

## Development and prevalence of castration-resistant prostate cancer subtypes

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### Abstract

**Background:** Castration-resistant prostate cancer (CRPC) occurs when prostate cancer (CaP) progresses under therapy-induced castrate conditions. Several mechanisms have been proposed to explain this acquired resistance, many of which are driven by androgen receptor (AR). Recent findings, however, sub-classified CRPC by downregulation/absence of AR in certain subtypes that consequently do not respond to anti-androgen therapies. To highlight the significance of CRPC sub-classification, we reviewed the development and treatment of CRPC, AR downregulation in CRPC, and summarized recent reports on the prevalence of CRPC subtypes.

**Methods:** Using a medline-based literature search, we reviewed mechanisms of CRPC development, current treatment schemes, and assessed the prevalence of AR low/negative subtypes of CRPC. Additionally, we performed immunohistochemical staining on human CRPC specimens to quantify AR expression across CRPC subtypes.

**Results:** In the majority of cases, CRPC continues to rely on AR signaling, which can be augmented in castrate-conditions through a variety of mechanisms. However, recently low/negative AR expression patterns were identified in a significant proportion of patient samples from a multitude of independent studies. In these AR low/negative cases, we postulated that AR protein may be downregulated by (1) promoter methylation, (2) transcriptional regulation, (3) post-transcriptional regulation by microRNA or RNA-binding-proteins, or (4) post-translational ubiquitination-mediated degradation.

**Conclusions:** Here, we discussed mechanisms of CRPC development and summarized the overall prevalence of CRPC subtypes; interestingly, AR low/negative CRPC represented a considerable proportion of diagnoses. Because these subtypes cannot be effectively treated with AR-targeted therapeutics, a better understanding of AR low/negative subtypes could lead to better treatment strategies and increased survival.

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**Keywords:** Castration-resistant prostate cancer, Double negative prostate cancer, AR low prostate cancer, Neuroendocrine prostate cancer, Therapy resistance

**Abbreviations:** ADT, androgen deprivation therapy, AR, androgen receptor, ARSI, androgen receptor signaling inhibitor, ARLPC, AR low prostate cancer, CaP, prostate cancer, CRPC, castration-resistant prostate cancer, DNPC, double negative prostate cancer, NEPC, neuroendocrine prostate cancer

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Contents

Introduction ..... 00

Methods ..... 00

    Meta-analysis ..... 00

    Immunohistochemistry ..... 00

    Statistical analysis ..... 00

Results ..... 00

    Androgens and AR in the prostate ..... 00

    Targeting the androgen axis ..... 00

    Treatment timeline and progression to castration-resistant prostate cancer ..... 00

    Mechanisms of AR-dependent CRPC ..... 00

        Increased AR expression ..... 00

        AR mutation ..... 00

        AR splice variants ..... 00

        Intra-tumoral steroid hormone synthesis ..... 00

        Co-factor activity modulation ..... 00

    AR-independent CRPC ..... 00

        Neuroendocrine prostate cancer (NEPC) ..... 00

        AR low/negative prostate cancer (ARLPC/DNPC) ..... 00

        AR heterogeneity ..... 00

    Regulation of AR in AR-independent CRPC ..... 00

        AR transcriptional regulation ..... 00

        AR post-transcriptional regulation ..... 00

        AR degradation ..... 00

Conclusions ..... 00

CRediT authorship contribution statement ..... 00

    Declaration of Competing Interest ..... 00

    Acknowledgments ..... 00

Funding ..... 00

Disclosures ..... 00

References ..... 00

Introduction

Prostate cancer (CaP) initiation and progression is driven by androgens through binding to the androgen receptor (AR).<sup>1</sup> This signaling cascade can be targeted with several types of androgen deprivation therapies (ADT), with initial success. However, 10–20% of cases progress after androgen deprivation to a more aggressive disease stage: castration-resistant prostate cancer (CRPC).<sup>2</sup> Several mechanisms for the development of CRPC have been proposed, including both AR-dependent and AR-independent processes.<sup>2,3</sup> While the majority of CRPCs remain dependent on AR and androgen signaling, the introduction of more effective AR-targeted therapies like enzalutamide and abiraterone have caused an increase of AR low/negative (AR low/-) CRPC subtypes.<sup>4,5</sup> This is clinically significant because AR-dependent CRPCs can be treated with AR-directed therapies, but there are few options for treatment of AR-

independent CRPC. Moreover, AR is a differentiation factor in the prostate, suggesting its presence or absence in advanced CaP underlies the plasticity between CRPC subtypes.<sup>6</sup> To further explore the role of the prostate differentiation factor, AR, in CRPC, our review summarizes (1) recent findings on mechanisms of AR-dependent and AR-independent CRPC development, (2) current CRPC treatment schemes, and (3) regulation of AR in all CRPC subtypes, with a particular focus on AR-independent CRPC.

Methods

Meta-analysis

A Medline-based literature search of peer-reviewed articles addressing CaP, CRPC, and AR was performed. For reviewing AR-negativity (Table 2), search results were narrowed based on the terms "prostate cancer" and "androgen receptor" and "IHC" to specifically interrogate AR protein expression. An additional criterion included filtering results for species specification (human). Of the 42 results on PubMed, 22 were included in Table 1 due to exclusion of studies that (1) were cell-line based, (2) did not explicitly quantify AR positivity or negativity in their analyses, or (3) did not show data for AR protein expression. For some studies, AR positivity was quantified as a percentage of total specimens stained; these cases were included in Table 1 after calculating AR negativity as the remainder of samples that were not considered "AR positive".

Immunohistochemistry

Tissues were acquired from the Prostate Cancer Biorepository Network (PCBN), through Dr. Colm Morrissey at the University of Washington.

Table 1. Median survival after treatment with second generation anti-androgens.

	Metastatic CRPC		Non-metastatic CRPC
	Chemotherapy naive	After chemotherapy	
Enzalutamide	2.2 mos median OS <sup>43</sup>	4.8 mos median OS <sup>42</sup>	21.9 mos median MFS <sup>44</sup>
Abiraterone	8.2 mos median PFS <sup>40</sup>	3.9 mos median OS <sup>41</sup>	28.7 mos median PFS <sup>45</sup>
Apalutamide	NCT02703623 trial ongoing (clinicaltrials.gov)		24.3 mos median MFS <sup>35</sup>
Darolutamide	NCT02933801 trial ongoing (clinicaltrials.gov)		22.0 mos median MFS <sup>37</sup>

Survival is represented as survival benefit over control for each study and was measured in median months (mos) of overall survival (OS), progression free survival (POS) or metastasis free survival (MFS). \* indicates values represented as raw median survival without normalization to placebo, as discussed in the primary source.

**Table 2.** Prevalence of AR low/negative Expression Pattern in CaP and CRPC.

Study	Year	Stage	AR low/-	n	Antibody
Becker et al. <sup>72</sup>	2020	CaP	18.4%	354	Ventana SP107
Bluemn et al. <sup>4</sup>	2017	CRPC	20.5%	44	Biogenex F39.4.1
Chodak et al. <sup>73</sup>	1992	CaP	68.4%	19	Rat monoclonal
Choucair et al. <sup>88</sup>	2012	CaP	26.6%	64	Neomarker AR 441
de Winter et al. <sup>74</sup>	1990	CaP	8.65%	81	F39.4.1
Hobisch et al. <sup>75</sup>	1996	Mets	33.3%	12	Paesel Lorei PG21
Husain et al. <sup>76</sup>	2016	CaP	40.0%	10	Biogenex F39.4.1
Kehr et al. <sup>77</sup>	2016	Mets	0.0%	6	Dako AR 441
Labrecque et al. <sup>5</sup>	2019	CRPC	27.6%	98	Biogenex F39.4.1
Li et al. <sup>78</sup>	2018	CRPC	26.7%	152	Santa Cruz N20
Miyamoto et al. <sup>79</sup>	1993	CaP	10.0%	10	AR52
Russo et al. <sup>80</sup>	2018	CRPC	33.3%	3	Millipore PG21
Sehgal et al. <sup>8</sup>	2019	CaP	32.0%	73	Biocare Medical AR 441
Sharp et al. <sup>89</sup>	2019	CRPC	24.3%	144	Santa Cruz AR 441
Steurer et al. <sup>81</sup>	2019	CaP	36.8%	7151	Novocastra 2F12
Suryavanshi et al. <sup>82</sup>	2015	CaP (Stage 2–3)	11.8%	34	Cell Marque SP107
		CaP (Stage 4)	35.0%	20	
Udager et al. <sup>83</sup>	2014	CRPC	10.0%	30	Cell Marque AR 441
Vagundova et al. <sup>84</sup>	2003	CaP	13.2%	53	Biogenex F39.4.1
Vellky et al. <sup>7</sup>	2019	CaP	42.6%	73	Spring Bioscience SP107
		Mets	27.0%	22	
Wang et al. <sup>85</sup>	2019	CaP	17.7%	62	Dako AR 441
		CRPC	25.0%	24	
Welti et al. <sup>86</sup>	2016	CaP	21.2%	33	Dako AR 441
		CRPC	14.3%	35	
Zhang et al. <sup>87</sup>	2011	Mets	12.0%	50	Biogenex F39.4.1

Quantification and scoring of AR in prostate cancer (CaP), metastases (Mets), and castration resistant prostate cancer (CRPC) showing the prevalence of AR low/negative expression pattern (noted either directly or by exclusion from AR positivity quantification). Each study has been listed by author, followed by year of publication, disease stage analyzed (CaP, Mets, CRPC), prevalence of AR low/- samples quantified as a percent of total samples, sample size (n), and the AR antibody used in the study.

This LuCaP patient derived xenograft tissue array includes CRPC subtypes AR+ (LuCaP23.1, 35, 77, 76CR, 73;  $n = 45$  cores), AR low/- (DNPC – LuCaP173.2A, 173.2B, ARLPC – LuCaP176;  $n = 27$  cores), and NEPC (LuCaP173.1, 145.1, 145.2, 93;  $n = 36$  cores). IHC was performed according to Biocare protocol, as previously described.<sup>7</sup> Briefly, tissues were hydrated using xylenes and ethanol, and primary antibody detecting the C-terminus of AR (Abcam Cat. No. 227678, Cambridge, UK) was incubated on tissue for 1 h at room temperature. AR expression was detected with Impact Vector Red substrate (Vector Laboratories Cat. No. 5105, Burlingame, CA, USA), and nuclei were counterstained with Mayer's hematoxylin (Millipore Sigma Cat. No. MHS16, St. Louis, MO, USA). Single stained slides were used to create a spectral library and the array was imaged using Vectra automatic image acquisition (Perkin Elmer, Waltham, MA, USA). Objective quantification was determined using InForm version 1.4 software (Perkin Elmer, Waltham, MA, USA) as previously described.<sup>7,8</sup>

### Statistical analysis

Graphpad Prism 5.04 (Graphpad Software, La Jolla, CA, USA) was used for statistical analysis. Differences among continuous variables were assessed with one-way ANOVA with Tukey's Multiple Comparison Test. For all analyses,  $p < 0.05$  was considered statistically significant, denoted with \*.

## Results

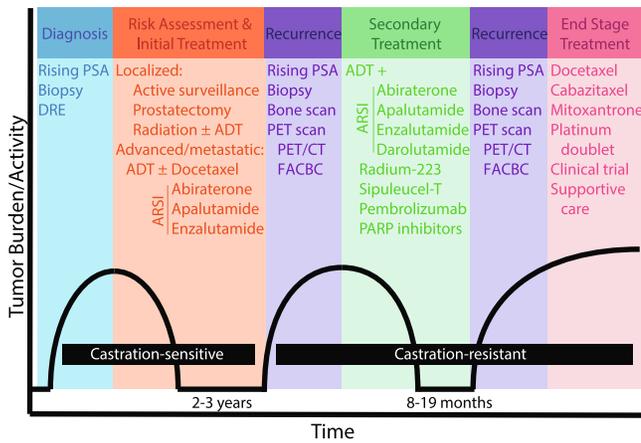
### Androgens and AR in the prostate

For decades, androgens have been implicated in prostate development, normal prostate homeostasis, CaP development, and CaP progression.<sup>1</sup> Mechanistically, androgen-mediated regulation works through AR, a ligand-dependent nuclear transcription factor.<sup>1</sup> In the normal prostate, it is generally accepted that signaling through stromal AR promotes cell

growth, while epithelial AR balances this stimulation by (1) acting as a suppressor of basal cell proliferation and (2) maintaining differentiated luminal cell survival.<sup>9,10</sup> However, in CaP initiation, epithelial AR can undergo a "malignancy switch", where it begins stimulating proliferation rather than maintaining differentiation.<sup>11</sup> Though this phenomenon is not well understood, several proposed mechanisms have been explored, including AR overexpression,<sup>12</sup> AR mutation,<sup>13</sup> and a shift from paracrine to cell-autonomous AR signalling.<sup>14,15</sup> Because androgens drive this signaling pathway, it was initially thought that increased androgens promoted disease progression, with some studies showing high serum levels of androgen were associated with increased risk of CaP.<sup>16,17</sup> However, several others have shown that there is either no association,<sup>18</sup> or an inverse association between serum androgens and CaP risk.<sup>19</sup> Importantly, several studies have shown that low serum testosterone levels at the time of CaP diagnosis is correlated with more aggressive disease.<sup>20,21</sup> Along this same line of thought, several studies have implicated loss of epithelial AR in prostatic disease to increased malignancy.<sup>9,22–24</sup> However, this phenomenon has been generally overlooked in CaP research, resulting in limited treatment options for men with AR low/negative disease. The loss of this differentiation marker may be particularly important as new, potent anti-androgens promote the development of AR low/- CRPC.<sup>4,5</sup>

### Targeting the androgen axis

Because androgens and AR play such a significant role in the prostate, many of the therapies used to treat CaP target this pathway by androgen deprivation therapy (ADT) via (1) surgical castration by orchiectomy or (2) chemical castration.<sup>25,26</sup> There are several categories of modern ADT drugs that have been successful in the clinic: luteinizing hormone-releasing hormone agonists/antagonists, AR antagonists, and androgen synthesis inhibitors (e.g. CYP17).<sup>25,26</sup> Normal androgen production in adult men is mediated through the hypothalamic-pituitary-gonadal (HPG) axis.<sup>27</sup> In this paradigm, luteinizing hormone-releasing hormone (LHRH; also known as gonadotropin releasing hormone, or GnRH) is



**Fig. 1.** Prostate Cancer Progression to CRPC Timeline and Treatments. Schematic for diagnosis and treatment of CaP through progression to CRPC. DRE = digital rectal exam, PSA = prostate-specific-antigen, ADT = androgen deprivation therapy (LHRH agonist/antagonists), ARSI = androgen receptor signaling inhibitor, abiraterone = abiraterone acetate, PET = positron emission tomography, CT = computed tomography, FACBC = anti-1-amino-3-18F-fluorocyclobutane-1-carboxylic acid (also known as fluciclovine F18), PARP = Poly (ADP-ribose) polymerase.

secreted from the hypothalamus to stimulate the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH).<sup>27</sup> LH then stimulates Leydig cells to produce testosterone. This circulating testosterone, when locally converted to DHT, can stimulate the AR-mediated transcriptional cascade in the prostate, while also inducing a negative feedback loop within the HPG axis to inhibit further secretion of LHRH, LH, and FSH, and testosterone production.<sup>27</sup> Because this negative feedback loop remains intact in CaP, LHRH agonists can be used to stimulate this negative feedback, ultimately resulting in a reduction in serum testosterone levels.<sup>26,27</sup> FDA-approved LHRH agonists include leuprolide, goserelin, triptorelin, and histrelin (cancer.org, Hormone Therapy for Prostate Cancer). However, LHRH agonists cause an initial flare in testosterone with adverse side effects; LHRH antagonists, on the other hand, can also shut down the production of testosterone by competitively binding the GnRH receptor, without the initial testosterone flare.<sup>28</sup> Currently, there is only one LHRH antagonist that is FDA approved – degarelix. Abarelix was used clinically before being removed from the market for adverse side effects, and relugolix is currently in clinical trial.<sup>29,30</sup>

ADT can also be achieved by directly inhibiting the androgen receptor using AR antagonists. There are several AR antagonists that are used clinically for both primary CaP and advanced disease. First generation AR antagonists were introduced to clinical practice in the US in the late 1980's, and include flutamide, nilutamide, and bicalutamide.<sup>31–33</sup> All three of these drugs are non-steroidal anti-androgens (NSAA) that act as reversible selective competitive silent antagonists.<sup>31</sup> Of the three, bicalutamide (BICA) has the strongest affinity for AR;<sup>32</sup> however, though BICA has a higher affinity for AR compared to other first generation anti-androgens, it is still about 30-fold lower than the affinity of native ligand, DHT.<sup>32</sup> Additionally, the survival benefit for men treated with BICA is modest at 3–6 months,<sup>33</sup> and at later stages in disease progression, BICA can exhibit partial agonistic effects when bound to AR.<sup>34</sup> In attempt to optimize the efficacy of AR antagonism, a second generation of drugs has been developed including enzalutamide, apalutamide, and darolutamide.<sup>35–37</sup> Enzalutamide (ENZ), introduced to clinical practice in 2012, is an NSAA that has a 5-8-fold increase of affinity for AR compared to BICA, which equates to a 2-3-fold reduced affinity for AR compared to DHT.<sup>36</sup> Similarly, NSAA apalutamide was approved by the FDA in 2018, and has a 5-10-fold increase of affinity for AR compared to BICA.<sup>35</sup> The

most recently developed NSAA, darolutamide, was approved by the FDA in 2019, and demonstrated the highest affinity to AR of any antagonist thus far (IC50 = 26 nM vs. 219 nM for ENZ and 200 nM for apalutamide).<sup>37</sup> In addition to higher affinity for binding to AR, the improved efficacy of these second-generation anti-androgens could be attributed to improved mechanism of action based on preventing translocation of AR to the nucleus, effectively preventing the AR-induced transcriptional program.<sup>38</sup>

Finally, the HPG axis can also stimulate the production of adrenal androgens, or in advanced disease, the tumor itself can produce androgen which can compensate for low levels of serum testosterone.<sup>27,39</sup> To circumvent this compensation, FDA-approved drug abiraterone acetate is used to inhibit the production of adrenal and intra-tumoral androgens by targeting CYP17, an enzyme involved in androgen steroidogenesis.<sup>40,41</sup> Second-generation AR antagonists and inhibitors of androgen production can be grouped together under the generalized category of androgen receptor signaling inhibitors (ARSI). Taken together, it is clear that targeting the androgen axis in CaP has been a mainstay of treatment for decades, and improved AR-targeted therapies are still progressing.

*Treatment timeline and progression to castration-resistant prostate cancer*

Men can be diagnosed with CaP based on several clinical tests including digital rectal exam (DRE), Gleason score from prostate biopsy, and/or rising levels of serum prostate-specific-antigen (PSA).<sup>25</sup> After diagnosis, initial interventions for localized disease include active surveillance (sometimes referred to as watchful waiting) or ADT via (1) surgery (prostatectomy) or (2) LHRH agonists/antagonists with or without radiation.<sup>25</sup> For locally advanced or metastatic cases, ADT can be combined with chemotherapy (docetaxel) or ARSI (abiraterone, apalutamide, enzalutamide) (Fig. 1).<sup>25,26</sup> Disease at this stage is thought to be castration-sensitive, where ADT results in reduced tumor burden and decreased serum PSA. While this androgen suppression is initially effective, nearly all patients undergoing hormone-based therapies stop responding within 2–3 years.<sup>39</sup> This recurrence, identified by a rise in PSA, biopsy, bone scan, and/or PET imaging of recurrent/new metastases, is known as castration-resistant prostate cancer (CRPC).<sup>39</sup>

Despite the nomenclature suggesting CRPC will not respond to hormone-based therapies, several second-generation anti-androgens have been successful for treatment of recurrent disease (Fig. 1). Enzalutamide for CRPC treatment has been shown to increase time to progression by 8.3 months in the AFFIRM trial, 11.2 months in the PREVAIL trial, and 19.4 months in the TERRAIN trial.<sup>33</sup> Median survival after treatment with second-generation anti-androgens (enzalutamide, abiraterone acetate, apalutamide, darolutamide) is summarized in Table 1.<sup>35,37,40–45</sup> Bicalutamide has also been used to treat CRPC with inconsistent efficacy. In one study, 17/38 cases of non-metastatic CRPC responded to BICA, with an increase of metastasis-free survival to 52.5 months compared to 15.7 months for non-responders.<sup>46</sup> Other studies have identified adverse effects from treatment with bicalutamide in advanced CaP due to the partial agonistic effect of binding to AR.<sup>34</sup> Treatment of CRPC with ADT can be combined with several other therapies including ARSI (abiraterone, apalutamide, enzalutamide, darolutamide), radium-223 to target bone metastases, immunotherapy-based approaches (sipuleucel-T, pembrolizumab), and PARP inhibitors (Olaparib, rucaparib) (Fig. 1).<sup>25,47</sup> This continued response to AR-targeted therapies suggests AR signaling plays a prolonged role in progression of CaP to CRPC; however, these treatments are not durable resulting in end stage treatment with chemotherapy (docetaxel, cabazitaxel, mitoxantrone, platinum doublet), enrollment in a clinical trial, or best supportive care (Fig. 1). Therefore, it is likely that there

are mechanisms regulating progression to CRPC that allow escape from the androgen axis.

### Mechanisms of AR-dependent CRPC

The role of the androgen/AR signaling axis in CaP progression is well-studied, with many studies purporting that castration resistance continues to rely on AR signaling. Data from these studies show AR-dependent CRPC can develop through multiple mechanisms: increased AR expression, AR mutation, emergence of AR splice variants, increased intra-tumoral steroid hormone synthesis or modulation of co-factor activity. These mechanisms are described briefly here, with more detailed explanations in reviews specifically focused on AR-dependent CRPC.<sup>48</sup>

#### Increased AR expression

Increased AR expression has been shown to occur by gene amplification, increased translation, and decreased degradation. AR gene amplification has been identified in (1) a subset of relapsed CaP tumors after ADT,<sup>49</sup> and (2) a subpopulation of cells within a hormone-naïve tumor.<sup>50</sup> Alternatively, AR expression can be affected by increased protein stability, which has been shown to occur through E3 ligase, MID1.<sup>51</sup> Conversely, another E3 ligase, SPOP, was mutated in 15% of CaP tumor vs. normal prostate resulting in increased persistence of AR expression due to decreased degradation.<sup>52</sup> Importantly, in all cases, the increased expression of AR may confer hypersensitivity to low levels of circulating androgens after ADT.

#### AR mutation

Several point mutations in the AR gene have been shown to increase AR activity. For example, mutations in the ligand binding domain of AR (H874Y, T877A, T877S) have been shown to relax ligand specificity of AR, allowing activation by alternate steroid hormone like 5 $\alpha$ -DHT, progesterone, and DHEA.<sup>53</sup> Other mutations including T878A, were found to be present in 15–20% of CRPC tumor samples, specifically in patients that showed agonistic effects of treatment with AR antagonists flutamide and bicalutamide.<sup>54</sup> More recently, specific mutations have also been implicated in therapy resistance to second generation AR antagonists, including F876L, which converted enzalutamide from antagonist to agonist in reporter-based mutagenesis screens.<sup>55</sup>

#### AR splice variants

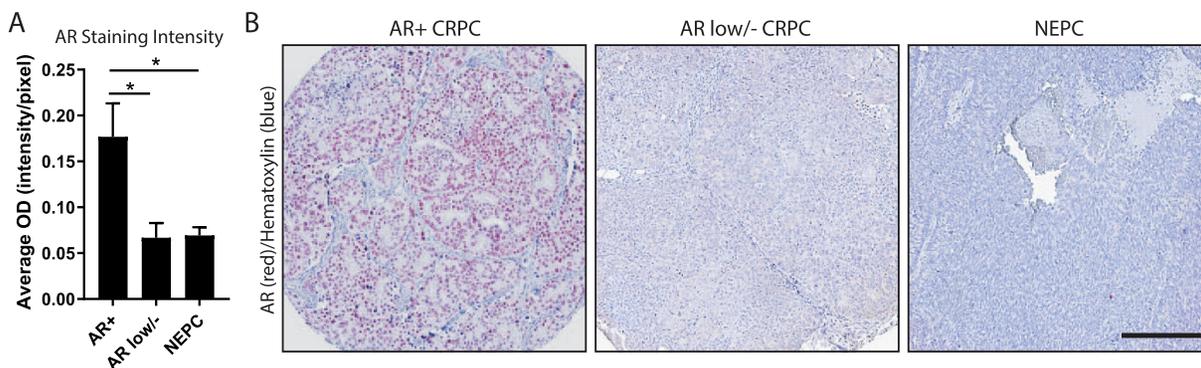
Recently characterized AR splice variants have been shown to emerge at later stages of CaP progression, including CRPC.<sup>56,57</sup> Some AR variants, including ARV7 and ARV567, lack the ligand binding domain present in the full-length receptor, resulting in constitutive transcription factor activity in the absence of ligand.<sup>56,57</sup> Additionally, AR and ARV7 require dimerization to translocate to the nucleus, and recent studies show that these receptors can heterodimerize and traffic to the nucleus together.<sup>58</sup> This is significant because full length AR requires ligand to dimerize, but ARV7 lacks the ligand-binding-domain, so heterodimerization may allow these receptors to translocate to the nucleus in a low androgen environment.<sup>58</sup> Based on the correlation between high levels of splice variant expression and poor prognosis, clinical implications may include targeting these variants in CRPC.<sup>56</sup> Clinical significance of targeting ARV7 in CRPC is currently under investigation with ARV7 inhibitor niclosamide.<sup>59</sup>

#### Intra-tumoral steroid hormone synthesis

While targeting the HPG axis results in near complete suppression of serum testosterone, several studies have shown that intra-prostatic levels of androgen (DHT) are only reduced by 70–80% after ADT.<sup>60,61</sup> This incomplete suppression of intra-prostatic androgens results in continued expression of AR target genes, suggesting that signaling remains active in castrate conditions.<sup>61</sup> Intra-tumoral synthesis of hormones can occur through (1) conversion of adrenal androgens (androstenedione, DHEA) to testosterone,<sup>62</sup> (2) conversion of cholesterol to testosterone,<sup>63</sup> or (3) *de novo* androgen synthesis by conversion of acetic acid to DHT.<sup>64</sup> In terms of therapy resistance, this intra-tumoral synthesis/retention of androgen could significantly alter the efficacy of ARSI by outcompeting AR antagonists for binding to AR, leading to development of CRPC.

#### Co-factor activity modulation

AR functions as a transcription factor in concert with several other factors that may play a role in the transcriptional targets and activity of AR.<sup>65</sup> These other factors have been categorized based on function as "co-activators" and "co-repressors", which in combination amount to >150 molecules that may influence the transcriptional activity of AR.<sup>66</sup> Importantly, these co-factors can activate AR transcriptional activity in low androgen conditions and have been implicated in the development of CRPC.<sup>65</sup> For example, CBP/P300 and GATA2 are co-activators of AR, and when inhibited, result in decreased AR expression and CaP growth.<sup>67,68</sup> On the other hand, SRC-1, -2, and -3, can influence AR reg-



**Fig. 2.** AR Expression in CRPC Subtypes. (A) AR protein expression was assessed in 3 subtypes of CRPC: AR positive (AR+,  $n = 45$  cores), AR low/negative (low/–,  $n = 27$  cores), and neuroendocrine (NEPC,  $n = 36$  cores). When quantified, AR optical density (OD) measured in intensity per pixel was significantly lower in AR low/– CRPC ( $p = 0.0486$ ) and NEPC ( $p = 0.0371$ ) compared to AR + CRPC. (B) Representative images of AR + CRPC, AR low/– CRPC, and NEPC cores, where AR expression is stained in red, and nuclei are counterstained with hematoxylin (blue). Scale bar represents 100  $\mu\text{m}$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ulation by promoting formation of promoter/enhancer complexes at the transcriptional start site of AR target genes.<sup>69</sup> While co-activators FKBP51 and SRCs are increased in CRPC, co-repressors are frequently decreased in CRPC, suggesting AR function can be influenced by co-factors that may allow persistent AR transcriptional activity in CRPC despite a dearth of circulating androgens.<sup>69</sup>

### AR-independent CRPC

For decades the CaP field has focused nearly exclusively on AR-dependent mechanisms for CRPC, and hence androgen-targeted therapies remain the primary treatment strategy; however, targeting AR invariably results in therapy resistance. To this end, an alternative to the gain of AR function philosophy is loss of AR function, which has only recently begun to be thoroughly investigated. In the prostate, there are several epithelial cell populations that are known to lack AR expression including basal-, stem-, neuroendocrine-, and some luminal cells.<sup>70,71</sup> Expansion of these AR negative cell populations, plasticity in differentiation states, and/or loss of AR in response to therapeutic pressures may give rise to a tumor (or part of a tumor) that is inherently resistant to AR antagonism due to the lack of targetable AR protein. The prevalence of an AR low/negative expression pattern has generally been underappreciated, but has been observed in a number of experiments (Table 2).<sup>4,5,7,8,72–89</sup> More generally, several studies have observed decreased AR protein expression/intensity in hormone-refractory patient metastases,<sup>90</sup> and in hormone-refractory patients when compared to normal, primary, or hormone-sensitive tissue.<sup>86,91</sup> CRPC subtypes that exhibit AR low/negative expression patterns (e.g. neuroendocrine prostate cancer – NEPC, double negative prostate cancer – DNPC, AR low prostate cancer – ARLPC) will be discussed here, and quantitative comparison of AR protein expression between subtypes will be assessed by immunohistochemical analysis (Fig. 2). AR heterogeneity, which implicates a subpopulation of AR-independent cell growth within a bulk AR+ population will also be discussed.

### Neuroendocrine prostate cancer (NEPC)

Historically, the primary subset of AR negativity in CRPC is NEPC, characterized by the loss of AR expression and gain of neuroendocrine differentiation markers chromogranin A and synaptophysin.<sup>92,93</sup> The normal prostate maintains a population of neuroendocrine cells, with 1% of primary CaP is considered NEPC; however, this differentiation is increased up to 30% of CRPC cases and is associated with poor clinical outcomes.<sup>94</sup> More, two recent studies have suggested that neuroendocrine subtypes of CRPC make up 9.1% and 10.2% of cases<sup>4,5</sup> (Table 3). When objectively compared to AR + CRPC, NEPC expressed significantly lower AR protein ( $p < 0.05$ ) (Fig. 2). Because NEPC lacks AR expression and signaling, ADT is not an effective treatment for this disease, resulting in a median survival rate of only 2 years after diagnosis.<sup>95</sup> Because these small-cell CaPs occur from expansion of the neuroendocrine cell population, resulting resistance to therapies can be considered an intrinsic resistance mechanism. Importantly, AR expression in NEPC is lost at both the RNA and protein level, suggesting potential epigenetic regulation of AR in this context.<sup>93</sup> Conventional clinical management for NEPC relies on cisplatin-based chemotherapy with recent data implicating an N-Myc/

EZH2 pathway in the development of NEPC, which may provide a basis for more targeted therapeutics.<sup>96</sup>

### AR low/negative prostate cancer (ARLPC/DNPC)

Recently, two subtypes of CRPC have been characterized based on decreased expression levels of AR, without the gain of neuroendocrine markers characteristic of NEPC.<sup>4,5</sup> As the names suggest, double negative prostate cancer (DNPC) stratify samples that are negative for both AR and neuroendocrine markers, while AR-low prostate cancer (ARLPC) indicate cases that lack neuroendocrine markers, but maintain low levels of AR.<sup>5</sup> In a cohort of 98 human metastatic CRPC samples from rapid autopsy, 8.2% were classified as DNPC and 9.2% were classified as ARLPC based on immunohistochemical and RNA-sequencing analyses<sup>5</sup> (Table 3). In another cohort of 44 CRPC patients, 11.4% were classified as DNPC<sup>4</sup> (Table 3). This increasing prevalence of subtypes of metastatic CRPC that lose AR expression could be due to the increased efficacy of AR antagonists. According to one study, the frequency of DNPC incidence has increased from 5.4% before FDA-approval of enzalutamide and abiraterone (years 1998–2011) to 23.3% since the approval of ENZ/abiraterone (years 2012–2016);<sup>4</sup> taken together, these data suggest a mechanism of acquired resistance in this context. Here, we quantified the expression of AR protein in LuCaP patient-derived xenograft specimens previously diagnosed as DNPC and ARLPC compared to AR + CRPC. In these sample, there was a significant decrease in AR expression in these AR low/- subtypes, compared to AR + CRPC ( $p < 0.05$ ) (Fig. 2), providing validation for AR negativity in some CRPC subsets. Importantly, because these subtypes lack AR protein, treating with AR antagonists is not a logical strategy.

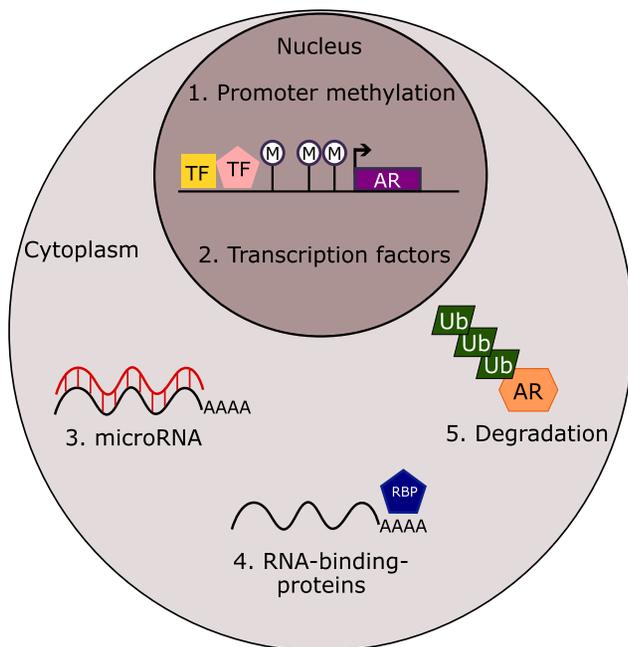
One interpretation of AR negativity in CaP is a loss of differentiation, or de-differentiation, to a state that lacks AR protein expression. In fact, a recent publications have investigated a multitude of phenotypes within metastatic CRPC noting that CRPC subtypes are differentiated on a disease continuum driven partially by AR.<sup>5,93,97</sup> Additionally, one paper showed that anti-androgen treatment could alter the differentiation state of CRPC, more specifically, DNPC to squamous cell carcinoma.<sup>5</sup> If it's true that DNPC represents a phase between differentiation states of CRPC, then it may be possible to induce differentiation of DNPC into a state that is easily targetable with current therapies: AR positive CRPC. Other therapeutic approaches have been suggested, including targeting the FGF/MAPK signaling axis.<sup>4</sup> Future investigation of the plasticity between differentiation states in CRPC, and the potential role for DDX3 in this context, should be considered.

### AR heterogeneity

In hormone naïve CaP and AR + CRPC, the majority of cells are AR positive; however, there is a subpopulation of cells within these subtypes that remains AR negative/low expressing. This variability of AR expression is known as AR heterogeneity, and has been recognized by the field for decades. Because AR-targeted therapy only effectively targets cells that express AR, increased AR heterogeneity is associated with poor clinical outcomes.<sup>78</sup> Though inter-experimental variability makes exact estimations of this heterogeneity difficult, several studies support the AR negative/low subpopulation can total up to 30% of cells within a primary tumor.<sup>7,8,74,91,98</sup> Furthermore, AR heterogeneity in human CaP bone metastases can be widespread, reaching up to 80% AR protein negativity in some patient cohorts.<sup>99</sup> In CRPC, heterogeneity of AR expression in 265 patients was observed; of these, 31% had AR positivity in <50% of the tumor, 41.5% had <10% AR positivity, and 1.8% of patients had tumors with <1% AR positivity.<sup>100</sup> Importantly, there is not a consensus for a threshold of AR positivity within a tumor that indicates positive response to ADT. This limited insight has led to widespread use of ADT for both castration-naïve

**Table 3.** CRPC Subtype Prevalence.

Study	Subtype	% of CRPC	n
Bluemn et al. <sup>4</sup> (2017)	AR+	79.5%	44
	NEPC	9.1%	
	DNPC	11.4%	
Labrecque et al. <sup>5</sup> (2019)	AR+	61.2%	98
	NEPC	10.2%	
	DNPC	8.2%	
	ARLPC	9.2%	



**Fig. 3.** Potential Mechanisms of AR Downregulation in CRPC. Schematic for potential mechanisms that downregulate AR in CRPC including (1) promoter methylation (M) (2) transcription factors (TF), (3) microRNAs, (4) RNA-binding-proteins (RBP), and (5) degradation mediated by ubiquitination (Ub).

and castration-resistant cases that may or may not express AR at biologically significant levels, potentially affecting efficacy.

#### Regulation of AR in AR-independent CRPC

Despite a relatively thorough understanding of recurrence that continues to rely on AR, there are only a few established mechanisms that are known to mediate the loss of AR expression in CRPC. Because AR low/- CRPC subtypes are becoming more prevalent, it is necessary to understand the regulation of AR in this context. Here, we will discuss several mechanisms that can cause downregulation of AR including promoter methylation, post-transcriptional targeting by microRNA and RNA-binding-proteins, and ubiquitin-mediated degradation (Fig. 3).

#### AR transcriptional regulation

Several studies have identified androgen receptor down-regulation at the transcriptional level in both CaP models and human samples.<sup>101–104</sup> In CaP cell lines DU145 and PC3, AR mRNA and protein are not expressed, suggesting a transcriptional repression of AR in these models.<sup>101</sup> In a seminal study assessing AR regulation in CaP, a repressive regulatory mechanism via promoter methylation was characterized.<sup>105</sup> Here, a 1.5 kb CpG island was identified by nucleotide sequence analysis in the AR gene landscape; this site for methylation was mapped to start in the promoter region for AR and span into exon 1, encompassing the transcriptional start site.<sup>103,105</sup> Using methylation-specific PCR, aberrant methylation was observed in this AR CpG island in several AR-negative cell line models, while AR-positive cells lines were unmethylated.<sup>105</sup> Additionally, demethylating the AR-negative cell lines using 5-aza-2'-deoxycytidine resulted in re-expression of AR mRNA.<sup>105</sup> These results indicate that AR can be transcriptionally downregulated by promoter methylation in models of CaP. To investigate the clinical incidence of this phenotype, AR expression was assessed in CaP patient samples – 4/15 samples lacked AR protein expression and 2/4 of those samples exhibited methylation at the AR promoter.<sup>103</sup> A similar study showed AR promoter methylation in

2/10 primary CaP patient samples and 4/14 CRPC samples.<sup>104</sup> In addition to downregulation by methylation, in a minority of cases AR can be transcriptionally down-regulated by gene deletion or frameshift mutations, which has been identified in androgen insensitivity syndrome.<sup>106</sup>

Another mechanism for transcriptional downregulation of AR is by expression changes of transcription factors that are known to regulate AR: TP53, RB1, and PTEN. TP53 and RB1 have been shown to transcriptionally regulate AR by binding the AR promoter either directly or through E2F to prevent transcription of AR.<sup>107,108</sup> Loss of these tumor suppressors at advanced stages of CaP may result in increased AR protein expression; however, recent data implicates TP53 and RB1 in disease recurrence, where TP53/RB1 loss is associated with diminished response to AR-targeted therapies and decreased survival.<sup>109</sup> Others have shown that alterations in TP53 and RB1 are associated with lineage plasticity and anti-androgen resistance in CRPC.<sup>110–112</sup> PTEN is another transcription factor that is known to downregulate AR; in this case, AR is suppressed via a PTEN/AKT signaling pathway,<sup>113</sup> with recent data suggesting PTEN loss may also be implicated in CRPC progression independent of the AKT pathway.<sup>114</sup>

Taken together, these data implicate several transcription factors that are known to suppress AR in the progression of CaP to CRPC.

#### AR post-transcriptional regulation

The current understanding of AR regulation at the post-transcriptional level implicates microRNAs (miR) and RNA binding proteins (RBP). miRs are short, untranslated RNAs that can cause degradation of target RNA, resulting in RNA silencing and post-transcriptional regulation of gene expression. Several miRs have been shown to target AR mRNA including miR-let7c.<sup>115</sup> More recently, research on miR targeting of AR has vastly increased, resulting in identification of 30 different miRs that can target AR and/or ARv7.<sup>116</sup> This list includes miR-205, miR-30c, miR-34c, miR-9, miR-135b, miR-149, and miR-193a.<sup>116</sup> Alternatively, RBPs can bind AR mRNA and either aid or disrupt translation. Several RBPs have been identified in post-transcriptional regulation of AR including HuR, PCBP1/2, and EBP1.<sup>117–119</sup> In some cases, RBPs positively regulate AR in CaP, as is the case for HuR and PCBP1/2; these RBPs have been shown to bind the AR mRNA 3'UTR and are suspected to play a role in mRNA stability or transport thereby enhancing mRNA translation.<sup>117</sup> On the other hand, EBP1, has been shown to negatively regulate AR translation in CaP. In this context, EBP1 bound to the UC-rich motif of the AR 3'UTR to promote mRNA decay.<sup>118</sup> In this same study, EBP1 was also identified to bind to a CAG-formed stem-loop in the 5' coding region of AR mRNA, resulting in translation inhibition.<sup>118</sup> A follow-up study assessed EBP1-mediated AR regulation in CRPC, where investigators observed EBP1 did not regulate AR mRNA levels, but reduced translation of AR mRNA in CRPC models.<sup>119</sup> This was purported to occur through a shift of AR mRNA towards translationally inactive ribosomes.<sup>119</sup>

#### AR degradation

Unlike most steroid hormone receptors, AR protein is not downregulated in a ligand-dependent fashion.<sup>120</sup> In fact, after ligand is removed, AR can be recycled back to the cytoplasm, where it can bind ligand and translocate to the nucleus for at least 4 cycles.<sup>121</sup> Despite the recycling of AR protein in response to ligand, protein levels can be regulated through the proteasome-mediated degradation pathway.<sup>122</sup> In this pathway, E3 ligase MDM2 is required for the ubiquitin-proteasomal degradation of AR, where MDM2 loss-of-function markedly decreased AR degradation.<sup>122</sup> This degradation pathway is of interest in AR-independent CRPC because if AR protein is being translated normally, then quickly degraded, AR-targeted therapies would not be effective. In fact, a recent study identified constant ubiquitination and subsequent degradation of AR through MDM2 in prostate cancer stem cells, which

exhibit an AR negative expression signature.<sup>71</sup> Additionally, there is precedent for this phenomenon in breast cancer therapy resistance, where degradation rates for estrogen receptor (ER) were 4-fold higher in anti-estrogen-resistant cells versus anti-estrogen sensitive cells.<sup>123</sup> This may be significant because it presents a potential pathway to increase survival under anti-hormone therapy; similar mechanisms may contribute to AR negativity in CaP and CRPC.

## Conclusions

Taken together, this review of the literature details the timeline for diagnosis and treatment of CaP and CRPC and highlights both AR-dependent and AR-independent mechanisms of CRPC development. While most of the literature focuses on AR-dependent CRPC, recent studies have highlighted the growing prevalence of AR-independent CRPC. Here, we described mechanisms through which the differentiation factor, AR, can be downregulated epigenetically, post-transcriptionally, or post-translationally, which may contribute to CRPC subtypes that cannot be effectively treated with AR-targeted therapies. We summarized the overall prevalence of CRPC that lacks AR expression, and further explored the incidence of specific CRPC subtypes. From these data, it is clear that AR-independent CRPC makes up a significant proportion of CRPC diagnoses, and that a better understanding of these AR low/negative subtypes could lead to better therapies and increased survival for men with this hormone-refractory disease.

## CRedit authorship contribution statement

**Jordan E. Vellky:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Funding acquisition. **William A. Ricke:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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