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ABSTRACT

Research on prostate cancer has extensively advanced in the past decade, through an improved understanding for its genetic basis and risk-stratification. Molecular classification of prostate cancer into distinct subtypes and the recognition of new histologic entities promise the development of tailored-made management strategies of patients. Nowadays, various alternatives are available for clinical management of localized disease ranging from observation alone through radical prostatectomy. In patients with castration-resistant prostate cancer, the approval of new drugs for the management of metastatic disease has offered promising results improving the survival of these patients. In this context, androgen receptors (AR) remain at the epicenter of prostate cancer research holding a prominent role in the biology and therapeutic regimens of prostate cancer. As many of castration-resistant tumors retain hormone-responsiveness, AR is a clinical relevant, druggable target. However, AR paradoxically remains neglected as a prostate cancer biomarker. The great advancements in prostate cancer preclinical and clinical research, imply further improvement in clinical and translational data, for patient selection and treatment optimization. For a precision medicine-guided clinical management of prostate cancer, AR evaluation has to be implemented in companion and complementary diagnostics, as discussed here. J. Cell. Biochem. 9999: 1–11, 2016.

KEY WORDS: PROSTATE CANCER; ANDROGEN RECEPTOR; CASTRATION-RESISTANT PROSTATE CANCER; ANDROGEN DEPRIVATION THERAPY; BIOMARKERS

Prostate cancer is the most common malignancy in men and a major cause of cancer deaths [WHO Classification, 2016]. The last decade has witnessed a great progress in our knowledge of prostate cancer biology, the potential of clinical management, and the classification of the disease. Thus, the recent 2016 World Health Organization (WHO) classification (aka WHO "blue book") introduced a new histologic entity (intraductal carcinoma), new variants of acinar adenocarcinoma and neuroendocrine carcinoma, and new immunohistochemical stains for diagnosis, grading, risk stratification, together with molecular genetics of acinar adenocarcinoma of the prostate [Humphrey et al., 2016; WHO Classification, 2016]. In addition to these, several new drugs have improved patient outcome and quality of life [Attard et al., 2016]. Recognition of prostate cancer hormone-responsiveness (e.g., androgen, estrogen, progesterone, corticosteroids) has heralded the translation of biological advances into clinical practice.

Androgen receptor (AR) axis has been early recognized and exploited as the cornerstone of prostate cancer treatment. Terms previously used for prostate cancer, such as "hormone refractory" or "androgen resistant," currently are considered inadequate. Instead, castration resistant prostate cancer (CRPC), a term describing lack or low response to androgen deprivation, is more appropriate to define these difficult-to-manage tumors. It is evident that AR holds a prominent role in this setting, although its assessment is not part of the routine pathology diagnosis for prostate cancer [WHO Classification, 2016]. The progress made has generated increasing demands for screening biomarkers, specific clinical management of identified molecular subtypes with different outcome, combined with predictive biomarkers for risk and response stratification, robust prognostic biomarkers and combined therapies and selection of the right treatment sequencing, which represent our major priorities. In this article, we provide an overview of advances—highlighting pitfalls and insights—and future directions for AR in prostate cancer.

PROSTATE CANCER AR TARGETING: SOMETHING OLD, GETTING NEW

The AR protein is a member of the steroid receptor family of transcription factors that share structurally conserved domains consisting of a DNA-binding domain (DBD), ligand-binding domain (LBD), an N′-terminal domain (NTD), and a hinge region that contains a nuclear localization sequence. The AR-LBD domain, is the

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hallmark of AR-targeting therapies, either directly (antiandrogens-binding LBD), or indirectly by reducing the levels of circulating and tissue androgens (LHRH/GnRH analogs and CYP17 inhibitors). Hence, AR-LBD is the epicenter of several mutations that have been associated with resistance to endocrine therapy of prostate cancer. The AR gene is located at chromosome X locus q11–12. The full-length receptor (FL-AR), consists of eight exons. Constitutively active AR variants (AR-Vs) have now been discovered, most of which contain the NTD and the DBD, but are deprived of LBD due to a truncation by a cryptic exon. As conventional androgen deprivation therapy (ADT) inhibits androgen-dependent activation of AR (mediated by the LBD), the presence of C-terminal truncated AR-Vs provides a compelling mechanism for CRPC cells to resist ADT. Their role in cancer initiation and progression remains contradictory. More than 20 different AR-Vs have been identified, usually with a truncated C′-terminal domain or hinge region [Lu et al., 2015].

AR as all other members of steroid hormone receptor family requires the functional activity of numerous chaperone and chaperone-associated proteins (including among others HSP90, HSP70, and p23), collectively termed as the foldosome. This AR-foldosome interaction regulates the activation, maturation, and stability of AR and maintains AR in a conformation that potentiates high-affinity ligand binding. However, it can also result in a proteasome-mediated AR turnover if the ligand is not available [Cano et al., 2013].

The primary agonists for AR are the androgens testosterone, and the more effective dihydrotestosterone (DHT) that bind to the AR-LBD, inducing a conformational change. This displaces the foldosome and triggers AR translocation into the nucleus. There, AR dimerizes, interacting with DNA androgen-responsive elements (AREs) that ultimately leads to cell growth, proliferation, and PSA secretion [Cano et al., 2013]. AR can additionally be trans-activated by growth factors (i.e., EGF, IGF, KGF) and cytokines (i.e., IL-6). The complexity of AR function and biology is also associated with AR signaling initiated from binding sites located at the plasma membrane (membrane-initiated steroid signaling, MISS). MISS has been described in several cell lines, including prostate cancer cells, and it is distinct from the ones observed in the traditional nuclear-associated effects (nuclear associated steroid signaling, NISS). Moreover, membrane androgen binding sites have been described in cell lines and prostate specimens, even in the absence of the full-length receptor FL-AR [Pelekanou et al., 2013]. Although, traditionally MISS has been considered as non-genomic (compared to NISS, genomic actions), it is now suggested that both MISS and NISS can trigger transcriptional effects, albeit with distinct signatures. AR signaling functions and possible blockade with novel agents are summarized in Figure 1. Reduction or blockade of gonadal androgen production or signaling, widely known as ADT, has been a part of the standard of care for patients with advanced-stage prostate cancer for more than 70 years [Huggins and Hodges, 2002]. Initially introduced as surgical castration, medical castration followed using gonadotropin-releasing hormone (GnRH) agonists (leuprolide and goserelin). These agonists saturate and suppress GnRH receptors, leading to decreased luteinizing hormone (LH), follicle-stimulating hormone (FSH) secretion, and subsequently decreased testicular production of testosterone. Additional suppression on adrenal androgen synthesis is often achieved using glucocorticoids. Androgen action blockade has also been made possible by the development of AR antagonists (antiandrogens), such as bicalutamide, flutamide, nilutamide (first generation antagonist), and enzalutamide (second-generation AR antagonist) (summarized in Figs. 1 and 2). Testosterone biosynthetic cytochrome P450 enzymes can also been inhibited, by ketoconazole, an antifungal drug impairing extratesticular androgen synthesis. Abiraterone is a quite recently introduced, more specific and potent inhibitor of androgen synthesis that restrains androgen synthesis in the testes, as well as in prostate cancer cells. New agents targeting AR axis are summarized in Figures 1 and 2 (including resistance mechanisms involved).

Unfortunately, despite the advances in prostate cancer drug development, most of prostate cancer patients develop castration resistant disease (CRPC, castration resistant prostate cancer). CPRC cells maintain androgen signaling and escape cell death or apoptosis despite robust androgen deprivation therapy (ADT). The term “castration-resistant prostate cancer” is used to describe this disease state, where even if circulating levels of testosterone are in castration range (50 ng/dl, instead of 280–1,100 ng/ml observed in normal males), AR signaling still drives tumor growth. Mechanisms leading to castration resistance could be defined based on their dependence on AR and ligand (cognate or not): AR and ligand dependent (androgen or non-androgen), AR dependent but ligand independent, and both AR and ligand independent (Figs. 2 and 3). The approval of abiraterone and enzalutamide for the treatment of advanced CRPC heralded a paradigm shift in the management of this disease, with demonstrated disease regression, clinical benefit, and good tolerance in a significant proportion of patients, validated in phase III clinical trials. ADT resistance remains a major issue for both enzalutamide and abiraterone. This has been shown in several reports and further confirmed by recent clinical trials [Narayanan et al., 2016]. Nevertheless, these two drugs have provided strong evidence on the importance of AR targeting, even under castration resistance [Narayanan et al., 2016]. The contribution of abiraterone and enzalutamide in prostate cancer management is not limited to their undeniable improved control of the disease. They have also shed light into the mechanisms involving androgen/AR axis and development of drug resistance and tumor cell survival despite use of androgen deprivation or even resistance of iatrogenic origin. The most striking paradigms include: (i) glucocorticoids interaction with AR, (ii) treatment induced-neuroendocrine differentiation, and (iii) constitutively activated AR variants.

INTERACTION OF SECOND GENERATION ANTIANDROGENS WITH GLUCOCORTICOIDS

Traditionally, glucocorticoid supplementation as part of systematic or palliative prostate cancer therapy has resulted in both subjective and objective responses in patients with advanced-stage disease. Introduction of the above-mentioned antiandrogens has unraveled, an unexpected iatrogenic stimulation of prostate cancer growth, mediated by glucocorticoids that could contribute to drug resistance and disease progression, even under good control of androgen

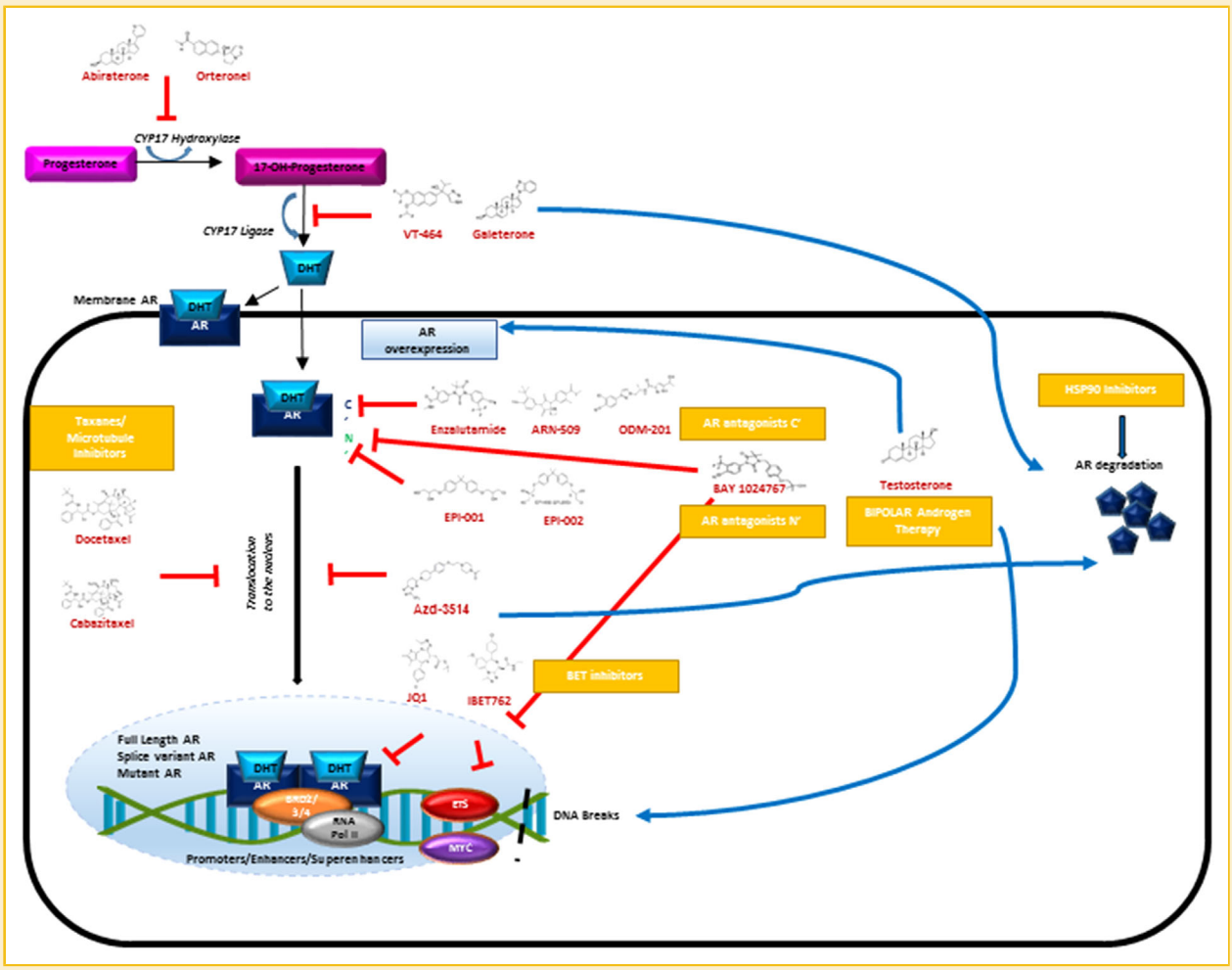

Fig. 1. New agents targeting AR axis in prostate cancer. In this figure, only agents introduced during the last decade are presented. Novel drugs can target several steps of the AR axis in prostate cancer. Firstly, inhibition of two steps of testosterone biosynthesis by abiraterone and orteronel or VT-464 and galeterone, respectively, reduces the levels of androgen. Binding of androgen to AR can be diminished by new generation AR antagonists like enzalutamide, AR-509 and ODM-501 that target the C′-terminal domain of the receptor (not present in AR-Vs). On the other hand, the N′-terminal domain a druggable target in AR-Vs (and AR-FL) can be blocked by EPI-001 and EPI-002. A new agent, BAY1024767, is efficient in targeting both AR-FL and AR-Vs. The AR-agonist/antagonist complex translocation to the nucleus can be controlled by AZD-3514 or drugs affecting microtubules, such as taxanes. Interaction of AR with downstream targets can be targeted by BET inhibitors, BAY1024767 or bipolar androgen therapy. Finally, AR turn-over can also be a druggable target through Hsp inhibition or induction by galeterone and AZD-3514. The effects of these new agents have not been thoroughly assessed for membrane-bound AR (MISS).

deprivation [Narayanan et al., 2016]. For example, the upregulation of glucocorticoid receptors (GRs) during enzalutamide therapy results in glucocorticoid–GR-mediated regulation of androgen target genes, leading to escape from enzalutamide blockade. On the other hand, abiraterone inhibits glucocorticoid synthesis and is usually accompanied by synthetic glucocorticoids to prevent adrenal insufficiency. These abiraterone-induced alterations in steroidogenesis patterns can induce resistance. On the other hand, as AR, GR, progesterone receptor (PR), and the mineralocorticoid receptor (MR) belong to class I nuclear receptors they share highly conserved DBDs. Hence, interactions within their pathways are facilitated. Thus, understanding the biological role of glucocorticoids in patients with prostate cancer is of major importance in the era of new and evolving antiandrogen therapies, as recently evidenced for baseline corticosteroids in a Phase 3 randomized trial of enzalutamide in patients with docetaxel-treated metastatic CRPC prostate clinical trial (COU-AA-301 study) [Montgomery et al., 2015]. This multifaceted androgen-glucocorticoids interaction provides important hints on the importance of the optimal regimen selection and appropriate drugs combinations. Of course, further studies are required to define under which condition glucocorticoids can be used with minimal or no negative impact on AR-targeting.

NEUROENDOCRINE DIFFERENTIATION INDUCED BY AR TARGETING THERAPY

Neuroendocrine histology in prostate cancer is a hallmark of highly aggressive disease with poor prognosis [Humphrey et al., 2016; WHO Classification, 2016]. Most patients develop visceral metastases, with

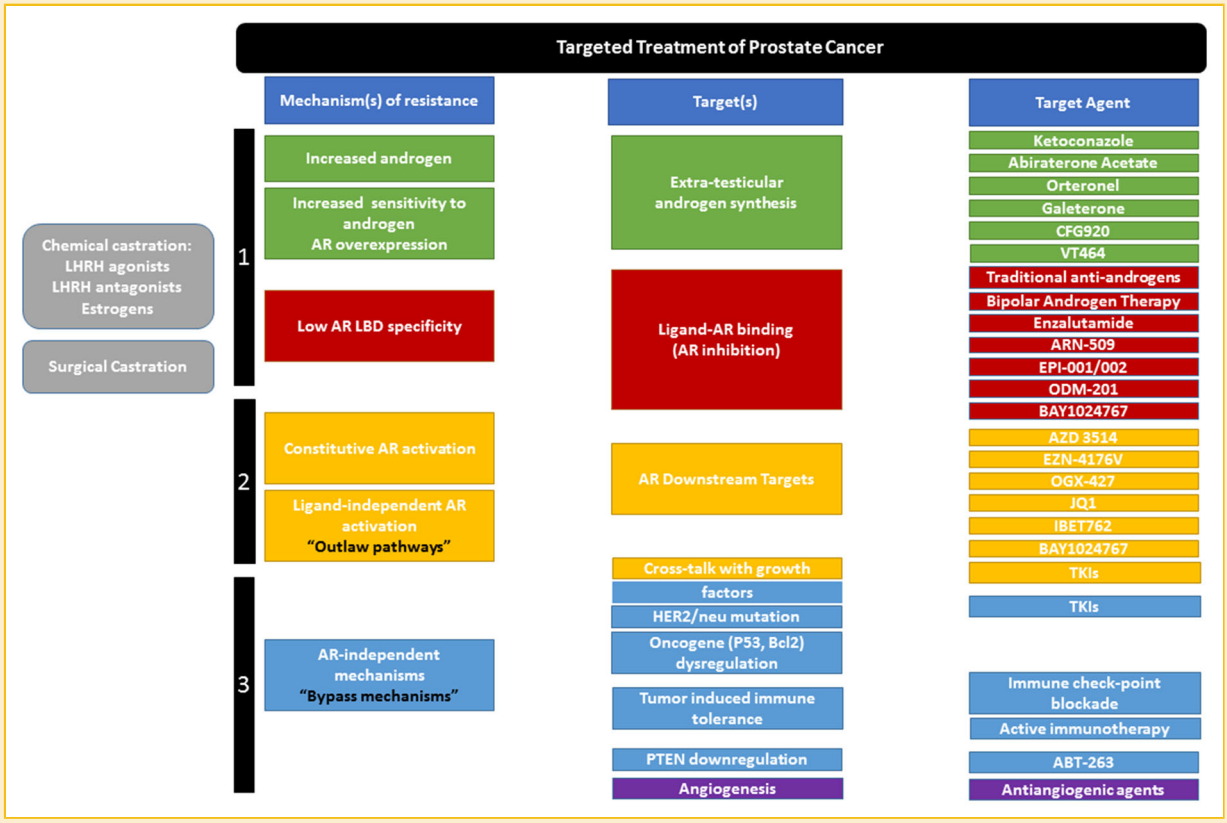

Fig. 2. ADT principles, mechanisms of resistance, and novel AR-related therapies. Resistance mechanisms can be divided into three groups: (1) this group is AR dependent and ligand dependent. It is due to high androgen levels (intratumor), increase in sensitivity to androgen, or AR overexpression and decreased specificity to AR LBD (through induction of mutations or AR-Vs). Inhibition of extratesticular androgen synthesis or antiandrogens and AR inhibitors can be useful to overcome this type of resistance. (2) AR-dependent and ligand-independent resistance mechanisms. The emergence of AR-Vs or mutated AR can constitutively activate AR. Cross-talk with growth factor receptors can also induce nonligand AR activation through cross-talk (outlaw pathways). Agents inhibiting downstream AR targets could be helpful for this type of resistance mechanism. (3) AR-independent resistance mechanisms (bypass pathways).

minor response to chemotherapy and survive less than a year. Neuroendocrine prostate cancer (NEPC) can arise de novo, but much more commonly arises after hormonal therapy for prostate adenocarcinoma. NEPC is characterized by the presence of small round blue neuroendocrine cells, which do not express androgen receptor (AR) or secrete prostate specific antigen (PSA), but usually express neuroendocrine markers such as chromogranin A, synaptophysin, and neuron specific enolase (NSE). Neuroendocrine differentiation was confirmed in tumor specimens of patients following enzalutamide or abetarone treatment, demonstrating AR positive and negative cells and TMPRSS2-ERG gene rearrangement [Beltran et al., 2011]. A new variant has been introduced by the WHO 2016 Classification: the large cell neuroendocrine carcinoma that arises following AR blockade [Humphrey et al., 2016; WHO Classification, 2016]. Recently, mast cell recruitment has been associated in neuroendocrine differentiation following enzalutamide (or bicalutamide) treatment. In this in vitro model, the aforementioned mechanism could be controlled by AR-siRNA or neutralizing anti-IL8 antibody, providing promising hints for new targets for this highly aggressive histotype of prostate cancer [Dang et al., 2015].

CONSTITUTIVELY ACTIVATED AR VARIANTS AND AR MUTANTS

Investigation on ADT resistance has shed light to mechanisms and generated many possible targets involved, directly or indirectly, at multiple levels of the AR axis. One of the most groundbreaking advancements was the identification of AR variants, deriving from mutations [Joseph et al., 2013] or alternative splicing [Maughan and Antonarakis, 2015a]. The expression of AR-V7 (or AR-3), a splice variant lacking the C′-domain, has been associated with resistance in enzalutamide and abiraterone [Antonarakis et al., 2014], while the effect of AR-V7 positivity in taxane resistance remains contradictory [Antonarakis et al., 2015]. In metastatic tumors following enzalutamide treatment the absence of AR-V7 was associated with better outcome [Efstathiou et al., 2015]. The fact that both enzalutamide and abiraterone target the LBD of the AR (absent in AR-V7 and mutated in several other variants) triggered the interest in development of inhibitors targeting the N′-domain, (such as EPI-001, ODM-201), as well as AR-foldosome targets (corregulators, as Hsp [Centenera et al., 2015; Wang et al., 2015; Seidel et al., 2016; Thakur et al., 2016], Bromodomain/BET [Asangani et al., 2014]). In this regard, detection of variant expression could be used as a criterion of exclusion for treatments with known resistance. It could

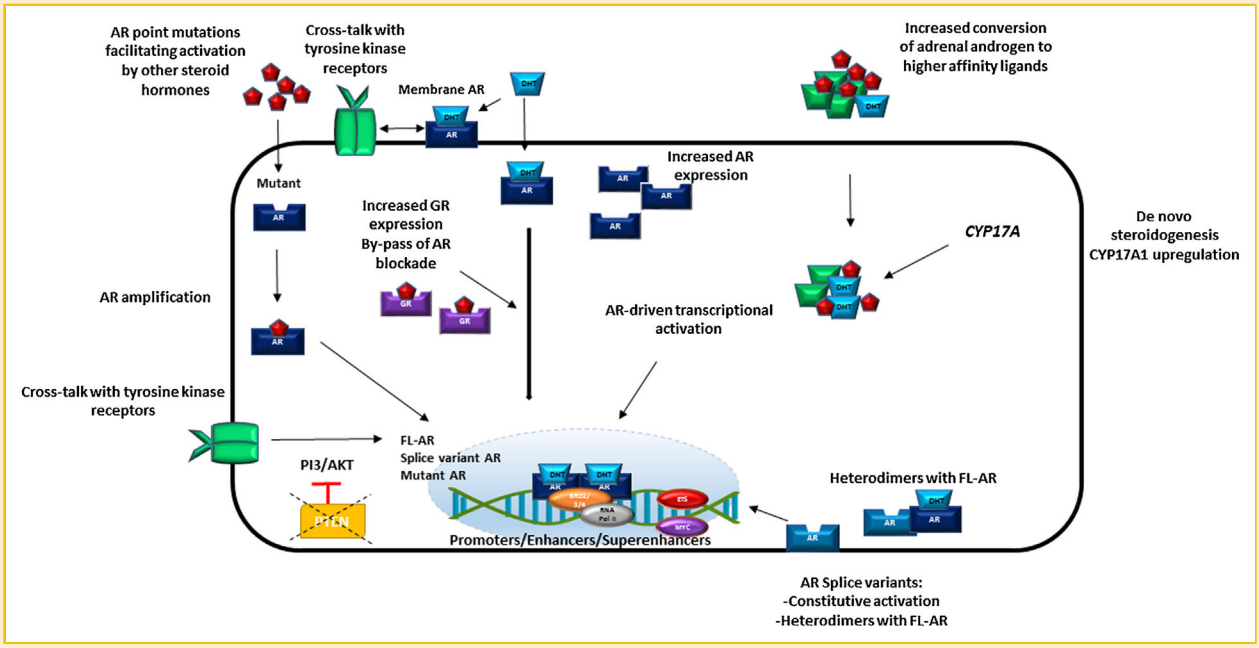

Fig. 3. Schematic representation of CRPC resistance mechanisms. CRPC resistance can be the result of several mechanisms, requiring or not AR. AR signaling can be increased by AR amplification, increased AR expression or increased steroidogenesis. Mutations of AR can trigger activation of AR by other steroid hormones, such as glucocorticosteroids. AR variants are constitutively activated and can form heterodimers with the AR-FL. Both cytosol and membrane-bound ARs can establish cross-talk with growth factor receptors. AR blockade can induce overexpression of glucocorticosteroid receptors (GR) that activate transcriptional targets in the nucleus (bypass mechanism).

also direct selection for targeted therapy independent of AR-LBD-independent treatment. This therapeutic strategy includes drugs inducing AR protein degradation, inhibiting AR splice variants or blocking their transcriptional activity. Galeterone (TOK-001), appears as good candidate, combining CYP17 blockade, AR signaling inhibition, as well as degradation of the AR protein (both the AR-FL and truncated AR variants). Interestingly, splice variants can form dimers with each other as well as with the FL-AR [Xu et al., 2015]. Dimerization is required for androgen-independent transcriptional activation of target genes. This observation further supports the targeting of N-domain, BET, HSPs (e.g., OGX-427, a Hsp27 Inhibitor, or Hsp90 inhibitors), as well as the combined targeting of both LBD and LBD-independent AR domains, when heterodimers are present [Maughan and Antonarakis, 2015b]. Similar strategy can be followed for AR mutants, often induced during endocrine treatment. Conversion of antagonism to agonism in the presence of different AR mutants has been observed for approved AR antagonists. A promising novel agent, BAY 1024767 is a competitive AR antagonist that exhibits strong activity against both FL-AR and mutated forms found in therapy-resistant patients. It retains antagonistic activity with increased androgen stimulation and in prostate cancer models with elevated AR protein levels. It also shows antiproliferative activity in a model expressing splice variants. Its potent antagonism when androgen or AR levels are high is most probably due to strong target engagement whereas its activity toward mutants may be linked to its extended structure that for AR mutants [Sugawara et al., 2016]. This may offer new therapy options by overcoming and/or delaying resistance, either by treating patients who do not respond or who are stimulated by approved antihormonal agents, or by sequential use of AR antagonists with different profiles. Another promising candidate for AR mutants targeting is ODM-201, a novel, nonsteroidal, orally administered active AR antagonist [Moilanen et al., 2015]. ODM-201 and its metabolite ORM-15341 are biochemical-structurally distinct from any known antiandrogens. As a full antagonist of AR mutants, it could play a role in treating cases of enzalutamide, ARN-509, bicalutamide, and flutamide resistance, especially by targeting AR mutation F876L, a driver of acquired resistance to ARN-509 and enzalutamide. A phase I/II clinical trial (ARADES) to assess ODM-201 in men with progressive CRPC has been started [Fizazi et al., 2015].

New combinations with drugs addressing other targets, such as chemohormonal therapy [Sweeney et al., 2015], combined with immunotherapy or tyrosine kinase inhibitors (TKIs) [Poovassery, 2015; Qi et al., 2015], which are under investigation. Another important step on the recognition of AR relevance, even in advanced CRPC, was bipolar androgen therapy (BAT). It was initiated by an observation that may sound paradoxical: supraphysiologic doses of testosterone can trigger CRPC cell death by AR overexpression through prevention of AR degradation (mandatory for DNA replication) and through double-strand DNA breaks, leading to apoptosis. A recently reported pilot clinical study on patients with mCRPC with testosterone cypionate combined with etoposide (to promote DNA double-strandbreaks), showed a prolonged benefit, an important decline of PSA and response confirmed by radiography [Schweizer et al., 2015]. Although the population included was small (16 patients) and heterogenous in terms of prior therapies received, further studies of this novel strategy are warranted. Comparison of

BAT versus enzalutamide is currently investigated in the TRANSFORMER trial, a randomized, open-label, multicenter phase II study in asymptomatic men with mCRPC (clinical-trials/prostate-cancer-NCT02286921).

Critical questions to be addressed that will foster design and selection of AR-tailored therapies in prostate cancer refer to (i) treatment sequence; (ii) clinical application of prostate cancer molecular taxonomy and the propensity of certain subtypes of prostate cancer to exhibit higher or lower AR activity; and (iii) multifocal disease and high heterogeneity that could reflect variability in AR-responses.

PROSTATE ANDROGEN RECEPTOR EVALUATION IN THE ERA OF NEXT GENERATION BIOMARKERS: ADVANCES AND CHALLENGES

The prostate is the prototype of androgen-responsive organ. Paradoxically, although AR targeting is the cornerstone of prostate cancer endocrine treatment, no companion diagnostics based on AR are established either at initial diagnosis or monitoring of the disease. However, the emergence of new prostate drugs approved for clinical use over the past several years, as detailed above, underlies the necessity for robust biomarkers to tailor therapies and to predict response to them, especially as AR remains the main target. Advanced CRPC is particularly challenging, as tissue is rarely available for molecular interrogation and the biology of the disease is quite different than at the baseline biopsy. Hence, non-invasive markers represent an important focus of ongoing research [Locke and Black, 2016]. Recent high throughput data analyses from The Cancer Genome Network and other groups have revealed the high heterogeneity of primary tumors and the importance of AR activity and its specific isoforms and splice variants, prior to disease progression into castration resistance [Boutros et al., 2015; Cancer Genome Atlas Research Network, 2015; Attard et al., 2016]. Below, we highlight some critical aspects of next generation prostate cancer biomarkers under development related to prostate cancer. We focus especially on advances and pitfalls of new technologies with clinical utility and emerging or unmet needs regarding AR evaluation.

ARs display high molecular heterogeneity as discussed above. Mutations, alternative splice variants, cryptic exons expression, constitutive ligand-independent activation, a high degree of epigenomic regulation, heterodimerization, cross-talk with growth factors, intracellular "shuttling" within various compartments associated with specific cellular and signaling effects, define a multifaceted constellation that renders the approach of ARs quite intriguing [Pelekanou et al., 2013]. Identification of AR forms and most of all, tracking of activity and responsiveness to ligands and inhibitors represent of major priority.

A wide spectrum of nonprotein- and protein-based biomarkers are under development, promising to revolutionize the care of prostate cancer patients. This is applied to tissue diagnostics, but also to "liquid biopsy approaches," such as blood and urine biomarkers. Indeed, mRNA, microRNA, cell-free DNA, prostasomes, and circulating tumor cells (CTCs) are under investigation in clinical trials, especially in the setting of metastatic CRPC, for their ability to predict response to novel therapies and patient survival. Promising results derive especially for measurement of splice variants, with AR-V7 being the best studied one, in view of prediction of resistance to enzalutamide, abiraterone, and taxanes [Antonarakis et al., 2014, 2015]. The meticulous testing of these biomarkers by incorporation into current clinical trials will aid in their widespread use and ability to guide prostate cancer management. These approaches aim to improve stratification and risk assessment. Moreover, they allow less invasive approaches to investigate tumor heterogeneity and progression of the disease.

Prostate cancer risk stratification after primary diagnosis is still based on traditional assessment of serum PSA, clinical staging, tumor's Gleason score and the extent of disease on prostate biopsy [WHO Classification, 2016]. However, all these assays may ultimately lead in over- or under-treatment. Multiple factors limit the prognostic and predictive capacities of these parameters, including the innate heterogeneity and multifocality of the disease, as well as incomplete sampling of cancer with current biopsy techniques [Boutros et al., 2015; Cancer Genome Atlas Research Network, 2015]. Added to the above, the suboptimal or complete absence of AR evaluation in routine clinical practice and diagnostics is quite striking. AR detection and monitoring have only been recently incorporated in several studies to track response/resistance to traditional or novel AR-targeting agents [Antonarakis et al., 2015]. These efforts are mainly focused on splice variants and mutant ARs. However, no established detection and evaluation criteria are applied for FL-AR, which remains the main therapeutic target and defines clinical management, especially at the initial stages of the disease. This means, that endocrine treatment is provided, accepting a priori the presence of responsive/functional ARs, without standardized, validated detection and evaluation criteria of receptor presence, as it is the case of estrogen and progesterone receptors in breast cancer. Even after emergence of resistance and induction of AR variants, heterodimerization with FL-AR seems to be critical to elicit their effects [Xu et al., 2015]. Interestingly, AR protein assay that could provide a more meticulous appreciation of functionality, epigenetic alterations, and response/resistance, remains elusive. This is due to many factors, such as: lack of adequate antibody validation, preponderance of studies with AR polyclonal antibodies [Guo et al., 2009], epitopes commonly shared between full-length receptor (FL-AR) and AR-Vs, or difficulties on tissue accession in cases of metastatic prostate cancer. In addition, the criteria of receptor positivity are still considering as positive/functional exclusively nuclear AR, although extranuclear actions and localization have been well described [Pelekanou et al., 2013]. A refined discrimination between different variants by antibody detection is quite difficult, due to epitope overlapping. Indeed, the N′-domain and partially the DBD are shared between AR-FL and variants, while C′ is truncated in variants. However, a rough distinction between FL-AR and many AR-Vs (lack of C′) could be performed by use of panel of antibodies targeting the C′ and the hinge or N′ domain of AR. To discriminate specific variants, use of mRNA in situ techniques to track different exons on normal/tumor and non-epithelial cells present in prostate specimens is a promising approach, as already reported [Antonarakis et al., 2014].

ARs have been traditionally assessed by semi-quantitative chromogenic-based assays for protein (immunohistochemistry) and mRNA detection (hybridization). Chromogenic assays are widely used in surgical pathology routine due to their ability to localize the antigen in a familiar morphological context, easy interpretation, and simple and not expensive equipment requirements. However, their potential to precisely assess intensity and expression levels is limited. Fluorescent assays can provide a combined multiplex target quantitative assessment, in a single sample, together with information on tissue distribution, marker co-localization, and synchronous level quantitation [Carvajal-Hausdorf et al., 2015]. These properties are very compelling especially in small biopsy specimens with minimal material. Quantitative fluorescence techniques (e.g. AQUA), can be applied in cells and archival formalin-fixed, paraffin-embedded (FFPE) tissue. Indeed, as our group has previously shown in several types of carcinomas, quantitative fluorescence, like AQUA technology, can provide objective measurement for both protein [Camp et al., 2002] and mRNA [Bordeaux et al., 2012]. Interestingly, AR assessment by fluorescent quantitative methods has not been applied yet in specimens or primary or metastatic prostate cancer. Regarding mRNA in situ detection, there is still major concern on the relatively lower level of expression of mRNAs compared to proteins and the sensitivity requirements for measuring low abundance transcripts. Moreover (as in protein detection), the use of different signal amplification and detection systems and the design of probes targeting transcript regions of variable size could impact the inter-assay reproducibility. Further studies are required to address these points prior to introduction of in situ RNA measurements in prostate cancer routine.

Another level of complexity of AR evaluation, derives from the formation of heterodimers between FL-AR and AR-variants or other nuclear receptors (such as the AhR) [Xu et al., 2015]. To date, dimerization has been demonstrated using BRET assay [Xu et al., 2015] in live cell models, but not in prostate cancer specimens. In situ proximity ligation assay (PLA) is an alternative technique, that could visualize formation of (hetero)dimers or functional interaction between various types of ARs and growth factors in both cells and FFPE tissue.

In addition, AR profiling in prostate stroma and immune infiltrate and/or blood cells, also require special attention: we need to define the full constellation of cells (tumor and microenvironment) that could be affected by precision therapeutic regimens. In addition, AR expression by immune cells, and especially lymphocytes should attract more concern in view of prostate immunotherapy approaches [Trigunaite et al., 2015]. Finally, it could be helpful to elaborate the source of expression or bias of circulating free DNA and CTCs.

In light of the above, the most important parameter in AR assessment in prostate cancer is to establish robust AR evaluation criteria and provide guidelines for pathologists training. Whether we have to be based on a traditional semi-quantitative assessment or more advanced assays based on quantitative microscopy or mutation profile, it is the pathologist who makes the final decision on tissue quality, assay performance, and correlation with rest of clinicopathological data.

TISSUE-BASED PROSTATE CANCER SIGNATURES AND MOLECULAR SUBTYPING

There is substantial heterogeneity among primary prostate cancers, evident in the spectrum of molecular abnormalities and its variable clinical course. The cancer genome atlas (TCGA), presented a comprehensive molecular analysis and taxonomy. However, this classification is still not adopted in oncology routine practice, although the discovery of specific genetic abnormalities has promoted our understanding of the molecular pathogenesis of prostate cancer and has demonstrated potentially therapeutically actionable molecular defects. In line with the above, although extensively discussed in the current 2016 WHO classification of tumors of the prostate, it is not yet suggested as a standard prostate cancer approach [Humphrey et al., 2016; WHO Classification, 2016]. Within the TCGA project, 333 primary prostate carcinomas were analyzed [Cancer Genome Atlas Research Network, 2015], introducing seven subtypes defined by specific gene fusions (ERG, ETV1/4, and FLI1) or mutations (SPOP, FOXA1, and IDH1). AR activity varied widely and in a subtype-specific manner, with SPOP and FOXA1 mutant tumors having the highest levels of AR-induced transcripts. Interestingly, SPOP and FOXA1 are highly associated with corregulator recruitment and AR epigenomics as well. A broad spectrum of AR activity was omnipresent in prostate tumors, as well as between genomic subtypes. Tumors with SPOP or FOXA1 mutations had the highest AR transcriptional activity of all genotypically distinct subsets of prostate cancer. This result could also be consistent with AR coactivators deregulation by SPOP mutations [Geng et al., 2013]. RNA sequencing revealed that several AR isoforms were expressed, most notably AR-V7 (detected at low levels in primary tumors and, in a few cases, in adjacent benign prostate tissue), without association with differential expression of known AR target genes or with the seven previously defined genomic subtypes. Most detected splice forms were truncated after DBD rather than at the exons encoding LBD. Truncated AR variants were previously assumed to be expressed predominantly in metastatic CRPC [Guo et al., 2009; Antonarakis et al., 2014], where, at least for AR-V7, their presence was associated with resistance to hormone therapy [Antonarakis et al., 2014]. However, in the latter study, only a minority of cases had tissue validated AR-V7 expression, as this was performed by mRNA in situ semi-quantitative method. While a subset of these mutations was found in tumors that also possessed SPOP mutations and had elevated levels of AR, FOXA1 mutations were mutually exclusive with all other alterations that define the genomic subclasses described in TCGA. While there were some truncating mutations near the AR C′- terminus and the C-terminal part of the forkhead domain, the majority of the mutations found in TCGA and in other prostate cancer cohorts were missense mutations that primarily affect the DNA binding domain of FOXA1. As suggested by the authors, the impact of FOXA1 mutations is mainly related to disrupting or altering interactions with other chromatin-bound cofactors. In metastatic CRPC, AR signaling was more frequently altered than in primary tumors, most often by amplification or mutation of AR (events essentially absent in primary samples). Interestingly, SPOP mutations were somewhat less frequent in the metastatic samples (8% vs. 11% in the primary samples).

In spite of the high quality of the TCGA data and analyses presented above, one should remain reserved as of their translational

validity and the application of these findings in clinical practice. Indeed, in the cohort of prostate cancer (termed PCAD in TCGA, including 52 paired normal and 498 cancer specimens), there exists a great heterogeneity of cancer/stroma/normal tissue (varying from 100/0/0 to 19/77/3 or 50/0/50) and a great disparity of lymphocyte (0–20% and monocyte (0–40%) infiltration, not related to the Gleason score. This element, is not taken into consideration by the authors examining the prostate cancer cohort and may introduce a potential bias in the analysis, especially in view of the expression of the AR by stroma or infiltrating cells.

High heterogeneity of prostate cancer was evidenced in another inspired study focused on multifocal prostate cancer [Boutros et al., 2015]. The investigators have shown that multifocal disease is highly heterogeneous for single-nucleotide variants (SNVs), copy number alterations (CNAs), and genomic rearrangements. They identified and validated a new recurrent amplification of MYCL (not evidenced in TCGA), associated with TP53 deletion, unique profiles of DNA damage and transcriptional dysregulation. Moreover, they demonstrated divergent tumor evolution in multifocal cancer and, in some cases, tumors of independent clonal origin were identified. These data represent the first systematic relation of intraprostatic genomic heterogeneity to predicted clinical outcome and prone the development of novel biomarkers that reflect individual prognosis [Boutros et al., 2015]. Both the TCGA and especially Boutros' reports highlight the fact, that primary tumors are dotted with a certain potential that is crucial to investigate and that CRPC cannot be the only priority and target of translational research.

Locally confined primary tumors represent the majority of diagnosed prostate cancer. Interestingly, cancers with similar Gleason scores show substantial interpatient heterogeneity in prostate cancer-specific mortality rates and progression of disease, as well as intraglandular biological heterogeneity. To date, biomarkers based on CNAs or mRNA abundance in primary tumor or blood samples have not yet reached maximal clinical application owing to a lack of understanding of inherent intraglandular and multifocal heterogeneity and their origin and degradation. Another important point suggested by the multifocal prostate cancer study is that all genome sequences derived from formalin-fixed, paraffin-embedded (FFPE) tumors show a marked reduction in the number of genomic rearrangements (likely attributable to the smaller insert sizes for these libraries) [Boutros et al., 2015]. This suggests that there are substantial false negative rates in sequencing such degraded DNA. On the other hand, despite nucleic acid degradation, we have to admit that the development of gene signature techniques (like Prolaris, tailored for archived FFPE tumors even in small bioptic material) have revolutionized the field and appear very promising in risk stratification, if further validated.

Studying metastatic (in most cases bone) prostate tumor is challenging, due to the difficulty to obtain metastatic specimens. Even the primary tumors themselves are small and mixed in with stromal and normal tissue, and so precise dissection is challenging. Prostate undersampling with the risk of missing a more threatening tumor is a critical consideration for tissue markers. The use of multiple repeat biopsies in active surveillance protocols presents significant morbidity for patients. In fact, 6.7% of low risk patients develop sepsis after biopsy and one-third of patients require self-medicating analgesia. Genomic tests can provide evidence to predict the presence of high stage and grade disease even if the concerned lesion is not sampled. As all proposed prostate cancer markers have been developed in retrospective studies, optimal clinical validation would require prospective clinical trials. Retrospective testing in prospectively conducted clinical trials could also be helpful. Translational success of these tests will likely only be achieved, only if they manage to address a specific clinical question (especially because of their high cost). When possible, laser capture microdissection (LCM) is a method to be recommended prior to any nucleic acid extraction to make sure that the tumor cells of concern are principally or exclusively interrogated.

BLOOD- AND URINE-BASED AR EVALUATION

Non-invasive markers to guide therapy of men with CRPC are a particularly important focus of ongoing research so as to overcome potential morbidity from repetitive biopsies or difficulty in accessing a metastatic site.

AR DETECTION IN CTCs

Promising results concerning the AR-V7 variant derive from CTC detection in patients with prostate cancer [Antonarakis et al., 2014]. However, detection of the same variant mRNA in whole blood was not of predictive value in another study, as the authors suggest possible AR-V7 expression by normal hemopoietic cells that could interfere with the low signal from tumor cells [Takeuchi et al., 2016]. Moreover, AR-V7 mRNA has also been detected in normal prostate and benign lesions. Other ARs have been detected in several organs, independently of prostate (including immune cells) [Pelekanou et al., 2013]. Several drawbacks of the CTC assay have to be considered: the assays on which prostate CTCs were processed, including the only FDA-cleared CTC method (CellSearch, Veridex), rely on immunomagnetic capture of CTCs, using antibodies against epithelial markers, like epithelial cell adhesion molecule (EpCAM) or cytokeratin, followed by additional characterization, to demonstrate that the detected event is a nucleated cell, but not a leukocyte. This means that the technique is based on antibody selection of epithelial-prostate markers. Hence, circulating cells profile is not necessarily reflecting adherent cells markers, as phenomena like epithelial-mesenchymal transition (EMT), could alter epithelial marker expression and generate bias. Indeed, AR-variants have been associated with higher expression of EMT markers, both in vitro and in vivo, but these results have not been validated in human samples [Wicha and Hayes, 2011]. Moreover, any CTCs that are EpCam-negative will be missed by these assays. Some of the newer CTC detection devices are based on mechanical features of CTCs (cell size, deformability) in order to enable an antigen-agnostic selection, the profile of single cells [Polzer et al., 2014] or the selection of CTC clusters instead of single cells [Aceto et al., 2014]. These approaches, however, can be limited by the inability to extract viable separated cells. Another point to underline is that prostate CTC studies on AR-variants, have been performed with different methods (CellSearch System and AdnaTest), a fact that does not allow direct comparison of results [Antonarakis et al., 2014, 2015]. As not all CTCs will lead to the formation of metastatic lesions, additional specific markers that can detect only CTCs with metastatic potential may be better for use

in clinical applications [Wicha and Hayes, 2011]. CTC performance in CRPC is well accepted, but this is not the case for early stage prostate cancer as sensitivity and specificity remain poor. CTC technique should be validated and compared with other viable methods in order to optimize AR detection. In addition, efforts focus on functional properties of prostate CTCs or disseminated cells in the bone-marrow (DTCs) by enrichment and propagation as short-term primary cell lines or mice xenografts. However, these efforts are not always successful, due to small number of viable isolated cells and reduced life-span [Cano et al., 2013]. To validate the predictive value, prospective large studies are required. Whether the detection of AR-Vs in CTCs could be a routinely applicable and cost-effective strategy remains an important issue. To date, the translation of this approach into clinical practice on a large scale seems to be still far away.

CELL FREE DNA ASSAYS

Cell free DNA (cfDNA) has been found to be useful in differentiating prostate cancer from benign prostate hyperplasia. In patients with prostate cancer AR cfDNA has been correlated with tumor stage and shorter recurrence free survival. Furthermore, cfDNA may have clinical relevance in CRPC in view of AR-directed therapies. Hence, AR gene aberrations have been correlated to radiographic/clinical disease progression on enzalutamide. Recently, cfDNA from patients with progressive disease, has been deeply sequenced, revealing already identified as well as newly described AR mutations. This mutation profiling was coupled with functional analysis and responses to established and new anti-AR agents, highlighting the need for new agents targeting areas other than the androgen binding site [Lallous et al., 2016]. This dual approach of molecular analysis coupled with pharmacological assays is a wonderful example of possibilities offered by new technologies. For example, a mutation in F876L detected in cfDNA has been associated to resistance to the novel AR competitive antagonist ARN-509. AR copy number gain and AR L702H mutation have been associated with resistance to abiraterone. These data indicate that cfDNA has a good potential to tailor treatment for patients with CRPC, although present at minimal quantities in blood. It is estimated that only up to 3% of tumor DNA is released into the circulatory system daily from the processes such as secretion, necrosis, and primarily apoptosis. This questions the percentage of DNA degradation and quality that define the cut-off for further downstream applications (e.g., whole exome sequencing and variant calling).

PROSTATE EXOSOMES (PROSTASOMES) ASSAYS

Similarly to healthy cells, prostate epithelial cancer cells produce extracellular vesicles (prostasomes) that can be isolated from seminal fluid, urine, and blood [Zijlstra and Stoorvogel, 2016]. They contain ubiquitously expressed and prostate-specific membrane and cytosolic proteins, as well as RNA. Protein, mRNA, long noncoding RNA, and microRNA composition of extracellular vesicles isolated from prostate cancer patients have been reported in quantitative and qualitative assays. It is to note, however, that sensitivity and specificity validation and confirmation of biomarkers needs large cohorts of prostate cancer patients. Most promising methods include comprehensive combinational screening for (mutant) RNA in prostasomes that are immunoisolated with antibodies targeting prostate-specific epitopes. The detection of prostasomes in blood is further complicated by the concomitant presence of exosomes from many other sources. So far, only a few studies have separated exosomes from other constituents in blood or urine, and no study has separated prostate epithelial cell-derived prostasomes from other exosomes within blood or urine. Plasma contains exosomes from nearly all tissues. This means that it is likely that prostasomes constitute only a minority population. Similarly, urine contains exosome vesicules from tissues within the entire urogenital tract. Prostasomes contain prostate-specific membrane proteins, and these could be used as targets for immune-isolation techniques to separate prostasomes from other constituents. Recently a 3-gene (ERG, PCA3, and SPDEF) assay of exosomal mRNA expression (ExoDx Prostate IntelliScore, ExoDx) has been tested in a study of 519 men aged 50 years or older. ExoDx plus standard of care (PSA, age, race, and family history) was reported more predictive of initial biopsy results in men with elevated PSA levels compared with standard of care alone. The ExoDx score successfully discriminated between Gleason score 7 and above (high risk of disease) versus Gleason score 6 and below [McKiernan et al., 2016]. This study is limited by the lack of central pathology review. Comparison with currently available blood-based assays, advanced imaging studies, which include MRI-targeted biopsy assessment, and validation of performance with respect to the pathologic abnormalities in prostatectomy specimens. It could also be of value to explore the role of the ExoDx Prostate IntelliScore in men enrolled in active surveillance protocols. Similar exosome/prostasome-based assays including AR expression profile have not been reported yet. However, as prostasomes can provide combined access to protein, mRNA, long noncoding RNA, and microRNA, they could be good candidates to approach the high complexity of AR in prostate cancer.

In conclusion, relevant, validated, and reproducible AR in situ assays are now, more than ever, required in prostate cancer, both in early, castration resistant and metastatic disease. AR protein and transcript detection and quantification within tumor and stromal compartment is required to better define clinical management and risk stratification. Molecular signatures should be accompanied by protein profiling of the main prostate cancer target, namely AR. Primary tumors can provide critical information on heterogeneity, mutation, and endocrine responses and are easily accessible, compared to metastatic sites. Multiplex in situ assays with validated monoclonal antibodies and sensitive probes can facilitate assessment of this highly complex biomarker. Blood markers and molecular assays still require rigorous validation and should not overcome the importance of tissue science.

CONCLUSIONS AND PERSPECTIVES

It is clear that AR, remains the principal target in the management of prostate cancer, in early and advanced disease. Either with "traditional" endocrine treatment, novel AR inhibitors, standard chemotherapeutic agents, as taxanes, small peptide inhibitors and lately immune therapies, AR remains at the epicenter. Whether it is the LBD, the ligand(s), corregulators recruitment, actin cytoskeleton, and translocation to the nucleus, AR, full-length or variant, are the

final target. This means, that in addition to optimization of drugs, clinical trials protocols and design, imaging, and next gen techniques in diagnosis, AR (full-length and variants) has to be implemented as a critical biomarker, both for prognosis and response to treatments. In order to optimize treatments and improve patients' outcome, we definitely need to work on development of state of the art companion and complementary, AR-based diagnostics.

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