Peer Review File

Article information: https://dx.doi.org/10.21037/tbcr-23-55

Reviewer Comments

Reviewer A

Comment 1: This review provides a comprehensive overview of the evolving landscape of liquid biopsy in breast cancer management. It emphasizes the limitations of focusing solely on one biomarker, such as circulating tumor cells (CTCs), due to their low abundance, especially in the early stages. The piece advocates for a broader approach, combining various circulating biomarkers like cell-free DNA, extracellular vesicles, and non-malignant cells, recognizing their potential synergy in providing a more comprehensive understanding of tumor biology.

The review rightly underscores the importance of viewing these biomarkers as complementary rather than competing, offering opportunities for improved prognosis, early detection, and treatment monitoring in breast cancer. It also highlights the necessity of overcoming technical challenges and establishing standardized protocols to unlock the full potential of liquid biopsy in reshaping breast cancer research and management.

Overall, this review provides a strong foundation for a comprehensive review of the clinical significance and challenges associated with integrating multiple biomarkers in liquid biopsy for breast cancer.

I would like to recommend to publish in this journal.

Reply 1: We are grateful Reviewer A for the kind comment on our manuscript.

Reviewer B

Comment 1: The authors present this review to discuss what other markers, combined with the detection of CTCs, might improve the sensitivity of detection of cancer, and its predictive and prognostic value. Low abundance of CTCs in blood particularly in early stages of the disease, despite the use of enrichment methods, is a challenge.

The authors explore the studies that analyze CTCs in blood along with other biomarkers, including cfDNA, extracellular vesicles, non-epithelial or dual positive CTCs, cell-free RNAs, and non-malignant cells examining their ability to contribute to a comprehensive disease profile.

This review is largely redundant with a recently published review (Clinical Chemistry 70:1 68–80, 2024) on the same topic in breast cancer titled “ Breast Cancer Circulating Tumor Cells: Current Clinical Applications and Future Prospects”. However, the descriptions of each study, and the combination of CTC with cfDNA, the small vs large vesicles, single microRNAs vs a group pf microRNAs and all white blood cell types is presented in more detail and sections are devoted to the different blood cell type analysis with CTC. These are often single papers that need yet to be validated. The review is comprehensive. It is obvious the author is devoted to CTCs, but it is important

to examine if any of the other analytes outperform CTCs and can stand alone.
Since so much detail is presented on each analyte, the three tables are redundant. The Figure is self-explanatory.
Reply 1: We would like to thank Reviewer B for acknowledging the value of our work and for bringing attention to the interesting review authored by A.K. Cani and D.F. Hayes published on Clinical Chemistry in January 2024, subsequent to the submission of the present work. As pointed out by Reviewer B, while Cani et al. provided a review of the state of the art of CTCs in breast cancer encompassing analytical validity, clinical validity and clinical utility, our article aims to delve into the advantages offered by the concurrently analyzing CTCs alongside with other circulating biomarkers. Together, these articles demonstrate the growing interest in CTCs within the contest of breast cancer research. Due to the comprehensive and up-to-date nature of this review, we have included it among our references (reference 5).
We recognize that each biomarker, including CTCs, has its own strengths and limitations. We firmly believe that their combined analysis holds the potential to unlock the full potential of liquid biopsy.
Regarding the inclusion of the two tables in our manuscript, we acknowledge that some of the information presented in the table is already discussed in the main text. However, we believe that the tables offer a concise and organized presentation of the data, that can enhance the reader's comprehension and facilitates a clearer overview (clinical setting, aim, main findings). Therefore, we consider the inclusion of these tables to be beneficial, enhancing the accessibility and utility of our manuscript.

Reviewer C
Comment 1: Introduction
The topic of liquid biopsy marker discovery, exploration, and combination that may culminate in the identification of biological patterns/signatures that are sufficiently specific to a disease or disease state is most certainly a hot topic in liquid biopsy research. The review has comprehended most LB markers including CTC, ct-DNA/mRNA, EV, and/or other cancer-associated circulating rare cells. Therefore, it is helpful in answering the question of what marker combinations (and platforms) could denote advancement in the field.
General comments:
(i) The authors do not state a search strategy implying the uselessness of the review to suggest a quality search strategy for the given topic to other investigators and implying that completeness of literature updates cannot be assumed by experts in the field.
(ii) Tables are referenced only in chapter 7.
Reply 1: We are grateful to Reviewer C for the comment on our manuscript and for providing valuable suggestions to improve the manuscript.
We appreciate the reviewer’s attention to the search strategy, however, given the narrative nature of our review and the fact this is not a systematic review nor a metanalysis, we believe that a search strategy is not needed. Our focus is on synthesizing existing literature and analyzing the current state of the field, to offer insights and perspectives on the topic of combination between CTCs and other

circulating biomarker. We have meticulously surveyed relevant literature across various databases and sources, ensuring that our review is comprehensive and encompasses a diverse array of perspectives and findings.
As details on the studies regarding each analyte are presented throughout the text, tables are referenced only in chapter 7 to offer the reader a summary of the data supporting the role of a multianalyte liquid biopsy approach before discussing the challenges associated with it.

Comment 2: The overall first paragraph (and also the abstract) paints a picture of established/routine use of CTCbased LB in breast cancer management. So they state: "Over the course of the past decade, liquid biopsy has gained increased prominence in the management of patients with breast cancer (BC)." ..Its validity extends to prognosis, prediction, and monitoring of treatment response in patients with BC."
→ The authors require to support this assessment by credible referencing stating adoptions of CTCbased liquid biopsy tests into breast cancer guidelines.
Reply 2: This sentence refers to liquid biopsy in general, encompassing various analytes such as ctDNA, and exosomes, not just CTCs. A reference providing an overview of the role of liquid biopsy in the diagnosis, treatment, and monitoring of breast cancer has been added.

Comment 3: The authors state: "Most of the liquid biopsy studies on BC have predominantly focused on the detection of circulating tumor cells (CTCs)."
→ This statement seems to ignore the overweight on studies conducted using cf/ct-DNA liquid biopsy.
Reply 3: We thank Reviewer C for giving us the possibility to clarify this point. We concur that nowadays most of the study conducted on liquid biopsy are including the evaluation of ctDNA. We have accordingly adjusted the text as follows and included a relevant reference.
Changes in the text: Even though nowadays the term liquid biopsy is predominantly associated with the detection of circulating tumor DNA (ctDNA), it was originally coined to describe the presence of circulating tumor cells (CTCs) (3). CTCs have been shown to play a pivotal role in the metastatic process, intravasating into the bloodstream and disseminating at distant sites (4). Many studies in patients with BC have focused on the detection of CTCs and CTCs detected using the Food and Drug Administration (FDA)-approved CellSearch® system have emerged as an independent and strong biomarker for survival in patients with early and metastatic BC (MBC).

Comment 4: Chapter 2; introduction seems irrelevant background knowledge
Reply 4: Each chapter includes an introduction to give the reader a concise overview of the individual liquid biopsy biomarker. Furthermore, as we only discuss studies involving the concomitant evaluation of specific biomarker with CTCs, we aim to provide the reader with references containing evidence on the single analyte that falls beyond the scope of our review

Comment 5: Chapter 2.1; the authors discuss CTC and cf-DNA as variably correlated and even the complementary nature between CTC and cf-DNA. The review could benefit from an explanation. A similar point can be made for all other markers.
Reply 5: In this section, we reported all the studies that included a simultaneous assessment of CTCs and ctDNA. We have explored the potential explanations between certain ctDNA alterations and CTC count. Moreover, we have discussed how the information gained from the simultaneous evaluation of these biomarkers can be integrated into the management of patients with BC. A similar approach has already been adopted for the other biomarkers, even though for other liquid biopsy analytes, the amount of studies addressing the combination with CTCs is much smaller.

Comment 6: Regarding the tumor-derived EVs in large size and size variation, I feel that the review could benefit from more care and detail. The review seems to be indiscriminate for the terms oncosomes and td-EV which may not be related after all. The term oncosomes seems to have been coined by Janus Rak's group in 2008 in investigations with brain tumors and the nature of the now-called td-EVs are basically CellSearch-derived epithelial objects lacking a nucleus formerly disregarded as epithelial debris with unknown nature/function and were initially reported by Coumans in 2010 to possess prognostic value. These objects seemed to be later simply termed extracellular vesicles. Since, the nature of the cell search derived td-EV seems to be based an assumptions, the authors should point out the unknown pathobiology of this marker.
Reply 6: We appreciate the comments of Reviewer B on this topic. Indeed, the field of extracellular vesicles faces challenges with nomenclature, which can lead to confusion. However, some clarity has been offered in a recent perspective published by D. C. I. Goberdhan in the British Journal of Cancer. The author states ‘large EVs (>1000 nm in diameter) are generated by the breaking off of parts of the cell, which can take place during apoptotic cell death. For this reason, the latter are often referred to as apoptotic bodies, though when produced by cancer cells, they are also called large oncosomes, with the
term ‘oncosome’ covering tumour EVs of all size’.
To clarify this point, as suggested by the Reviewer, we have revised the text to specify that tdEVs have not been fully characterized because of the limitations of the isolation methods and the assumption that they are oncosomes is primarily based on the size of these objects. In the revised version of the manuscript, we also emphasize the need to perform downstream analyses to characterize the properties of tdEV.

Comment 7: Confusing statement chapter 4.2 page 16 lines 345
The authors write: “disease progression and death was observed among patients with < 5 CTCs and without CAMLs, patients with < 5 CTCs and with CAMLs, with ≥ 5 CTCs but without CAMLs, or with ≥ 5 CTCs and with CAMLs (PFS: HR =0.84, 3.42 and 4.04 respectively, $P < 0.0001$; OS: HR = 2.66, 6.14, and 9.13, respectively, $P < 0.0001$).
“

→ One would understand 4 possible marker combinations yet giving only 3 values of HR per two
clinical endpoints.
Reply 7: We have rephrased this sentence to make it clearer. There are only 3 HR per PFS and OS because the HR is calculated based on the comparison of "< 5 CTCs and without CAMLs" (i.e. reference group) vs. the other 3 groups: 1) "< 5 CTCs and with CAMLs", 2) "≥ 5 CTCs but without CAMLs", and 3) "≥ 5 CTCs and with CAMLs".

Comment 8: the authors conclude the chapter: "In summary, these studies highlight that beyond canonical CTCs other subpopulations of cells, either tumor or stroma-derived cells, can be detected in the bloodstream and might offer additional insights into the management of patients with MBC."
→ Worth mentioning could be a further outlook of the realization of the holistic approach in liquid
biopsy given by Schreier and Co-workers in 2020 and 2021 coining the term systemic cytology
wherein the circulating rare cell population is analyzed as the ultimate realization of multi-type cellbased liquid biopsy.
Reply 8: We thank the Reviewer for the suggestions. This reference has been added to the manuscript along with a sentence supporting this work (reference 101).

Comment 9: Chapter 6 could benefit from an extra compilation of synergistic effects to support the purpose of this review.
Reply 9: Chapter 6 focuses on studies that have evaluated the association between non-malignant cells and CTCs. Due to the limited availability of data on individual analytes, with often only single papers available, further validation is necessary and it is currently not possible to evaluate their synergistic effect. Despite the limited amount of data on this topic, we believe that this section provides valuable insights for future research, especially given the growing recognition of the tumor microenvironment's critical role.

Comment 10: Conclusion
(i) The conclusion should be more elaborate summarizing the benefit of specific combinations by
naming clear synergistic effects that could guide the design of unified multi-parameter LB detection platforms.
(ii) the authors state: "Although these various analytes seem to be partly related to CTCs, they mostly do not overlap, suggesting different biological processes. Therefore, they should be considered synergistic rather than strictly competitive."
→ the review could really benefit from a deeper analysis/explanations then "different biological
processes" since the essence of the synergistic effect is marker complementation rather than
correlation.
Reply 10: We appreciate Reviewer C's comment. The benefits of specific combinations

of CTCs are detailed in each section. We have rephrased our conclusions to enhance clarity. We have focused on the combination of CTCs with ctDNA, being these the most studied circulating biomarker, highlighting the advantage offered by ctDNA in terms of sensitivity and by CTCs in terms of providing insight into tumor biology (possibility to perform analysis at RNA, protein level and functional studies). Since the synergistic effect of combining multiple analytes has not been fully demonstrated yet, we rephrased our sentence to include the concept of complementarity. In the revised version of the manuscript, we also provide clarification on the term 'biological process'. Below is how the first part of the conclusions has been modified.