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DOTTORATO DI RICERCA IN

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CICLO XXXI

Androgen receptor status and its changes during prostate cancer treatments

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INDEX

1.INTRODUCTION p. 1

1.1 Prostate cancer p. 1

1.1.1 Epidemiology p. 1

1.1.2 Classification and staging p. 1

1.1.3 Therapeutic approaches p. 4

1.1.4 Monitoring of the disease p. 9

1.2 Prostate cancer biomarkers p. 9

1.2.1 Diagnostic and monitoring biomarkers p. 9

1.2.2 Androgen receptor and its signaling p. 10

1.2.3 Germline mutations in PCa and association with ^{177}Lu-PSMA therapy p. 13

1.3 Liquid biopsy in prostate cancer p.13

2. AIMS p. 16

3. MATERIALS AND METHODS p. 17

3.1 Patients cohorts p. 17

3.2 Plasma collection and processing p. 18

3.3 PCR analyses p. 18

3.4 Next Generation Sequencing (NGS) p. 21

3.5 Statistical analyses p. 21

4. RESULTS p. 23

4.1 Plasma AR CN in docetaxel-treated patients p. 23

4.2 Plasma AR CN in cabazitaxel-treated patients p. 26

4.3 Plasma AR changes in enzalutamide and abiraterone-treated patients p. 28

4.4 Plasma AR CN in ^{177}Lu-PSMA-treated patients p. 37

4.5 Genomic analyses in ^{177}Lu-PSMA-treated patients p. 38

5. DISCUSSION p. 40

6. REFERENCES p. 43

1. INTRODUCTION

1.1 Prostate cancer

1.1.1 Epidemiology

Prostate cancer (PCa) represents the 7.1% (1,276,106) of the total cases of diagnosed cancers for incidence and is the fifth cause of cancer death among men worldwide in 2018, accounting for 358,989 number of death (Figure 1) [1].

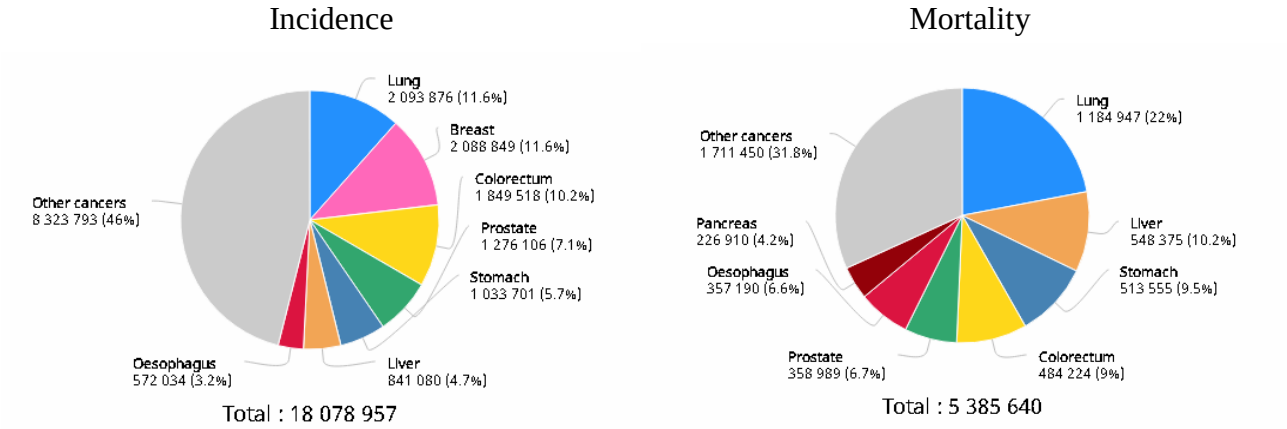

Figure 1. Incidence and mortality (Adapted from https://gco.iarc.fr/)

1.1.2 Classification and staging

Patients with diagnosis of PCa present a highly variable clinical behavior, with a large number of tumors remaining latent for decades following diagnosis. PCa patients are risk-stratified on the basis of clinical and pathological features, but foci with more aggressive features than adjacent areas may give rise to distant and lethal metastases [2].

Although the majority of patients have indolent and low risk tumor, a percentage of PCa develops high risk tumor characteristics that benefit from disease treatments. PCa is divided in three categories based on the stage of disease: localized disease, locally advanced disease and metastatic disease. In localized PCa, radical prostatectomy is considered the gold standard treatment and consists of complete removal of the prostatic gland, despite it is not side-effect free, whereas radiotherapy could be used in inoperable patients with radical purpose [3].

PCa tissues deriving from radical prostatectomy or biopsies are usually characterized in terms of clinical TNM, Gleason score and serum prostate specific antigen (PSA) levels. Clinical TNM is a classification in which: "T" letter identifies the primary tumor and is divided in clinical (from T0 to T4) and pathological (from pT0 to pT4) classification; "N" letter highlights regional lymph node involvement and is divided in clinical (N0 or N1) and pathological (pN0 or pN1) classification based on absence/presence of lymph node metastasis; "M" letter shows absence (M0) or presence of metastasis (M1a – M1c), as described in Table 1 [4].

Table 1. TNM classification of PCa

T - Primary Tumour		
TX	Primary tumour cannot be assessed	
T0	No evidence of primary tumour	
T1	Clinically inapparent tumour that is not palpable	
	T1a	Tumour incidental histological finding in 5% or less of tissue resected
	T1b	Tumour incidental histological finding in more than 5% of tissue resected
	T1c	Tumour identified by needle biopsy (e.g. because of elevated prostate-specific antigen (PSA) level)
T2	Tumour that is palpable and confined within the prostate	
	T2a	Tumour involves one half of one lobe or less
	T2b	Tumour involves more than half of one lobe, but not both lobes
	T2c	Tumour involves both lobes
T3	Tumour extends through the prostatic capsule[1]	
	T3a	Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement
	T3b	Tumour invades seminal vesicle(s)
T4	Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall	
N - Regional Lymph Nodes[2]		
NX	Regional lymph nodes cannot be assessed	
N0	No regional lymph node metastasis	
N1	Regional lymph node metastasis	
M - Distant Metastasis[3]		
M0	No distant metastasis	
M1	Distant metastasis	
	M1a	Non-regional lymph node(s)
	M1b	Bone(s)
	M1c	Other site(s)

[1]Invasion into the prostatic apex or into (but not beyond) the prostatic capsule is not classified as T3 but as T2

[2]Metastasis no larger than 0.2 cm can be designated pNmi

[3]When more than one site of metastasis is present, the most advanced category is used. M1c is the most advanced category

(Adapted from EAU – ESTRO – ESUR – SIOG Guidelines on Prostate cancer)

In clinical practice, the method used to assess prognostic outcome of the carcinoma of the prostate is histopathological grading (Gleason score) which is based on assessment of architectural pattern considering the two predominant patterns (primary and secondary pattern), thus highlighting morphological heterogeneity of PCa [5]. Figure 2 shows hyperplasia (a) and PCa tissues (b,c,d).

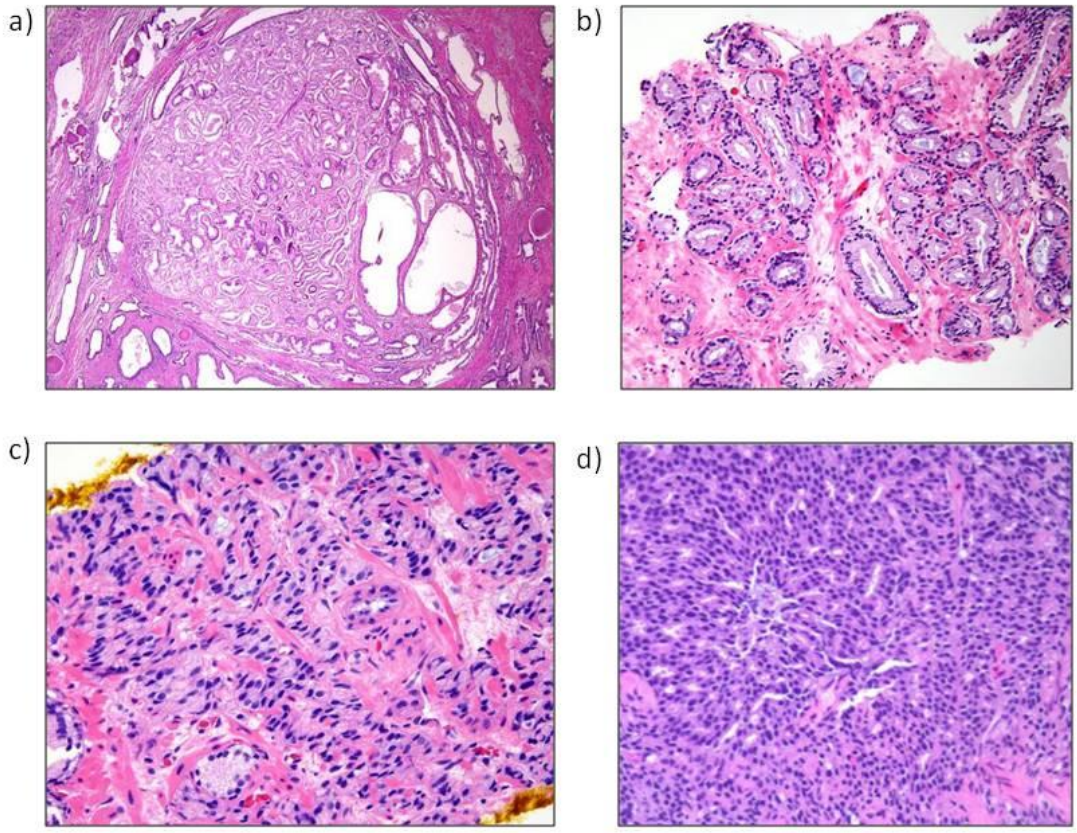

Figure 2. a) Adenomyomatous prostatic hyperplasia nodule, b) Grade 3 prostate cancer, c) Grade 4 prostate cancer, d) Grade 5 prostate cancer.

Tumor grading is divided in 5 grades based on the recommendations of the International Society of Urological Pathology (ISUP) Consensus Conference of 2014, as shown in Table 2.

Table 2. ISUP 2014 grades

Gleason score	ISUP grade
2-6	1
7 (3+4)	2
7 (4+3)	3
8 (4+4 or 3+5 or 5+3)	4
9-10	5

1.1.3 Therapeutic approaches

Androgen deprivation therapy (ADT) is the mainstay treatment used for patients who underwent radical prostatectomy but presented residual disease; patients who underwent radical prostatectomy but were lymph node positive and who presented metastatic disease at the onset and did not undergo radical prostatectomy.

Androgen deprivation can be performed by suppressing the secretion of testicular androgens or blocking androgens receptor. The combination of these two methods achieve the complete androgen blockade [6]. ADT includes first of all surgical castration that consists of a considerable decline of testosterone levels, but the most used ADTs involve androgen receptor (AR) inhibitors, luteinizing hormone-releasing hormone (LHRH) agonists, LHRH antagonists, 5α reductase inhibitors and anti androgens inhibitors of dihydrotestosterone (DHT).

AR inhibitors act blocking the negative feedback to stimulate LHRH and luteinizing hormone (LH) release, thus LHRH agonists increase testosterone levels. However, after a variable period of time, LHRH agonists down regulate LHRH receptors, thus reducing LH release and testosterone production as well as LHRH antagonists and surgical castration (testes removal). Several drugs can also inhibit androgen production by adrenal glands, while 5α Reductase inhibitors prevent conversion of testosterone to DHT. Lastly, there are anti androgens that are inhibitors of DHT with the binding to AR in normal and PCa cells [7]. Testosterone monitoring is part of clinical practice

during LHRH treatment because 13-38% of patients receiving LHRH analogues does not achieve castrate serum testosterone levels (<50 ng/mL) [8]. Patients with advanced PCa can only benefit temporarily from ADT because it entails a transitory decrease in testosterone and DHT synthesis, leading to biochemical recurrence (two consecutive PSA rising > 0.2 ng/ml) and progression to a castration-resistant prostate cancer (CRPC) condition. Figure 3 shows PCa evolution from castrate-sensitive condition to advanced CRPC and the treatments that can be used in each step.

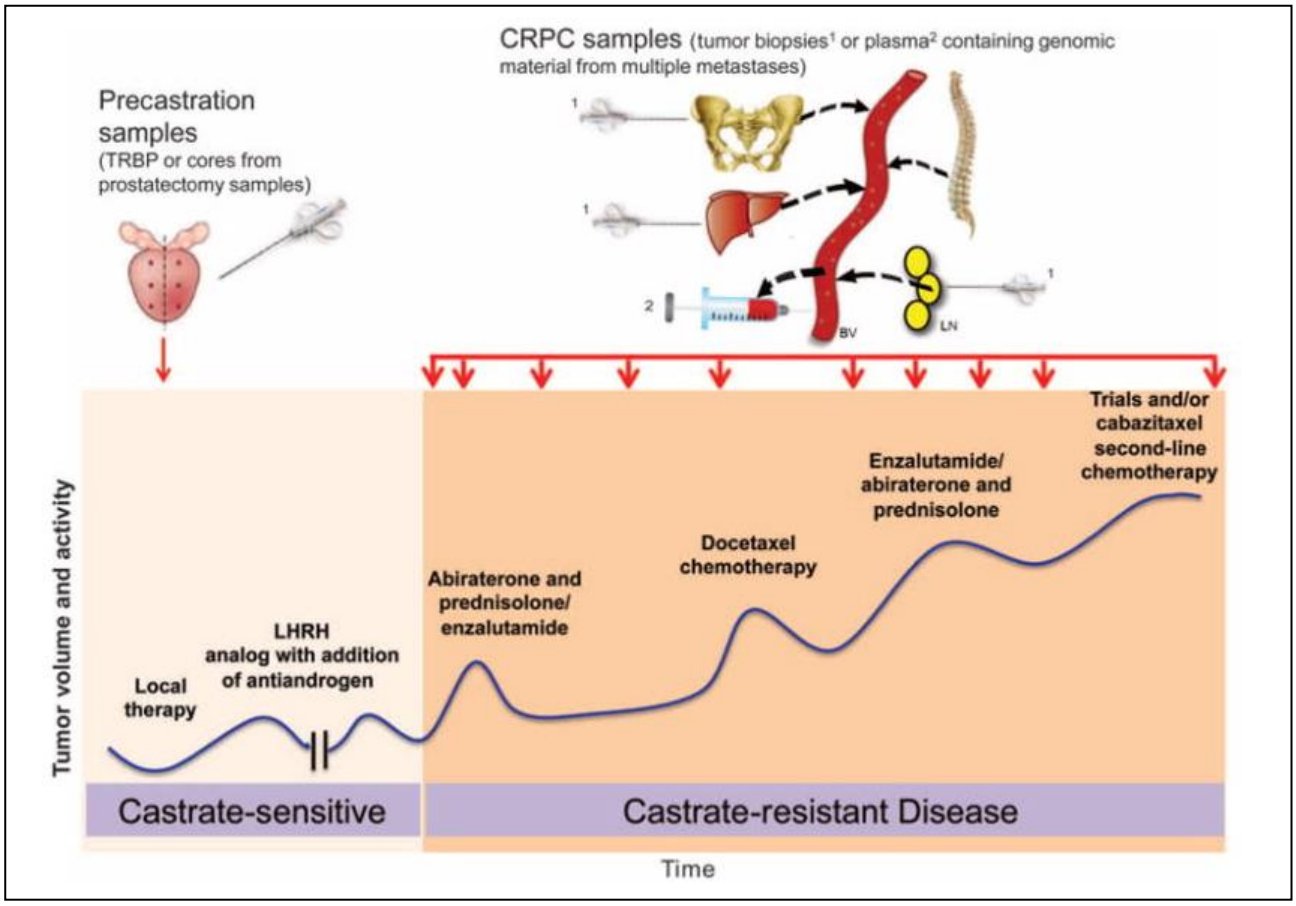

Figure 3. Prostate cancer evolution and associated treatments (adapted from Carreira S et al. *Sci Transl Med* 2014).

The CRPC condition is characterized by either biochemical progression with three consecutive rises in PSA or radiological progression with the appearance of new lesions, despite the state of castrate serum testosterone < 50 ng/dL; serum testosterone test must be performed to confirm all CRPC cases [7].

Since androgen signaling continue to be active, therapies targeting AR pathway remain a valid chance for CRPC patients [9]. Enzalutamide and abiraterone acetate therapies belong to next generation anti-androgen axis that showed clinical efficacy in CRPC in terms of progression free survival (PFS) and overall survival (OS), both in pre and post chemotherapy settings [10-13] .

Enzalutamide binds avidly the ligand-binding domain and inhibits AR, acting as an antagonist of AR and preventing AR translocation into the nucleus followed by transcriptional activation of target genes [14].

Abiraterone acetate is a selective inhibitor of the enzyme cytochrome P450 17 α-hydrolase (CYP17A1) that depletes androgen synthesis in testicles, andrenal glands and tumor microenvironment [15,16]. Figure 4 shows how enzalutamide and abiraterone block the androgen signaling.

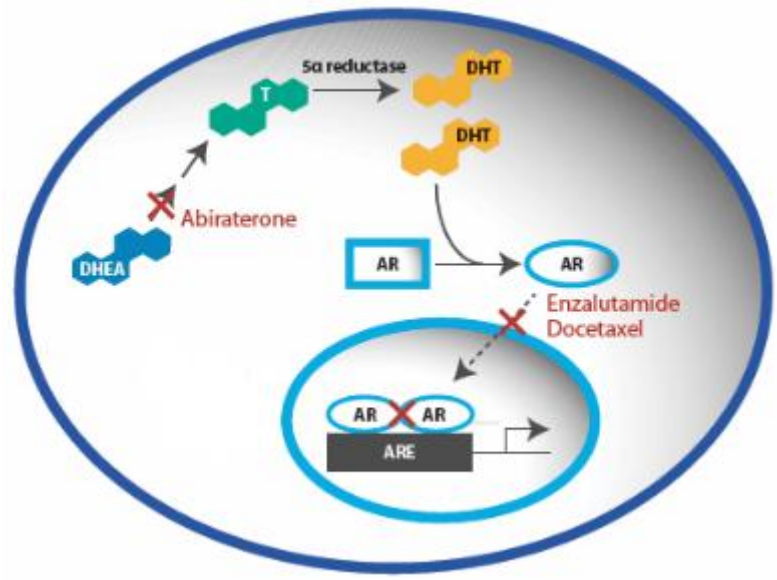

Figure 4 Intracellular androgen signaling and mechanisms of blockade of enzalutamide and abiraterone (adapted from Hurwitz M et al. *Oncology* 2013)

Approximately all metastatic CRPC (mCRPC) patients go to progression to first line treatments after a period of time and this pave the way for studies understanding the mechanisms of resistance. Enzalutamide and abiraterone could modify the biological characteristics of CRPC, suggesting to investigate the molecular bases of treatment resistance. One of the major cause of mechanisms of

resistance to enzalutamide and abiraterone is the reactivation of androgen-AR axis through AR gain or mutations [17,18]. Kira et al. described three main mechanisms of resistance to these two drugs: persistant AR transactivation, bypassing AR and AR indifference [19]. In the first case, AR is active even if castration maintains low testosterone levels, thus downstream genes are expressed. In this category gain and overexpression of AR are included leading to progression to CRPC in patients treated with ADT. Bypassing AR consists of the transcription of AR downstream genes via glucocorticoid receptor and progesterone receptor. Lastly, AR indifference allows tumor progression without AR activation or transcription of AR downstream genes. This mechanism of resistance involves neuroendocrine PCa which seems to be a clonal evolution from prostatic adenocarcinoma [20].

Recent studies also highlighted the possibility to use enzalutamide and abiraterone in combination rather than in sequential use [21].

A second option for metastatic hormone refractory PCa is chemotherapy, in particular docetaxel is used as first or second line of treatment and cabazitaxel is used after docetaxel failure.

Docetaxel is a second-generation taxane chemotherapeutic agent that binds to tubulin on microtubules of the cytoskeleton and promotes cell cycle arrest [22]. A study reported that docetaxel increases OS in mCRPC patients [23]. Moreover, some literature data showed that taxanes can also interact with AR in PCa cells, reducing nuclear translocation [24].

Cabazitaxel is a novel tubulin-binding taxane that demonstrated to be useful for the treatment of mCRPC progressing after docetaxel chemotherapy. In this setting of patients, cabazitaxel demonstrated OS benefit in phase III TROPIC study [25].

Nowadays, there is not a validated sequence of therapies for advanced metastatic PCa. The clinicians tend to administer abiraterone or enzalutamide as first line treatment unless the patient have a high tumor burden at diagnosis in which chemotherapy with docetaxel is preferred. The choice between enzalutamide and abiraterone is currently arbitrary even if there are some recommendations: enzalutamide is preferred in cardiopathic or diabetic patients because abiraterone

is associated with cortisone. In the absence of co-morbid medical conditions the choice of these two drugs is age based, in fact enzalutamide is preferred for patients older than 80-85 years old, whereas abiraterone is preferred in younger patients for who it is better tolerated than older patients, thus enzalutamide could be an option in third line after chemotherapy treatment.

Radium-223 is a novel alpha-emitting radiopharmaceutical and calcium mimetic indicated for the treatment of mCRPC with bone metastases that affect about 90% of metastatic PCa patients. Radium-223 contributes to bone-related palliation but it also provides OS benefit in patients without visceral metastases and lymphadenopathy greater than 3 cm. Currently, the right timing of the use of this radiopharmaceutical is not clear in the treatment sequence of mCRPC: its efficacy is independent of prior use of docetaxel, with similar safety profile in the pre-docetaxel and post-docetaxel setting. For this reason radium-223 can be used in patients either in progression disease (PD) after docetaxel or in patients who are not eligible for cytotoxic chemotherapy. Currently, clinicians tend to use radium-223 in the first-line setting as well as post docetaxel [26].

Prostate-specific membrane antigen (PSMA) is a type II transmembrane glycoprotein belonging to the M28 peptidase family [27]. It acts as a glutamate carboxypeptidase and it is located in the cytosol in normal prostate cells. In PCa cells, PSMA shifts to a membrane-bound protein, being highly expressed on the surface of cancer cells [28]. Interestingly, PSMA expression increases in metastatic and hormone-refractory PCa [29], suggesting to be a good target both for imaging and therapy of PCa. ^{177}Lu-PSMA-617, a molecule inhibitor of PSMA, has been increasingly used for radioligand therapy of mCRPC patients as compassionate use, that is in cases of PD after all lines of therapies approved. Several cases of response to ^{177}Lu-PSMA-617 therapy have been reported [30, 31]. Moreover, systematic reviews showed that ^{177}Lu-PSMA-617 is an effective treatment, since it often causes PSA decline $\geq$ 50% [32,33].

1.1.4 Monitoring of the disease

PCa patients with less aggressive disease can be included in monitoring programs without undergoing definitive therapy. The programs are divided in: watchful waiting and active surveillance. Watchful waiting involves treating symptoms with palliative purpose; active surveillance consists of disease monitoring with serial PSA measurements, prostate biopsies, physical examinations or a combination of both [34].

On the other hand, clinical follow up such as PSA levels and digital rectal examination (DRE) are routinely used after radical prostatectomy or radiotherapy [35]. In advanced CRPC, patients undergo repeat serum PSA levels every two to three months with computed tomography (CT) scans and bone scintigraphy at least every six months [36]. At least two of three criteria should be satisfy to define PD and stop the current treatment.

However, during therapy, the fractions of DNA alterations may vary under treatment selection giving rise to alterations originally present in a very small number of cancer cells [37]. The difficulty of obtaining biopsies paved the way to analyze different sources of biomarkers: serum PSA measurements help clinician to identify treatment related PD but, because it does not report on the biological mechanisms, it is not reliable enough for monitoring disease [38,39], so there is now an urgent need to identify specific prognostic and predictive biomarkers.

1.2 Prostate cancer biomarkers

1.2.1 Diagnostic and monitoring biomarkers

PSA is a glycoprotein produced by both benign and malignant prostatic cells. Serum PSA levels could be a useful biomarker for early detection of PCa reducing the percentage of patients with high-risk disease at diagnosis, for monitoring response to treatments and detecting recurrence in an early phase [40]. However, since it could be elevated also in prostatitis, benign prostatic hypertrophy and other non-malignant conditions, it is not a specific marker and PCa patients may present low serum PSA levels [41].

During PCa treatments, rising of PSA level identifies treatment failure, defined as biochemical recurrence. However, biochemical recurrence alone after radical prostatectomy or other treatments does not always indicate clinical recurrence or disease progression. Moreover, even if metastases are always preceded by a rising of PSA, a measurable PSA is not necessary accompanied by metastatic disease [42].

It has been shown that several markers are able to increase sensitivity and specificity to PSA, such as kallikreines which are included in 4Kscore or Phi tests [43,44], Transmembrane protease serine 2 (TMPRSS2)-Erg fusion and urine Prostate Cancer Antigen 3 (PCA3) [45,46]. However, these markers have not been entered clinical practice yet because too limited literature data are available or the results were not impressive.

1.2.2 Androgen receptor and its signaling

Androgen receptor gene, located on chromosome Xq11-12, consists of eight exons and encodes a multidomain protein of 110 kDa. It is a multi-domain protein composed of a N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), a hinge region and a C-terminal ligand binding domain (LBD) (Figure 5).

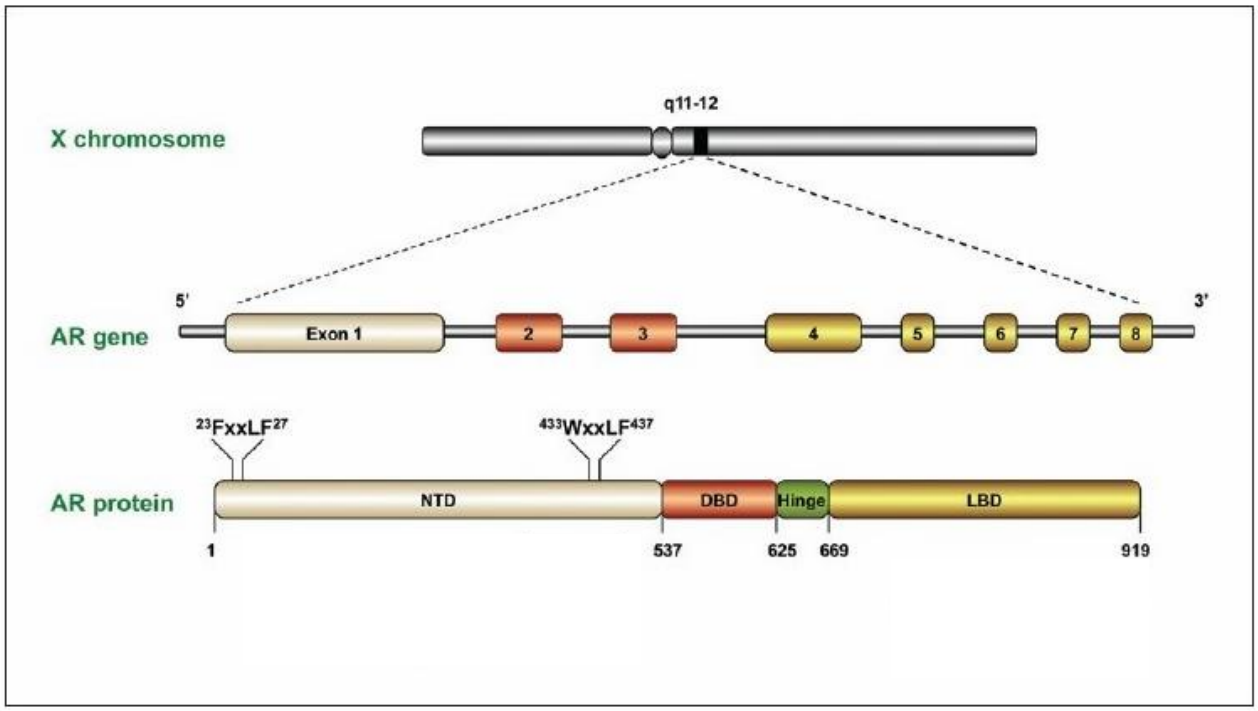

Figure 5. Schematic representation of AR gene and protein (adapted from Peter E Lonergan et al. *J Carcinog* 2011)

AR is a nuclear steroid hormone receptor and it is a ligand-dependent transcription factor. When it binds its ligand, it dimerizes and translocates from the cytosol to the nucleus where it cooperates with co-regulators and binds to androgen responsive elements controlling target gene expression (e.g. PSA and TMPRSS2) [13].

The androgen signaling (Figure 6) starts in the hypotalamus with pulsatile release of LHRH that arrives to the pituitary gland and binds to LHRH receptors stimulating the release of LH. LH binds to its receptors in testes, leading to testosterone production. In addition to testes, also andrenal gland produces low levels of androgens. In prostate cells, the 5α reductase enzyme converts testosterone to DHT which binds to AR that translocates into the nucleus activating the transcription of downstream genes involved in cell survival and growth. When testosterone levels increase, LHRH and LH decrease without modifying physiological levels of serum testosterone (negative feedback). AR plays a key role in development, differentiation, secretion and homeostasis in normal prostate [47] but also in carcinogenesis [48,49].

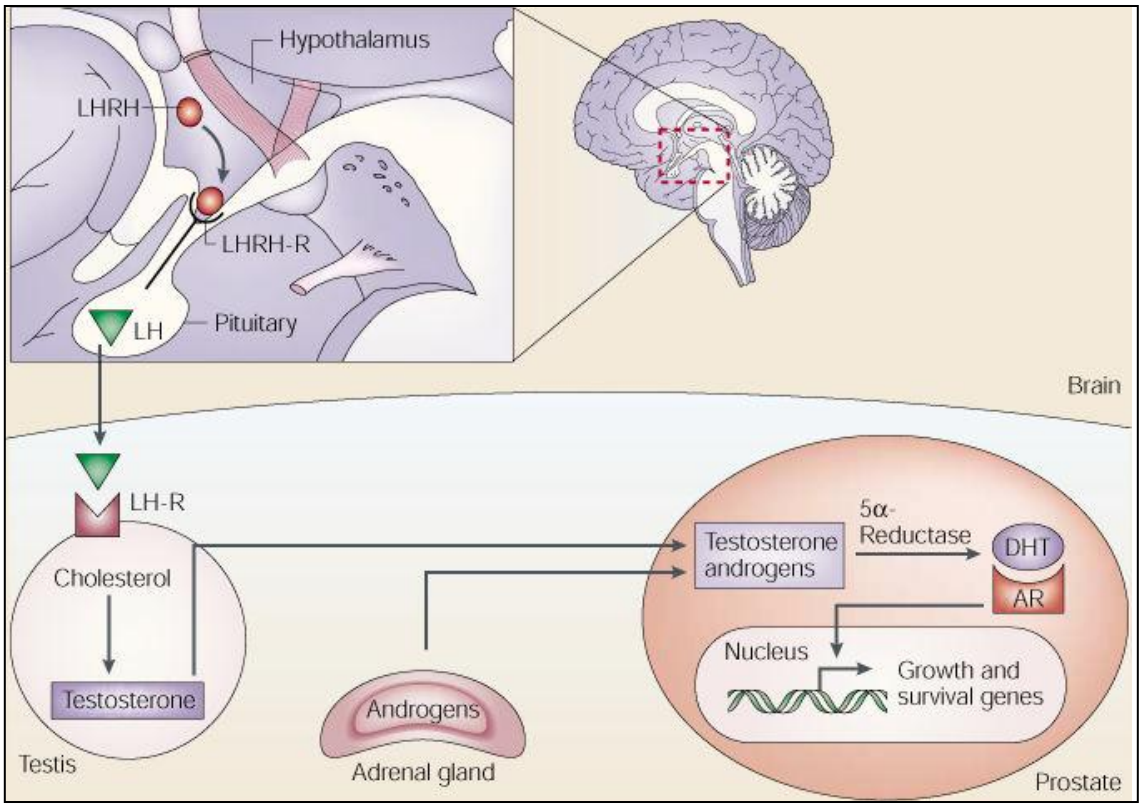

Figure 6. Androgen signaling (adapted from Samuel R. Denmeade et al. *Nat Rev Cancer* 2002)

Structural variants comprise duplications, insertions, deletions, inversions and translocations. Duplications (gain) and deletions (loss) of DNA sequences result in structural genomic variants known as copy number variations (CNV). CNV can alter coding and non coding regions of genes, some of these influence different signaling pathways and are often associated with cancer risk and development [50].

CNV of AR has been well investigated and is considered to be one of the main mechanisms of hormone-sensitive to hormone-resistant transition [51]. AR gain is a frequent aberration in advanced PCa, particularly in CRPC setting [52]. Moreover, AR gain is known to be also associated with high protein expression, thus suggesting its involvement in mechanisms of resistance to AR targeting therapies [53]. In CRPC patients treated with abiraterone, CNV of AR and CYP17A1 detected in serum DNA seem to be associated with clinical outcome [54]. Besides AR copy number (CN) status, AR splice variants detection on circulating tumor cells (CTCs) of CRPC patients treated with enzalutamide and abiraterone is also associated with shorter PFS and OS [55,56]. We

also showed that CTCs positive patients had higher AR CN even though AR CN gain can also be observed in CTCs negative patients [57].

1.2.3 Germline mutations in PCa and association with ^{177}Lu-PSMA therapy

Literature data showed an incidence of 11.8% of germline mutations in genes involved in DNA repair pathways in men with metastatic PCa [58]. *BRCA1*, *BRCA2*, *ATM*, *PALB2*, *CHEK2*, *HDAC2* and *FANCA* alterations are linked to sensitivity to platinum and poly (adenosine diphosphate [ADP] -ribose) polymerase (PARP) inhibitors, such as Olaparib that inhibits PARP enzyme and blocks the repair of DNA strand breaks, leading to synthetic lethality and cell death [59]. A case report by Ahmadzadehfar et al. showed that *BRCA2* mutations could be the cause of poor response to ^{177}Lu-PSMA radionuclide therapy, whereas the subsequent therapy with PARP inhibitor was effective with a PFS of 5 months [60]. This suggest that, although 80% of mCRPC patients respond to ^{177}Lu-PSMA therapy, the 20% of non responder patients could present a more aggressive disease and/or germline mutations.

1.3 Liquid biopsy in prostate cancer

A major limitation of PCa is obtaining tumor samples from biopsies because it is an invasive and costly test, difficult to perform especially in advanced cancer patients. Blood, urine, saliva, cerebrospinal fluid and other biological fluids can be used as alternative source of molecular biomarkers. In the human blood a variety of materials derived from different tissues are present: circulating cell-free DNA (cfDNA), circulating free RNA (microRNA and RNA included in extracellular vesicles), CTCs. Circulating markers analysis seems to represent an optimal instrument for personalized medicine [61].

In this contest, cfDNA has emerged as a minimally invasive and good source of biomarkers able to bypass the issue of obtaining tumor biopsies. Indeed cfDNA could be informative of genomic material deriving from multiple metastases (liquid biopsy), providing information on tumor

characteristics at the moment of sample collection and suggesting its role in monitoring tumor heterogeneity [62,63].

A number of studies highlighted that cfDNA contains specific tumor-related alterations, such as methylation, CNV and mutations, thus confirming the existence of circulating tumor DNA (ctDNA) [54,64,65]. The percentage of ctDNA varies from 0.1% to approximately 60% of cfDNA, related to tumor burden and type [66].

The variation of cfDNA concentration during treatments represents a useful biomarker in different cancer types [67,68]. Moreover, cfDNA can also monitor clonal evolution and identify resistance to treatment [69,70]. A study identified RAS mutation in the blood of colorectal cancer patients but not in the matched tumor tissue samples, suggesting dynamic clonal competition [71].

As described in Figure 7, cfDNA derives mainly from apoptosis in healthy individuals as well as in cancer patients, but also from necrosis especially in cancer patients [72].

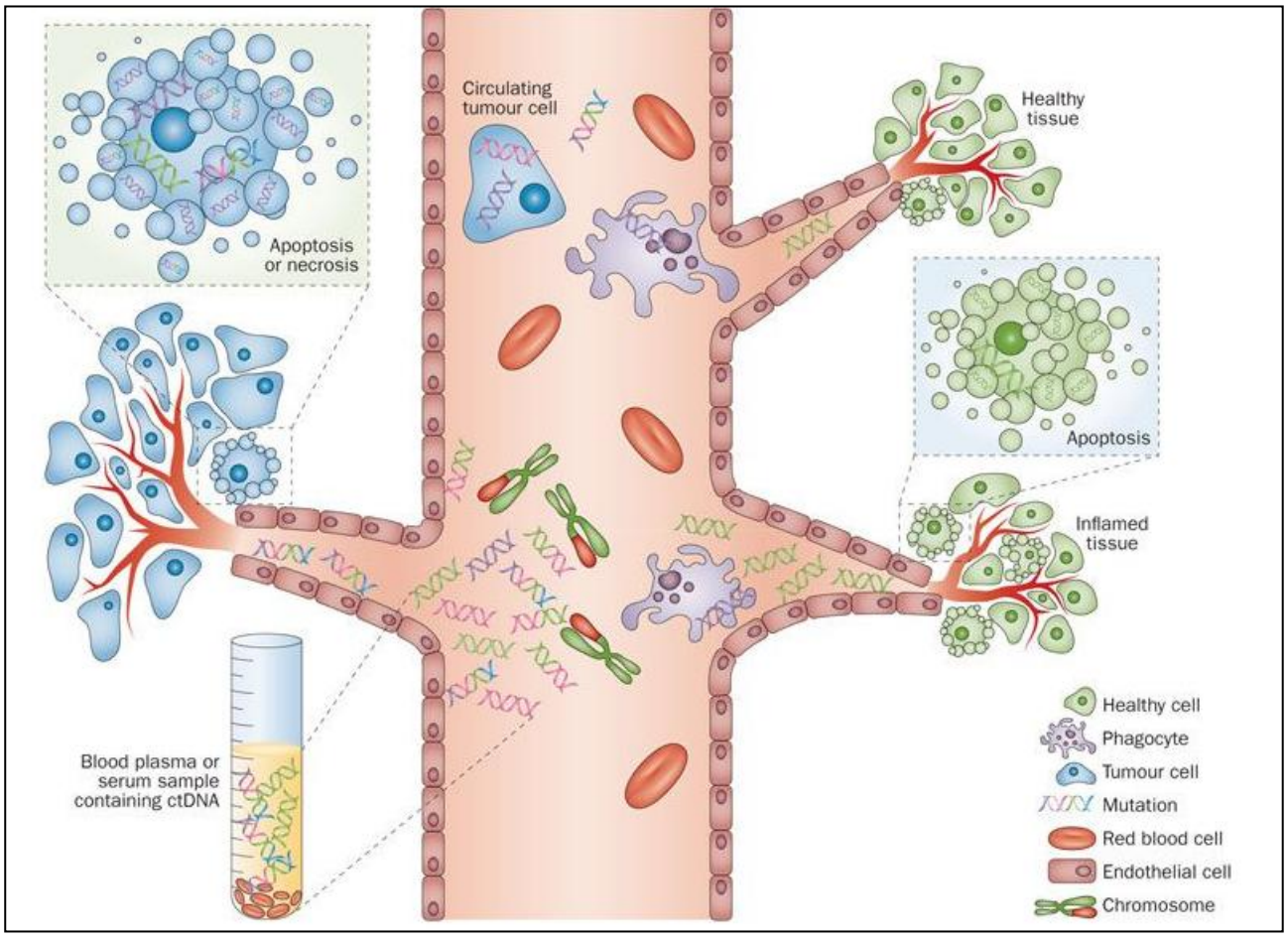

Figure 7. Cell free DNA in blood vessels (adapted from Crowley E et al. *Nat Rev Clin Oncol* 2013)

The release of cfDNA by the tumor can be attributed to cancer cells, normal cells and cells of the tumor microenvironment.

Although ctDNA is limited in cfDNA and diluted by high molecular genomic DNA from leukocytes, the detection of CNV is quite easy because each tumor CNV contributes to a large number of ctDNA fragments to the overall pool of cfDNA [73].

CfDNA in serum and plasma samples is best characterized: several studies have shown that DNA deriving from plasma is better than that deriving from serum because the higher quantity of cfDNA in serum is due to the clotting of white blood cells that dilutes tumor DNA present in the samples [74,75]. The release of cfDNA in healthy individuals is lower (1.8-35 ng/mL) [76,77] than that found in cancer patients (5-1500 ng/mL) [78,79]. Most of literature data show concordance between analyses on plasma cfDNA and those on tumor tissue [66,80]. For these reasons, plasma cfDNA could be used before and during treatment for monitoring treatment response and to identify tumor relapse thanks to its noninvasive and easy to perform nature, as well as cost-effective and highly sensitivity.

2. AIMS

Metastatic PCa patients can temporarily benefit from ADT, but then become castration-resistant. There is currently no validated sequence of therapies for metastatic castration-resistant PCa patients. Chemotherapy with docetaxel and cabazitaxel, or next generation anti-androgen treatments such as those based on abiraterone and enzalutamide, are the recognized therapeutic options. A major hurdle remains the lack of reliable biomarkers for predicting clinically relevant treatment responses in these patients. In fact, for patients progressing after ADT therapy there are no clinical guidelines or a specific further treatment recommended. These deficits could be overcome, at least in part, by identifying novel prognostic factors.

AR is considered one of the main actor in PCa inciting investigation of this receptor. Our previous work has demonstrated that AR CNV before the start of treatment (baseline) of enzalutamide and abiraterone was associated with resistance to enzalutamide and abiraterone, both in pre- and post-chemotherapy with docetaxel [81].

In this study we aim to evaluate the prognostic role of AR CNV at baseline of docetaxel and cabazitaxel and to verify if changes of AR CN status during treatment with enzalutamide and abiraterone may be useful for disease monitoring and clinical decision making. These molecular analyses will be correlated with clinical pathological characteristics and clinical outcomes, such as PFS and OS. We will also determine whether AR CNV before ^{177}Lu-PSMA radionuclide therapy and mutations in a panel of 26 genes involved in DNA repair mechanisms correlate with clinical features.

3. MATERIALS AND METHODS

3.1 Patient cohorts

Docetaxel treated patients

A cohort of 163 mCRPC patients treated between May 2011 and January 2017 with standard-dose intravenous docetaxel 75 mg/m^2 every 3 week with prednisone 5 mg twice daily for a maximum of 10 cycles were included. Pre-treatment plasma samples were collected. Additional selection criteria included Eastern Cooperative Oncology Group (ECOG) performance status ≤2, adequate cardiac, hepatic, renal and bone marrow function, and severe comorbidities.

Cabazitaxel treated patients

A cohort of 155 mCRPC patients treated between September 2011 and January 2018 with a second or third-line cabazitaxel treatment at standard doses (25mg/m^2 every three weeks together with prednisone 5mg twice daily) for a maximum of 10 cycles until evidence of PD or unacceptable toxicity were included. Patients with histologically confirmed prostate adenocarcinoma and no sign of PD, despite serum testosterone levels below 50 ng/dL, were enrolled in the study. Pre-treatment plasma samples were collected. Additional selection criteria included Eastern Cooperative Oncology Group (ECOG) performance status ≤2, adequate cardiac, hepatic, renal and bone marrow function, and severe comorbidities.

Enzalutamide and abiraterone patients

Patients diagnosed for mCRPC without neuroendocrine differentiation in the primary tumor were included in this study. We enrolled 154 patients treated with enzalutamide or abiraterone between August 2012 and June 2016: 30 abiraterone pre-docetaxel, 32 abiraterone post-docetaxel, 25 enzalutamide pre-docetaxel and 67 enzalutamide post-docetaxel. Plasma samples during treatments were collected for 79 patients approximately at first radiological evaluation: 24 treated with abiraterone pre-docetaxel, 27 with abiraterone post-docetaxel, 11 treated with enzalutamide pre-docetaxel and 17 with enzalutamide post-docetaxel. Two time points samples were analyzed for 10 patients. Additional selection criteria included ECOG performance status ≤ 2, adequate cardiac,

hepatic, renal and bone marrow function; serum potassium level ≥ 3.5 mmol/L and ongoing ADT with serum testosterone < 50 ng/dL. Enzalutamide was administered 160 mg once a day, abiraterone 1 g once a day and prednisone 5mg twice daily, continuously until evidence of PD or unacceptable toxicity. Baseline serum PSA levels were measured a week before starting therapy. During treatment, all recorded PSA test and scan results were collected: PSA was evaluated every 4 weeks for serologic response and imaging investigations as clinically indicated. The Prostate Cancer Working Group 2 (PCWG2) criteria were used to define response and progression [82].

^{177}Lu-PSMA patients

Between April 2017 and November 2018, 43 mCRPC patients were treated with ^{177}Lu-PSMA-617. Patients younger than 75 years and not heavily pretreated received 5.5 Gigabecquerel (GBq) of ^{177}Lu-PSMA-617, while other patients received 4.2 GBq per cycle, for a total of 4-6 cycles for 8 weeks. Pre-therapy whole blood and plasma samples were collected.

3.2 Plasma collection and processing

Approximately 10 ml of peripheral blood were collected in plasma EDTA tubes, which can be maintained at 4°C for a maximum of 4h until processing. Serial samples were collected at baseline and during treatment (approximately at first radiological evaluation) with enzalutamide or abiraterone. Blood samples were centrifuged at 1,000 x g for 15 minutes to obtain plasma then aliquoted in 2 ml cryovials and stored at -80°C. Transferring only the upper part of the supernatant reduces the risk of cell or cell debris contamination.

3.3 PCR analyses

DNA isolation and quantification

One milliliter of plasma was thawed and DNA was extracted using QIAamp Circulating Nucleic Acid Kit (Qiagen, Milan, Italy) according to the manufacturer's instruction. DNA was quantified by a spectrophotometer (Nanodrop ND-1000, Celbio, Milan, Italy) using 2 µl of DNA.

digital PCR analysis

DNA samples were diluted to 2.5 ng/µl in a final volume of 6.8 µl. CN analyses were performed by QuantStudio3D digital PCR (dPCR) System (Thermo Fisher Scientific) in a duplex assay using FAM and VIC fluorescent probes. AR CN was evaluated with two assays located within the ligand binding domain of the gene (*AR1*: Hs04107225; *AR2*: Hs04511283) and two reference genes were selected as control genes: *RNaseP*, TaqMan™ Copy Number Reference Assay, and *AGO1*(Hs02320401), modified with VIC-labeled probe. DNA samples from three healthy male donors were pooled and used as calibrator.

A mix of the reagents was prepared using: 7.25 µl of QuantStudio 3D MM2 2X (Life Technologies), 0.725 µl of TaqMan Copy Number Assay, 0725 µl of TaqMan Reference Assay per sample, for a total volume of 8.7 µl. Then 14.5 µl of the mixture sample-mix was aliquoted into a loading blade in the loader in order to prepare the QuantStudio 3D Digital PCR 20K Chip. PCR reaction was performed on GeneAmp PCR system 700 (Applied Biosystem). PCR steps in digital PCR procedures were as follows: 10 min at 96°C, 2 min at 60°C and 30 sec at 98°C (45 cycles), 2 min at 60°C. After PCR was completed, the chips were loaded into QuantStudio 3D reader (Applied Biosystem).

Data were analyzed using QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific). The average number of copies per reaction microlitres was determined using Poisson distribution. A ratio of target copies and reference copies was measured for each sample, then a ratio between sample and calibrator was calculated. Cutoff value identified was >2.01 for gain, as reported in our previous work [57]. Figure 8 summarizes dPCR steps.

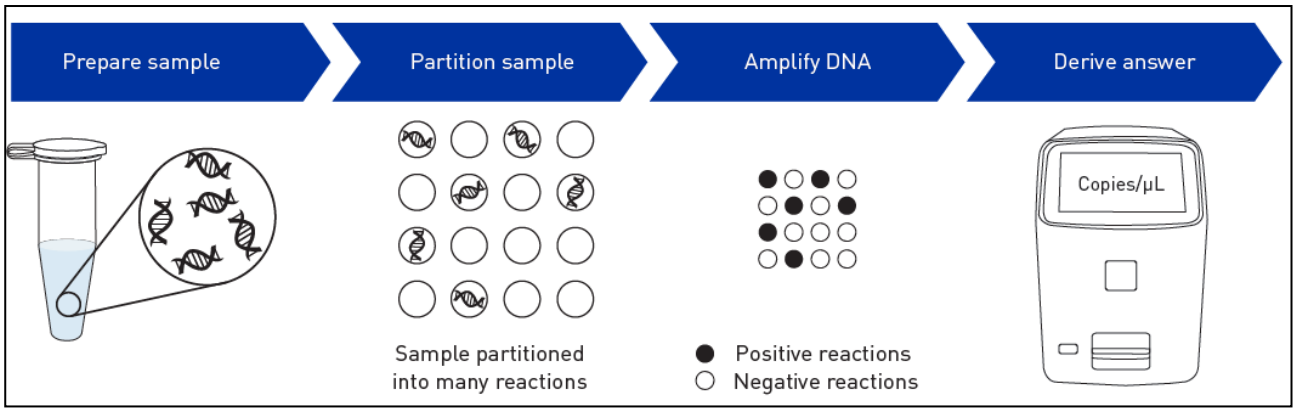

Figure 8. digital PCR steps consists of preparation of the sample, partition of the sample into positive and negative reactions, PCR amplification and reading of the chip (adapted from QuantStudio 3D Digital PCR System, Applied biosystem).

droplet digital PCR analysis

Multiplex droplet digital PCR (ddPCR) on a QX200 ddPCR system (Bio-Rad) was performed to validate the results obtained on dPCR. Eleven samples were analyzed with ddPCR in the Institute of Cancer Research (London, UK).

AR CN (Hs04121925) was evaluated with at least two reference genes: *ZXDB* (Hs02220689), *NSUN3* (dHsaCP2506682), *ElF2C1* (dHsaCP1000002,), and *AP3B1* (dHsaCP1000001). Multiple assays were performed by varying the concentration of the fluorescent probes to discriminate droplets positive according to fluorescent intensity. Every run contained two negative control wells and two wells with germline sample DNA characterized by normal AR CN.

A mix of the reagents was prepared with 10 µl of 2xSupermix, a volume of primer probe assays of 2 µl and 1-2 ng DNA, and in a total volume of 20 µl. Automated Droplet generator (Bio-Rad) partitioned PCR reactions into ~20,000 droplets per sample. PCR reaction was performed on Mastercycler Nexus GSX1 (Eppendorf). PCR steps of ddPCR procedures were as follows: 10 min at 99°C, 15 sec at 95°C and 60 sec at 60°C (40 cycles), 10 min at 98°C. Bio-Rad QX200 droplet reader was used to read samples using QuantaSoft software. The average number of copies per reaction microlitres was determined using Poisson distribution [83].

3.4 Next generation sequencing (NGS)

DNA isolation and quantification

Germline DNA was extracted from 200 µl of thawed whole blood using QIAamp DNA Mini kit (Qiagen) according to the manufacturer's instruction. DNA was quantified by a fluorometer (Qubit, ThermoFisher Scientific, Milan, Italy) using 2 µl of DNA.

Procedure for NGS

Sequencing libraries were created using 50 ng of genomic DNA and the enrichment protocol Hereditary Cancer Solution (SOPHiA GENETICS) for simultaneous sequencing of a panel of 26 genes. The Hereditary Cancer Solution (HCS) panel covers the coding regions and splicing junctions (± 25bp) of 26 genes (target region of 105 kb), associated with DNA repair systems, breast and ovarian cancer susceptibility, Lynch Syndrome (HNPCC) and intestinal polyposis syndromes. The genes are listed below: *ATM*, *APC*, *BARD1*, *BRCA1*, *BRCA2*, *BRIP1*, *CDH1*, *CHEK2*, *EPCAM*, *FAM175A*, *MLH1*, *MRE11A*, *MSH2*, *MSH6*, *MUTYH*, *NBN*, *PALB2*, *PIK3CA*, *PMS2*, *PTEN*, *RAD50*, *RAD51C*, *RAD51D*, *STK11*, TP53, XRCC2. The sequencing was performed using MiSeq platform (Illumina) with MiSeq Reagent Kit v3 configured 2×150 cycles, according to the manufacturer's instructions. HCS panel was used to identify germline mutations associated with DNA repair systems in 43 patients treated with ^{177}Lu-PSMA radionuclide therapy.

3.5 Statistical analyses

The description of the case series was made through the use of descriptive statistics such as frequency and percentage for nominal and ordinal variables, average, standard deviation, median and range for continuous variables. OS was calculated in months as the difference between the date of start of therapy and the death for deceased patients and as the difference between the date of start of therapy and the date of last follow-up for living patients. Events are represented by deceased patients.

PFS was calculated in months as the difference between the date of start of therapy and the date of progression for progressing patients and as the difference between the date of start of therapy and the date of last tumor evaluation for patients not in progression.

Events are represented by those who have had disease progression or died without evidence of disease.

OS, PFS and 95% confidence intervals (CI) were calculated using the Kaplan-Meier limit product method [84] and compared with the logrank test [85].

Hazard Ratio (HR) and the corresponding 95% CI were estimated using the Cox multiple regression model [86]. Odds ratios (OR) and 95% CI of PSA response were assessed using a logistic regression analysis.

All p values <0.05 were obtained considering 2-sided tests and statistical analyses were performed with the SAS statistical software, version 9.4 (SAS Institute, Cary, NC, USA).

4. RESULTS

4.1 Plasma AR CN in docetaxel-treated patients

In a case series of 163 mCRPC patients treated with docetaxel prior to first- or second-line treatment, we identified AR gain in plasma samples of 50 patients (31%). The median number of docetaxel cycles did not differ between AR-gain and AR-normal patients, with a median of 8 cycles (range 6-10). OS was significantly shorter in AR-gain versus AR-normal patients (hazard ratio [HR] 95% CI 1.08–2.39, p = 0.02). The median OS was 14 months (95% confidence interval [CI] 12–23) for AR-gain patients and 22 months (95% CI 20–29) for AR-normal. No significant difference was observed for PFS (HR 1.04, 95% CI 0.74– 1.46, p = 0.8). The median PFS was 7 months (95% CI 5–8) in AR-gain patients and 7 months (95% CI 6–8) in AR-normal patients. No significant difference was also observed for PSA decline >50% (OR = 1.14, 95% CI 0.65–1.99, p = 0.7).

We also compared the cohort of patients treated with first-line docetaxel (n=115) with patients treated with first-line abiraterone or enzalutamide (n=73) of our previous study [57]. Patients characteristics are summarized in Table 3.

Table 3. Characteristics of patients treated with docetaxel

	No. (%)
Age, years Median (IQR)	70 (65-75)
ECOG Performance Status, n (%)	
0-1	141 (87.6)
≥2	20 (12.4)
Unknown/missing	2
Gleason score, n (%)	
<8	54 (35.1)
≥8	100 (64.9)
Unknown/missing	9
Bone metastases, n (%)	
No	20 (12.3)
Yes	143 (87.7)
Visceral metastases, n (%)	
No	130 (79.7)
Yes	33 (20.3)
Liver metastases, n (%)	
No	149 (91.4)
Yes	14 (8.6)
Nodal metastases, n (%)	
No	91 (56.5)
Yes	70 (43.5)
Unknown/missing	2
Serum PSA, mg/l	
Median (IQR)	47.4 (15.7-160.1)
Serum LDH, n (%)	
<225 U/l	64 (41.3)
≥225[#] U/l	91 (58.7)
Unknown/missing	8
Hemoglobin, n (%)	
<12.5 g/l	67 (41.1)
≥12.5[#] g/l	98 (58.9)
ALP, n (%)	
<129 U/l	63 (40.9)
≥129[#] U/l	91 (59.1)
Unknown/missing	9
Plasma *AR* status, n (%)	
Normal	113 (69.3)
Gain*	50 (30.7)
Median follow-up, months (IQR)	33 (20-46)
Death events, n	109
Median OS, months (95% CI)	21.6 (18.7-26.0)
Median PFS, months (95% CI)	7.0 (6.4-7.7)

Abbreviations. IQR, interquartile range; ECOG, Eastern Cooperative Oncology Group; PSA, prostate-specific antigen; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; *AR*, androgen receptor; OS, overall survival; CI, confidence interval; PFS, progression-free survival.

In an exploratory analysis we found that patients with normal AR CN that were treated with abiraterone or enzalutamide presented a significantly lower risk of progressive disease (HR = 2.60, 95% CI 1.75–3.86, p < 0.001) and death (HR = 1.93, 95% CI 1.19–3.12, p = 0.008) compared to docetaxel-treated patients. On the other hand, there was a trend toward a lower risk of progressive disease (HR = 0.82, 95% CI 0.40–1.69, p = 0.6) and death (HR = 0.53, 95% CI 0.24–1.16, p = 0.11) in AR-gain patients treated with docetaxel compared with abiraterone or enzalutamide-treated patients [87].

Figure 9 shows an example of a sample with AR CN normal using dPCR by QuantStudio 3D AnalysisSuite Cloud Software, whereas Figure 10 shows an example of a sample with AR CN gain.

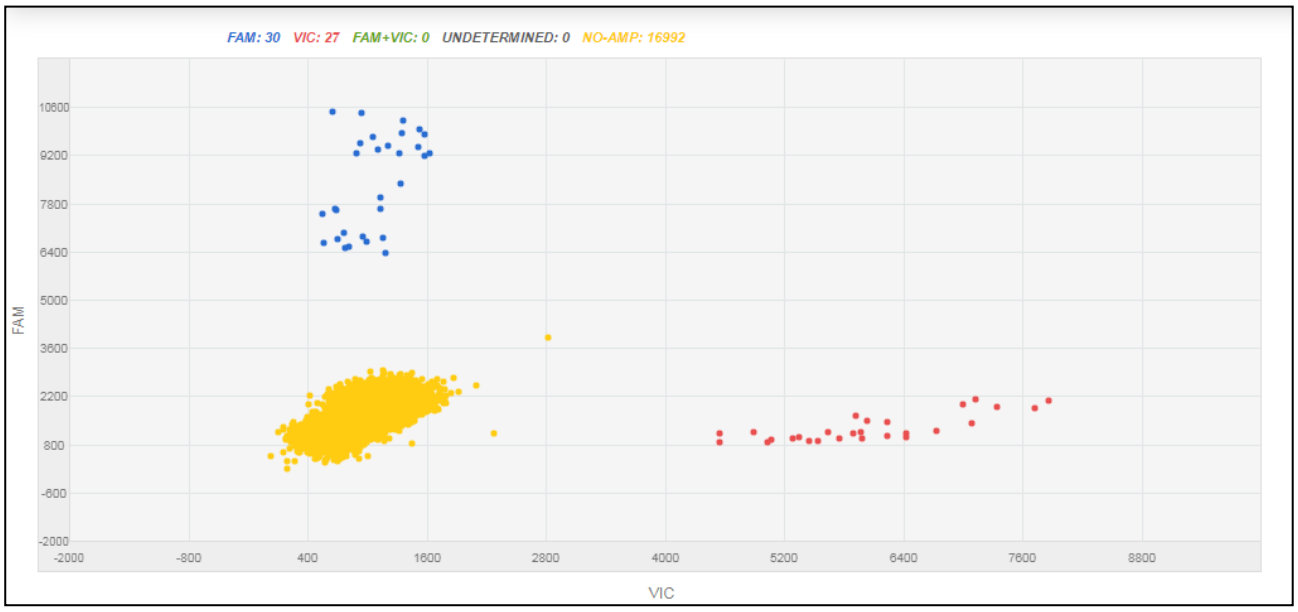

Figure 9. Example of AR CN normal in dPCR. dPCR plot for the duplex of *AR2* (blue dots) and *RNaseP* (red dots) assays. A threshold of 5000 for *AR2* (FAM threshold) and 3500 for *RNaseP* (VIC threshold) was set. Yellow dots represented no amplified DNA.

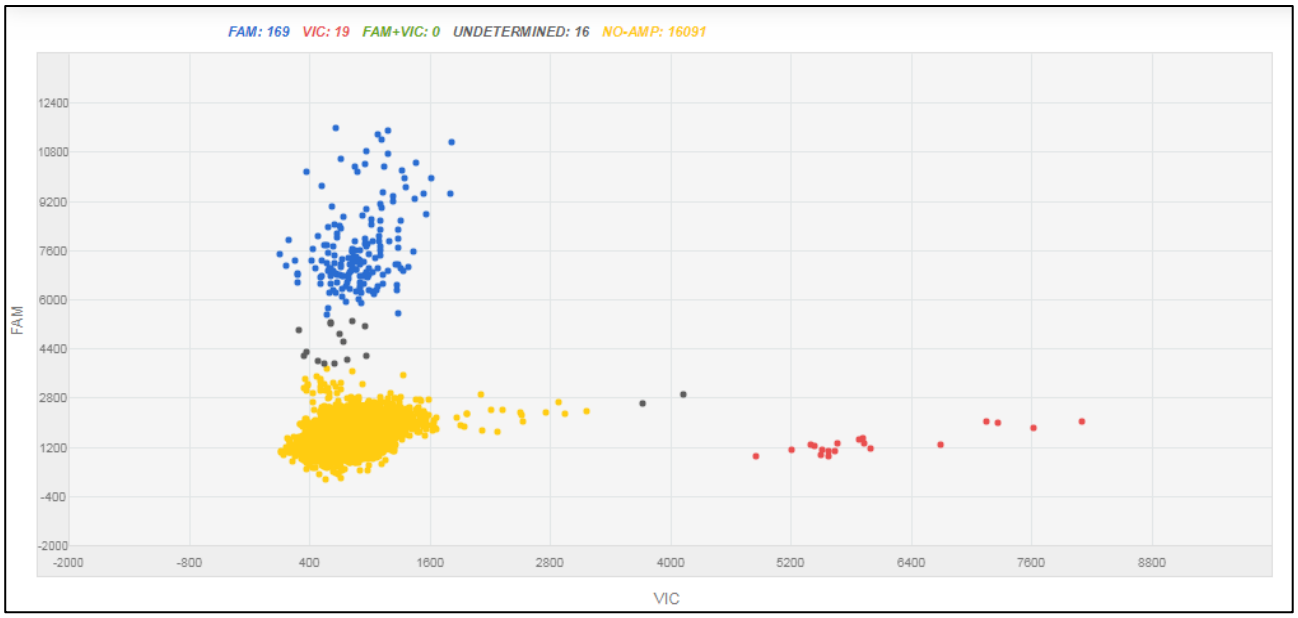

Figure 10. Example of AR CN gain in dPCR. dPCR plot for the duplex of *AR2* (blue dots) and *RNaseP* (red dots) assays. A threshold of 5000 for *AR2* (FAM threshold) and 3500 for *RNaseP* (VIC threshold) was set. Yellow dots represented no amplified DNA. Grey dots represented undefined dots.

4.2 Plasma AR CN in cabazitaxel-treated patients

We analyzed plasma AR CN status of 155 mCRPC at baseline of cabazitaxel treatment. Of these patients, 49 (31.6%) received cabazitaxel as a second-line treatment and 106 (68.4%) as third-line therapy. Characteristics of cabazitaxel-treated patients are summarized in Table 4.

Table 4. Characteristics of cabazitaxel-treated patients

	Total (n = 155)	***AR* normal (n = 90)**	***AR* gain (n = 65)**	**p value**
Age, years Median (range)	70 (43-87)	70 (43-84)	70 (55-87)	0.952
ECOG PS, n (%)				
0-1	118 (84.3)	73 (85.9)	45 (81.8)	
2	22 (15.7)	12 (14.1)	10 (18.2)	
Unknown/missing	15	5	10	0.520
Gleason score, n (%)				
<8	33 (23.6)	19 (22.9)	14 (24.6)	
≥8	107 (76.4)	64 (77.1)	43 (75.4)	
Unknown/missing	15	7	8	0.820
Bone metastases, n (%)				
No	10 (6.5)	9 (10.0)	1 (1.5)	
Yes	145 (93.5)	81 (90.0)	64 (98.5)	0.046
Visceral metastases, n (%)				
No	124 (80.5)	77 (85.6)	47 (73.4)	
Yes	30 (19.5)	13 (14.4)	17 (26.6)	
Unknown/missing	1	0	1	0.062
Liver metastases, n (%)				
No	142 (91.6)	87 (96.7)	55 (84.6)	
Yes	13 (8.4)	3 (3.3)	10 (15.4)	0.008
Nodal metastases, n (%)				
No	73 (47.1)	45 (50.0)	28 (43.1)	
Yes	82 (52.9)	45 (50.0)	37 (56.9)	0.396
Serum PSA, mg/l				
Median (range)	80 (0.05-5000)	60 (0.05-5000)	123 (0.18-2871)	0.001
Serum LDH, n (%)				
<225 U/l	52 (42.6)	37 (52.1)	15 (29.4)	
≥225[#] U/l	70 (57.4)	34 (47.9)	36 (70.6)	
Unknown/missing	33	19	14	0.013
Hemoglobin, n (%)				
≥12.5[#] g/l	73 (47.1)	48 (53.3)	25 (38.5)	
<12.5 g/l	82 (52.9)	42 (46.7)	40 (61.5)	0.068
ALP, n (%)				
<129 U/l	49 (41.2)	37 (53.6)	12 (24.0)	
≥129[#] U/l	70 (58.8)	32 (46.4)	38 (76.0)	
Unknown/missing	36	21	15	0.001
Previous abi or enza, n (%)				
No	49 (30.7)	26 (28.9)	23 (35.4)	
Yes	106 (69.3)	64 (71.1)	42 (64.6)	0.392

Abbreviations. *AR*, androgen receptor; ECOG, Eastern Cooperative Oncology Group; PS, performance status; PSA, prostate-specific antigen; LDH, lactate dehydrogenase; ALP, alkaline phosphatase; abi, abiraterone; enza, enzalutamide.

We found that 65 (41.9%) had AR CN gain. Median follow up was 24 months (range 0.5-47), median OS was 12.2 months (95% CI 10.1-15.2) and median PFS was 4.4 months (95% CI 3.7-5.4). AR-gain patients presented a significantly shorter median PFS (4.0 versus 5.0 months, HR 1.47, 95% CI 1.05-2.07, p = 0.026) and a trend toward a shorter median OS (10.5 versus 14.1

months, HR 1.44, 95% CI 0.98-2.13, p = 0.064). No significant difference was also observed for PSA decline >50% (OR 1.00, 95% CI 0.99-1.00, p = 0.882).

We compared the cohort of second-line cabazitaxel-treated patients (n=49) with a cohort of patients treated with second-line abiraterone and enzalutamide (n=85) and reported in a previous study [57]. Exploratory analyses showed a significant difference in PFS and OS between patients with AR-normal or AR-gain, suggesting that AR-normal patients could benefit more from abiraterone or enzalutamide than cabazitaxel second-line cohort [88].

4.3 Plasma AR changes in enzalutamide and abiraterone-treated patients

Digital PCR analyses were performed in duplex to identify AR CN status on plasma DNA of all 154 patients at baseline of enzalutamide or abiraterone pre- and post-chemotherapy. Baseline AR CN status was necessary to define AR CN changes during enzalutamide or abiraterone treatment. Baseline AR CN status of all 154 patients is reported in Table 5.

Table 5. Enzalutamide/abiraterone patients characteristics

	Abi pre-docetaxel (n=30)	**Abi post-docetaxel (n=32)**	**Enza pre-docetaxel (n=25)**	**Enza post-docetaxel (n=67)**
Age, years: median value (IQR)	75 (69-81)	73 (69-77)	72 (70-80)	76 (71-81)
	n (%)	**n (%)**	**n (%)**	**n (%)**
Gleason score				
6-7	15 (51.7)	6 (21.4)	13 (54.2)	9 (39.1)
8-10	14 (48.3)	22 (78.6)	11 (45.8)	14 (60.9)
Unknown	1	4	1	44
Metastatic sites				
Bone	23 (76.7)	28 (87.5)	4 (16.0)	23 (88.5)
Visceral	2 (6.7)	14 (43.7)	0	3 (11.5)
Liver	1 (3.3)	4 (12.5)	0	3 (11.5)
Lymph nodes	15 (50.0)	14 (43.7)	6 (24.0)	14 (53.8)
Previous abi or enza treatment				
No	30 (100)	25 (78.1)	25 (100)	26 (100)
Yes	0	7 (21.9)	0	0
Unknown	0	0	0	41
Previous docetaxel treatment				
No	30 (100)	0	25 (100)	0
Yes	0	32 (100)	0	26 (100)
Unknown	0	0	0	41
Previous cabazitaxel treatment				
No	30 (100)	27 (84.4)	25 (100)	23 (88.5)
Yes	0	5 (15.6)	0	3 (11.5)
Unknown	0	0	0	41
***Baseline AR* copy number**				
Normal	27 (90.0)	21 (65.6)	22 (88.0)	50 (76.9)
Gain	3 (10.0)	11 (34.4)	3 (12.0)	15 (23.1)
Unknown	0	0	0	2
Baseline ALP, U/L median value (IQR)	97 (85-121)	113 (80-199)	67 (55-88)	94 (79-241)
<129	25 (83.3)	18 (56.2)	22 (88.0)	17 (65.4)
≥129	5 (16.7)	14 (43.8)	3 (12.0)	9 (34.6)
Unknown	0	0	0	41
Baseline LDH, U/L median value (IQR)	150 (132-189)	183 (157-216)	164 (145-180)	167 (136-198
<225	28 (93.3)	25 (78.1)	23 (92.0)	24 (92.3)
≥225	2 (6.7)	7 (21.9)	2 (8.0)	2 (7.7)
Unknown	0	0	0	41
Baseline NLR median value (IQR)	2.69 (2.17-3.31)	2.75 (2.13-4.78)	2.51 (1.84-3.21)	2.46 (1.78-3.04)
<3	17 (56.7)	17 (53.1)	15 (60.0)	17 (65.4)
≥3	13 (43.3)	15 (46.9)	10 (40.0)	9 (34.6)
Unknown	0	0	0	41
Baseline Neutrophil, median value (IQR)	3575 (3140-5140)	3915 (3195-5005)	3200 (2790-4390)	3560 (2880-5230)
Baseline Lymphocyte, median value (IQR)	1440 (1150-1690)	1300 (975-1665)	1540 (1030-1620)	1515 (1260-1860)
Baseline dsDNA, median value (IQR)	24.50 (14.32-37.80)	32.01 (13.79-69.70)	19.80 (15.40-24.63)	28.08 (14.00-67.00)
Baseline PSA, ng/mL median value (IQR)	20.54 (4.91-59.42)	67.5 (20.02-177.05)	28.00 (11.83-45.21)	27.62 (11.09-234.60)

Abbreviations: Abi, abiraterone; Enza, enzalutamide; IQR, interquartile range; AR, androgen receptor; ALP, Alkaline phosphatase; LDH, lactate dehydrogenase; NLR, neutrophil:lymphocyte ratio; dsDNA, double strand DNA; PSA, prostate specific antigen.

Regarding OS we found that patients with AR CN gain presented a lower median OS than patients with AR CN normal at baseline (17.9 months *vs* 29.8 months respectively, HR 2.23, 95% CI 1.46-3.40; p = 0.0002). Figure 11 shows OS in relation to AR CN.

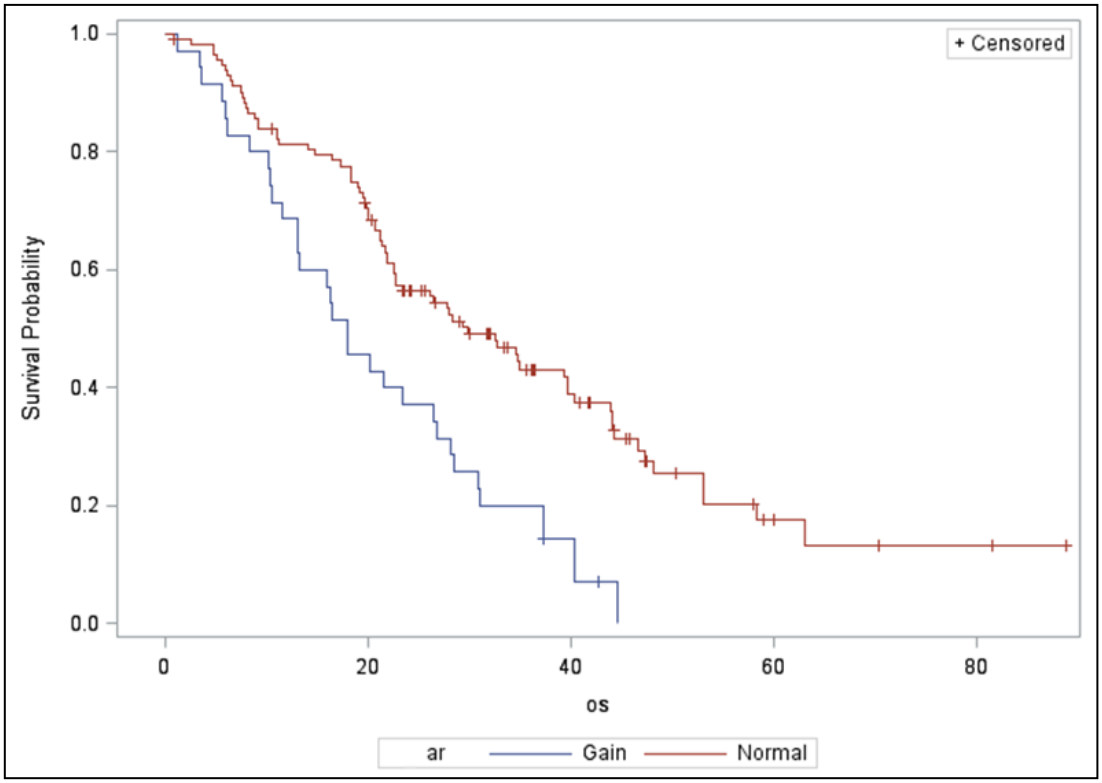

Figure 11. Association of plasma AR CN at baseline with OS.

We found that patients with AR CN gain presented a lower median PFS than patients with AR CN normal at baseline (9.6 months *vs* 12.2 months respectively, HR 1.80, 95% CI 1.21-2.68; p = 0.003). Figure 12 shows PFS in relation to AR CN.

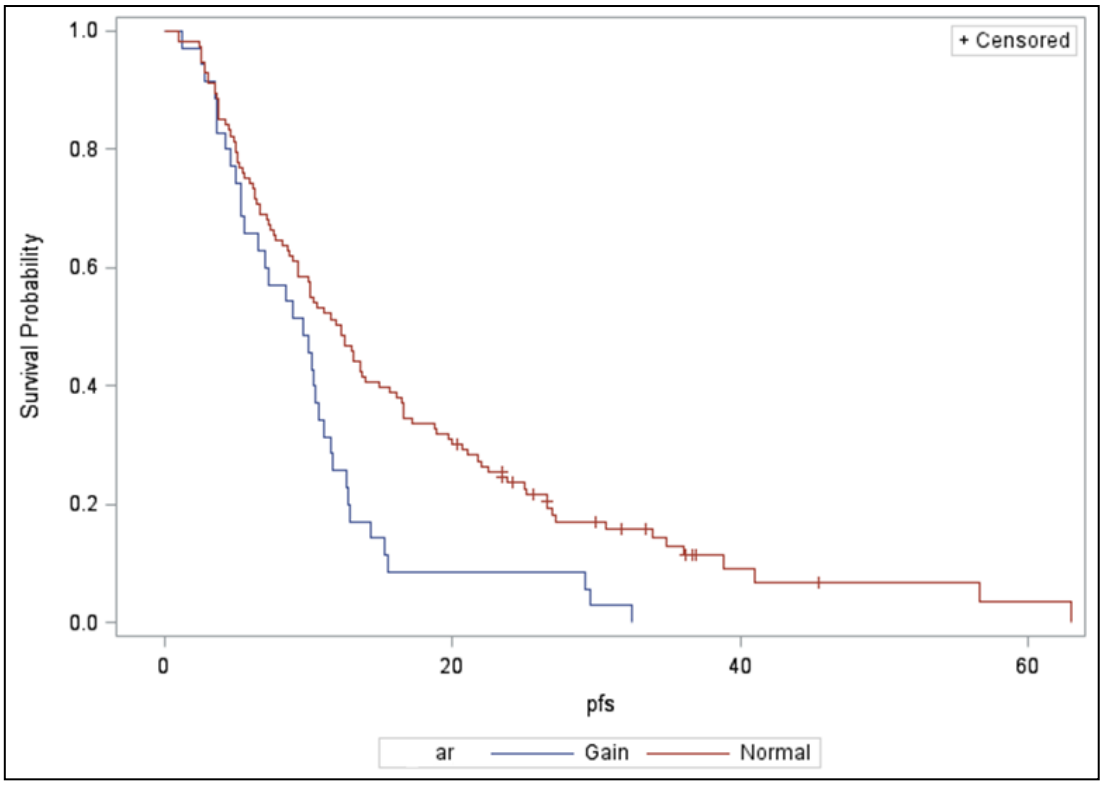

Figure 12. Association of plasma AR CN at baseline with PFS

We were able to collect samples during one of these treatments from 79 patients to identify AR CN changes. In particular we examined one time point for all 79 patients and two time points for 10 patients during therapy. Eleven patients were analyzed both in dPCR and ddPCR to confirm dPCR results. Concordance of 100% was found between the methods, as indicated in Table 6.

Table 6. Verification of concordance between dPCR and ddPCR analyses

Patients ID	Raw AR CN (dPCR)	Category (dPCR)	Raw AR CN	Category (ddPCR)
ES1	0,97	N	1	N
ES8	1,3	N	0,9	N
ES10	56	G	32	G
ES14	27	G	3	G
ES16	0,8	N	1,2	N
ES27	1,34	N	1,4	N
ES29	0,88	N	1	N
ES32	6	G	10	G
ES34	1,1	N	0,6	N
ES37	5,4	G	3,6	G
ES39	0,9	N	0,9	N

Abbreviations: AR, androgen receptor; CN, copy number; dPCR, digital PCR; ddPCR, droplet digital PCR

Table 7 shows that among seventy-nine patients evaluated for AR CN status in one time point during treatment, eleven (14%) changed AR CN status from baseline time point. Nine patients changed from normal to gain AR CN status (14.1%): two patients were treated with abiraterone pre-chemotherapy, three patients were treated with abiraterone post-chemotherapy, two patients were treated with enzalutamide pre-chemotherapy and two with enzalutamide post-chemotherapy. Two patients changed from gain to normal status (13.3%): one patient was treated with abiraterone pre-chemotherapy and one with enzalutamide post-chemotherapy. These results suggested that AR CN change is independent of the type of treatment. Three patients were treated with abiraterone pre-chemotherapy, three with abiraterone post-chemotherapy, two with enzalutamide pre-chemotherapy and three with enzalutamide post-chemotherapy. Sixty-eight patients did not changed AR CN status from baseline time point. AR CN status changed after a median of 7 months (range: 3-17 months).

Table 7. AR CN changes during treatment

	***AR* copy number during therapy**	
	Normal N (%)	Gain N (%)
OVERALL		
***AR* copy number (baseline)**		
Normal	55 (85.9)	9 (14.1)
Gain	2 (13.3)	13 (86.7)
Abi pre-docetaxel		
***AR* copy number (baseline)**		
Normal	19 (90.5)	2 (9.5)
Gain	1 (33.3)	2 (66.7)
Abi post-docetaxel		
***AR* copy number (baseline)**		
Normal	18 (85.7)	3 (14.3)
Gain	0	6 (100)
Enza pre-docetaxel		
***AR* copy number (baseline)**		
Normal	6 (75.0)	2 (25.0)
Gain	0	3 (100.0)
Enza post-docetaxel		
***AR* copy number (baseline)**		
Normal	12 (85.7)	2 (14.3)
Gain	1 (33.3)	2 (66.7)

Abbreviations: AR, androgen receptor; Abi, abiraterone; Enza, enzalutamide

Among ten patients evaluated for AR CN status in two time points during treatment, four (40%) changed AR CN status during treatments. Two patients treated with abiraterone pre-chemotherapy experienced a variation of AR CN from normal to gain status after 8 and 11 months. A patient treated with abiraterone post-chemotherapy experienced two AR CN changes from gain to normal to gain once again after 3 and 9 months. A patient treated with enzalutamide post-chemotherapy experienced two AR CN changes from gain to normal to gain once again after 4 and 8 months. Six patients did not changed AR CN status from baseline time point.

Considering OS and PFS, we found that patients changing AR CN from normal to gain status had a median OS of 19.8 months (95% CI 8.0 – 27.7) compared to patients with stable AR CN normal that presented a median OS of 22.7 months (95% CI 19.0 – 30.5), patients with stable AR CN gain had a median OS of 9.1 (95% CI 3.8 – 17.9) whereas patients changing AR CN from gain to normal status not reached (p = 0.001).

Table 8 and Figure 13 show OS in relation to AR CN changes considering the four possible categories of AR CN status.

Table 8. Overall Survival in relation to AR CN changes

	N. patients	**N. deaths**	**Median OS (months) (95% CI)**	**p**
Normal→Gain	9	8	19.8 (8.0-27.7)	
Gain→Normal	2	1	not reached	
Normal→Normal	55	38	22.7 (19.0-30.5)	
Gain→Gain	13	12	9.1 (3.8-17.9)	0.001

Abbreviations: OS, overall survival; CI confidence intervals

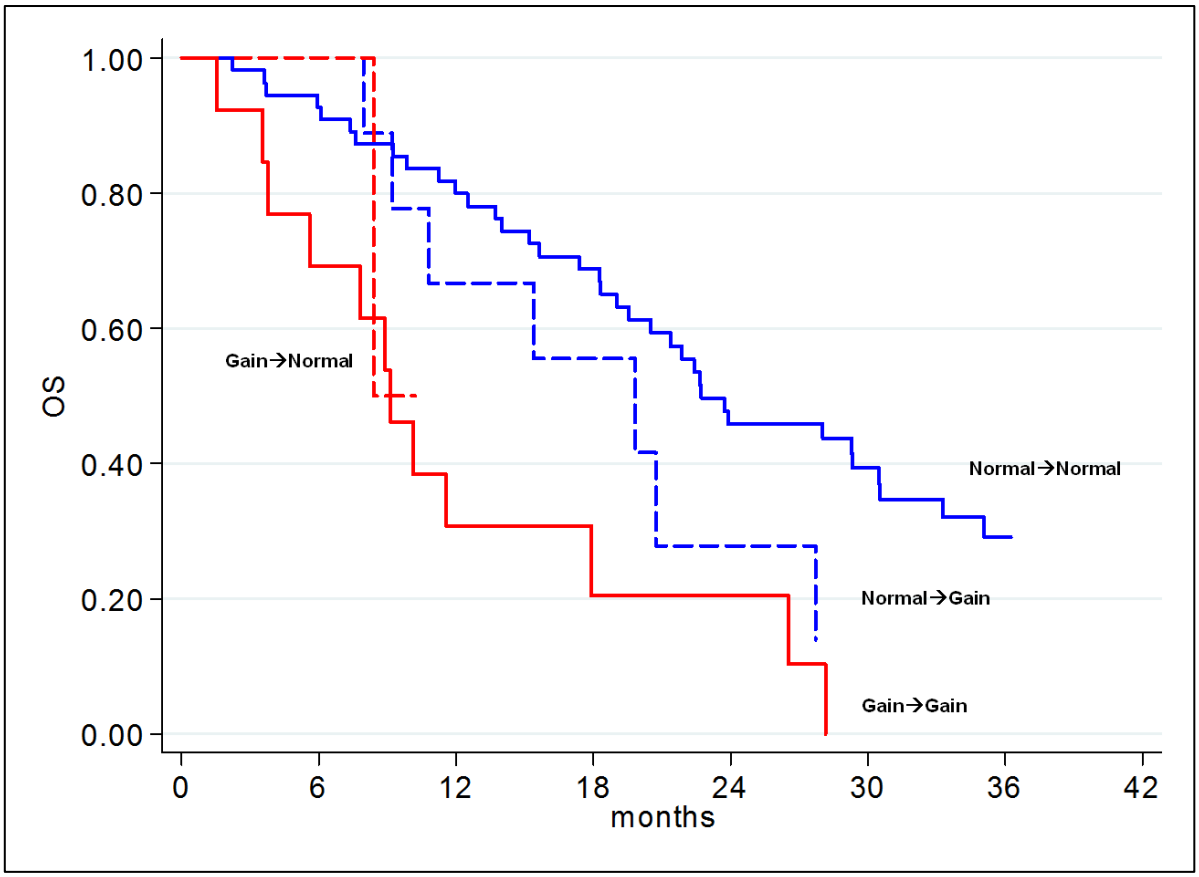

Figure 13. Correlation between plasma AR CN changes and OS for AR variation from Gain to Normal, Gain to Gain, Normal to Gain and Normal to Normal in both chemotherapy naïve or post chemotherapy CRPC patients treated with enzalutamide or abiraterone.

We found that patients changing AR CN from normal to gain status had a median PFS of 9.2 months (95% CI 2.0 – 15.7) compared to patients with stable AR CN normal that presented a median PFS of 7.3 months (95% CI 4.6 – 9.0), patients with stable AR CN gain had a median PFS of 5.4 (95% CI 3.6 – 6.5) whereas patients changing AR CN from gain to normal status had a median PFS of 6.1 (95% CI 3.8 – 8.4) (p= 0.141) (Table 9). However, a trend was observed for AR CN normal status to have better PFS than AR CN gain status (Figure 14).

Table 9. PFS in relation to AR CN changes

	N. patients	N. deaths	Median PFS (95% CI)	p
Normal→Gain	9	9	9.2 (2.0-15.7)	
Gain→Normal	2	2	6.1 (3.8-8.4)	
Normal→Normal	55	51	7.3 (4.6-9.0)	
Gain→Gain	13	13	5.4 (3.6-6.5)	0.141

Abbreviations: PFS, progression free survival; CI confidence intervals

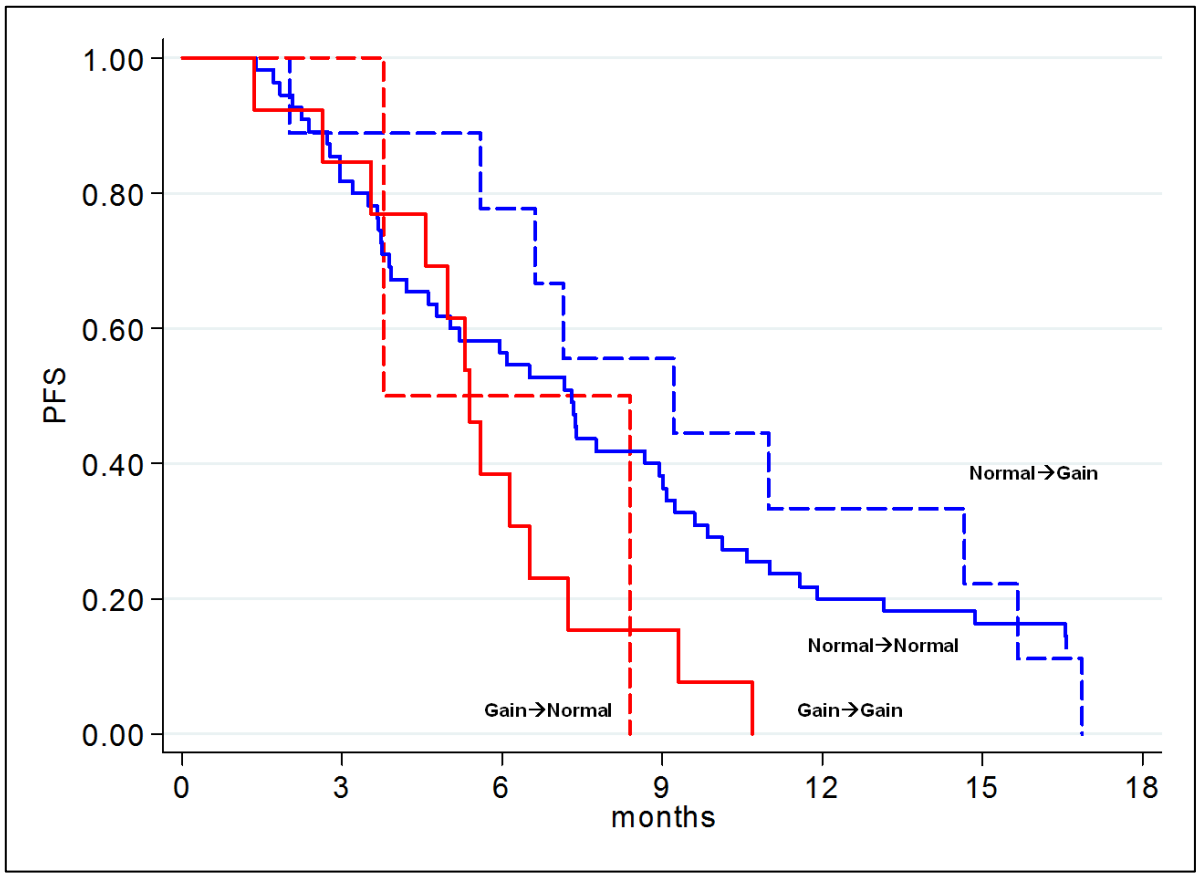

Figure 14. Association of plasma AR CN changes with outcome. Overall survival for AR CN changes from Gain to Normal, Gain to Gain, Normal to Gain and Normal to Normal in both chemotherapy naïve or post chemotherapy CRPC patients treated with enzalutamide or abiraterone.

Plasma AR CN status and 10 patients characteristics already known to be clinically significant [89] were evaluated by both univariate and multivariable analysis.

Normal to gain status presented HR, 3.34 (95% CI: 1.22 - 9.13) for OS compared to persistent normal status and HR, 0.80 (95% CI: 0.33 – 1.95) for PFS; gain to normal status presented an HR, 4.86 (95% CI: 0.47-50.49) for OS and 0.58 (95% CI: 0.06-5.49) for PFS; gain to gain AR status presented an HR, 5.61 (95% CI: 1.83 – 17.13) for OS and 2.87 (95% CI: 1.07-7.70) for PFS. Multivariable analysis shows that AR CN change is an independent negative prognostic factor (Table 10).

Table 10. Multivariable analysis

	OS		**PFS**	
	HR (95% CI)	**p**	**HR (95% CI)**	**p**
AR CN change				
(Normal→Gain vs Normal→Normal)	3.34 (1.22-9.13)	0.003	0.80 (0.33-1.95)	0.132
(Gain→Normal vs Normal→Normal)	4.86 (0.47-50.49)		0.58 (0.06-5.49)	
(Gain→Gain vs Normal→Normal)	5.61 (1.83-17.13)		2.87 (1.07-7.70)	
Gleason score (8-10 vs 6-7)	0.98 (0.46-2.09)	0.966	0.69 (0.35-1.35)	0.275
Bone metastasis (yes vs no)	4.20 (1.86-9.46)	0.0005	3.13 (1.54-6.35)	0.002
Visceral metastasis (yes vs no)	0.81 (0.33-1.99)	0.648	1.21 (0.54-2.73)	0.649
Lymph nodes metastasis (yes vs no)	2.89 (1.44-5.78)	0.003	1.90 (1.05-3.47)	0.035
LDH (≥225 vs <225)	0.88 (0.30-2.59)	0.818	1.26 (0.49-3.21)	0.631
NLR (≥3 vs <3)	2.24 (1.03-4.84)	0.041	1.28 (0.68-2.43)	0.447
ALP (≥129 vs <129)	0.48 (0.21-1.08)	0.076	1.04 (0.51-2.09)	0.921
Age (continuous variable)	0.97 (0.92-1.03)	0.364	0.90 (0.86-0.96)	0.0003
Log PSA baseline (continuous variable)	1.28 (0.96-1.69)	0.090	1.20 (0.95-1.52)	0.117
Log dsDNA baseline (continuous variable)	2.49 (1.49-4.15)	0.0005	1.94 (1.29-2.92)	0.001

Abbreviations: OS, overall survival; PFS, progression free survival; HR, hazard ratio; CI, confidence intervals; AR, androgen receptor; LDH, lactate dehydrogenase; NLR, neutrophil:lymphocyte ratio; ALP, Alkaline phosphatase; PSA, prostate specific antigen; dsDNA, double strand DNA.

4.4 Plasma AR CN in ^{177}Lu-PSMA-treated patients

A total of 40 of 43 patients treated with ^{177}Lu-PSMA were evaluable for AR CN. Patients characteristics are shown in table 11.

Table 11. Characteristics of ^{177}Lu-PSMA treated patients

Characteristics	N (%)
Median age (range), years	72 (54-86)
Median PSA (ng/ml) (IQR range)	66.1 (21.5-125.2)
ECOG Performance status	
0	17 (42.5%)
1	19 (47.5%)
2	4 (4%)
Sites of disease on Ga-PSMA PET	
Bone	38 (95%)
Lymph node	25 (62.5%)
Lung/Liver	14 (35%)
Received prior treatments	
Radical prostatectomy or radiotherapy	23 (57.5%)
Castration	40 (100%)
Enzalutamide or abiraterone	31 (77.5%)
Docetaxel	31 (77.5%)
Cabazitaxel	25 (62.5%)
Plasma AR status	
Normal	25 (62.5%)
Gain	15 (37.5%)

Abbreviations: PSA, prostate specific antigen; IQR, interquartile range; ECOG, Eastern Cooperative Oncology Group; Ga-PSMA PET, Gallium prostate specific membrane antigen Positron Emission Tomography; AR, androgen receptor.

Fifteen patients (37.5%) showed AR CN gain. A PSA response was reported in 15 (37.5%) of the 40 patients, 3 of 15 (20%) with AR-gain, and 12 of 25 (48%) with no gain (p = 0.080). Early

progressive disease, defined as treatment interruption occurring within 4 months of the start of treatment, was observed in 17 (42.5%) of the 40 patients, 12 of 15 (80%) with AR-gain and 5 of 25 (20%) with no gain (p = 0.0002). The OR for patients without PSA response (decline <50%) having AR-gain was 3.69, 95% CI 0.83-16.36, p = 0.085. Patients with early PD having AR-gain showed OR of 16.00, 95% CI 3.23-79.27, p=0.0007.

4.5 Genomic analyses in ^{177}Lu-PSMA-treated patients

We evaluated the HCS panel in 43 mCRPC patients treated with ^{177}Lu-PSMA. Pathogenetic variants were found in *ATM*, *BRCA2*, *PMS2* genes. Unclassified variants were identified in *BRCA1*, *BRCA2*, *ATM*, *NBN*, *CHEK2*, *APC*, *RAD51D*, *CDH1*, *MSH6*, *XRCC2*, *RAD51C*, *BARD1*, *PALB2*, *PIK3CA*, *MSH2*, *STK11*, *BRIP1*. Moreover, a *BRIP1* deletion was found in one of the patients.

We observed that one of the two patients with *ATM* pathogenetic variants exhibited an early PD and AR CN gain, whereas the other presented AR CN normal and no early PD. We did not find early PD in the patient who presented pathogenetic variants of the *BRCA2* gene and AR CN normal. Moreover, no early PD was observed in the patient with pathogenetic variant of the *PMS2* gene and AR CN gain. Table 12 shows pathogenetic (C4/C5) and unclassified (C3) variants found using HCS panel in ^{177}Lu-PSMA treated patients.

Table 12. Pathogenetic and unclassified variants of ^{177}Lu-PSMA treated patients

Patient ID	BRCA1 variants	BRCA2 variants	Pathogenetic variants (other genes)	Unclassified variants in other genes	CNVs in all genes
0287			**ATM, NM_000051** c.7000_7003delTACA; p.Tyr2334Glnfs*4		
LU 3				**ATM, NM_000051** c.5890A>G;p.Lys1964Glu **NBN, NM_002485** c.643C>T; p.Arg215Trp	
LU 8				**NBN, NM_002485**: c.633T>A;p.Asp211Glu	
0121				**ATM, NM_000051** c.5975A>C; p.Lys1992Thr	
0122				**CHEK2, NM_007194**: c.1227C>T; p.Asp409Asp	
0120	**BRCA1 NM_007294** c.5333-21A>C;p.? **C3**				
0124				**APC,NM_000038** c.6297T>C; p.Phe2099Phe	
0147				**ATM, NM_000051** c.6067G>A; p.Gly2023Arg	
0200				**RAD51D NM_002878** c.26G>C; p.Cys9Ser	
0292				**CHEK2, NM_007194** c.1427C>T;p.Thr476Met	
0225		**BRCA2 NM_000059** c.9934A>G; p.Ile3312Val **C3**		**CDH1, NM_004360** c.2232A>G; p.Pro744Pro	
0199				**MSH6, NM_000179** c.-8C>T	
0146				**XRCC2, NM_005431** c.398T>C; p.Leu133Pro	
LU 11			**PMS2, NM_000535** c.1021delA;p.Arg341Glyfs*15	**RAD51C, NM_058216:** c.114C>A; p.Leu38Leu	
LU 2				**BARD1, NM_000465** c.2233T>G; p.Tyr745Asp **NBN, NM_002485** c.643C>T; p.Arg215Trp	
0239				**PALB2, NM_024675** c.3428T>A;p.Leu1143His **PIK3CA, NM_006218** c.1747-8A>G	
0285				**MSH2,NM_000251** c.482T>A; p.Val161Asp **STK11, NM_000455** c.1159C>T; p.Pro387Ser	
0145				**BRIP1, NM_032043** c.3262C>T; p.His1088Tyr	
0203				**MSH2,NM_000251:** c.815C>T; p.Ala272Val **MSH6, NM_000179:** c.998C>T; p.Thr333Ile **PIK3CA, NM_006218** c.1827T>A; p.Pro609Pro)	
0149				**STK11, NM_000455:** c.1105C>T; p.Pro369Ser **MSH6, NM_000179** c.2963G>T; p.Arg988Leu	
0157				**MSH6, NM_000179** c.998C>T; p.Thr333Ile	
0242				**NBN, NM_002485:** c.1849G>A; p.Glu617Lys	
0166				**NBN, NM_002485** c.506G>A;p.Arg169His	
0151					**BRIP 1 del**
0192	**BRCA1 NM_007294** c.1834A>G; p.Arg612Gly **C3**		**ATM, NM_000051**: c.7563C>G;p.Tyr2521*	**BRIP1, NM_032043** c.3275C>T; p.Pro1092Leu	
0183		**BRCA2 NM_000059:** c.9676delT; p.Tyr3226Ilefs*23 **C5**			
LU 5				**ATM, NM_000051** c.7475T>G; p.Leu2492Arg **BRIP1, NM_032043**: c.139C>G; p.Pro47Ala	

Abbreviations: CNVs, copy number variations

5. DISCUSSION

In this study we have investigated the putative value of AR CN in stratifying patients in subsets benefitting from one type of therapy rather than another. We report that AR CN gain was associated with a significantly shorter OS in first-line docetaxel-treated patients compared to AR CN normal patients. However, when we compare first-line enzalutamide or abiraterone-naïve docetaxel-treated patients with first-line docetaxel- naïve enzalutamide/abiraterone-treated patients we found that patients with AR gain may respond better to docetaxel than enzalutamide or abiraterone as a first-line treatment.

We found that AR gain was associated with significantly shorter OS also in cabazitaxel-treated cohort. However, our exploratory analysis suggests that patients with AR gain may respond better to cabazitaxel as a second-line treatment compared with treatments with enzalutamide or abiraterone. These results are in line with what has been found in a randomized trial involving cabazitaxel therapy *vs* AR-targeting agents [90].

One of the major clinical issues for oncologists remains to be able to predict which drugs can be more effective in mCRPC patients. There is therefore a pressing need to identify predictive biomarkers able to identify the responsive patients. In this contest, liquid biopsy has emerged as a particularly useful biological source of biomarkers for molecular analyses, also given the fact that obtaining tissue specimens from metastatic lesions is often difficult. Wyatt and coworkers highlighted that there is high concordance between cfDNA analyses and matched metastatic tissue analyses when performing exome sequencing on PCa [91]. Annala and collaborators have documented the potential usefulness of liquid biopsy specimen for guiding the use of AR-targeted therapies in clinical practice [92].

We also evaluated the role of plasma AR CN changes in enzalutamide or abiraterone treatments monitoring which is a clinically relevant issue in PCa patients with metastatic advanced disease. We found AR changes during disease treatment in 11 patients and this could be partly due to the presence of different clones of tumor cells selected under the pressure of enzalutamide and

abiraterone. To date no clinically validated biomarkers are available for monitoring response to enzalutamide and abiraterone. Those patients who changed AR CN status from normal to gain could not respond to enzalutamide and abiraterone, as reported previously [57]. In these cases it may be clinically relevant re-evaluating AR CN during treatment to monitor tumor evolution and possibly modify the treatment with chemotherapy. Our results suggested that AR CN changes from normal status is an independent negative prognostic factor and confirm that AR CN status at baseline influences PCa prognosis, as demonstrated by our previous studies [57, 81].

Actually, it is unclear whether lethal phenotype derive from multiple foci with different genomic patterns that metastasize or it depends on a single clone that maintains dominance during the course of the disease. Probably, circulating cfDNA may be released from multiple metastatic lesions so that tumor aberrations, that commonly occur in PCa, can be detected in liquid biopsy specimen [18, 62]. In treatment-naïve patients, intra-tumoral heterogeneity increases as the tumor burden increases, and individual metastatic lesions are affected by their local microenvironment, even though they are relatively genetically homogeneous. CRPC cells modify themselves during treatments, as represented by lineage plasticity (metachronous heterogeneity) and because of the presence of distinct clones selected by therapies. These heterogeneities form the basis of treatment resistance. We also reported that AR CN status identifies mCRPC resistant to ^{177}Lu-PSMA. This suggests a potential better activity of ^{177}Lu-PSMA in earlier phases of PCa. Prospective evaluation of treatment decision making based on plasma AR status is warranted.

The analysis of HCS panel identified several pathogenetic and unclassified variants of genes involved in DNA repair mechanisms. We found that a patient with a pathogenetic variant in *ATM* gene early progressed, suggesting that patients with germline mutations should not respond to ^{177}Lu-PSMA, as reported in literature data [60]. Larger prospective studies are warranted to elucidate and verify the impact of gene variants in clinical outcome of patients treated with ^{177}Lu-PSMA.

We confirmed the results obtained on dPCR with multiplex AR CN ddPCR which is one of the most used method to analyze plasma CNVs. We obtained 100% of concordance between the two techniques, confirming our previous results [57]. Digital PCR and ddPCR allow to detect cfDNA alterations with high sensitivity starting from low volume of plasma sample. They are both capable of detecting low percentage CN differences to be identified (as it is DNA from liquid biopsy) or determining CN differences where the ratios between the target and reference are very small. However, both these technologies present a limited multiplexing capacity (1-2 target by dPCR, up to 5-10 by ddPCR), whereas NGS allows to analyze several alterations simultaneously [93].

We recognized that the present study has some limitations that are linked to the modest sample size and to the non-randomized, retrospective nature of the study. Indeed, prospective randomized studies are needed to confirm these first observations. Moreover, we have considered the total circulating DNA rather than the plasma DNA tumor content as done previously [81]. Based on a bioinformatic algorithm, it combines control sample data features, in-silico simulation and Mann-Whitney statistics. This test allows to estimate the fraction of DNA deriving from the tumor, thereby avoiding a bias introduced by dilution of tumor-derived DNA with healthy DNA. Indeed, cfDNA is about 5 ng/ml in healthy population deriving mainly from hematopoietic cells [94], whereas PCa patients present higher concentrations because of the combination of tumor-derived and healthy DNA [95]. Lastly, we only considered AR CN alterations, but other concurrently AR aberrations, such as somatic point mutations or splice variants expression, could provide further information and could be associated with clinical outcome in mCRPC.

In conclusions, the present study highlights the prognostic role of AR CN in docetaxel and cabazitaxel treated patients and that AR CN change during treatment is an independent unfavorable prognostic factor, further underlining the prognostic role of AR CN at baseline of enzalutamide and abiraterone. Prospective studies to validate these findings and further define the clinical utility of molecular analyses performed on liquid biopsies are warranted for CRPC patients treated with systemic therapies.

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