Editor-in- Chief, August 21, 2020

World Journal of Clinical Oncology

Round 1

Resubmission of Manuscript: NO: 55936

Dear Sir,

Enclosed please find the revised manuscript entitled "Forkhead box P3 and indoleamine 2,3-dioxygenase co-expression in Pakistani triple negative breast cancer patients" (Manuscript: NO. 55936) and our responses to the reviewers and editor comments. We appreciate the time and details provided by the reviewer and editorial office, which enabled us to improve the manuscript.

We hope the revised manuscript is now acceptable for publication in *World Journal of Clinical Oncology.*

All amendments in the text are marked in purple.

We appreciate your consideration of our manuscript.

Yours sincerely

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Point-by-point response

1. AIM: The aim should be more specific, e.g. co-expression of FOXP3 and IDO in TNBC with respect to hormone-positive BC or with respect to the aggressiveness of the tumor.

Ans: The aim of our study has been amended as per suggestion in the main text.

2. RESULTS: The authors must add a comparison, e.g. with respect to hormone-positive BC.

Ans: It has been amended as per suggestion in the main text.

3. FOXP3 has prognostic significance in triple-negative breast cancer (TNBC)[8]. The authors must specify what kind of prognostic significance, e.g. good or worse.

Ans: Lee et al. observed the prognostic significance of FOXP3- positive T-regs in TNBC. They suggested that assessment of FOXP3- positive T-regs in combination with other risk factors could improve the prognostic stratification of TNBC. Therefore, further studies were required to categorize FOXP3- positive T-regs for good or worse prognosis.

4. The authors analyzed in a retrospective manner the expression of the biomarkers in relation to patients' survival. I point out that the correlation with the PFS could be also very interesting and should be added as an investigation in the paper.

Ans: we observed that there was no statistically significant difference in the overall survival between patients with FOXP3-positive or -negative tumors (p-value = 0.73) as shown in (Figure 2). Progression free survival was not the scope of our current study.

5. A blind histopathologic assessment was conducted by pathologists. How many pathologists?? The number should be indicated, as well as the choice in case of discordance.

Ans: The two pathologist were involved in the study. The mean score of both pathologists was considered as final score.

6. The scores were further categorized as low, medium, and high. This description must be specified, the authors are invited to define what they mean for low, medium, and high with respect to the assigned scores. For example, 0-1 low, 2 medium and 3 high

Ans: The amendments have been incorporated in the main text as per suggestion "The scores were further categorized as low (1-3), medium (4-6) and high (7-9)".

7.The baseline characteristics of female breast cancer patients (n=100) are summarized in Table I. The mean age at the time of diagnosis was 48.28 ± 11.83. The table I must be completely revised. The authors must describe the two groups of the study, i.e. TNBC and hormone-positive BC, with the clinicopathological characteristics. It is important to add the number of patients analyzed for each group (the sum of the two groups should be 100). Moreover, the mean age must be indicated for the two groups (TNBC versus hormone-positive BC) to point out the homogeneity of the population of the study.

Ans: Our first table (baseline characteristics) is showing the overall characteristics of study population which cannot be omitted. We have added another table (Table-2) as per suggestion to compare the TNBC versus hormone-positive breast cancer population.

8. Based on IHC analysis, the FOXP3 expression had nuclear localization. I think that FOXP3 protein expression has been already evaluated in the literature, the authors should underline if the localization is as expected or not.

Ans: According to the literature, the FOXP3 expression had nuclear localization and we found the same results.

9. Out of 100 patients, 25 expressed FOXP3-positive T-regs and 75 expressed FOXP3-negative T-regs (Figure 1). The authors must provide a table, also as supplementary materials, where they show positive and negative FOXP3 cases with the main clinicopathological characteristics, or, alternatively, describe this in the text.

Ans: The data of 75 out of 100 FOXP3 negative cases is provided in supplementary data (Table 1).

10. There is contradictory data regarding the involvement of FOXP3+ T-regs in breast cancer patients. The fact that in literature there are contradictory results about the role of FOXP3 should be discussed extensively, giving more detail and in comparison, with the results obtained by the authors. Moreover, in my opinion, this could be cited as an aim.

Ans: Several studies identified that FOXP3^{+} T-regs infiltration in tumor microenvironment may affect the breast cancer progression. Our results are in compliance with the results published previously. Bates et al. demonstrated that high ratio of FOXP3 cells anticipate worse relapse-free survival and shorten overall survival in patients with invasive breast carcinoma. In another study the researchers observed no difference in overall survival among patients expressing high

or low FOXP3. Our results are more in the favor of above-mentioned study as compared to the study demonstrated by Bates et al. that has been discussed in detail in discussion section.

11. FOXP3 expression was identified in 25 breast cancer patients and the majority of these patients displayed TNBC phenotype. The authors must discuss the FOXP3 positive cases on the basis of: the % of positive vs negative in TNBC and % of positive vs negative in hormone-positive BC. The authors are invited once more, to compare their results with those of literature.

Ans: The percentages have been incorporated in the main text as per suggestion: "36.73% TNBC patients expressed FOXP3 positive cells while 13.72% hormone positive breast cancer patients expressed FOXP3 positive cells. On the other hand, FOXP3 expression was not detected in 63.26% TNBC patients and 86.27% hormone positive breast cancer patients. Our findings of FOXP3 T-regs infiltration in TNBC patients is similar to several studies published before which identified the involvement of FOXP3 positive cells in breast cancer progression" [7,24].

12. FOXP3+ T-regs have prognostic implications in TNBC[8]. IDO expression is also associated with TNBC[26]. The authors must specify what kind of prognostic implications FOXP3 positive T-regs have, and to discuss their results in comparison with those of literature. Moreover, the kind of implications of IDO expression in TNBC must be discussed, as the request above indicated for FOXP3.

Ans: As described previously, Lee et al. observed the prognostic significance of FOXP3- positive T-regs in TNBC. They suggested that assessment of FOXP3- positive T-regs in combination with other risk factors could improve the prognostic stratification of TNBC. Therefore, further studies were required to categorize FOXP3- positive T-regs for good or worse prognosis. Furthermore, high IDO expression was detected in the TNBC patients. Previously FOXP3 and IDO co-expression has been detected in breast cancer patients but the co-expression of IDO and FOXP3 in TNBC has recently been identified.

13. Finally, our study did not compare results of the IDO enzymatic activity with IDO protein expression due to retrospective nature of study. However, this is the subject of our ongoing prospective study. The sentence is confusing. The authors must explain in a more detailed and clear form why IDO enzymatic activity measurement should be interesting and its relation with protein expression on the basis of literature data.

Ans: These are future prospects to analyze enzymatic activity of IDO in breast cancer patients, which is not part of the current study and was creating confusion. Therefore, the relevant lines have been removed from main text.

14. FOXP3 monitoring and IDO manipulation in TNBC patients may be an effective therapeutic strategy. The term manipulation is not very clear; what the authors do mean?

Ans: It has been amended as per suggestion in the main text. "Evaluation of FOXP3 and IDO expression in TNBC patients may provide an effective therapeutic strategy".

15. Figure1. The authors are invited to show examples of low, medium, and high scores for both markers. The staining of IDO seems to be very diffuse and the co-expression with FOXP3 showed in the figure is not completely sound. For example, I noted that the orientation of the slice seems to be not right. Moreover, the authors must provide for comparison the pictures of IHC of normal breast tissue (e.g. tissue derived from reductive mammoplasty) analyzed for the two biomarkers.

Ans: The focus of our manuscript was to investigate the co-expression of FOXP3 and IDO in braest cancer patients. The images have been taken based on required criteria. Nevertheless we have provided the IDO expression (low, medium, and high) images in the supplementary data (Figure1). Furthermore, we have replaced the FOXP3 and IDO co-expression images in the main figures keeping appropriate slice orientation in view. Positive controls were run for both the

biomarkers. According to the literature, IDO is not expressed in the normal breast tissues (Théate et al. 2015).

Ref:

Théate I, van Baren N, Pilotte L, et al. Extensive profiling of the expression of the indoleamine 2,3-dioxygenase 1 protein in normal and tumoral human tissues. Cancer Immunol Res. 2015;3(2):161–172.

16. Figure: 2 Kaplan-Meier curve of overall survival for total patients diagnosed with breast cancer. TNBC is a subgroup of BC that generally accounts for 10-15% of BC, therefore a table with about 50% of TNBC can lead to misinterpretation. I suggest analyzing separately the survival curve for TNBC versus hormone-positive BC.

Ans: Our hypothesis was to quantify the FOXP3 expression in relation with IDO expression and to study the overall survival in patients diagnosed with breast cancer from Pakistan. We observed that there was no statistically significant difference in the overall survival between patients with FOXP3-positive or -negative tumors (p-value = 0.73) as shown in (Figure 2). TNBC versus hormone-positive BC was not the scope of our current study.

17.Finally: An immunosuppressive enzyme, indoleamine 2, 3 dioxygenase (IDO) catabolizes the tryptophan in to kynurenines. Maybe, there is a typo "in to"

Ans: The typo error has been corrected in the main text.

Note:

1. Article highlights have been provided in the main text as per suggestion.
2. This study was funded by Shaukat Khanum Memorial Cancer Hospital and Research Centre, Institute Award no: IRB-16-08. Approval from IRB is considered as grant approval. IRB letter is considered as final grant letter in our hospital (which has been attached). According to hospital policy the separate grant letter is not required.

Article Highlights

Forkhead box P3 (FOXP3) and indoleamine 2,3 dioxygenase (IDO) are expressed in triple negative breast cancer (TNBC). IDO upregulation and FOXP3 infiltration in tumor microenvironment may inhibit the local immune responses. FOXP3 positive cells might be associated with high expression of IDO in TNBC patients. Evaluation of FOXP3 and IDO expression in TNBC patients may offer an effective therapeutic strategy.

Round2

Resubmission of Manuscript: NO: 55936

Dear Jia-Ru Fan,

Enclosed please find the revised manuscript entitled "Forkhead box P3 and indoleamine 2,3-dioxygenase co-expression in Pakistani triple negative breast cancer patients" (Manuscript: NO. 55936) and our responses to the reviewers. We appreciate the time and details provided by the reviewer and editorial office, which enabled us to improve the manuscript.

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All amendments in the manuscript are marked in yellow.

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Yours sincerely

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Point-by-point response

1. In the Abstract (Result section): A significant association of FOXP3 and IDO co-expression was observed among patients with TNBC (P= 0.01) as compared to the hormone-positive breast cancer. **New query:** The authors did not explain what kind of association… or the English language is not clear for me (may be the author's means: a positive association between FOXP3 and IDO in TNBC or a significant co-expression of FOXP3 and IDO?

   ANS: The line has been modified as per suggestion of the reviewer. "A significant co-expression of FOXP3 and IDO was observed among patients with TNBC (*P*= 0.01) as compared to the hormone-positive breast cancer."

2. Query 3. FOXP3 has prognostic significance in triple-negative breast cancer (TNBC)[8]. The authors must specify what kind of prognostic significance, e.g. good or worse. Ans: Lee et al. observed the prognostic significance of FOXP3- positive T-regs in TNBC. They suggested that assessment of FOXP3- positive T-regs in combination with other risk factors could improve the prognostic stratification of TNBC. Therefore, further studies were required to categorize FOXP3- positive T-regs for good or worse prognosis. **New query:** The above considerations cannot be a reply to the reviewer: They must be inserted in the context.

   ANS: The paragraph has been inserted in the main text as suggested "but Lee et al. observed the prognostic significance of FOXP3-positive T-regs as compared to FOXP3-negative T-regs in TNBC. Furthermore, they identified that improved survival was linked with FOXP3-postive T-regs in TNBC. This finding was in contrast with other types of cancers [8]. Therefore, further studies were required to categorize FOXP3- positive T-regs for good or worse prognosis."

3. Query 5. A blind histopathologic assessment was conducted by pathologists. How many pathologists?? The number should be indicated, as well as the choice in case of

discordance. Ans: The two pathologists were involved in the study. The mean score of both pathologists was considered as final score. **New query:** Firstly, the above answer has not been introduced in the materials and methods. Furthermore, I do not completely agree with the method used to assign the score. This method could be acceptable only if the two pathologists have assigned the same score or a different score in the same category (low, medium or high). On the contrary, there should be a third pathologist for the assignment of the score and the final score should be a consensus of all pathologists. Thus, the authors must follow this recommendation and, if necessary, revise the database. The description of the assigned score is crucial and must be carefully explained in the materials and methods.

ANS: The description carefully explained and incorporated in the main text (Materials and Methods). The two pathologists were involved in the study. Pathologists conducted a blind histopathologic assessment. The discrepancies between the two pathologists were reviewed mutually to reach the consensus. The mean score of both pathologists was considered as final score."

All the studies conducted before mentioned that two pathologist has evaluated expression independently. We have followed the same. For further clarification, I am giving you the reference. These references are the part of our manuscript as well.

1. Merlo A, Casalini P, Carcangiu ML, Malventano C, Triulzi T, Menard S, Tagliabue E, Balsari A. FOXP3 expression and overall survival in breast cancer. J Clin Oncol. 2009 Apr 10;27(11):1746-52.
2. Soliman H, Rawal B, Fulp J, Lee JH, Lopez A, Bui MM, Khalil F, Antonia S, Yfantis HG, Lee DH, Dorsey TH. Analysis of indoleamine 2-3 dioxygenase (IDO1) expression in breast cancer tissue by immunohistochemistry. Cancer immunology, immunotherapy. 2013 May 1;62(5):829-37.

4. **Query** 8. Based on IHC analysis, the FOXP3 expression had nuclear localization. I think that FOXP3 protein expression has been already evaluated in the literature, the authors should underline if the localization is as expected or not. Ans: According to the literature, the FOXP3 expression had nuclear localization and we found the same results. **New**

query: The authors must introduce this concept in the text and add the appropriate reference.

ANS: As per reviewer suggestion, the reference is added and explained in the main text. "FOXP3 expression had nuclear localization [6]."

5. **Query** 9. Out of 100 patients, 25 expressed FOXP3-positive T-regs and 75 expressed FOXP3-negative T-regs (Figure 1). The authors must provide a table, also as supplementary materials, where they show positive and negative FOXP3 cases with the main clinicopathological characteristics, or, alternatively, describe this in the text. Ans: The data of 75 out of 100 FOXP3 negative cases is provided in supplementary data (Table 1). **New query:** In the table 1, I would like to know, at least, which molecular subtype resulted positive or negative to FOXP3.

ANS: The major issue was raised before to provide the data of FOXP3-negative T-regs (n=75). The data was provided in the supplementary materials. Now the new query has been raised about the molecular subtypes of TNBC. In our hospital setting, we do not sub-categorized TNBC. Due to this reason, we are unable to provide you this information.

6. **Query** 12. FOXP3+ T-regs have prognostic implications in TNBC[8]. IDO expression is also associated with TNBC[26]. The authors must specify what kind of prognostic implications FOXP3 positive T-regs have, and to discuss their results in comparison with those of literature. Moreover, the kind of implications of IDO expression in TNBC must be discussed, as the request above indicated for FOXP3. Ans: As described previously, Lee et al. observed the prognostic significance of FOXP3- positive T-regs in TNBC. They suggested that assessment of FOXP3- positive T-regs in combination with other risk factors could improve the prognostic stratification of TNBC. Therefore, further studies were required to categorize FOXP3- positive T-regs for good or worse prognosis. Furthermore, high IDO expression was detected in the TNBC patients. Previously FOXP3 and IDO co-expression has been detected in breast cancer patients but the co-expression of IDO and FOXP3 in TNBC has recently been identified. **New query:** The authors must insert in the text a summary of the above answer.

ANS: The paragraph has been inserted in the main text as suggested, "Lee et al. observed the prognostic significance of FOXP3-positive T-regs as compared to FOXP3-negative T-regs in TNBC. Furthermore, they identified that improved survival was linked with FOXP3-postive T-regs in TNBC. This finding was in contrast with other types of cancers [8]. Therefore, further studies were required to categorize FOXP3- positive T-regs for good or worse prognosis."

7 **Query 16**. Figure: 2 Kaplan-Meier curve of overall survival for total patients diagnosed with breast cancer. TNBC is a subgroup of BC that generally accounts for 10-15% of BC, therefore a table with about 50% of TNBC can lead to misinterpretation. I suggest analyzing separately the survival curve for TNBC versus hormone-positive BC. Ans: Our hypothesis was to quantify the FOXP3 expression in relation with IDO expression and to study the overall survival in patients diagnosed with breast cancer from Pakistan. We observed that there was no statistically significant difference in the overall survival between patients with FOXP3-positive or -negative tumors (p-value = 0.73) as shown in (Figure 2). TNBC versus hormone-positive BC was not the scope of our current study. **New query:** I don't agree with the answer of the authors because the aim of the study is the expression of FOXP3 in TNBC, also the Kaplan-Meier curve of all BCs can be confusing.

ANS: To avoid any further confusion, we have removed the data of Kaplan-Meier curve of overall survival for total patients diagnosed with breast cancer.

8 **New query:** Finally, in the abstract and discussion sections the sentence "FOXP3 and IDO expression monitoring in TNBC patients may provide an effective therapeutic strategy." is not acceptable in this form. The authors must carefully explain how, in their opinion, the monitoring of FOXP3 and IDO expression could be useful to provide effective therapeutic strategy.

ANS: IDO inhibitors are currently the part of clinical trials for different cancers. Therefore, we used the sentence of effective therapeutic strategy. To keeping in view of your suggestion, we are going to change the sentence. "Evaluation of FOXP3 and IDO expression in TNBC patients may offer a new therapeutic option."