Author’s response to reviews

Title: Immunostaining with D2-40 improves evaluation of lymphovascular invasion, but may not predict sentinel lymph node status in early breast cancer

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Author’s response to reviews: see over
To Professor Melissa Norton  
*Editor-in-Chief of BMC Cancer*

Ref.: Submission of the revised manuscript “**IMMUNOSTAINING WITH D2-40 IMPROVES EVALUATION OF LYMPHOVASCULAR INVASION, BUT MAY NOT PREDICT SENTINEL LYMPH NODE STATUS IN EARLY BREAST CANCER**”

Dear Sir,

On behalf of all authors, I submit the revised manuscript “**IMMUNOSTAINING WITH D2-40 IMPROVES EVALUATION OF LYMPHOVASCULAR INVASION, BUT MAY NOT PREDICT SENTINEL LYMPH NODE STATUS IN EARLY BREAST CANCER**” for publication in *BMC Cancer*.

We are grateful for the attention given to the manuscript and for the constructive suggestions made by the reviewers. We think that they have contributed to the improvement of the manuscript and hope that we achieved the expectations reflected by their comments.

All authors declare that they have participated enough in the preparation of this manuscript, in order to take public responsibility for it. They also declare that no substantial part of this manuscript has been published elsewhere, nor is under evaluation in any other scientific journal. In case of acceptance, the authors entirely agree with the copyright transfer policy of the Editors of this Journal.

The present report has been approved by the Ethics Committee of our institution.

Sincerely yours,

José Vassallo
IMMUNOSTAINING WITH D2-40 IMPROVES EVALUATION OF LYMPHOVASCULAR INVASION, BUT MAY NOT PREDICT SENTINEL LYMPH NODE STATUS IN EARLY BREAST CANCER (Britto AV et al)

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RESPONSES TO THE REVIEWERS’ COMMENTS

REVIEWER # 1

1- About the term “(lympho-)vascular invasion”...
RESPONSE: The authors also agree that, for the detection of vascular invasion on H&E stained sections, the best terminology is “vascular invasion”. We did not mean that distinction between blood- and lymph-vessels, and even artifacts, is easily done on routine sections. We included this modification, in order to make it clear that both methods, H&E and D2-40, may deal with somewhat different structures.

2. “… maybe the enhanced detection of LI is more harmful than useful and could shift patients to higher risk groups without substantial evidence…”
RESPONSE: We fully agree with this concern on the basis of our findings, and had already discussed the issue very briefly in the original manuscript. In the revised manuscript, the issue was discussed more clearly, and justifies the need of D2-40, not to alter the clinical procedures for the time being, but to allow that larger numbers of cases are evaluated worldwide, until this technique is included in the risk categories. The analogy with the use to cytokeratin in the detection of minimal infiltration of SLN is appropriate, as it is not yet being considered to change patients’ management in most centers.

3. “Abbreviation of “sentinel lymph node as SN or SLN instead of SL…””
RESPONSE: In the revised manuscript, the abbreviation SLN was adopted.

4. “In the methods there is no need to start with 126 patients; just start with the 92 patients with invasive breast cancer”…
RESPONSE: In the revised manuscript, we started with the description of the 92 patients.

5. Clarify “clinicopathological stage”
RESPONSE: In the revised manuscript, we state that staging for T and N is pathological and for M, clinical, that is, pT pN cM.

6. “Detail the method of tracing SLN and the pathological processing”…
RESPONSE: The method of intraoperative detection of SLN was described, as well as the pathological procedures (see Methods).

7. “… only nodal foci greater than 2mm (i.e., greater than micrometastasis) were considered positive. Mistyping? Should it be 0.2mm?”
RESPONSE: For the purpose of the present study, only macrometastasis (≥ 2mm) was considered. We did not want to include in the same category patients with more established risk with others, in which the clinical value is not yet well established (see also Schwartz et al, 2002, Hum Pathol 33:579-589; Lyman et al, 2005, J Clin Oncol 23:7703-7720, and Motomura et al, 2007, Breast Cancer 14:25-20). The value of macrometastasis (but not micrometastasis) had already been found in other studies: Viale et al (2005; ref # 36 of the revised manuscript) and Marinho et al (2008; ref # 17 of the revised manuscript). As there is no solid information concerning the value of LVI in predicting micrometastasis, we stated clearly in the revised text and “abstract”, that we are just considering “lack of correlation between LVI and SLN macrometastasis”.

8. “… data on the number of slides stained… If multiple slides were stained, provide median or mean and SD…”
RESPONSE: As stated in the revised manuscript, all immunostainings were performed in two levels of the paraffin blocks. The final figures represent the simple mean of both counts, reason for what no SD was presented.

9. “… prognosis (e. g., disease free or overall survival) was not directly assessed.”
RESPONSE: In the revised manuscript, no mention to a possible prognostic value of our findings is present.

10. “I would strongly recommend reducing the manuscript to describe only findings related to MVD, LVI, VEGF and SLN status”
RESPONSE: Only the results above were included. However, as some details were added, and the reduction of the “Discussion” section would leave some important points without enough explanation, the manuscript persists with around 3000 words. The paragraphs were rearranged, so that it became clearer.

11. “… risk categories, … statistics…”
RESPONSE: The risk categories were briefly defined. The statistical tests and results were reviewed. The number of observers was provided in the “Methods” section.

12. “For tumor sizes please provide further details…”
RESPONSE: The figures shown in the study correspond to the median size and range. This was included in the corrected manuscript.

13. “Table 1 would benefit from the addition of further rows: e. g. mean MVD, LVD, presence of LVI on H&E, IHC, VEGF results…”
RESPONSE: As Referee #2 asked to omit all these data, Table 1 of the original manuscript was omitted. All important results were included in the text. In order to attend to the request of Referee #1, only the results of MVD, LVD, VEGF and SLN status were summarized in an additional table (Table 5 of the revised manuscript).
14. “Table 2 is not really necessary. This can be mentioned in the text. As a strange finding, this would have merited more discussion.”

**RESPONSE:** Table 2 from the original manuscript was omitted and the corrected data were included in the revised manuscript. In fact, not LVI, but SLN status was correlated with menopausal status (SLN+ correlated with pre-menopausal status), which is in accordance with the younger age of patients with SLN+.

15. Corrections of references # 12, 24, 32.

**RESPONSE:** In the revised manuscript, these references correspond to # 12, 28 and 38, respectively. The corrections suggested were included.

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**REVIEWER # 2**

1. “… the histological evaluation of the study specimens appears as carefully performed and the study report is worth of rewriting with focus on the clinical feasibility of D2-40 staining, not on ‘adding some discussion’ …”

**RESPONSE:** The suggestion was accepted and the objective of the study was changed to: “In the present study it was our purpose to evaluate the feasibility of vascular invasion assessed by H&E and by immunostaining with D2-40, as well as LVD, MVD and VEGF-A expression in early breast carcinoma, and its correlation to sentinel lymph node (SLN) status and to other clinicopathological parameters.”

2. “Omit the results of VEGF-A and CD34 stainings.”

**RESPONSE:** As Referee #1 asked for the inclusion of these data in Table 1 of the original manuscript, we included part of both suggestions omitting the original Table 1, and referring the results of VEGF-A and CD34 very briefly in Table 5. The according discussion was maintained short.
3. “Describe shortly the “risk groups” in Methods section.”

RESPONSE: The risk categories were briefly described in “Methods” according to Goldhirsh et al (2005) [reference # 16 of the revised manuscript]:

*Low risk*: Node negative AND all of the following features: \( pT \leq 2 \text{cm} \), AND histological grade 1, AND absence of peritumoral vascular invasion, AND HER2/neu gene neither overexpressed nor amplified, AND age \( \geq 35 \text{ years} \).

*Intermediate risk*: IF node negative AND at least one of the following features: \( pT \geq 2 \text{ cm} \), OR histological grade 2-3, OR presence of peritumoral vascular invasion, OR HER2/neu gene overexpressed or amplified, OR age < 35 years. OR IF less than four nodes are positive, AND HER2/neu gene neither overexpressed nor amplified.

*High risk*: One to three nodes positive AND Her2/neu gene overexpressed or amplified, OR four or more nodes positive.

4. “Include patient and tumor features in the Methods section (that is Table 1 and corresponding text in the beginning of the Results section).”

RESPONSE: The change was made and Table 1 of the original manuscript was omitted as justified previously.

5. Suggestions for presentations of the Results in Tables and analysis using the exact Fisher’s test.

RESPONSE: The following four suggestions to present the results in tables were made as indicated by Referee # 2. Tables 1 to 4 of the revised manuscript correspond to these changes. They are accordingly commented in the “Discussion” section.

REVIEWER # 3

1. “Abstract, last line: The expression “SL invasion” should be replaced by “SL metastasis”.”
RESPONSE: The suggestion was included in the revised manuscript.

2. “Background, 3rd line: correct the expression that refers to stage ‘T1 to 3a’ to ‘T1 to 3A’.”
   RESPONSE: The suggestion was included in the revised manuscript.

3. “Methods, 1st paragraph, 7th line: the expression ‘Nottingham modification’ is preferred to ‘Elston-Ellis modification’.”
   RESPONSE: The suggestion was included in the revised manuscript.

4. “Methods, item ‘Microdensity and vascular invasion assessment’ – the authors should cite the reference of the definition of tumoral lymphatic vessel invasion. Is the criteria of Yamauchi et al (ref. 22?)”
   RESPONSE: Definition was used according to Yamauchi et al. In the revised manuscript the reference is # 24. The modification was included in the revised manuscript.

5. “Results: …correlation between LVI and menopausal status… / Table 2 can be excluded…”
   RESPONSE: As stated above, Table 2 from the original manuscript was omitted and the corrected data were included in the revised manuscript. In fact, not LVI, but SLN status was correlated with menopausal status (SLN+ correlated with pre-menopausal status), which is in accordance with the younger age of patients with SLN+.

6. “Reference 24 must be completed. There is a capital letter B at the end of title of ref # 33 that must be deleted.”
   RESPONSE: Both corrections were made. The corresponding numbers of the references in the revised manuscript are 28 and 39, respectively.