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THESIS

**PROGNOSTIC AND PREDICTIVE ROLE OF
miRNAs, CTCs AND AR-V7+ CTCs EXPRESSION
IN ADVANCED PROSTATE CANCER TREATED
WITH NEW HORMONAL AGENTS:
A FEASIBILITY STUDY**

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ABSTRACT

Background. Circulating tumor cells (CTC) counts of 5 or more/7.5 mL predict shorter survival in men with mCRPC. Moreover, the presence of androgen receptor splice variant-7 mRNA (AR-V7) in CTCs is thought to play a relevant role in the development of primary or acquired resistance to enzalutamide or abiraterone.

MiRNAs, small endogenous mRNA, have been identified as associated to the presence and aggressiveness of prostate cancer and for instance, overexpression of some miRNAs contributes to resistance to docetaxel and cabazitaxel.

We developed a new immunofluorescence-based assay for AR-V7 expression in CTCs and tested its prognostic association with PFS and OS.

Co-primary aims are to investigate AR-V7 with a new integrated assay and to study role of miRNAs in mCRPC patients treated with abiraterone or enzalutamide, and to study the relationship between AR-V7 expression, CTCs, and miRNAs with PFS or OS.

Methods. We performed a single-centre, observational, prospective trial enrolling patients with mCRPC candidate to receive enzalutamide or abiraterone. CTC samples have been collected at baseline, after 1 month and every three months thereafter until progression or at 12 months without progression. CTCs have been enumerated with CellSearch System. We integrated the standard assay with a monoclonal antibody able to recognize the AR-V7 protein. Slides created from pts' samples underwent automated immunofluorescent staining and AR-V7+ CTCs were enumerated. MiRNAs have been evaluated at the same time point.

Results. Since Sep 2016, 31 patients have been enrolled. We compared CTC counts between standard assay and the integrated assay and found no statistical differences in the mean total CTC number \pm SD (Wilcoxon Signed Rank test, $p= 0.313$).

Sixteen out of 28 evaluable patients (57%) had 1 or more CTCs at baseline, 9 pts (32%) had more than 5 CTCs/7.5 ml, and 4 pts (14.3%) were AR-V7+ before any exposure to abiraterone or enzalutamide.

After a median follow-up time of 8.1 months, 6 patients have progressed and 4 have died. No association has been found between CTCs $\geq 5/7.5$ mL and survival. The presence of at least one AR-V7+ CTC at baseline did not correlate with PSA response, but had a weak association with shorter PFS (log-rank test, $p = 0.055$) and a statistically significant impact ($p = 0.02$) on OS.

MiRNA failed in this analysis to correlate with survival outcome.

Conclusion. We developed a new integrated assay for detecting AR-V7+ CTCs, based on an automated platform that permits serial sampling with low inter- and intra-test variability. The clinical utility of this test in anticipating the resistance to abiraterone or enzalutamide is under study.

1. INTRODUCTION.

Prostate cancer (PCa) is the most prevalent malignancy in Western countries, and the second leading cause of cancer-related mortality in men [1,2]. Nine out of ten patients are diagnosed with localized disease and managed by primary curative treatment (either surgery or radiotherapy) or active surveillance. In European countries, during the last decade, the 5-year relative survival rate for PCa steadily increased up to 84% [3]. This result is undoubtedly due to the extensive use of PSA screening, despite the other side of the coin being over-diagnosis and over-treatments [4]. However, the risk of developing metastatic disease during long-term follow-up is not negligible, and can range cumulatively from 26% to 38% after radical prostatectomy or other curative approaches. Moreover, around 4% of the patients are initially diagnosed with metastatic disease [5] and morbidity and mortality by PCa are mainly caused by metastases. Therefore, there is an unmet need to improve our ability to stratify patients according their risk and to predict the treatment outcome.

The aim of treatment for metastatic prostate cancer (mPCa) is to halt, or at least to slow down, the disease progression: medical castration, using a luteinizing-releasing hormone (LHRH) agonist or antagonist, or bilateral orchiectomy is the most common first-line therapy (androgen deprivation therapy, ADT) [6]. At first disease progression, intense androgen blockade, by adding bicalutamide, could be considered [7], even if this second hormonal manipulation has never demonstrated to impact on survival.

Recently, data presented at American Society of Medical Oncology (ASCO) Annual Meeting in 2015 showed a significant benefit in overall survival (OS) from adding six courses of docetaxel to ADT at diagnosis in patients with mPCa at presentation and high volume disease [8]. Based also on the subsequent results of STAMPEDE and FETUG AFU15 trials [9, 10] the addition of docetaxel to ADT should now be considered standard

care for men with metastatic hormone-sensitive prostate cancer who are newly diagnosed with metastatic disease [11].

More recently, data from 2017 ASCO Annual Meeting indicated a survival gain from the addition of abiraterone acetate to ADT in mPCa at first presentation [12, 13].

When disease progresses to first line therapy, it becomes a metastatic castration-resistant prostate cancer (mCRPC). In a hormone-sensitive disease, multiple mechanisms have been described in the attempt to explain the development of CRPC. Despite castrate level of androgens, *AR* signalling still remains the key of the development of resistance. Constitutively active *AR* splicing variants, *AR* gene amplification and/or overexpression, *AR* gene mutation, and an unclear role for *AR* cofactors [14] have led to the knowledge that CRPC is not androgen independent, and continues to rely on androgen signalling [15].

In the castration-resistant setting, treatment strategies have traditionally consisted of hormonal therapy, chemotherapy, bisphosphonates, radioisotopes, and best supportive care (BSC), with several new treatment options approved in the last few years.

Chemotherapy may lead to PSA level decrease and symptomatic improvements: docetaxel is the standard first line chemotherapy with a median OS gain of 2.4 months compared to mitoxantrone [16]; cabazitaxel, a different drug belonging to the taxane family, has been approved after docetaxel failure since it improves OS [17]; mitoxantrone continues to be a further chemotherapy option although with unknown impact on survival.

Furthermore, several anti-hormonal drugs have recently emerged for the treatment of mCRPC based on the new knowledge on *AR* signalling. Abiraterone is a cytochrome P450 17A1 (CYP17A1) inhibitor that impairs androgen-receptor signalling by depleting adrenal and intratumoral androgens [18]. It has been approved for second or further line therapy after docetaxel [19] in mCRPC patients, and in the chemo-naïve setting [20], thanks to the relevant survival gain demonstrated in large randomized trials conducted against placebo.

Enzalutamide is an inhibitor of androgen-receptor signalling that exerts its activity by binding avidly to the ligand-binding domain of the androgen receptor, competing with and displacing the natural ligands of this receptor (testosterone and dihydrotestosterone) while also inhibiting translocation of the androgen receptor into the nucleus and impairing transcriptional activation of androgen-responsive target genes. Randomized trials provided solid data of OS improvement, therefore it has been firstly approved for second or further line of therapy after docetaxel [21], and later also for first line treatment in chemo-naïve patients [22].

More recently, the radiometabolic agent radium 223 has been introduced for the treatment of mCRPC [23] with bone only metastatic disease.

Since different drugs can be used in first or second line therapy and some of them can be used in both settings, the best treatment sequence is still debated and no definitive data are available. The treatment choice should be done by an oncologist trained in PCa's management and, possibly, by a Prostate Unit, and should take into account the presence of visceral vs bone disease, symptoms related to the disease, the need for disease shrinkage, comorbidities, and finally patients' preference.

Although enzalutamide and abiraterone represent breakthroughs in the treatment of mCRPC, approximately 20 to 40% of patients show immediate resistance to these agents [19, 21]. Among patients who initially respond, virtually all acquire secondary resistance. One of the plausible explanation for the resistance could be the presence of androgen receptor splice variants (AR-V) [24, 25]. These alternatively variants encode a truncated AR protein that lacks the C-terminal ligand-binding domain but retains the transactivating N-terminal domain [26]. The truncated proteins are unable to bind ligand, but they are constitutively active as transcription factors capable of promoting activation of target genes, such as KLK3 (PSA), KLK2 (HK2), TMPRSS2 and NKX3-1. Although supported by preclinical studies, the clinical significance of AR-V in patients receiving enzalutamide or

abiraterone has been long unknown until the results of the first study by Antonarakis and colleagues [27] which showed that the presence of AR-V7 transcript in circulating mCRPC cells may be associated with resistance to enzalutamide or abiraterone.

Further studies showed that levels of AR-V correlate with PCa progression [28], and expression of AR-V7 increases markedly upon androgen deprivation [29]. These findings suggest that AR-Vs may contribute to clinical progression of PCa.

Besides, subsequent studies indicated that AR-Vs not only supports the broad androgen/*AR* transcriptional program, but may also uniquely transcriptionally activate alternative targets genes associated the regulation of the cell [30]. AR-V7 is the most clinically relevant variant, because it is the most frequently observed and the most abundant AR-V in clinical specimens, and the only one that can be detected reproducibly at both the mRNA and protein levels. From the first Antonarakis's report [27] we learnt that the prevalence of AR-V7 was higher in enzalutamide-treated men who had previously received abiraterone and vice versa; AR-V7 prevalence was lowest in men who did not receive either agent. In addition, authors reported that all men with baseline detection of AR-V7 remained positive during the therapy, while 14% of patients with negative AR-V7 status at baseline converted to AR-V7-positive during treatment. The baseline presence of AR-V7 is thought to be a negative predictive factor for treatment with enzalutamide or abiraterone.

However, more data on biomarkers in patients with mCRPC are needed. An assay measuring a tumour marker must be evaluated in order to demonstrate its analytic and clinical validity, and at the end its clinical utility (the results of the assay should lead to a clinical decision that evidently improves patient outcome) [31]. Thus, a marker must encompass several levels of evidence in the attempt to demonstrate its clinical utility, with the final aim to improve patients' outcome, in terms of OS, disease-free survival and quality of life, and obtain a more cost-efficient application of effective therapies. The Tumor

Marker Utility Grading System (TMUGS) establishes an agenda for evaluating the clinical utility of tumour markers, and describes five levels of evidence as “categories that define the quality of data available on which the utility score is based”. These levels range from the weak level V evidence, derived from case reports and clinical examples, to the strongest level I evidence, “the definitive demonstration of clinical utility, obtained by a single, high-powered, prospective, randomized, controlled trial or from a meta-analysis or overview of multiple, well-designed studies”, passing through intermediate degrees of evidence in levels II–IV.

Many studies have illustrated the potential of liquid biopsy as tumour marker. The concept of liquid biopsy has gradually evolved reaching nowadays a wider field of application from the initial idea of “leukemic phase of solid tumours” [32]. Circulating tumour cells (CTCs) were described for the first time in 1869 by Ashworth, who documented the presence of cells “identical with those of the cancer itself” in the blood of a metastatic cancer patient. In 2001, Pachmann et al. combined laser scanning cytometry with immunomagnetic bead enrichment to detect and quantify rare tumour cells in peripheral blood and bone marrow [33]. A few years later, the association of CTC number and patient outcome was demonstrated in metastatic breast cancer using the CellSearch® platform (Janssen Diagnostics, LLC, Raritan, NJ, USA), a semi-automated system that immunomagnetically enriches epithelial cell adhesion molecule (EpCAM)-positive CTCs and enumerates them as (EpCAM+) CK+ DAPI+ CD45– cells [34].

Despite the great number of technologies developed to enrich, isolate and characterize these metastatic seeds of neoplasm, only the CellSearch® technology has demonstrated the clinical validity of CTC quantification (level I evidence) as a prognostic factor for metastatic breast cancer, by a pooled analysis of individual patient data published in 2014 [35].

The CellSearch® CTC Kit has been cleared by the Food and Drug Administration (FDA) for detection and enumeration of CTCs of epithelial origin in whole blood of metastatic colon, prostate and breast cancers. Specifically, for mCRPC, the EpCAM+ CTC number strongly correlates to prognosis: a cut-off value of 5 CTC/7.5 mL of peripheral blood has been validated, and patients whose CTC number is above this cut-off are at high risk of progression and shorter OS when treated with chemotherapy or abiraterone [36-38]. Since 2015, the CTC level has been included in the international guidelines for its prognostic value in metastatic breast cancer and, later, in prostate cancer. However, its predictive value and clinical utility are still under discussion.

More in detail, current limits to an extensive use of CTC assays in the clinical setting include: the lack of a consensus about the features that are necessary and sufficient to define an event as CTC; the poor ability to track tumour cells undergoing epithelial-to-mesenchymal-transition (EMT), responsible for the loss of epithelial markers in favour of the mesenchymal ones; the sensitivity of the techniques with a limit of detection of CTCs in no more than 50% of metastatic patients; the lack of a comprehensive genomic characterization of the circulating compartment together with a reliable assessment of its heterogeneity [39].

More recently, the identification of AR-V7 as a potential marker of resistance for treatment with abiraterone or enzalutamide has increased the interest in CTC as a tool for promoting personalized treatment [40], since AR-V7 has been isolated from CTCs.

MicroRNAs (miRNAs) are endogenous, short (18-25 nucleotides) single-stranded noncoding RNAs found in eukaryotic cells, synthesized and processed in the nucleus (pre-miRNA) before being exported to the cytoplasm (mature miRNA). Depending on the degree of complementarity with the 3' untranslated region (UTR), miRNAs can degrade or block the translation of their target mRNAs, and thus play a significant regulatory role in various biological processes, such as gene expression, cell proliferation, apoptosis, viral

defence, metabolism, hematopoietic differentiation and tumorigenesis [41-43]. Some of them have been identified as associated to the presence and aggressiveness of prostate cancer [44-47]: miR141, a member of miR-200 family, is abnormal in a wide range of common human cancers and it is over-expressed in prostate carcinoma, raising a controversial issue about the role of miR141 in cancer progression [48, 49]. As far as for miR141, significantly higher expression levels of miR-375 were depicted in PCa patients with higher Gleason score and more advanced pathological stage, as well as with regional lymph nodes metastases [50]. Some miRNAs are also predictive factors: for instance, overexpression of mir-181a in PCa cells contributes to their resistance to docetaxel and cabazitaxel [51].

Apart from and association with a more aggressive clinical and biological behaviour, the role of miR141 and miR375 in prostate cancer remains largely unknown. A recent work performed at our Institution has demonstrated that miR-106a/miR-103b and miR-106a/miR-223 in patients treated with curative intent may correlate with the development of metastases [52].

Co-primary aims of the present study are to investigate AR-V7 with a new integrated assay and to study role of miRNAs in mCRPC patients treated with abiraterone or enzalutamide, and to study the relationship between AR-V7 expression, CTCs, and miRNAs with PFS or OS.

2. MATERIALS AND METHODS.

Population. We designed an exploratory, prospective trial, enrolling patients with mCRPC who are candidate to receive abiraterone or enzalutamide with or without previous treatment with docetaxel. Key inclusion criteria included life expectancy greater than 6 months; disease progression after therapy with LHRH agonist or antagonist, with or without anti-androgen drugs (bicalutamide, flutamide); previous treatment with docetaxel administered for either castration-sensitive or resistant CRPC was allowed but not mandatory; absence of clinical contraindications to abiraterone or enzalutamide, adequate compliance to study procedures. All patients had to sign an informed consent form. All anti-neoplastic drugs have been administered according to current label authorisation in Italy and administered until progression, or unacceptable toxicity. No change of treatment or dose was recommended based on the results of this study. The choice between abiraterone or enzalutamide was made according to patients' comorbidities and potential contraindications to steroids, which should be given along with abiraterone but not with enzalutamide. Adverse events have been treated according to the best knowledge available at the time of their appearance. Disease progression has been defined as radiological (according to PCWG3 or RECIST criteria), or clinical (deterioration of general conditions).

Detection of miRNA. The expression of miRNAs has been evaluated by quantitative PCR of miRNAs selected from total RNA extracted. miR141 and miR375 have been tested. At each time point, 5 ml of blood anticoagulated with EDTA was collected from patients and then processed at our laboratory at Istituto Oncologico Veneto. Plasma was separated on Ficoll Plus (GE Healthcare) in 13 ml tubes (5 ml of blood on 4ml of Ficoll) and centrifuged at 581g (1800 rpm) for 30 minutes. Then, supernatant was removed (plasma approximatively 2 ml) 1ml above PBMC without mixing or touching it and then plasma was collected in 1.5ml eppendorf tubes. Subsequently, plasma was spinned at 2.500g for 15

minutes at room temperature and divided in 2ml eppendorf tubes. The tubes were stored at -80°C before RNA extraction. RNA extraction was conducted with NucleoSpin miRNA plasma kit (Macherey-Nagel©) according to manufacturer instruction, then extracted RNA was diluted in 30 µl of DNase/RNase free H₂O. 5 µl of total RNA were used for first-strand cDNA synthesis in 15 µl reaction volume using the TaqMan miRNA Reverse Transcription kit (Applied Biosystems©) and miRNA specific stem-loop primers. TaqMan specific miRNA assay and Light Cycler 480 Probe Master (Roche©) was used according to the manufacturers' protocol. All samples were run at least in duplicate including no-RT and no-template negative controls in a total volume of 20 µl reaction. A total of 45 amplification cycles were run. Absolute quantification was carried out on LightCycler 480 II (Roche©) with the automatic settings using the second derivative maximum method. This method permits to identify the threshold cycle (Ct) of a sample as the point where the slope of the sample's fluorescence curve exhibits the highest flexus (Software Version, handbook, Roche), thus minimizing biases due to the operator. All amplification curves were carefully controlled for artifacts, and inadequate samples were discarded from the analysis. Only Ct<40 values were considered. Mean values from duplicate/triplicate runs were calculated for each miRNA. Values of the miRNA obtained have been normalized with normal expressed miRNA 130b in order to analyse the data [52].

Detection of CTCs and AR-V7. The CTCs have been assessed with CellSearch™ System (Veridex©), which is the first automated platform for the capture, enrichment, identification and enumeration of CTCs in the peripheral blood [34]. We used this system according to manufacturer's instruction. An event was classified as a CTC when its morphological features were consistent with those of a tumour cell, and it exhibited the phenotype EpCAM+, CK+, DAPI+ and CD45- [53]. Quantitative results were expressed as the total number of CTCs per 7.5 ml of blood. In order to develop a new CS assay for the detection of AR-V7 in CTCs, we also integrated the standard CS assay with a monoclonal antibody

(mAb) that recognizes the variant 7 of the Androgen Receptor (AR-V7 EPR15656 abcam final concentration 0.87mg/ml), as previously performed for developing other CS integrated assays by our research group [53, 54]: slides created from mCRPC patient samples underwent automated immunofluorescent staining for AR-V7.

Timepoints. miRNA, CTC, AR-V7 have been evaluated on day 1 and after 28 days, thereafter at 3 months, 6 months, 9 months during treatment and at disease progression or at 12 months without progression, whichever comes first.

Statistical consideration. Progression was measured from the start of treatment until radiological or clinical progression (defined by PCWG3 criteria) or death; overall survival was calculated from start of treatment to death. Survival of patients lost to follow-up was checked with the national registry. PFS and OS were estimated using the the Kaplan-Meier method, and subgroups with different values of CTCs, AR-V7+ CTCs, and miRNAs at baseline and during treatment were compared with log-rank test. Fisher's exact Chi-squared test was used to evaluate the predictive accuracy of miRNAs levels, PSA response and CTC and AR-V7 positivity with progression and survival.

Ethical consideration. The study has been approved by local Ethics Committee at Istituto Oncologico Veneto, before any patient was enrolled.

3. RESULTS.

Population. Between September 2016 and August 2017, thirty-two patients have been prospectively enrolled onto this study at Istituto Oncologico Veneto. One patient refused to continue the study immediately after the signature of the consent form due to personal logistic reasons. Considering the cohort of the thirty-one male patients who have at least a baseline assessment, median age was 75.2 years (range from 57.7 to 89.6 years). All the patients had a histologic diagnosis of PCa: 14 of them (45%) underwent radical prostatectomy, 14 (45%) patients underwent radical radiotherapy, 17 (55%) received only prostatic biopsy on primary tumor. Median Gleason Score was 8 (range from 6 to 10). Most of the patients (23, 74%) had bone metastases, 17 patients (55%) had lymph nodes disease, and 2 of them (6%) had also local relapse. No patients had visceral disease in this cohort. Most of the patients had ECOG performance status 1 (74%), all the others are performance status 0 (26%).

The majority of patients were at first line therapy for mCRPC (26 patients, 84%), while 5 patients (16%) had already received a standard first line chemotherapy with docetaxel. Eight patients (26%) received abiraterone, while the other 26 (74%) received enzalutamide, reflecting investigators' choice.

At the baseline assessment, mean PSA level was 19.2 ng/ml (range from 0.5 to 1550 ng/ml).

Population characteristics are summarized in the following table:

Patients' characteristics	(n=31)
Median age (range)	75.2 years (57.7-89.6)
ECOG Performance Status	
- 0	8 (26%)
- 1	23 (74%)
Previous treatment of primary tumor	
- prostatectomy	14 (45%)
- radiotherapy	3 (10%)
- only biopsy	14 (45%)

Site of disease	
- bone metastases	23 (74%)
- lymph node metastases	17 (55%)
- visceral disease	none
- local relapse	2 (6%)

Previous treatment for mCRPC	
- none	26 (84%)
- docetaxel	5 (16%)

Response. Based on serial PSA levels, 20 patients (65%) had a PSA decrease greater than 50% within the first assessment at three months after enrolment; 10 patients (32%) had no PSA response (3 had PSA progression according to PWCG3 criteria [Scher, JCO 20016], 7 had stable PSA levels); 1 patient (3%) is still not evaluable.

On the radiologic side, 8 patients (25%) are not yet evaluable. Out of 23 evaluable patients for best response, 6 (26%) had a radiological response according to RECIST criteria, 15 patients (65%) had stable disease, and 2 patients (9%) had disease progression.

CTC counts and AR-V7 expression and development of an integrated assay.

Initially, we enumerated CTCs with the standard CS assay and with our new integrated CS assay able to identify AR-V7+ CTCs by means of a new monoclonal antibody (mAb) recognizing the variant 7 of the Androgen Receptor. Firstly, we compared the CTC levels measured by standard assay and the integrated assay in the firstly enrolled patients. Eleven patients were considered. The graph in figure 1 shows the mean total CTC number \pm SD in the standard and integrated assay and we did not find any statistically significant difference (Wilcoxon Signed Rank test, $p= 0.313$). Thus, we can stat that any difference obtained using the two different tests could be attributed to chance. Therefore, after the first eleven patients, we used only one test to enumerate at the same time both total CTCs and AR-V7+ CTCs from peripheral blood sample.

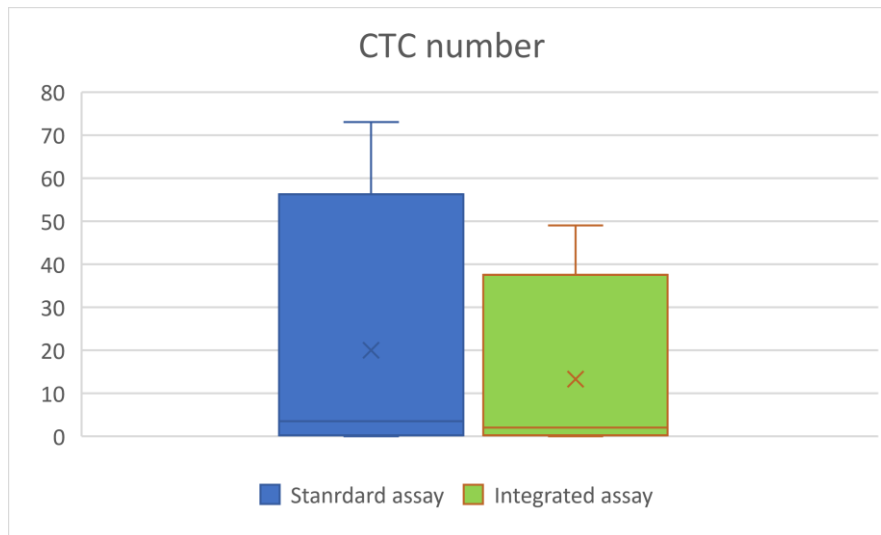


Figure 1. Mean total CTC number \pm SD in the standard and integrated assay (Wilcoxon Signed Rank test, $p=0.313$)

With the integrated CS assay, CTCs expression with the determination of the eventual presence of AR-V7+ CTCs has been tested at baseline and in all the following assessments for all the patients of the cohort.

Cells with morphological features consistent with those of a tumour cell, and exhibiting the phenotype EpCAM+, CK+, DAPI+ and CD45- were enumerated as CTCs. Those cells staining also for AR-V7 were classified as AR-V7+ CTCs.

For three patients, basal value of CTC was not available due to technical issue. Sixteen out of 28 evaluable patients (57%) had CTCs at baseline, of whom 9 (32%) had a basal value of CTC higher than 5/7.5 ml which (prognostic cut-off for mCRPC patients). Among all these patients, 4 (14.3%) also expressed AR-V7+ CTCs at baseline, before any exposure to either abiraterone or enzalutamide.

miRNA expression. miRNA expression has been tested at the baseline and at all the following assessments for the first 15 patients among the study population. miR141 and miR375 were expressed at baseline in all the patients. A trend to statistical significance has been found in the correlation between prospective changes of these two miRNAs and PSA response according to PCWG3 criteria (Fisher's exact test, $p=0.057$). Instead, no

statistically significant correlation has been found between changes of levels of these two miRNAs with radiological response. Data on the value of miR141/130b ratio and miR375/130b are shown in figure 2 and 3 respectively.

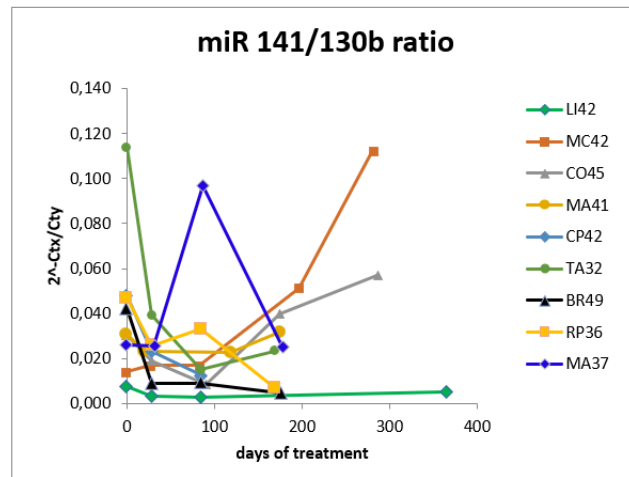


Figure 2. miR141/130b ratio during the study. Linear graphic for each patient (each line represents a patient).

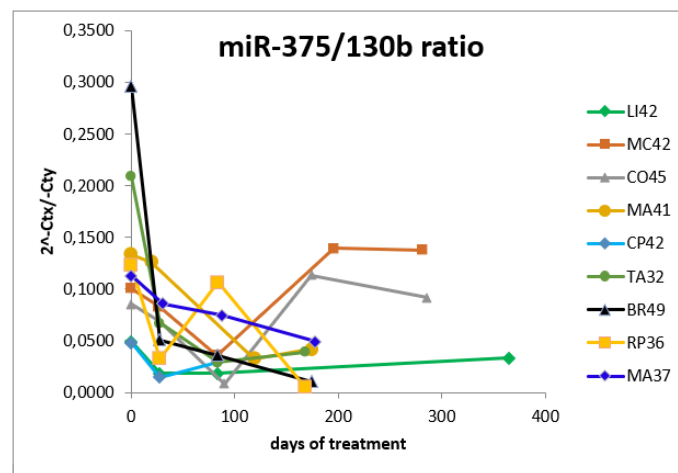


Figure 3. miR375/130b ratio during the study. Linear graphic for each patient. (each line represents a patient).

Survival analyses. After a median follow-up time of 8.1 months, median OS or PFS have not been reached: 6 patients (19%) have progressed and 4 (13%) died.

The curves of OS and PFS are shown in figure 4.

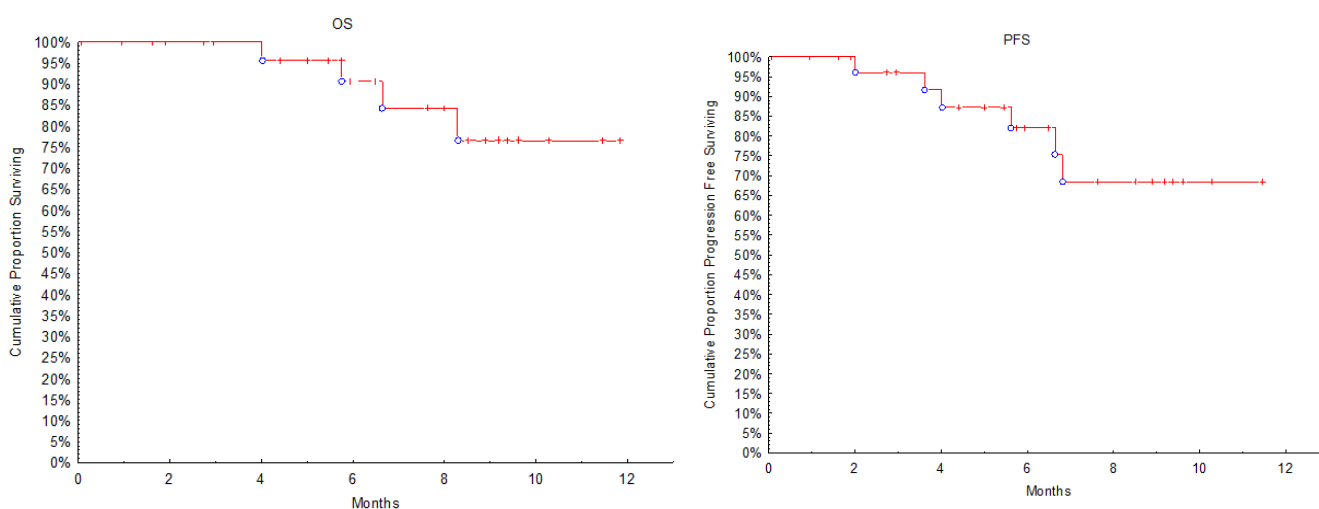


Figure 4. OS (a) and PFS (b) for the whole population.

As previously reported [37] we stratified all the patients according to CTC number of 5 or more cells. In the evaluable population (n=28), at the time of this analysis 94.7% of patients with CTC counts negative or below the cut-off are alive, while 88.9% of patients with at least 5 CTCs are alive, this difference being not statistically significant (Kaplan-Meier, log-rank test, $p = 0,453$).

PFS was slightly superior for patients with less than 5 CTC at baseline compared to the other group, but again there was not a statistically significant difference (log-rank test, $p = 0,910$).

We also analysed the changes of CTC number and of AR-V7+ CTC number during the treatment. As the whole population is numerically small, the differences among groups are not great enough to exclude statistically a random sampling variability and the level of expression does not vary over the time (Kruskal Wallis On Way analysis of variance on ranks, $p=0.414$ and $p=0.562$ respectively) (figure 5).

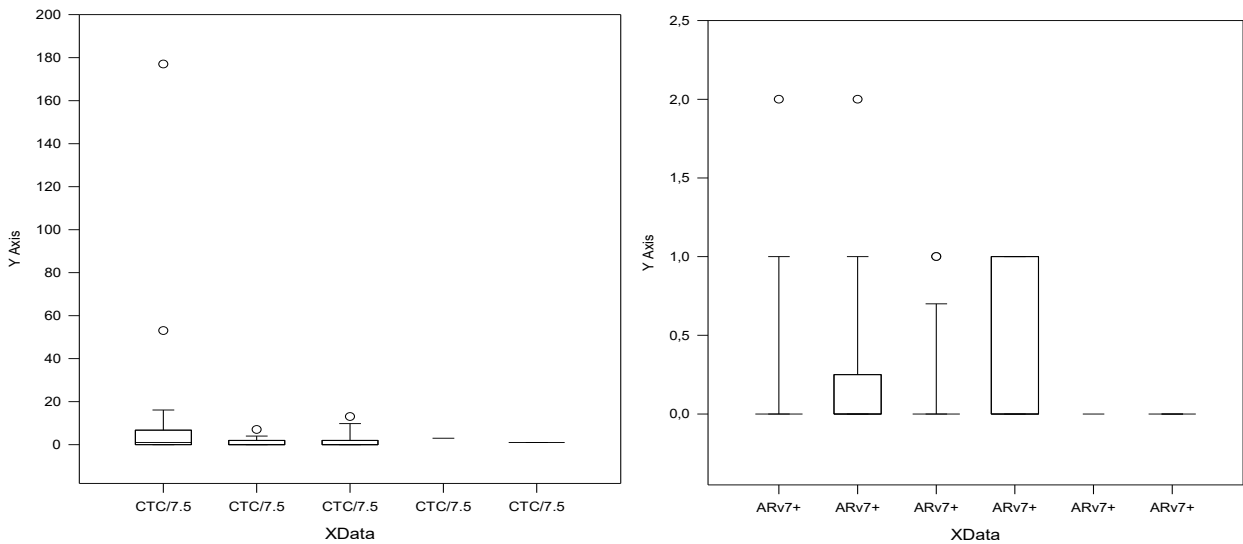


Figure 5. Changes of CTC number and of AR-V7+ CTC number during the treatment (Kruskal Wallis On Way analysis of variance on ranks, $p=0.414$ and $p=0.562$ respectively).

We also stratified the patients according to AR-V7+ CTC number of 1 or more cells. In our cohort, we found a weak association between AR-V7+ CTCs in peripheral blood at baseline and shorter PFS (log-rank test, $p = 0,055$) (figure 6) and a statistically significant impact on OS (log-rank test, $p = 0,02$) (figure 7), between the two groups.

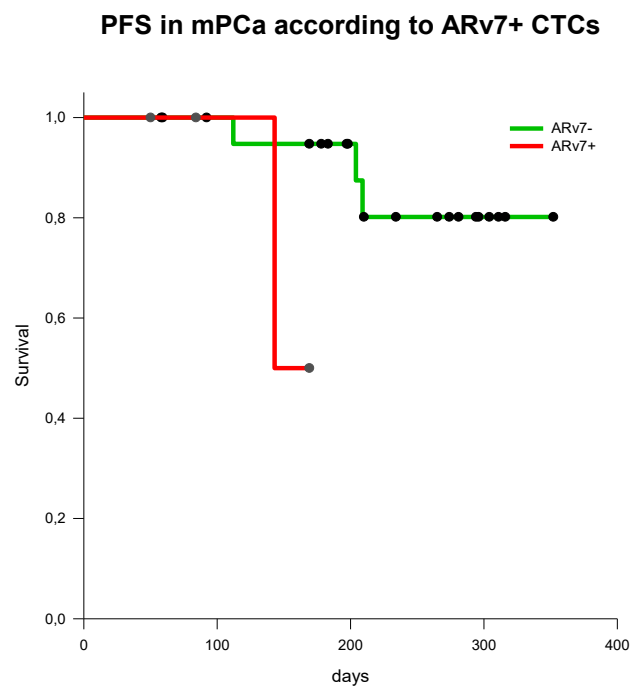


Figure 6. PFS for AR-V7+ CTC vs AR-V7- patients

OS in mPCa according to ARv7+ CTCs

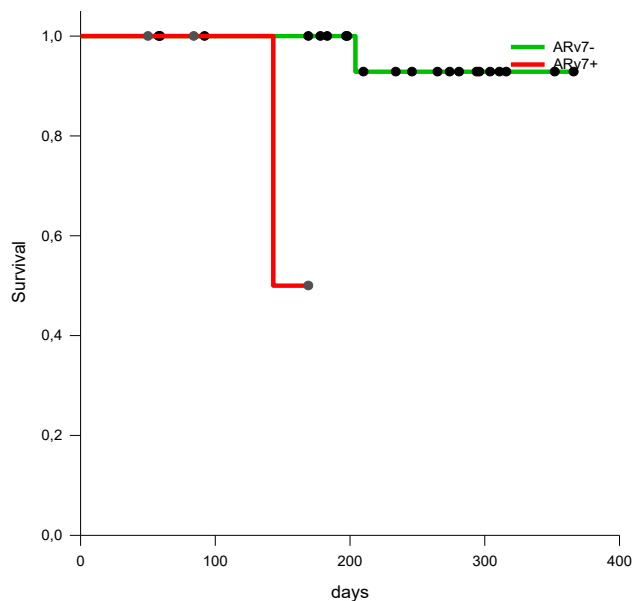


Figure 7. OS for AR-V7+ CTC vs AR-V7- patients

Representative cases. Some interesting cases have been identified during the analyses.

During the first year of follow-up, 4 patients died out of 32 enrolled patients and 2 of them had more than 5 CTCs at baseline blood draw.

Three out of 4 deceased patients did not show AR-V7+ CTCs at baseline, and initially responded to the treatment, showing important reduction of tumor burden (2 out of 4) and persistence of CTC level lower than 5 cells; then one of them showed ARV7+ CTCs at the time of progression (data shown in figure 8).

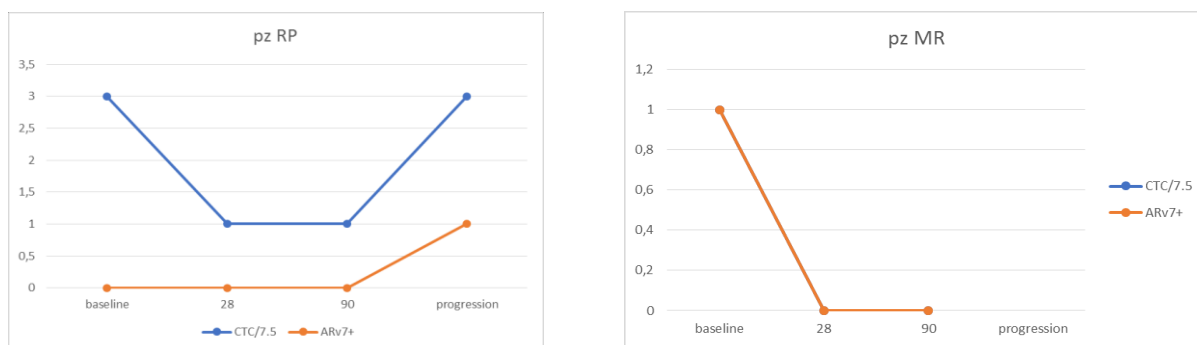


Figure 8. Representative cases for CTCs and AR-V7+ CTCs variation over time (longitudinal graphic)

Regarding miRNAs, one patient showed concordant variations over time of PSA, miR141/103b ratio and miR375/103b ratio. On the other side, another patient with increasing value of such miRNAs achieved a radiological and biochemical response (figure 9).

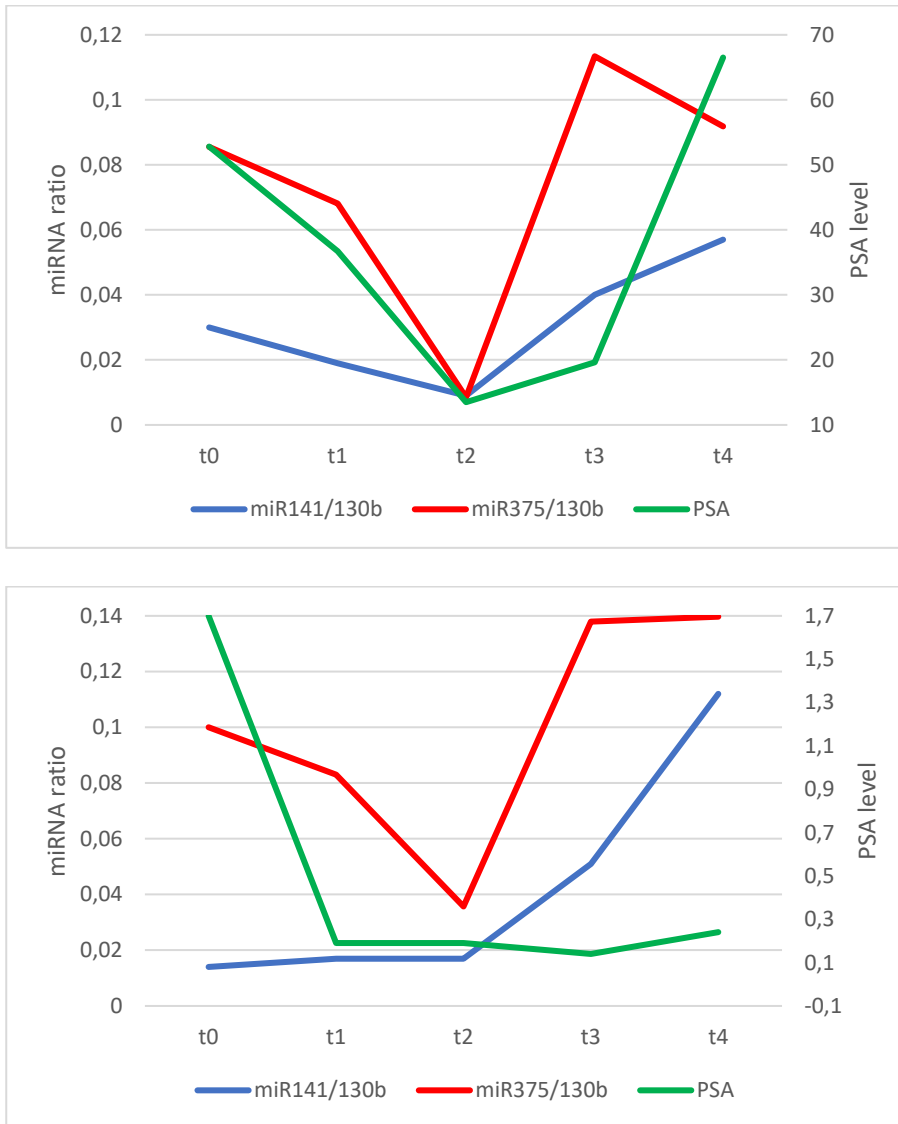


Figure 9. Representative cases for miRNA variation over time (longitudinal graphic).

4. DISCUSSION.

Thirty-two consecutive patients with mCRPC seen at our institution have been prospectively enrolled into an academic clinical trial aiming to assess the prognostic and predictive role of AR-V7+ CTCs and the two miR141 and miR375 during treatment with either abiraterone or enzalutamide. All of them had bone and/or lymph nodal disease and no visceral disease; median age was 75.2 years and a good proportion of them had not undergone any treatment on the primary tumor but only biopsy (45%). All patients were ECOG performance status 0 or 1. Population characteristics are very close to what has been yet reported in the registrative trials of abiraterone and enzalutamide. In the PREVAIL study [20] median age of the enzalutamide subgroup was 72 years and Gleason score was the same of our study; in the same way, there were no patients with ECOG performance status greater than 1. In the abiraterone pre-chemotherapy trial [19] the population has the same general characteristics.

Since one of the main aims of the study was to investigate AR-V7 with a new integrated assay able to enumerate total CTCs and AR-V7+ CTCs at the same time, firstly we have validated the test. In a previously reporter paper, Antonarakis et al. [27] identified AR-V7 through mRNA expression. The analyses were performed using the ProstateCancerDetect kit with multiplexed reverse-transcription polymerase-chain-reaction (qRT-PCR) primers to detect the presence of CTCs, and custom primers designed to detect the full-length-AR (AR-FL) and AR-V7. The relative AR-V7 transcript abundance was determined by calculating the ratio of AR-V7 to AR-FL. In our study, to quantify the fraction of AR-V7+ CTCs, we integrated the standard CS assay with a monoclonal antibody that recognizes the variant 7 of the Androgen Receptor, analysed with the fourth filter of the CellSearch System. Results were expressed as the total number of CTCs and AR-V7+ CTCs per 7.5 mL of peripheral blood. For the first enrolled patients, we have enumerated the CTCs with both the assays. Since we did not find any statistically

significant difference between the two groups, any difference obtained using the two different tests could be attributed to chance; therefore, we proceeded to use the integrated assay to enumerate CTCs and AR-V7+ CTCs in serial samples with lower inter- and intra-test variability than other reported tests detecting AR-V7 mRNA.

Regarding CTCs, it is already proven that CTC is a validated biomarker for prostate cancer [37]: a value equal or greater 5 CTC/7.5 ml is a negative prognostic factor. At the time of this writing, baseline CTC values show only a trend to significance in our cohort but this may be attributed to the low number of events and needs to be re-assessed with longer follow-up. We will try going to confirm the prognostic role of the basal value of CTC with a longer period of follow-up and a greater number of event, hopefully having reached median value of OS and PFS.

It is widely known that AR-V7 is associated with resistance to enzalutamide and abiraterone [25]. AR-V7 has been found to be significantly upregulated during PCa progression; moreover, expression of AR-V7 in primary prostate tumours has been demonstrated predictable for survival outcome after radical prostatectomy [55]. In a previous study, Hornberg et al. [56] the AR-V7 transcripts were demonstrated to increase in mCRPC compared with metastatic hormone-sensitive PCa and to be associated with a poor prognosis. Moreover, a recently published analysis confirms that patients positive for AR-V7+ CTCs before starting abiraterone or enzalutamide experience more PSA increases, shorter time on therapy and PFS, and also shorter OS compared to patients without AR-V7 expression [57]. In our cohort, AR-V7 was present in 14.3% of patients at baseline. This is in line with the expectation, since AR-V7 expression before treatment with enzalutamide or abiraterone ranged from 9% to 15% in other published reports [58].

Analysing the subgroup of patients with at least one AR-V7+ CTCs we found a statistically significant impact on OS and a weak association with PFS, but the small numbers do not allow us to compare patients according to relevant clinical and

pathological prognostic factors and to carry out a multivariate analysis. A longer period of observation and a higher number of events are necessary to confirm if the difference is statistically robust. A larger, and possibly multicenter study is warranted in order to confirm the clinical utility of this test with the final aim of incorporating it in the clinical practice of mCRPC management.

It has already been demonstrated that miR-141 and miR-375 are associated with a more aggressive behaviour of PCa. No data are available on predictive or prognostic role of miRNAs in mCRPC treated with hormonal agents. We hypothesized a possible role of the levels of these miRNAs in predicting shortened PFS and OS, but data collected up to now in our cohort are conflicting and do not allow us to draw any conclusion. In fact, no correlation has been found between baseline level of miR-375 and miR-141 expression and time to radiological or biochemical response, as well as with survival. Most likely, more preclinical data are needed to assess the role of miRNAs in developing of hormone resistance and/or alternative miRNAs should be studied.

Major limitation of the present study is the small number of patients and the short period of observation allowing only a quite small number of events to happen. In the published data, median PFS and OS with abiraterone or enzalutamide are around 18 and 35 months, respectively [22, 59].

5. FUTURE PERSPECTIVE.

As already discussed, the main limitation of the present study is the short follow-up: we therefore need to follow patients for a longer period of time in order to collect a greater number of events and assess the correlation between CTC number, AR-V7+ CTC number and control of disease and survival.

Moreover, we have planned to investigate the pathological samples of the primary tumours in the attempt to demonstrate with immunohistochemistry and/or molecular biology the presence of AR-V7 in the primary prostate tumor of the 4 patients with baseline expression of AR-V7.

Finally, we are going to perform droplet digital PCR in the AR-V7+ CTCs sample collected in our study to have molecular identification of the splicing variant of androgen receptor.

6. CONCLUSION.

We developed a new integrated assay for detecting ARv7+ CTCs by combining the standard CS assay (IVD) with a specific mAb detecting the splicing variant 7 of the Androgen Receptor. In comparison with previously reported assays, our test is based on an automated platform that permitted serial sampling with low inter- and intra-test variability, characteristics that are mandatory to plan clinical prospective studies and to introduce companion diagnostics in the clinical routine.

We found that the new assay is a feasible tool for detecting AR-V7+ CTCs and to study their dynamic changes under the pressure of hormonal drugs.

The clinical utility of this test in anticipating the resistance to enzalutamide and abiraterone is under study.

7. BIBLIOGRAPHY

1. Siegel RL, Miller KD, Jemal A. *Cancer Statistics 2017*. CA Cancer J Clin. 2017;67:7-30
2. Basch E, Loblaw DA, Oliver T, et al. *Systemic therapy in men with metastatic castration-resistant prostate cancer: American Society of Clinical Oncology and Cancer Care Ontario clinical practice guideline*. J Clin Oncol 2014;32(30):3436-48
3. De Angelis R, Sant M, Coleman MP, et al. *Cancer survival in Europe 1999-2007 by country and age: results of EUROCARE--5-a population-based study*. Lancet Oncol 2014 Jan;15(1):23-34
4. Bill-Axelson A, Holmberg L, Garmo H, et al. *Radical prostatectomy or watchful waiting in early prostate cancer*. N Engl J Med 2014 Mar 6;370(10):932-42
5. Seal BS, Aches CV, Puto K, et al. *Efficacy, patient-reported outcomes (PROs), and tolerability of the changing therapeutic landscape in patients with metastatic prostate cancer (MPC): a systematic literature review*. Value Health 2013;16(5):872-90
6. Loblaw DA, Virgo KS, Nam R, et al. *Initial hormonal management of androgen-sensitive metastatic, recurrent, or progressive prostate cancer: 2006 update of an American Society of Clinical Oncology practice guideline*. J Clin Oncol 2007 Apr 20;25(12):1596-605
7. Merseburger AS, Alkazar A, von Klot CA. *Androgen deprivation therapy as backbone therapy in the management of prostate cancer*. Onco Targets Ther 2016 Nov 29;9:7263-7274
8. Sweeney CJ, Chen YH, Carducci M, et al. *Chemohormonal Therapy in Metastatic Hormone-Sensitive Prostate Cancer*. N Engl J Med 2015 ;373(8):737-46
9. James ND, Sydes MR, Clarke NW, et al. *Addition of docetaxel, zoledronic acid, or both to first-line long-term hormone therapy in prostate cancer (STAMPEDE): survival results from an adaptive, multiarm, multistage, platform randomised controlled trial*. Lancet. 2016 Mar 19;387(10024):1163-77

10. Gravis G, Fizazi K, Joly F, et al. *Androgen-deprivation therapy alone or with docetaxel in non-castrate metastatic prostate cancer (GETUG-AFU 15): a randomised, open-label, phase 3 trial*. Lancet Oncol 2013;14(2):149-58
11. Vale CL, Burdett S, Rydzewska LHM, et al. *Addition of docetaxel or bisphosphonates to standard of care in men with localised or metastatic, hormone-sensitive prostate cancer: a systematic review and meta-analyses of aggregate data*. Lancet Oncol 2016 Feb;17(2):243-25
12. James ND, De Bono S, Spears MR, et al. *Abiraterone for Prostate Cancer Not Previously Treated with Hormone Therapy*. N Engl J Med. 2017; 377: 338-51
13. Fizazi K, Tran N, Fein LE, et al. *LATITUDE: Abiraterone plus Prednisone in Metastatic, Castration-Sensitive Prostate Cancer*. N Engl J Med. 2017, 377(4):352-360
14. Hu R, Denmeade SR, Luo J. *Molecular processes leading to aberrant androgen receptor signaling and castration resistance in prostate cancer*. Exp Rev Endocr Metab 2010; 5(5): 753–764
15. Longo DL. *New therapies for castration-resistant prostate cancer*. N Engl J Med 2010 Jul 29;363(5):479-81
16. Tannok IF, de Wit R, Berry WR, et al. *Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer*. N Engl J Med 2004 Oct 7;351(15):1502-12
17. de Bono JS, Oudard S, Ozguroglu M, et al. *Prednisone plus cabazitaxel or mitoxantrone for metastatic castration-resistant prostate cancer progressing after docetaxel treatment: a randomised open-label trial*. Lancet. 2010 Oct 2;376(9747):1147-54.
18. Attard G, Reid AH, Yap TA, et al. *Phase I clinical trial of a selective inhibitor of CYP17, abiraterone acetate, confirms that castration-resistant prostate cancer commonly remains hormone driven*. J Clin Oncol 2008;26(28):4563-71

19. Ryan CJ, Smith MR, de Bono JS, et al. *Abiraterone in metastatic prostate cancer without previous chemotherapy*. N Engl J Med. 2013;368(2):138-48
20. De Bono JS, Logothetis CJ, Molina A, et al. *Abiraterone and increased survival in metastatic prostate cancer*. N Engl J Med 2011;364(21):1995-2005.
21. Scher HI, Fizazi K, Saad F, et al. *Increased survival with enzalutamide in prostate cancer after chemotherapy*. N Engl J Med. 2012;367(13):1187-97
22. Beer TM, Armstrong AJ, Rathkopf DE, et al. *Enzalutamide in metastatic prostate cancer before chemotherapy*. N Engl J Med. 2014;371(5):424-33
23. Parker C, Nilsson S, Heinrich D, et al. *Alpha emitter radium-223 and survival in metastatic prostate cancer*. N Engl J Med 2013 18;369(3):213-23
24. Mostaghel EA, Marck BT, Plymate SR, et al. *Resistance to CYP17A1 inhibition with abiraterone in castration-resistant prostate cancer: induction of steroidogenesis and androgen receptor splice variants*. Clin Cancer Res 2011;17(18):5913-25
25. Nadiminty N, Tummala R, Liu C, et al. *NF- κ B2/p52 induces resistance to enzalutamide in prostate cancer: role of androgen receptor and its variants*. Mol Cancer Ther 2013;12(8):1629-37
26. Dehm SM, Schmidt LJ, Heemers HV, et al. *Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance*. Cancer Res 2008 Jul 1;68(13):5469-77
27. Antonarakis ES, Lu C, Wang H, et al. *AR-V7 and Resistance to Enzalutamide and Abiraterone in Prostate Cancer*. N Engl J Med 2014; 371:1028-1038
28. Hu R, Dunn TA, Wei S, et al. *Ligand-independent androgen receptor variants derived from splicing of cryptic exons signify hormone-refractory prostate cancer*. Cancer Res 2009 Jan 1;69(1):16-22

29. Yu Y, Liu L, Xie N, et al. *Expression and function of the progesterone receptor in human prostate stroma provide novel insights to cell proliferation control.* J Clin Endocrinol Metab 2013 Jul;98(7):2887-96
30. Hu R, Lu C, Mostaghel EA, et al. *Distinct transcriptional programs mediated by the ligand-dependent full-length androgen receptor and its splice variants in castration-resistant prostate cancer.* Cancer Res 2012; 15;72(14):3457-62
31. Teutsch SM, Bradley LA, Palomaki GE, et al. *The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Initiative: methods of the EGAPP Working Group.* Genet Med 2009; 11(1):3-14
32. Mocellin S, Keilholz U, Rossi CR, et al. *Circulating tumor cells: the 'leukemic phase' of solid cancers.* Trends Mol Med. 2006 Mar;12(3):130-9
33. Pachmann K, Heiss P, Demel U, et al. *Detection and quantification of small numbers of circulating tumour cells in peripheral blood using laser scanning cytometer (LSC).* Clin Chem Lab Med 2001;39(9):811-7
34. Cristofanilli M, Budd GT, Ellis MJ, et al. *Circulating tumor cells, disease progression, and survival in metastatic breast cancer.* N Engl J Med 2004;351(8):781-91
35. Bidard FC, Peeters DJ, Fehm T, et al. *Clinical validity of circulating tumour cells in patients with metastatic breast cancer: a pooled analysis of individual patient data.* Lancet Oncol 2014;15(4):406-14
36. Danila DC, Heller G, Gignac GA, et al. *Circulating tumor cell number and prognosis in progressive castration-resistant prostate cancer.* Clin Cancer Res. 2007;13(23):7053-8
37. De Bono JS, Scher HI, Montgomery D, et al. *Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer.* Clin Cancer Res 2008;14(19):6302-9

38. Onstenk W, de Klaver W, de Wit R, et al. *The use of circulating tumor cells in guiding treatment decisions for patients with metastatic castration-resistant prostate cancer.* Cancer Treat Rev 2016;46:42-50
39. Manicone M, Poggiana C, Facchinetti A, et al. *Critical issues in the clinical application of liquid biopsy in non-small cell lung cancer.* J Thorac Disease 2017
40. Maas M, Hegemann M, Rausch S, et al. *Circulating tumor cells and their role in prostate cancer.* Asian J Androl 2017.
41. Garcia-Schwarz G, Santiago JG. *Rapid high-specificity microRNA detection using a two-stage isotachopheresis assay.* Angew Chem Int Ed Engl 2013;52(44):11534-7.
42. Liu C, Kelnar K, Vlassov AV, et al. *Distinct microRNA expression profiles in prostate cancer stem/progenitor cells and tumor-suppressive functions of let-7.* Cancer Res 2012;72(13):3393-404
43. Zhou X, Wang X, Huang Z, et al. *Prognostic value of miR-21 in various cancers: an updating meta-analysis.* PLoS One. 2014;9(7):e102413
44. Mitchell PS, Parkin RK, Kroh EM, et al. *Circulating microRNAs as stable blood-based markers for cancer detection.* Proc Natl Acad Sci U S A. 2008 Jul 29;105(30):10513-8
45. Waltering KK, Porkka KP, Jalava SE, et al. *Androgen regulation of micro-RNAs in prostate cancer.* Prostate 2011;71(6):604-14
46. Moltzahn F, Olshen AB, Baehner L, et al. *Microfluidic-based multiplex qRT-PCR identifies diagnostic and prognostic microRNA signatures in the sera of prostate cancer patients.* Clin Cancer Res 2011;71(2):550-60
47. Selth LA, Townley SL, Bert AG, et al. *Circulating microRNAs predict biochemical recurrence in prostate cancer patients.* Br J Cancer 2013;109(3):641-50
48. Forman D, Burley VJ. *Gastric cancer: global pattern of the disease and an overview of environmental risk factors.* Best Prat Res Clin Gastr 2006;20(4):633-49

49. Iorio MV, Visone R, Di Leva G, et al. *MicroRNA signatures in human ovarian cancer*. *Cancer Res* 2007 Sep 15;67(18):8699-707.
50. Costa-Pinheiro P, Ramalho-Carvalho J, Vieira FQ et al. *MicroRNA-375 plays a dual role in prostate carcinogenesis*. *Clin Epigenetics* 2015;7:42.
51. Armstrong CM, Liu C, Lou W, et al. *MicroRNA-181a promotes docetaxel resistance in prostate cancer cells*. *Prostate* 2017;77(9):1020-1028
52. Sharova E, Grassi A, Marcer A, et al. *A circulating miRNA assay as a first-line test for prostate cancer screening*. *Br J Cancer* 2016;114(12):1362-6
53. Rossi E, Basso U, Celadin R, et al. *M30 neoepitope expression in epithelial cancer: quantification of apoptosis in circulating tumor cells by CellSearch analysis*. *Clin Cancer Res* 2010 ;16(21):5233-43
54. Rossi E, Facchinetti A, Zamarchi R. *Customizing CellSearch platform*. *Cytometry* 2013;83(7):595-8
55. Guo Z, Yang X, Sun F, et al. *A novel androgen receptor splice variant is up-regulated during prostate cancer progression and promotes androgen depletion-resistant growth*. *Cancer Res* 2009 Mar 15;69(6):2305-13
56. Hörnberg E, Ylitalo EB, Crnalic S, et al. *Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival*. *PLoS One* 2011 Apr 28;6(4):e19059.
57. Scher HI, Lu D, Schreiber NA, et al. *Association of AR-V7 on Circulating Tumor Cells as a Treatment-Specific Biomarker With Outcomes and Survival in Castration-Resistant Prostate Cancer*. *JAMA Oncol* 2016; 2(11):1441-1449.
58. Antonarakis ES, Lu C, Luber B, et al. *Clinical Significance of Androgen Receptor Splice Variant-7 mRNA Detection in Circulating Tumor Cells of Men With Metastatic Castration-Resistant Prostate Cancer Treated With First- and Second-Line Abiraterone and Enzalutamide*. *J Clin Oncol* 2017 Jul 1;35(19):2149-2156

59. Friedlander TW, Graff JN, Zejnullahu K, et al. *High-Dose Abiraterone Acetate in Men With Castration Resistant Prostate Cancer*. Clin Genitourin Cancer 2017; S1558-7673(17)30163-5