nature portfolio

Peer Review File

Assessment of blood-based tumor mutational burden on clinical outcomes in advanced breast and prostate cancer treated with immune checkpoint inhibitors

Corresponding Author: Dr Pedro Barata

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Dear Authors,

Thank you for submitting your manuscript for review. I appreciate the effort and thoroughness demonstrated in your study. Your paper is well-written and addresses a significant gap in our understanding of using tumor mutational burden (TMB) as a biomarker for therapy selection with immune checkpoint inhibitors (ICIs) in "immune-cold" tumors.

Below are my comments and suggestions for revision:

Abstract:
The abstract provides a comprehensive overview of your study, but it could benefit from a clearer statement of the main findings. Consider emphasizing the clinical relevance of bTMB in the context of ICI therapy for advanced breast and prostate cancer.

Methods:
The methodology is detailed and well-explained. However, the reference used for the definition of plasma-based microssatelite status uses only two possible results: MSI-H or MSS, which I recommend to be used in your paper wherever this test result is mentioned. It is stated on the statistical analysis that "blood TMB ≥ 10 but/Mb defined bTMB high", however, in other places of the manuscript, the same term "bTMB high" is used to describe other subpopulations.

Results:
The results section is comprehensive, but consider reviewing some parts. For example: it is not clear what was analyzed on this statement: "There was no association between median bTMB and mPFS (HR 0.99 CI 0.99-1.0; p=0.56)", and adding the definition for the second group used for comparison in the following: "16.5 months [CI 95%, 14-19.1] MSI-H/ MMR defect vs 10.3 months [CI 95%, 0-27.2], p=0.906".
Consider reviewing what is written In sentences like: "In bTMB-10 GATA3 was significantly higher than in patients with a bTMB <10, but there was no significance observed in the bTMB-16 group.", as the groups described refers to patients with TMB equal or higher than the threshold considered, so when describing them as a group you are not including patients with results below the threshold.
It is not clear the comparison performed for the following sentence "CTNNB1 showed no significant difference in both bTMB-10 and bTMB-16.".

Figures and tables are well-presented. However, ensure that all abbreviations used in the tables and figures are defined in the legends for ease of understanding.
Consider reviewing what is described and presented in the figures. For example, in Figure 3A, the legend suggests that the cohort analyzed included only patients with TBM ≥ 10 mut/Mb, however the figure presents the results of both bTMB ≥ and < 10 mut/Mb.

Discussion:
The discussion provides a good interpretation of your findings. It would benefit from a more detailed comparison with previous studies on tTMB and bTMB, highlighting how your results align or differ from existing literature.
Consider discussing how bTMB might be affected by the moment when the analysis is performed, since we might see higher circulating tumor fraction during disease progression and accumulation of mutations as a result of oncologic evolutive

process under treatment selection pressure.

Minor Points:
There are minor typographical errors and inconsistencies in the use of abbreviations and definitions throughout the manuscript. A thorough proofreading would be beneficial.

Overall, your study provides valuable insights into the potential role of bTMB as a biomarker for ICIs in advanced breast and prostate cancers. With the suggested revisions, your manuscript will be even more impactful and informative to the oncology community.

Congratulations.

Reviewer #2

(Remarks to the Author)
This study reports an analysis of 51 patients with high bTMB (defined here as 10/mb or higher). Although this is not stated anywhere, as far as I can understand this was a real world study, retrospective, in 11 different centers. It is not clear how the cohort was identified.
The cohort includes breast and prostate cancer patients, that had a blood NGS test at some point (after 1-9 lines of treatment – very large heterogeneity) and at some time later (no information about additional intervening lines of treatment), got ICI treatment. The ICI received was mostly pembrolizumab, but included also combination ICI and even one patient got an investigational ICI and one got BiTE (presumably also investigational).
Median bTMB was around 16. In 8 pts MSI was identified. These patients had a higher bTMB than the rest and longer PFS but not a longer ICI treatment (or a not-statistically-significant difference – this data is not shown, but the numbers are small).
No correlation was found between bTMB and PFS. No correlation was found between MSI and PFS, despite multiple testing in subgroups with various bTMB cutoffs (although a trend for longer PFS is seen in the k-m graphs (fig 2).
A statistically non-significant correlation was found between MSI and OS (a correlation between bTMB and OS is not mentioned).
All responses (persumably to CPI) were seen in patients with bTMB of 16 or higher.

Additionally this study reports a retrospective analysis of Guardant dataset of 14k breast and prostate Ca patients. 25% had a bTMB 10 or higher, 15% had a bTMB of 16 or higher.
bTMB 10 or higher included almost all of the bMSI-H patients, some of which had a bTMB in the range of 10-16. The genes most commonly mutated in these patients, were more commonly mutated in the patients with high bTMB.

Major comments
Regarding the first part of the study: it is not clear if this is a retrospective real-world study or not. If so, this must be stated clearly. It is not clear how the patients were identified to be included in the cohort. The statistical analysis plan (SAP) is not clear. Lack of a SAP reduces the quality of the study.
Regarding the 2nd part, and the analysis of Guardant dataset: besides providing very basic information about bTMB, there is not much that can be learned from this analysis. Since the definition of high TMB is based on the rate of mutations found, it is not surprising that mutations in the commonly mutated genes are more common in the high TMB group. Considering the multiple testings done, the meaning of finding any different pattern regarding a specific gene or group of patients cannot be too significant.

Discussing bTMB and the cutoffs must acknowledge doi.org/10.1038/s41591-018-0134-3
and doi: 10.1038/s41591-022-01754-x and doi: 10.1038/s41591-022-01933-w.

Minor comments: in the methods section, details are given about numbers of patients identified for specific tests done. This should be removed to “results”.
Response is reported to have been assessed by RECIST method. Since this method is defined and validated only prospective studies, this issue should be clarified.
ICI treatment prior to the guardant test was an exclusion criteria, how come one patient did get ICI treatment prior to the test and was included?
Line 209: is it mentioned that some genes were higher, while it is meant (I believe) that the rate of mutations in these genes was higher. This is a recurring error.
Line 213: “no significance observed in the bTMB 16 or higher group” – I assume this is meant to be “no significant difference was seen comparing the patients with bTMB higher vs lower than 16”. This is a recurring error.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Congratulations to the authors for their excellent conceptualization and development of this manuscript. This article

addresses a crucial and emerging topic within clinical oncology: the use of circulating tumoral biomarkers for prognostic and predictive analysis. The study provides valuable insights into this rapidly evolving area of research, especially in the context of personalizing cancer treatments.

The authors rightly emphasize that while blood-based tests are being clinically implemented, often by extrapolating from tissue-based assays, many of these tests lack robust prospective validation for guiding treatment decisions. This is a critical point, as it underscores the potential risks of applying unvalidated biomarkers in clinical practice, which could lead to unnecessary safety issues for patients and introduce inefficiencies and excess costs into healthcare systems.

Moreover, the focus on "immune-cold" tumors—such as breast and prostate cancers—characterized by low expected tumor mutational burden, highlights the significance of this study. Given that these subpopulations are generally small, the modest patient sample in this research already provides a valuable glimpse into the real-world efficacy of immunotherapy within these contexts. Despite the relatively limited cohort size, the study offers practical insights into the challenges and opportunities in treating these traditionally non-immunogenic tumor types.

The conclusions drawn by the authors are well-supported by their data and reflect the potential and limitations of their findings. As such, it is clear that further research is warranted in this field. Continued efforts to validate circulating biomarkers for predicting the benefit of immunotherapy-based approaches in "immune-cold" tumors are crucial for optimizing treatment strategies and improving patient outcomes.

Once again, congratulations to the authors for contributing to this important area of investigation. I look forward to seeing how future studies build upon this work to better integrate circulating biomarkers into clinical practice.

Daniel Vargas Pivato de Almeida, MD
Assistant Attending
Oncoclinicas&Co
Executive Director for Brazil of Genitourinary Board of Latin American Cooperative Oncology Group

Reviewer #2

(Remarks to the Author)
Evaluation of revised version, 14th Aug 2024

Major comments in the previous review:
- Lack of clarity of the nature of the study being a retrospective analysis – this is still not mentioned in the key points nor in the abstract. It is however mentioned in the methods now.
- Description of the clinical cohort creation is now more detailed but still not clear. : "Given low attrition of patients with ovarian and pancreatic cancers only breast and prostate cancer was included in the final analysis" was that meant to be high attrition? Or low numbers?
- The statistical analysis plan (SAP) is now described.
- The clinical cohort includes breast and prostate cancer patients, that had a blood NGS test at some point and at some time later got ICI treatment. There is still no information about additional intervening lines of treatment between the blood test and the ICI treatment, not even the number of treatment lines.
-

Major comments relating the current version:
- The hypothesis of this study is now stated as (in the abstract) : "We hypothesized that a TMB ≥10 mut/Mb as detected in blood (bTMB) is being used as equivalent to TMB in tissue (tTMB) to offer ICI in the cases where tTMB is not available." The study is not designed at all to prove or refute this hypothesis.
- Methods (abstract): "The Guardant Health genomic database (GHGD) was then queried for patients with breast (N=7899) or prostate (N=6093) cancers who had a bTMB of ≥10 and 16 mut/Mb, identified by ctDNA NGS (N=13,992) for associations of bTMB with genomic alterations." From this sentence it can be understand that only highTMB were analyzed, but in the methods section now: "The Guardant Health genomic database was queried (2020-2023) for patients with breast or prostate cancer who had ctDNA NGS (Total n=13,992; Guardant360, Redwood City, CA) performed as part of clinical care". Which is correct?
- The retrospective analysis of Guardant dataset of 14k breast and prostate Ca patients is presented in figure 3. There is unclarity about what is presented in figures 3b and 3d. It is stated that the patients are stratified by MSI status, but the titles of these figures relates to the bTMB cutoffs of 10 and 16. The corresponding figures 3a and 3d have the same titles; they include patients both above and below this cutoff. It is not clear if figures 3b and 3d also include both the high and low bTMB patients, stratified by MSI status (as the corresponding fig 3a and 3c) or only the high bTMB stratified by MSI status (as can be implied from the title of these figures).
- Results (abstract): "High bMSI was associated with higher bTMB (correlation test, $r=0.66$, $p< 0.001$) in all responses except one." This sentence is not clear. The association between two parameters is a statistical measure, mentioning here that (what I think is meant) one of the responders did not have both high bMSI and high bTMB is not relevant here and is very confusing to the reader.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
Thank you for replying to most of my comments. However, a few issues still remain open.
*"The median time between G360 test and initiation of ICI was 81 (0- days). "
This needs to be completed (range?)

*" Authors: Thank you for the opportunity to clarify. We have made edits to clarify inclusion criteria as follows: We included all patients with breast and prostate cancer who had an NGS test as SOC that included assessing for a TMB score. We then looked at TMB-H cut-offs of ≥10 and ≥16 mut/Mb within this group."
Thank you for this corrections. However, this issue is not described accordingly in the abstract. Also in the methods (bottom of page 5): "Per protocol, patients with metastatic breast (excluded triple negative), ovarian, pancreatic, and prostate cancer who received treatment with an ICI following a bTMB 10 mutations per megabase (mut/Mb) detected by Guardant360, were eligible to be included in this study.

Dear Editorial Team,

Thank you for the opportunity to publish this manuscript in the journal. We thank the editorial team for their recommendations, and please find below our answer to the reviewers' comments and edits. Thank you very much.

Reviewer 1:

1. Abstract: The abstract provides a comprehensive overview of your study, but it could benefit from a clearer statement of the main findings. Consider emphasizing the clinical relevance of bTMB in the context of ICI therapy for advanced breast and prostate cancer.

Authors: Thank you for this comment. We have added the following language in the conclusion section to further clarify our results: "While bTMB can be informative for patient care, this data shows it is likely not independently beneficial for predicting response to ICI therapy in breast and prostate cancers."

2. Methods: The methodology is detailed and well-explained. However, the reference used for the definition of plasma-based microsatellite status uses only two possible results: MSI-H or MSS, which I recommend to be used in your paper wherever this test result is mentioned. It is stated on the statistical analysis that "blood TMB ≥ 10 but/Mb defined bTMB high", however, in other places of the manuscript, the same term "bTMB high" is used to describe other subpopulations.

Authors: We appreciate this feedback. For language identifying MSI results, we have changed the abbreviations in the manuscript to bMSI-H (blood MSI-H) and bMSI-Not Detected consistently throughout the document. Due to liquid biopsy assays being unable to definitively call a result MSS for biological reasons, such as the potential for lower ctDNA shed to impact MSI detection, we chose to use bMSI-Not Detected to accurately represent the results (this is also how MSI results are reported on the clinical reports).
We have also clarified the terminology for bTMB-H throughout the manuscript, so thank you for your thoughts here.

3. Results: The results section is comprehensive, but consider reviewing some parts. For example: it is not clear what was analyzed on this statement: "There was no association between median bTMB and mPFS (HR 0.99 CI 0.99-1.0; p=0.56)" and adding the definition for the second group used for comparison in the following: "16.5 months [CI 95%, 14-19.1] MSI-H/ MMR defect vs 10.3 months [CI 95%, 0-27.2], p=0.906".
Consider reviewing what is written in sentences like: "In bTMB-10 GATA3 was significantly higher than in patients with a bTMB <10, but there was no significance observed in the bTMB-16 group.", as the groups described refers to patients with TMB equal or higher than the threshold considered, so when describing them as a group you are not including patients with

results below the threshold. It is not clear the comparison performed for the following sentence "CTNNB1 showed no significant difference in both bTMB-10 and bTMB-16.".

Authors: Thank you for your feedback regarding needing clarification about comparator groups. We have rectified this throughout the manuscript.

4. Figures and tables are well-presented. However, ensure that all abbreviations used in the tables and figures are defined in the legends for ease of understanding. Consider reviewing what is described and presented in the figures. For example, in Figure 3A, the legend suggests that the cohort analyzed included only patients with TBM ≥ 10 mut/Mb, however the figure presents the results of both bTMB ≥ and < 10 mut/Mb.

Authors: Thank you very much, we have updated the figure legends and abbreviations where appropriate.

5. Discussion: The discussion provides a good interpretation of your findings. It would benefit from a more detailed comparison with previous studies on tTMB and bTMB, highlighting how your results align or differ from existing literature. Consider discussing how bTMB might be affected by the moment when the analysis is performed, since we might see higher circulating tumor fraction during disease progression and accumulation of mutations as a result of oncologic evolutive process under treatment selection pressure.

Authors:
We have added language explaining limitations of how blood collection timing may impact bTMB scores and added reference (Reference 22): “Limitations of our study include the retrospective design and the inclusion of a heavily pretreated population which might have impacted bTMB results by inducing additional mutations. The timing of blood collection can also be a limitation: if drawn too soon after a patient receives therapy, this can suppress ctDNA shed and thus impact bTMB results. Similarly, if drawn during disease progression, bTMB is more likely to be observed at higher levels.”

6. Minor Points: There are minor typographical errors and inconsistencies in the use of abbreviations and definitions throughout the manuscript. A thorough proofreading would be beneficial.

Authors: Thank you, we have thoroughly proofread and corrected errors in the document.

Reviewer 2:
Major:
1. Regarding the first part of the study: it is not clear if this is a retrospective real-world study or not. If so, this must be stated clearly. It is not clear how the patients were identified to be included in the cohort. The statistical analysis plan (SAP) is not clear. Lack of a SAP reduces the quality of the study

Authors: Thank you very much for your critique here, which we know will help us create a more impactful manuscript. We have clarified that this is a retrospective real-world study and further detailed how patients were identified for this study. We have also updated our statistical plan.

2. Regarding the 2nd part, and the analysis of Guardant dataset: besides providing very basic information about bTMB, there is not much that can be learned from this analysis. Since the definition of high TMB is based on the rate of mutations found, it is not surprising that mutations in the commonly mutated genes are more common in the high TMB group. Considering the multiple testings done, the meaning of finding any different pattern regarding a specific gene or group of patients cannot be too significant.

Authors: Thank you for your feedback related to the genomic analyses. We have added some stronger language explaining that the increase in genomic alterations is expected in bTMB-H patients: "This is not unexpected, as an increased TMB score confers an increase in genomic alterations." This is in addition to the paragraph reviewing this in the discussion.

Minor:

1. In the methods section, details are given about numbers of patients identified for specific tests done. This should be removed to "results".

Authors: Thank you, we have moved these numbers to the results where appropriate.

2. Response is reported to have been assessed by RECIST method. Since this method is defined and validated only in prospective studies, this issue should be clarified.

Authors: Thank you. All investigators are cancer trialists and experienced in performing imaging evaluation using RECIST which is a validated criteria to document tumor response. RECIST is in fact used similarly in prospective and retrospective analysis since it defines complete, partial response, stable and progressive disease.

3. ICI treatment prior to the guardant test was an exclusion criterion, how come one patient did get ICI treatment prior to the test and was included?

Authors: Thank you for the opportunity to clarify this patient's eligibility. Indeed, one patient that was exposed to IO prior to Guardant360 also received IO treatment following a bTMB ≥10 mutations per megabase (mut/Mb), meeting eligibility criteria: "patients who received treatment with an ICI following a bTMB ≥10 mutations per megabase (mut/Mb) detected by Guardant360 are eligible."

4. Line 209: is it mentioned that some genes were higher, while it is meant (I believe) that the rate of mutations in these genes was higher. This is a recurring error.
Line 213: "no significance observed in the bTMB 16 or higher group" – I assume this is meant to be "no significant difference was seen comparing the patients with bTMB higher vs lower than 16". This is a recurring error.

Authors: We appreciate your valuable feedback here. We have corrected the language surrounding an increase in alteration rates throughout the manuscript. Thank you for suggesting this improvement and we agree with the reviewer that the manuscript reads more clearly now. Similarly, we have also corrected the discrepancy of the comparator groups (bTMB-H vs bTMB-L) throughout the manuscript.

Thank you,

Pedro C. Barata, MD, MSc, FACP
Miggo Family Chair in Cancer Research
Co-Leader Genitourinary (GU) Disease Team
Director of GU Medical Oncology Research Program
University Hospitals Seidman Cancer Center
Associate Professor of Medicine
Case Western Reserve University
Case Comprehensive Cancer Center

Dear Editorial Team,

Thank you for the opportunity to publish this manuscript in the journal. We thank the editorial team for their recommendations. We have worked on our manuscript and please find below our answer to the reviewers' comments point by points and edits made in the manuscript specifically from reviewer 2. We believe we have address them all successfully. Thank you again.

Reviewer 1:

1. Congratulations to the authors for their excellent conceptualization and development of this manuscript. This article addresses a crucial and emerging topic within clinical oncology: the use of circulating tumoral biomarkers for prognostic and predictive analysis. The study provides valuable insights into this rapidly evolving area of research, especially in the context of personalizing cancer treatments.

The authors rightly emphasize that while blood-based tests are being clinically implemented, often by extrapolating from tissue-based assays, many of these tests lack robust prospective validation for guiding treatment decisions. This is a critical point, as it underscores the potential risks of applying unvalidated biomarkers in clinical practice, which could lead to unnecessary safety issues for patients and introduce inefficiencies and excess costs into healthcare systems.

Moreover, the focus on "immune-cold" tumors—such as breast and prostate cancers—characterized by low expected tumor mutational burden, highlights the significance of this study. Given that these subpopulations are generally small, the modest patient sample in this research already provides a valuable glimpse into the real-world efficacy of immunotherapy within these contexts. Despite the relatively limited cohort size, the study offers practical insights into the challenges and opportunities in treating these traditionally non-immunogenic tumor types.

The conclusions drawn by the authors are well-supported by their data and reflect the potential and limitations of their findings. As such, it is clear that further research is warranted in this field. Continued efforts to validate circulating biomarkers for predicting the benefit of immunotherapy-based approaches in "immune-cold" tumors are crucial for optimizing treatment strategies and improving patient outcomes.

Once again, congratulations to the authors for contributing to this important area of investigation. I look forward to seeing how future studies build upon this work to better integrate circulating biomarkers into clinical practice.

Authors: Thank you for your kind words and the time you took to review our manuscript. We appreciate your insights in this process and are glad you saw a similar need in this field. We agree that further research is needed in this area and hope larger studies will be designed in the future to answer lingering questions!

Reviewer 2:

1. Lack of clarity of the nature of the study being a retrospective analysis – this is still not mentioned in the key points nor in the abstract. It is however mentioned in the methods now.

Authors: Thank you for your clarification; we have added this language to the abstract and key points as well.

2. Description of the clinical cohort creation is now more detailed but still not clear: "Given low attrition of patients with ovarian and pancreatic cancers only breast and prostate cancer was included in the final analysis" was that meant to be high attrition? Or low numbers?

Authors: Thank you for pointing out this and the opportunity to clarify in the manuscript. We have clarified the methods section to justify that 1) ovarian and pancreatic cancers were eligible to be included according to approved protocol and 2) the very low number of patients with ovarian and pancreatic cancers identified among participating institutions (n=1 pancreatic; n=0 ovarian carcinoma), we clarified the inclusion criteria of final cohort.

3. The clinical cohort includes breast and prostate cancer patients, that had a blood NGS test at some point and at some time later got ICI treatment. There is still no information about additional intervening lines of treatment between the blood test and the ICI treatment, not even the number of treatment lines.

Authors: This reviewer raises a very good question around the number and type of systemic therapies between G360 test and initiation of ICI treatment. We have added language to provide that information as follows: "The median time between G360 test and initiation of ICI was 81 (0- days). Less than one third of patients (n=15) received any systemic therapy between G360 test and ICI and included chemotherapy [taxanes n=9, platinum n=2, capecitabine n=2), novel hormonal therapies [enzalutamide n=5, abiraterone acetate n=1, exemestane n=1], mTOR inhibitor (n=1) and sipuleucel-T (n=1)."

4. The hypothesis of this study is stated as (in the abstract): "We hypothesized that a TMB ≥10 mut/Mb as detected in blood (bTMB) is being used as equivalent to TMB in tissue (tTMB) to offer ICI in the cases where tTMB is not available." The study is not designed at all to prove or refute this hypothesis.

Authors: Thank you for your feedback here and the opportunity to clarify. This study was designed to confirm or refute previous observations that TMB results in liquid biopsy were being used frequently in clinical practice to consider ICI in patients with advanced solid tumors where ICI is not normally considered. This is stated in the abstract "We hypothesized that TMB score as detected in blood (bTMB) is being used in clinical practice to offer ICI to patients with advanced cancers in the cases where tTMB is not available. We further hypothesized that bTMB ≥10 mut/Mb is not a strong predictor of response to ICI." Indeed, we were able to

investigate the activity of ICI in cases of bTMB > 10 which was one of the objectives of the study, as stated in the abstract (Objective). While we take the findings of this study as not definitive, we identified a relatively small cohort of < 50 patients (among a pool of 596 patients) which might refute our hypothesis. In fact, the practice of offering an ICI in these tumor types, solely based on bTMB, appears infrequent in academic practices. We describe and discuss these findings in paragraphs 2-3 of the Discussion. We made edits to the text (abstract and key points also) to address and clarify the hypothesis and findings.

5. Methods (abstract): "The Guardant Health genomic database (GHGD) was then queried for patients with breast (N=7899) or prostate (N=6093) cancers who had a bTMB of ≥10 and 16 mut/Mb, identified by ctDNA NGS (N=13,992) for associations of bTMB with genomic alterations." From this sentence it can be understand that only highTMB were analyzed, but in the methods section now: "The Guardant Health genomic database was queried (2020-2023) for patients with breast or prostate cancer who had ctDNA NGS (Total n=13,992; Guardant360, Redwood City, CA) performed as part of clinical care". Which is correct?

Authors: Thank you for the opportunity to clarify. We have made edits to clarify inclusion criteria as follows: We included all patients with breast and prostate cancer who had an NGS test as SOC that included assessing for a TMB score. We then looked at TMB-H cut-offs of ≥10 and ≥16 mut/Mb within this group.

6. The retrospective analysis of Guardant dataset of 14k breast and prostate Ca patients is presented in figure 3. There is unclarity about what is presented in figures 3b and 3d. It is stated that the patients are stratified by MSI status, but the titles of these figures relates to the bTMB cutoffs of 10 and 16. The corresponding figures 3a and 3d have the same titles; they include patients both above and below this cutoff. It is not clear if figures 3b and 3d also include both the high and low bTMB patients, stratified by MSI status (as the corresponding fig 3a and 3c) or only the high bTMB stratified by MSI status (as can be implied from the title of these figures).

Authors: We fully agree with this reviewer. To clarify, figures 3b and 3d are looking at TMB-H patients only (with the noted cut-off) and then stratifying by MSI status. We have updated language in the figure legend as well as updated the figure title to make it clear we are only looking at TMB-H patients. Figures 3a and 3c are looking at all TMB patients and comparing TMB-H and TMB-L groups, as described in the title/legend of figures.

7. Results (abstract): "High bMSI was associated with higher bTMB (correlation test, r=0.66, p< 0.001) in all responses except one." This sentence is not clear. The association between two parameters is a statistical measure, mentioning here that (what I think is meant) one of the responders did not have both high bMSI and high bTMB is not relevant here and is very confusing to the reader.

Authors: Thank you, we agree that this is confusing and appreciate you pointing it out. We have made changes and now reads: High bMSI was associated with higher bTMB (correlation test, r=0.66, p< 0.001).

We would like to thank Reviewer 1 and 2 for their time, and we appreciate such a detailed inspection and critique of our manuscript. This feedback has undoubtedly made our manuscript better and clearer for the reader.

Thank you,

Pedro C. Barata, MD, MSc, FACP
Miggo Family Chair in Cancer Research
Co-Leader Genitourinary (GU) Disease Team
Director of GU Medical Oncology Research Program
University Hospitals Seidman Cancer Center
Associate Professor of Medicine
Case Western Reserve University
Case Comprehensive Cancer Center

Dear Editorial Team,

Thank you for the opportunity to publish this manuscript in the journal. We thank the editorial team for their recommendations. We have worked on our manuscript and please find below our answer to the reviewers' comments point by points and edits made in the manuscript specifically from reviewer 2. We believe we have address them all successfully. Thank you again.

Reviewer 2:

1. "The median time between G360 test and initiation of ICI was 35 (0- days)." This needs to be completed (range?).

Authors: Thank you for catching this typo! The average time was 81 days and the median time was 35 days (0-514 days). We have added this information to the text.

2.1 - Original reviewer comment: Methods (abstract): "The Guardant Health genomic database (GHGD) was then queried for patients with breast (N=7899) or prostate (N=6093) cancers who had a bTMB of ≥10 and 16 mut/Mb, identified by ctDNA NGS (N=13,992) for associations of bTMB with genomic alterations." From this sentence it can be understand that only highTMB were analyzed, but in the methods section now: "The Guardant Health genomic database was queried (2020-2023) for patients with breast or prostate cancer who had ctDNA NGS (Total n=13,992; Guardant360, Redwood City, CA) performed as part of clinical care". Which is correct?

Authors: Thank you for the opportunity to clarify. First, we included all patients with breast and prostate cancer who had an NGS test as SOC that included assessing for a TMB score (n=13,992) included in The Guardant Health genomic database (GHGD). We then looked at TMB-H cut-offs of ≥10 and ≥16 mut/Mb within this group. We have made edits to clarify this inclusion criteria.

2.2 - Reviewer follow-up comment: Thank you for these corrections. However, this issue is not described accordingly in the abstract. Also, in the methods (bottom of page 5): "Per protocol, patients with metastatic breast (excluded triple negative), ovarian, pancreatic, and prostate cancer who received treatment with an ICI following a bTMB ≥10 mutations per megabase (mut/Mb) detected by Guardant360, were eligible to be included in this study.

Authors: In this project, we included both a clinical cohort (n=48) and a large genomic database (n=13,992). Our response above to 2.1 focus on the criteria for the Guardant Health database query (which was breast and prostate cancers only, to align with the final clinical cohort). The most recent reviewer comment 2.2 is addressing the clinical cohort, for which the protocol included breast, ovarian, pancreatic, and prostate cancers. Given the low

numbers of patients with pancreatic and ovarian tumors (n=1 pancreatic cancer; n=0 ovarian cancer), the final clinical cohort included included only patients with breast or prostate cancers. We have adjusted the methods section (see excerpt below) to hopefully better clarify this clinical cohort inclusion criteria. Figure 1 also gives a consort diagram to better explain the flow of how patients were included/excluded in this cohort. We have adjusted language in the abstract to better coincide with this as well.

"This retrospective, real-world study was IRB-approved at University Hospitals (STUDY20230043) and local IRBs at all eleven participating sites. Per protocol, patients with metastatic breast (excluded triple negative) ovarian, pancreatic, and prostate cancer who received treatment with an ICI following an NGS result with Guardant360 that included a bTMB score, were eligible to be included in this study. However, given the very low attrition of patients with ovarian and pancreatic cancers identified among the participating institutions (n=1 pancreatic cancer; n=0 ovarian cancer), only breast and prostate cancer was included in the final analysis. Different bTMB cut-offs were assessed, including ≥10 mut/Mb and ≥16 mut/Mb, to determine if implementing a higher bTMB-H benchmark would improve survival outcomes."
"The Guardant Health genomic database was then queried (2020-2023) for patients with breast or prostate cancer who had ctDNA NGS, which included evaluation for a TMB score (Total n=13,992; Guardant360, Redwood City, CA), performed as part of clinical care."

Thank you,

Pedro C. Barata, MD, MSc, FACP
Miggo Family Chair in Cancer Research
Co-Leader Genitourinary (GU) Disease Team
Director of GU Medical Oncology Research Program
University Hospitals Seidman Cancer Center
Associate Professor of Medicine
Case Western Reserve University
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