Peer Review File

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Reviewer Comments

Reviewer A

Comment 1: "The discussion section, particularly the part discussing the potential role of TILs in guiding therapy choices, could be expanded further. This section could delve into more details about ongoing clinical trials, their designs, and their implications for personalized treatment strategies. The article dedicates a substantial portion to the prognostic role of TILs, while the predictive role is comparatively shorter. To provide a more balanced perspective, the predictive role could be given more in-depth coverage. To further enrich the discussion, consider integrating the findings from the following study: Dieci MV, Frassoldati A, Generali D, et al. Tumor-infiltrating lymphocytes and molecular response after neoadjuvant therapy for HR+/HER2- breast cancer: results from two prospective trials. Breast Cancer Res Treat. 2017 Jun;163(2):295-302. doi: 10.1007/s10549-017-4191-y. Epub 2017 Mar 13. Erratum in: Breast Cancer Res Treat. 2017 Jun;163(3):637. PMID: 28289852. This study focused on the relationship between TILs and molecular response following neoadjuvant therapy in HR+/HER2- breast cancer patients. By incorporating these findings into your review article, you can enhance the discussion around the specific impacts of TILs in different breast cancer subtypes and their relevance in guiding treatment strategies. This integration would provide a more comprehensive perspective on the topic, demonstrating the broader significance of TILs in various breast cancer contexts.

Reply 1: We appreciate the comments and suggestion to clarify the points above mentioned, however, we have not considered the suggested reference because it deals with hormone receptor-positive disease, which is not the focus of our review. Expanding the focus to other subtypes would compromise our ability to address specific information on TNBC, arguably the subtype of BC that has more information and seems to be more responsive to ICI. We feel that one of the strengths of our work is exactly concentrating on the specific subtype of TN tumors.

Reviewer B

Comment 1: Some terms, like estrogen receptor (ER), progesterone receptor (PgR), and Human Epidermal Growth Factor Receptor-type 2 (HER-2), are introduced with acronyms. It is essential to maintain consistency in acronym use once they are introduced to avoid confusion.

Reply 1: Thank you for this comment, we reviewed all acronyms and reorganized them in a standardized way throughout the text.

Comment 2: In the section "Current status of systemic therapy in TNBC": The authors reported the pCR and EFS results from the first interim analysis. Yet, it is crucial to note that by the third interim analysis, the pCR benefit had decreased to a 7.5% difference (lines 134-135)

Reply 2: Thank you for calling attention to this important fact. We updated this information and it currently reads: "The pCR rate (63% vs. 55.6%, p=0.0005) and event-free survival at 3 years (84.5% vs. 76.8%; HR: 0.63; p=0.0003)"

Comment 3: The authors provide a well-structured overview of immunotherapy in early TNBC. However, there are several unresolved questions regarding the application of immunotherapy in TNBC. For a comprehensive understanding, kindly refer to some of the reviews on this subject, which provide in-depth insights (1. Jacobs et al, Hope and Hype around Immunotherapy in Triple-Negative Breast Cancer, Cancers 2023; 2. Agostinetto et al, Progress and pitfalls in the use of immunotherapy for patients with triple-negative breast cancer. Expert Opin. Investig. Drugs 2022; Agostinetto et al, Nat Rev Clin Oncol. 2022).

Reply 3: We appreciate the comment and the suggested references. We discuss these unresolved questions in this latest version. The following excerpts were added to the text.

"Despite a rough start with several phase III trials failing to meet key survival endpoints (25–27) and withdrawn of initially approved agents (Atezolizumab), immune checkpoint inhibitors have been incorporated in the treatment of TNBC. Although initially evaluated in the metastatic setting, early-stage disease represents a promising scenario for the adoption of these agents, since tumor burden is limited and the tumor microenvironment is less impacted by previous systemic treatments."

"The chemotherapy backbone consisted of weekly paclitaxel plus carboplatin followed by anthracycline plus cyclophosphamide every 3 weeks. After surgery, patients continued on adjuvant pembrolizumab or placebo for up to 9 cycles.".

"These results, have established the KEYNOTE-522 regimen as the standard of care for patients with stage II and III eTNBC (30)."

"However, some caveats and difficulties remain regarding the potential toxicity and the selection of patients who benefit from the addition of PD1-blockade (31). The unique side-effect profile of immunotherapeutic agents is particularly relevant for patients with curable disease. In KEYNOTE-522, almost 13% of patients in the pembrolizumab arm experienced grade 3–5 immune-related adverse events (irAEs), versus only 1% in the placebo arm (29). Recommendations for a standardized approach to evaluate and treat irAEs have been published and patients should be monitored closely for these events (32).

Importantly, the prognosis of patients who achieve a pCR is highly favorable whether or not they receive immunotherapy (3-year EFS: 92.5% in the control arm vs 94.4% in the pembrolizumab arm). Although this analysis was exploratory and not powered to make a definitive conclusion, it questions whether adjuvant pembrolizumab adds additional benefits post-pCR (33). The toxicity of adjuvant pembrolizumab was not negligible, with a 6.3% of high-grade irAEs. The OptimICE-PCR study (NCT 05812807), is an ongoing clinical trial, that will address the continuation of adjuvant Pembrolizumab in patients with pCR. Until the results of this trial are available, a shared decision process should be used to determine whether to continue adjuvant pembrolizumab post-pCR in an individual patient (34).
Patients with residual disease had 3-year EFS rates of 56.8% and 67.4% in the control and experimental arms, respectively. In this setting, there is no room for treatment de-esclation and adjuvant pembrolizumab should be prescribed if no contraindication exists. Furthermore, other adjuvant therapies must be considered to improve the outcomes in these patients”

Comment 4: The section “Definition of TILs and standard evaluation”, offers a comprehensive explanation of TILs and their significance in TNBC. However, slight simplification might enhance readability and understanding for better clarity. Furthermore, it is crucial to note that not all cited studies followed the WP-defined evaluation criteria for TILs, potentially leading to biases in interpretation, especially in older publications. This distinction should be emphasized when relevant.

Reply 4: The paragraph has been rewritten for greater clarity of information. In the “Prevalence of TILs in early TNBC” section, the phrase " Not all studies cited follow the evaluation rules defined by the TILs working group, which hampers comparative analyses. Despite this, the studies agree regarding the prevalence of sTILs in the studied populations" has been added.

Comment 5: In the section “TILs as a prognostic biomarker in eTNBC” the author does an excellent job of citing various studies to support evidence. However, there are some redundancies when discussing the benefits of high TIL levels in TNBC. Consider merging similar points to enhance readability. Moreover, it would be beneficial also to discuss TILs' dynamic prognostic role, emphasizing how they evolve during neoadjuvant therapy, leading to uncertainties about the baseline value observed. For this point, kindly consider referencing the Neotrip trial from ESMO 2022 by Bianchini et al.
Reply 5: Thank you for pointing this out. We considered the suggested reference, and the followed sentences were added to the section.
“It is important to highlight the dynamic characteristic of TILs density during the

evolution of the disease. The cellular population in the TME is impacted by systemic treatment. An increase in TILs during neoadjuvant treatment appears to be associated with better outcomes in TNBC. The survival benefit of higher levels of infiltration was demonstrated in a meta-analysis that analyzed studies that performed paired analyses of TILs density before and after NACT (64). The NeoTRIP study also demonstrated an increase in TILs after 1 cycle of neoadjuvant systemic therapy (65)."
"Importantly, better definition of the international standards for assessment of TILs in residual disease and in surgical specimens with pCR is needed to validate the potential role of dynamic changes in TILs after neoadjuvant therapy."
"Although some studies were carried out prior to the standardization of TILs assessment, the positive correlation between TILs and better prognosis has been consistent."

Comment 6: In the section "TILs as a predictive biomarker in eTNBC", it is crucial to emphasize that the predictive role of TILs has not been rigorously assessed in dedicated prospective studies. As a result, many of the present assertions remain only speculative. This aspect should be accentuated when discussing the clinical utility of TILs as predictive markers. Please consider citing the paper by D Hayes, JCO 2021 which delves into defining the clinical utility of tumor biomarkers.

Reply 6: Thank you again for this critical aspect in the interpretation of the available data. We considered the suggested reference, and the sentence "we recognize that the current data are mostly based on retrospective exploratory analyses and do not meet the criteria for clinical utility, defined by Hayes, et al, which consider the analytical validity of the test, the significance of related results, and the magnitude of impact, in addition to the level of evidence that determines the applicability of the test (92)." was added to the section.

Comment 7: In the "Future perspective" paragraph, please consider exploring deeper the potential difficulties in translating the knowledge of TILs into clinical practice or the challenges in using them as reliable biomarkers. Authors should also consider dedicating proper emphasis and citations to cutting-edge aspects like Spatial Transcriptomics and other spatial immunophenotyping techniques, such as multiplex immunofluorescence. These methods may offer enhanced capacities for interpreting immune-engagement. In this context, please refer to the work by D. Hammerl, Nature Communications 2021 for a more comprehensive understanding.
Reply 7: We appreciate your comments and the suggested reference. We discuss these questions through additional reference.
"Translating knowledge of TILs into clinical practice and their use as an effective and reliable biomarker faces several difficulties related to tumor heterogeneity, dynamic variability of TILs, data interpretation, different assessment techniques and the

complexity of the TME. In addition to the proposal of the TILs working group, new technologies, such as automated methods of immunoflorescence image analysis, next-generation sequencing (NGS) and the use of transcriptomic data, can also contribute to a greater understanding and precision in the quantification of TILs and potentially improve clinical applicability. Among other references, a transcriptomic signature was correlated with TILs assessed by histology in a cohort of patients with early breast cancer. The declared signature was found to be a good biomarker associated with DFS and OS in an analysis adjusted for molecular and clinical variables, with better survival in basal and HER2 tumor types (93). The use of multiplexed immunofluorescent imaging and next-generation sequencing (NGS) that can determine the spatial distribution of specific immunophenotypes has also showed an impact on TNBC. A study evaluated the development and validation of a gene classifier for spatial immunophenotype and found positive results with response to checkpoint inhibitor (anti-PD1) treatment independently of currently used clinical markers (94).”

Comment 8: In the “Conclusion”, I would suggest summarizing the primary findings on TILs in early breast cancer, emphasizing their clinical relevance. Authors should avoid any repetitive information to maintain clarity and focus on the manuscript's core objectives. The subject of this review is of utmost importance, providing a comprehensive and insightful dive into the relevance of TILs in early-stage TNBC. The manuscript requires minor revisions for better clarity and completeness.

Reply 8: In the “Conclusion” section, the paragraph “The expression of TILs in early TNBC has shown clinical relevance in several studies that are consistent in showing the association of higher density of TILs and favorable outcomes in terms of both survival and response to neoadjuvant treatment. This impact supports the development of studies that evaluate the expression of TILs as stratification factors or the conduct of studies that can evaluate the usefulness and clinical validation of the biomarker.” was added to the text to summarize the findings, and some sentences were omitted to avoid redundant information