

Role of Androgen Receptor Variants in Prostate Cancer:

Report from the 2017 Mission Androgen Receptor Variants Meeting

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ABSTRACT

Context: Although a number of studies have demonstrated the importance of constitutively-active androgen receptor variants (AR-Vs) in prostate cancer, questions still remain about the precise role of AR-Vs in the progression of castration-resistant prostate cancer (CRPC).

Objective: Key stakeholders and opinion leaders in prostate cancer convened on May 11th 2017 in Boston to establish the current state of the field of AR-Vs.

Evidence Acquisition: The meeting “Mission Androgen Receptor Variants (MARS)” was the second of its kind sponsored by the Prostate Cancer Foundation (PCF). This invitation-only event was attended by international leaders in the field and representatives from sponsoring organizations (PCF and industry sponsors). Eighteen faculty members gave short presentations, which were followed by in-depth discussions. Discussions focused on three thematic topics: (1) potential of AR-Vs as biomarkers of therapeutic resistance, (2) role of AR-Vs as functionally active CRPC progression drivers, and (3) utility of AR-Vs as therapeutic targets in CRPC.

Evidence Synthesis: The three meeting organizers synthesized this meeting report, which is intended to summarize major data discussed at the meeting and identify key questions as well as strategies for addressing these questions. There was a critical consensus that further study of the AR-Vs is an important research focus in CRPC. Contrasting views and emphasis, each supported by data, were presented at the meeting, discussed among the participants, and synthesized in this report.

Conclusions: This article highlights the state of knowledge and outlines the most pressing questions that need to be addressed to advance the AR-V field.

Patient Summary: Although further investigation is needed to delineate the role of AR variants in metastatic castration-resistant prostate cancer (CRPC), advances in measurement science have enabled development of blood-based tests for treatment selection. Detection of AR variants (e.g., AR-V7) identified a patient population with poor outcomes to existing AR-targeting therapies, highlighting the need for novel therapeutic agents currently under development.

Introduction: Because prostate cancer is an androgen-dependent disease, the androgen receptor (AR) is the primary molecular target for systemic prostate cancer therapy. Despite initial robust responses to first-line androgen deprivation therapies (ADT), nearly all patients with advanced prostate cancer progress to lethal castration-resistant prostate cancer (CRPC). Importantly, in CRPC, the AR continues to be the primary molecular driver, as evidenced by efficacy of novel hormonal therapies, abiraterone and enzalutamide, in CRPC patients (1-4). While effective, therapies targeting AR are not curative, due to intrinsic and acquired resistance to first-line ADT and novel hormonal therapies. Molecular mechanisms of resistance are largely driven by AR aberrations including AR protein overexpression, AR gene amplification, AR gene mutations, and AR variants (AR-Vs) (5).

AR-Vs are truncated AR proteins lacking the AR ligand-binding domain (AR-LBD) (6). While AR-Vs have been frequently detected in CRPC, their expression and functional role in benign prostate tissues and primary prostate cancers is not readily apparent. Structural rearrangements in the AR gene and alternative AR mRNA splicing are at least two mechanisms for expression of AR-Vs in CRPC (6). Multiple AR-Vs arising from AR gene rearrangements and/or alternative splicing have been characterized. To date, AR splice variant-7 (AR-V7) has been studied in greatest detail owing to its relative abundance and frequency of detection in CRPC (7, 8), as well as its potential clinical utility as a marker for treatment selection in men with metastatic CRPC (mCRPC) (9). However, in-depth studies have also been conducted on other AR-Vs, including AR-V1, AR-V3, AR-V7, AR-V9 and ARv567es (10-12). Structural differences of these AR-Vs are illustrated in Figure 1. Since AR-Vs contain the AR DNA-binding domain and the AR transcriptional activation domain, they are capable of transcriptional regulation, in spite of the loss of the AR-LBD. Further, since the AR-Vs lack the AR-LBD, they are not regulated by either first-line or novel hormonal therapies currently used in the clinic. At the MARS2 meeting, our efforts were streamlined to evaluate the role of AR-Vs as biomarkers, molecular drivers, and as therapeutic targets. The authors identified key consensus, discussion points, and critical future work needed to advance the field.

Methods and Evidence Synthesis: The three meeting organizers (JL, SMD, GVR) conceived the idea of an invitation-only meeting focusing on androgen receptor variants. Three thematic topics were predefined prior to the meeting: (1) potential of AR-Vs as biomarkers of therapeutic resistance, (2) role of AR-Vs as functionally active CRPC progression drivers, and (3) utility of AR-Vs as therapeutic targets in CRPC. The meeting was sponsored by the Prostate Cancer Foundation (PCF), Sanofi, Astellas, Janssen Research and Development LLC, and Sun Pharma, and held in Boston, MA, prior to the American Urological Association annual meeting. Academic physicians and scientists from the US, UK, Canada, Australia, and Japan, as well as representatives from four sponsoring pharmaceutical companies attended this meeting. Eighteen faculty members gave short presentations, which were followed by in-depth discussions. The three meeting organizers summarized major data discussed at the meeting, identified 26 key questions in the field, and synthesized this meeting report. The 26 key questions were included in an online survey sent to all nonindustry participants after the meeting. Voting results (% and number of approval votes) were included in the three boxes summarizing general consensus reached at the meeting. Detailed voting results were included in Supplemental Document.

Section 1: Measurement science pertaining to AR-Vs and other AR aberrations

Tissue-based testing of AR-Vs. Prior studies had established that AR-Vs can be detected at the RNA and protein level in CRPC samples. Expression of several AR-Vs including AR-V7 has been reported in benign prostate tissue and primary prostate cancers (9). Although AR-vs were detected in untreated prostate tumors and benign prostate tissues, their levels were substantially lower and likely represented background splice events detectable by RT-PCR and RNA-seq that may not lead to robust detection by RISH and IHC. However, expression of AR-V7 is higher in CRPC, potentially due to AR gene amplification and/or induction of AR-V7 by ADT (13-15), which occurs in CRPC cell lines and xenografts. Therefore, amplification of the AR gene, a frequent event in CRPC, is likely to underlie the increased expression of

AR-Vs at this disease stage. Whether the selective advantage for AR gene amplification is overexpression of AR or AR-Vs (or both) remains to be established.

Dr. Richard Lee reported the development of a branched chain RNA *in situ* histochemistry (RNA ISH) assay for the detection of AR-V7 mRNA within archival prostate cancer tissue. This branched chain RNA ISH assays and other similar assays reported in the literature utilized 20mer probes tiled across 500-1000 base pairs of the unique cryptic exon 3 (CE3) at the 3' terminus of AR-V7 mRNA (16-18). Using an automated ISH system, AR-V7 was detected in FFPE samples from radical prostatectomy (n=30, detection rate 1/30), metastatic, castration-sensitive prostate cancer (n=22, detection rate 10/22), and metastatic CRPC (n=12, detection rate 12/12), suggesting that AR-V7 expression dramatically increases with disease progression (18). In this pilot study, AR-V7 mRNA detection was also associated with duration of response to first-line ADT. Dr. Lee presented an individual case with serial tissues collected before, during, and after ADT showing progressively higher AR-V7 expression. Thus, automated RNA-ISH assay is feasible for AR-V7 evaluation in archival FFPE prostate cancer tissue, and this small-cohort study suggests baseline AR-V7 by this method is a negative predictive marker for treatment with ADT.

Dr. Johann de Bono reported on advances in tissue-based measurements of AR-V7 mRNA and protein. The primary drawback of RNA ISH- and IHC-based detection of AR-V7 mRNA and protein has been the lack of specificity (19, 20). Dr. de Bono presented data on a novel RNA ISH method, as well as a new AR-V7 antibody developed by RevMab shown to be analytically valid with a single band on Western blot. The novel RNA ISH method detected a single splice junction specific to AR-V7 (20), and the new AR-V7 antibody did not detect false positive signals reported in a previous study with the Abcam antibody (19). These validated *in situ* detection methods enabled precision measurement of AR-V7 in morphologically intact clinical tissue specimens. A recent study reported a novel RISH detection method and compared the RISH results with IHC by the RevMab antibody (20). The findings further confirmed

the improved specificity of the new antibody. Detection of AR-V7 by the novel RNA ISH method in a cohort of CRPC biopsies was generally associated with poorer response to abiraterone and enzalutamide (20). Dr. de Bono suggested that AR-V7 mRNA detection alone may not correlate with AR-V7 protein levels, due to altered kinetics of AR-V7 protein degradation (21).

Synthesis: AR-V7 mRNA and protein can be detected in morphologically intact tissues. Tissue-based studies support the feasibility of measuring AR-V7 in metastatic tissue biopsies. As AR-V7 is infrequently detected in untreated patients by RISH or IHC, elevated AR-V7 expression may be an acquired event after hormonal therapies although detection in untreated cases may theoretically have prognostic or predictive value. Both RISH and IHC detection values are continuous variables. Thus, differences in detection rate between studies could reflect the different cut-off values. In addition, difference in techniques, the reliance on RNA vs protein, and sampling criteria may result in different detection rates.

Blood-based detection of AR-Vs and other AR aberrations

Previous studies have utilized circulating tumor cells (CTC) (16, 22-25), plasma exosomes (26), peripheral blood mononucleated cells (PBMC) (27) and even whole blood samples (28-31) for detection of AR-Vs (mainly AR-V7) in men with mCRPC. Among these, the CTC-based AR-V7 test has been analytically validated and implemented in CLIA-certified labs on the basis of clinical correlative findings (25, 32). However, CTC-based AR-V7 tests may be limited. Critically, determination of the AR-V7 status and its quantification were not possible in a significant proportion of mCRPC patients who were CTC-negative. In addition, many other relevant molecular targets are compatible with blood-based measurements, including AR amplification/mutation, AR-Vs other than AR-V7, as well as non-AR genomic alterations implicated in CRPC progression.

Novel CTC platforms may partially address this limitation. Dr. Joshua Lang demonstrated the feasibility of the VERSA system to detect mRNAs of multiple AR variants using CTCs captured using antibodies targeted to cell surface proteins EpCAM and TROP2. Overall AR-V7 detection rate was 6/26 (26%) in this study, in-line with the literature. In addition to AR-V7, AR-V9 was also detected at high frequency (11). Using this platform, ARv567es was detected at lower frequency (~1% of patients). The system allowed analysis of additional genes involved in EMT and neuroendocrine differentiation.

Dr. Mayuko Kanayama reported the Juntendo University pilot study, where they evaluated the detection of AR-V7 positive CTCs along with examination of PSMA in 19 Japanese mCRPC patients treated with different CRPC therapies. Overall AR-V7 detection rate was 26% (5/19). In addition to AR-V7, Dr. Kanayama discussed the potential negative predictive value of PSMA detection (positive rate 47%, 9/19) in this small cohort.

Drs. Martin Gleave and Gerhard Attard presented data on blood-based measurements through the isolation of circulating tumor DNA (CtDNA). Published data have established the feasibility of detecting genomic alterations in CtDNA samples in patients with CRPC, and evolution in these genomic alterations over time under selective pressures of treatment (33-35). A wide spectrum of genomic alterations (AR, DNA repair genes, TP53, PIK3CA, RB1) detected in CtDNA samples are also detected in matched metastatic tissues. In addition, CtDNA assays may reveal greater heterogeneity of alterations in some patients than is possible through biopsy of a single metastatic site, suggesting that metastatic tissue biopsy may not be required to determine the somatic status of clinically-actionable prostate cancer driver genes (36). CtDNA assays therefore may be utilized for development of prognostic and predictive biomarkers. There is a growing need to optimize panels & develop CLIA certified assay for prospective validation of CtDNA markers.

Synthesis: It is feasible to measure AR aberrations (AR-V7, AR-V9, AR amplification, AR mutation) as well as other disease drivers using blood-based assays. However, no studies have integrated these measurements. The challenge ahead is to perform analytical as well as clinical validation of individual and integrated assays with concerted efforts.

Box I: Session I general consensus and key discussion points (% and number of votes from 27 meeting participants)

Tissue-based measurement science

1. Robust methods, including RNA-ISH and IHC, have been developed for measurement of AR-V7 mRNA and protein in morphologically intact prostate tissue specimens, enabling correlative analysis using tissue specimens and cross-validation of alternative test platforms (96.3%, 26/27).
2. Current data confirms AR-V7 positive cells are always AR-FL positive (92.6%, 25/27).
3. Infrequent detection in untreated prostate cancer specimens suggest limited clinical utility of detecting AR-Vs in hormone naïve settings (81.5%, 22/27).
4. In situ detection methods for other AR-Vs need to be developed in order to assess the overall AR-V burden and potential tissue heterogeneity (100%, 27/27).

Blood-based measurement Science

1. Many studies have confirmed the prognostic value of blood-based AR-V7 detection in mCRPC (85.2%, 23/27).
2. CTC-based AR-V7 tests have been analytically validated and implemented in CLIA-certified laboratories (85.2%, 23/27)..
3. Beyond AR-V7, other AR-Vs can also be detected using novel CTC testing platforms, and somatic DNA alterations can be measured in circulating tumor DNA samples without the need for metastatic biopsies (85.2%, 23/27)..
4. Blood-based detection of AR-V7 or other AR alterations may reflect high disease burden. As such biomarker studies do not always reflect underlying biology (96.3%, 26/27).
5. Analytical and clinical validation of individual or integrated blood-based tests are required before clinical implementation (100%, 27/27).

Session 2: AR-V functional role and regulation

Regulation of gene expression by AR-V

At the functional level, ADT causes increased expression of AR and AR-Vs due to relief of androgen/AR-mediated suppression of AR gene transcription (37). Thus, AR-Vs (and AR-FL) induced by ADT may regulate the same genes regulated by androgen-activated AR-FL. An alternative model is that elevated AR-Vs may confer a distinct transcriptional program and cellular phenotype. Dr. Nancy L. Weigel used

an inducible AR-V7 system to show that while AR-V7 induces canonical AR genes such as FKBP5 and KLK3, RNA-seq analysis revealed expression of distinct genes that were associated with AR-V7 induction. For example, EDN2, ETS2, SRD5A1, ORM1, BIRC3, HSP27, and HES1 were specifically induced following induction of AR-V7 expression, while SGK1 was specifically induced by AR-FL activation (38). Interestingly, the AR-V7-specific target genes, EDN2 and ETS2 are genes that can be regulated by AR if the pioneer transcription factor, FOXA1, is depleted. Conversely, AR can induce RASSF3 only when FOXA1 is present, but AR-V7 does not induce RASSF3 under either condition. When ARv567es was investigated, there also appeared to be significant overlap with AR-V7 and AR-FL targets. However, there may also be ARv567es-specific target genes owing to the retention of the AR hinge region in ARv567es as opposed to AR-V7 (Fig. 1).

Dr. Laura Cato showed LNCaP95 cells (LNCaP cells derived from long-term passage under castrate conditions) may depend on both AR-FL and AR-V7 for short-term proliferation, as evidenced by slowed LNCaP95 cell growth after dox-inducible knock-down of either AR-FL or AR-V7 (14). Using the AR-V7 RevMab and an AR C-terminal antibody for ChIP-seq, they demonstrate that AR-V7 and AR-FL bind to the same genomic location in this dox-inducible model. AR-V7 chromatin binding was reduced in response to AR-FL knock-down and vice versa, knock-down of AR-V7 reduced AR-FL chromatin binding. Further work is ongoing to identify similarities and differences in the transcriptional activities of the two receptors.

Synthesis: Laboratory research using cell-line model systems may be used to dissect gene expression and chromatin binding programs directed by AR-FL and AR-Vs. The majority of chromatin binding events and transcriptional targets of AR-Vs and androgen-activated AR-FL display significant overlap.

However, genome-wide analyses have suggested an AR-V-specific transcriptional program may exist.

The interplay between AR-FL, AR-V7, and other AR-Vs deserves further in-depth investigation, particularly in the setting of the native molecular context of clinical specimens.

Role of AR dimerization and AR co-regulators

An important question in the AR-V field is whether AR-V functions as a dimer, and if so, how AR-V/AR-FL homo- and hetero-dimers influence therapeutic targeting (39). Published studies from Dr. Yan Dong's lab focused (40-42) on interactions between AR-FL and various AR-Vs. Using tagged forms of AR-FL and AR-Vs, Dr. Dong suggested that AR-Vs form dimers through DBD/DBD interactions, and that this dimerization is required for function but not nuclear localization. AR-Vs can modulate the function and localization of AR-FL through formation of AR-FL/AR-V heterodimers, which are mediated by the DBD/DBD interactions, but also binding of the AR-V N-terminus with the AR-FL C-terminus. Importantly, this AR-V/AR-FL heterodimer was not inhibited by enzalutamide. The critical function of the dimer was supported by mutually-dependent co-occupancy of genomic sites by AR-FL and AR-V7.

AR coregulators are important for AR-FL transcriptional activity (43) and may drive context-specific AR functions. The AR-V cistrome consists of canonical AREs, overlaps with AR-FL, and AR-V specific genes may reflect the biphasic nature of AR transcriptional activation of certain target genes (10, 44), indicating the same or similar set of co-regulators may be involved in AR-FL and AR-V function. However, AR-Vs such as ARv567es and AR-V7 have reduced affinity for AREs compared with AR-FL (10) and altered kinetics of DNA binding may require different sets of co-regulators. Dr. Luke Selth presented his work on dissecting the sets of co-regulators bound by AR and ARv567es using Rapid Immunoprecipitation Mass-spectrometry of Endogenous proteins (RIME) (45). This work showed a high degree of overlap between the AR-FL and ARv567es interactomes, but also yielded differences that may arise from co-regulators specific to the AR-LBD and/or unique binding surfaces on the variant protein. One new example of an AR-FL and ARv567es-shared co-regulator recently identified by Dr Selth's group is GRHL2 (45), which was shown to participate in a feed-forward transcriptional loop with active AR-FL

and AR-Vs that likely drives their activity in CRPC. Dr. Selth also noted there are likely to be differences in regulation of AR/AR-V by the ubiquitin-proteasome system (46), as AR-Vs lose interaction and dependency on HSP90 (47).

Synthesis: Current data indicates that chromatin binding and transcriptional activation mediated by AR-Vs requires homo-dimerization. While evidence also exists to support a role for heterodimerization between AR-Vs and AR-FL, the relative contributions of AR-V homo-dimers and AR-FL/AR-V heterodimers to activation of AR-FL/AR-V target genes remains to be characterized. RIME represents an effective approach to study complexes nucleated by these homo-and hetero-dimers, and will be useful for characterizing AR-FL and AR-V interactomes in clinical specimens.

AR-V as potential disease driver

Whether AR-Vs drive therapeutic resistance in CRPC remains an unresolved topic. Studies in favor of AR-Vs functioning as drivers of resistance have come from models where AR-Vs are endogenously-expressed at high levels, and their knock-down restores sensitivity to castration and/or antiandrogens (44, 48). In contrast, studies arguing against AR-Vs functioning as drivers of resistance have come from models where AR-Vs are introduced ectopically, or are expressed at extremely low levels relative to AR-FL. For instance, Dr. Charles Sawyers discussed his early published work indicating that AR-Vs lack the key properties of drug resistance drivers. In these studies, overexpression of AR-V7 in LNCaP cells was able to confer gain-of-function in terms of ligand-independent growth, but did not impart resistance to enzalutamide, suggesting that AR-Vs may require full length AR for gain-of-function (49). In this context, Dr. Sawyers indicated that rapid induction of AR-V7 by ADT may be a by-product of the increased transcription of the AR gene and simply reflect a mechanism for rapid induction of AR-FL expression by ADT (14, 49). Another argument against a driver role for AR-Vs came from his observations with a prostate cancer cell line (Myc-Cap) derived from the Hi-Myc mouse, which expressed

AR-Vs but remained sensitive to castration and enzalutamide. However, he also noted the Myc transgene in this model was under control of an androgen-responsive promoter, which clouds interpretation. The murine AR-V, although structurally different from the human AR-V, demonstrated *in vitro* functional activities similar to human AR-V. Additionally, the 3' terminal exons in these murine AR-Vs were not located within the AR gene locus as is the case for human AR-V7. Instead, these 3' terminal exons were located hundreds of kb upstream or downstream of the AR locus, indicating that underlying AR gene rearrangements were responsible for their splicing into AR mRNA.

Dr. Steven Balk presented data on progressive increases of AR-V7 expression in VCaP cells treated with enzalutamide *in vitro* and combination of abiraterone/enzalutamide *in vivo*. Using these cells with high AR-V7 expression, he showed that AR activity was inhibited by knock-down with a siRNA targeted to AR exon 1 (which encodes the AR NTD) or the 3' terminal exon CE3 of AR-V7. Interestingly, AR activity in these cells was not inhibited by siRNA that selectively knocked down AR-FL. In contrast to this adaptive model, VCaP cells treated acutely with enzalutamide and displaying rapid induction of AR-V7 displayed very low AR activity, suggesting that additional cofactors may be important for AR-V function in settings of acute vs. adapted AR-V7 expression.

Synthesis: Discrepancies have been noted in the contribution of AR-Vs to the phenotype of therapeutic resistance in CRPC. It is possible that these discrepancies may be due to whether knock-down or overexpression approaches were used to interrogate AR-V function. Additionally, several of the models used for AR-V knock-down experiments and displaying AR-V-driven resistance phenotypes also harbor structural rearrangements in the AR gene and/or were adapted to long-term treatment with enzalutamide. This suggests that alterations in AR gene structure or adaptive changes cofactor milieu may be important determinants of AR-V function. Additionally, it has recently been shown that AR-V9, AR-V1, and additional AR-Vs utilizing cryptic exons in AR intron 3 are coordinately expressed in CRPC and

susceptible to knock-down with siRNAs/shRNAs that had been previously-thought to target AR-V7 exclusively (11, 50). Thus, AR-V7 knock-down studies reported in the literature were actually inhibiting expression of multiple AR-Vs simultaneously. Further work is required to elucidate the impact of these parameters on AR-V function as drivers of resistance in CRPC.

Box II: Session 2 general consensus and key discussion points (% and number of votes from 27 participants)

Regulation of gene expression by AR-V

1. Transcriptional programs directed by AR-Vs overlap with those directed by androgen-activated AR-FL, providing a mechanism by which AR-Vs mediate resistance to AR-targeted therapies (77.8%, 21/27).
2. Differences in transcriptional programs exist between AR-FL and AR-Vs, providing a mechanism for AR-Vs as drivers of disease progression and drug resistance beyond those driven by AR-FL (88.9%, 24/27).
3. Additional studies are needed to further dissect the genomic functions mediated by AR-FL and AR-Vs, particularly in the native molecular context in clinical specimens (100%, 27/27).

Role of AR dimerization and AR co-regulators

1. Existing data indicates AR-Vs function as homodimers when mediating genomic functions (81.5%, 22/27).
2. Existing data indicates AR-Vs heterodimerize with AR-FL and promote ligand-independent transcriptional activity of AR-FL (85.2%, 23/27).
3. AR-Vs are dependent on co-regulators to transcriptionally activate target genes (92.6%, 25/27)
4. There is a pressing need to characterize AR and AR-V dimerization as well as their bound co-regulators in clinical specimens (100%, 27/27).

AR-V as disease drivers

1. In some in vitro models and a subset of CRPC patients, AR-V7 is functioning as a driver of resistance to AR-targeted therapy (96.3%, 26/27).
2. AR-Vs beyond AR-V7 are important for driving resistance to AR-targeted therapy (77.8%, 21/27).
3. AR-V7 and other AR-Vs function as drivers of resistance to AR-targeted therapy only under specific contexts, such as alterations in AR gene structure or adaptive changes in cofactor milieu (74.1%, 20/27).
4. Additional investigation is required to further delineate AR-V as drivers of resistance in CRPC (96.3%, 26/27).

Session III: Therapeutic targets and strategies

Utility of targeting AR amino terminus

The AR amino terminus (NTD) (amino acids 1–538) is structurally unique among steroid receptors: it is much longer, has a stronger transactivation domain and is critical for the transactivation and function of

the AR. Experimental and bioinformatic analyses reveal that the AR-NTD is intrinsically disordered in solution and exists as an ensemble of interconverting conformations. In response to environmental stresses, the AR-NTD may rapidly and reversibly fluctuate between conformations. These alterations in domain structure may enable transient interactions between AR-NTD and protein coregulators that may allosterically regulate AR function. Analyses by circular dichroism, Fourier transform infrared spectroscopy, secondary structure prediction, and mutagenesis have revealed variations in the degree of intrinsic disorder of different regions of the AR-NTD: some domains may adopt more stable secondary structure than others. While the flexibility and intrinsic disorder of the AR-NTD in solution and the lack of a crystal structure hamper rational design of drugs using virtual docking approaches, the relative stability of some domains makes them potentially targetable. In addition, the lack of sequence homology between the NTDs of AR and other members of the steroid hormone receptor family suggests that drugs targeting the AR-NTD will be more selective for the AR, yielding fewer side effects.

The primary advantage of drugs targeting the AR-NTD is the potential to fundamentally target all forms of the AR, including those that drive resistance to AR-LBD targeting therapies. Since the AR-NTD is retained in all forms of biologically active AR, including amplified AR-FL, AR-LBD mutations (e.g., W741C for bicalutamide and F876L for enzalutamide) and AR-Vs, drugs targeting AR-NTD should be effective against all these AR forms. The addition of such drugs to the CRPC armamentarium is likely to have significant clinical utility to prevent and overcome drug resistance.

A bisphenol A derivative, EPI-001 has been shown to bind the AF-1 region of the AR-NTD and inhibit AR function. EPI-001 has been shown to inhibit AR-NTD transactivation, inhibit proliferation of cell lines expressing various forms of AR, and to selectively block AR-protein interactions and recruitment of the AR to DNA response elements (51). A derivative of EPI-001 is currently in phase I clinical trials.

Importantly, EPI-001 provided proof of principle of the translatability of drugs targeting the AR-NTD: however, its utility as a therapeutic agent remains to be proven.

Development of novel agents

Dr. Artem Cherkasov performed *in silico* screening of 150 million compounds to identify drugs targeting the AR DNA binding domain (52). Because the AR DNA binding domain is shared between AR-FL, AR-LBD mutations and AR-Vs, such an approach is likely to target all forms of the AR. Indeed, their lead compound VPC-14449 inhibits binding of AR-FL as well as AR-V7 and ARv567es to chromatin, and consequently blocks transcriptional activity driven by AR (53). Dr. Cherkasov and colleagues also developed VPC-17005 to block AR dimerization and this compound resulted in abrogation of AR function. Importantly, VPC-17005 selectively inhibited AR DNA binding without affecting other steroid hormone receptors, including progesterone receptor (PR), estrogen receptor (ER), and glucocorticoid receptor (GR). Further development of these drugs could pave the way forward for rational design of drugs targeting the undruggable - transcription factors.

Dr. Allen Gao showed that niclosamide, a FDA approved drug to treat tapeworm infections displayed activity against AR-V7 function (54-56). A gene expression signature associated with treatment with niclosamide overlapped significantly with AR knock-down. This activity against AR-V7 may be driven by AR degradation as evidenced by MG132, a 26S proteasome inhibitor, inhibiting niclosamide-mediated AR-V7 protein degradation. Niclosamide inhibited growth of enzalutamide-resistant C4-2B cells and synergized with treatment with abiraterone and enzalutamide (55). Phase Ib/II clinical trials combining niclosamide with abiraterone/enzalutamide in mCRPC patients is currently ongoing.

Additional indirect strategies to interfere with AR-V function are to target either their interactions with protein coregulators or downstream transcriptional targets. Dr. Kerry Burnstein proposed indirectly

targeting AR-V activity by disrupting interactions with key AR-V coactivators such as VAV3 and other AR amino terminal-interacting regulators (57). She showed that a coactivator-enhanced AR-V transcriptional target could be exploited therapeutically in CRPC xenograft models. Similarly, another indirect approach to targeting AR-Vs rose out of an observation that certain kinase inhibitors inhibited growth of AR positive cells but not AR negative cells. Dr. Stephen Plymate presented data showing that bumped kinase inhibitors (BKI) could inhibit prostate cancer cells that are driven by the constitutively active AR-V7. One proposed mechanism of action was inhibition of serine 81 phosphorylation on both AR-FL and AR-V7. Of note, in the absence of androgen, serine 81 is phosphorylated in cells expressing AR-FL and AR-V7. They also demonstrated that their candidate BKIs inhibited growth of the AR-FL driven LuCaP35 human PDX model in non-castrate mice as well as AR-V7 driven LNCaP95 xenografts in castrate mice. Their current BKI PK data demonstrate that they can reach effective EC50 levels in mice with a single daily oral dose and no observable toxicity after 6 weeks of treatment. Importantly the BKIs have a narrow kinase target range. They will continue to modify the BKI to achieve increased potency.

Dr. Robert Matusik showed that NFkB induces expression of AR-V7 and that inhibition of NFkB using Bortezomib reduced AR-V7 levels and restored CRPC responsiveness to antiandrogens in cell line and xenografts models using CRW22RV1 and C4-2B cells. Further, neuropeptides released by neuroendocrine prostate cancer, activate the Gastrin Releasing Peptide Receptor to induce NFkB and AR-V7 expression resulting in CRPC (58, 59). Other inhibitors of NFkB, such as methotrexate or LC-1 (dimethylamino Parthenolide, DMAPT), would reduce AR-V7 expression in CRPC cell lines and restore responsiveness to antiandrogens.

Dr. Amina Zoubeidi suggested that a significant fraction (9/35) of AR-positive enzalutamide-resistant xenografts are potentially AR-indifferent as indicated by lack of PSA expression (60) and have biologic similarity to pluripotent and neuroendocrine tumors. Stem-cell factors likely emerge early during

therapeutic inhibition of the AR pathway, which can then be followed later by expression of neuroendocrine markers. Inhibition of EZH2 can reverse the process back to an AR-driven state. Dr. Zoubeidi discussed BRN2, which was found to be overexpressed in CRPC tumors associated with low serum PSA. She further demonstrated that BRN2 was a transcription factor that likely functioned as a master regulator of enzalutamide-induced neuroendocrine trans-differentiation required for expression of neuroendocrine markers. Mechanistically, AR suppressed BRN2, and this negative feedback can be relieved by enzalutamide. Thus, BRN2 is a potential target in advanced CRPC and inhibition of BRN2 in combination with enzalutamide is being explored.

Synthesis: The unresolved question of whether AR-Vs function as the primary molecular drivers in CRPC has led to the question of whether AR-Vs are viable therapeutic targets for CRPC. Since AR-Vs lack the AR LBD, novel drugs would be required to target AR-Vs. Since the amino terminus of AR-Vs is identical to the amino terminus of AR-FL, therapeutic strategies developed against AR-Vs are likely to have effect against all forms of the AR. This rationale has spawned significant interest from multiple investigators to target the amino terminus of AR or to down-regulate expression of AR-Vs. However, approaches to target the AR N-terminus are limited by the intrinsic disorder and the lack of a crystal structure of this domain.

Box III: Session 3 general consensus and discussion points (% and number of votes from 27 participants)

Utility of targeting AR amino terminus

1. Targeting the amino terminus of the AR represents a novel approach to overcome resistance to AR targeting agents which target the AR-ligand binding domain (88.9%, 24/27).
2. Drugs targeting the AR amino terminus have the potential to target all forms of the AR, including AR-FL and multiple forms of AR-Vs (92.6%, 25/27).
3. Drugs targeting the AR amino terminus may overcome the resistance mediated by AR- LBD mutations (92.6%, 25/27).

Development of novel agents

1. Drugs specifically targeting AR-Vs would be useful for establishing the role and biological importance of AR-Vs in CRPC (96.3%, 26/27).
2. Transcription factors are challenging therapeutic targets; however, novel AR-targeted agents that specifically block AR DNA binding or AR dimerization should be evaluated (96.3%, 26/27).
3. Drugs targeting AR-V co-regulator proteins may specifically block AR-Vs (81.5%, 22/27).

Discussion and Conclusions

To realize the potential of translating laboratory discoveries to patient benefit, it is important to understand the biology, measurement science, and relevant experimental therapeutic approaches from multiple perspectives. In the nine years since the first report of AR variants, critical advances have been made. Importantly, testing platforms have been developed to facilitate AR-V measurements for biomarker-driven or biomarker-stratified clinical trials, and AR-Vs are being explored as a therapeutic target. In spite of these advances, there remains a need to conduct prospective trials to further assess the clinical utility of AR-Vs in mCRPC, and future efforts are also needed to improve blood-based testing platforms beyond AR-V7 by integrating multiple AR aberrations to enable robust treatment selection and patient selection. In addition, how AR-Vs mediate genomic function as a transcription factor, particularly in homo- vs. hetero-dimer contexts remains incompletely characterized. Additionally, an unresolved question that remains is whether AR-Vs drives therapeutic resistance in CRPC, and if so, under which specific contexts. Blood-based detection of AR-V7 identified a patient population with poor outcomes to existing therapeutic agents,

highlighting the need to develop novel therapeutic approaches for mCRPC. Finally, there is a pressing need to develop markers and therapeutic approaches targeting AR-indifferent prostate cancers.

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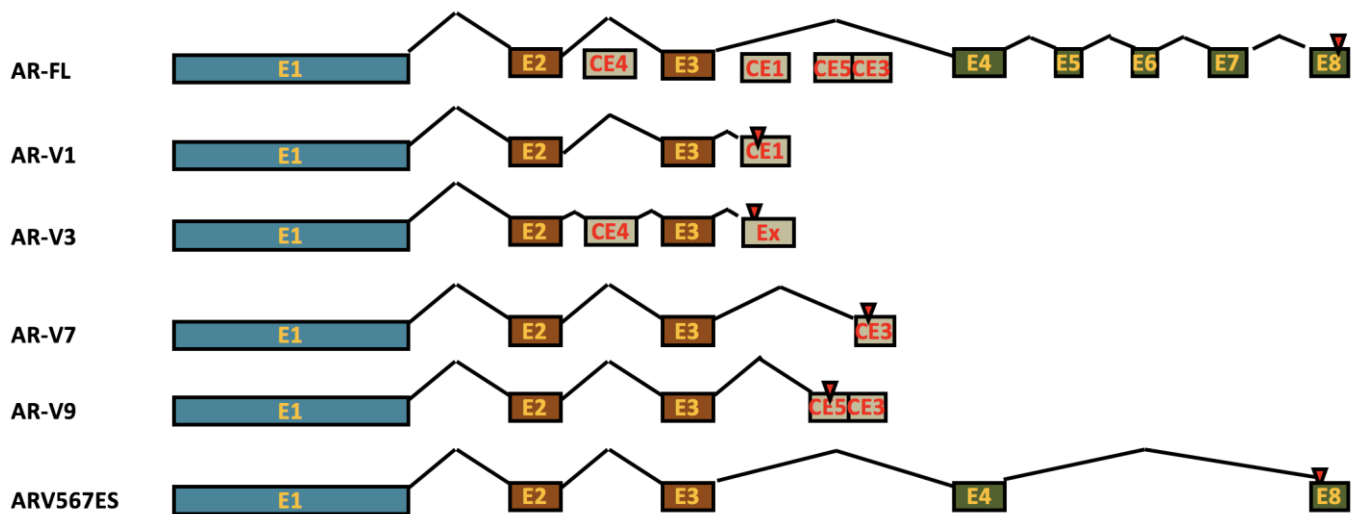
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Figure 1 legend: Transcript structure for representative AR-Vs. E1-E8: canonical AR exons 1-8; CE1-5: cryptic exons 1-5; Ex: unknown exon in AR-V3. Stop codon positions are marked for each AR transcript.



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