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**Reviewer A**

The reviewer appreciates the importance and quality both of Cho et al's study and the short review of the literature in the present Editorial Commentary.

A few minor edits are recommended as follows, which may help readers grasp the significance of Cho's findings more easily:

***1.*** *Lines 28-30 and 45 to 48 seem somewhat redundant. They both make the argument that examining molecular markers on CTC will offer an improvement over examining the same markers in serum on the one hand, or over plain enumeration of CTC on the other hand. Cho at al. impressively demonstrate this, and the author very clearly summarizes Cho's work in the present article. However, it would be helpful to give more background to readers regarding previous studies of molecular markers on CTC. The reviewer would recommend to add a paragraph referencing studies that have examined expression of molecular markers on individual CTC and their predictive potential on patient outcomes before the study of Cho et al. in somewhat more depth.'*
*Line 58 to 68 seem to attempt this, however, it is not immediately clear (without actually reading the complete references) which of the studies cited here examine CTC-based molecular markers and which rely on serum-based molecular markers only. It might be useful to separate the review of serum-based molecular markers, CTC enumeration and CTC-based molecular markers in a clearer way. The reviewer would also suggest to double-check if other previous studies looking at prognostics and patient outcomes deserve to be added and discussed here.*

**Response 1:** We appreciate the Reviewer's suggestion and have now updated the discussion adding a paragraph with details of most relevant studies that have examined expression of molecular markers on individual CTC and their predictive potential on patient outcomes, as follows:
"Several studies have investigated the expression of molecular markers on individual CTCs and their predictive potential on patient outcomes. Androgen Receptor Splice Variant-7 (AR-V7) was one of the first markers studied in association with taxane sensitivity over ARPIs (10). PSA responses and clinical outcomes were superior with taxanes compared with enzalutamide or abiraterone therapy in AR-V7-positive men, while they did not differ by treatment type in AR-V7-negative men (10). Overall, CTC+/AR-V7+ patients compared to CTC- or CTC+/AR-V7- were significantly more likely to have Gleason scores ≥ 8, metastatic disease at diagnosis, higher PSA, prior abiraterone or enzalutamide use, prior taxane use, and Eastern Cooperative Oncology Group ≥ 1 (11). Interestingly, TP53 inactivation was shown to outperform the predictive and prognostic significance of AR in another study (12). Overall, CTC-based WNT5a and AURKA expression combined with known clinical prognosticators

namely Halabi score (13), or multigene signatures including AR, AR-V7, PSA, PSCA, TSPAN8, NKX3.1, WNT5B. and SPINK1 (14) seem to better capture the biology of this heterogeneous disease, predicting clinical outcomes in patients with aggressive and ARPI-resistant prostate cancer. Overall, the transcriptional profile including AR-Vs, AR targets, and neuroendocrine prostate cancer markers such as RB1, TP53, and PTEN, obtained from CTCs may detect neuroendocrine differentiation and can serve as an independent, prospectively validated prognostic marker (15). Furthermore, expression of the DNA/RNA helicase schlafen family member 11 (SLFN11) may identify patients with CRPC with a better response to platinum chemotherapy independent of histology or other genomic alterations (16)."

Added references:

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14. De Laere B, Oeyen S, Mayrhofer M, Whitington T, van Dam PJ, Van Oyen P, Ghysel C, Ampe J, Ost P, Demey W, Hoekx L, Schrijvers D, Brouwers B, Lybaert W, Everaert EG, De Maeseneer D, Strijbos M, Bols A, Fransis K, Beije N, de Kruijff IE, van Dam V, Brouwer A, Goossens D, Heyrman L, Van den Eynden GG, Rutten A, Del Favero J, Rantalainen M, Rajan P, Sleijfer S, Ullén A, Yachnin J, Grönberg H, Van Laere SJ, Lindberg J, Dirix LY. *TP53* Outperforms Other Androgen Receptor Biomarkers to Predict Abiraterone or Enzalutamide Outcome in Metastatic Castration-Resistant Prostate Cancer. Clin Cancer Res. 2019 Mar 15;25(6):1766-1773. doi: 10.1158/1078-0432.CCR-18-1943.

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14. Sperger JM, Emamekhoo H, McKay RR, Stahlfeld CN, Singh A, Chen XE, Kwak L, Gilsdorf CS, Wolfe SK, Wei XX, Silver R, Zhang Z, Morris MJ, Bubley G, Feng FY, Scher HI, Rathkopf D, Dehm SM, Choueiri TK, Halabi S, Armstrong AJ, Wyatt AW, Taplin ME, Zhao SG, Lang JM. Prospective Evaluation of Clinical Outcomes Using a Multiplex Liquid Biopsy Targeting Diverse Resistance Mechanisms in Metastatic Prostate Cancer. J Clin Oncol. 2021 Sep 10;39(26):2926-2937. doi: 10.1200/JCO.21.00169.

16. Zhao SG, Sperger JM, Schehr JL, McKay RR, Emamekhoo H, Singh A, Schultz ZD, Bade RM, Stahlfeld CN, Gilsdorf CS, Hernandez CI, Wolfe SK, Mayberry RD, Krause HM, Bootsma M, Helzer KT, Rydzewski N, Bakhtiar H, Shi Y, Blitzer G, Kyriakopoulos CE, Kosoff D, Wei XX, Floberg J, Sethakorn N, Sharifi M, Harari PM, Huang W, Beltran H, Choueiri TK, Scher HI,

Rathkopf DE, Halabi S, Armstrong AJ, Beebe DJ, Yu M, Sundling KE, Taplin ME, Lang JM. A clinical-grade liquid biomarker detects neuroendocrine differentiation in prostate cancer. J Clin Invest. 2022 Nov 1;132(21):e161858. doi: 10.1172/JCI161858.
17. Conteduca V, Ku SY, Puca L, Slade M, Fernandez L, Hess J, Bareja R, Vlachostergios PJ, Sigouros M, Mosquera JM, Sboner A, Nanus DM, Elemento O, Dittamore R, Tagawa ST, Beltran H. SLFN11 Expression in Advanced Prostate Cancer and Response to Platinum-based Chemotherapy. Mol Cancer Ther. 2020 May;19(5):1157-1164. doi: 10.1158/1535-7163.MCT-19-0926.

***2.*** *Line 49 to 57: it does not seem necessary to quote Cho et al. 3 times in the same paragraph.*

**Response 2:** We omitted one out of 3 quotations of Cho et al. reference.

**Reviewer B**

***1.*** *Clarity and Structure:*
*The article could benefit from clearer organization, particularly in distinguishing the different stages of prostate cancer and the specific roles of PSMA and PSA in each stage.*
*The introduction could more clearly define the scope and aims of the article to set expectations for the reader.*

**Response 1:** The Reviewer's comments were taken into serious consideration and addressed by explicitly describing the different stages of prostate cancer and what are the specific roles of PSMA and PSA in each stage (prior to the study of Cho et al.). Additionally, the introduction was reorganized and re-written to better define the scope and aims of the article by Cho et al., as follows:
"In localized prostate cancer, which involves disease confined to the prostate, elevated PSA levels indicate disease presence or/and recurrence after definitive therapy (6). In metastatic hormone-sensitive prostate cancer (mHSPC), which describes the disease that has spread beyond the prostate but is still responsive to hormone therapy, high PSA levels predict worse progression-free survival (PFS) (7). In metastatic castration-resistant prostate cancer (mCRPC) which involves the disease that continues to grow despite hormone therapy, PSA levels are critical for monitoring treatment response to life-prolonging therapies as well as an independent prognostic marker (8). PSMA is overexpressed in mCRPC and has been associated with tumor aggressiveness and progression (97). More importantly, PSMA-targeting for diagnostic (PSMA-PET) and therapeutic purposes (PSMA radioligand therapies) has opened new avenues in personalized management of prostate cancer patients, culminating in the approval of 177Lu-PSMA-617 in 2022 for men with PSMA-PET-positive metastatic castration-resistant prostate cancer (107,9). However, not all patients respond to PSMA-radioligand therapy, in part owing to inter- and intra-patient heterogeneity of PSMA expression among different sites of metastases."
"Cho et al. analyzed the relationship between mRNA gene expression of PSMA and PSA in

CTC counts, and patient outcomes in prostate cancer patients. The aim of their study was to assess whether a circulating tumor cell (CTC)-based transcript gene signature, including PSMA mRNA, could help inform prognostication in various stages of prostate cancer, including localized, mHSPC and mCRPC. For this purpose, the authors analyzed 247 patients with prostate cancer at various stages, consisting of 94 subjects with localized prostate cancer, 44 diagnosed with mHSPC, and 109 suffering from mCRPC. They also used 21 healthy individuals as a control group. Six genes including PSMA, PSA, AR, AR-V7, EpCAM, and KRT 19 were measured for mRNA expression in CTCs of these patients. The impact of CTC-based PSMA and PSA mRNA expression on four distinct clinical outcomes was examined, including biochemical recurrence-free survival in localized disease, mCRPC progression-free survival (PFS) in mHSPC state, PSA-PFS and radiological-PFS time in mCRPC state."

***2.*** *Detail and Depth:*
*More detailed explanation of the methodologies used to measure PSMA and PSA expression in CTCs would enhance understanding, especially for readers unfamiliar with these techniques.*
*The discussion on the limitations of the study could be expanded to explore alternative methods or additional research needed to address these limitations.*

**Response 2:** We appreciate the Reviewer's comment which was now addressed in detail by adding a full explanation of the methodologies used to measure PSA and PSMA expression CTCs. We also added a paragraph discussing the limitations of the study and also included though sans suggestions for additional/future research to address these limitations, as follows:
"From a methodology perspective, the authors first conducted CTC enumeration after isolating them from peripheral blood with use of EpCAM labelling and high-gradient magnetic flux. Then, reverse transcription polymerase chain reaction (PCR) and digital-droplet PCR were performed in CTCs to quantify the mRNA expression of the six genes (PSMA, PSA, AR, AR-V7, EpCAM and KRT-19) in number of copies per μL per CTCs. Finally, using the median values as cut-offs to separate in low and high-expressing subgroups, they evaluated statistical associations between mRNA expression of every separate gene and the afore-mentioned clinical outcomes (18)."
"The study of Cho et al. (180) was limited by a relatively limited number of patients per disease stage, which could impact the generalizability of the findings. Thus, future studies should prospectively test a larger sample from each distinct clinical state within the prostate cancer continuum. Additionally, mechanisms leading to tumor recurrence, progression to mCRPC, and drug resistance may not be fully detectable using droplet digital PCR alone. Multiplex PCR which allows for evaluation of expression across multiple cancer-related genes or even single-cell RNA profiling could provide additional insights into tumor heterogeneity, treatment resistance and potential therapeutic targets. Another important limitation which is inherent to the methodology of PSMA assessment is the lack of special information provided by PSMA imaging. To tackle this, future studies combining CTC-based and nuclear assessment of PSMA expression are needed."

***3.*** *Data and Evidence:*

*Incorporate more quantitative data or statistical results from Cho et al.'s study to support claims, such as specific survival rates or response rates linked to PSMA and PSA expression levels.*
*It would be helpful to include a comparison of results with other studies to contextualize findings within the broader field of prostate cancer research.*

**Response 3:** We have now added more quantitative data and statistical results, linked to PSMA and PSA expression levels. We also added a comparison with prior studies providing the specific place of this study within the broader context of clinically useful markers for prostate cancer prognostication and treatment selection, as follows:
"Specifically, the two-year BCR-free survival was significantly longer in those with low versus high PSMA- and PSA-mRNA levels in their CTCs ($p=0.018$ and $p=0.037$, respectively). Additionally, high mRNA expression levels of PSMA and PSA was were significantly linked to a 12-month shorter mCRPC-free survival ($p=0.011$ and $p= 0.028$, respectively). These mHSPC patients had almost 7-times higher measured level of PSMA compared to those who did not progress to mCRPC state. as well as progression-free survival Furthermore, high mRNA levels of PSMA and PSA were associated with a 2-fold and 3.5-fold higher risk of PSA- and radiographic progression, respectively ($p=0.045$ and $p=0.001$, respectively) in patients with mCRPC undergoing treatment with ARPIs and docetaxel (1018). Likewise, high PSMA- and PSA-expressors were at least 3-times more likely to experience resistance to docetaxel evidenced by shorter PSA-PFS and radiographic PFS. Notably, patients unresponsive to ARPIs had 10-times higher numbers of PSMA mRNA copies/μL/CTCs compared to responders ($p<0.001$)."
"In comparison to these prior studies, it is interesting that the results of Cho et al. (18) with respect to the prognostic and predictive value of AR- and AR-V7 mRNA expression in CTCs for ARPI or docetaxel response and CRPC-PFS were non-significant or inconclusive. Perhaps, the low number of CTCs in several cases with mHSPC and mCRPC may have affected the validity of their findings. It can be postulated that even with fewer CTCs, aggressive prostate cancer may still rapidly progress from mHSPC to mCRPC state or/and manifest unresponsiveness to ARPIs and taxanes. This rapid progression and treatment resistance is better represented by copies of PSMA and PSA in CTCs, according to Cho et al (18)."

***4.*** *Language and Terminology:*
*Simplify some of the technical jargon for accessibility to a broader audience, particularly in explaining complex biological processes or molecular techniques.*
*Ensure consistent use of terminology throughout the article, particularly when referring to stages of prostate cancer and specific biomarkers.*

**Response 4:** Technical jargon referring to biological processes and molecular techniques was simplified throughout the manuscript for accessibility to a broader audience.

***5.*** *Conclusion and Implications:*
*The conclusion could more explicitly summarize the potential clinical implications of using*

*CTC-based PSMA and PSA expression as biomarkers, including specific examples of how this might change current clinical practice.*
*Suggest future research directions or clinical trials that could further validate the findings or explore new aspects of CTC biomarker utility.*

**Response 5:** We have added a whole new discussion within the conclusion section summarizing the clinical implications of CTC-based PSMA and PSA mRNA assessment as biomarkers as well as providing suggestions for future studies validating and expanding CTC-based biomarker utility, as follows:
"Incorporating CTC-based PSMA and PSA assessments into clinical practice could enhance personalized treatment strategies and improve patient outcomes in prostate cancer management. First, high levels of PSMA and PSA mRNA in CTCs can indicate resistance to androgen receptor signalling inhibitors (ARSIs) and taxane-based chemotherapy. This information can guide clinicians to consider alternative therapies, such as radioligand therapy (e.g., 177Lu-PSMA-617) for patients with high PSMA expression. Second, regular assessment of CTC PSMA and PSA levels can help in monitoring treatment efficacy. A lack of significant decrease in these biomarkers may suggest treatment resistance, prompting a change in therapeutic strategy. Third, patients with elevated CTC PSMA and PSA levels may be at higher risk for rapid disease progression and poorer outcomes. This stratification can inform more aggressive treatment approaches or closer monitoring for these high-risk patients."
"Future research directions and clinical trials focusing on CTC biomarkers in prostate cancer could include large-scale, multicenter clinical trials to validate the prognostic and predictive value of CTC-based PSMA and PSA expression in diverse patient populations. This could help establish standardized thresholds for clinical decision-making. Additionally, implementing trials that assess the pharmacodynamic utility of CTCs could test their value as real-time biomarkers for treatment response. Exploring the potential of advanced molecular profiling of CTCs could be used to identify specific mutations and gene expression patterns that predict treatment responses. This could lead to more personalized therapeutic approaches. Developing and testing new CTC isolation and analysis technologies could improve capture efficiency and purity, enabling better characterization of CTCs and their heterogeneity. Finally, conducting longitudinal studies to monitor CTC dynamics over time, could help explore their role in disease progression and treatment resistance, which could inform adaptive treatment strategies."