Author’s response to reviews

Title: MONITORING THE RESPONSIVENESS OF T AND ANTIGEN PRESENTING CELL COMPARTMENTS IN BREAST CANCER PATIENTS IS USEFUL TO PREDICT CLINICAL TUMOR RESPONSE TO NEOADJUVANT CHEMOTHERAPY

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REVIEWERS RESPONSE

Dear BMC Cancer Editor

We would like to thank you for the in-depth review of our manuscript by the reviewers. We found all the comments by the three reviewers pertinent and relevant to improve the quality of our paper. In the first place, we want to clarify a point raised by the three reviewers in regard to the misdiagnosis we ascribed to the patients involved in this prospective study, the reviewers are right, all patients had Ductal Invasive Carcinoma and not Ductal Cancer in situ, we apologize for this mistake. We take into account all the points mentioned by each reviewer in this new version of the manuscript; we consider it as an improved version compared to the initial submission.

Please find below, for your perusal, point by point our response to all the inquiries of the reviewers.

Kind Regards,

Carlos A. Parra-López

Sylvain Ladoire (Reviewer 1): In the manuscript entitled "Monitoring the responsiveness of T and antigen presenting cell compartments in breast cancer patients is useful to predict clinical tumor response to neoadjuvant chemotherapy", Bernal-Estevéz et al. reported their clinical experience of peripheral immunomonitoring before and after neoadjuvant anthracyclines-based chemotherapy in a small cohort of breast cancer patients.
The authors claimed in their conclusions that their model of immunomonitoring is suitable to predict tumor response, and to monitor the recovery of immune surveillance during chemotherapy.

The specific topic of the importance of immunogenic effects of some chemotherapies like anthracyclines is generally well described and the fact that these immune effects are poorly explored in human patients is also discussed. Thus, the manuscript appears to be one of the rare that is questioning the modulation of peripheral immune response during chemotherapy (especially during immunogenic chemo). Moreover, the setting of neoadjuvant chemotherapy leads to correlate many immunologic parameters with the response to chemotherapy (by pathological examination of surgical specimen after chemo).

However, many major experimental/methodological problems exist, disabling the results presented here:

First of all, some points need to be highlighted and/or clarified concerning the cohort of breast cancer patients:

1. How much patients were REALLY immunomonitored before AND after chemotherapy? (this number should appear in the abstract): 23 or 15? this should be précised for each experiments.
   - Seventeen patients were immunomonitored before and after chemotherapy, this number is now mentioned in the abstract.
   - In the reviewed version of the manuscript, we specified the total number of patients on each experiment. We included in the analyses only paired data.
   - The data of two new patients before and after chemotherapy are included in the revised text of the results.

2. How long before the 1st chemo et after the 3rd chemo were obtained the blood samples?
   - The blood samples collected before the 1st chemo was collected between 1-3 days before treatment.
   - The second blood sample (pos-chemotherapy) was collected 8-10 days after the third chemo.

This information is now clarified in the revised version of the manuscript in the materials and methods section

3. It is not clear in the present form, if blood samples have been collected prospectively, or if it is a retrospective analysis of previously biobanked frozen PBMCs? please clarify this point.
- The samples obtained from the breast cancer patients were included in a prospectively study, not from a biobanked PBMCs samples.

4. Clinico-pathologic data (Table 1) are presented in a very unusual form: please use usual cutoff values concerning: age (>50 vs <50), axillary lymph node status (pN1 vs pN2 vs pN3, or cN1, cN2, cN3), ER (positive (eg >10%) vs negative) and same for PR expression, Ki67 (> or <14%), and HER2 (0,1,2 vs overexpressed (eg 3+ or 2+ and FISH+).

- We have fully modified data on Table 1 attending the reviewer’s suggestion.

5. The cohort of breast cancer patients is very small and very heterogeneous: authors must precise the number of each molecular subtype (eg: luminal non HER2, HER2 overexpressing, and triple negative breast cancer). Moreover, can they explain why there is 4 stage IV patients according to TNM classification, and only 1 patient considered as metastatic? please exclude metastatic patient(s) from all analyses.

- The molecular subtype (Luminal A, Luminal B, Her2/neu overexpressing and basal-like) of the patients is now included in the new version of Table 1 (Clinico-pathologic data).

- We excluded the data of the metastatic and stage IV patients.

6. Another clear limitation of this work is the absence of patients treated with neoadjuvant chemo, and non-responding to AC schedule: such cohort of patients would allow the authors to really compare responding vs non-responding patients (and not responding patients vs healthy donors)

- We agree with the reviewer’s comment regarding the small size of the sample and that would be fantastic to have a nonresponding group to compare. However, in this prospective study, we follow up the behavior of the clinical response of the breast cancer patients to A/C chemotherapy and the results presented are a clear reflection of how neoadjuvant chemotherapy works in our patients. We had to screen 560 patients in three years period to include 17 in our cohort. The reviewer is right 16/17 patients had a very similar RCB with only one with progressive disease (Panel A Figure 1). The similar pattern of responsiveness exhibited by most of our patients to chemotherapy was a kind of unexpected result for us and would predict that it would have taken to us a very long time to have a non-responder group of patients.

Attending to the results though we still consider our study fully valid because based on RCB classification (included now), our immunomonitoring system was able to correlate the immune responsiveness of the patients with the clinical response to chemotherapy which is the point of our paper.

Secondly, concerning the results reported here:
1. Concerning the quality of the response to chemotherapy: the gold standard to appreciate this is pathological complete response (pCR): however, pCR has not been observed in this cohort...nevertheless, the quality of response could not be evaluated on the median tumor shrinkage observed on 15-20 patients, which are not representative of the population of breast cancer patients. Authors could use other endpoints (reduction of > 50%, or better: the Residual Cancer Burden (RCB), which is the new gold standard to appreciate response to primary systemic chemo).

- In the new version of Table 1 (clinic-pathological data), we include the suggested classification based on the Residual Cancer Burden (RCB) using the web-based calculator of the MD Anderson Center, this process was supported by a trained pathologist at the medical school of Universidad Nacional de Colombia as suggested.

2. Is there a correlation between the level of Treg / MDSCs and the stage of disease?

- We explored several analyses between Tregs, MDSCs with clinical stages of the breast cancer patients, but we were unable to establish a statistically significant correlation.

3. The absence of difference in level of Treg/MDSCs between cancer patients and HD can be also explained by lack of power (small cohort)...

- The reviewer is right, we did not find differences in Tregs/MDSCs between HD and patients as has been reported in other studies, although, this may be explained by the small cohort of patients included in our study as pointed by the reviewer, we consider this can also be explained because variability in markers and phenotypes used to characterize MDSCs by flow cytometry - including ours - may make difficult to compare MDSCs among studies.

4. Line 229-230: "together, these results suggest….that is partially recovered after 3 cycles of AC chemotherapy": this sentence should be removed, as results presented here in Fig 3 do not support this conclusion.

- We agree, the results showed in figure 3 does not support this statement, we remove the final part “partially recovered after three cycles of A/C chemotherapy” in this result.

5. Concerning the last part of the manuscript: conclusions derived from a PCA conducted on 15-20 patients must be only considered as exploratory results, and many caution should be taken regarding such results (which must be in my opinion published only in supplementary data). Moreover, these preliminary data reached low statistical significance, and can only differentiate BC patients from HD (and not responder vs non responder patients to chemo: thus in my opinion, there is no clinical utility from these results).
We consider that the reviewer is right, in consequence, we move the results concerning the PCA (Figure 4) to a supplementary figure (Figure S1) and we reduced the importance of PCA in the results section regarding PCA.

6. In the last paragraph of results, by the same, a multivariate analysis conducted with more variable than patients should be regarded with caution…and the readout (tumor area smaller than 2.4 cm²) is not clinically relevant: prefer the RCB, after working with a trained breast pathologist.

As mentioned above we use now the RCB classification with the support of a trained pathologist (see table 1). Attending to what the reviewer suggests, we conducted a new multivariable analysis using RCB instead TNM and as we expected the new results fully sustain, hence, we organized a new version of Figure 4 and revised the description of the results accordingly in the last paragraph of the results section.

Finally the results presented here suffer from the small number and the heterogeneity of patients included in this cohort, and from high number of non-clinically relevant results to achieve the initial goal of this study: highlighting immunological differences in the profile of responding and non-responding patients to immunogenic chemotherapy. Additionally, other immunological parameters could be interesting to monitor in such cohort of patients, like immune checkpoints molecule expression by T cells or APCs. This could reinforce the results for further submission.

- We appreciate the reviewers’ comment, we have considered several assays and phenotype analyses along those suggested to monitor in our breast cancer patients. We published some of this process in a breast cancer patient (Bernal-Estevez D. et al., 2016 BMC Cancer), but based on the small blood volume allowed by the ethics committee to be drawn (20mL) for the present study, the PBMCs sample was a limiting factor for assessing other phenotypes. Despite this, we attempted the analysis of the memory phenotype of Tumor Associated Antigens (TAA) HLA-A* 02:01 tetramer stained specific T cells without conclusive results in over 12 patients of the cohort not included in the present study. We are currently running a clinical trial with a new cohort of breast cancer patients in which we have access to a higher blood sample. In this clinical trial, we are exploring the expression of immune checkpoints such as PD-1, CTLA-4, and BTLA in TAAs in antigen-specific T cells, we address the issue of T follicular helper cells and stem-like memory T cells, that although are giving promising results we consider these phenotypes are beyond the scope of the present study.

General comments—minor points:

1. The abstract must be re-written, because in the present form, parts of the results appear to be in the conclusion, and no real conclusion exists.

- We have adjusted the abstract to improve the difference between results and conclusions sections.
2. Line 96 (patients and blood samples): the pathological diagnosis must be "ductal invasive carcinoma" (and not DCIS), because DCIS are never treated with chemotherapy (only surgery +/- radiation therapy)

- We apologize for the typo error regarding the diagnosis. These patients have a diagnostic of "ductal invasive carcinoma" – DIC and not DCIS.

3. Line 167: "there is no published evidence….": please remove this, as this type of data is published (for example Limagne et al, Cancer Res 2016)

- Attending to the kind suggestion of the reviewer, we modified this statement attending this suggestion, and include the reference Limagne et al., Cancer Res 2016, for colorectal cancer; however, we still did not find a particular paper in breast cancer and A/C chemotherapy.

Nicoletta Biglia (Reviewer 2): The Authors have done an impressive work on an interesting point of view concerning the interaction between chemotherapy, chemoresistance and the immune system.

To my opinion some issues must be addressed.

1) In the material and methods section the Authors speak of total of 560 breast cancer patients with pathological diagnosis of Ductal Carcinoma in Situ (DCIS). This must be a misunderstanding because there is no indication to Chemotherapy at all in patients with DCIS.

- As addressed by the first reviewer, we apologize for the typo, it is Ductal Invasive Carcinoma, and regard to the 560 patients this number is in reference to all types of breast cancer. From this universe, we included in this study only those who were eligible for A/C chemotherapy. This is now clarified in the materials and methods section.

2) To better understand and classify the study population, the patient's clinical characteristics should be expressed not only as deviation from the median, but also according to the international classifications: namely ER and PR as negative or positive according to the % of positive staining with the cut-off at 1% or 10% of positive staining (according to the reference they decide to choose), Her2/neu should be defined as positive only if staining +++ or fish test positive in case of staining ++ or --, Ki67 as positive or negative according to a cut off value (20% in most of the recent publications).

- We have fully revised Table 1 attending the reviewer’s suggestion. We use standard nomenclature for Clinico-pathologic data and included the RCB classification as also was suggested by Reviewer 1.

3) It should be analyzed whether there is any association between the tumor response to chemotherapy in the individual case and the behavior of the immunological variables. The analysis in fig 5 (ABCD) would be more informative taking into account the %
schrinkage of tumor in the individual patient instead of the final tumor size (> or < than 2.4 cm\(^2\)) without reference to the initial tumor burden.

- We agree with the reviewers’ comment and make several analyses based on the immune response and the RCB score (these results were similar to the data analyzed by the percentage of tumor shrinkage). The new analyses are now shown in the new version of Figure 4.

4) The number of patients before and after chemotherapy considered in the different analysis (Fig 1,2,3) is sometimes different because only in 15 out of 23 patients it was possible to obtain post treatment blood sample for paired and correlation analysis. This introduces a considerable bias in the comparison of the values. To my opinion the comparison should be made between the pre- and post-chemotherapy values measured in each patient and the results analyzed according to the pathological response of the tumor.

- We fully agree with both reviewers on this point, hence, we now excluded the data of samples that were not paired between before and after chemotherapy. All the new results presented in the revised version only include paired samples.

Agnieszka Kołacińska (Reviewer 3): Before I review this manuscript, please clarify: Materials and Methods line 96: 560 patients with DCIS (ductal carcinoma in situ), of these patients 36% were eligible to be treated with doxorubicin and cyclophosphamide. WE DO NOT TREAT DCIS PATIENTS WITH CHEMO

This is a clear mistake on our part; we apologize by this the patients were diagnosed with ductal invasive carcinoma.

The authors’ response letter has been included as a supplementary file