally found genes may harbor specific characteristics that are relevant for tumor subtyping. A closer examination of the top 50 genes identified in this analysis showed that almost 80% of those had not been studied at that time. Interestingly, more than 75% of these unstudied genes were associated with some other cancer types. However, several of the genes found to be deregulated like SPAG5[217,218] were already extensively studied. In contrast to that, other genes that are recommended for their application as biomarker or already established for diagnostic purposes, such as FOLH1/PSMA, were less conspicuous in these analyses. In consequence, approx. 500 genes identified by our approach are still unconsidered on this list of prominent potential biomarkers[34]. A significant limitation of this analysis is the use of the surrogate endpoint BCR and the relatively short follow up time of median 16 months. Clearly, validation of these findings in better characterized cohorts is warranted.

### 3.1.3 Conclusion

The present current methodical approaches, newly developed technologies and acquired insights into molecular oncology, allow to explore the field of biomarker research from a completely new angle. Exact details remain to be established, thus requiring further investigations in the fields of of DNA (epigenetics, mutations, copy number variations), RNA (mRNA and lncRNA expression) and protein (posttranslational modifications) research[26,219–221]. Our systematic analysis revealed a large number of potential transcripts that have not been considered relevant for diagnostic and prognostic biomarker research, yet. Pointing out these dimensions, it becomes more and more clear that the unmet need for further innovative genetic biomarkers is enormous and the limit to biomarker research is far from being exhausted[34].

## 3.2 Chapter II: The impacts of AR splice variants on DNA Repair

Yuri Tolkach*, **Anika Kremer***, Gábor Lotz, Matthias Schmid, Thomas Mayr, Sarah Förster, Stephan Garbe, Sana Hosni, Marcus V. Cronauer, Ildikó Kocsmár, Éva Kocsmár, Péter Riesz, Abdullah Alajati, Manuel Ritter, Jörg Ellinger, Carsten-Henning Ohlmann, Glen Kristiansen

* equal contribution

Androgen Receptor Splice Variants Contribute to the Upregulation of DNA Repair in Prostate Cancer[207]

Cancers (Basel). 2022 Sep 13;14(18):4441

(publication provided in section 6.2.2)

### 3.2.1 Introduction

Canonical AR signaling and DNA repair are firmly interconnected and consequently used for therapeutic measures in PCa. This exploitation of the interaction of distinct DNA repair mechanisms resulting in the concept of synthetic lethality is applied in approx. 25% of CRPC patients. A status of “BRCAness” is induced by the presence of pathogenic mutations, the defectiveness of genes involved in DSB repair, or by ADT[199,222–224]. In this background, use of PARPi disables tumor cells to effectively repair DNA damages, hence leading to the accumulation of eventually lethal mutations[222,223]. The application of this concept becomes increasingly inappropriate as the disease advances, apparently due to AR alterations such as splice variants, amplifications, mutations, that are provoked by ADT[127,204,205]. In the wake of AR blockage by AA application, constitutively active AR-Vs such as AR-V7 might preserve DNA repair activity[200]. In consequence, both AR-FL and AR-Vs have the capacity to directly mediate the expression of key genes essential for DNA repair[199,200,225], which was further supported *in vitro* using cell line models[200,206]. This study aimed to elucidate the effects of AR-V signaling on DNA repair genes and AR pathway in CRPC tumors *in vitro* and in a retrospective clinical study at different stages of the disease[207].

### 3.2.2 Results

In this study, 273 PCa tissue samples including primary hormone-naïve tissues, primary metastases, hormone-sensitive tissues under ADT as well as CRPC tissues were analyzed.

Within this project, preparation and experimental analysis of clinical samples was performed by Anika Kremer. She also participated in the development of the bioinformatic pipeline, data analysis and visualization of clinical data. *In vitro* experiments were performed by Dr. Sarah Förster, Anika Kremer performed the analysis and visualization of the *in vitro* data. Anika Kremer was involved in writing of the original draft, review, editing and submission of the final draft.

The study substantiated the association between AR pathway activation, AR-V expression and deregulation of the DDR system by analyzing clinical tumor samples and validation of the clinical finding in an *in vitro* tumor model. All samples were examined according to their AR-V status (expression of AR-V1, AR-V3, AR-V7, AR-V9), which correlated with the progression of the disease. Primarily AR-V7 was detected under ADT and in CRPC tumors, always being accompanied by at least one of the other splice variants.

Enhanced expression of AR-Vs in CRPC tumors leads to an increased AR pathway activity, indicated by the reactivation of DNA repair machinery in advanced PCa as observed in several studies[200,206]. Cellular proliferation, measured via *MKI67 (Ki-67)* and *UBE2C* expression, tightly associated with the expression of AR-Vs. *PCNA* expression, however, is strongly correlated with *MKI67* expression without any evidence of dependence on AR-V expression. In combination, AR proliferation and signaling appeared to be dependent on the presence of AR-Vs in both ADT and CRPC tumors, indicating progredient dedifferentiation of tumors in the course of the disease.

In the presence of AR-Vs, DDR was significantly elevated as measured by the expression of HR DNA repair genes. In advanced PCa, the DNA-R score and AR score were correlated. Regarding PARPi, this study may have clinical implications as the presence of AR-Vs might be predictive for the effectiveness of synthetic lethality-based therapies. However, there was no evidence for the dependence of AR isoform expression on PARP gene expression, pointing out that the determination of PARP expression levels does not reflect PARylation activity, as shown by Asim et al.[223].

Verification of these results via linear regression resulted in a similar positive association between AR-Vs and DNA repair, independent of the association between AR-Vs and proliferation.

In ADT and CRPC samples, DNA repair gene analyses revealed an altered signature of DDR gene expression in presence of AR-Vs compared to primary tumors. In accordance with the work of Taylor et al[172] and Grasso et al[32], seven genes were upregulated in both ADT and CRPC samples.

Verification of the specific regulation of DDR genes in CRPC tumors as found in clinical samples was conducted in an *in vitro* tumor cell line model. In the clinical treatment of PCa, X-ray irradiation is a common therapeutic method used after primary tumor resection. Thus, γH2A.X foci formation, provoked by X-ray irradiation, was measured in LNCaP cells overexpressing AR-V7 (LNCaP/V7) as model for CRPC (performed by Dr. Sarah Förster). 24h after irradiation, a significantly reduced number of residual foci was observed in the presence of AR-V7 compared to AR-FL, pointing towards accelerated DDR activity[226] as a consequence of AR-V7 expression. Analysis of clinical CRPC samples corroborated the modulation of DDR gene activity in the presence of constitutively active AR-Vs. *In vitro* expression analyses confirmed and validated the modified expression of several of these DDR genes. The de-regulated genes classified in either the group of DNA damage sensors or HR genes and thus corroborated former studies that reported a causal link between AR-Vs and DDR after irradiation[200]. The present *in vitro* analysis also confirmed the results of the comparison of clinical CRPC and primary tumors (CRPC vs. PRIM): a subset of these genes exhibited a substantial shift in DDR gene expression *in vitro* in the LNCaP/V7 CRPC model. Specifically, a set of three genes (ATM, NBN, MCPH1) was found downregulated in clinical CRPC samples and in the *in vitro* models. These genes are functionally tightly interconnected and associated with the recruitment of DDR foci. Functional loss of these genes is also associated with the sensitization to PARPi therapy. In contrast, AR-V expression as induced by ADT, is known to reactivate DDR[207].

### 3.2.3 Conclusion

Our retrospective study corroborates the interconnection between AR signaling, altered AR expression, and DDR in a large cohort of clinical PCa tumor samples. Reactivation of the DNA repair machinery is associated with the expression of AR splice variants and AR pathway activity in ADT refractory tumors. Thus, AR-Vs might be causative for the diminished or absent effect of therapeutic principles like synthetic lethality via ADT in combination with PARPi. The impact of AR-Vs appeared to be independent of their effect on the proliferation of tumor cells. In an androgen-sensitive PCa cell line, adapted to mimic the castration-resistant phenotype of CRPC tumors by overexpression of AR-V7, these clinical findings were validated. Here, the modulated DDR gene expression that occurs in the presence of AR splice variants was found to be directly linked to an increased DNA repair activity. This finding indicates novel therapeutic approaches for CRPC. Hence, determination of AR-V expression is pivotal for the course of the disease, has therapeutic implications and thus should be taken into consideration as predictive biomarker[207].

## 3.3 Chapter III: The complexity of resistance mechanisms following long-term antiandrogen treatment of PCa tumor models *in vitro* (unpublished)

### 3.3.1 Introduction

The androgen signaling axis pivotally determines the pathogenesis of PCa and is commonly targeted by ADT for the treatment of PCa. As virtually all tumors eventually become castration refractory, resistance towards ADT and AAs poses a major problem in the long-term therapy of advanced PCa patients[227]. However, the mechanisms underlying long-term AA treatment resistance are not fully understood yet. Clinical studies demonstrated that the emergence of resistance is linked to accelerated metastatic spreading. However, treatment is generally continued, even after the emergence of drug resistance[228]. Other studies show that even upon changes in treatment regimens, time to tumor progression will be shortened for tumors once they developed resistance mechanisms in first-line treatments. So-called cross-resistances are described for the successive therapeutic approaches of PCa with varying AAs, irrespective of the generation of the applied AA[179,229]. Amongst a multitude of mechanisms that lead to the development of cross-resistances, most adaptations involve upregulation of AR expression, the emergence of AR-Vs[230] as well as the occurrence of mutations in the AR locus. Less studied is the role of the GR in PCa, which is suggested to take over the role of the AR using commonalities in their structure and mechanism of action[231]. Overlapping sets of gene targets and transcriptomes[232] suggest that the GR may be able to promote resistance to novel generations of AAs and even drive cell proliferation[233], as described for treatment with second-generation AAs *in vivo* and *in vitro*[120,234,235].

It is known that ARSIs targeting different components of the AR signaling pathway will develop drug-specific molecular adaptations in the course of resistance formation[236]. This study aimed to investigate the development of resistance in long-term AA-treated AR-expressing PCa cell lines *in vitro* and their potential re-sensitization with complementary antiandrogens. To date, only few *in vitro* CRPC models have been established[237,238]. Experimental setup and treatment regimens aimed at reasonably mirroring the clinical situation of long-term AA treatment in PCa patients. This study

aimed to answer the following questions: Is resistance formation similarly achieved for all AAs in all tumor models? Are resistance mechanisms specific for a chosen AA? Do specific molecular alterations appear upon long-term treatment with different AAs?

For the investigation of advanced, androgen-deprived PCa, *in vitro* tumor models were established. Two AR-expressing PCa cell lines (androgen-sensitive LNCaP and the androgen-insensitive CRPC cell line 22Rv1) were long-term treated with antiandrogens to facilitate resistance to either the 1st generation (bicalutamide, BICA), 2nd generation (apalutamide, APA) or latest generation (Darolutamide, DARO) of antiandrogens. In general, the clinical application of AAs follows a preceding chemical or surgical castration. These castrate conditions were mimicked *in vitro* by the utilization of full media[239]. As reported by Sedelaar and Issacs, and supported by others[239,240], culture in standard growth media provides testosterone concentrations sufficiently low to be defined as androgen-deprived as in patients' sera upon ADT[17], while other steroids are retained. To closely mimic these physiological conditions, AR-expressing PCa cell lines were subjected to long-term treatment with the antiandrogens bicalutamide (BICA), apalutamide (APA) and darolutamide (DARO), to achieve resistance formation. Long-term treated sublines were investigated for cross-resistance formation or re-sensitization and the regulation of potential resistance-mediating genes like the AR or a compensatory upregulation of the GR under androgen deprivation.

Understanding the mechanisms and biochemical pathways that underlie treatment resistance following long-term AA deprivation might allow for further therapy options in AA-induced CRPCs.

### 3.3.2 Results

**Generation, morphological and primary biochemical characterization of AA long-term treated PCa cell lines**

*Generation of AA-tolerant PCa sublines*

Androgen-sensitive LNCaP cells were cultured in full media under a gradual increase in AAs (BICA, APA or DARO) concentration to allow for continuous growth for six to nine months. 22Rv1 cell treatment follows LNCaP treatment. Accounting for the limited AA responsiveness of 22Rv1 cells, BICA, APA and DARO concentrations were raised no higher than 40 to 60 µM (Table 6).

***Table 6**: Antiandrogen treatment concentrations (BICA, APA, DARO) to induce formation of resistance in the PCa cell lines LNCaP$^{LT}$ and 22Rv1$^{LT}$.*

| | LNCaP$^{LT}$ | 22Rv1$^{LT}$ |
|---|---|---|
| **BICA** | 60 µM | 60 µM |
| **APA** | 10 µM | 40 µM |
| **DARO** | 5 µM | 40 µM |

Control cell lines were cultured in parallel in the absence of AAs. Over a period of two years of cultivation, stable growth of control and long-term treated LNCaP $^{LT}$ / 22Rv1$^{LT}$ sublines was achieved. A schematic overview of the treatments and analyses performed in this chapter is given below (Figure 5).

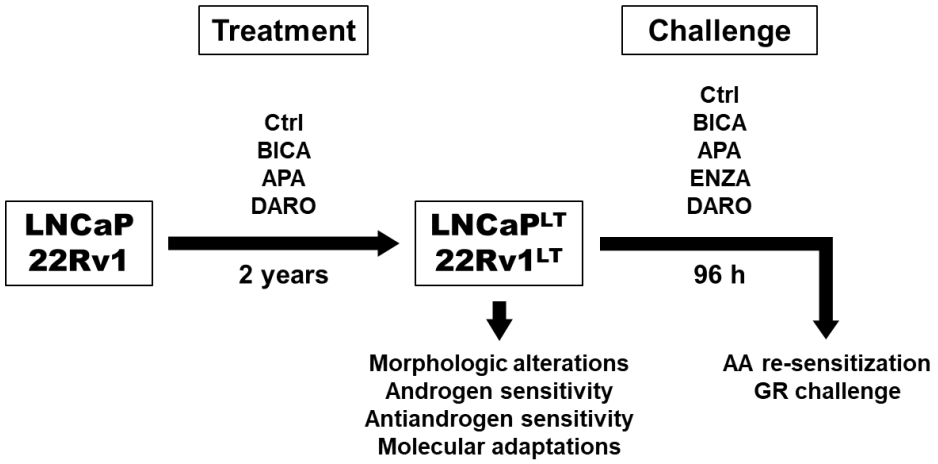



***Figure 5**: Flow chart of the treatments and analyses performed to generate and characterize the long-term AA treated LNCaP$^{LT}$ and 22Rv1$^{LT}$ sublines.*

*Morphological characterization*

LNCaP is a common PCa cell line known to be androgen-sensitive based on their sole expression of AR-FL. The long-term cultured LNCaP control cell line (**LNCaP-Ctrl$^{LT}$**) shows elongated projections (Figure 6) that resemble the morphology of parental LNCaP cells as depicted by the provider (ACC-256, German Collection of Microorganisms and Cell Cultures DSMZ, Braunschweig, Germany). Additionally, long-term culture of LNCaP is known to induce changes in androgen sensitivity[241].

The described phenotype is also seen in LNCaP cells long-term treated with BICA (**LNCaP-Bica$^{LT}$**) with elongated and finer projections compared to LNCaP-Ctrl$^{LT}$ cells (Figure 6). BICA is known for its ambivalent antagonistic and agonistic effect on AR-FL function. In the presence of the AR T878A mutations, BICA exhibits entirely agonistic properties. In contrast, the antagonistic effect on AR-FL activity predominates for APA. Phenotypically, LNCaP cells long-term treated with APA (**LNCaP-Apa$^{LT}$**) display only short protrusions, whereas the size and aggregation of the rounded cell bodies are similar to LNCaP-Ctrl$^{LT}$ and LNCaP-Bica$^{LT}$ cell bodies. In contrast, DARO long-term treated LNCaP cells (**LNCaP-Daro$^{LT}$**) appear flattened, pointing towards an improved attachment to the culture surface. However, cell diameters and sizes do not change significantly between the different LNCaP sublines or 22Rv1 cells (Table 7). Protrusions of the LNCaP-Daro$^{LT}$ subline (black arrowhead in Figure 6), however, appear morphologically similar to LNCaP-Ctrl$^{LT}$.

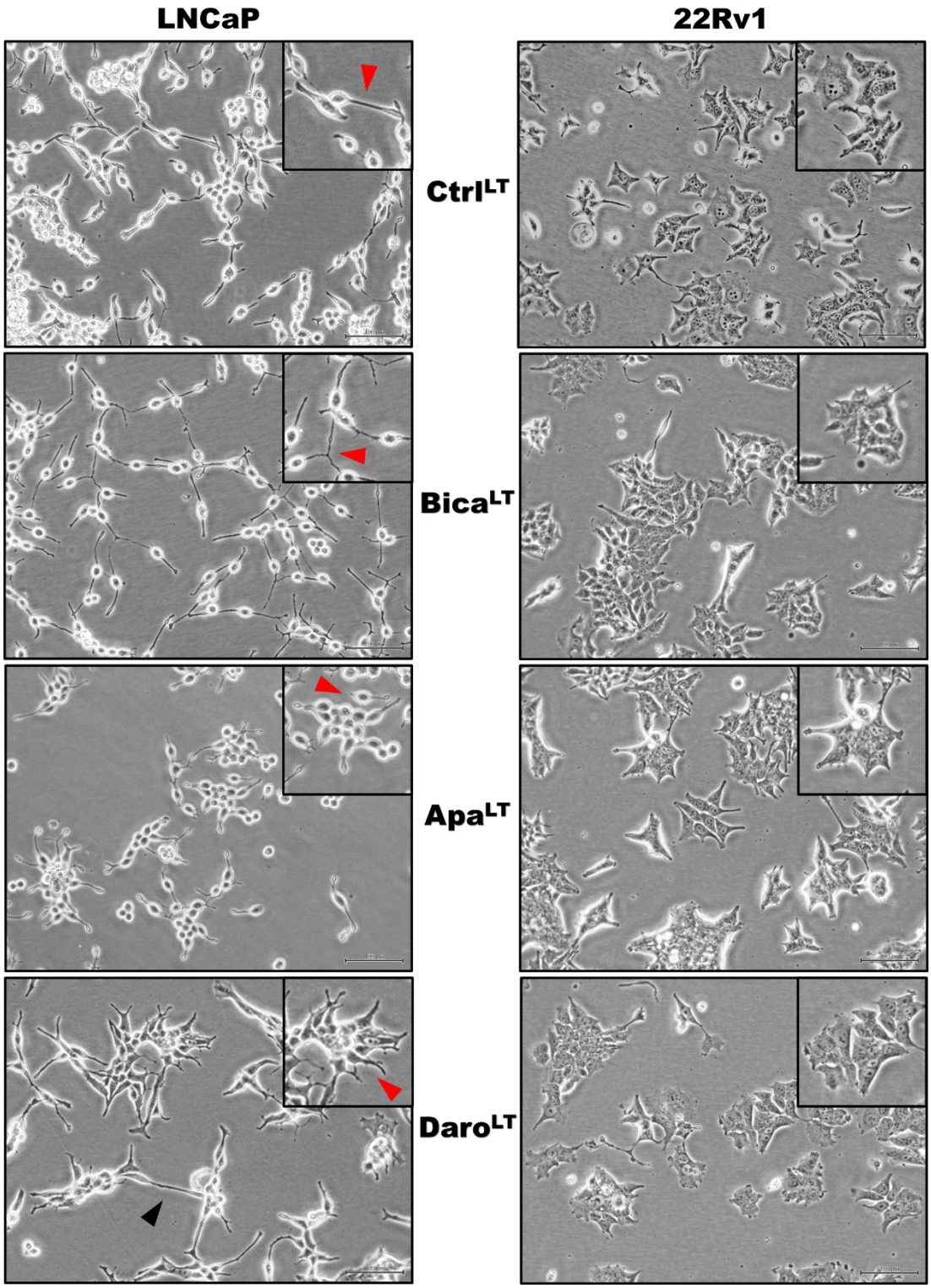



***Figure 6**: **Cell morphology of the untreated control (Ctrl$^{LT}$) and long-term antiandrogen (AA )-treated LNCaP and 22Rv1 cell lines at 20X magnification.** Cells were cultured in growth media containing 10% FBS and 1% Pen/Strep. Additionally, the respective AA concentration used for the resistance formation was added to full media (Table 6). Bar corresponds to 100 µm. Inserts represent magnifications of specific morphological features, arrowheads (red) therein point at morphologies of projections and cellular features. A black arrowhead marks corresponding projections in the LNCaP-Daro$^{LT}$ subline.*

***Table 7: Cell diameters of PCa sublines.*** *AA treatment does not affect cell diameters of PCa sublines. Diameters were determined from freshly trypsinized cells on the Nucleocounter NC-200.*

| Cell line | Diameter [µm] |
|---|---|
| **22Rv1-Ctrl$^{LT}$** | 15.2 |
| **LNCaP-Ctrl$^{LT}$** | 15.0 |
| **LNCaP-Bica$^{LT}$** | 14.5 |
| **LNCaP-Daro$^{LT}$** | 16.0 |
| **LNCaP-Apa$^{LT}$** | 15.3 |

22Rv1 is an AR-expressing PCa cell line of epitheloid morphology, with a castration-resistant behavior due to its expression of AR splice variants (AR-Vs), in particular consitutively active AR-V7[185]. In our experiments, AA non-responsive sublines were generated by long-term treatment with BICA (**22Rv1-Bica$^{LT}$**), APA (**22Rv1-Apa$^{LT}$**) or DARO (**22Rv1-Daro$^{LT}$**). In line with the classification as CRPC line, no gross morphological differences were observed in the long-term AA-treated cell lines compared to long-term cultured 22Rv1-Ctrl$^{LT}$ (Figure 6, right panel).

*Biochemical characterization*

To answer the question whether changes in AR expression may account for the gross morphological alterations observed after long-term AA treatment in LNCaP cells, AR-FL and AR-V7 protein expression was analyzed in the described LNCaP sublines (Figure 7A). AR-FL protein expression was maximally up to 2-fold increased (LNCaP-APA$^{LT}$) and hence remained largely constant in all LNCaP sublines (Figure 7B). AR-V7 protein expression was not detectable in all LNCaP$^{LT}$ sublines. In summary, no AR-V7 expression was detectable in the LNCaP$^{LT}$ sublines, while AR-FL expression was elevated in LNCaP-APA$^{LT}$ cells.

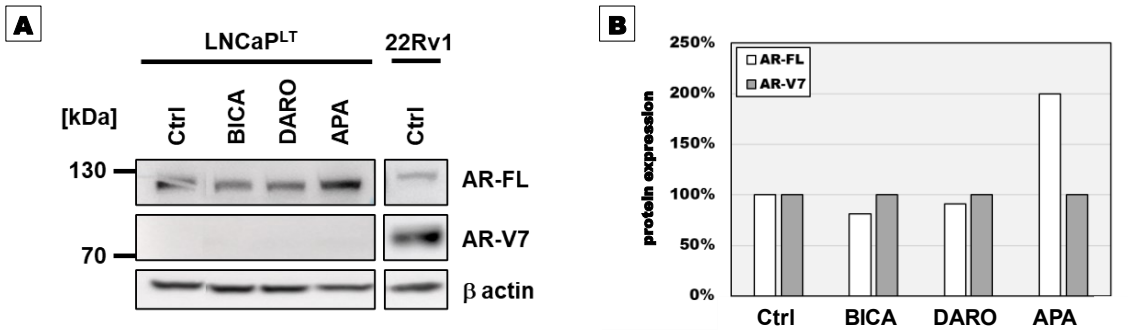



***Figure 7: Protein expression of AR-FL and its major splice variant AR-V7 in AA long-term treated LNCaP cells.*** *A) Representative immunoblot of three independent experiments showing AR-FL and AR-V7 expression in untreated LNCaP$^{LT}$ (Ctrl) cells and long-term BICA (Bica$^{LT}$), DARO (Daro$^{LT}$) and APA (Apa$^{LT}$) treated LNCaP cells. Cells were treated for 96 h with AAs at the concentrations used for long-term culture (Table 6). Long exposure was selected for AR-V7 to detect minimal protein levels in LNCaP$^{LT}$ sublines. AR expression of 22Rv1$^{LT}$ cells (Ctrl) is shown for reference (same blot, same exposure times), β actin serves as loading control. B) Normalized relative AR-FL and AR-V7 protein quantification of LNCaP sublines, derived from the representative immunoblot shown in (A). Untreated LNCaP cells were chosen as reference (Ctrl). AR-FL and AR-V7 protein expression was normalized to β actin. The plot shows protein expression in relation to LNCaP-Ctrl$^{LT}$ (Ctrl).*

## Features of resistance formation in long-term AA-treated PCa cell lines

### *Antiandrogen sensitivity and tolerance in naïve and AA-treated PCa cell lines*

The following experiments were designed to answer the question if long-term AA treatment of PCa cell lines is reflected by increase of antiandrogen tolerance. To study the impact of the antiandrogens (BICA, APA, DARO), the following and further experiments below were performed in steroid-depleted media. In the absence of AAs, only minimal discrepancies in the proliferative index were observed for all parental and long-term treated sublines after 96 h of incubation (Supplementary Figure 1).

AA sensitivity was tested by continued treatment with BICA, APA and DARO, the AAs used for long-term treatment of LNCaP and 22Rv1 cell lines. A remarkable feature of the AA impact on proliferation of PCa cell lines is their significant reduction in proliferative index in the clinically relevant concentration range[238]. However, complete cell loss is only achieved at AA concentrations that are independent of AR activity[242]. In the first set of experiments, parental AR-expressing LNCaP and 22Rv1 as well as AR-non-expressing PC-3 cells were subjected to increasing amounts of AAs to

determine $IC_{50}$ concentrations for specific AAs on proliferation in the described control and AA-treated sublines.

Long-term cultured **LNCaP-Ctrl$^{LT}$** cells show an $IC_{50}$ for BICA of approx. 60 µM. Published data by Ryu et al.[243] for cell viability in the presence of BICA in low passage LNCaPs determined approx. 30 µM indicating a shift to higher BICA tolerance upon long-term culture of LNCaP cells. While no data are published für APA and DARO challenge of LNCaP cells, $IC_{50}$ values of 30 µM and 10-20 µM were estimated for APA and DARO challenge in these experiments, respectively. Taken together, these data suggest that higher generation AAs show a trend toward higher anti-proliferative potency at lower concentrations (Figure 8A, LNCaP-Ctrl$^{LT}$) in the presence of mutant AR T878A compared to lower generation AAs.

Continued long-term culture of **22Rv1-Ctrl$^{LT}$** cells also showed a trend towards increased potency of DARO ($IC_{50}$: 50 µM) over BICA treatment ($IC_{50}$: 100 µM). APA treatment on the other hand had no effect ($IC_{50}$: not reached) on 22Rv1-Ctrl$^{LT}$ proliferation over the entire range of APA concentrations (Figure 8A).

Similar results were obtained by AA challenge of AR-negative **PC-3** cells (Figure 8A, PC-3). While DARO tended to be more effective than BICA ($IC_{50}$: 50-60 µM vs. 100 µM), APA hat no effect ($IC_{50}$: not reached) on this cell line. Similar AA tolerances determined for 22Rv1-Ctrl$^{LT}$ and PC-3 cells and are likely based on AR-independent off-target effects of the AAs on proliferation in the cell lines[242].

The presence of the AR T878A mutation predisposes LNCaP cells to a broadened ligand responsiveness and differential sensitivity with respect to a number of AAs[149,244–246]. In line with a described agonistic effect of BICA in the presence of this mutation, a strongly diminished efficacy of BICA was observed in short-term challenge. In the presence of T878A, APA is known to display only partially agonistic effects on the AR, mirrored by an increased inhibitory capacity. The results here show that the presence of the AR T878A mutation does affect AR inhibition by DARO, thus confirming findings by others[164].

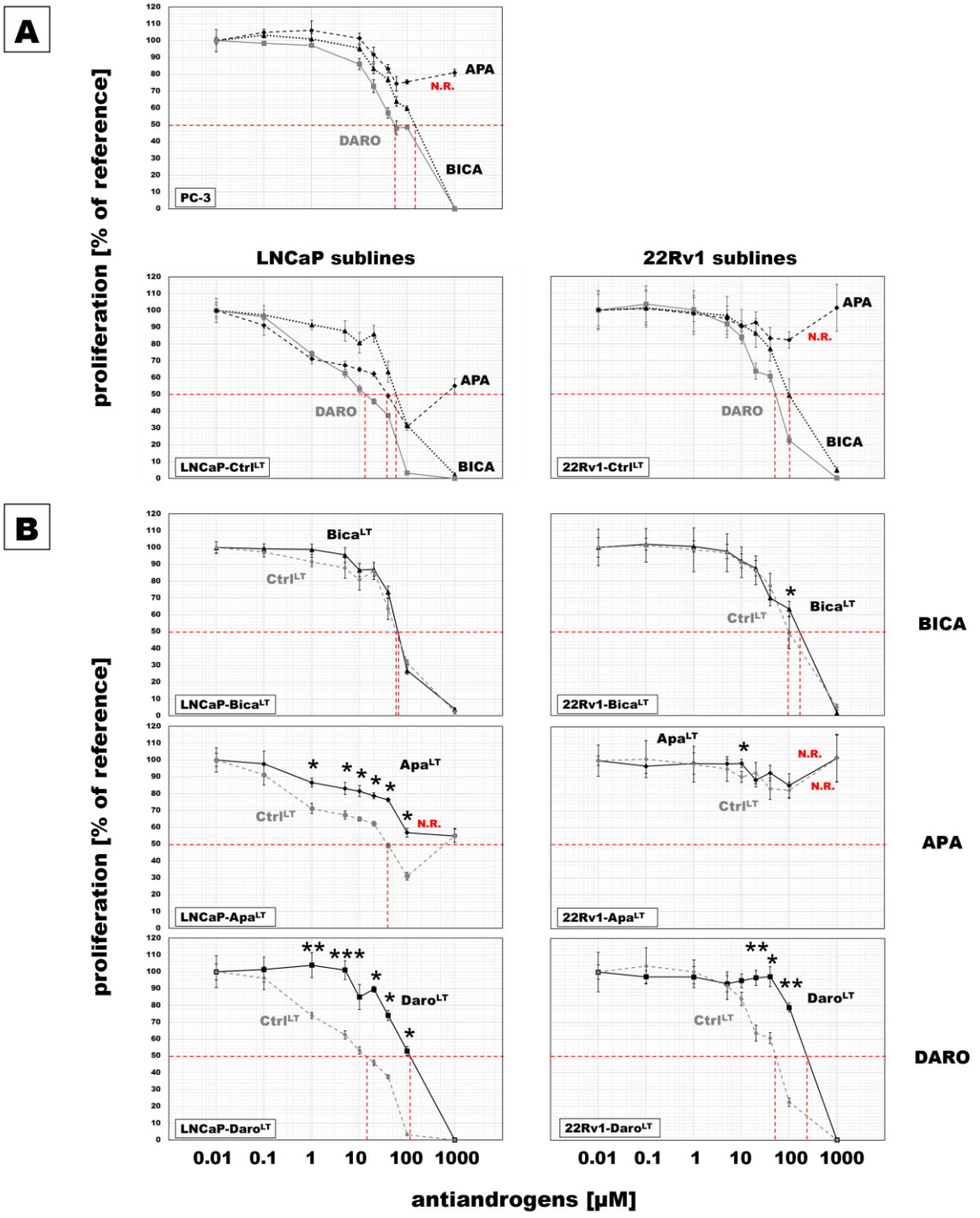



***Figure 8: AA sensitivity in PCa cell lines and long-term AA-treated sublines.*** *A) Effect of AAs on proliferation of AR-FL expressing (LNCaP-Ctrl$^{LT}$), AR-FL and AR-V expressing (22Rv1-Ctrl$^{LT}$) and AR-non-expressing (PC-3) cell lines. B) Effect of corresponding antiandrogen treatment on proliferation of long-term AA-cultured LNCaP and 22Rv1 sublines (black curves) under BICA (Bica$^{LT}$; upper panel), APA (Apa$^{LT}$, middle panel) or DARO treatment (Daro$^{LT}$, lower panel). Proliferation of LNCaP/ 22Rv1 control cell lines under no or minimal treatment served as reference (Ctrl$^{LT}$, grey dashed lines). Cells were cultured for 96 h in steroid-depleted media containing increasing amounts of AAs (0.01, 0.1, 1, 10, 100, 1000 µM). Proliferative index was measured by MTS reaction. Data are shown as % of AA treated reference. Horizontal red dashed line: half maximal growth compared to reference. Vertical red dashed lines indicate $IC_{50}$ values; N.R.: $IC_{50}$ not reached. Error bars depict standard deviations of three independent experiments (n = 3). Independent two-sample t tests were used for the comparison of long-term AA treated sublines (Bica$^{LT}$, Apa$^{LT}$, Daro$^{LT}$) vs. Ctrl$^{LT}$, respectively. p values: * = p < 0.05, ** = p < 0.005, *** = p < 0.0005 (exact p values: Supplementary Table 2).*

To answer the question if the differential AA responses determined in the presence of the AR T878A mutation might also affect resistance development upon long-term AA treatment, $IC_{50}$ values were determined analogously for long-term BICA, APA and DARO-treated **LNCaP$^{LT}$** cells. The AR H875Y mutation present in 22Rv1 cells[185] is known to affect AA activities similarly as T878A. As additional resistance mechanism, these cells also express ligand-independent AR-Vs which possess a third zinc-finger in their DBD[247]. These data may indicate that AR alterations would counteract resistance formation upon long-term AA treatment in 22Rv1 cells.

As expected, long-term treatment of 22Rv1 cells with BICA and APA (**22Rv1-Bica$^{LT}$** and **22Rv1-Apa$^{LT}$**) did not yield resistant cells with increased AA tolerance (BICA: $IC_{50}$ >100 µM; APA: $IC_{50}$: not reached). Long-term **22Rv1-Daro$^{LT}$** cells, however, do display increased tolerance to the respective AA at higher dosage (DARO: $IC_{50}$: >100 µM) in comparison to 22Rv1-Ctrl$^{LT}$ (DARO: $IC_{50}$: 50 µM). The short-term challenge of the latter cell line at high DARO dosage results in a significant albeit AR-independent growth inhibition, since growth inhibition of the AR-negative **PC-3** cell line with DARO yields similar data. Hence, increased tolerance in the 22Rv1-Daro$^{LT}$ for the respective AA might also affect AR-independent signaling (Figure 8B, right panel).

Long-term treated **LNCaP-Bica$^{LT}$** cells showed no increase in BICA tolerance (BICA: $IC_{50}$: 60-70 µM vs. 60 µM), indicating that prolonged BICA treatment did not induce resistance to this respective AA (Fig 8B, upper panel). A partial agonistic activity of APA long-term treatment further increased the tolerance of **LNCaP-Apa$^{LT}$** towards APA ($IC_{50}$: not reached) as compared to the parental cell line ($IC_{50}$: 30 µM). The sensitivity towards DARO calculated in **LNCaP-Daro$^{LT}$** cells is even more reduced (DARO: $IC_{50}$: >100 µM vs. 10-20 µM), indicating 5 to 10-fold lower sensitivity of long-term treated LNCaP cells for this AA. For reasons of congruence with the following dataset, $IC_{50}$ values discussed here are listed in Table 8A-C.

The failure to generate resistant 22Rv1 sublines by long-term treatment with AAs is best explained by the presence of two independent AR alterations in 22Rv1 that affect AA binding and function. Even resistance formation by the long-term application of DARO would yield a subline whose increased tolerance does presumably not entirely depend on AR signaling. On the other hand, the AR T878A mutation in LNCaP cells does differentially affect treatment with AAs of different generations: in accordance with an agonistic/ partial-agonistic/ antagonistic impact of this AR mutation on binding of

BICA/ APA/ DARO, respectively, no resistance/ partial-resistance/ complete resistance to these AAs in long-term LNCaP$^{LT}$ cells was achieved. Interestingly and in line with the literature, even the control cell lines do not completely die in clinically relevant AA concentrations that equate to approx. 10 µM *in vitro*[238].

*Androgen sensitivity in naïve and AA-treated PCa cell lines*

Androgen sensitivity of long-term cultured AR-expressing cell lines (LNCaP-Ctrl$^{LT}$ and 22Rv1-Ctrl$^{LT}$) was functionally assessed by testing the impact of DHT application in increasing concentrations in steroid-depleted media on proliferation as described above (Supplementary Figure 1). Proliferation of AR-negative **PC-3** cells was analyzed accordingly, but was largely unaffected by DHT (Figure 9A, PC-3). Corroborating published data[248], a dose-dependent increase of LNCaP cell growth was observed with a peak maximum reached at 10 nM DHT (Figure 9A, **LNCaP-Ctrl$^{LT}$**), indicative of a progressive androgen de-sensitization as described by Pfeil et al.[249]. AR signaling can only be minimally activated by DHT in 22Rv1 cells[250] due to the predominance of ligand-independent AR-V expression (AR8)[251,247], an AR-V7 related isoform that mediates androgen-independent growth. The presence of DHT at high concentration (100 nM) negatively affected all tested cell lines, indicating an emerging anti-proliferative effect of the substance applied in this assay[252]. As long-term culturing of LNCaP cells induces partial androgen de-sensitization even in the absence of AAs, this effect is taken into account by comparing AA-treated LNCaP sublines with LnCaP-Ctrl$^{LT}$ cells to clearly determine AA-derived effects on LNCaP survival and proliferation.

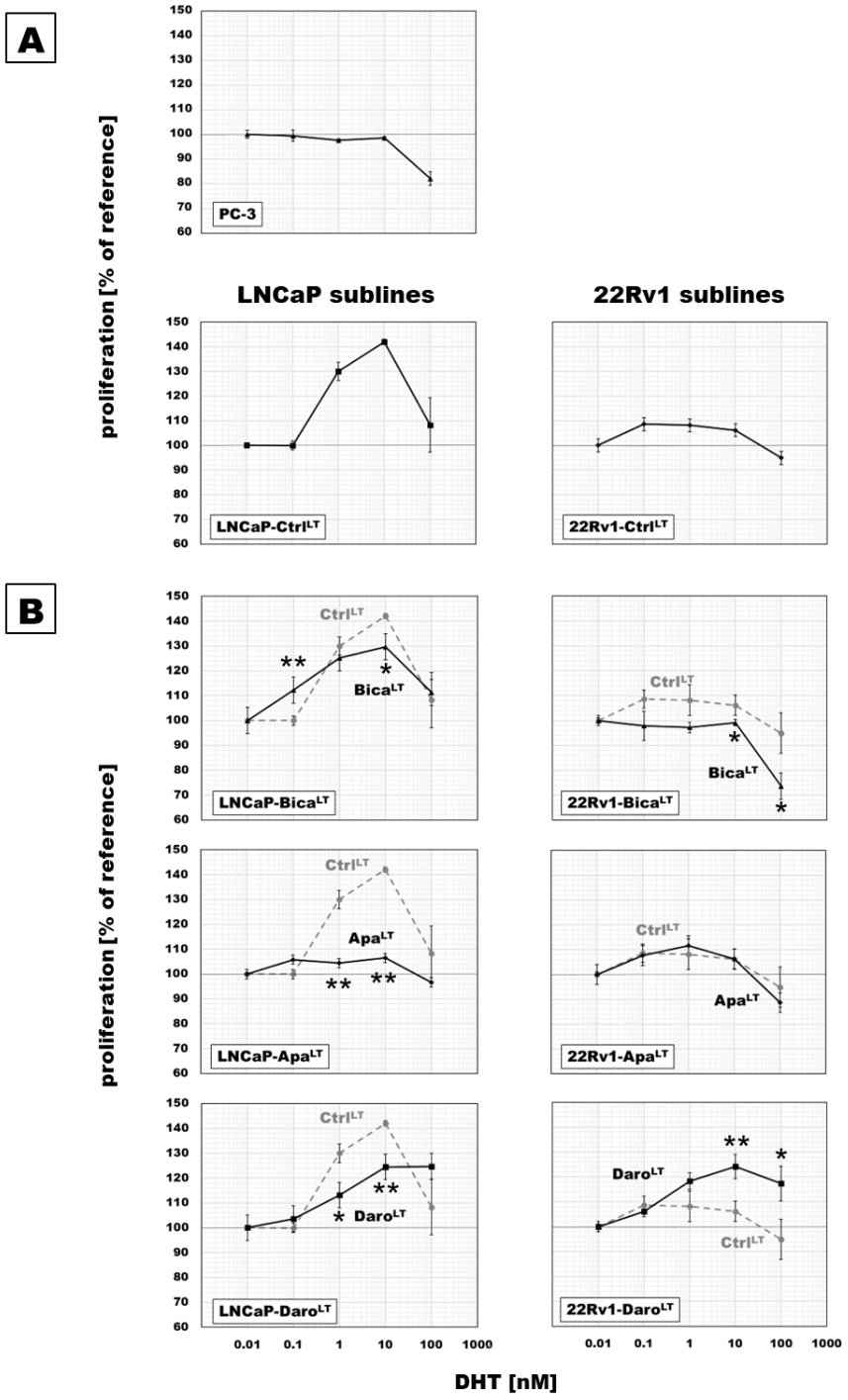



***Figure 9: Androgen sensitivity in PCa cell lines and long-term AA-treated sublines.*** *A) Effect of androgens on proliferation of untreated AR-expressing (LNCaP-Ctrl$^{LT}$, 22Rv1-Ctrl$^{LT}$) and AR-non-expressing (PC-3) cell lines. Note the dose-dependent impact of DHT on LNCaP-Ctrl$^{LT}$ cells, while the presence of AR-Vs induces androgen insensitivity in 22Rv1-Ctrl$^{LT}$. The absence of AR expression renders PC-3 cells non-responsive over the entire range of DHT concentrations used. B) Effect of androgens on proliferation of long-term cultured LNCaP and 22Rv1 sublines (black curves) under BICA (Bica$^{LT}$; upper panel) or APA (Apa$^{LT}$, middle panel) or DARO treatment (Daro$^{LT}$, lower panel). Proliferation of LNCaP/ 22Rv1 control cell lines under no or minimal treatment served as reference (Ctrl$^{LT}$, grey dashed lines). Cells were cultured for 96 h in absence of AAs in steroid-depleted media containing increasing amounts of DHT (0.01, 0.1, 1, 10, 100 nM). Proliferative index was measured in triplicates by MTS reaction. Data are shown as % of DHT treated reference. Error bars depict standard deviations of triplicate measurements of one independent experiment (n = 1). Independent two-sample t tests were used for the comparison of long-term AA treated sublines (Bica$^{LT}$, Apa$^{LT}$, Daro$^{LT}$) vs. Ctrl$^{LT}$, respectively. p values: * = p < 0.05, ** = p < 0.005 (exact p values: Supplementary Table 1).*

Applying this approach, we observed that long-term treated **LNCaP-Bica$^{LT}$** cells retained responsiveness to DHT, similar to LNCaP-Ctrl$^{LT}$ cells. Likewise and similar to the observation in untreated **22Rv1-Ctrl$^{LT}$** cells (Figure 9A, 22Rv1-Ctrl$^{LT}$), **22Rv1-Bica$^{LT}$** cells appeared completely unresponsive to DHT treatment (Figure 9B, upper panel, black curve). These data indicate that both sublines showed only minor alterations in androgen sensitivity upon long-term BICA treatment. An analogous behavior was observed for **22Rv1-Apa$^{LT}$** cells under DHT treatment. In contrast to control cells (Figure 9B, middle panel, grey curve), long-term application of APA rendered the cells virtually unresponsive to androgens. Long-term APA treated LNCaP cells (**LNCaP-Apa$^{LT}$**) also lost their responsiveness to DHT, indicating destructive interference with ligand-induced AR signaling (Figure 9B, middle panel, black curves). Both parental LNCaP and 22Rv1 cells as well as long-term DARO treated sublines are similarly responsive towards DHT treatment at low concentrations. At high DHT concentrations, **LNCaP-Daro$^{LT}$** and **22Rv1-Daro$^{LT}$** retained similar dose-dependent androgen sensitivities with half-maximal proliferation at approximately 1 nM DHT for both sublines, irrespective of their AR-V status. (Figure 9B, lower panel, black curves). This finding suggests a distinct mode of action of this AA, irrespective of the cell line and its AR mutational/variant status. Responsiveness of 22Rv1-Daro$^{LT}$ cells at high androgen concentrations (Figure 9, lower panel) puts a purely AR-independent resistance formation of this cell line into question.

The data presented here indicate that long-term AA treatment with different generations of androgen competitors yields distinct adaptations to ensure continued survival and growth. While long-term BICA treatment shows minimal signs of altered androgen sensitivity in this assay, APA treatment renders cells largely unresponsive to androgens. In contrast, **LNCaP-Daro$^{LT}$** and **22Rv1-Daro$^{LT}$** sublines both show hyposensitivity to androgen application, suggesting that long-term DARO tolerance is induced by mechanisms that still involve the AR signaling pathway. These modes of resistance will be studied at a molecular level in the next chapter.

*Molecular adaptations of LNCaP cells to long-term AA treatment*

ADT in PCa treatment is widely used and is known to upregulate AR-FL and AR-Vs mRNA and protein expression *in vivo*[207] and *in vitro*[253,254]**.** Moreover, upregulation of GR (NR3C1) as a compensatory mechanism associated with APA resistance was described by Arora et al.[120].

The data presented above indicate AA-specific tolerance development and varying DHT sensitivity in the LNCaP$^{LT}$ sublines. In this chapter, expression of proteins known to be involved in common AA resistance pathways was checked by immunoblotting.

Short-term challenge of LNCaP-Ctrl$^{LT}$ cells in steroid-depleted medium and long exposure times generates minimal signals for GR and AR-V7 with the antibodies used. In contrast to incubation in full medium (Figure 7A), no changes in AR-FL expression were detected in short-term (**LNCaP-Ctrl$^{LT}$**) or long-term (**LNCaP$^{LT}$**) AA-treated cells (Figure 10A, lower panel; Figure 10B, white bars). Differences in protein expression, however, appeared specific for long-term APA treatment: here a dramatic increase in GR expression is observed (Figure 10B, black bars). This finding corroborates data by Arora et al. 2013 who introduced upregulation of GR as resistance mechanism upon prolonged APA treatment[120]. In contrast to AR-FL protein expression, levels of AR-V7 protein are specifically upregulated in long-term BICA-treated LNCaP$^{LT}$ cells (Figure 10B, grey bars). Minimal AR-V7 expression was observed upon APA long-term treatment of LNCaP cells. Comparison with the levels of AR-V7 present in castration-resistant 22Rv1 cells, however, marks AR-V7 upregulation as moderate. In line with these findings, upregulation of AR-V7 in LNCaP cells under experimental conditions was recently described[193,255]. In line with this, AR-V7 upregulation in long-term BICA-treated LNCaP$^{LT}$ cells does not confer any alteration in sensitivity for either androgen or the AA BICA, which likely excludes adaptive AR-V7 expression in this subline as potential resistance mechanism (Figure 10A, lower panel).

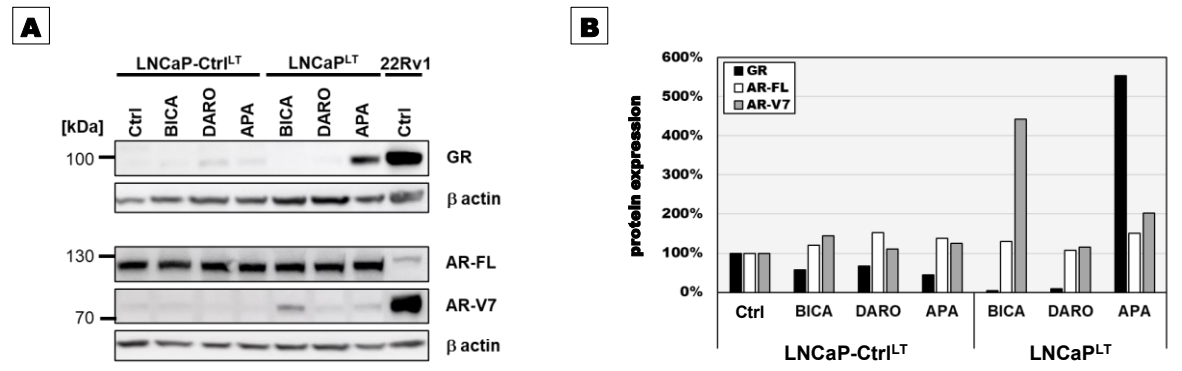



***Figure 10**: **LNCaP cells respond to long-term treatment by AA-specific molecular adaptations.** Long-term cultivated LNCaP-Ctrl$^{LT}$ cells and long-term AA-treated LNCaP$^{LT}$ cells were subjected to AA treatment (BICA, DARO, APA) used for long-term culture (Table 6) for 96 h under serum-deprived conditions. Untreated LNCaP-Ctrl$^{LT}$ cells served as treatment reference. A) The panel shows a representative immunoblot for GR (NR3C1) in the upper panel (three independent experiments, n = 3). β actin served as loading control. The lower panel shows immunoblots for full-length AR (AR-FL) and its major splice variant (AR-V7). β actin served as loading control. 22Rv1 cells were used as positive control for their expression of GR, AR-FL and AR-V7. B) Normalized relative GR, AR-FL and AR-V7 protein quantification of LNCaP sublines, derived from the representative immunoblot shown in (A). Untreated LNCaP cells were chosen as reference (Ctrl). GR, AR-FL and AR-V7 protein expression was normalized to β actin. The plot shows protein expression in relation to LNCaP-Ctrl$^{LT}$ (Ctrl). AR: androgen receptor, GR: glucocorticoid receptor.*

*Short-term challenge with complementary AAs undermines resistance formation in long-term treated PCa cells*

As shown in the last chapter, specific AAs evoke specific molecular mechanisms that lead to increased AA tolerance and resistance formation in PCa cells. To answer the question whether resistance triggered by one AA can be overcome by treatment with a complementary AA, untreated control and long-term AA-treated PCa cells were subjected to treatment with a panel of AAs (BICA, APA, ENZA, DARO). Androgen-sensitive LNCaP and castration-resistant 22Rv1 were analyzed for proliferation in the absence and presence of the listed AAs. $IC_{50}$ for treatments referred to in Figures 9 and 11 are listed for LNCaP (Table 8A), 22Rv1 (Table 8B) sublines and PC-3 cells (Table 8C). Long-term AA-treated LNCaP and 22Rv1 sublines were individually treated with complementary AAs and plotted against control cells and the long-term AA-treated subline treated with the respective AA (Supplementary Figure 2-3).

***Table 8: Estimated AA $IC_{50}$ values and AA treatment concentrations for AR-expressing and AR-non-expressing cell lines and derived sublines.***
*A) AR-expressing LNCaP and derived sublines ($LNCaP^{LT}$) were subjected to increasing amounts of antiandrogens (BICA, APA, ENZA, DARO) in steroid-deprived media. AA treatment concentrations used to generate $LNCaP^{LT}$ sublines are listed for reference (treatment). B) AR-V expressing 22Rv1 and derived sublines ($22Rv1^{LT}$) were subjected to increasing amounts of antiandrogens (BICA, APA, ENZA, DARO) in steroid-deprived media. AA treatment concentrations used to generate $22Rv1^{LT}$ sublines are listed for reference (treatment). C) AR non-expressing PC-3 cells were subjected to increasing amounts of antiandrogens (BICA, APA, DARO) in steroid-deprived media. $IC_{50}$ values were extracted from proliferation data plotted against applied AA (Figure 9 + 11) and are presented as mean [±. standard deviation] from three independent experiments (n = 3), not reached: $IC_{50}$ not reached; >100 µM: $IC_{50}$ estimation outside the range of common AA usage, N.A.: Treatment not applied.*

**A**

| | BICA | APA | ENZA | DARO | Treatment |
|---|---|---|---|---|---|
| **LNCaP-Ctrl** | 60 µM [± 6.2] | 30 µM [± 1.5] | 30 µM [± 3.7] | 10 µM [± 2.2] | N.A. |
| **LNCaP-Bica$^{LT}$** | 60 µM [± 3.6] | 70 µM [± 2.8] | 70 µM [± 3.2] | 40 µM [± 2.7] | 60 µM |
| **LNCaP-Apa$^{LT}$** | 60 µM [± 8.1] | not reached | 90 µM [± 3.7] | 40 µM [± 1.1] | 10 µM |
| **LNCaP-Daro$^{LT}$** | 60 µM [± 12.0] | not reached | not reached | > 100 µM [± 2.7] | 5 µM |

**B**

| | BICA | APA | ENZA | DARO | Treatment |
|---|---|---|---|---|---|
| **22Rv1-Ctrl** | 100 µM [± 9.7] | not reached | not reached | 50 µM [± 3.3] | N.A. |
| **22Rv1-Bica$^{LT}$** | > 100 µM [± 4.5] | not reached | not reached | 40 µM [± 3.9] | 60 µM |
| **22Rv1-Apa$^{LT}$** | 70 µM [± 2.7] | not reached | not reached | 40 µM [± 4.3] | 40 µM |
| **22Rv1-Daro$^{LT}$** | > 100 µM [± 2.4] | not reached | not reached | > 100 µM [± 2.6] | 40 µM |

**C**

| | BICA | APA | ENZA | DARO | Treatment |
|---|---|---|---|---|---|
| **PC-3** | > 100 µM [± 1.5] | not reached | N.A. | < 60 µM [± 4.2] | N.A. |

For androgen-sensitive **LNCaP-Ctrl$^{LT}$** cells (Figure11A, left), efficacy of AA challenge followed the known responsiveness to the AR T878A mutation[149] with BICA ($IC_{50}$: 30 µM) < APA ($IC_{50}$: 60 µM)/ ENZA ($IC_{50}$: 60 µM) < DARO ($IC_{50}$: 10-20 µM). Challenge within the second generation of AAs confirmed the effect similarity of APA and ENZA[256], pointing towards cross-resistance formation upon application of these AAs**.** On the other hand, castration-resistant **22Rv1-Ctrl$^{LT}$** cells (Figure11A, right) hardly respond to BICA, APA and ENZA challenge with $IC_{50}$ values only reached for BICA (100 µM). In these cells, however, DARO treatment is still effective at high concentrations ($IC_{50}$: 50 µM). These data indicate that DARO treatment, in contrast to the other AAs, is functional even in the presence of AR T878A or H875Y mutations, and despite the expression of ligand-independent AR-Vs.

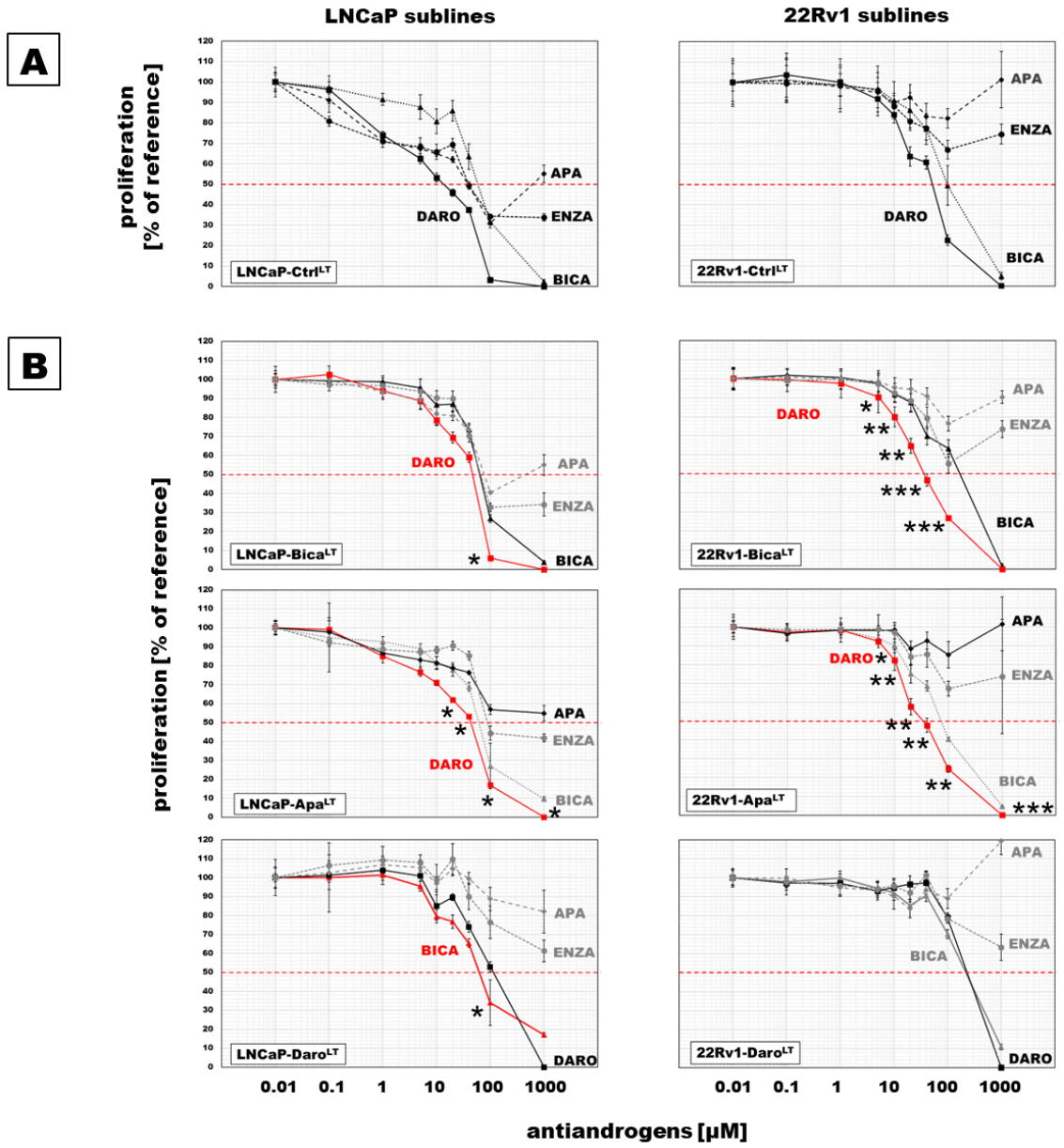



***Figure 11**: **Cross-resistances in AR-expressing PCa cell lines and long-term AA-treated sublines.** A) Effect of antiandrogen challenge (BICA, APA, ENZA, DARO) on proliferation of AR-FL expressing (LNCaP-Ctrl$^{LT}$) and AR-FL/ AR-V expressing (22Rv1-Ctrl$^{LT}$) cell lines. B) Effect of antiandrogen challenge on proliferation of long-term AA-cultured LNCaP and 22Rv1 sublines (black curves) under BICA (Bica$^{LT}$; upper panel), APA (Apa$^{LT}$, middle panel) or DARO treatment (Daro$^{LT}$, lower panel). Cells were cultured for 96 h in steroid-depleted media containing increasing amounts of antiandrogens (0.01, 0.1, 1, 10, 100, 1000 µM). Proliferative index was measured by MTS reaction. Data are shown as % of reference. Black curves indicate LT-sublines treated with their respective AA. Grey dashed curves display treatment with complementary AAs. AA challenge still effective in each LT-subline is shown as red curve. Horizontal red dashed line: half maximal growth compared to reference. Error bars depict standard deviations of three independent experiments (n = 3). Independent two-sample t tests were used to compare the most effective challenge of each LNCaP/ 22Rv1 subline (red curve) vs. treatment with the respective AA (black curve). p values: * = p < 0.05, ** = p < 0.005, *** = p < 0.0005 (exact p values: Supplementary Table 3).*

The efficacy of DARO treatment was also evident in LNCaP and 22Rv1 long-term challenged with either BICA (Figure 11B, upper panel) or APA (Figure 11B, middle panel) with $IC_{50}$ values ≈ 40 µM. On the other hand, AA treatment of **22Rv1-Daro$^{LT}$** showed no changes in efficacy for any of the AAs applied compared to DARO. Though not reaching significance, BICA challenge appears to be mildly effective in **LNCaP-Daro$^{LT}$** cells ($IC_{50}$: 60 µM) compared to DARO challenge. Short-term DARO challenge of AR-expressing **LNCaP$^{LT}$** and **22Rv1 Bica$^{LT}$** and **Apa$^{LT}$** sublines reveals an effective approach ($IC_{50}$ ≈ 40 µM) to overcome resistance mediated by first- and second-generation AAs. This effect appears to be AR-dependent, AR-negative **PC-3** cells subjected to DARO challenge required higher effective doses ($IC_{50}$ > 50 µM, Figure 9A, right) to achieve an equivalent and probably unspecific, toxic impact on proliferation.

*Molecular adaptations to short-term AA challenge of LNCaP-APA$^{LT}$ cells*

Unchanged androgen sensitivity in combination with unchanged responsiveness to BICA as compared to control cells led to the assumption that LNCaP-Bica$^{LT}$ cells are non-resistant. However, the failure to induce growth inhibition of these cells upon short-term APA or ENZA treatment in the last set of experiments questions this idea. Whether the moderate expression of AR-V7 observed for **LNCaP-Bica$^{LT}$** cells (Figure 10A) might play a role to achieve tolerance in response to second-generation AAs remains an open question.

Given the combination of detectable but decreased androgen sensitivity and increased tolerance towards second-generation AAs, **LNCaP-Daro$^{LT}$** cells cannot be classified as resistant in a classical sense since no common molecular resistance mechanisms were deciphered to be activated in these cells. As the molecular mechanism for increased tolerance in long-term DARO-treated sublines is not clear, the observed re-sensitization by under BICA challenge requires further investigation.

**LNCaP-Apa$^{LT}$** cells, on the other hand, are completely androgen insensitive and resistant to BICA, APA and ENZA treatment. Only DARO challenge further inhibits growth of this subline. As known from the literature, prolonged APA and ENZA treatment mediates AA resistance by upregulation of GR protein expression[120]**.** This resistance mechanism was also observed in LNCaP-Apa$^{LT}$ cells. Challenge of this

subline with complementary AAs is suspected to modulate this compensatory mechanism. To understand the molecular changes upon short-term AA treatment, GR and AR variant expression was checked in immunoblots (Figure 12A) at AA concentrations of 20 µM that showed discernable alterations in proliferation assays (Figure 9). No major alterations in protein expression of AR-FL were observed in non-treated (Ctrl) cells and upon challenge with AAs (BICA, DARO, APA, ENZA) in LNCaP-Apa$^{LT}$ (Figure 12B, white bars).

Under short-term APA and ENZA treatment, LNCaP-Apa$^{LT}$ cells produce detectable levels of GR protein expression. GR expression, however, is diminished by around 50% in untreated cells and upon BICA and DARO challenge (Figure 12B, black bars). Interestingly, untreated and BICA challenged cells upregulate AR-V7, a finding that is not observed under DARO challenge (Figure 12B, grey bars). It is tempting to speculate that the AR-V7 upregulation observed here might counteract the diminished GR expression, resulting in unchanged proliferative behavior. Exceptional in our analysis was again DARO treatment of LNCaP-Apa$^{LT}$ cells: in the absence of compensatory AR-V7 upregulation, downregulation GR expression by 60% appears to mediate a 20% growth inhibition at 20 µM.

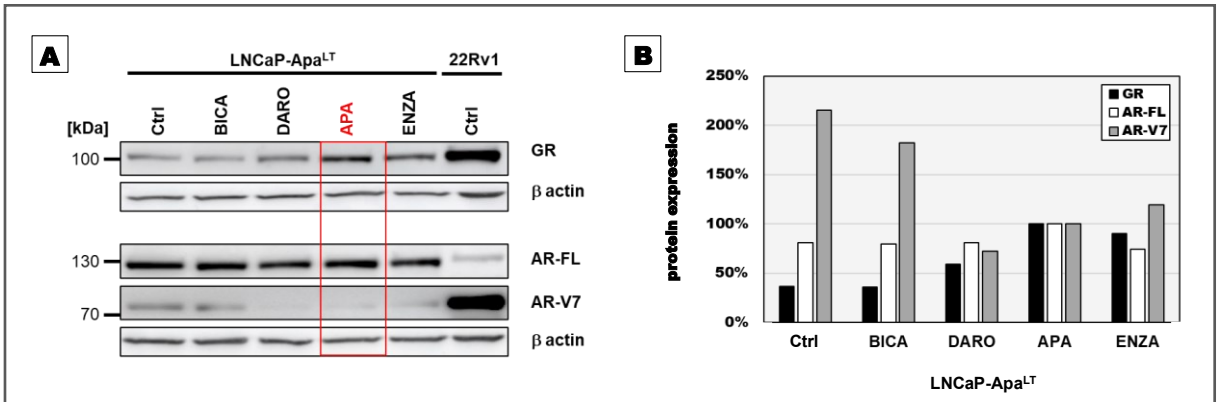



***Figure 12: LNCaP-Apa$^{LT}$ cells respond to short-term challenge by AA-specific molecular adaptations.*** *Long-term cultivated LNCaP-Apa$^{LT}$ cells were either not treated (Ctrl) or subjected to short-term challenge with 20 µM AA (BICA, DARO, APA, ENZA) for 96 h under serum-deprived conditions. APA-treated LNCaP-Apa$^{LT}$ cells served as treatment reference (red frame). A) The panel shows immunoblots for glucocorticoid receptor (GR, NR3C1) in the upper panel. β actin served as loading control. The lower panel shows immunoblots for full-length androgen receptor (AR-FL) and its major splice variant (AR-V7). β actin served as loading control. 22Rv1 cells were used as positive control for their expression of GR, AR-FL and AR-V7. B) Normalized relative GR, AR-FL and AR-V7 protein quantification of LNCaP sublines, derived from the representative immunoblot shown in (A). APA-treated LNCaP-Apa$^{LT}$ cells served as reference. GR, AR-FL and AR-V7 protein expression was normalized to β actin. The plot shows protein expression in relation to APA-treated LNCaP-APA$^{LT}$.*

Taken together, resistance to second-generation AAs (APA, ENZA) is shown by others[120] and in this work to confer AA resistance by upregulation of GR (Figure 10). Challenge by second-generation AAs maintains GR expression, accompanied by maintained resistance to ENZA (Figure 11). However, the application of first-generation AAs cannot maintain GR protein expression. In contrast, reduced GR expression is accompanied by upregulation of AR-V7 and a maintained resistance to BICA. Interestingly, the same mechanism is observed when AA application is terminated. Only therapy switch to DARO appears promising, as GR expression is downregulated without compensatory AR-V expression. This setting led to a re-sensitization as shown by growth inhibition (Figure 11) of the resistant LNCaP-Apa$^{LT}$ cells upon DARO application within 96 h. These results presented here will allow for a more detailed molecular analysis at the transcriptomic and genomic level to analyze for (target) gene deregulation and the establishment of a mutational landscape of long-term AA treated PCa cell lines, respectively.

### 3.3.3 Discussion

In the clinical treatment of PCa, tumor progression toward castration resistance poses a major problem. Understanding the mechanisms underlying treatment resistance following long-term AA application will allow for suitable therapy options and treatment regimens for AA-induced CRPCs. To investigate the effects of long-term AA treatment, PCa cell lines (LNCaP and 22Rv1) with at least partially intact AR signaling pathway were cultured according to the specifications of the german S3 guideline for clinical treatment of prostate carcinoma[17].

PCa cell lines were cultured under conditions that mimicked the castrate levels of PCa patients upon ADT[239] and were subjected to long-term AA treatment to investigate resistance to either first (BICA), second (APA) or latest generation (DARO) AAs. Various criteria were selected to determine resistance development in the generated cell lines: decline in cell proliferation with subsequent recovery after AA treatment increase[257], residual androgen sensitivity, tolerance for either the corresponding or complementary AAs and occurrence of molecular resistance mechanisms (AR, AR-V7, GR). Simultaneously and for each cell line, an untreated (parental) control was

propagated under similar culture conditions to distinguish specifically AA-induced impacts from unintended side effects of long-term culturing.

Many PCa cell lines including LNCaP and 22Rv1 harbor activating *AR* mutations that occur upon long-term androgen ablation to provide survival advantage[258]. These mutations allow for the promiscuous activation of mutated AR by non-androgenic steroids or AAs[259], thus reducing efficacy of AA treatment[32,234,260,261]. In consequence, 22Rv1 cells were selected as castration refractory cell line due to the expression of ligand-independent AR-Vs, apart from the presence of the AR H875Y mutation[262]. In comparison, LNCaP cells possess the most frequently occurring ADT-associated mutation of the AR (AR T878A) that renders them at least partially resistant to a selection of AAs and increases the susceptibility to other steroids like glucocorticoids to activate the AR[263].

*Morphological and biochemical characterization of long-term treated PCa sublines*

In line with its classification as CRPC cell line, 22Rv1 sublines did not show prominent morphological alterations following long-term AA treatment as compared to the long-term cultivated parental cell line. In contrast, the morphology of long-term DARO-treated LNCaP cells resembled the epithelial-like phenotype of androgen-independent C4-2 cells[242,264] which were derived from LNCaP cells to acquire CRPC characteristics[265]. Long-term BICA treated LNCaP cells showed elongated projections and thus resembled the phenotype of the control cell line. LNCaP cells subjected to long-term APA treatment, however, displayed only short protrusions. Taken together, only the long-term DARO treated subline showed morphologic adaptations to long-term AA treatment that are not reflected by changes in AR-FL or AR-V expression.

Further experiments targeted potentially modulated biochemical features in the long-term AA treated *in vitro* models. As basis for all subsequent experiments, a pilot experiment confirmed similar proliferative behavior of control and AA-treated LNCaP$^{LT}$ and 22Rv1$^{LT}$ sublines in the absence of AA treatment. The absence of androgens, glucocorticoids and, in general, steroid precursors in steroid hormone-depleted media allowed to prevent promiscuous AR activation despite the presence of AR mutations[262].

*Mutations in the AR predispose to differential AA tolerance upon long-term treatment of PCa cells*

Mutations of the AR which frequently occur upon long-term ADT predispose cells to differential sensitivity and a broadened ligand specificity for several AAs[149]. Both the AR T878A mutation found in LNCaP cells and the AR H875Y of the 22Rv1 cell line confer full agonistic properties for BICA, while APA is converted into a partial agonist that retains residual AA characteristics[266]. Only DARO maintains full AA properties in the presence of these AR mutations[267,268]. In accordance, development of tolerance was only achieved upon long-term treatment with APA or DARO in the LNCaP and 22Rv1 sublines. The ineffectiveness of BICA to develop AA tolerance in both long-term treated sublines is best explained by the presence of AR mutations within the ligand-binding domain, which convert BICA into an AR agonist, *per se* beneficial for PCa cell growth. Interestingly, even though 22Rv1 cells are considered AA-resistant, they still exhibit responsiveness to high concentrations of DARO upon long-term treatment, which measurably increased tolerance in the respective subline. The presence of ligand-independent AR-Vs in 22Rv1 cells or the absence of AR in PC-3 cells show similar tolerances for high concentrations of each AA in both cell lines, suggesting that the observed effects may not exclusively depend on AR-signaling.

*AA-specific changes in androgen sensitivity occur upon long-term treatment of PCa cells*

Androgen sensitivity is a crucial factor in resistance development as it allows for the evaluation of residual AR-FL activity, with the dependence on androgens to mediate proliferation as read-out. Both AR-negative PC-3 cells, as well as the AR-V7 expressing 22Rv1 cells were non-responsive to DHT treatment, which corroborated the classification of long-term cultured 22Rv1 cells as AA resistant[262]. Upon long-term culture, especially under androgen-deprived conditions, a reduced androgen sensitivity that is no direct impact of long-term AA treatment is induced through activation of the PI3K/ AKT pathway[241,269–271]. Reduced DHT sensitivity was observed for long-term cultured, parental LNCaP cells as compared to the results obtained for early passage LNCaP cells[252]. Similar observations of long-term cultured LNCaP cells under androgen-depleted conditions were assumed to be associated with non-

genomic AR signaling[251]. The interaction of SRC and AR is suspected to induce the activation of the MAPK and PI3K/Akt pathways which hence modulates androgen dependence and proliferation[272].

Apart from this, long-term BICA treatment had no sustained impact on androgen sensitivity, while APA treatment rendered long-term treated LNCaP and 22Rv1 sublines independent of the impact of DHT. Interestingly, long-term DARO treatment led to an increased proliferation under elevated DHT concentrations. This finding suggests that DARO treatment rendered both LNCaP and 22Rv1 cell lines insensitive to induction of apoptosis under high-dosage androgen treatment, whereas reduced proliferation of LNCaP, 22Rv1 and PC-3 cells hinted at the described induction of apoptosis in parental cell lines at high DHT concentrations[273].

*Only darolutamide challenge re-sensitizes resistant PCa cells after long-term AA treatment*

Short-term challenge of the *in vitro* resistance models using complementary AAs undermined the previously observed efficacy of DARO in both PCa sublines. Only short-term DARO challenge revealed its potential to overcome insensitivity to long-term treatment with first and second-generations AAs, while BICA and APA treatment failed to achieve comparable re-sensitization within the therapeutically relevant AA range. These findings are in line with the literature, as advance in AA generation is associated with increasing AR affinity and enhanced AA potency[237]. Surprisingly, the long-term DARO-treated LNCaP subline was still minimally responsive to short-term BICA challenge. The mechanisms underlying the observation of a first-generation AA to sensitize cells resistant to the latest generation AA require further investigation.

*Re-sensitization by darolutamide challenge correlates with reduced expression of GR*

To investigate the underlying molecular mechanisms of castration resistance in advanced, androgen-deprived PCa, overexpression of AR-FL, occurrence of ligand-independent AR-V7[274] or the compensatory upregulation of GR[275] were determined. Long-term BICA treatment induced moderate AR-V7 protein expression, which is known to confer resistance to second-generation AAs[256], hence possibly explaining

the non-responsiveness of this subline towards APA or ENZA treatment. In contrast, APA treatment provoked enhanced GR expression. Long-term DARO treated sublines, however, failed to modulate any of the compensatory mechanisms mentioned above. Their mode of resistance development remains elusive, and possibly does not exclusively rely on steroid receptor activity.

AA-induced expression of GR is suspected to predict a poor clinical outcome and an increased risk for bone fractures[266]. A reversibility of GR expression caused by second-generation AAs was described by withdrawal of AR inhibitors[120]. In accordance with these findings, GR expressing LNCaP-Apa$^{LT}$ cells demonstrated treatment-specific molecular adaptations upon therapy switch. Exclusively AA replacement within the second-generation of AAs maintained GR expression. While first-generation AA (BICA) or treatment termination resulted in an upregulation of AR-V7 protein expression indicative of a compensation for the decreased amount of GR protein, DARO challenge did not induce any of the mentioned compensatory mechanisms while reducing GR expression. Based on these findings, the question arose whether AAs affect GR expression itself or may have an impact on its protein stability as indicated by Dong et al.[276].

Taken together, these findings demonstrate that determination of resistance development following long-term AA treatment highly depends on various criteria[257] that need to be considered thoroughly. While 22Rv1 cells demonstrated castration resistance for all previously defined criteria, the LNCaP sublines exhibited AA-specific adaptations to long-term treatment (Table 6).

Despite the induction of a neuroendocrine morphology and the expression of AR-V7, long-term BICA treatment failed to impair androgen sensitivity or enhance tolerance for the respective AA. In consequence, the generated LNCaP subline cannot be classified as AA-resistant due to its maintained dependence on AR signaling.

In contrast, long-term APA treatment resulted in diminished or even loss of androgen responsiveness and AR activity. In combination with the occurrence of GR expression as compensatory mechanism for the functional loss of AR[277], this LNCaP subline can be classified as AA-resistant and androgen independent.

***Table 9: AA-specific resistance characteristics of LNCaP sublines acquired upon long-term treatment.*** *Overview of acquired AA-specific characteristics classified into previously determined resistance criteria: recovery after proliferative crisis, morphology, androgen-sensitivity, antiandrogen (AA)-sensitivity, AA allowing for the most effective re-sensitization, compensatory mechanism, and final resistance classification. LNCaP cells were long-term subjected to either BICA, APA or DARO treatment.*

| | BICA | APA | DARO | reference |
|---|---|---|---|---|
| **recovery** | yes | yes | yes | |
| **morphology** | elongated protrusions | shortened protrusions | CRPC-like | Figure 7 |
| **AA sensitivity** | sensitive | partly sensitive | insensitive | Figure 8 |
| **androgen sensitivity** | unaffected | insensitive | sensitive | Figure 9 |
| **most effective re-sensitization** | DARO | DARO | (BICA) | Figure 11 |
| **compensatory mechanism** | AR-V7 | GR | unknown | Figure 10 |
| **resistance** | **no** | **yes** | **undefined** | |

Interestingly, therapy switch to DARO re-sensitized both long-term BICA and APA-treated LNCaP sublines for AA treatment as shown by growth inhibition. Additionally, DARO demonstrated its potential for re-sensitization during the short-term challenge of long-term APA treated LNCaP cells by downregulating GR expression without compensatory AR-V expression. Further unique characteristics of DARO treatment were revealed in the long-term sublines: DARO was functional even in the presence of AR mutations and ligand-independent AR-V7. It did not induce any of the investigated compensatory mechanisms but rendered the respective sublines virtually insensitive to complementary AAs and de-sensitized them for the apoptotic impact of high-dosage androgens. The effects occurring upon long-term DARO treatment may not exclusively be AR-dependent but could be explained by the modulation of, for example, apoptosis-associated genes like Bcl-2[278] or SGK1[119,279,280]. The ladder was already described in the context of the pro-survival, antiapoptotic activity of the GR[119,279,280]. Alternatively, an increased expression of the enzyme aromatase (CYP19A1) may lead to an augmented reduction of testosterone and increased production of estrogens instead[281].

In summary, different AAs appear to cause specific resistance mechanisms[266] leading to “resistance phenotypes” that may be defined by the biochemical pathways activated to escape ADT[229]. Identification of these mechanisms and pathways could allow for tumor re-sensitization with AA-specific treatment regimens that exhibit a broadened effect spectrum by additionally targeting alternative pathways such as the GR or DARO resistance-inducing genes to prevent resistance formation. Provided that different generations of AAs result in specific resistance characteristics[257], patients could benefit from mechanism-based treatment regimens that result in tumor re-sensitization.

# 4. Synopsis

PCa is one of the most frequently occurring cancers in men in the western world and is typically known for its heterogeneity. It is classified as well-treatable if diagnosed at an early stage. Consequently, identifying appropriate biomarkers is essential to drive and support important clinical decisions. The broad field of biomarker research is crucial, however, regarding the frequent morphological and molecular heterogeneity of PCa, it is also complex and challenging.

To define the manifold possibilities left for biomarker research that are still undiscovered, a bioinformatic transcriptome analysis of the publicly available prostate adenocarcinoma cohort (PRAD) provided by The Cancer Genome Atlas Program TCGA[15] was performed. This general approach aimed at a systematic, ideally unbiased investigation of potential biomarkers based on the mRNA expression of approximately 18,000 genes and further clinical parameters of 499 PCa and 53 normal tissue samples. A wide selection of potential target genes was identified by analyses with increasing stringency: correlation analysis of gene expression with ISUP grading, univariate and multivariate cox regression analysis. Of the genes most significantly associated with the included criteria, 75% / 87% / 87% of genes had already been described in the context of PCa or DNA repair which, in consequence, leaves 25% / 13% / 13% of these genes uninvestigated. An overview of the identified genes and the respective literature is provided as Supplementary Table 4.

Recapitulating these findings three years after publication generally corroborated this overall approach of a holistic, bioinformatic analysis. As shown above, stricter criteria resulted in the identification of genes that appear to be evidently associated with ISUP grading and patient survival, and hence are more likely to be considered for their potential role as PCa biomarker.

Surprisingly, multiple genes that are recommended or even established for diagnostic purposes such as FOLH1/PSMA, KLK3/PSA or AR[33,214] did not appear among the best-ranked genes analyzed above.

If only 20% of the originally assumed 500 unexploited genes had the potential to be druggable or to show biomarker traits, still a total of 100 target genes remain unconsidered. Additionally, several of these genes could be combined to a gene signature that, in turn, would allow for the application as biomarker and for further disease stratification[282]. Interestingly, a number of the identified target genes have been studied independently in PCa after appearance of the publication (Supplementary Tables 4-6). In consequence, this re-analysis validates the potential of the genes described in this publication. Further analyses using “omic” technologies or cohorts with fewer limitations will give the prospect to validate the described targets and identify even more potential biomarkers with this approach in the future.

Extrapolating this transcriptome analysis to the broad field of “omics” that address the genome, methylome, proteome, acetylome, lipidome, gylcome, etc., for the identification of new biomarkers reveals the tremendous potential that might be left for PCa biomarker research. Bioinformatic approaches may be a promising strategy for a virtually unbiased evaluation of large datasets which could pave the way for improved PCa treatment throughout disease progression.

Interestingly, many of the previously mentioned genes are associated with one of the key figures in PCa development and progression: the AR. Moreover, approximately 40% of the identified genes were mentioned in the context of DNA repair and PARP inhibition (Supplementary Table 4). These findings provided a first indication for the interconnection between AR/ AR-Vs and DNA repair in PCa. Since expression and activity of the AR appears to be decisive for the success of ADT and the subsequent progression to castration-resistance[283], it appeared plausible to further investigate the impact of the AR on DNA repair in PCa specimens.

*In vivo,* the tight interconnection between canonical AR signaling and DNA repair in PCa had already been demonstrated[180]. For the first time, the impact of altered AR pathway activity, especially of AR-Vs, on DNA repair at different stages of the disease was confirmed in PCa patient tumor samples. Additionally, these findings were validated in *in vitro* tumor models that mimicked the hormone-sensitive and CRPC phenotype. The observed deregulation of several DDR genes in the presence of AR-Vs, put into context of findings of other research groups[197,284,285], confirmed the idea of a potential “DNA repair gene signature” that may serve as a novel biomarker or that may be used as an alternative therapy approach for CRPC. The exact members of this

signature, however, remain to be elucidated. Besides, the efficiency of therapeutic concepts exploiting the principles of synthetic lethality appeared diminished in the presence of AR-V7.

In consequence, data published by many labs including our lab point towards the crucial role of AR-Vs in the progression of hormone-sensitive to castration-resistant PCa. The occurrence of ligand-independent AR-Vs appeared to be decisive for the efficacy of therapeutic measures by rendering CRPC tumors irresponsive to common clinical therapies such as ADT or PARPi. Moreover, a recent report of the DDR promoting role of AR-V7 under severe DNA damage in PCa cells also confirmed the impaired synergistic effects between AR antagonists and PARPi therapy[286]. These findings substantiate the predictive biomarker potential of AR-V7 to determine the efficacy of novel PARPi-based therapies in PCa.

The consideration of androgen axis components, especially AR-Vs[180,287], as a starting point for novel therapeutic approaches is supported by several publications[283]. In particular, the utility of AR-V7 as a biomarker was extensively validated in preclinical and clinical studies[288,289], determining hormone-independent, constitutively active AR-V7 to be one of the key drivers of AA resistance in CRPC[286,290].

As most tumors eventually relapse and insensitivity towards ADT can be attributed to several effectors besides AR-V7[291–293], castration-resistance poses a major problem in the treatment of PCa and hence appeared mandatory to investigate. While AR-FL and AR-V7 expressions are commonly considered causal for resistance development, only a few studies referred to the investigation of GR as a compensatory mechanism for the functional loss of AR due to ADT[293]. To examine the mechanisms underlying the development of AA-resistance *in vitro* under physiological conditions, androgen-responsive PCa cell lines (LNCaP and 22Rv1) were cultured under androgen-deprived conditions[239] with different generation of AAs (BICA, APA, DARO).

In line with the literature, long-term treatment with the different generations of AAs induced distinct resistance mechanisms exploiting specific underlying biochemical pathways[229,256,266,294].

Comparable to our described findings of AR-V7 emergence in CRPC *ex vivo*[207], long-term BICA treatment of LNCaP cells induced a moderate upregulation of AR-V7 expression. However, BICA application failed to generate an androgen-independent, AA-resistant cell line. Long-term APA treatment, however, virtually completely diminished androgen responsiveness and AR activity. In combination with an induced GR expression to compensate for the functional loss of AR, also described by Arora et al.[120], all criteria allowed for a classification of this LNCaP subline as AA-resistant. Upon treatment of 22Rv1 sublines with BICA and APA, no gross alterations were observed compared to the parental cell line, indicating AA-resistance based on the expression of AR-V7 and the presence of the AR H878Y mutation as described in the literature[185]. Interestingly, the effect mechanism of DARO appeared unique, as it was functional even upon expression of ligand-independent AR-V7 and exhibited exclusively antagonistic properties despite the presence of AR mutations in LNCaP and 22Rv1 cells. The special characteristic of DARO to impede any of the above-mentioned compensatory mechanisms during first- and second-line treatment in LNCaP cells makes it a powerful tool for the treatment of CRPC. On the other hand, and in contrast to BICA and APA, long-term DARO treatment rendered the respective sublines virtually insensitive to any AA indicating that its application may preferably not be recommended as first-line treatment. The mechanisms underlying DARO resistance appear to involve criteria that are currently under investigation. Whether one of the above-mentioned mechanisms also affects DNA repair gene regulation or has a direct impact on PARPi will be subject to further studies.

A deeper understanding of resistance effectors and the underlying pathways may unveil great opportunities for the treatment of CRPC. As shown for BICA, the expression of a compensatory mechanism alone is not suitable to determine castration resistance. A selection of diverse criteria could allow for the determination of “resistance phenotypes”[229] that specify for a treatment regimen targeting biochemical pathways beyond AR signaling. Targeting these resistance phenotypes, in turn, may likely not only prevent tumor progression, but could allow for a re-sensitization of tumors considered as therapy resistant.

The diagnosis of prostate cancer is still mainly based on morphological criteria but is supported by biomarker analysis, which is currently mainly performed on an IHC basis. Newly developed techniques progressively extend biomarker analyses on oncogenic driver mutations at the genomic level. Transcriptome analyses provide a new way to comprehensively analyze biomarker expression at mRNA level and offers the opportunity to identify and establish a whole set of novel biomarkers. A confirmed diagnosis enables correct therapeutic intervention and thus a reliable prognosis. The study presented in **chapter I** explored and estimated the number of potential biomarkers to be expected from a transcriptomic analysis of primary PCa samples. At least 250 genes that are positively associated with prognosis were identified. About 50% of the top 15 identified genes are already in the focus of PCa research, which demonstrates the good prospects of biomarker search by transcriptome analysis. The importance of biomarkers is of undeniable relevance for the early detection and clinical treatment of PCa. More precise diagnoses and prognoses based on specific biomarkers can lead to an improved patient stratification and eligibility. **Chapter II** explored the predictive nature of the AR splice variant “V7” expression in irradiation of PCa primary tumors. As AR-V7 expression promotes the repair of DNA double strand breaks induced by irradiation, its presence would predict a limited success of this therapeutic intervention. While AR-V7 expression is rare in primary tumors, it emerges after prolonged systemic androgen deprivation therapy, rendering the tumor successively resistant to AR inhibition. Besides AR-V7, further resistance mechanisms involving AR signaling occur upon long-term ADT. In **chapter III**, an analysis of *in vitro* PCa models long-term treated with different generations of AAs to mimic ADT in patients revealed AA-specific molecular adaptations that result in resistance formation. However, resistance development involving AA treatment deserves and requires a deeper molecular characterization, which will unveil treatment algorithms that render PCa diagnosis and therapy more reliable. Unravelling the complex interconnections between disease drivers, drug targets and resistance effectors will allow for the identification of specific biomarkers. Considering the multitude of opportunities that are left in this research area, novel approaches such as the investigation of novel gene signatures and the development of new treatment regimens are needed. An overall deeper understanding of the connections between mechanisms and pathways underlying distinct therapy approaches will allow for improved therapy strategies that clearly benefit PCa patients.

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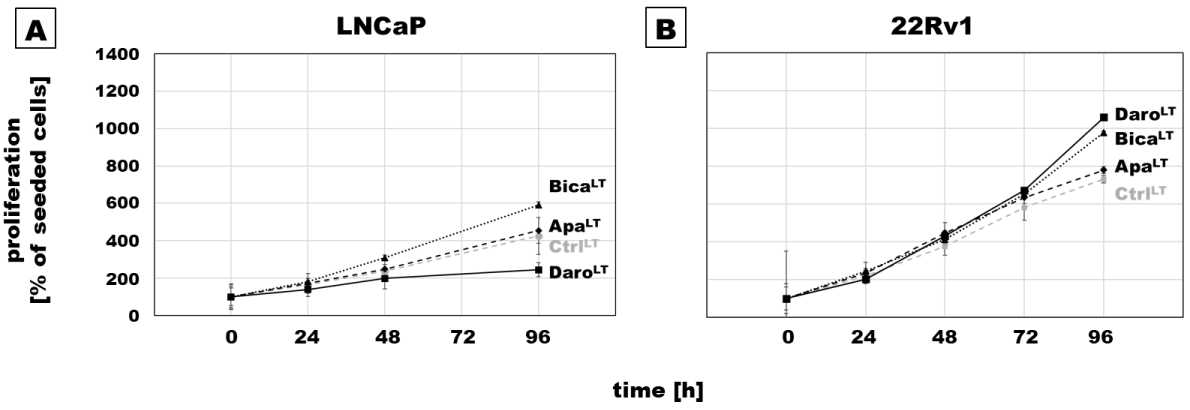
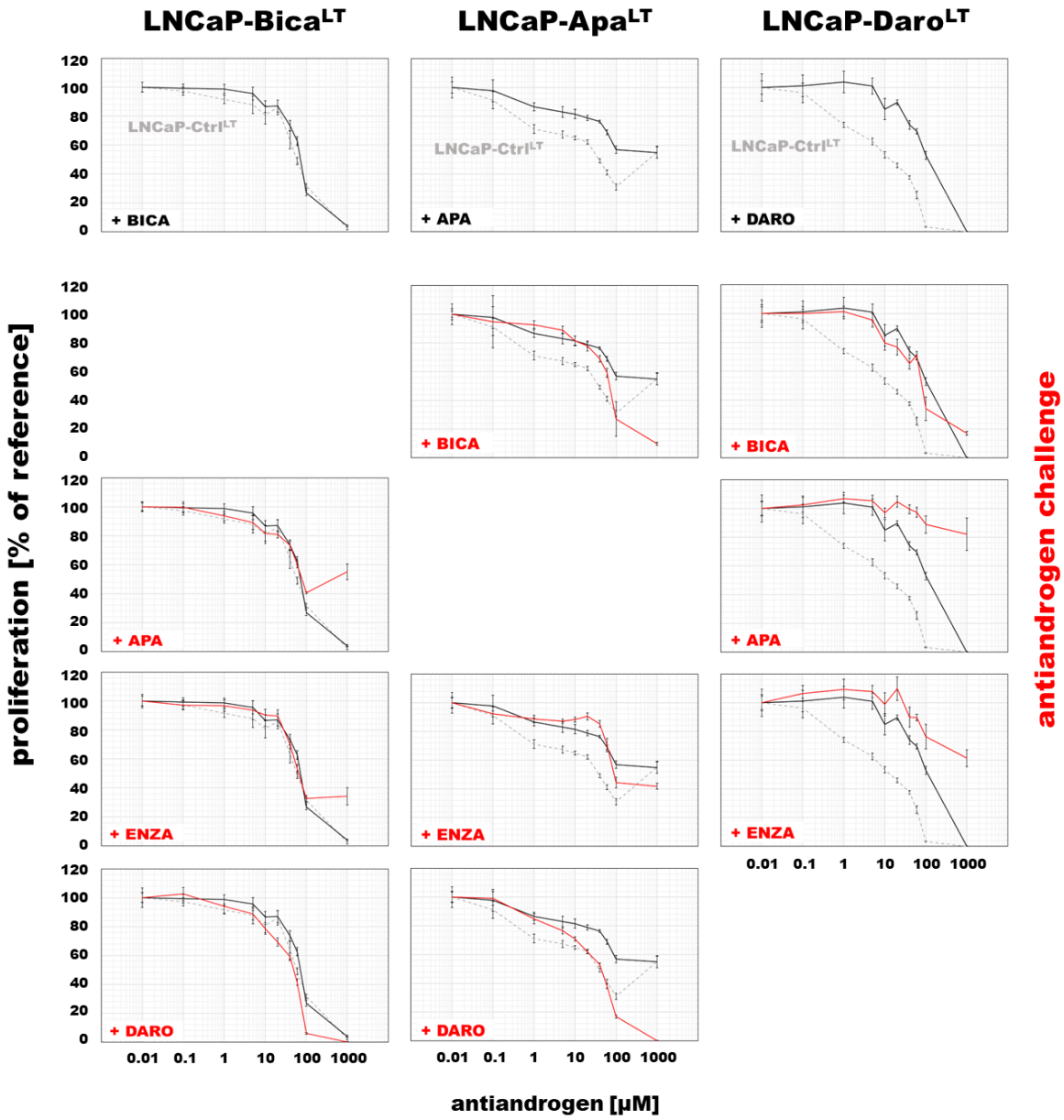
# 6. Appendix

## 6.1 Supplementary Materials

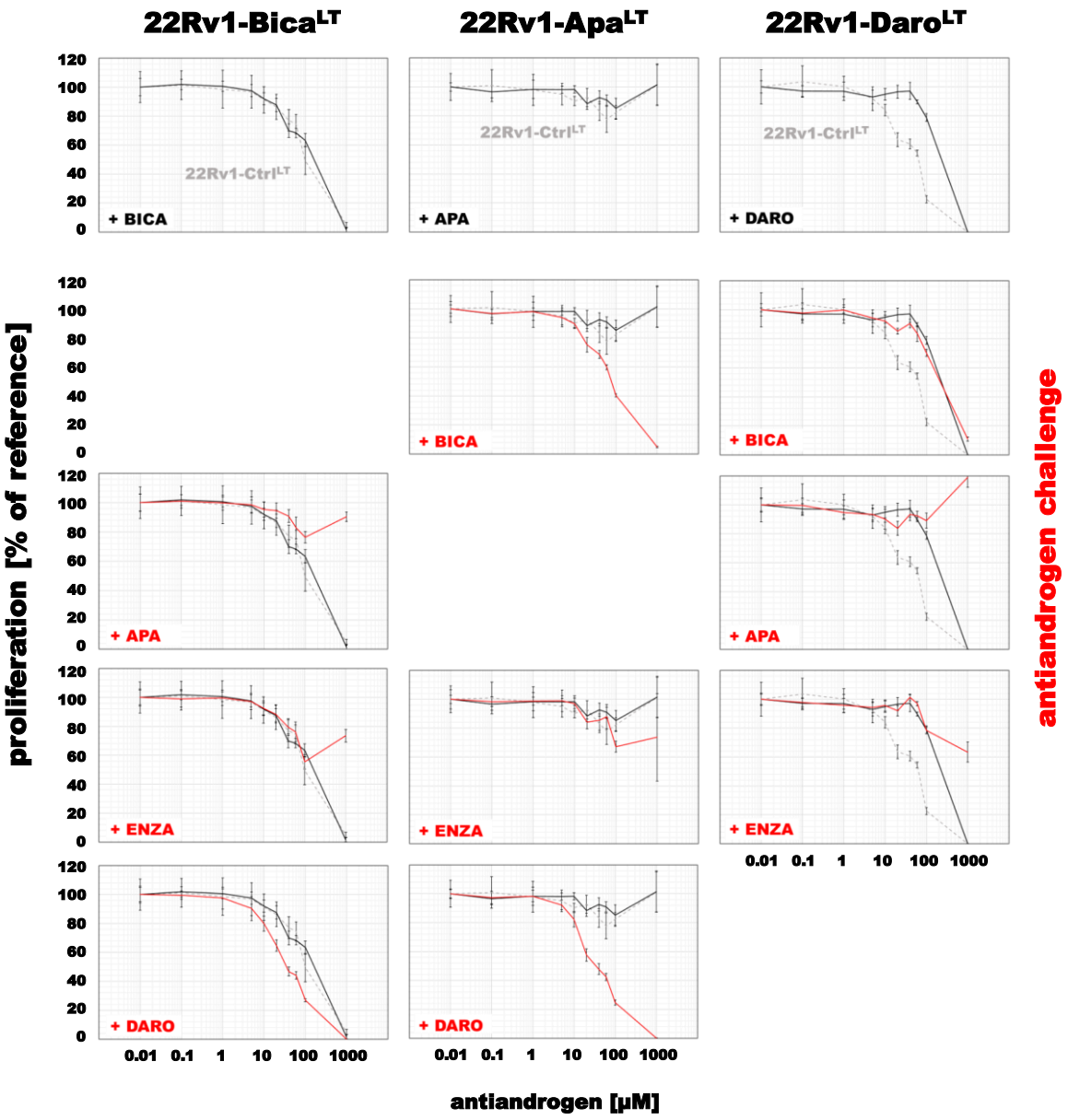
### 6.1.1 Supplementary Figures




***Supplementary Figure 1: Changes in proliferation index of AR-expressing PCa cell lines and long-term AA treated sublines manifest after 96 h.*** *A) Time course of proliferation calculated for hormone-sensitive AR-FL expressing LNCaP (Ctrl$^{LT}$) and long-term treated sublines (LNCaP-Bica$^{LT}$, -Apa$^{LT}$, -Daro$^{LT}$) in the absence of AAs. B) Time course of proliferation calculated for castration-resistant AR-V7 expressing 22Rv1 (Ctrl$^{LT}$) and long-term treated sublines (22Rv1-Bica$^{LT}$, -Apa$^{LT}$, -Daro$^{LT}$) in the absence of antiandrogens. Significant differences in proliferation were observed for 22Rv1 only after 96 h. In consequence, all further proliferation data were determined after 96 h of incubation. Cells were cultured for 0 h to 96 h in steroid-depleted media. Proliferative index was measured by MTS reaction. Data are shown as % of seeded cells as determined at the day after seeding (0 h). Black curves indicate LT-sublines. Grey dashed curves show parental (Ctrl$^{LT}$) cells. Error bars depict standard deviations of one independent experiment (n = 1).*




***Supplementary Figure 2: Impact of individual complementary antiandrogens on long-term AA treated LNCaP sublines.*** *Effect of complementary antiandrogens (BICA, APA, ENZA, DARO; red curve) on proliferation of LNCaP$^{LT}$ sublines in comparison to short-term challenge of parental LNCaPs (grey curve) and LNCaP$^{LT}$-subline treated with its corresponding AA (black curve). Cells were cultured for 96 h in steroid-depleted media containing increasing amounts of antiandrogens (0.01, 0.1, 1, 10, 100, 1000 µM). Proliferative index was measured by MTS reaction. Data are shown as % of reference. Error bars depict standard deviations of three independent experiments (n = 3).*




***Supplementary Figure 3: Impact of individual complementary antiandrogens on long-term AA treated 22Rv1 sublines.*** Effect of complementary antiandrogens (BICA, APA, ENZA, DARO; red curve) on proliferation of 22Rv1$^{LT}$ sublines in comparison to short-term challenge of parental 22Rv1s (grey curve) and 22Rv1$^{LT}$-subline treated with its corresponding AA (black curve). *Cells were cultured for 96 h in steroid-depleted media containing increasing amounts of antiandrogens (0.01, 0.1, 1, 10, 100, 1000 µM). Proliferative index was measured by MTS reaction. Data are shown as % of reference. Error bars depict standard deviations of three independent experiments (n = 3).*

### 6.1.2 Supplementary Tables

***Supplementary Table 1: Significances assessed for the comparison of DHT treatment in long-term AA treated (Bica$^{LT}$, Apa$^{LT}$, Daro$^{LT}$) vs. Ctrl$^{LT}$ LNCaP (A) and 22Rv1 (B) sublines*** *using independent two-sample t tests. For reasons of clarity, only significant values (p < 5.00e-02) are shown.*

**A**

| sublines | | DHT [nM] | | | | |
|---|---|---|---|---|---|---|
| | | 0.01 | 0.1 | 1 | 10 | 100 |
| LNCaP-Bica$^{LT}$ | LNCaP-Ctrl$^{LT}$ | | 3.26e-03 | | 8.81e-03 | |
| LNCaP-Apa$^{LT}$ | LNCaP-Ctrl$^{LT}$ | | | 2.14e-02 | 3.33e-03 | |
| LNCaP-Daro$^{LT}$ | LNCaP-Ctrl$^{LT}$ | | | 3.58e-03 | 1.20e-03 | |

**B**

| subline | | DHT [nM] | | | | |
|---|---|---|---|---|---|---|
| | | 0.01 | 0.1 | 1 | 10 | 100 |
| 22Rv1-Bica$^{LT}$ | 22Rv1-Ctrl$^{LT}$ | | | | 3.34e-02 | 3.26e-02 |
| 22Rv1-Apa$^{LT}$ | 22Rv1-Ctrl$^{LT}$ | | | | | |
| 22Rv1-Daro$^{LT}$ | 22Rv1-Ctrl$^{LT}$ | | | | 4.83e-03 | 3.29e-02 |

***Supplementary Table 2: Significances assessed for the comparison of AA treatment in long-term AA treated (Bica$^{LT}$, Apa$^{LT}$, Daro$^{LT}$) vs. Ctrl$^{LT}$ LNCaP (A) and 22Rv1 (B) sublines*** *using independent two-sample t tests. For reasons of clarity, only significant values (p < 5.00e-02) are shown.*

**A**

| sublines | | antiandrogen [µM] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AA | 0.01 | 0.1 | 1 | 5 | 10 | 20 | 40 | 100 | 1000 |
| LNCaP-Bica$^{LT}$ | LNCaP-Ctrl$^{LT}$ | BICA | | | | | | | | | |
| LNCaP-Apa$^{LT}$ | LNCaP-Ctrl$^{LT}$ | APA | | | 4.90e-02 | 4.58e-02 | 3.11e-02 | 1.23e-02 | 1.28e-02 | 5.47e-03 | |
| LNCaP-Daro$^{LT}$ | LNCaP-Ctrl$^{LT}$ | DARO | | | 3.24e-03 | 2.95e-04 | | 7.56e-03 | 9.72e-03 | 7.64e-03 | |

**B**

| sublines | | antiandrogen [nM] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | AA | 0.01 | 0.1 | 1 | 5 | 10 | 20 | 40 | 100 | 1000 |
| 22Rv1-Bica$^{LT}$ | 22Rv1-Ctrl$^{LT}$ | BICA | | | | | | | | 8.19e-03 | |
| 22Rv1-Apa$^{LT}$ | 22Rv1-Ctrl$^{LT}$ | APA | | | | | 2.20e-02 | | | | |
| 22Rv1-Daro$^{LT}$ | 22Rv1-Ctrl$^{LT}$ | DARO | | | | | | 2.59e-03 | 7.28e-03 | 1.78e-03 | |

***Supplementary Table 3: Significances assessed for the comparison of the most effective AA challenge vs. AA treatment used to generate the respective LNCaP (A) and 22Rv1 (B) sublines*** *using independent two-sample t tests. For reasons of clarity, only significant values (p < 5.00e-02) are shown.*

**A**

| subline | antiandrogen [µM] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | challenge | treatment | 0.01 | 0.1 | 1 | 5 | 10 | 20 | 40 | 100 | 1000 |
| LNCaP-Bica$^{LT}$ | DARO | BICA | | | | | | | | 6.35e-03 | |
| LNCaP-Apa$^{LT}$ | DARO | APA | | | | | | 1.87e-02 | 9.31e-03 | 3.56e-04 | |
| LNCaP-Daro$^{LT}$ | BICA | DARO | | | | | | | | 3.63e-02 | |

**B**

| subline | antiandrogen [nM] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | challenge | treatment | 0.01 | 0.1 | 1 | 5 | 10 | 20 | 40 | 100 | 1000 |
| 22Rv1-Bica$^{LT}$ | DARO | BICA | | | | 2.11e-02 | 5.09e-04 | 2.98e-03 | 2.17e-06 | 3.15e-04 | |
| 22Rv1-Apa$^{LT}$ | DARO | APA | | | | 2.30e-02 | 3.17e-03 | 2.19e-03 | 5.30e-04 | 2.66e-03 | 7.72e-04 |
| 22Rv1-Daro$^{LT}$ | BICA | DARO | | | | | | | | | |

***Supplementary Table 4: Top 20 genes found during the correlation analysis of mRNA gene expression vs. ISUP grading.*** ** genes described in the context of PARPi*

| investigated | | | not investigated |
|---|---|---|---|
| PCa | DNA repair | PCa + DNA repair | |
| GEMIN4 (PMID 22506892 | | CBX1 * (PMID: 23536649, PMID: 18438399, PMID: 25769025)* | NCAPG5 |
| VPS36 (PMID28197629) | | SPAG5 * (PMID: **27037000**, PMID: 30736840) | SH3RF2 |
| TROAP (PMID **30431120)** | | ACP2 (PMID: 2477829, PMID: **31865061)** | KIAA0319L |
| DONSON (PMID 33739968) | | KIF20A * (PMID: 31565099, PMID: 30038716) | CCDC149 |
| FAM72D (PMID 32566639) | | KIF23 (PMID 32624708) | KCNK6 |
| RNF185 (PMID 35111198) | | SMC4 * (PMID: **26631616,** PMID: 9789013, PMID: **16543152**) | |
| DPP4 (PMID 32296640, PMID: 15735018) | | ABCC5 (PMID 26708806, PMID: 16508919, PMID 34271023) | |
| EPHX2 (PMID 32687069) | | | |

***Supplementary Table 5: Top 15 genes found during the univariate cox regression analysis.*** ** genes described in the context of PARPi*

| investigated | | | not investigated |
|---|---|---|---|
| PCa | DNA repair | PCa + DNA repair | |
| FAM72B (PMID 25519703) | EXOSC10 (PMID: 25632158) | ABCC5 (PMID 26708806, PMID: 16508919, PMID 34271023) | CCDC18 |
| RCF4 (PMID 11326315 ) | | CCNA2 (PMID 26708806, PMID: 21044312, PMID: **34497666**) | MPDU1 |
| PRC1 (PMID 16258266) | | NEK2 (PMID 16024624) | |
| CDC20 (PMID 19530225) | | SPAG5 * (PMID: **27037000**, PMID: 30736840) | |
| PXK (PMID 23900074) | | TPX2 * (PMID 22761906) | |
| FAM72D (PMID 32566639) | | KIF15 (PMID 31454442) | |

***Supplementary Table 6: Top 15 genes found during the multivariate cox regression analysis.*** ** genes described in the context of PARPi*

| investigated | | | not investigated |
|---|---|---|---|
| PCa | DNA repair | PCa + DNA repair | |
| DLK1 (PMID 18375047) | ATP6V1D (PMID 18497997) | DKK2 (PMID 27431620) | NCKIPSD |
| PXK (PMID 23900074) | AKAP8 (PMID 29930011) | ZNF414 (PMID 35318951) | C22orf32 |
| RALB* (PMID 16103060, PMID 20619549) | GBAS (PMID 12969785) | | EPO |
| PDGFA (PMID 19562724) | P2RY12 (PMID 24349080) | | |
| BLK (PMID 16497524) | RFXAP (PMID 33093461) | | |

## 6.2 Publication prints:

### 6.2.1 Where is the limit of prostate cancer biomarker research? Systematic investigation of potential prognostic and diagnostic biomarkers

Kremer et al. BMC Urology (2019) 19:46
https://doi.org/10.1186/s12894-019-0479-z

BMC Urology

RESEARCH ARTICLE Open Access

# Where is the limit of prostate cancer biomarker research? Systematic investigation of potential prognostic and diagnostic biomarkers

Anika Kremer[1], Tobias Kremer[2], Glen Kristiansen[1†] and Yuri Tolkach[1*†]

## Abstract

**Background:** The identification of appropriate biomarkers is essential to support important clinical decisions in patients with prostate cancer. The aim of our study was a systematic bioinformatical analysis of the mRNA expression of all genes available for the prostate adenocarcinoma cohort of The Cancer Genome Atlas (TCGA), regarding their potential prognostic and diagnostic role.

**Methods:** The study cohort comprises 499 patients (TCGA prostate cancer cohort). mRNA expression data were available for approx. 20,000 genes. The bioinformatical statistical pipeline addressed gene expression differences in tumor vs. benign prostate tissue (including gene set enrichment analysis, GSEA) in samples from tumors with different aggressivenesses (Gleason score), as well as prognostic values in multistep survival analyses.

**Results:** Among all genes analyzed, 1754 were significantly downregulated and 1553 genes were significantly upregulated in tumor tissue. In GSEA, 16 of 30 top enriched biological processes were alterations of epigenetic regulation at different levels. Significant correlation with Gleason Score was evident for 8724 genes (range of Pearson r-values 0.09–0.43; all $p < 0.05$). In univariate Cox regression analyses, mRNA expression of 3571 genes showed statistically significant association with biochemical recurrence-free survival with a range of hazard ratios 0.3–3.8 ($p$-value 7.4e− 07 to 0.05). Among these, 571 genes were independently associated with biochemical recurrence in multivariate analysis. Access to the full database including results is provided as supplement.

**Conclusions:** In our systematic analysis we found a big number of genes of potential diagnostic and prognostic value, many of which have not been studied in prostate cancer to date. Due to the comprehensive nature of this analysis and free access to the results, this study represents a reference database for prostate cancer researchers which can be used as a powerful tool for validation purposes and planning of new studies.

**Keywords:** Prostate cancer, Biomarkers, Bioinformatics, mRNA expression, Prognostic, Diagnostic

## Background

Prostate cancer (PCa) is one of the most common cancers in men worldwide [1]. Once the prostate carcinoma is diagnosed, it is considered well treatable if recognized at an early stage. Though, over the past years, PCa therapy came into the focus of criticism due to its potential for overtreatment by e.g. radical prostatectomy [2, 3]. However, rendering reliable diagnoses and prognoses of the progression of the disease based on tumor grading and modern classifications is impeded by its high degree of morphological and molecular genetic heterogeneity [4, 5]. Therefore, its high rate of occurrence and frequent heterogeneity make PCa not only a crucial but also a complex and challenging research target in clinical and research settings.

The identification of appropriate biomarkers is therefore essential to drive important clinical decisions in patients with prostate cancer [6]. Hundreds

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of studies focusing on new biomarkers are being published every year for more than 40 years now [7]. Three main biomarker branches have been established: diagnostic markers, that identify patients at risk of prostate cancer using serum/ urine as substrate, or diagnostic immunohistochemistry during biopsy evaluation, prognostic markers, which give an idea of a certain clinical outcome, e.g. biochemical recurrence after radical treatment and predictive markers, predicting a response to specific, usually medicament-based therapy.

Prognostic biomarkers, even after their almost 40-year-long research way, are still not recommended for utilization in clinical routine although several of them (in form of commercial gene expression signatures) are considered as potential candidates. However, with no data currently available about their clinical relevance at long term, also prospective validation is lacking [8].

Given that after 40 years of research multiple studies appear constantly in the literature addressing single genes or their combinations in the prognostic setting, the aim of our study was to define the limits of biomarker research in patients with prostate cancer, primarily through the identification of potential targets not yet investigated. This comprehensive, holistic approach cannot only serve as an outline of the immense possibilities that are still left for prognostic and diagnostic biomarker research but could also be used as a starting point for important discussions such as the prioritization of research targets and changes in research methodology for future prostate cancer research.

## Methods

### Patient cohort

This study comprised a total of 499 patients from the prostate adenocarcinoma cohort of The Cancer Genome Atlas (TCGA). Mean age of the patients was 61.0 years (range 41–78 years). Clinicopathological information was available for all patients (Fig. 1). Follow-up information with biochemical recurrence as an endpoint was available for 452 patients, 83 of which have developed a biochemical recurrence. Median follow-up time was 16.9 months (range 1–153 months).

### Quality control of clinical data

The raw clinical data (version 28.01.2016) was preprocessed and organized in a database as follows: 1) selection of relevant clinicopathological parameters (age, serum PSA level, pathological staging and grading, follow-up information); 2) All parameters were controlled for consistency, duplicates were removed.

### mRNA expression data

mRNA expression data were generated using the Illumina HiSeq 2000 RNA Sequencing platform (Version 2; data version 28.01.2016). RNA expression was available for tumor samples of all patients and for additional 53 samples with normal tissue normalize according to TCGA protocol. Using barcode as an identifier, tumor and normal tissue samples were extracted separately with further merging of RNA expression data for tumor samples to clinical data. After excluding the genes with duplicate names and missing expression, mRNA expression of 20,500 genes was available for analysis. From this list, further 2819 genes were excluded due to absent or very low mRNA expression values (median = 0 reads), leaving 17,681 genes in the final analysis.

### Bioinformatical approach / statistics

All statistical analyses were performed using R (R Foundation for Statistical Computing; version 3.5.0). The packages used were pastecs, TCGAbiolinks, limma, edgeR, KMsurv, survMisc, rms, stringi, Hmisc, tidyverse and doParallel.

The fully automatized bioinformatical pipeline is outlined in Fig. 2. In brief, for survival analyses, dichotomization of mRNA expression was carried out using 1) median level of mRNA expression and 2) cut-off optimization (best cut-off). The best cut-off was selected using the survMisc package (automatized systematic univariate Cox regression-based analysis of all available cut-offs for mRNA expression of single genes). Survival analyses were conducted using univariate and multivariate Cox proportional hazards regression. Kaplan-Meier estimates were calculated using both the best cut-off and median for each gene with accompanying log-rank test and automatic generation of Kaplan-Meier curves for all genes. The inclusion criterion for multivariate analysis was a $p$-value < 0.05 in univariate analysis.

Correlation analyses were performed to identify the associations of clinical variables (Gleason Score) with the mRNA expression of single genes (Pearson correlation coefficient (r) and p-level). Pairwise comparison of the gene expression of normal and tumor tissue was carried out using a negative binomial generalized log-linear model and correction for false discovery rate (FDR).

### Gene set enrichment analysis (GSEA)

GSEA for tumor versus normal tissue was performed using the GSEAPreranked tool in javaGSEA Application (The Broad Institute, Inc., Massachusetts Institute of Technology and Regents of the University of California). Single genes were ranked based on the logFC parameter stemming from differential gene expression analysis. Gene sets from the Hallmark collection (well-defined biological states or processes, $n = 50$) and the Gene

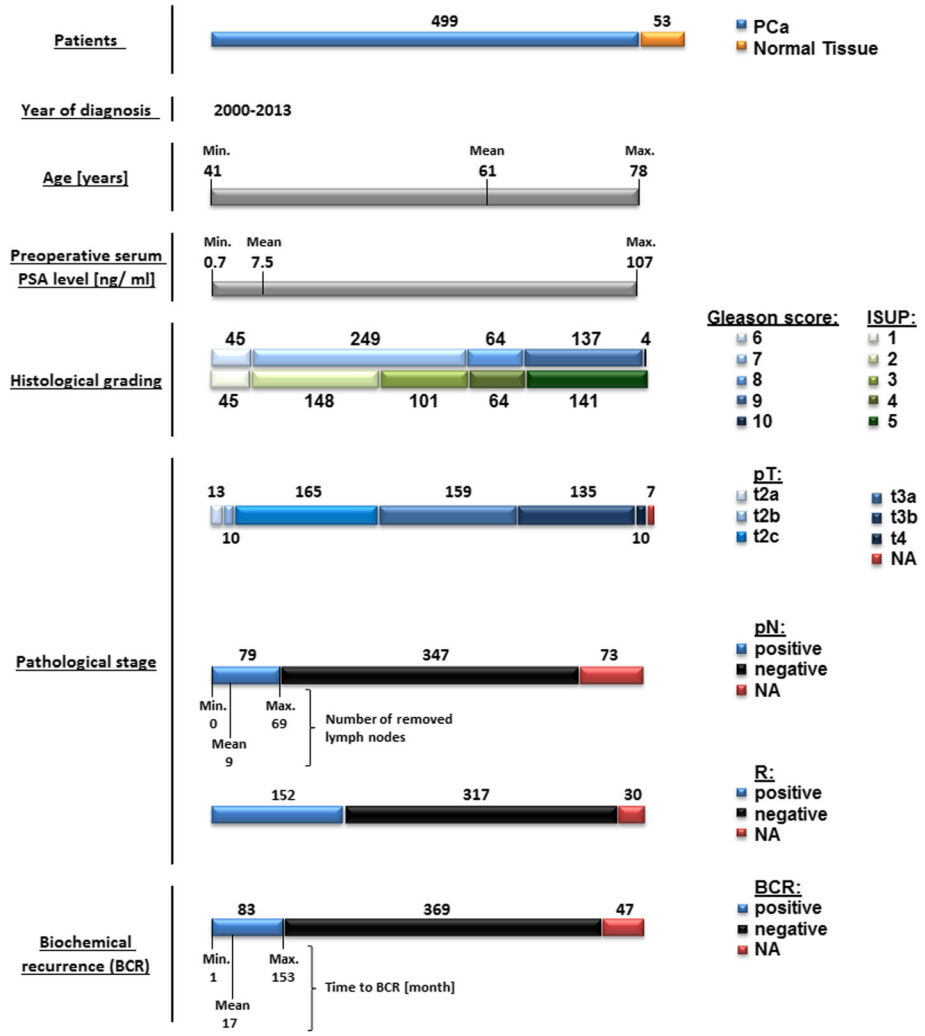



**Fig. 1** Clinicopathological characteristics of patients

Ontology database (GO biological processes, $n = 4436$) were used for GSEA as provided by Molecular Signatures Database (MSigDB) v6.2 (The Broad Institute, Inc., Massachusetts Institute of Technology and Regents of the University of California). GSEA was performed using the following setup: number of permutations – 1000, enrichment statistic – weighted, gene set size restriction – 15-500 genes, FDR cut-off – 0.25.

## Results

### Tumor vs normal tissue

Among all genes analyzed, 1754 were significantly downregulated in tumor tissue (fold change (FC) > 2, logFC < – 1.0; FDR range from 0.05 to 3.3e-275). 133 of these genes showed profound downregulation with logFC < – 3.0 (maximal logFC -9.7; FDR 1.4e– 09 – 3.3e-275).

Another 1553 genes were significantly upregulated in tumor tissue (FC > 2, logFC > 1; FDR range from 0.047 to 1.6e-51) with very high levels of upregulation (logFC > 3; maximal logFC 9.9) in 123 genes (FDR 0.02–4.1e-36). For full information see Additional file 2: Table S1.

In GSEA analysis, 10 Hallmark gene sets and 809 gene sets from the GO Biological Processes collection were enriched in tumor tissue with FDR < 0.25 (Fig. 3, Additional file 3: Table S2). Multiple Biological Processes enriched in tumor tissue were related to altered epigenetic regulation (chromatin organization, gene silencing).

### Correlation with Gleason score (tumor aggressiveness)

Of 17,681 genes, 8724 genes showed significant correlation levels to the International Society of Urological Pathology (ISUP) histological grading group of the tumor (based on the Gleason Score) with *p*-values

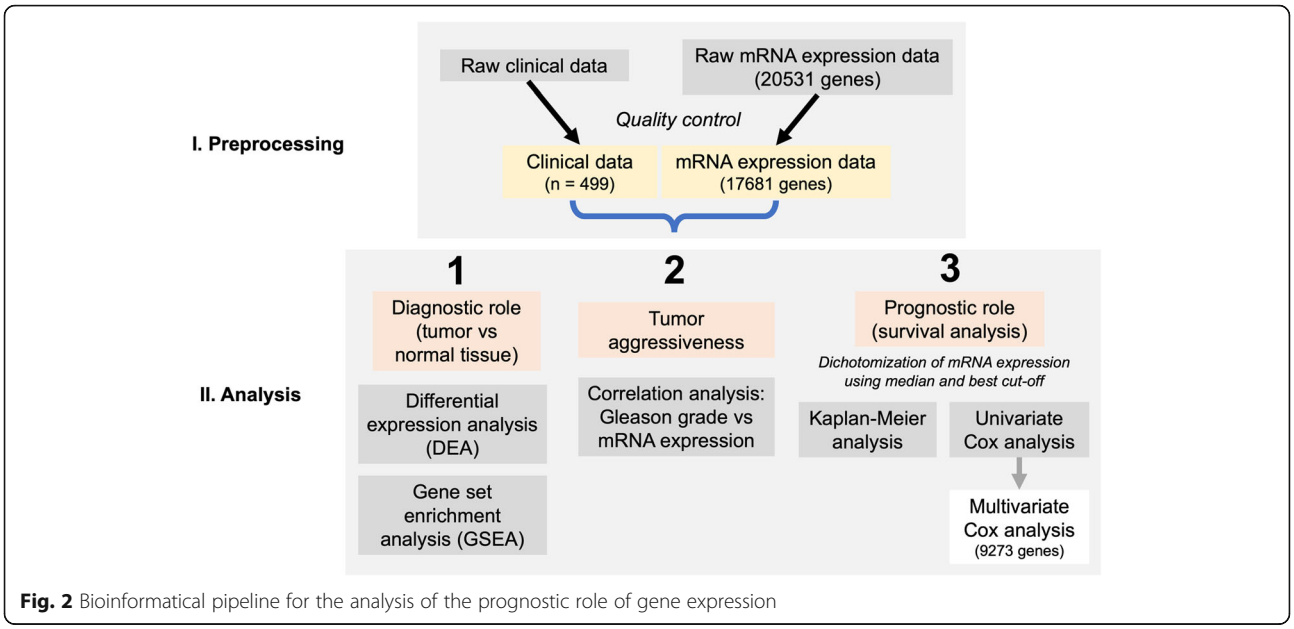



**Fig. 2** Bioinformatical pipeline for the analysis of the prognostic role of gene expression

ranging from 10e-24 up to 0.05 and range of Pearson r-values of 0.09–0.43. From this list, 5557 genes were positively correlated to ISUP grouping, while 3167 genes were negatively correlated. Top 20 genes with the highest levels of positive and negative correlation are presented in Table 1 (for full analysis see Additional file 4: Table S3; for correlation analysis high grade (≥4 + 4) vs low grade tumors see Additional file 5: Table S4).

### Prognostic role of mRNA expression (survival analyses)

#### *Univariate cox regression*

In univariate Cox regression analyses (Fig. 4), 3571 of 17,681 genes showed a statistically significant association with biochemical recurrence (BCR)-free survival of patients with a range of hazard ratios (HR) 0.3–3.8 ($p$-values 7.4e− 07 to 0.05), when dichotomized using median of expression (additional 5719 genes demonstrated statistical significance with $p < 0.05$ using best cut-off for dichotomization). Of 3571

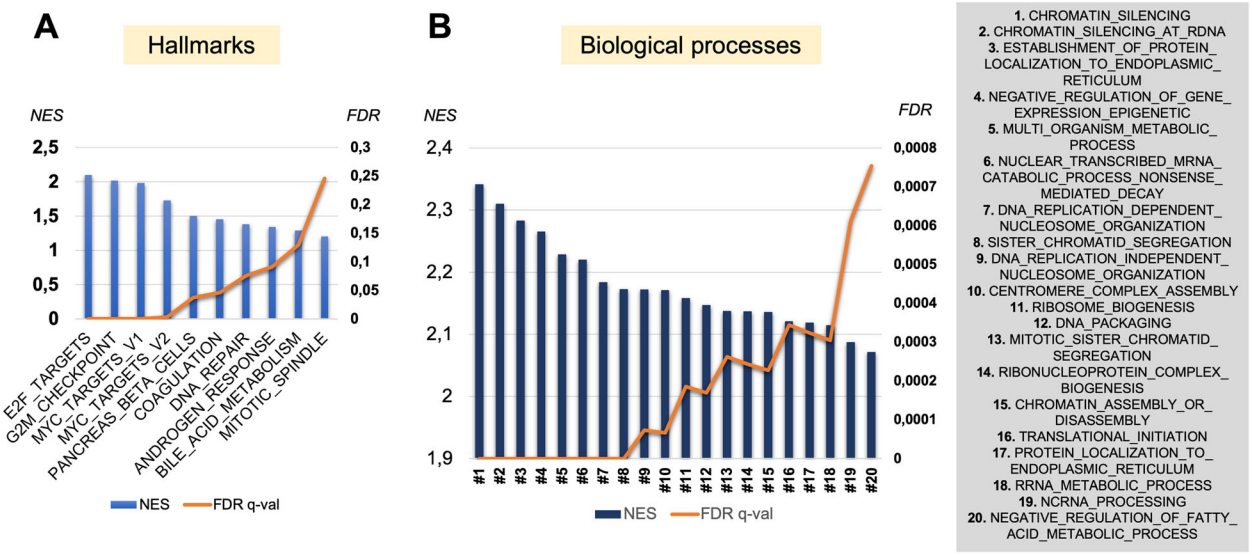



**Fig. 3** The results of Gene Set Enrichment Analysis (GSEA) in tumor tissue compared to normal tissue. Two collections of gene sets from Molecular Signatures Database (MSigDB) were analyzed: **a**) Hallmarks of well-defined biological states of processes (10/10 enriched signatures for tumor tissue are shown). **b** Gene Ontology database: biological processes (top 20/809 enriched signatures are shown). False discovery rate was set at a cut-off of 0.25; 1000 permutations were made for every analysis. Abbreviations: NES – normalized enrichment score (main metrics of GSEA); FDR – false discovery rate

**Table 1** Top 20 genes with the highest levels of mRNA expression correlation to ISUP grading group of the tumor

| Positive correlation | | | Negative correlation | | |
|---|---|---|---|---|---|
| Gene | ISUP / Pearson r | ISUP / p-value | Gene | ISUP / Pearson r | ISUP / *p*-value |
| TROAP | 0,408 | 2,21e-21 | SH3RF2 | −0,429 | 9,99e-2 |
| CBX1 | 0,408 | 2,55e-21 | RNF185 | −0,405 | 4,85e-21 |
| ABCC5 | 0,404 | 6,19e-21 | VPS36 | −0,400 | 1,74e-20 |
| DONSON | 0,403 | 8,80e-21 | ACP2 | −0,397 | 3,60e-20 |
| SMC4 | 0,400 | 1,55e-20 | GEMIN4 | −0,388 | 2,70e-19 |
| KIF20A | 0,400 | 1,65e-20 | KIAA0319L | −0,387 | 3,03e-19 |
| SPAG5 | 0,399 | 2,29e-20 | KCNK6 | -0,384 | 6,28e-19 |
| NCAPG2 | 0,396 | 4,57e-20 | DPP4 | -0,383 | 7,49e-19 |
| KIF23 | 0,395 | 5,42e-20 | CCDC149 | -0,381 | 1,33e-18 |
| FAM72D | 0,394 | 6,57e-20 | EPHX2 | -0,380 | 1,65e-18 |

genes significantly associated with BCR (dichotomization using median), 827 were not significantly correlated with the ISUP grading group of the tumor. Higher mRNA expression was prognostically unfavorable for 2390 genes and favorable for 1181 genes (top 20 genes outlined in Fig. 4). Full information about the prognostic significance of mRNA expression of single genes in univariate analysis using median and best cut-off for dichotomization is available as Additional file 4: Table S3.

### *Multivariate cox regression*

Pathological staging of the tumor (pT: pooled pT3/4 vs pT2), ISUP histological grade group of the tumor, presence of lymph nodes metastases (pN1 vs pN0) and status of the resection margins (R1 vs R0) were included into multivariate Cox regression models, together with the expression of single genes which showed statistically significant association with BCR-free survival in univariate Cox regression analysis (3571 genes with dichotomization using median of expression and 9273 genes with dichotomization using the optimized cut-off).

Among 3571 (median as cut-off) / 9273 (best cut-off) included genes, 571 / 2435 genes, respectively, showed statistically significant association with biochemical recurrence (Fig. 5). With median as cut-off, multivariate Cox regression *p*-values for single genes ranged from

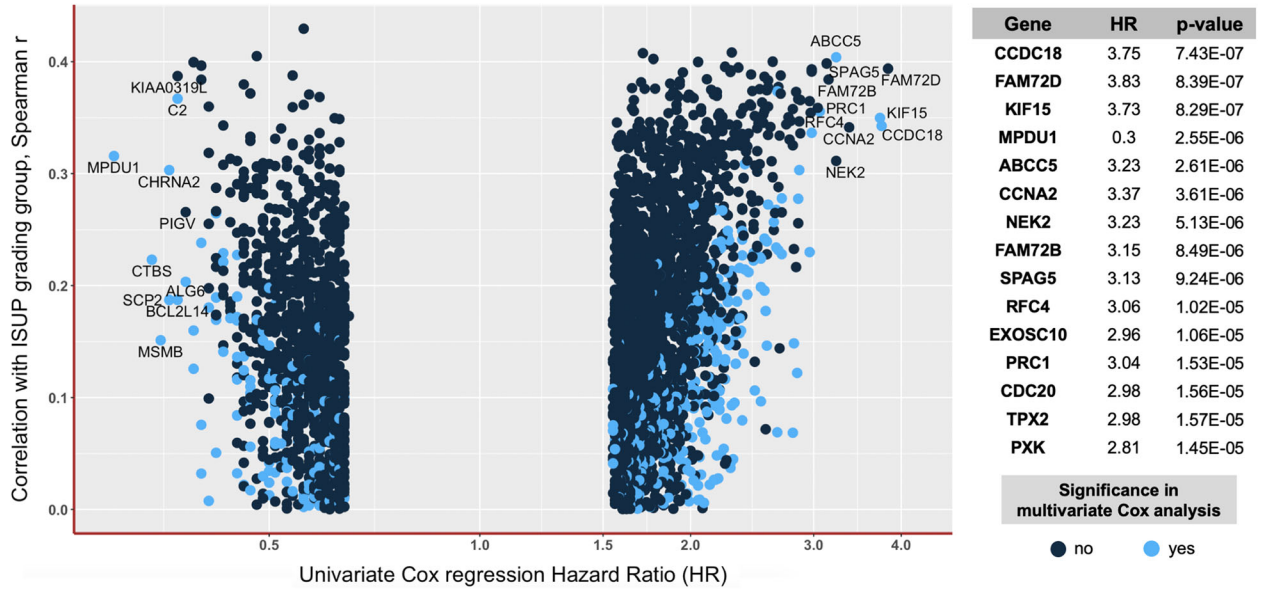



**Fig. 4** All genes with statistically significant association of mRNA expression with biochemical recurrence in univariate Cox regression analysis (all $p < 0.05$, median of expression as dichotomization cut-off) stratified according to univariate Hazard Ratio (HR) and correlation level between ISUP grad group of the tumor and mRNA-expression of the gene. Blue spots represent the genes which are independently associated with biochemical recurrence-free survival in multivariate Cox regression analysis. Top 15 genes with highest levels of statistical significance in univariate Cox analysis are highlighted with detailed outputs from univariate Cox regression analysis

0.0005 to 0.05 and hazard ratios from 0.40 to 2.47 (best cut-off p-value range 2.1e− 05 – 0.05, HR range 0.10–12.31). Kaplan-Meier curves for genes most significantly and independently associated with BCR-free survival in multivariate analysis are presented in Fig. 6. Full information about the prognostic significance of mRNA expression of all single genes in the multivariate analysis with dichotomization using median and best cut-off is available as additional file 6: Table S5.

Of 571 genes independently associated with BCR-free survival (median as cut-off), mRNA expression of 276 genes was significantly correlated with the ISUP grade group of the tumor (Pearson $r > 0.10$, $p < 0.05$).

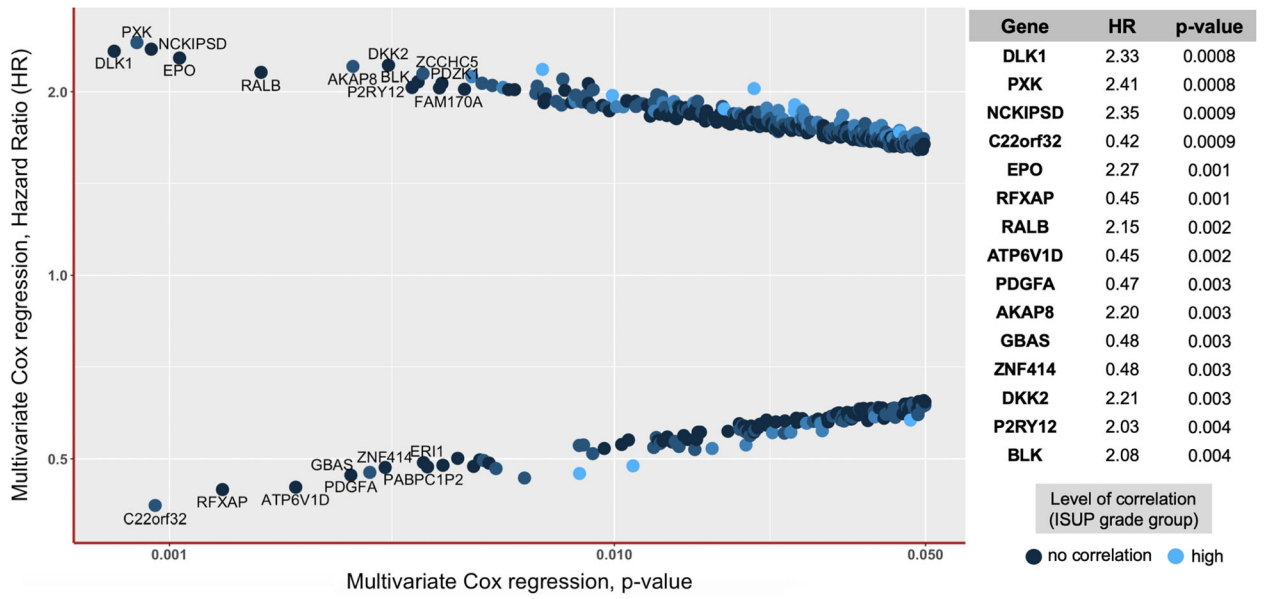
Characterization of the top 50 genes independently associated with BCR-free survival with analysis of their potential for further investigations in patients with prostate cancer is presented in Additional file 7: Table S6.

## Discussion

The identification of appropriate biomarkers is essential to drive important clinical decisions in patients with prostate cancer [7]. Hundreds of studies focusing on new biomarkers are being published every year since the 1980th, addressing three main biomarker branches (diagnostic, prognostic, and predictive). Even though diagnostic markers have found their niche in the clinical practice (selection of patients at risk of prostate cancer for biopsy based on serum (e.g. prostate-specific antigen, four kallikrein score) or urine analysis (RNA expression of PCA3 [9], or of gene pair HOXC6 / DLX1 [10]) [8], immunohistochemistry during primary diagnosis or metastatic disease [11], theranostic targets for imaging, such as prostate-specific antigen [12]), prognostic biomarkers are still not recommended by professional guidelines, despite 40 years of intensive research in patients with prostate cancer.

The aim of our study was to delineate the limits of prostate cancer prognostic and diagnostic biomarker research and to show the extent of perspective targets have not been studied in prostate cancer yet. For this purpose, we used a well-characterized primary hormone-naïve prostate cancer cohort from TCGA with 499 patients, representing 499 tumor and 53 normal tissue samples. The limits of diagnostic and prognostic biomarker research could be investigated with the use of this cohort as it includes data regarding the status of almost all genes. We have selected RNA expression data of all genes called using RNAseq approach as a surrogate for their functional relevance, and systematically approached the questions of the diagnostic and prognostic role of single genes using our automatized bioinformatical pipeline (Fig. 2).

As for the prognostic role of these genes during the statistical stages of Kaplan-Meier, univariate and multivariate Cox-regression analysis, we have cleared out 571 genes with independent prognostic significance for biochemical recurrence after radical prostatectomy using strict rules prescribed by REMARK criteria [13] (especially, using the median of expression as cut-off for dichotomization). Even more genes (overall 2435 genes) carried independent prognostic value under relaxed




| Gene | HR | p-value |
|---|---|---|
| DLK1 | 2.33 | 0.0008 |
| PXK | 2.41 | 0.0008 |
| NCKIPSD | 2.35 | 0.0009 |
| C22orf32 | 0.42 | 0.0009 |
| EPO | 2.27 | 0.001 |
| RFXAP | 0.45 | 0.001 |
| RALB | 2.15 | 0.002 |
| ATP6V1D | 0.45 | 0.002 |
| PDGFA | 0.47 | 0.003 |
| AKAP8 | 2.20 | 0.003 |
| GBAS | 0.48 | 0.003 |
| ZNF414 | 0.48 | 0.003 |
| DKK2 | 2.21 | 0.003 |
| P2RY12 | 2.03 | 0.004 |
| BLK | 2.08 | 0.004 |

**Fig. 5** All genes with statistically significant association of mRNA expression with biochemical recurrence in multivariate Cox regression analysis (all $p < 0.05$, median of expression as dichotomization cut-off) stratified according to multivariate Hazard Ratio (HR) and multivariate analysis p-level. Colors represent correlation levels to the ISUP grade group of the tumor (from dark blue = Spearman $r < 0.1$ and no significant correlation up to light blue = Spearman $r > 0.3$, $p > 0.05$). Detailed outputs for top 15 genes from multivariate Cox regression analysis are provided on the right side

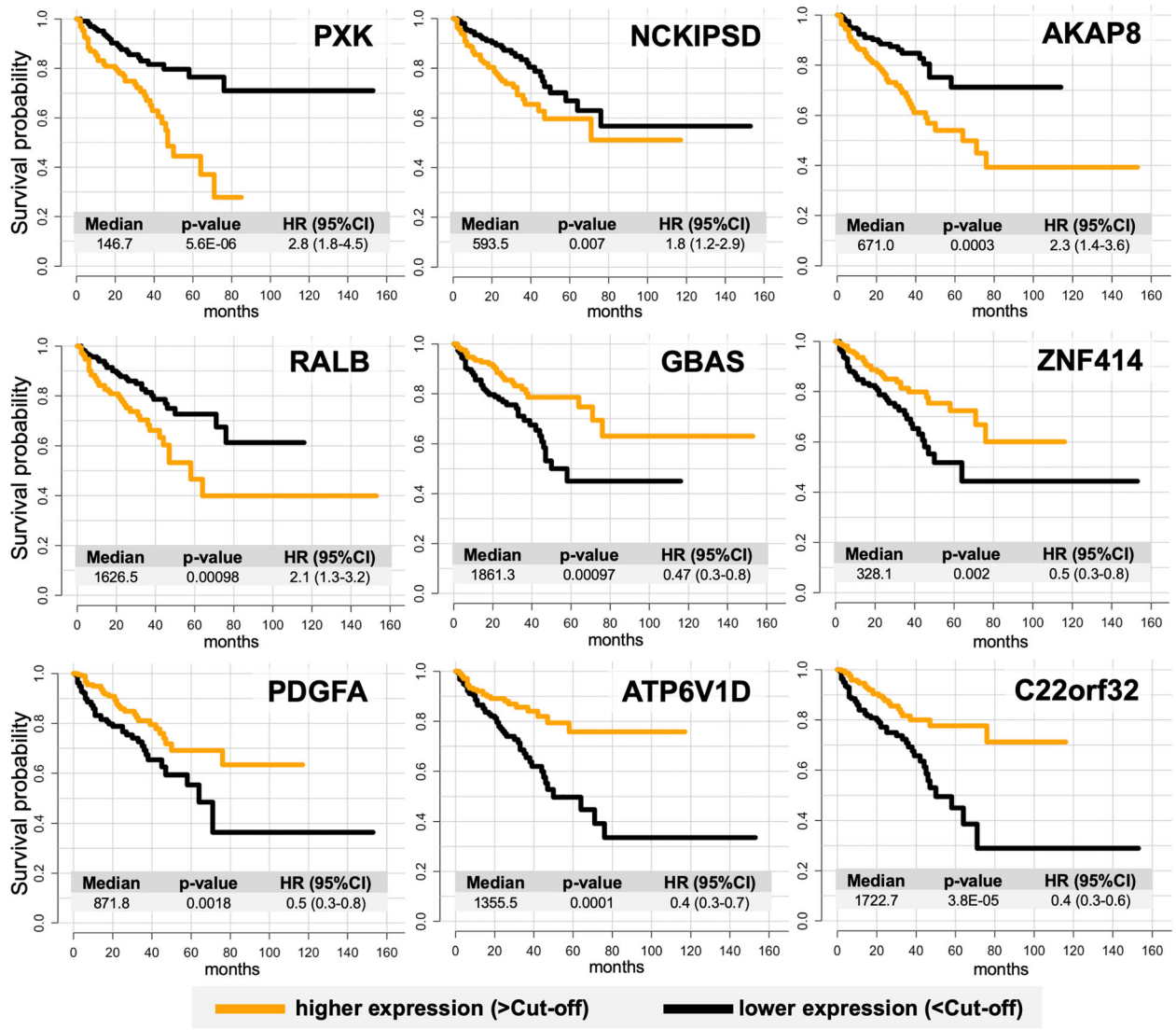



**Fig. 6** Kaplan-Meier curves for nine top-ranked genes with substantial expression in prostate cancer, statistically significant and independently associated with biochemical recurrence-free survival of the patients with prostate cancer in multivariate Cox regression analysis (median of expression as dichotomization cut-off). Abbreviations: Median – median of mRNA expression, HR – hazard ratio, l95% CI and u95% CI – lower and upper 95% percentile of the confidence interval (CI), low expression – expression levels below cut-off, high expression – expression levels above cut-off

criteria (optimized cut-off for dichotomization). Although prone to statistical bias, using the best cut-off for dichotomization is logical from a biological point of view (e.g., only a small part (not the half) of carcinomas could have special aggressiveness features delineated by gene expression). Therefore, these genes also could and should be considered as potential candidates for further characterization.

Interestingly, when performing a detailed analysis of the top 50 genes showing independent prognostic value (Additional file 7: Table S6), 40 of them (80%), as for the actual state of published research, have not been studied in prostate cancer, yet. 31 of these genes were shown to be of some significance for other cancer types. This gives us a broad perspective of how much research effort and time we should invest to cover all potentially relevant targets which have not been in the scope of prostate cancer researchers yet.

As for the diagnostic role of these genes, we hereby provide a comprehensive analysis based on two approaches: 1) differential expression in tumor vs. normal tissue, and 2) correlation of mRNA expression with Gleason-Score / ISUP-grade group of the tumor. The first part of this analysis provides a big number of potential targets which are highly upregulated ($n = 123$) or downregulated ($n = 133$) in tumor tissue with fold change (FC) more than 8 (logFC > 3) with multiple genes showing less pronounced, however, still significant up- and downregulation.

Some of these genes were already extensively studied in prostate cancer, mostly those upregulated in tumor tissue: SPINK1 [14] (Top17, logFC 4.8), ETV4 [15] (Top63, logFC 3.6), PCA3 [9] (Top79, logFC 3.5), TDRD1 [16, 17] (Top86, logFC 3.4), AMACR [18] (Top105, logFC 3.2), DLX1 [10] (Top110, logFC 3.1), HOXC6 [10] (Top268, logFC 2.3). However, others are still representing potential diagnostic targets at different stages of clinical decision making (before diagnosis, after first negative biopsy, control of recurrence, immunohistochemical diagnosis of prostate cancer on biopsy, theranostic targets). Between the above mentioned highly upregulated genes and several genes used as targets in clinical practice such as FOLH1 coding PSMA (logFC 1.7, Top622 among all upregulated genes), there is a gap of approx. 500 genes which could represent potential diagnostic targets.

Interestingly, several well-known genes, despite being recommended for their utility in the identification of patients at risk of prostate cancer at RNA expression level, are by far not in the top of this list (e.g. in urine; PCA3, HOXC6, DLX1 [9, 10]). Hence, these results once again outline the need for further research on diagnostic biomarkers.

Gene set enrichment analysis (GSEA) for differentially expressed genes allowed us to detect many biological processes/pathways altered in tumor tissue. Interestingly, among the top 30 biological processes altered in prostate cancer, 16 (53.3%) were related to epigenetic mechanisms, such as chromatin functioning/ organization, and epigenetic gene silencing (Fig. 3, Additional file 3: Table S2). The epigenetics of prostate cancer are well studied at the level of DNA methylation [19], however, more studies investigating broader epigenetic mechanisms related to chromatin organization and functioning are warranted for a further comprehension of the prostate cancer biology.

In general, our study and associated supplementary materials including full results of the performed analyses represent are a very useful reference database for those researchers willing to validate the results of their studies involving different levels of gene expression (mRNA, methylation analysis, protein expression) in patients with prostate cancer.

The limitations of our study are mainly associated with the inherent limitations of the TCGA cohort and should be considered using this material as a reference point. Due to the relatively short follow-up period (median 17 months, range 1–153 months), the findings of our analyses are more useful for patients with higher Gleason scores, as they develop BCR earlier. Among 45 patients with Gleason Score 3 + 3 = 6, only 3 patients have developed BCR to the end of follow-up compared to 80 patients with BCR among patients with other Gleason scores. To address this point, we have carried out a multivariate Cox regression analysis separately for patients of all ISUP grade groups and for patients of ISUP grade groups ≥2 (Additional file 8: Table S7). In conclusion, the results showed minimal discrepancies in the resulting set of independent prognostic biomarkers and therefore, the robustness of our initial findings.

Furthermore, despite the high quality of the TCGA cohort, a validation cohort would be needed to verify the results, as it was shown earlier that a significant number of genes (mainly from small studies) do not pass the validation landmark [20]. Also, it is important to mention, that the patient cohort that was used in this study is a post-prostatectomy cohort of patients with primary hormone-naïve prostate cancer and the results, therefore, could be only in the restrictive manner extended to the patients with metastatic and castration-resistant prostate cancer.

Moreover, the analyses carried out involved only RNA expression levels of the genes. In the modern era of multi-omics and presence of multiple aspects of alternative regulation of gene function from transcription to protein function, this should be respectively interpreted. Only one tumor sample per patient was analyzed by TCGA which can introduce a bias related to undersampling of the tumor, given high levels of prostate cancer morphological and molecular genetic heterogeneity [5]. Accordingly, validation studies should address this important aspect of prostate cancer biology.

We believe that our study should also be a starting point for an important discussion. Although the particular advantage of our study is a comprehensive outline of the immense possibilities that are still left for prognostic and diagnostic biomarker research, it poses one important question (taking in account the almost 40-year-long portfolio of translational biomarker research which still didn't find its way into clinical practice): what should be the priority of the future prostate cancer research and how do we need to change our research methodology to overcome the above mentioned issues of failing clinical relevance of biomarkers (especially prognostic) studied before? There is still no definitive answer to this question. However, it is more or less clear that single targets will probably only make sense in a diagnostic, but not in a prognostic setting. The optimal methodological approach for the development of prognostic biomarkers remains to be established and probably will account for pathway-oriented genetic and epigenetic changes at different levels (mutations, copy-number rearrangements, DNA methylation, chromatin remodeling, mRNA and ncRNA expression, posttranslational modifications, protein expression) and at different time points to account for tumor evolution and tumor heterogeneity [4, 21–23].

## Conclusions

Our study provides a comprehensive overview of the prognostic and diagnostic mRNA biomarkers in patients with primary prostate cancer, both already studied and, more importantly, not yet addressed in prostate cancer. Interestingly, several of them show a great potential for further research. These findings could be used as a reference point for further biomarker research and validation data for ongoing projects by most prostate cancer researchers. A full database including the results is provided as Supplement to be used as an everyday tool.

## Additional files

**Additional file 1:** Supplementary Data. List of abbreviations. (PDF 29 kb)

**Additional file 2: Table S1** Pairwise comparison of the gene expression of normal and tumor tissue using negative binomial generalized log-linear model and correction for false discovery rate (FDR). (TXT 2100 kb)

**Additional file 3: Table S2** Full results of Gene Set Enrichment Analysis (GSEA) for tumor vs normal tissue using Gene Ontology Biological Processes database. (TXT 131 kb)

**Additional file 4: Table S3** Correlation between ISUP histological grade group of the tumor (Grade groups 1–5) and mRNA expression of single genes, univariate Cox-regression analysis with biochemical recurrence as an endpoint. Full list of genes ($n = 17,681$). (TXT 3078 kb)

**Additional file 5: Table S4** Correlation between ISUP histological grade group of the tumor (dichotomized as high-grade, ≥4 + 4, vs low-grade tumors) and mRNA expression of single genes. Correlation analysis for ISUP grade groups (Groups 1–5) is provided in parallel for comparison. Delta of Pearson *r* correlation levels is calculated for these 2 different settings. (TXT 2104 kb)

**Additional file 6: Table S5** Full results of the multivariate Cox regression analysis for single genes. (TXT 1949 kb)

**Additional file 7: Table S6** Top 50 genes with statistically significant and independent association with biochemical recurrence-free survival in multivariate Cox regression analysis (dichotomization using median) in patients with prostate cancer. Estimates of their perspectivity for further research are provided. (PDF 65 kb)

**Additional file 8: Table S7** Results of the multivariate Cox regression analysis for single genes separately for patients of ISUP grade groups 2–5. (TXT 1940 kb)

### Abbreviations

BCR: Biochemical recurrence; FC: Fold change; FDR: False discovery rate; ISUP: International Society of Urological Pathology; PCa: Prostate cancer; TCGA: The Cancer Genome Atlas

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None.

### Authors' contributions

Data curation / Bioinformatics: YT, AK, TK. Data analysis: AK, TK, YT. Visualization: AK, YT. Writing - original draft: AK, YT. Writing - review & editing: GK. Supervision: YT, GK. Critical revision for important intellectual content: all authors. The authors have read and approved this information. All authors read and approved the final manuscript.

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### Availability of data and materials

Clinical dataset and mRNA expression database was downloaded from the Broad GDAC Firehose homepage (gdac.broadinstitute.org). Comprehensive databases with stratified analyses generated during this study are included in this published article as Additional file 1: Supplementary data.

### Ethics approval and consent to participate

Not applicable. This study represents a secondary analysis of existing public data.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note

**Supplementary Data: List of abbreviations,**

used in the Supplementary materials

| | |
|---|---|
| Best/ Best_coff | Best cut-off |
| Cor_delta | Difference in correlation levels calculated for ISUP grade groups 1-5 vs dichotomized low-/high-grade tumors |
| Cor_isup_p_val | Correlation between ISUP grading group and p-Value |
| Cor_isup_r | Correlation between ISUP grading groups (1-5) and gene expression; Pearson correlation coefficient (r) |
| Cor_isup_hg_r | Correlation between high-grade/low-grade tumors and gene expression; Pearson correlation coefficient (r) |
| ES | Enrichment score (GSEA) |
| FDR | False discovery rate |
| GSEA | Gene Set Enrichment Analysis |
| GO | Gene ontology |
| HR | Hazard ratio |
| ISUP | International Society of Uropathology (ISUP grade groups) |
| isup_hg_lg_ttest_p_val | Comparisons of expression in low- vs high-grade tumors, t-test value |
| isup_hg_lg_wil_p_val | Comparisons of expression in low- vs high-grade tumors, Wilcox p value (Mann Whitney U test) |
| isup_lg_mean | Mean of expression in patients with low-grade tumors |
| isup_hg_mean | Mean of expression in patients with high-grade tumors |
| L95 | Lower 95% quantile |
| LogCPM | Log counts per million |
| LogFC | Log fold-change |
| LR | Left–right asymmetry |
| Med | Median |
| Multi | Multivariate cox analysis |
| NES | Normalized enrichment score (GSEA) |
| Normal | Normal Tissue |
| P/ PValue | p-Value |
| Tumor | Tumor Tissue |
| U95 | Upper 95% quantile |
| Uni | Univariate cox analysis |

**Suppl. Table VI:**
Top 50 genes with statistically significant and independent association with biochemical recurrence-free survival in patients with prostate cancer (multivariate Cox analysis; dichotomization using median)

| Gene | Name | Function | Publications for cancer* | Publications for prostate cancer* |
|---|---|---|---|---|
| **AKAP8** | A-Kinase Anchoring Protein 8 | Recruitment of PKA and other signaling molecules, chromosome condensation during mitosis | 11 | 0 |
| **ARMC9** | Armadillo Repeat Containing 9 | basal body protein encoded | 0 | 0 |
| **ATP6V1D** | ATPase H+ Transporting V1 Subunit D | component of vacuolar ATPase, associated with melanomas | 1 | 0 |
| **BLK** | B Lymphoid Tyrosine Kinase | B-cell receptor signaling and development | 96 | 6 |
| **C17orf67** | Chromosome 17 Open Reading Frame 67 | Uncharacterized | 0 | 0 |
| **C18orf32** | Chromosome 18 Open Reading Frame 32 | Activation of NF-kappa-B signaling pathway | 1 | 0 |
| **C22orf32** | Chromosome 22 Open Reading Frame 32 | regulatory subunit of the mitochondrial calcium uniporter complex (uniplex) | 1 | 0 |
| **CBR4** | Carbonyl Reductase 4 | mitochondrial fatty acid biosynthesis | 3 | 0 |
| **CCDC18** | Coiled-Coil Domain Containing 18 | nucleotide binding | 1 | 0 |
| **CHST7** | Carbohydrate Sulfotransferase 7 | generate sulfated glycosaminoglycan (GAG) during chondroitin sulfate biosynthesis, transfer sulfate to N-acetylgalactosamine | 6 | 0 |
| **CSTF3** | Cleavage Stimulation Factor Subunit 3 | polyadenylation and 3' end cleavage of pre-mRNAs | 2 | 0 |
| **CYSLTR1** | Cysteinyl Leukotriene Receptor 1 | receptor for cysteinyl leukotrienes mediating bronchoconstriction | 9 | 0 |
| **DBH** | Dopamine Beta-Hydroxylase | Catalyzation of the conversion of dopamine to norepinephrine | 41 | 0 |
| **DGAT2** | Diacylglycerol O-Acyltransferase 2 | Catalyzation of covalent binding of diacylglycerol to long chain fatty acyl-CoAs | 13 | 1 |
| **DKK2** | Dickkopf WNT Signaling Pathway Inhibitor 2 | embryonic development, agonist or antagonist of Wnt/beta-catenin signaling | 65 | 3 |
| **DLK1** | Delta Like Non-Canonical Notch Ligand 1 | regulator of cell growth, differentiation of several cell types including adipocytes | 174 | 11 |
| **DNM3** | Dynamin 3 | guanosine triphosphate (GTP)-binding protein associated with microtubules and vesicular transport | 12 | 0 |

| | | | | |
|---|---|---|---|---|
| **E2F3** | E2F Transcription Factor 3 | Regulation of the expression of cell-cycle genes | 298 | 24 |
| **EMR4P** | Adhesion G Protein-Coupled Receptor E4, Pseudogene | leukocyte adhesion and migration | 0 | 0 |
| **EPO** | Erythropoietin | promotes red blood cell production or erythropoiesis | 1087 | 19 |
| **ERI1** | Exoribonuclease 1 | histone mRNA decay after replication | 2 | 0 |
| **FAM170A** | Family With Sequence Similarity 170 Member A | Regulation of the expression of heat shock genes | 0 | 0 |
| **FZD2** | Frizzled Class Receptor 2 | Receptor for Wnt proteins,<br>coupled to the beta-catenin canonical signaling pathway | 45 | 1 |
| **GBAS** | Glioblastoma amplified sequence | vesicular transport, oxidative phosphorylation | 6 | 0 |
| **KIAA0196** | WASH Complex Subunit Strumpellin | inhibiting WASH nucleation-promoting factor (NPF) activity<br>recruitment and activation of the Arp2/3 complex | 5 | 4 |
| **KIF19** | Kinesin Family Member 19 | Response to elevated platelet cytosolic Ca2+ and Golgi-to-ER retrograde transport | 1 | 0 |
| **KLHL38** | Kelch Like Family Member 38 | Contributes to ubiquitin-protein transferase activity | 0 | 0 |
| **LAIR1** | Leukocyte Associated Immunoglobulin Like Receptor 1 | anchor for tyrosine phosphatase SHP-1,<br>may induce cell death in myeloid leukemias | 4 | 0 |
| **LILRA1** | Leukocyte Immunoglobulin Like Receptor A1 | regulation of immune responses | 1 | 0 |
| **LILRA3** | Leukocyte Immunoglobulin Like Receptor A3 | soluble receptor for class I major histocompatibility complex (MHC) antigens | 4 | 2 |
| **MAPK15** | Mitogen-Activated Protein Kinase 15 | transferase activity,<br>transferring phosphorus-containing groups,<br>protein tyrosine kinase activity | 10 | 0 |
| **NBEAL2** | Neurobeachin Like 2 | role in megakaryocyte alpha-granule biogenesis | 6 | 0 |
| **NCKIPSD** | NCK Interacting Protein With SH3 Domain | signal transduction,<br>function in the maintenance of sarcomeres and in the assembly of myofibrils into sarcomeres | 5 | 0 |
| **P2RY12** | Purinergic Receptor P2Y12 | platelet aggregation,<br>target for the treatment of thromboembolisms and other clotting disorders | 20 | 0 |
| **PABPC1P2** | Poly(A) Binding Protein Cytoplasmic 1 Pseudogene 2 | pseudogene, uncharacterized | 0 | 0 |
| **PCDHGA9** | Protocadherin Gamma Subfamily A 9 | establishment and function of specific cell-cell connections in the brain | 2 | 0 |
| **PDGFA** | Platelet Derived Growth Factor Subunit A | binding and activation of PDGF receptor tyrosine kinases,<br>developmental processes | 88 | 5 |
| **PDZK1** | PDZ Domain Containing 1 | regulation of the HDL receptor (cholesterol metabolism) | 36 | 0 |

| | | | | |
|---|---|---|---|---|
| **PIWIL4** | Piwi Like RNA-Mediated Gene Silencing 4 | development and maintenance of germline stem cells | 16 | 0 |
| **PPIL2** | Peptidylprolyl Isomerase Like 2 | protein folding, immunosuppression by cyclosporin A and infection of HIV-1 virions | 1 | 0 |
| **PSORS1C3** | Psoriasis Susceptibility 1 Candidate 3 (Non-Protein Coding) | RNA Gene, non-protein coding | 0 | 0 |
| **PXK** | PX Domain Containing Serine/Threonine Kinase Like | synaptic transmission, ligand-induced internalization and degradation of epidermal growth factors | 5 | 1 |
| **RALB** | RAS Like Proto-Oncogene B | mediation of the transmembrane signaling initiated by certain cell surface receptors | 86 | 2 |
| **RFXAP** | Regulatory Factor X Associated Protein | central role in development and control of the immune system | 11 | 0 |
| **RMRP** | RNA Component Of Mitochondrial RNA Processing Endoribonuclease | cleavage of mitochondrial RNA at a priming site of mitochondrial DNA replication | 23 | 1 |
| **RPL18** | Ribosomal Protein L18 | component of the 60S ribosome subunit | 3 | 0 |
| **RPL27A** | Ribosomal Protein L27a | component of the 60S ribosome subunit | 8 | 0 |
| **SCP2** | Sterol Carrier Protein 2 | oxidation of branched chain fatty acids | 30 | 0 |
| **STC2** | Stanniocalcin 2 | regulation of renal and intestinal calcium and phosphate transport, cell metabolism, cellular calcium/phosphate homeostasis | 63 | 1 |
| **SYBU** | Syntabulin | activity-dependent presynaptic assembly during neuronal development | 0 | 0 |
| **TEKT3** | Tektin 3 | exact function of this gene is not known | 0 | 0 |
| **TFR2** | Transferrin Receptor 2 | mediates cellular uptake of transferrin-bound iron, iron metabolism, hepatocyte function and erythrocyte differentiation | 39 | 1 |
| **TNKS** | Tankyrase | NAD+ ADP-ribosyltransferase activity | 126 | 1 |
| **UBE2L3** | Ubiquitin Conjugating Enzyme E2 L3 | ubiquitination of p53, c-Fos, and the NF-kB precursor p105 in vitro | 34 | 1 |
| **WRAP53** | WD Repeat Containing Antisense To TP53 | telomere synthesis | 30 | 0 |
| **ZCCHC5** | Zinc Finger CCHC-Type Containing 5 | transcriptional regulation, postnatal myogenesis, regulation of satellite cells self-renewal | 1 | 0 |
| **ZNF414** | Zinc Finger Protein 414 | transcriptional regulation | 1 | 0 |

*At manuscript submission date.

### 6.2.2 Androgen Receptor Splice Variants Contribute to the Upregulation of DNA Repair in Prostate Cancer

cancers

MDPI

*Article*

# Androgen Receptor Splice Variants Contribute to the Upregulation of DNA Repair in Prostate Cancer

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**Simple Summary:** Ligand-independent androgen receptor splice variants emerge during androgen deprivation therapy and are suspected to render prostate carcinomas castration-resistant. In a retrospective analysis of a large cohort of primary and advanced prostate tumors, we observed increased expression of androgen receptor splice variants in therapy refractory tumors. Our hypothesis was that AR splice variants exert their tumor-promoting activity by modulating the intrinsic DNA repair machinery. In the sequence from primary over advanced tumors under androgen-deprivation therapy to castration resistance, AR splice variant expression increases and is linked to increased expression of DNA repair genes. This effect of AR splice variants appeared independent of their known impact on tumor cell proliferation. These clinical findings were validated in an androgen-sensitive prostate cancer cell line that mimics a castration-resistant phenotype by overexpression of AR-V7. Modulated DNA repair gene expression in the presence of AR splice variants is linked to increased DNA repair activity, pointing at a novel therapeutic approach for castration-resistant prostate cancer.

**Abstract:** Background: Canonical androgen receptor (AR) signaling regulates a network of DNA repair genes in prostate cancer (PCA). Experimental and clinical evidence indicates that androgen deprivation not only suppresses DNA repair activity but is often synthetically lethal in combination with PARP inhibition. The present study aimed to elucidate the impact of AR splice variants (AR-Vs), occurring in advanced or late-stage PCA, on DNA repair machinery. Methods: Two hundred and seventy-three tissue samples were analyzed, including primary hormone-naïve PCA, primary metastases, hormone-sensitive PCA on androgen deprivation therapy (ADT) and castration refractory PCA (CRPC group). The transcript levels of the target genes were profiled using the nCounter platform. Experimental support for the findings was gained in AR/AR-V7-expressing LNCaP cells subjected to ionizing radiation. Results: AR-Vs were present in half of hormone-sensitive PCAs on androgen deprivation therapy (ADT) and two-thirds of CRPC samples. The presence of AR-Vs is highly correlated with increased activity in the AR pathway and DNA repair gene expression. In AR-V-expressing CRPC, the DNA repair score increased by 2.5-fold as compared to AR-V-negative samples. Enhanced DNA repair and the deregulation of DNA repair genes by AR-V7 supported the clinical data in a cell line model. Conclusions: The expression of AR splice variants such as AR-V7 in PCA patients following ADT might be a reason for reduced or absent therapy effects in patients on additional PARP inhibition due to the modulation of DNA repair gene expression. Consequently, AR-Vs should be further studied as predictive biomarkers for therapy response in this setting.

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## 1. Introduction

Androgen receptor (AR) signaling and DNA repair are tightly interconnected in prostate cancer (PCA) [1–8]. The presence of pathogenic mutations in genes responsible for homologous recombination (HR) DNA repair opens up the possibility of therapy with PARP inhibitors (PARPi) in up to 25% of patients with metastatic castration-refractory PCA (CRPC) [9,10]. In cases of insufficiency in HR DNA repair, PARP is a reserve system that operates through base excision. Blocking base excision repair with PARPi renders tumor cells incapable of effectively repairing DNA damage, which then eventually accumulates lethal mutations [5,6]. However, there is early evidence that PARPi might be effective in CRPC in the absence of HR DNA gene mutations [5,6,11]. Androgen deprivation therapy (ADT) on PCA cells induces a so-called functional "BRCAness". The term BRCAness defines an insufficiency in the HR DNA repair system [2,5,6], originally caused by the functional loss of the BRCA1 and BRCA2 genes. BRCA-deficient cells use error-prone DDR pathways that consequently increase their genome instability [12]. ADT treatment mimics this loss of BRCA1/2 expression. In this situation, PARPi deepens the DNA repair insufficiency, resulting in synthetic lethality for tumor cells. It has also been shown in vitro that both *PARP1* and *PARP2* are critical effectors of the AR pathway activity. Besides their function in DNA repair, PARP enzymes are known to be transcriptional coactivators of the AR. Notably, PARP-1 appears to activate AR function and affect downstream signaling [13,14], which is another rationale for targeting PARP in PCA [5–8,15,16].

The synthetic lethality of ADT and PARPi might, however, be dependent on AR alterations (splice variants, amplification, mutations) often present in advanced PCA [17–20] and responsible for sustained AR pathway activity during ADT. The upregulation of AR-V7 in clinical samples of advanced PCA patients was reported by Sharp et al. [21]. DNA repair in PCA cell lines exposed to ionizing radiation was found to be diminished following AR blockade with enzalutamide but largely preserved in the case of AR-V7 and ARv567es splice variant expression [3]. There is limited evidence that both full-length AR (AR-FL) and AR splice variants (AR-Vs) directly activate the expression of key genes necessary for DNA repair [2,3,8,22]. Apparently, both non-homologous end-joining and HR DNA repair are the main effectors of AR-FL and AR-Vs in prostate cancer [1,2,4]. Blocking AR-FL using antiandrogens has been shown to retain AR-V activity with regard to supporting the DNA repair system. However, so far, this has mainly been shown in cell line models [3,8,23].

The present study aimed to clarify, in clinical PCA samples, whether the expression of key DNA repair genes is affected by ADT, particularly in the presence of AR-Vs. A cohort of patients in different stages of PCA was analyzed to characterize AR pathway activity, and these data were correlated with the expression of DNA repair genes. Luo et al. reported that DNA repair is modulated by androgen receptor splice variant 7 [23]. The present study provides evidence that DNA repair is partly dependent on AR pathway activity in PCA. As enhanced DNA repair is induced in the presence of AR-Vs through the modulation of DNA repair gene expression, the application of synthetic lethality concepts, such as the combination of ADT and PARPi, might be questionable.

## 2. Materials and Methods

### *2.1. Patient Cohort*

The study cohort consisted of 184 patients: 167 patients with PCA in different disease stages and 17 patients from control groups (Table 1). All patients received ADT (LHRH analogs/antagonists) alone or in combination with abiraterone or antiandrogens (bicalutamide, etc.). Two patients with primary small-cell carcinoma of the prostate and sarcomatous carcinoma lacking prostate epithelial differentiation after ADT were also

included. Materials on twenty-two patients with CRPC were provided by the Department of Pathology, Forensic and Insurance Medicine, Semmelweis University, Budapest, Hungary (2005–2016). All other patients were diagnosed at the Institute of Pathology of the University Hospital Bonn, Bonn, Germany (2003–2018).

**Table 1.** Clinical characteristics of the study cohort.

| | Number of Patients | Number of Samples |
|---|---|---|
| PCA, primary tumor, hormone-naïve | 77 | 136 |
| ***pT-stage*** | | |
| pT1b (TURP) | 20 | |
| pT2 | 14 | |
| pT3a | 9 | |
| pT3b | 21 | |
| pT4 | 3 | |
| unknown | 10 | |
| ***pN stage*** | | |
| pN0 | 32 | |
| pN1 | 12 | |
| pNx | 33 | |
| ***Prostatectomy*** | | |
| *ISUP/WHO grade group* | | |
| ISUP 1 | 3 | |
| ISUP 2 | 6 | |
| ISUP 3 | 1 | |
| ISUP 4 | 37 | |
| ISUP 5 | 30 | |
| ***Morphology*** | | |
| Acinar adenocarcinoma | 52 | |
| Ductal/mixed adenocarcinoma [§] | 25 | |
| PCA, metastases, hormone-naïve | 23 | 28 |
| PCA ADT * | 42 | 55 |
| PCA CRPC *,[#] | 32 | 35 |
| BPH | 10 | 10 |
| Benign prostate tissue without hyperplasia | 7 | 7 |
| Neuroendocrine carcinoma of the prostate | 1 | 1 |
| Sarcomatoid carcinoma [$] | 1 | 1 |
| OVERALL | 184 | 273 |

Comments: *—CRPC samples from primary (untreated) tumor in the prostate or from metastases; [§]—only samples containing ductal adenocarcinoma were included in the analysis in case of mixed ductal/acinar morphology; [#]—four samples of CRPC bone metastases that failed quality control are not showed here. [$]—primary tumor sample, after androgen deprivation therapy.

### 2.2. Samples

All samples were harvested from formalin-fixed paraffin-embedded (FFPE) tissue blocks (flow chart: see Figure 1). Multiple samples (up to 4) from tumors of selected patients were analyzed to address heterogeneity of primary and metastatic tumors with an overall number of 273 samples passing quality control (Figure 2A). All tumor samples had a purity of >90% tumor cells and were macrodissected. Among included CRPC samples, 29 were from primary tumor (no treatment with curative intent) and 6 from metastatic lesions (2 from bone and 2 from soft tissue metastases, 1 from liver and 1 from retroperitoneal lymph node).

### 2.3. RNA Extraction

One or several 10 µm sections from paraffin block were used for macrodissection and total mRNA extraction. PureLink™ FFPE RNA Isolation Kit (ThermoFisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions. NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) was applied for mRNA quality control and quantification.

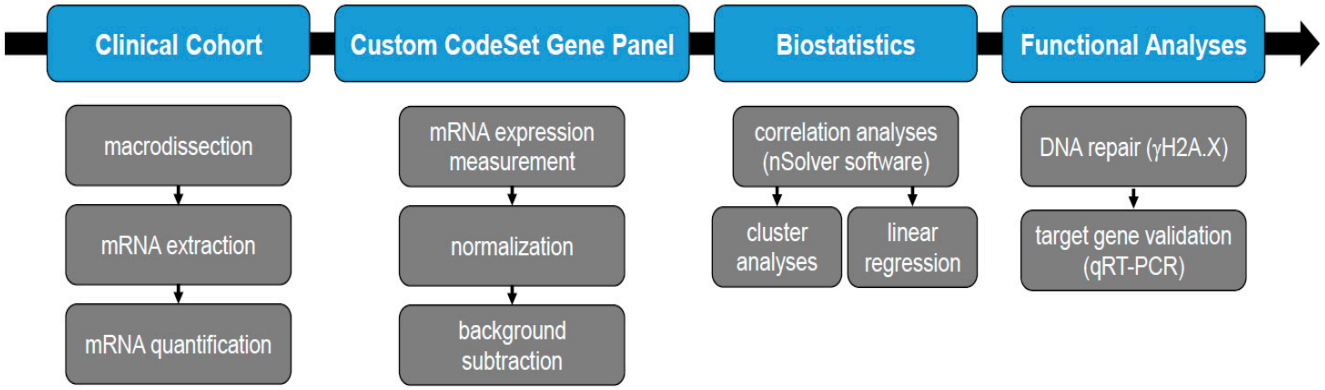



**Figure 1.** Flow chart of the main steps in this study: material processing, data acquisition, biostatistical analyses and experimental validation.

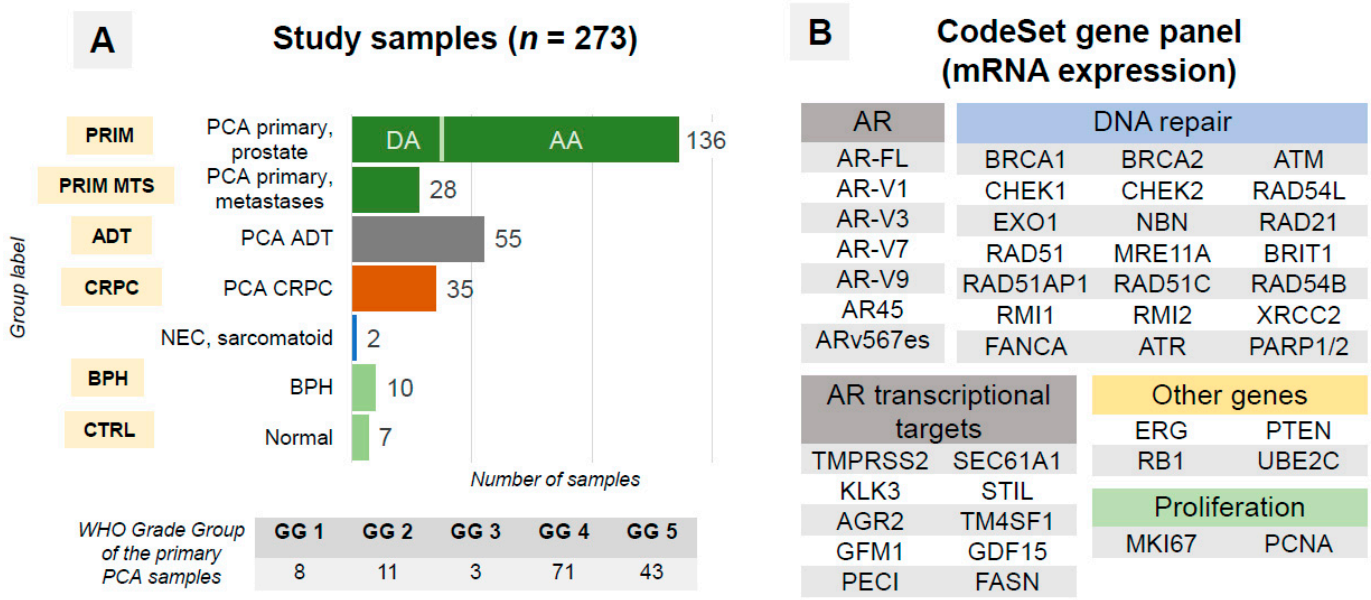



**Figure 2.** (**A**) Structure of the samples in study cohort and group labels used in further Figures. Among primary prostate cancer samples, 33 ductal adenocarcinoma (DA) samples were analyzed. Abbreviations: AA—acinar adenocarcinoma, ADT—androgen deprivation therapy (primary tumor and metastases), BPH—benign prostatic hyperplasia, CRPC—castration-refractory prostate cancer (primary tumor and metastases), CTRL—normal prostate tissue, GG—grade group, NEC—neuroendocrine carcinoma, PCA—prostate cancer, WHO—World Health Organization. (**B**) Composition of the gene panel for mRNA expression analysis using nCounter technology. AR—androgen receptor, FL—full-length. Additionally, four housekeeping genes (HPRT1, ALAS1, ARF1, PGK1) were included in the panel.

### *2.4. RNA Expression Analysis*

All RNA expression analyses were performed using the nCounter platform (NanoString Technologies, Inc.; Seattle, WA, USA). A custom CodeSet gene panel (Supplementary Table S1) included 45 target genes: (1) AR-FL and splice variants (AR-Vs; junction-specific probes), (2) AR transcriptional targets, (3) DNA repair-associated genes, (4) proliferation-related genes, and (5) further genes relevant for PCA (Figure 2B). Four housekeeping genes (*HPRT1, ALAS1, ARF1, PGK1*) were included. All samples were titrated to 100 ng of the total mRNA amount. Internal nCounter negative and positive controls as well as other internal metrics (RNA quantity, binding density) were used for quality control.

### 2.5. Statistical Analysis

Raw RNA expression data were processed by nSolver Analysis Software v. 4.0.70 (Nanostring Technologies, Seattle, WA, USA). Negative controls were used for background subtraction (geometric mean) of the called expression values. Internal positive controls and reference genes were used for normalization of expression levels. All further analyses were carried out in R (v. 3.6.0, The R Foundation for Statistical Computing). AR pathway score (AR score) was calculated from 10 genes representing transcriptional targets of AR (Figure 2B), as described in [19], with benign non-hyperplastic prostatic tissue used as reference. In brief, for each gene, a z-score was calculated by subtracting the pooled mean of expression in reference tissue samples divided by the pooled standard deviation of expression in reference tissue samples. AR score output was calculated as the sum of z-scores for all ten target genes divided by the number of genes. The HRDNA repair score (DNA-R score) was calculated using the same principle using all genes ($n$ = 20) from our panel related to HR DNA repair (excluding *PARP1* and *PARP2*). The expression of AR-Vs was evaluated both quantitatively (correlation analyses, heatmap) and qualitatively. For qualitative estimation of AR-Vs (present/not present), a threshold of 20 normalized counts (approximately 5 standard deviations above mean AR-V expression for control tissue samples) was used after background subtraction of the geometric mean of negative control samples. Appropriate parametric ($t$-test), non-parametric (Mann–Whitney U test) and correlation statistical tests (Pearson's $r$) were used for comparison between groups and parameters. Expression heatmaps were created in nSolver 4.0 analysis software (nanoString, Seattle, WA, USA) using Pearson's correlation for clustering.

### 2.6. Linear Regression Analysis

To analyze the joint effects of AR-Vs and proliferation on DNA repair, score variables that quantified either AR-Vs or the proliferation of tumor cells were constructed. These scores were set up in the same way as the DNA-R score using z-scores of the AR splice variants (AR-V1, -V3, -V7, -V9) and proliferation genes (MKI67, PCNA) for both the splice variant (SV score) and proliferation (P score) scores, respectively. Values of multiple samples from single patients were averaged. In the next step, the scores were used to fit linear regression models with the DNA-R score as the dependent variable and the SV and P scores as independent variables. The fits of the linear regression models were used to investigate whether AR-Vs had an effect on DNA repair when accounting for the effect of tumor cell proliferation on DNA repair (Supplementary Figure S9).

### 2.7. Cell Culture and Irradiation

LNCaP cells were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). LNCaP cells stably overexpressing AR-FL (LNCaP/AR) or AR-V7 (LNCaP/V7) were generated by lentiviral transduction. Lentivirus was generated in HEK 293T cells via co-transfection of VSV-G, Gag-Pol and AR-FL/ V7 expressing plasmids using jetPRIME (Polyplus, Illkirch, France). pLENTI6.3/AR-GC-E2325 (lentiviral vector for AR-FL expression [24]) was a gift from Karl-Henning Kalland, and AR-V7-pcw107, described in Martz et al., 2014 [25], was a gift from David Sabatini and Kris Wood. LNCaP/AR cells were selected with 5.5 µg/mL Blasticidin (Life Technologies, Paisley, UK) for 2 weeks, and LNCaP/V7 cells were selected with 1 µg/mL Puromycin (Cayman Chemical, Ann Arbor, USA) for 5 days.

Cells were cultured in RPMI1640 medium containing GlutaMax supplemented with 1% penicillin/streptomycin (10,000 U/mL) and 10% fetal bovine serum (Life Technologies, Paisley, UK) or 10% charcoal-stripped fetal bovine serum (DCC) (Biowest, Nuaillé, France) at 37 °C in a humidified atmosphere containing 5% $CO_2$. Cells were routinely checked to exclude mycoplasma contamination.

Radiation experiments were performed on a linear accelerator (Truebeam Stx, Varian Medical System, Palo Alto, CA, USA) using 6 MeV photon energy at a dose rate of 4 Gy/min at dose maximum ($D_{max}$ = 20 mm). Depending on the experimental setting, the applied

dose to the cells varied between 2 to 6 Gy. For this purpose, cells seeded in 6-well plates (for Western Blot and RNA analysis) or on glass coverslips placed in 12-well plates (for IF) were positioned in a tissue-equivalent RW3-plasticphantom (PTW, Freiburg, Germany) at a depth of $D_{max}$. The field size was adapted according to the number of plates irradiated.

### 2.8. γH2A.X Assay

DNA double-strand breaks were determined by γH2A.X staining cells [26]. Cells were grown for 24 h in androgen-deprived medium and then subjected to 2 Gy irradiation. Immunofluorescence was performed 24 h post-irradiation, as previously described [27]. Foci were visualized using phospho-Histone H2A.X (catalog number 05-636; Millipore, Temecula, CA, USA) and anti-mouse IgG/IgM Alexa Fluor 488-conjugated secondary antibodies (Dianova, Hamburg, Germany). Subsequently, cells were embedded in Fluoromount-G with DAPI (Life Technologies, Carlsbad, CA, USA). Fluorescent images were acquired on an Olympus CKX53 microscope (Tokyo, Japan) and foci were counted using QuPath Software v0.3.2 [28]. At least 200 cells per condition were counted.

### 2.9. Immunoblot

Cell lysates were prepared using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Complete Protease Inhibitor Cocktail, Roche, Basel, Switzerland and Halt Protease and Phosphatase Inhibitor Thermo Scientific, Rockford, IL, USA) as previously described [29]. Primary antibodies used included AR-V7 (31-1109-00, RevMab Biosciences, San Francisco, CA, USA), γH2A.X (05-636, Merck Millipore, Darmstadt, Germany), and β-Actin (ab6276, Abcam, Cambridge, UK). As a secondary antibody, a horseradish peroxidase-conjugated antibody (ab6789, Abcam) was used. Signals were detected using ECL Western Blot Substrate or SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA) on a Fusion S imaging system (Vilber Lourmat, Radolfzell, Germany).

### 2.10. Quantitative qRT-PCR

To study the mRNA expression of DNA repair genes (Supplementary Table S1) in LNCaP cells stably overexpressing AR-FL or AR-V7, cells were seeded 24 h prior to irradiation in androgen-deprived medium. Six hours after irradiation (6Gy), either protein lysates or total RNA were recovered from the cells, followed by qRT-PCR as previously described [29]. Oligonucleotide primers specific for DNA repair genes and PPIA (peptidylprolyl isomerase A, used as housekeeping gene) were purchased from biomers.net (Ulm, Germany). Primer sequences are provided in Supplementary Table S2. Sequence verification of the amplification products was performed with Sanger sequencing. Gene expression was measured in triplicates per gene. Relative gene expression was assessed using the $\Delta\Delta C_\tau$ method with PPIA as a housekeeping gene (Supplementary Tables S3 and S4).

### 2.11. Ethical Considerations

The study was approved by the ethical committees of the University of Bonn (Votum 124/19) and Semmelweis University (#177/2016).

## 3. Results

### 3.1. Quality Control (QC)

Three and five samples were excluded at the QC stage from the ADT and CRPC groups, respectively, due to low RNA quality (the final composition is in Table 1, excluding samples failing QC). The excluded samples were small, decalcified bone biopsies and transurethral resections.

### 3.2. AR-Vs Appear Mostly as a Response to ADT

An analysis of AR-FL and AR-V mRNA expression (AR-V1, AR-V3, AR-V7, AR-V9, AR45, ARv567es) was performed on the nCounter platform (Figures 1 and 3). AR-Vs were

detected in approximately two-thirds of CRPC samples and half of ADT samples but in only 14% of hormone-naïve cases (84% with Gleason Score > 4 + 3; no statistical association with ductal/acinar morphology). We detected no expression of AR-Vs in normal tissue and BPH samples (Figure 3A,B). AR-V1 was the most common AR-V in hormone-naïve PCA. AR-V7 was most common during ADT and in the CRPC stage (Figure 3B–D). In fact, in CRPC samples, AR-V7 was present in 100% of the samples expressing any of the other AR-Vs (Figure 3D). One of two CRPC patients on abiraterone therapy in our cohort expressed AR-Vs.

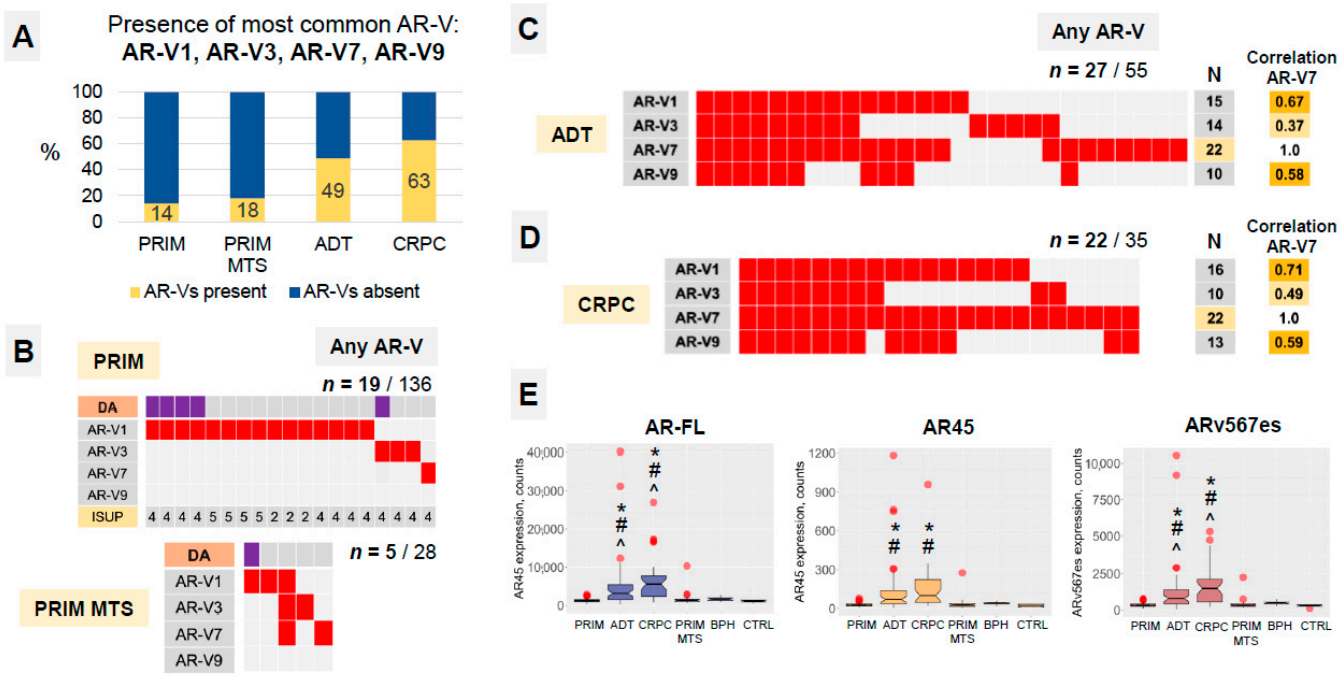



**Figure 3.** (**A**) Analysis of samples expressing any of the four androgen receptor splice variants in the four main study groups. BPH and CTRL groups were negative for AR-Vs. Numbers represent percentages of samples containing AR-Vs. (**B**–**D**) Stratification of the samples expressing any of the AR-V splice variants (V1, V3, V7, V9) in study groups. Boxes represent single samples. Stratification according to histological subtype (acinar or ductal (DA) adenocarcinoma) in primary hormone-naïve tumor samples. As other AR-Vs are co-expressed with AR-V7 in CRPCs, the latter appears a reasonable surrogate marker for the presence of AR-Vs. Correlation (co-existence) measure is presented for AR-V1, -V3 and -V9 compared to AR-V7 splice variant. "*n*" represents the number of samples positive for single AR splice variants. (**E**) Expression of full-length AR (AR-FL) and two other splice variants (AR45 and ARv567es) in tumor and benign study groups. Statistical significance ($p < 0.05$): * vs. PRIM group, ^ CRPC vs. ADT group, # vs. CTRL group (Mann–Whitney U-test).

Both AR45 and ARv567es mRNA were detectable in tumor and benign samples (Figure 3E). AR-FL, AR45 and ARv567es mRNA expressions were significantly higher in ADT and CRPC samples (all $p < 0.001$). AR45 and ARv567es mRNA expressions were highly correlated with the expression of AR-FL (Pearson's $r$ 0.86 and 0.99, respectively, both $p < 1.0 \times 10^{-10}$). ARv567es mRNA expression was approximately three and four times higher (both $p < 0.0001$) in presence of any of the other AR-V splice variants in the CRPC and ADT groups, correspondingly.

A certain level of intra-patient heterogeneity was evident regarding the presence of AR-Vs. The AR-V status of multiple samples from single patients was heterogeneous in 9 out of 41 patients in the PRIM group, 1 of 4 patients in PRIM MTS, and 3 of 10 patients in the ADT group, but not in the CRPC group.

Both samples with primary small cell neuroendocrine carcinoma of the prostate and sarcomatoid carcinoma (post-ADT) showed no or almost undetectable expression levels of AR-Vs and AR-FL, respectively [30].

### *3.3. AR Signaling and Proliferation Depend on the Presence of AR-Vs in ADT and CRPC Tumors*

To measure the activation of the AR pathway, we calculated an AR score from cumulative levels of expression for 10 established transcriptional targets of AR (Figure 2B) with CTRL samples as a reference.

The median AR-FL expression increased with the progression of the disease (Figure 3E). However, AR target gene (positive AR score) induction was significantly reduced in the ADT and CRPC samples (despite ongoing ADT), while AR signaling was significantly activated in samples from both the PRIM and PRIM MTS groups (Figure 4A). In fact, a smaller part of the ADT and CRPC samples revealed the downregulation of known AR target genes (negative AR score) (Figure 4A).

The decrease in the AR score, as observed for ADT and CRPC, however, does not translate into a significant change in the proliferative index. Similar to the PRIM MTS group, MKI67 expression was significantly elevated in ADT and CRPC compared to the PRIM samples (Figure 4B), but only in ADT and CRPC (Figure 4D, Supplementary Figure S1A,B), but MKI67 expression correlated with AR-Vs only in ADT and CRPC samples (Figure 4D, Supplementary Figure S1A,B). A similar upregulation of the PRIM MET, ADT and CRPC cohort was detected for the ubiquitin-conjugating enzyme E2C *(UBE2C)* in comparison to the CTRL, BPH and PRIM groups (Supplementary Figure S2A). The progressive loss of AR target gene expression, in combination with an increasing proliferative index, may hint at a progredient dedifferentiation of tumors ranging from the PRIM to CRPC cohorts.

The expression of UBE2C is driven by AR-V- and not by AR-signaling in CRPC tumors [31]. Our analyses support this finding, as UBE2C expression was significantly upregulated only in CRPC tumors expressing AR-Vs (Figure 4E, Supplementary Figure S2B). In the same group, we found a significant association between AR-V expression with increased proliferation (MKI67) and the AR-V$^+$-dependent elevation of AR target gene expression, while in AR-V negative CRPCs, AR target gene induction was in the range of the CTRL group ($p = 0.003$; Figure 4C). We did not find evidence for a correlation between AR pathway activity and *PTEN* or *RB1* expression in any of the groups (all $p > 0.05$).

In both ADT and CRPC groups (Figure 4D), significantly higher *MKI67* mRNA expression was evident in tumors expressing AR-V splice variants. *PCNA* expression strongly correlated with the *MKI67* expression (Pearson's r 0.52, $p < 2.2 \times 10^{-16}$; not shown) without any evidence of dependence on AR-Vs (Supplementary Figure S1C,D).

### *3.4. Homologous Recombination DNA Repair Activity Depends on the Presence of AR-Vs and AR Pathway Activation*

Overall, the mRNA expression of 20 genes associated with HR DNA repair was analyzed in our study. Unsupervised heatmap clustering analysis showed evidence of two major clusters of DNA repair genes, one of them containing *BRCA1* and the other containing *BRCA2* expression. With the introduction of AR-Vs in this analysis as a quantitative parameter, AR-V expression was preferentially associated with the CRPC phenotype (11/45 in the AR-V low-expressing group vs. 23/45 in the AR-V high-expressers, Figure 5). In the AR-V high-expressing group, DNA repair gene expression was more abundantly deregulated as compared to the AR-V low-expressing group, where a more stable expression of DNA repair genes was observed.

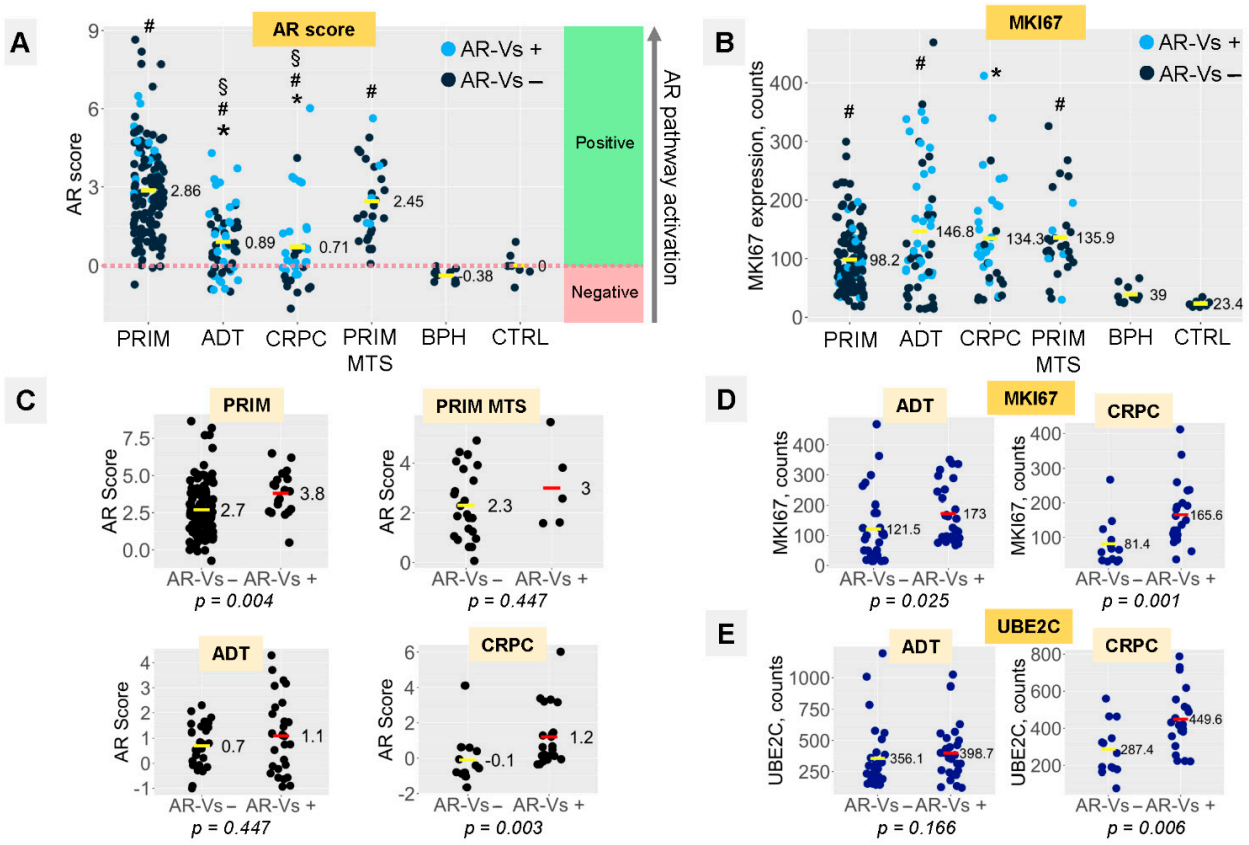



**Figure 4.** (**A**) Distribution of AR scores in study groups (cumulative score of AR pathway activation, calculated based on mRNA expression of 10 transcriptional targets of AR). Light blue points express any of the AR-V splice variants (V1, V3, V7, V9); dark blue points do not express AR-V splice variants. "Positive" area represents AR score in "activated" range compared to reference group (CTRL: benign non-hyperplastic prostate tissue). "Negative" area represents depression in AR signaling. (**B**) mRNA expression of proliferation marker MKI67. (**C**) Analysis of AR score distribution in four "tumor" study groups in relation to dependence on AR-V splice variant expression. (**D**) Analysis of MKI67 mRNA expression in ADT and CRPC groups in relation to dependence on AR-V splice variant expression. (**E**) Analysis of UBE2C mRNA expression in ADT and CRPC groups in relation to dependence on AR-V splice variant expression. *p*-levels calculated using Mann–Whitney U-test. Statistical significance ($p < 0.05$): * vs. PRIM group, # vs. CTRL group, § vs. PRIM MTS group.

To quantify DNA repair gene expression, we calculated a DNA repair activity score (DNA-R score) from the expression levels of 20 genes (excluding *PARP1* and *PARP2*) using CTRL samples as a reference, analogous to the AR score introduced above (Figures 6 and S3). The DNA-R score significantly increased in all groups, including BPH, compared to the reference group (Figure 6A). In the presence of AR-Vs, the DNA-R score increased significantly in CRPCs. We observed a similar trend in the ADT group (Figure 6B). In the PRIM and ADT groups, as well as in the CRPC group (statistical trend), the DNA-R score significantly correlated with the AR score (Figure 6C). In linear regression models, the effect of proliferation on DNA repair was clearly visible (Table 2, lower panel). However when accounting for this effect, residual coefficient estimates revealed a positive association between the presence of AR-Vs and enhanced DNA repair in both primary tumors (ADT, $p = 0.0377$) and tumors that that underwent androgen deprivation therapy (ADT, $p = 0.0297$). Statistical analysis revealed a strong trend for CRPC within the latter group (Table 2, upper panel). An analysis of the CRPC group ($p = 0.0551$) showed a strong trend in the same direction in the respective regression models. The DNA-R score was not found to be correlated with *RB1*, *PTEN* or *ERG* mRNA expression (all $p > 0.05$).

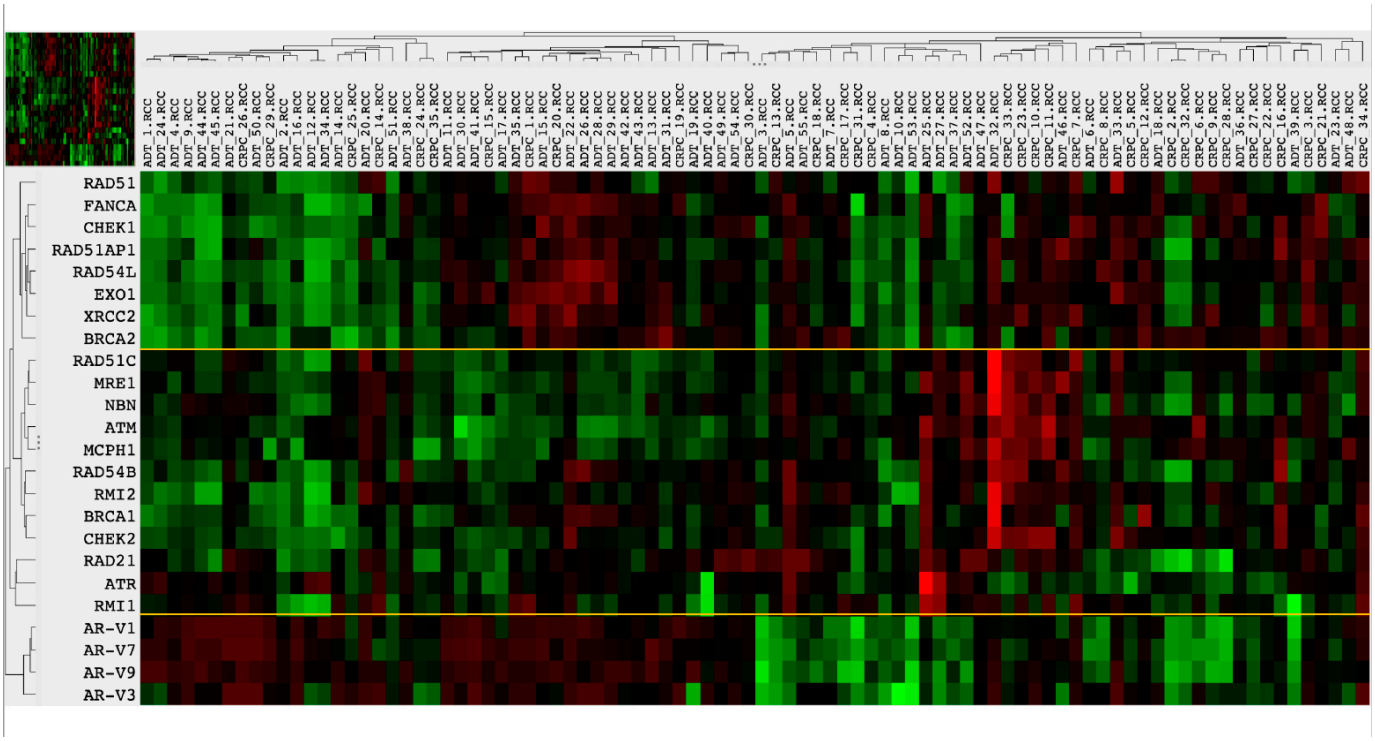



**Figure 5.** Heatmap plot of mRNA expression levels of DNA repair genes and AR-V splice variants as a quantitative parameter (**left side**). Samples represent pooled tumor samples from ADT and CRPC groups. Note similarities in the expression levels of the upper cluster of DNA repair genes (RAD51 to BRCA2) and AR-V splice variants, with the exception of 15 samples on the **left side** (mostly ADT samples). Clusters are separated by yellow lines.

To quantify DNA repair gene expression, we calculated a DNA repair activity score (DNA-R score) from the expression levels of 20 genes (excluding *PARP1* and *PARP2*) using CTRL samples as a reference, analogous to the AR score introduced above (Figures 6 and S3). The DNA-R score significantly increased in all groups, including BPH, compared to the reference group (Figure 6A). In the presence of AR-Vs, the DNA-R score increased significantly in CRPCs. We observed a similar trend in the ADT group (Figure 6B). In the PRIM and ADT groups, as well as in the CRPC group (statistical trend), the DNA-R score significantly correlated with the AR score (Figure 6C). In linear regression models, the effect of proliferation on DNA repair was clearly visible (Table 2, lower panel). However when accounting for this effect, residual coefficient estimates revealed a positive association between the presence of AR-Vs and enhanced DNA repair in both primary tumors (ADT, $p = 0.0377$) and tumors that that underwent androgen deprivation therapy (ADT, $p = 0.0297$). Statistical analysis revealed a strong trend for CRPC within the latter group (Table 2, upper panel). An analysis of the CRPC group ($p = 0.0551$) showed a strong trend in the same direction in the respective regression models. The DNA-R score was not found to be correlated with *RB1*, *PTEN* or *ERG* mRNA expression (all $p > 0.05$).

An analysis of expression and dependence on the presence of AR-Vs for single HR DNA repair genes is presented in Supplementary Figures S4–S7. The trends in mRNA expression for the individual DNA repair genes were similar to the ADT and CRPC groups. Some genes (*ATM*, *RAD51C*, *BRCA2*, *MRE1*, *RMI1*) were more profoundly downregulated in the CRPC group (Figure 7A). A number of DNA repair genes showed statistically significant altered expression in the PRIM, ADT and CRPC groups in the presence of AR-Vs (Figure 7B).

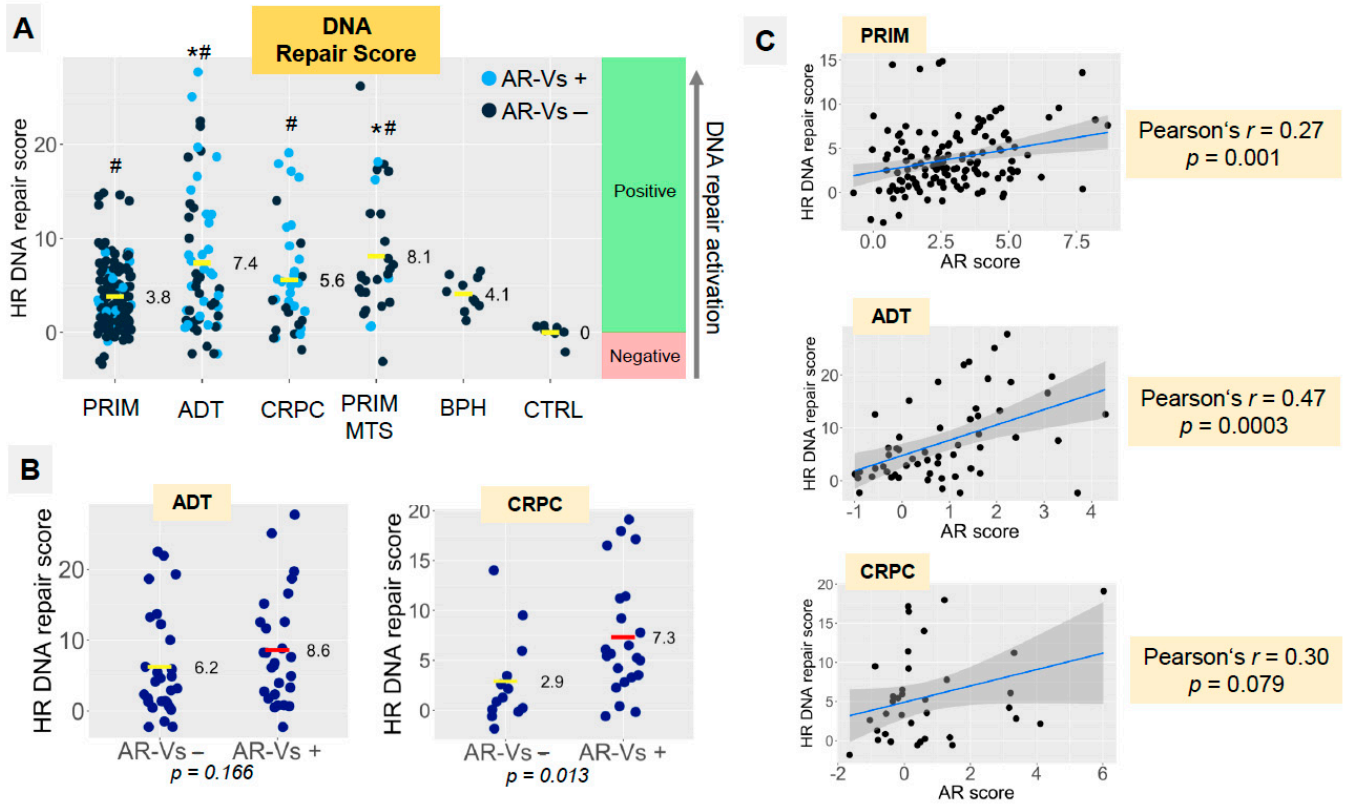



**Figure 6.** (**A**) Distribution of DNA repair scores in study groups (cumulative score based on mRNA expression of 20 DNA repair genes). Light blue points express any of the AR-V splice variants (V1, V3, V7, V9); dark blue points do not express AR-V splice variants. (**B**) Dependence of DNA repair score on the presence of AR-V splice variants (V1, V3, V7, V9) in ADT and CRPC groups. *p*-levels calculated using Mann–Whitney U-test. Statistical significance ($p < 0.05$): * vs. PRIM group, # vs. CTRL group. (**C**) Correlation analysis shows dependence of the AR score and DNA repair score in PRIM, ADT and CRPC groups.

**Table 2.** Estimates of associations between AR splice variants, proliferation and DNA repair, as obtained from fitting group-wise linear regression models with the DNA-R score as a dependent variable and splice variant and proliferation scores as independent variables. * $p < 0.05$, *** $p < 0.0005$.

| **AR splice variants vs. DNA repair.** | | | | |
|---|---|---|---|---|
| | **Patients** | **Coefficient Estimate** | **SD** | ***p*-Value** |
| ADT | 88 | 0.008013 | 0.003624 | 0.0297 * |
| ADT nonCRPC | 53 | 0.007468 | 0.004472 | 0.101 |
| CRPC | 35 | 0.012147 | 0.006103 | 0.0551 |
| Prim | 136 | 0.080467 | 0.038329 | 0.0377 * |
| **AR splice variants vs. proliferation.** | | | | |
| | **Patients** | **Coefficient Estimate** | **SD** | ***p*-Value** |
| ADT | 88 | 0.028671 | 0.002070 | $<2 \times 10^{-16}$ *** |
| ADT nonCRPC | 53 | 0.030869 | 0.002317 | $<2 \times 10^{-16}$ *** |
| CRPC | 35 | 0.023398 | 0.003974 | $1.5 \times 10^{-6}$ *** |
| Prim | 136 | 0.030435 | 0.002304 | $<2 \times 10^{-16}$ *** |

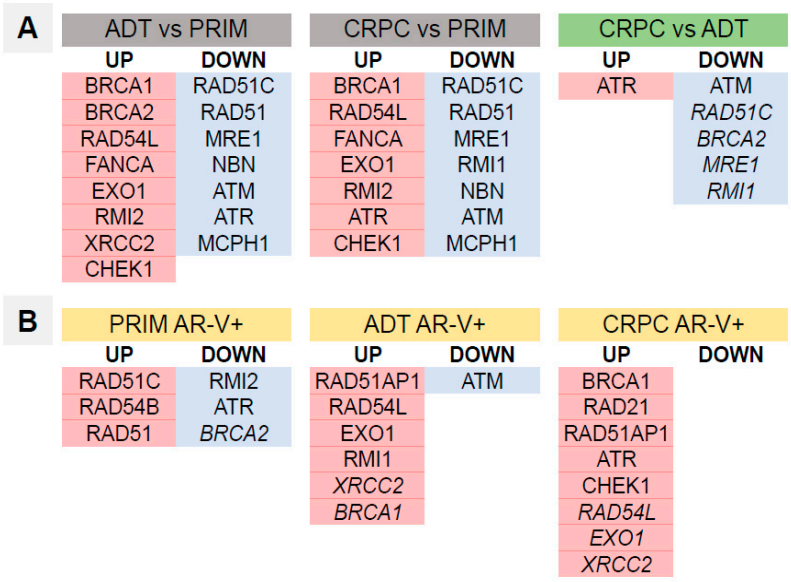



**Figure 7.** DNA repair genes differentially expressed in samples from different ADT, CRPC and PRIM groups ($p < 0.05$; in italic—$p = 0.05$–$0.1$) in relation to AR-V expression. Detailed expression analysis of individual DNA repair genes is provided in Supplementary Figures S4–S7. Upregulated genes are shown with a red background and downregulated genes with a blue background. (**A**) Cumulative analysis of all samples in study groups independent of AR-V status. (**B**) Analysis of genes affected in samples positive for any of AR-V splice variants (V1, V3, V7, V9) compared to those without AR-V splice variant expression.

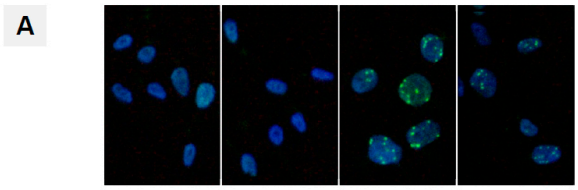
It is known that DNA repair genes are also involved in various aspects of cell cycle progression. Gene ontology (GO) analyses of our gene set further validated the involvement of our gene set in DNA repair, as compared to the cell cycle and mitotic processes (Supplementary Figure S11A). We found BRCA1 genes as well as the BRCA2 cluster upregulated in processes related to DNA repair, but only to a low extent were they involved in cell cycle-related pathways. Ranking the pathways using GO Panther hierarchical cluster analysis (Supplementary Figure S11B) revealed our gene set to be significantly linked to multiple DNA repair pathways (DNA repair, double-strand break repair, double-strand break repair via homologous recombination, cellular response to DNA damage stimulus, recombinational repair, DNA recombination), followed by cell cycle pathways. It is striking that DDR pathways showed up to five times higher "fold enrichment" (13.91–62.22) compared to cell cycle pathways (9.25–13.34).

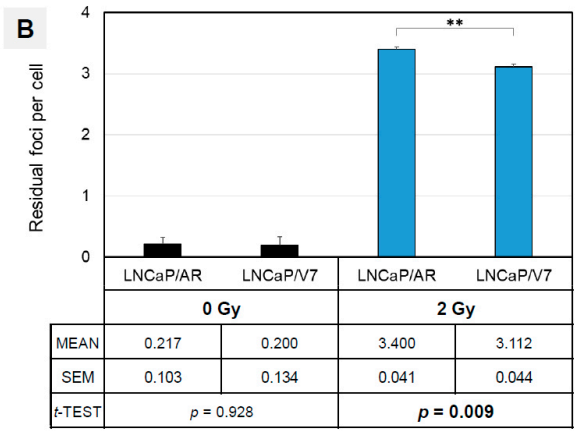
*PARP1* and *PARP2* mRNA expression was not found to correlate with the AR score in any of the groups. Both *PARP1* and *PARP2* were statistically significantly downregulated in the CRPC group compared to primary tumors (both $p < 0.05$; Supplementary Figure S8), where were not dependent on the presence of AR-Vs.

### *3.5. AR-V7 Enhances DNA Double-Strand Break Repair in an In Vitro PCA Model*

The induction of DNA repair genes by AR splice variants can be measured by γH2A.X foci formation after the introduction of double-strand breaks. We used the LNCaP/V7 PCA in vitro tumor model for irradiation and assayed for γH2A.X foci formation over time. In contrast to primary foci, which correlate in number with DSBs, residual foci indicate the number of DBSs in the repair process [32]. We screened for foci formation at three timepoints (1 h, 24 h and 48 h after irradiation) and verified a strong induction of γH2AX focus formation at the early timepoint (1 h), a gradual decline of foci after 24 h and an almost complete loss of detectable foci 2 days after irradiation (Supplementary Figure S12). Compared to LNCaP cells overexpressing AR-FL (Supplementary Figure S10), we observed a modest but significantly reduced number of residual foci 24 h after irradiation (−8.5%,

$p < 0.01$) in the presence of AR-V7, indicating accelerated DNA repair in LNCaP cells containing this AR splice variant [32] (Figure 8). Consequently, the presence of AR-V7 in PCA cells improves DNA repair provoked by X-ray irradiation.




| | 0 Gy | | 2 Gy | |
|---|---|---|---|---|
| MEAN | 0.217 | 0.200 | 3.400 | 3.112 |
| SEM | 0.103 | 0.134 | 0.041 | 0.044 |
| *t*-TEST | $p = 0.928$ | | **$p = 0.009$** | |

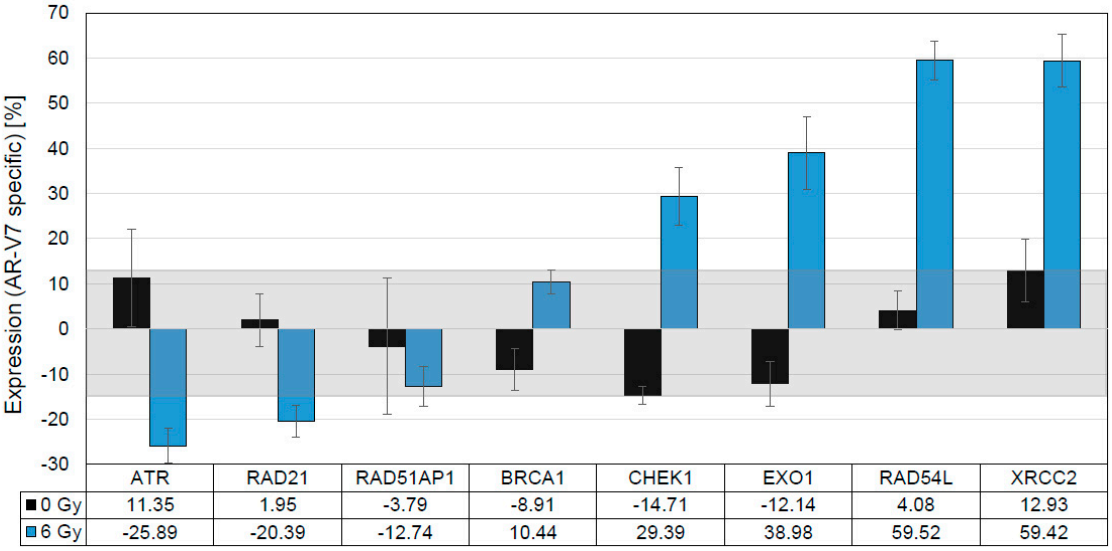
**Figure 8.** (**A**) The presence of AR-V7 enhances DNA repair in vitro. Increased DNA repair is visualized by a diminished number of residual γH2Ax foci 24 h after irradiation. Nuclear counterstain with DAPI from left to right: LNCaP/AR (0 Gy), LNCaP/V7 (0 Gy), LNCaP/AR (2 Gy), LNCaP/V7 (2 Gy). (**B**) Quantification of residual γH2A.X foci 24 h after irradiation (2 Gy, blue). Non-irradiated cells were used for comparison (0 Gy, black). In total, >200 nuclei were counted per cell line, irradiative condition and experiment, with a mean of three independent experiments. ** = $p < 0.01$.

### *3.6. In Vitro Validation of Findings in Clinical Samples*

With the first proof that AR-V7 expression in tumor cells enhances DNA repair, our next step was to investigate the AR-V7-specific regulation of DNA repair gene expression (mRNA). DNA repair gene upregulation was observed in CRPC tumors (Figure 7B) expressing androgen receptor splice variants (CRPC AR-V+) in comparison to ADT refractory tumors in the absence of AR-Vs. We translated the CRPC AR-V+ phenotype in our tumor model to LNCaP cells expressing AR-V7, while androgen-resistant LNCaP cells overexpressing AR-FL mimicked the reference cohort (CRPC) [33]. We induced DNA damage in order to study the transcriptional regulation of DNA repair genes in vitro 6 h after irradiation. We observed only a minimal alteration of DNA repair genes in unirradiated reference samples of both LNCaP/AR and LNCaP/V7 cells. This finding disproves the assumption of a gross impact of AR-V7 on repair genes prior to DNA damage (Figure 9). Upon irradiation, however, a set of four genes (CHEK1, EXO1, RAD54L, XRCC2) was strongly upregulated, specifically in cells expressing AR-V7, confirming and validating the expression data generated from clinical samples, as described above. These similarities point to the representative nature of LNCaP/V7 cells as an in vitro model of CRPC tumor cells that express AR splice variants (CRPC AR-V+, Figure 7B).

A similar in vitro analysis was performed to corroborate the results achieved by comparing clinical CRPC and PRIM sub-cohorts (CRPC vs. PRIM; Figure 7A), where alterations in DNA repair gene expression were associated with the CRPC phenotype. *In vitro*, clinical CRPC was phenocopied by our LNCaP/V7 cell line, while the PRIM phenotype was represented by androgen-responsive LNCaP cells. As a primary result in the absence of DNA damage, we observed minor alterations in DNA repair gene mRNA expression. DNA repair induced by double-strand breaks resulted in a substantial shift in a

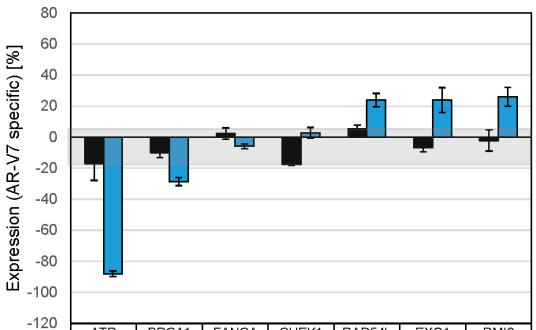
subset of genes (RAD54L, EXO1, RMI2; Figure 10A), earlier identified as upregulated in clinical CRPC samples. Three additional genes (ATM, NBN, MCPH1) were confirmed as downregulated in vitro in the LNCaP/V7 CRPC model (Figure 10B). The genes identified by in vitro analyses can be equally assigned to either the group of DNA damage sensors (ATM, CHEK1/CHK1, MCPH1, NBN) or HR repair genes (EXO1, RAD54L, RMI2, XRCC2), in accordance with published data [3,5,8,34,35].




| | ATR | RAD21 | RAD51AP1 | BRCA1 | CHEK1 | EXO1 | RAD54L | XRCC2 |
|---|---|---|---|---|---|---|---|---|
| 0 Gy | 11.35 | 1.95 | -3.79 | -8.91 | -14.71 | -12.14 | 4.08 | 12.93 |
| 6 Gy | -25.89 | -20.39 | -12.74 | 10.44 | 29.39 | 38.98 | 59.52 | 59.42 |

**Figure 9.** Validation of upregulated DNA repair genes of the AR splice variant expressing CRPC. LNCaP overexpressing AR-V7 served as a surrogate for CRPCs expressing AR-Vs. DNA repair genes upregulated in CRPC+AR-Vs (Figure 7B) were validated by qRT-PCR under irradiated (6 Gy, IR, blue bars) and non-irradiated (0 Gy, Ø, black bars) androgen-deprived conditions. The latter condition, with generally lower AR-V7-specific expression, served as the threshold (light gray box) to identify genes (CHEK1, EXO1, RAD54L, XRCC2) strongly upregulated by irradiation.

**A**




| | ATR | BRCA1 | FANCA | CHEK1 | RAD54L | EXO1 | RMI2 |
|---|---|---|---|---|---|---|---|
| 0 Gy | -17.07 | -9.99 | 2.26 | -17.63 | 5.29 | -6.71 | -2.19 |
| 6 Gy | -88.12 | -28.61 | -5.86 | 2.71 | 23.89 | 23.86 | 26.01 |

**Figure 10.** *Cont.*

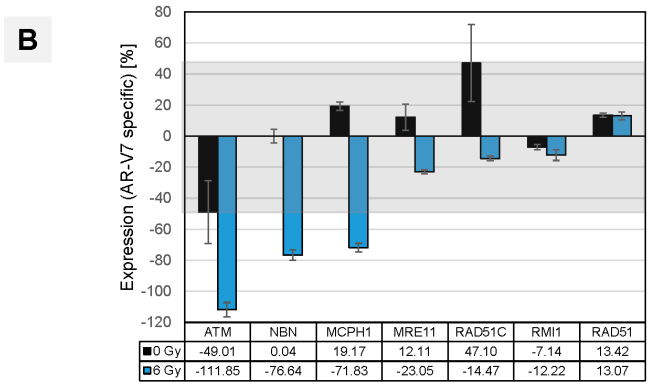



**Figure 10.** (**A**) Impact of AR splice variants in clinical CRPC via the validation of deregulated DNA repair genes in vitro. LNCaP cells overexpressing AR-V7 served as a surrogate for CRPCs expressing AR splice variants. DNA repair genes deregulated in CRPC vs. PRIM (Figure 7A) were validated by qRT-PCR under irradiated (6 Gy, IR, blue bars) and non-irradiated (0 Gy, Ø, black bars), androgen-deprived conditions. The latter condition, with generally lower AR-V7-specific expression, served as the threshold (light grey box) to identify genes visibly upregulated by irradiation such as RAD54L, EXO1, RMI2. (**B**) Under the same experimental conditions, genes such as ATM, NBN, MPCH1 were confirmed as downregulated.

## 4. Discussion

The recognition of the commonly impaired DNA damage response by defects in HR has enabled new, targeted therapeutic interventions in many malignant tumors, including PCA [9,36]. The connection between the AR pathway and DNA repair in PCA proved to be so tight that, even in the absence of pathogenic mutations in DNA repair genes, there is a possibility of targeted therapeutic interventions using PARPi. ADT causes the significant downregulation of DNA repair genes given direct and indirect transcriptional regulation of the latter through AR [1–3,5–8,15,37]. This functional impairment of HR is sufficient to induce synthetic lethality under treatment with PARPi. Several major studies [5,6] provided a proof of principle for such synthetic lethality in cell line experiments, and two clinical trials have been conducted to date on patients with CRPC. A pilot study (NCI 9012) showed no differences in response rates between CRPC cohorts receiving abiraterone versus abiraterone/veliparib [38]. Abiraterone/olaparib, however, was effective in unselected patients with CRPC compared to abiraterone only [11], in particular, in a subgroup of patients with pathogenic mutations in HR DNA repair genes (NCT03732820) [39].

These studies [5,6] of synthetic lethality [11,38] analyzed AR function in the context of mutant DNA repair genes in CRPC tumors. AR splice variants showed activating effects in DNA repair genes similar to full-length androgen receptors [3,8], thus provoking the examination of the effect of AR-Vs on DNA repair genes in a clinical setting.

An appropriate in vitro model system has to fulfill several aspects to closely mimic the physiological situation of AR-V7-expressing PCA tumor cells [40]. The expression of AR-V7 is only observed in the context of full-length AR. The choice of an LNCaP cell model with high endogenous AR-FL expression caused by lentiviral transduction avoids clonal effects. To compensate for higher total AR expression in LNCaP/V7, we generated an LNCaP control cell line that overexpresses full-length AR (LNCaP/AR). Original LNCaP cells harbor the AR T878A mutation [41], which is known to cause aberrant AR behavior. However, the use of steroid-depleted media minimizes the effect of mutant AR signaling, and comparison with the control cell line LNCaP/AR, which overexpresses the wild-type full-length androgen receptor, ensures the analysis of AR-V7-specific effects in this in vitro tumor model. Hence, we are confident that our experimental setup allows for the analysis of AR-V7-specific effects in DDR even in the presence of the endogenous AR T878A mutation in LNCaP cells.

Cell lines such as VCaP might be used to analyze endogenous AR-V7 effects on DNA repair. However, VCaP cells are infected with and secrete the retrovirus Bxv-1, a xenotropic

mouse leukemia virus, as shown by Sfanos et al. [42]. In the course of retroviral infection, γH2AX foci are detectable at sites of proviral integration [43], limiting the use of this assay to study DNA repair in VCaP cells.

Thus, we cannot exclude the impact of this virus on cellular physiology and AR-FL/AR-V7-signaling in these cells. We chose the transgenic approach using LNCaP cells, as these cells are not known to produce any viruses, closely mimicking the clinical situation.

To the best of our knowledge, this is the first study to analyze the link between AR pathway activation, the presence of AR splice variants and the transcription of DNA repair genes in multiple subgroups of clinical PCA tumor samples that reflect the development of ADT resistance. While the upregulation of AR-V7 under ADT treatment in patient samples was already reported by Sharp et al. [21], our approach provides data for those subgroups, including a CRPC specimen. Additionally, these data were substantiated by cell line models, mimicking the CRPC phenotype of the clinical samples. Importantly, we provide evidence that crucial components of the DNA repair machinery might be induced in the presence of AR-Vs in CRPC. Thus, approximately two-thirds of patients with CRPC and half of the patients on ADT (but still hormone-sensitive) in our study harbored AR-V1, -V3, -V7, and -V9. Additionally, our study demonstrated the presence of AR-Vs is correlated to the increased activity of the AR pathway measured by the AR score in CRPC. Furthermore, the cellular proliferation index doubled in CRPC in the presence of AR-Vs as measured by MKI67 expression (Figure 4C,D).

This study shows that the expression of DNA repair genes, summarized as a DNA repair score, is 2.5 times higher in the presence of AR-Vs in CRPC (Figure 6B,C). This validates the results of previous experimental studies, which showed that, even in the absence of AR-FL, AR splice variants can provide the necessary transcriptional support for DNA repair genes [3] and the loss of AR-Vs sensitizes them to ionizing radiation [8]. As was demonstrated by Luo et al. [23], AR-V7 significantly promotes the DDR of PCA cells under severe DNA damage. The impact of AR-Vs on the DDR machinery may have clinical implications, as the activity of DNA repair in CRPC in the presence of AR-V splice variants seems to be similar or even higher than in hormone-naïve tumors. This may explain the rather inconclusive findings concerning the combination therapy of ADT with PARPi [11,38], implying that AR-Vs may be predictive of the efficacy of synthetic lethality-based therapeutic regimens (in a negative way), and this should be clarified in further studies.

The expression signature of DNA repair genes in primary tumors differs from that of tumors under ADT and CRPC (Figure 7). Most of the genes analyzed are similarly affected in ADT and CRPC samples: among upregulated genes (BRCA1, RAD54L, FANCA, EXO1, RMI2, XRCC1, CHEK1), the majority were found to have the same tendency in CRPC tumors in studies by Taylor et al. [44] and Grasso et al. [45] Only RAD51 was downregulated in our study, but it was upregulated in the aforementioned studies. Interestingly, the majority of regulated genes in our study (ADT and CRPC) were shown to be transcriptionally downregulated by androgen blockade in experimental studies [1–3,6–8,15,37], contradicting the findings of Taylor et al. [44], Grasso et al. [45] and our results. Our study provides evidence that AR-Vs may account for this discrepancy, as the genes induced by AR-Vs largely overlap with regulated genes in tumors under androgen depletion or CRPCs (compared to hormone-naïve tumors; Figure 7A,B).

Specifically, BRCA1, RAD54L, EXO1 and CHEK1 are identically upregulated. It is now tempting to draw the following conclusions. First, elevated levels of AR-Vs detectable during ADT provide transcriptional support for DNA repair gene activity. This is corroborated by two experimental studies utilizing PCA cell lines [3,8]. Second, the activating effect of AR-Vs applies to some, but not all, DNA repair genes. Indeed, AR-Vs and AR-FL transcriptomes are, to some extent, different [31], even in regard to DNA damage response genes [1,8,34,35]. However, the precise differences in the transcriptional effects of AR-FL and AR-Vs concerning DNA repair are, to date, understudied by far and warrant further investigations.

*PARP1* and *PARP2* were characterized earlier as important effectors of AR, functioning in positive regulatory loops [6–8,15]. In our study, mRNA expression in both genes was, to some extent, downregulated in CRPC samples compared to hormone-naïve tumors (Supplementary Figure S8) but did not show any dependency on AR splice variant expression. This could be related to the fact that mRNA expression in these genes is not a reliable measure of PARylation activity, which was also shown earlier [6].

Our analysis of clinical CRPC samples demonstrates the specific upregulation of DNA repair genes in two clinical settings: CRPC vs. PRIM and CRPC AR-V+ vs. CRPC AR-V-. This points to the initiation of DNA damage repair (DDR) in CRPC tumors. However, the precise mechanisms leading to AR-V-mediated modulations in DDR remain largely unknown. In our study, we constructed two in vitro cell line models simulating clinical CRPC tumor phenotypes with and without AR-V7 overexpression, derived from the initially hormone-sensitive LNCaP cell line (corresponding to the hormone-naïve phenotype in our experimental setup). In an in vitro CRPC model using LNCaP cells that overexpress AR-V7, DDR was induced by ionizing radiation. The presence of AR-V7 yielded a significant reduction in γH2A.X foci, which mark positions in the genome with actively ongoing DDR [32]. The resulting superior DDR has a beneficial impact on AR-V7 tumor cells. Upon DNA damage induced by irradiation, AR is known to translocate to the nucleus and initiate the expression of DNA repair genes such as XRCC2 [2]. AR splice variants such as AR-V7 are already located at a high fraction in the nuclei of primary PCA cells [46], while AR-FL remains cytoplasmatic in the absence of activating ligands. An augmented transcriptional activation of DNA repair genes in the presence of AR-Vs, therefore, appears plausible and was substantiated by our PCA tumor model.

DNA repair genes with de-regulated expression in CRPC clinical samples can either be classified in the group of DNA damage sensors (ATM, CHEK1/CHK1, MCPH1, NBN) or HR genes (EXO1, RAD54L, RMI2, XRCC2). Their altered expression was confirmed by our in vitro analyses. These data indicate that AR-Vs indeed play a multidirectional role in augmenting DDR in CRPC tumors (Figures 8–10) and mirror the findings of Yin et al., 2017 [3], who reported a causal link between AR-Vs and DNA repair after irradiation.

Furthermore, we confirmed three genes (ATM, NBN and MCPH1) to be downregulated in clinical CRPC tumor samples in our in vitro models. Various publications reported a downregulation of genes with a potential tumor-suppressive function in DNA repair [40,47], with ATM being one of the crucial and most thoroughly studied. One publication showed an association between NBN mutations and high-grade PCA in a Polish patient cohort [48], suggesting a tumor-suppressive function in this gene in normal prostate tissue. MCPH1, also downregulated in our clinical CRPC tumor samples, was previously reported to be downregulated in prostate carcinoma [49]. MCPH1 is functionally tightly associated with ATM and NBN. MCPH1 recruits ATM and NBN to DNA damage repair foci as part of the early DNA damage response [50]. The functional loss of ATM or NBN, two genes that are downregulated in our study, is associated with poor survival in PCa patients [50–52] and sensitizes PARP inhibitor therapies. Androgen receptor splice variant expression, in contrast, appears to reactivate DNA damage repair gene expression previously thwarted by ADT. In summary, our experiments using the AR-V7 in vitro PCA model suggest that the PARPi sensitization of CRPC tumors will likely not occur in the presence of AR-Vs [8], despite the observed loss of ATM expression. To the best of our knowledge, for the first time, the AR-V-dependent alteration of DDR gene expression has been shown in CRPC patients (Figure 7), and accelerated DDR in an AR-V7-dependent CRPC tumor model was confirmed.

In contrast to our expectations, we found an independent impact of AR-V expression on DDR gene expression in the clinical cohort. The employment of a linear regression model provided not only a significant positive association of AR-V expression with proliferation but also independently for DNA repair gene expression in primary tumors (Table 2). Calculations for the other groups resulted in corresponding associations, concluding that AR-Vs have an independent impact on DDR gene expression. Corroborating these findings

in vitro, analogous alterations of DDR gene expression, as found in the clinical samples, appeared within a timeframe that excludes the involvement of proliferative aspects of the functionality of AR-V7.

This study is not devoid of limitations, as DNA repair is a very complex process. Other important components of the DNA repair system, such as Ku70 protein [53], DNA protein kinase catalytic subunit [54] and some other genes, which have been shown to be AR-dependent, were not studied. Several other AR rearrangements (gene amplification and mutations) were also not targets of our study.

## 5. Conclusions

This study confirms the tight interconnection between AR signaling, alterations in AR expression and the transcription of DNA repair genes in clinical tumor samples and in vitro prostate cancer models. Of particular importance is the modulation of DNA repair gene expression in the presence of AR splice variants in CRPC. The expression of AR splice variants might be a reason for the reduced or absent effect of therapeutic concepts exploiting the principle of synthetic lethality between ADT and PARP inhibition. Thus, AR-Vs show potential as predictive biomarkers for the efficacy of PARPi therapy, as previously suggested [23].

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cancers14184441/s1. Table S1: CodeSet gene panel used in the study with probe sequences for RNA expression analysis. Table S2: Primer sequences used for qPCR analysis. Table S3: Validation of DNA repair genes by qRT-PCR analysis that were significantly deregulated in clinical samples (CRPC + AR-V). Table S4: Validation of DNA repair genes by qRT-PCR analysis that were significantly deregulated in clinical samples (CRPC vs PRIM). Figure S1: MKI67 and PCNA mRNA expression in study groups. Figure S2: UBE2C mRNA expression in study groups. Figure S3: mRNA expression of 20 DNA repair genes in study groups and correlation of DNA repair and AR scores. Figure S4: mRNA expression of 20 DNA repair genes in dependence on AR-V status in the PRIM group. Figure S5: mRNA expression of 20 DNA repair genes in dependence on AR-V status in the CRPC group. Figure S6: mRNA expression of 20 DNA repair genes in dependence on AR-V status in the ADT group. Figure S7: mRNA expression of 20 DNA repair genes in dependence on AR-V status. Figure S8: mRNA expression of PARP1 (A) and PARP2 (B) in study groups. Figure S9: Separation of the impact of AR splice variants on proliferation and DNA repair using a linear regression model. Figure S10: Expression of total androgen receptor and splice variant 7 in the LNCaP/AR in vitro tumor models. Figure S11: Gene ontology analysis of genes de-regulated in clinical samples reveals association with DNA repair. Figure S12: AR-V7 improves DNA repair after irradiation in an in vitro tumor model.

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**Informed Consent Statement:** This is a retrospective study with tissue samples from anonymized patients who are not identifiable from the data set. Written informed consent is not available for the patients of this data set.

**Data Availability Statement:** Data generated in this study are available upon request.

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**Table S1: CodeSet gene panel used in the study with probe sequences for RNA expression analysis.** Genes involved in this analysis are listed below with symbols, position of the Nanostring probe within the mRNA sequence and probe sequence. NCBI accession numbers are used for genes, ensemble transcript identifiers are used for AR isoforms. Housekeeping genes are marked in green, genes involved in DNA repair are in blue. All other genes, including AR-V genes, are labeled in black.

| Gene symbol | Accession # | Position | Sequence |
|---|---|---|---|
| ABCF2 | NM_007189.1 | 1541-1640 | TCTCACCTTTGGAGTACATGATGAAGTGCTACCCAGAGATCAAGGAGAAGGAAGAAATGAGGAAGATCATTGGGCGATACGGTCTCACTGGGAAACAACA |
| AGR2 | NM_006408.3 | 581-680 | GACCCATCTCTGACAGTTAGAGCCGATATCACTGGAAGATATTCAAATCGTCTCTATGCTTACGAACCTGCAGATACAGCTCTGTTGCTTGACAACATGA |
| ALAS1 | NM_000688.4 | 396-495 | AGAAAGCAGGCAAATCTCTGTTGTTCTATGCCCAAAACTGCCCCAAGATGATGGAAGTTGGGGCCAAGCCAGCCCCTCGGGCATTGTCCACTGCAGCAGT |
| AR-V9 | AR_V9.1 | 30-129 | AAATGTTATGAAGCAGGGATGACTCTGGGAGGACAACTTACCTGAGCAAGCTGCTTTTTGGAGACATTTGCACATCTTTTGGGATCACGTT |
| AR-V3 | ENST00000514029.1 | 2050-2149 | CTCACATGTGGAAGCTGCAAGGTCTTCTTCAAAAGAGCCGCTGAAGGATTTTTCAGAATGAACAAATTAAAAGAATCATAATCAGACACTAACCCCAAGC |
| AR-V7 | ENST00000514029. | 12446-2545 | GTCCATCTTGTCGTCTTCGGAAATGTTATGAAGCAGGGATGACTCTGGGAGAAAAATTCCGGGTTGGCAATTGCAAGCATCTCAAAATGACCAGACCCTG |
| AR-FL | NM_000044.4 | 3081-3180 | TGCTCAAGACGCTTCTACCAGCTCACCAAGCTCCTGGACTCCGTGCAGCCTATTGCGAGAGAGCTGCATCAGTTCACTTTTGACCTGCTAATCAAGTCAC |
| AR45 | NM_001011645.3 | 2278-2377 | TGCTCCTGACATTGCCTGTCACTTTTTCCCATGATACTCTGGCTTCACAGTTTGGAGACTGCCAGGGACCATGTTTTGCCCATTGACTATTACTTTCCAC |
| AR-V1 | NM_001348063.1 | 2368-2467 | GTCGTCTTCGGAAATGTTATGAAGCAGGGATGACTCTGGGAGCAGCTGTTGTTGTTTCTGAAAGAATCTTGAGGGTGTTTGGAGTCTCAGAATGGCTTCC |
| ARv567es | GU208210.1 | 2139-2238 | ACTGGGAGAGAGACAGCTTGTACACGTGGTCAAGTGGGCCAAGGCCTTGCCTGATTGCGAGAGAGCTGCATCAGTTCACTTTTGACCTGCTAATCAAGTC |
| ARF1 | NM_001024227.1 | 1371-1470 | CAATTCTGCATGGTCACAGTAGAGATCCCCGCAACTCGCTTGTCCTTGGGTCACCCTGCATTCCATAGCCATGTGCTTGTCCCTGTGCTCCCACGGTTCC |
| ATM | NM_138292.3 | 1324-1423 | TGAAGATAAAGAACTTCAGTGGACCTTCATAATGCTGACCTACCTGAATAACACACTGGTAGAAGATTGTGTCAAAGTTCGATCAGCAGCTGTTACCTGT |
| ATR | NM_001184.2 | 566-665 | AAGACTTGGTTTACCTCCATAGAAGAAATGTGATGGGTCATGCTGTGGAATGGCCAGTGGTCATGAGCCGATTTTTAAGTCAATTAGATGAACACATGGG |
| BRCA1 | NM_007305.2 | 1276-1375 | CATTAGATGATAGGTGGTACATGCACAGTTGCTCTGGGAGTCTTCAGAATAGAAACTACCCATCTCAAGAGGAGCTCATTAAGGTTGTTGATGTGGAGGA |
| BRCA2 | NM_000059.3 | 116-215 | GGGGACAGATTTGTGACCGGCGCGGTTTTTGTCAGCTTACTCCGGCCAAAAAAGAACTGCACCTCTGGAGCGGACTTATTTACCAAGCATTGGAGGAATA |
| CHEK1 | NM_001114121.1 | 2226-2325 | AGGGTGATGGATTGGAGTTCAAGAGACACTTCCTGAAGATTAAAGGGAAGCTGATTGATATTGTGAGCAGCCAGAAGATTTGGCTTCCTGCCACATGATC |
| CHEK2 | NM_001005735.1 | 895-994 | GGAGAGGTAAAGCTGGCTTTCGAGAGGAAAACATGTAAGAAAGTAGCCATAAAGATCATCAGCAAAAGGAAGTTTGCTATTGGTTCAGCAAGAGAGGCAG |
| DMC1 | NM_007068.2 | 901-1000 | GCAGCAAAAATTGGCCCAGATGTTGTCACGACTCCAAAAAATCTCAGAAGAATATAACGTGGCTGTTTTTGTGACCAATCAAATGACTGCCGATCCAGGA |
| ECI2 | NM_006117.2 | 941-1040 | AGTTAACAGCGGGAGAGGCATGTGCTCAAGGACTTGTTACTGAAGTTTTCCCTGATAGCACTTTTCAGAAAGAAGTCTGGACCAGGCTGAAGGCATTTGC |
| ERG | NM_001136155.1 | 342-441 | GCGGTGAAAGAATATGGCCTTCCAGACGTCAACATCTTGTTATTCCAGAACATCGATGGGAAGGAACTGTGCAAGATGACCAAGGACGACTTCCAGAGGC |
| EXO1 | NM_003686.3 | 2716-2815 | GCCAGAGCCAGTGGGCTGAGCAAGAAGCCGGCAAGCATCCAGAAGAGAAAGCATCATAATGCCGAGAACAAGCCGGGGTTACAGATCAAACTCAATGAGC |
| FANCA | NM_000135.2 | 799-898 | CTGAGAAGAACTGTGGAGCCTGAAAAAATGCCCGCAGGTCACGGTTGATGTACTGCAGAGAATGCTGATTTTTGCACTTGACGCTTTGGCTGCTGGAGTAC |
| FASN | NM_004104.4 | 5388-5487 | GAGGTGCTTGGCTACGCACGGTCGCTTCCTGGAAATTGGCAAATTCGACCTTTCTCAGAACCACCCGCTCGGCATGGCTATCTTCCTGAAGAACGTGACA |
| GDF15 | NM_004864.2 | 181-280 | ACTCCAGATTCCGAGAGTTGCGGAAACGCTACGAGGACCTGCTAACCAGGCTGCGGGCCAACCAGAGCTGGGAAGATTCGAACACCGACCTCGTCCCGGC |
| GFM1 | NM_024996.5 | 1611-1710 | GAAATCTATGCTCAGAGGCTGGAAAGAGAGTATGGCTGTCCTTGTATCACAGGAAAGCCAAAAGTTGCCTTTCGAGAGACCATTACTGCCCCTGTCCCGT |
| HPRT1 | NM_000194.1 | 241-340 | TGTGATGAAGGAGATGGGAGGCCATCACATTGTAGCCCTCTGTGTGCTCAAGGGGGGCTATAAATTCTTTGCTGACCTGCTGGATTACATCAAAGCACTG |
| KLK3 | NM_001030049.1 | 434-533 | GTGTGTGGACCTCCATGTTATTTCCAATGACGTGTGTGCGCAAGTTCACCCTCAGAAGGTGACCAAGTTCATGCTGTGTGCTGGACGCTGGACAGGGGGC |
| MCPH1/ BRIT1 | NM_024596.2 | 643-742 | CCACCTCTTCCCAAATGATTCAGCAGTCTCATGATAATCCAAGTAACTCTCTGTGTGAAGCACCTTTGAACATTTCACGTGATACTTTGTGTTCAGATGA |
| MKI67 | NM_002417.2 | 4021-4120 | AGCAGATGTAGAGGGAGAACTCTTAGCGTGCAGGAATCTAATGCCATCAGCAGGCAAAGCCATGCACACGCCTAAACCATCAGTAGGTGAAGAGAAAGAC |
| MRE1 | NM_005591.3 | 506-605 | TTCCATGGGTGAACTATCAAGATGGCAACCTCAACATTTCAATTCCAGTGTTTAGTATTCATGGCAATCATGACGATCCCACAGGGGCAGATGCACTTTG |
| NBN | NM_002485.4 | 1061-1160 | GACTACAAAGAATTACTGTGATCCTCAGGGCCATCCCAGTACAGGATTAAAGACAACAACTCCAGGACCAAGCCTTTCACAAGGCGTGTCAGTTGATGAA |
| PARP1 | NM_001618.3 | 3017-3116 | AAGGTTTGGGCAAAACTACCCCTGATCCTTCAGCTAACATTAGTCTGGATGGTGTAGACGTTCCTCTTGGGACCGGGATTTCATCTGGTGTGAATGACAC |
| PARP2 | NM_005484.3 | 1155-1254 | GTTATGAGTTCAAAGTGATTTCCCAGTACCTACAATCTACCCATGCTCCCACACACAGCGACTATACCATGACCTTGCTGGATTTGTTTGAAGTGGAGAA |
| PCNA | NM_002592.2 | 281-380 | GGTGTTGGAGGCACTCAAGGACCTCATCAACGAGGCCTGCTGGGATATTAGCTCCAGCGGTGTAAACCTGCAGAGCATGGACTCGTCCCACGTCTCTTTG |
| PGK1 | NM_000291.2 | 1031-1130 | GCAAGAAGTATGCTGAGGCTGTCACTCGGGCTAAGCAGATTGTGTGGAATGGTCCTGTGGGGGTATTTGAATGGGAAGCTTTTGCCCGGGGAACCAAAGC |
| PTEN | NM_000314.6 | 5345-5444 | CTTCAGATACTCTTGTGCTGTGCAGCAGTGGCTCTGTGTGTAAATGCTATGCACTGAGGATACACAAAAATACCAATATGATGTGTACAGGATAATGCCT |
| RAD21 | NM_006265.2 | 1081-1180 | GATGAGGATGATAATGTATCAATGGGTGGGCCTGATAGTCCTGATTCAGTGGATCCCGTTGAACCAATGCCAACCATGACTGATCAAACAACACTTGTTC |
| RAD51 | NM_133487.2 | 567-666 | AGACCACCAGACCCAGCTCCTTTATCAAGCATCAGCCATGATGGTAGAATCTAGGTATGCACTGCTTATTGTAGACAGTGCCACCGCCCTTTACAGAACA |
| RAD51AP1 | NM_001130862.1 | 1126-1225 | TCTGTGAAGTCTCCCAATCAGAGTCTCCGCCTTGGCTTGTCCAGATTAGCACGAGTTAAACCTTTGCATCCAAATGCCACTAGCACCTGAGTGTGGTACA |
| RAD51C | NM_002876.2 | 301-400 | GCACTGGAACTTCTTGAGCAGGAGCATACCCAGGGCTTCATAATCACCTTCTGTTCAGCACTAGATGATATTCTTGGGGGTGGAGTGCCCTTAATGAAAA |
| RAD54B | NM_012415.2 | 827-926 | TAGACAGAATGATTTCCAAAATTGCAAACCACGCCATGACCCATATACGCCAAATTCCCTCGTTATGCCACGACCAGATAAGAATCACCAGTGGGTATTC |
| RAD54L | NM_003579.2 | 1436-1535 | ATGAACCAGCGTGGAGCCAGGGTGTCTTCTCCCATCCTCATCATTTCCTATGAGACCTTCCGCCTTCATGTTGGAGTCCTCCAGAAAGGAAGTGTTGGTC |
| RB1 | NM_000321.1 | 2111-2210 | CCTATCTCCGGCTAAATACACTTTGTGAACGCCTTCTGTCTGAGCACCCAGAATTAGAACATATCATCTGGACCCTTTTCCAGCACACCCTGCAGAATGA |
| RMI1 | NM_024945.2 | 528-627 | TGTTAACTTGAGTCAGGCCCAAATGAATAAACAAGTGTTTGAGCAGTGGCTCCTTACTGATCTGAGGGATTTGGAGCATCCTCTTTTACCCGATGGCATT |
| RMI2 | NM_152308.1 | 891-990 | AAGACAGACTGTGTAAAAAAGGAATGACATCCTGGCTCCTCATCTTCTTCATCAGCAACTACCATAACCAGTTTGCGAGTCAAATGGCATTTCCTAACGG |
| SEC61A1 | NM_013336.3 | 2246-2345 | TCTGTGCACCTATTGGCTCTTCTAGCTGACTCTTCTGGTTGGGCTTAGAGTCTGCCTGTTTCTGCTAGCTCCGTGTTTAGTCCACTTGGGTCATCAGCTC |
| STIL | NM_001048166.1 | 2477-2576 | GGAAGCACAGTCTTCCCCTGGCTTGCACATGAGAAAAGGTGTAAGCATTGCTGTGAGCACAGGTGCTAGCTTGTTTTGGAATGCAGCAGGTGAGGATCAA |
| TM4SF1 | NM_014220.2 | 96-195 | AATCGCAGTATTTAAGAGGTAGCAGGAATGGGCTGAGAGTGGTGTTTGCTTTCTCCACCAGAAGGGCACACTTTCATCTAATTTGGGGTATCACTGAGCT |
| TMPRSS2 | NM_005656.3 | 1080-1179 | AGACAATCTTTCATGTTCTATGGAGCCGGATACCAAGTAGAAAAAGTGATTTCTCATCCAAATTATGACTCCAAGACCAAGAACAATGACATTGCGCTGA |
| UBE2C | NM_181803.1 | 270-369 | CTTTTAAGAAGTACCTGCAAGAAACCTACTCAAAGCAGGTCACCAGCCAGGAGCCCTGACCCAGGCTGCCCAGCCTGTCCTTGTGTCGTCTTTTTAATTT |
| XRCC2 | NM_005431.1 | 537-636 | CTGTCAGCTTTTTACTGGATAGACCGCGTCAATGGAGGAGAAAGTGTGAACTTACAGGAGTCTACTCTGAGGAAATGTTCTCAGTGCTTAGAGAAGCTTG |

# Table S2: Primer sequences used for qPCR analysis

| Gene | FW-Primer | RV-Primer |
|---|---|---|
| ATM | TGGTGCTATTTACGGAGCTGA | AGCCTGAAGTACACAGAGAACA |
| ATR | AGGCCAAAGGCAGTTGTATTG | CAAATGACAGGAGGGAGTTGCT |
| BRCA1 | TGCGGGAGGAAAATGGGTAG | CTGGGATTCTCTTGCTCGCT |
| BRCA2 | GACTCTGCCGCTGTACCAAT | GTGGACAGGAAACATCATCTGC |
| CHEK1 | CATGGCAGGGGTGGTTTATCT | CGAAATACTGTTGCCAAGCCA |
| EXO1 | TGCGTGGGATTGGATTAGCA | TGGCCCGAATAAACCCGTTG |
| FANCA | GGACCTGAATGCCCTTTTGC | AGGCTTGATCCTGCAAAGCA |
| MCPH1/ BRIT1 | AAATCTTTCCCCCACCTCTTCC | ATGAGTGTAAGCCACCAGCA |
| MRE11 | TCCCAGAGGAGCTTGACTGA | CCTCTGACTGCATCTTTCTCCA |
| NBN | GCAGAAATTGGATTGGCGGT | AAGGCTTGGTCCTGGAGTTG |
| RAD21 | CAAAGCCCATGTGTTCGAGTG | GTCCTGATGTCCGTAATGCC |
| RAD51 | TCAACACAGACCACCAGACC | CTGAAAGCTCACCTCGACCC |
| RAD51AP1 | TGCGGCCTGTGAGACATAAG | TCCTTTGGTGCTGTTCTGGA |
| RAD51C | AAACCCTCCGAGCTTAGCAA | TGTGACTCAGATGTACCAGCA |
| RAD54B | ACTGTTTCCCTCTTGTGGATGT | AGCTCCACATCTGCCATTCA |
| RAD54L | GCTGGCCAAGAGAAAACCTG | ATCTGGGTCTCACTGCTGGA |
| RMI1 | TGTCATCAAATCCTGTGCTGCT | TGCAGCTAAAAGCCAAGTTTCA |
| RMI2 | CGGCCCTGTCTAGTCCCA | CCTCCAGTTCCCACATACTTTC |
| XRCC2 | GGGCGATGTGTAGTGCCTT | CTTCTACCTTCAAGTCGGGCA |
| PPIA | GCTGGACCCAACACAAATGG | GGCCTCCACAATATTCATGCCT |

**Table S3: Validation of DNA repair genes by qRT-PCR analysis that were significantly deregulated in clinical samples**. Comparison of androgen-resistant AR-V7 overexpressing cells (LNCaP/V7) with androgen-resistant AR-FL overexpressing cells (LNCaP/AR) mimics the results of the clinical data obtained for CRPC AR-V+ (**Fig. 6B**). DNA repair was provoked by irradiation and AR-V7 specific gene regulation (DCC IR) was analyzed by qRT-PCR using the $\Delta\Delta C_\tau$ method. Non-irradiated cells (DCC) were checked in parallel. Genes significantly upregulated in the CRPC AR-V+ analysis were marked.

| Gene | DCC | DCC IR | CRPC + AR-V |
|---|---|---|---|
| ATM | 28% | -57% | |
| ATR | 11% | -26% | UP |
| BRCA1 | -9% | 10% | UP |
| BRCA2 | -2% | 26% | |
| CHEK1 | -15% | 29% | UP |
| EXO1 | -12% | 39% | UP |
| FANCA | 20% | 43% | |
| MCPH1 | -12% | -58% | |
| MRE11 | 9% | -30% | |
| NBN | -13% | -49% | |
| RAD21 | 2% | -20% | UP |
| RAD51 | -23% | 28% | |
| RAD51AP1 | -4% | -13% | UP |
| RAD51C | 15% | -3% | |
| RAD54B | 9% | 23% | |
| RAD54L | 4% | 60% | UP |
| RMI1 | 2% | 16% | |
| RMI2 | 0% | 50% | |
| XRCC2 | 13% | 59% | UP |

**Table S4: Validation of DNA repair genes by qRT-PCR analysis that were significantly deregulated in clinical samples**. Comparison of androgen-resistant AR-V7 overexpressing cells (LNCaP/V7) with androgen-sensitive LNCaP wt cells mimics the results of the clinical data obtained for CRPC vs PRIM (**Fig. 6A**). DNA repair was provoked by irradiation and the impact of the AR-V7 splice variant on the CRPC phenotype (DCC IR) was analyzed by qRT-PCR using the $\Delta\Delta C_\tau$ method. Non-irradiated cells (DCC) were checked in parallel. Genes significantly deregulated in the CRPC vs PRIM analysis were marked.

| Gene | DCC | DCC IR | CRPC vs PRIM |
|---|---|---|---|
| ATM | -49% | -112% | DOWN |
| ATR | -17% | -88% | UP |
| BRCA1 | -10% | -29% | UP |
| BRCA2 | -2% | -24% | |
| CHEK1 | -18% | 3% | UP |
| EXO1 | -7% | 24% | UP |
| FANCA | 2% | -6% | UP |
| MCPH1 | 19% | -72% | DOWN |
| MRE11 | 12% | -23% | DOWN |
| NBN | 0% | -77% | DOWN |
| RAD21 | 4% | -25% | |
| RAD51 | 13% | 13% | DOWN |
| RAD51AP1 | 39% | 7% | |
| RAD51C | 47% | -14% | DOWN |
| RAD54B | -9% | -7% | |
| RAD54L | 5% | 24% | UP |
| RMI1 | -7% | -12% | DOWN |
| RMI2 | -2% | 26% | UP |
| XRCC2 | 3% | 23% | |

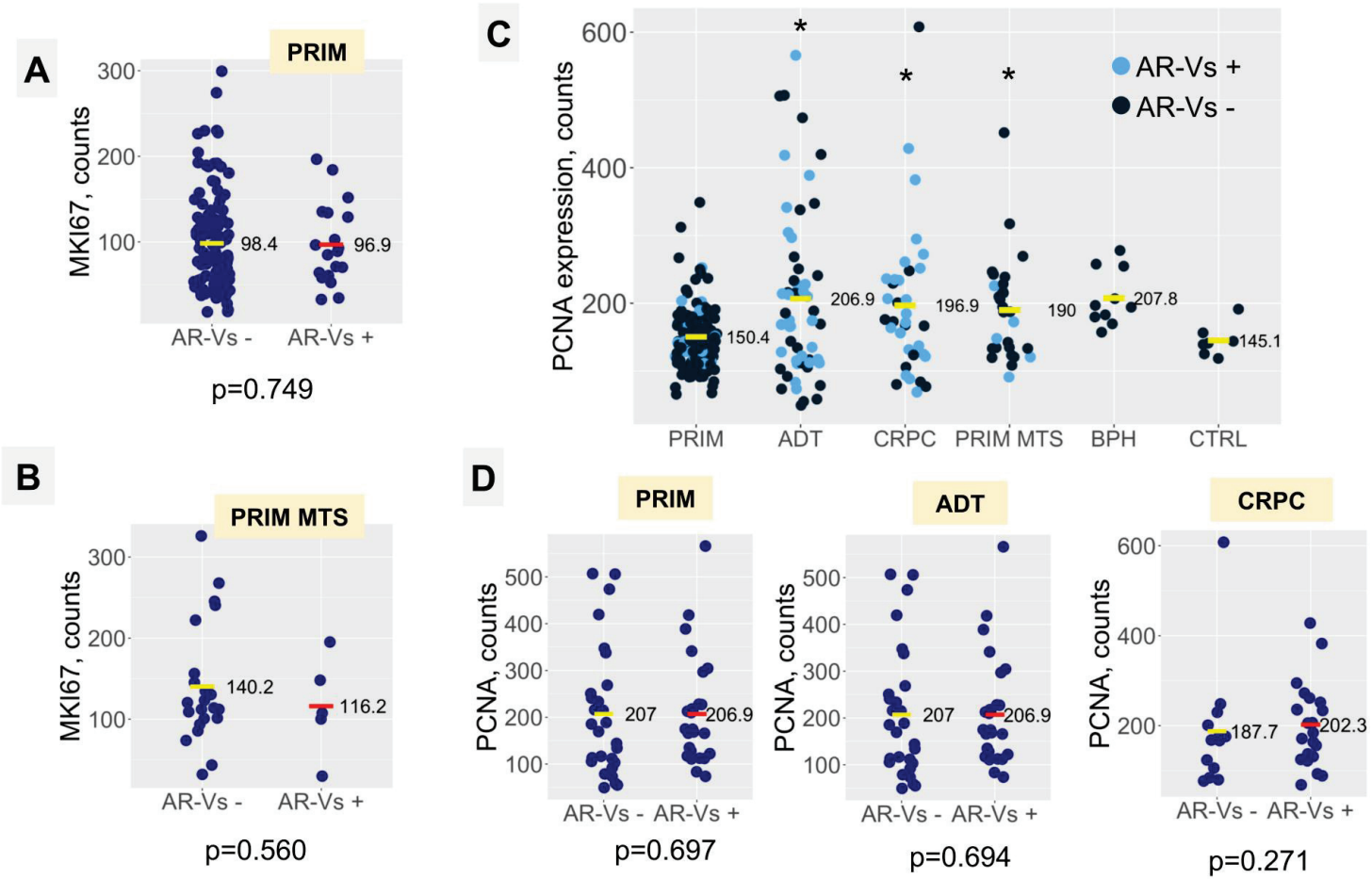



**Figure S1: MKI67 and PCNA mRNA expression in study groups**. Light blue points express any of AR-V splice variants (V1, V3, V7, V9), dark blue points do not express AR-V splice variants. *p*-levels were calculated using the Mann-Whitney U-test. * vs PRIM

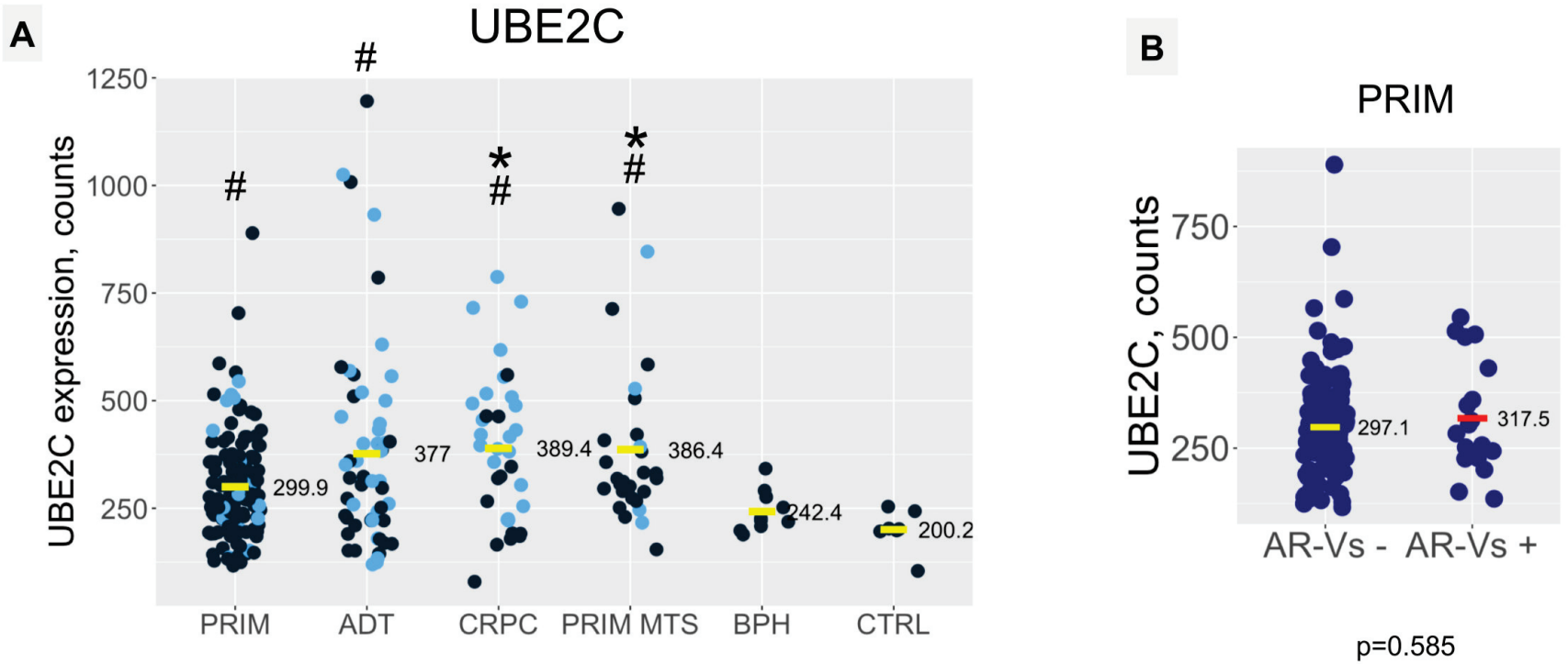



**Figure S2: UBE2C mRNA expression in study groups.** Light blue points express any of AR-V splice variants (V1, V3, V7, V9), dark blue points do not express AR-V splice variants.p-levels were calculated using the Mann-Whitney U-test. Statistical significance ($p < 0.05$): * vs PRIM group, # vs CTRL group.

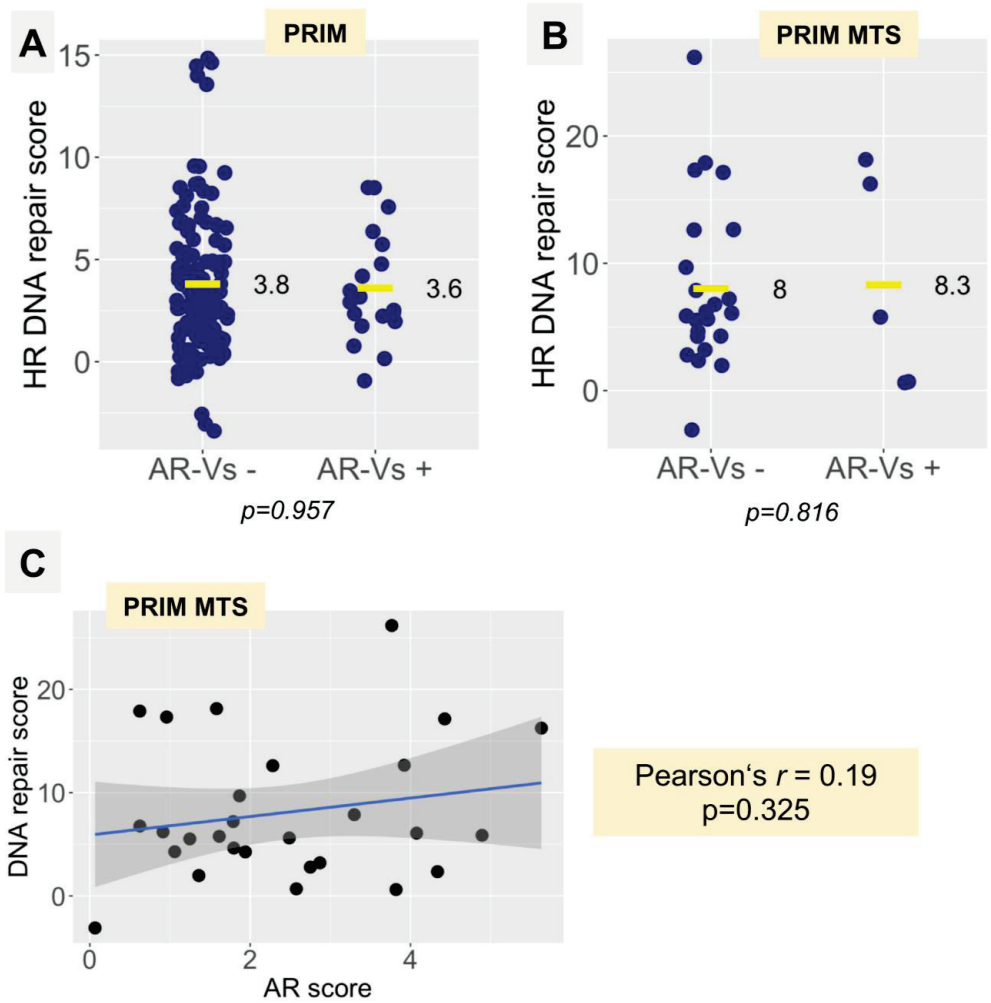



**Figure S3: mRNA expression of 20 DNA repair genes in study groups and correlation of DNA repair and AR scores.** *p*-levels were calculated using the Mann-Whitney U-test.

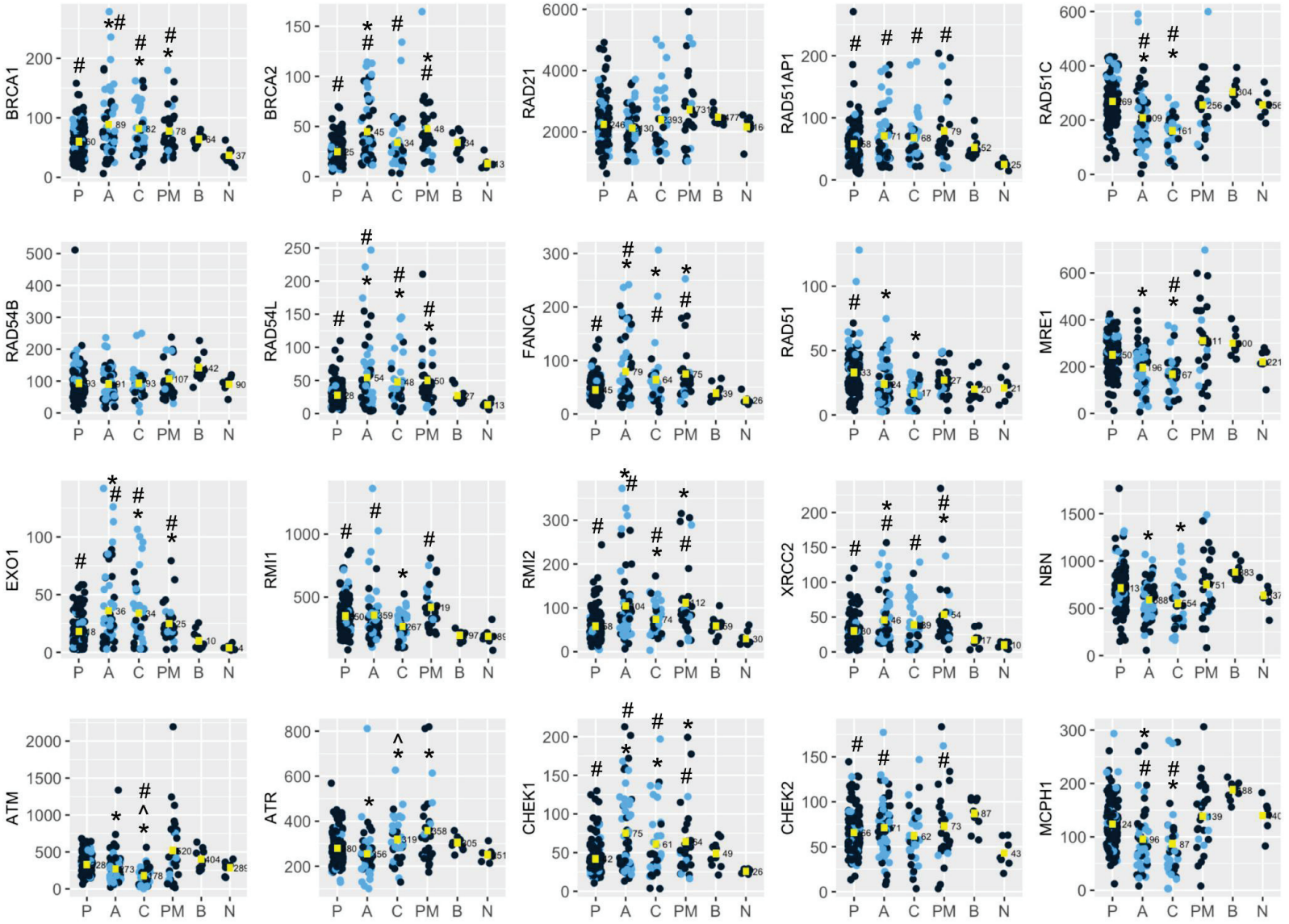



**Figure S4: mRNA expression of 20 DNA repair genes in dependence on AR-V status** (expression of any of four AR-V splice variants: V1, V3, V7, V9; light blue in all examined tissue types (PRIM, ADT, CRPC, PRIM MTS, BPH, NORM). Dark blue dots represent samples devoid of AR splice variants. p-values were calculated using the Mann-Whitney U-test. Groups were statistically significant: * vs PRIM group, ^ CRPC vs ADT group, # vs CTRL group, § vs PRIM MTS group.

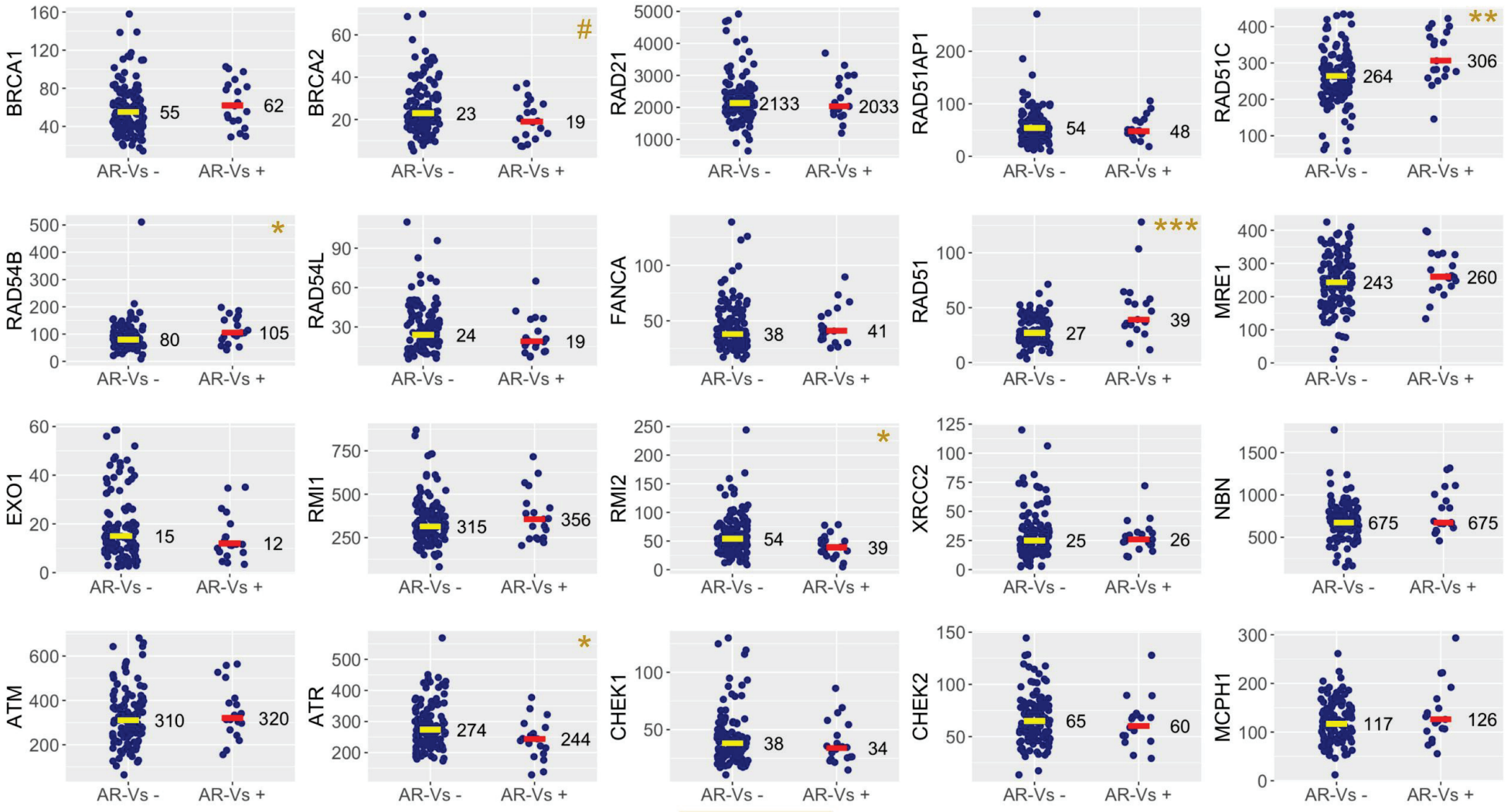



**Figure S5: mRNA expression of 20 DNA repair genes in dependence on AR-V status in the PRIM group.**
Expression of any of four AR-Vs: V1, V3, V7, V9. *p*-values were calculated using the Mann-Whitney U-test. * $p = 0.01 - 0.05$, ** $p = 0.01 - 0.001$, *** $p < 0.001$, # $p = 0.05 - 0.1$.

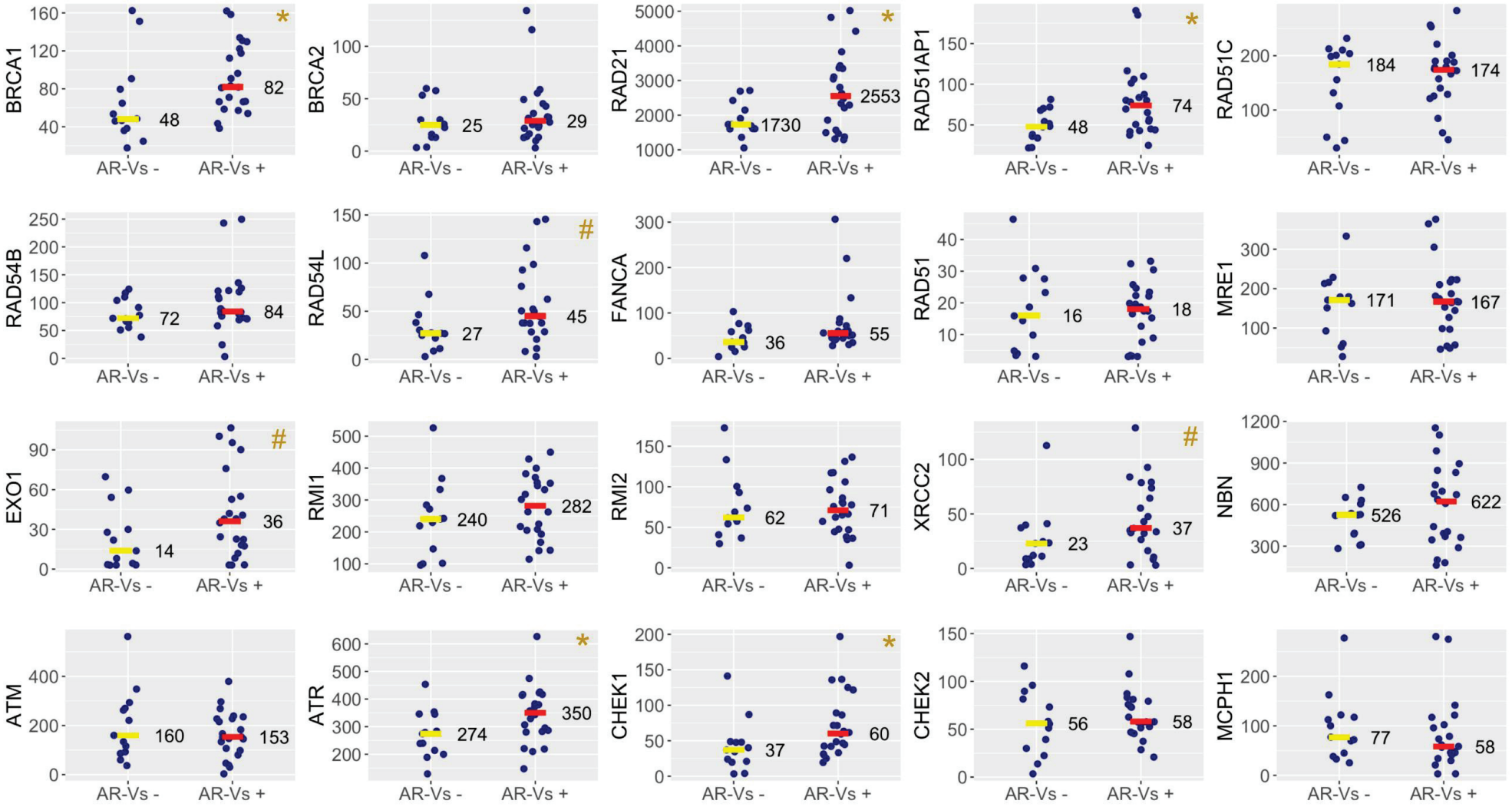



**Figure S6: mRNA expression of 20 DNA repair genes in dependence on AR-V status in the CRPC group.**

Expression of any of four AR-Vs: V1, V3, V7, V9. *p*-values were calculated using the Mann-Whitney U-test. * $p = 0.01 - 0.05$, # $p = 0.05 - 0.1$.

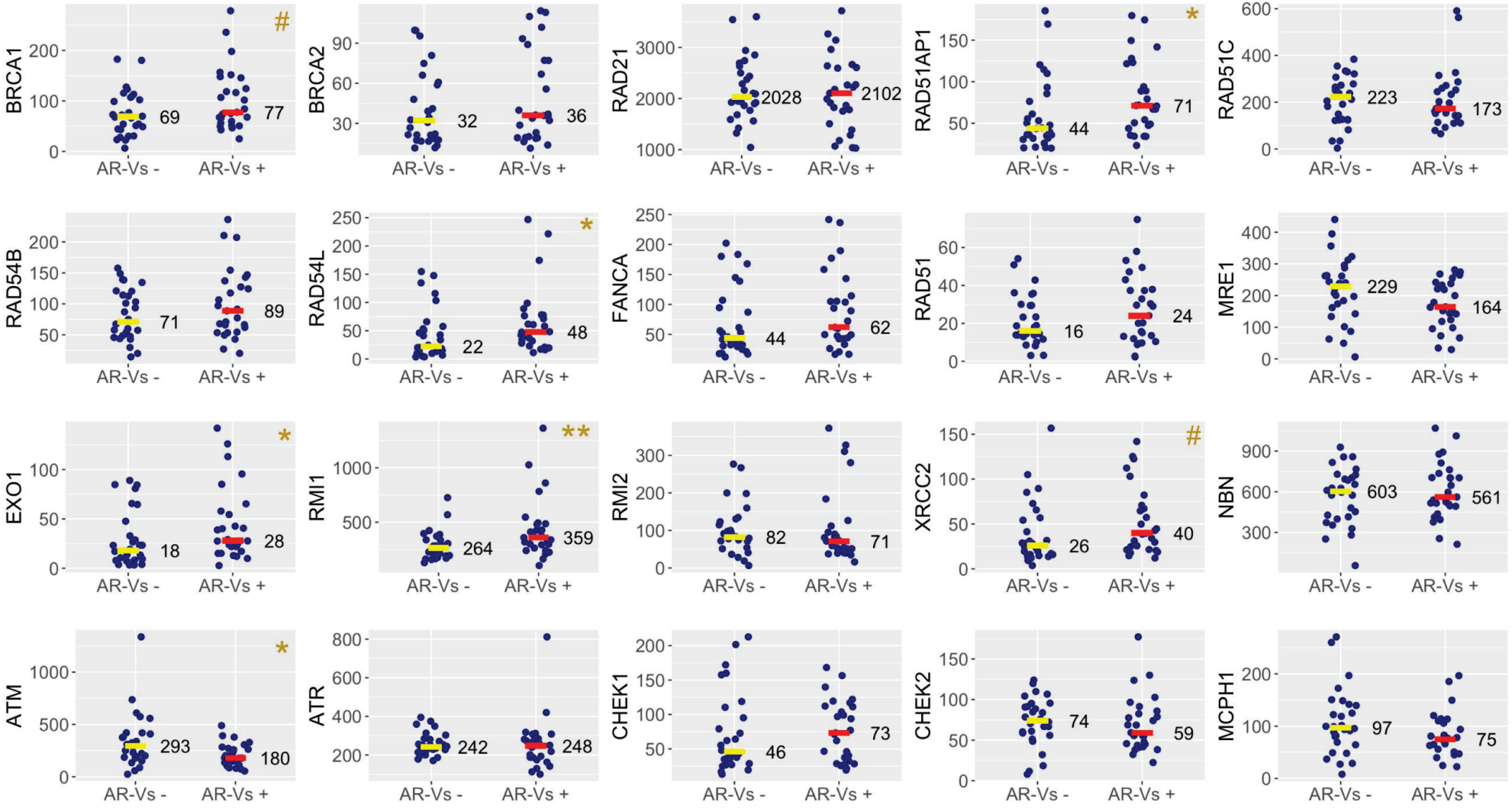



**Figure S7: mRNA expression of 20 DNA repair genes in dependence on AR-V status in the ADT group.**

Expression of any of four AR-Vs: V1, V3, V7, V9. *p*-values were calculated using the Mann-Whitney U-test. * $p = 0.01 - 0.05$, ** $p = 0.01 - 0.001$, # $p = 0.05 - 0.1$.

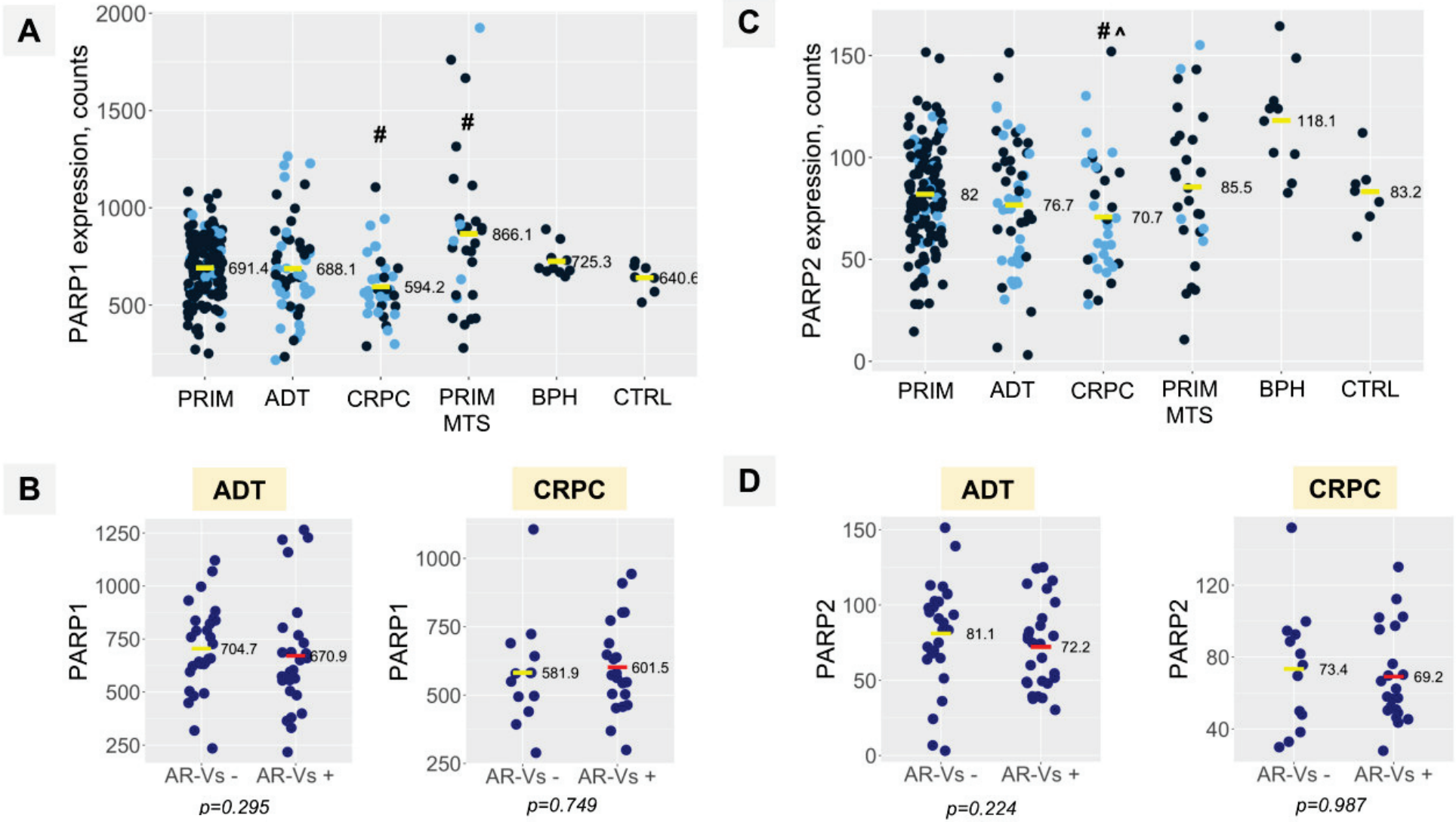

A
PARP1 expression, counts
2000
1500
1000
500
691.4
688.1
594.2
866.1
725.3
640.6
#
#
PRIM
ADT
CRPC
PRIM MTS
BPH
CTRL
C
PARP2 expression, counts
150
100
50
0
82
76.7
70.7
85.5
118.1
83.2
#^
PRIM
ADT
CRPC
PRIM MTS
BPH
CTRL
B
ADT
CRPC
PARP1
1250
1000
750
500
250
704.7
670.9
581.9
601.5
AR-Vs -
AR-Vs +
p=0.295
p=0.749
D
ADT
CRPC
PARP2
150
100
50
0
120
80
40
81.1
72.2
73.4
69.2
AR-Vs -
AR-Vs +
p=0.224
p=0.987

**Figure S8: mRNA expression of PARP1 (A) and PARP2 (B) in study groups.** Light blue points express any of AR-V splice variants (V1, V3, V7, V9), dark blue points do not express AR-V splice variants. PARP1 (**C**) and PARP2 (**D**) expression in ADT and CRPC groups in dependence on AR-V status (expression of any of four AR-V splice variants: V1, V3, V7, V9). p-levels were calculated using the Mann-Whitney U-test. Groups were statistically significant ($p<0.05$): ^ CRPC vs ADT group, # vs CTRL group.

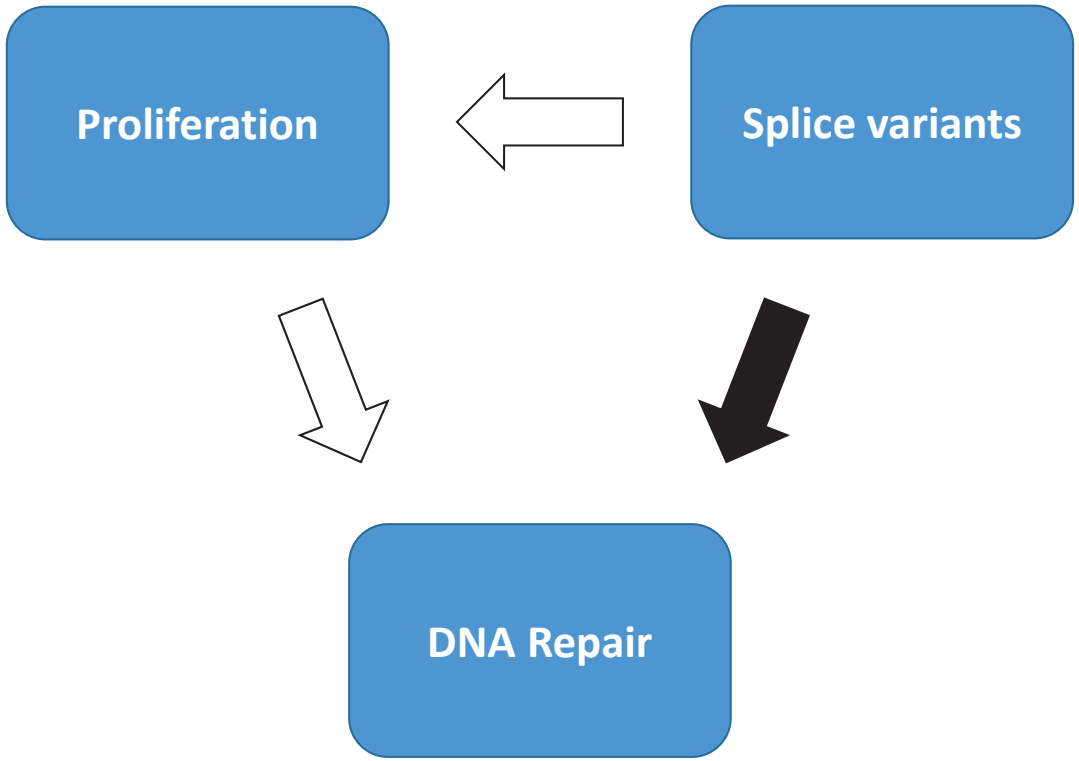



**Figure S9: Separation of the impact of AR splice variants on proliferation and DNA repair using a linear regression model.** Directed acyclic graph visualizing the relationships between AR splice variants, proliferation and DNA repair. Our linear regression model allowed to calculate an effect of AR splice variants on DNA repair gene regulation (black arrow) while accounting for the effect of proliferation on DNA repair (white arrow).

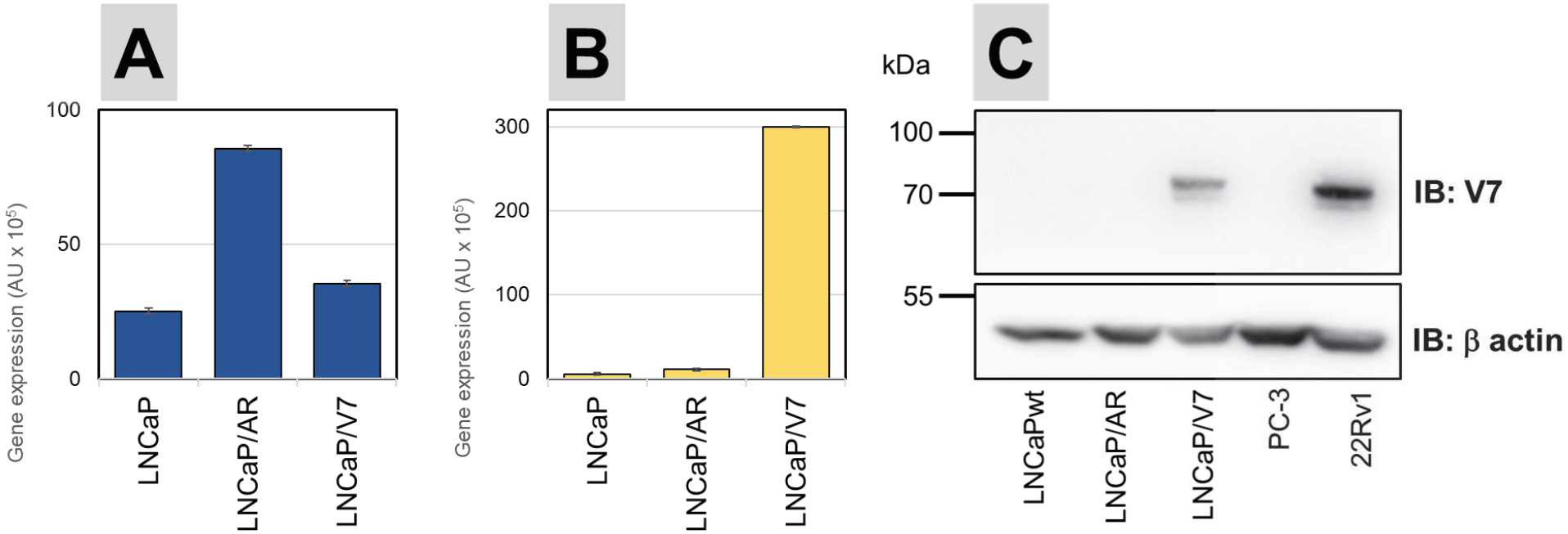
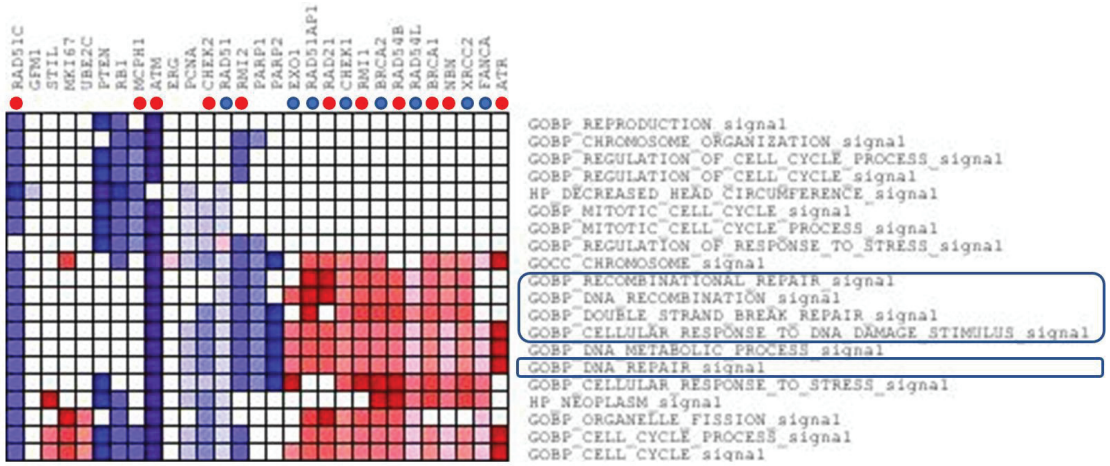



**Figure S10: Expression of total androgen receptor and splice variant 7 in the LNCaP/AR *in vitro* tumor models.** Parental LNCaPwt and derived cell lines overexpressing either full length AR (LNCaP/AR) or the AR-V7 splice variant (LNCaP/V7) were analyzed for total AR (A) or AR-V7 gene expression (B). AR-V7 protein was also detected by AR-V7 specific immunoblotting in LNCaP/V7. 22Rv1 cells, known to express AR-V7, served as control. None of the other cell lines expressed this splice variant, β actin was used as loading control (C). (AU – arbitrary units)




A

| GO biological process complete | Homo sapiens - REFLIST (20589) | upload_1 (41) | upload_1 (expected) | upload_1 (over/under) | upload_1 (fold Enrichment) | upload_1 (raw P-value) | upload_1 (FDR) |
|---|---|---|---|---|---|---|---|
| DNA repair (GO:0006281) | 508 | 22 | 1.01 | + | 21.75 | 1,05E-24 | 1.64E-20 |
| double-strand break repair (GO:0006302) | 203 | 17 | 0.4 | + | 42.05 | 1,93E-23 | 1.51E-19 |
| double-strand break repair via homologous recombination (GO:0000724) | 113 | 14 | 0.23 | + | 62.22 | 1,65E-21 | 8.60E-18 |
| recombinational repair (GO:0000725) | 117 | 14 | 0.23 | + | 60.09 | 2,59E-21 | 1.01E-17 |
| cellular response to DNA damage stimulus (GO:0006974) | 756 | 22 | 1.51 | + | 14.61 | 4,48E-21 | 1.40E-17 |
| DNA metabolic process (GO:0006259) | 794 | 22 | 1.58 | + | 13.91 | 1,25E-20 | 3.27E-17 |
| DNA recombination (GO:0006310) | 245 | 15 | 0.49 | + | 30.75 | 1,01E-18 | 2.27E-15 |
| cell cycle (GO:0007049) | 1249 | 23 | 2.49 | + | 9.25 | 8,44E-18 | 1.65E-14 |
| cell cycle process (GO:0022402) | 841 | 20 | 1.67 | + | 11.94 | 2,43E-17 | 4.24E-14 |

B

**Figure S11: Gene ontology analysis of genes de-regulated in clinical samples reveals association with DNA repair.** (A) Twenty pathways passed the threshold and are listed in the matrix. Pathways related to DNA repair (recombinational repair, DNA recombination, DSB repair, cellular response to DNA damage stimulus, DNA repair) are highlighted by blue frames and are associated with the majority of genes de-regulated in our analysis, but are not primarily involved in cell cycle pathways (right side). BRCA1 cluster genes are labeled by red dots, BRCA2 cluster genes by blue dots. (B) GO Panther hierarchical clustering ranks gene set analyzed in clinical samples most significantly with DNA repair pathways.

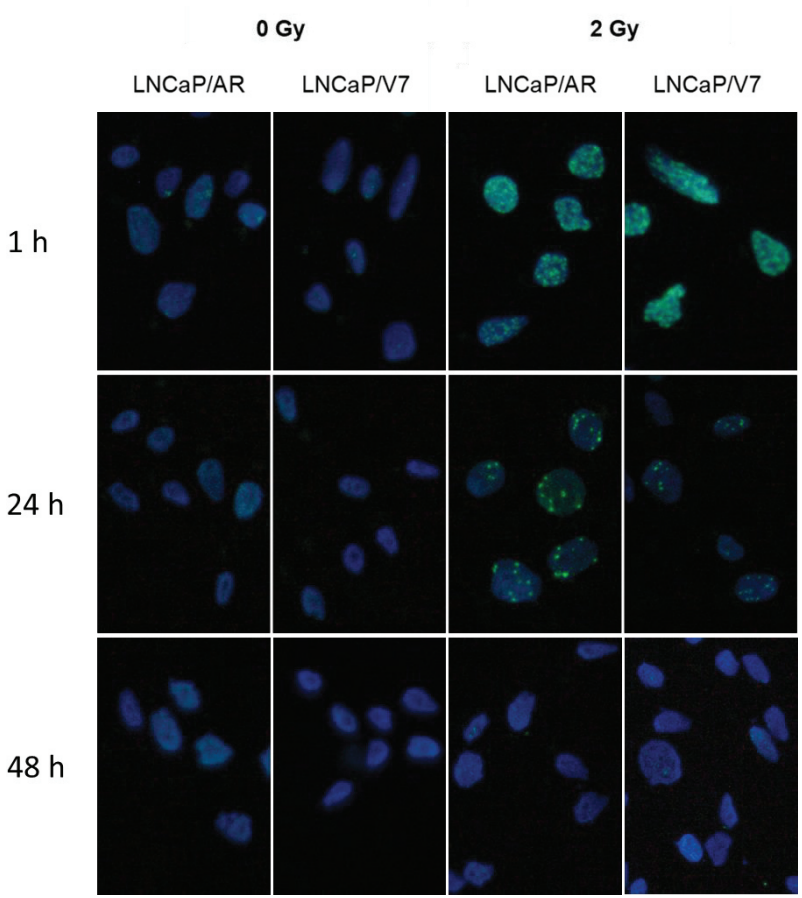



**Figure S12: AR-V7 improves DNA repair after irradiation in an in vitro tumor model.** Vizualization of gH2Ax foci. Nuclear counterstain with DAPI from left to right: LNCaP/AR (0 Gy), LNCaP/V7 (0 Gy), LNCaP/AR (2 Gy), LNCaP/V7 (2 Gy). Time points are 1 h, 24 h and 48 h after irradiation. Note the high number of foci immediately after irradiation compared to the almost complete loss of visible foci after two days.

# 7. List of publications

Publications involved in this dissertation:

2022 **Androgen Receptor Splice Variants Contribute to the Upregulation of DNA Repair in Prostate Cancer.**
Tolkach Y*, **Kremer A***, Lotz G, Schmid M, Mayr T, Förster S, Garbe S, Hosni S, Cronauer MV, Kocsmár I, Kocsmár É, Riesz P, Alajati A, Ritter M, Ellinger J, Ohlmann CH, Kristiansen G.
*equal contributions.
Cancers (Basel). 2022 Sep 13;14(18):4441.

2019 **Where is the limit of prostate cancer biomarker research? Systematic investigation of potential prognostic and diagnostic biomarkers.**
**Kremer A**, Kremer T, Kristiansen G, Tolkach Y.
BMC Urol. 2019 Jun 6;19(1):46.

Additional publications:

2019 **Prostate-specific membrane antigen expression in hepatocellular carcinoma: potential use for prognosis and diagnostic imaging.**
Tolkach Y, Goltz D, **Kremer A**, Ahmadzadehfar H, Bergheim D, Essler M, Lam M, de Keizer B, Fischer HP, Kristiansen G.
Oncotarget. 2019 Jun 25;10(41):4149-4160.

2019 **Apelin and apelin receptor expression in renal cell carcinoma.**
Tolkach Y, Ellinger J, **Kremer A**, Esser L, Müller SC, Stephan C, Jung K, Toma M, Kristiansen G, Hauser S.
Br J Cancer. 2019 Mar;120(6):633-639.

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