Dear Editor,

We are grateful for the opportunity to resubmit our Manuscript entitled “A lactate shuttle system between tumour and stromal cells is associated with poor prognosis in prostate cancer”, Ms. 2369854151138229. We have considered the reviewer’s comments and have made the suggested changes. We believe this will improve the manuscript.

Our detailed response to the reviewers’ comments is attached below.

We look forward to hearing from you again soon with your decision.

Yours sincerely,

Fátima Baltazar

Assistant Professor, PhD

Life and Health Sciences Research Institute (ICVS), School of Health Sciences,
University of Minho
4710-057 Braga
Portugal
In reply to Reviewer Judith J Jans:

Major comments:

1. In figure 2, the authors describe and visualize in a heatmap the differences in three proteins across malignant transformation. It is not immediately obvious to the reader how to estimate the value of this observation: please prove the claims made with statistical analysis showing differences between the groups.

R: We agree with the reviewers comment and in order to address this major comment we replaced the previous heatmap by a sorted heatmap (i.e. sorted according to one protein column within each fibroblast group). Figures were partially made using R statistical computing environment, R version 3.0.0. The stronger expression (3) is represented by the most intense colour. The $p$ values representing the differences between groups were indicated.

2. Please also comment on why only these three proteins were chosen.

R: We agree with the reviewer’s comment that the reason why only these proteins were chosen for the heatmap construction might not be very clear. Thus, we introduced a sentence explaining that in Figure 1 we show the percentage of positive cases for the expression of each protein studied and because only MCT4, PDK1 and CAIX showed some positivity in the fibroblasts, these proteins were the ones chosen to present in a more detailed way using the heatmap representation.

Minor comments:

3. In table 2, significant correlations between multiple factors are shown. In the results and discussion section, a non significant correlation between PDK1 and perineural invasion is discussed. This correlation is too suggestive given the high $p$-value and multiple testing and should not be discussed.

4. Similarly, the correlation between MCT1/4 and biochemical recurrence should not be discussed. Or if discussed, please indicate how independent the correlation with recurrence is of the pT staging.

R: The reviewer’s comments are pertinent, however since we are describing for the first time the expression of metabolic proteins in fibroblasts in relation to tumour in a comprehensive casuistic of prostate cancer patients with reliable clinic-pathological information we thought it was pertinent to include and describe not only direct associations but also all the tendencies found in this study. However, we removed the discussion on PDK1 since the $p$-value is quite high.
In reply to Reviewer Céline Pinheiro:

Major Compulsory Revisions

1. The question and rationale of the work posed by the authors is either not clear or not completely answered as the expression of some of the markers (LDHV, AMACR, ACOX-3 and DBP) is not at all explored in the Discussion section. Please discuss the relevance of the data found for these markers and how they fit in the hypothesis.

R: As suggested by the reviewer, the Discussion section was improved in order to include some discussion on LDHV, AMACR, ACOX-3 and DBP expression, according to the theory defended on this work.

2. Both the Introduction and Discussion sections lack important information by other authors. Please describe in more detail the metabolic symbiosis between CAFs and cancer cells, by including in the Introduction section information related to other studies, such as the studies in prostate (already included in the Discussion), lung (Cancer Biol Ther. 2007 Sep;6(9):1476-9) and colorectal cancers. Include these 2 last articles also in the Discussion.

R: As suggested by the reviewer, the two new references were included in the paper and the Discussion was improved based on that (references 14-18).

3. Some information included in the manuscript is not supported by references: the regulation of MCT1 in the Introduction section, GLUT expression in cancer cells previously observed by the authors in the Discussion section and prognostic value of perineural invasion in the Discussion section.

R: We agree with the reviewer’s comment and new references were included in the paper to support the information included in the manuscript.

4. The Results section may be improved if the data obtained is more extensively described. PDK1 expression frequency in tumour cells and stroma is not quantitatively compared while the expression frequencies of the markers exclusively found in cancer cells is not further described: are they high frequencies, low frequencies?

R: The comment of the reviewer is pertinent and important. However, the major aim of this work was to present evidence for differences between the expression of key-metabolic proteins in fibroblasts when comparing to tumour cells, as so, the expression found in cancer cells is being deeply explored by us but in the context of another study.

5. The second paragraph of the Results section describes an analysis without any result description or figure/table. Please clarify this analysis by including the result description concerning this analysis in the same paragraph. Also, this paragraph refers “the same proteins”, however, the proteins analysed are only 3, instead of the 10 initially studied. Please clarify this issue in the Results section as others expressions suggest analysis of the 10 proteins while the figures show only part of them (ex. first part of fourth paragraph and description of Figure 3).

R: We agree with the reviewers comment and the text was modified accordingly. Also, as already suggested by the reviewer Judith Jans, we introduced a sentence explaining the selection of the proteins.

6. The third paragraph of the Results section is essentially the legend to Figure Please remove it from the Results section.
R: We agree with the reviewers comment and the suggested alterations were performed.

7. Please be cautious when stating that “no expression/staining” is found in some cases (Results, fourth paragraph, line 6 and line 10). The cut-off used in the present study, where only moderate and strong final scores were considered positive, should not create the confusion that negative cases show no protein expression.
R: We agree with the reviewers comment and the suggested alterations were performed.

8. The Discussion section is poorly explored. The results obtained can be further discussed, based on results by other groups. For instance, the Reverse Warburg effect is mentioned in the abstract but left behind in the rest of the manuscript. The Discussion can be much more improved if a discussion, based on Reverse Warburg effect studies by Lisanti’s group, is included. Similarly, as mentioned before, studies on the metabolic symbiosis between cancer cells and CAFs by Koukouraki’s group should be included in this section. Additionally, the expression frequencies of these proteins in cancer cells should be compared with studies by others.
R: We agree with the reviewer’s comment and the Discussion section was improved in order to include the suggestions.

Minor Essential Revisions

1. The manuscript shows some English incoherence and other small typing errors such as: “tumour” appears both as “tumour” and “tumor”, the Results section shows a mixture of verbal conjugations (past and present), “rim” instead of “kidney” in Table 1, p (for p value) must be in italic, lack of commas, etc. Please review.
R: We agree with the reviewer’s comments and the suggested alterations were performed.

2. Is the last sentence of Introduction’s 1st paragraph in the correct place? Should it not be at the end of the Introduction, following the main aim of the work?
R: We agree with the reviewer’s comment and the suggested alterations were performed.

3. The section Patient sample selection should begin by stating the type, number and origin of the samples. Also, please list in this section the clinic-pathological information used (transfer the sentence “Preoperative serum total PSA,...” from the Results section to the Material and Methods section).
R: We agree with the reviewer’s comment and the suggested alterations were performed.

4. Please confirm if the version of SPSS used was 17.0, as the last version is 21.0.
R: We confirm that the version of SPSS used was 17.0 at the time of the study.
5. The 3rd sentence of the Results section is a repetition of what was stated in the 2nd sentence. Please review.
R: This comment was taken into consideration and the sentence was reviewed.

6. p values in Table 2 that do not reach significance should not be presented in bold.
R: This comment was taken into consideration and the sentence was reviewed.

7. p=0.052 is not significant, therefore, the association of MCT1 and MCT4 co-expression, in cancer cells and CAFs respectively, with biochemical recurrence after surgery does not exist.
R: We agree with the reviewer that the value obtained in this study is not indeed significant. However, it represents a strong tendency which is worth to be mentioned and discussed from the point of view of this study. To note that other combinations studied did not show any significant association or tendency to correlate with clinico-pathological data, in contrast to MCT1 and MCT4 co-expression, in cancer and CAFs, respectively. Also, further studies are needed to provide evidence on the possible in vivo/in vitro significance of this result and the results obtained here using human samples would be taken as a strong basis to develop further work exploring this subject.

8. In the Discussion section, paragraph 5, which proteins show changes along malignant transformation?
9. The Conclusion is a summary of the last paragraph of the Discussion. Replace the Conclusion by the last paragraph of the Discussion.
10. Provide a more detailed legend for Figure 1, including the meaning of “*”.

R (8-10): The discussion and conclusion sections were changed according to the reviewer’s suggestions in order to improve and clarify the ideas presented.

Discretionary Revisions:

1. Please clarify why there are only 203 non-neoplastic samples, if the 408 patients were only included if both normal and tumour tissues were available.
R: The pathologist involved in this study selected also non-neoplastic glands adjacent to tumour that showed no evidence of being infiltrated by malignant glands. Also, only the glands without any evidence of morphology alterations were selected. As a result, non-neoplastic samples showing any level of transformation were not included, decreasing the number of non-neoplastic samples used but increasing the rigor of the study.

2. The abbreviations BAFs and PAFs are introduced in the Results section, but no longer used in the rest of the results description. The continued use of these abbreviations may help to understand sentences that are in the present form somewhat confusing, for example: fourth paragraph of the Results section.
R: We agree with the reviewer’s comment and the suggested alterations were performed.
3. Figure 3 includes representative immunohistochemical reactions of only part of the proteins studied. Among the proteins that were only expressed in cancer cells, why only MCT1 is represented in Figure 3? Why the other proteins are not also included in Figure 3? Also, Figure 3 should be the first Figure of Results section.

R: The reviewer’s comment is pertinent. The reason for MCT1 being the only one showed in more detail in contrast to the other proteins that are also only expressed in the tumour is justified by the fact that MCT1 expression in tumour glands was really evident on the plasma membrane, a result that fits with the theory of the Reverse Warburg effect, as so, we though that showing MCT1 expression in the membranes of the cells provides evidence for the existence of a lactate shuttle between fibroblasts and cancer cells.

4. Why use 2 CAFs in Figure 4? An image with only one tumour cell and one CAF would be clearer. Also, this Figure would be more adequate in the Discussion section, to illustrate the hypothesis.

R: We agree with the reviewer’s comment and the Figure 4 was moved to the discussion section.

5. Was the expression of the different proteins in cancer cells associated with the clinico-pathological data? Such data would greatly improve the manuscript.

R: The comment of the reviewer is pertinent and important and in fact the association between different proteins expression in prostate cancer cells and the clinicopathological value was studied by our group and the results were already submitted in the context of another study. In the present study, we aimed to focus on the possible interplay between cancer cells and fibroblasts with an emphasis for the clinicopathological significance of the expression of these proteins in fibroblasts, as so, the correlations suggested by the reviewer is part of another study.