Author’s response to reviews

Title: Differential Proteomic Comparison of Breast Cancer Secretome Using a Quantitative Paired Analysis Workflow

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Dear Editor,

As per your request, we provide below a point-by-point rebuttal regarding the issues raised by reviewer 2.

Reviewer 2 comments:

“The additional validation using SRM does add strength, however, the fact that of the 12 selected to validate, only 25% (3/12) could be validated. This is surprising, since SRM specifically looks for a given set of targets. Quite why the remaining 9/12 could be significantly detected in a non-targeted approach, but unable to be confirmed in a targeted approach is concerning if this were ever to be translated to a diagnostic setting.”
In response to the reviewer’s discrepancy concern, it is worth mentioning that non-targeted and targeted approaches were performed under different experimental conditions: in the first case, peptides were fractionated using much longer column and gradients lengths and MS/MS data were acquired on an Orbitrap Elite mass spectrometer. SRM experiments, on the other hand, were performed on a TSQ Quantiva mass spectrometer, following a less resolutive chromatographic step. Nevertheless, as we have pointed out in the revised manuscript, from the 12 proteins assayed, 9 displayed fold-changes that corroborated the shotgun results. However, following statistical analysis with Paired Analyzer, only three of them presented statistical significance (p-value < 0.05). One possible reason was the smaller sample number available for SRM experiments (i.e., 6 sample pairs instead of 10). As detailed in the manuscript, the p-value of a peptide is calculated based on the total number of pairs of samples analyzed and the number of pairs of samples where that peptide either presents a significant fold change (> 1.5) or is found absent in both samples of a pair (both samples of a patient) or is found present in only one sample of a pair (present only in the cancer sample or only in the non-diseased one). Therefore, the higher the number of paired samples, the greater the chance of reaching lower p-values.

Regarding the three proteins that were discarded from the comparative analysis, it is important to add that we have used stringent criteria regarding peak picking and data processing, sample preparation, and MS measurement variability sources. The gold standard recommendations for targeted proteomics state that data not abiding to such quality control checks [such as signal below limit of quantification (LOQ), peptide co-elution, peak truncation, poor co-elution and peak shape of transitions from same precursor, inconsistent retention time across replicates etc] should be discarded (Picotti et al 2009; Reiter et al 2011). Hence, since some of these technical issues were observed for peptide representatives of these 3 proteins, their results were considered unreliable, and reported as such.

Finally, it is important to stress that this study analyzed the differential proteome of NAF paired samples from unilateral breast cancer patients - i.e., comparing samples subjected to the same physiological conditions (same patient), in the hope of contributing to a deeper understanding of this pathology. Using a discovery proteomic pipeline, we were able to find differences in cellular metabolism and immune system activation, which were partially corroborated by a preliminary SRM assay. Before any of these findings can be used in clinical practice, additional verification/validation experiments will be needed, which will involve larger and independent cohorts. This is clearly beyond the scope of this paper.