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Immune escape mechanisms and immunotherapy of urothelial bladder cancer

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Immune Escape and Immunotherapy in Bladder Cancer
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Dear Dr. Yang,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be

pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below. Please understand that your manuscript requires an entire overhaul in terms of restructuring and language editing in addition to the points raised by the reviewers. The editorial board will focus on this heavily after receiving a revised draft.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by Feb 22, 2021.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: The authors of this paper attempt to provide an overview of immunotherapy for bladder cancer, providing some background on mechanisms of immune escape, history of immunotherapy and of immunotherapy for bladder cancer, and then a broad overview various immunotherapies used in bladder cancer, both approved and investigational.

While the manuscript is clearly thoroughly researched, I think it could be improved by taking a bit of a more narrow focus. Starting from the title, it is not clear what the true goal or focus of the paper is, and that theme persists throughout. The title suggests discussions of immune escape/resistance mechanisms, but that is not really addressed in any great detail in the paper. The introduction talks about bladder cancer in general, and then immunotherapy with checkpoint blockade in general, but does not clearly let the reader know what the paper will be about. Then there is a section on various immune micorenvironmental pathways involved in tumor immunity, but each is 1-2 sentences, so they do not provide a very in depth understanding of any of these pathways, and its not clear that these connect to the discussions on individual therapies. The next section describes some history of therapies for bladder cancer, as well as some of the history of immunotherapy, but other than the part about BCG, I'm not sure it fits the rest of the paper. Much of the history section is just a repeat of Figure 1 from line form to sentence form. And I'm not sure all the important highlights of the Hx of immunotherapy are truly addressed.

Next the paper moves to systemic tx for bladder cancer. It includes approved immunotherapies (aPD1, BCG) as well as investigational therapies. Again, I think the decision to be inclusive is to the detriment of new understanding of any of these therapies. Particularly in the anti-PD1 section, the authors spend time reviewing results from the initial

phase I studies, yet do not comment on larger more contemporary Phase 3 single agent or combo studies, including leaving out data on maintenance avelumab which is now the standard of care. Following this, they move on to more investigational therapies, which are exciting, but details about where these therapies stand in context to the landscape of trials in BC is given short shrift.

I recommend to the authors deciding what they would like to most focus on. Is this a review article about current immunotherapies for bladder cancer? If so, more detail about the history of BCG and checkpoint blockade might be helpful, and then i would suggest more updated data including phase 3 studies and combo treatments would be needed. Then one might choose to speculate at how combinations will move in the future, and/or maybe include some info about pathways and discuss potential strategies to overcome resistance as the title implied. Another route would be to focus on novel therapies for bladder cancer, with more time given to NK cells, TAMs, DCs, etc. Thus, more detail about the preclinical and clinical work that has been done would be informative, and then where things stand currently in the clinic and how the authors see things moving forward.

As is, I can not recommend the paper for publication. Please see my attached specific edits for more detailed comments.

Reviewer #3: Dear editor, thank you for giving me the opportunity to review this paper.

The authors propose to review the importance of immunity in bladder cancer, the interplay between immunity and cancer, and immune therapy associated. Authors were very cautious about developing exhaustively all the approaches using immune therapies against bladder cancer.
However, some comments have to be done (the abbreviation ICI is for immune checkpoint inhibitors).

Major comments :

Rather than speaking about bladder cancer, it is better to speak about urothelial carcinoma, from bladder or upper urinary tract.

Line 38 : the level of evidence of neo adjuvant chemo prior to radical cystectomy is really higher than adjuvant chemo. This sentence has to be more accurate and rephrased.
Immune therapies such as BCG therapy and ICI (in urothelial carcinoma) need to be more developed in introduction in order to highlight their importance compared to other approaches.

Line 93 : this sentence has to be rephrased, and it is supposed to introduce this paragraph.

Paragraph 4 :
Results of studies have to be more accurately reported ; it is necesary to understand why BCGtherapy was approved. Which results based on ?

Paragraph 5 may be shortened, since there is no major advance in this lead.
Add a ref to the last sentence lines 182-183. I am not sure about this statement.

Paragraph 6
Line 197 : the adverb ubiquitously has to be verified.

Paragraph 6.1 has to be lengthened ! This is the major milestone reached in urothelial carcinoma !

1- Discuss results in fit and unfit patients (to cisplatin). Mutational load and response (Balar Lancet 2017)
2- Discuss in 2nd line, the link between objective response and duration of response (Fradet Ann Oncol 2019).
3- Speak about the rationale of treatment of maintenance, and cite and discuss Javelin Bladder 100 (Powles NEJM 2020). mOS is 15Mo in standard arm, 21 Mo in exp arm (Survival benefit ever seen before !)
4- MSI phenotype +/- in Lynch sd (especially in upper urinary tract carcinoma) and efficacy of ICI.

Paragraphs 7, 8 and 9 are interesting but need clarification/ synthesis at the end. They may be shortened.

Add a paragraph of combination : rationales and results. BCGtherapy and ICI ; ICI - ICI ; ICI chemo (phases III have arleady given some results)…

Add a paragraph on bladder cancer and molecular subtypes : what is the most responsive ? Basal ? Neuronal subgroup TCGA 2017 and response to atezo (Kim Eur Urol 2019)

Discussion has to be developed : discuss BCGtherapies, rationale of combination with immune checkpoint, why maintenance immune therapies are > combo (chem + immune checkpoints no benefit in OS at the moment).

Minor comments :
Not necessary to repeat each time Fig1. To add once at the begining of the paragraph.
Line 376 : Platinum based chemo rather than cisplatin because roughly 40% of patients are unfit for cisplatin.

Sincerely yours

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

Authors' response

Response to Editor and Reviewer Comments

Dear Editor-in-Chief Dr. Michal Heger:

Attached please find the revised version of our manuscript entitled "**Immune Escape Mechanisms and Immunotherapy of Urothelial Bladder Cancer**" (JCTRes-D-20-00138). We appreciated very much for those valuable comments and helpful suggestions from you and the reviewers, which have guided us to significantly improve the quality of our manuscript. We have thoroughly revised the manuscript accordingly, and the changes were highlighted in blue in the revised version.

Responses to Editor's comments

Question 1. For your guidance, reviewers' comments are appended below. Please understand that your manuscript requires an entire overhaul in terms of restructuring and language editing in addition to the points raised by the reviewers. The editorial board will focus on this heavily after receiving a revised draft.

Response: Thank you very much for your and the reviewers' comments, we have thoroughly revised the manuscript accordingly, and restructured and rephrased the language meticulously.

Question 2. If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Response: As suggested, the Response letter to Editor and Reviewer Comments and the revised manuscript with Tracked Changes have been submitted to the system.

Question 3. I would argue neoadjuvant is the standard of care. Suggest making it say "(neo)adjuvant". I find this confusing. You mention several therapies, so I am not sure what is meant by response rate. Do you mean sure rate when all of these are added together? Suggest rephrasing.

Response: As suggested, this sentence has been phrased to "Currently, transurethral resection of bladder tumor (TURBT), radical cystectomy, neoadjuvant systematic chemotherapy, and intravesical chemotherapy are the main treatment options for UBC [7]." (p4, lines 66-68).

The original sentence "However, the overall response rate (ORR) to these treatments is only 30% [3]." has been revised to "However, above standard therapies have remained unchanged for three decades [3]." (p4, lines 68-69).

Question 4. This is confusing. These drugs are also approved for bladder, but also many other

diseases not mentioned. Atezo is a 2 PD-L1 targeted Tx. Avelumab and durvalumab are also approved for bladder. Not sure what these other cancers have to do with it.

Response: As suggested, the structure of the manuscript has been revised thoroughly, especially the introduction section. In the paragraph 3 of the introduction section, we describe the history of immunotherapy for UBC. The original sentence has been phrased to "Immunotherapy has revolutionized cancer treatment in recent years, which enhances or inhibits the immune function of the body to achieve the purpose of the treatment of diseases." (p4, lines 72-74)
Additionally, the unrelated information about other tumor types has been deleted.

Question 5. These papers you cite are lung cancer papers. The relative risk of death is different by disease and setting. I am not sure how this relates to bladder cancer.

Response: As suggested, the irrelevant information in the manuscript has been deleted. And the original sentences "Three PD-1 inhibitors, pembrolizumab, nivolumab, and atezolizumab, have been approved by the US Food and Drug Administration (FDA) for the treatment of melanoma, non-small-cell lung cancer, and Hodgkin's lymphoma. PD-1 inhibitors reduce the risk of death by approximately 40% and significantly improve overall survival compared with chemotherapy (7, 8)." have been deleted.

Question 6. This is one long run-on sentence.

Response: Due the limit evidence of Loss of antigenicity in tumor cells supporting the immune escape mechanisms of UBC, the original **2.1 Loss of antigenicity in tumor cells** section has been deleted.

Question 7. Do you mean JAK inhibitors?

Response: Sorry for this confusion. "JNK" has been revised to "C-Jun N-terminal kinases (JNK)". (p8, lines 191-192)

Question 8. Consider expanding on this. Just knowing that Fas expression increases on BC cells compared to wt does not prove it acts as a regulator of immune escape.

Response: As suggested, The original sentence has been phrased to "Furthermore, gene expression studies demonstrated that the expression of Fas ligand (FasL) in UBC patients was higher than those of healthy individuals, regardless of grading and staging of tumors [42]. FasL-expressing UBC cells can induce Fas-mediated killing of autologous T lymphocytes both in vitro and in vivo. These findings suggested that FasL acted as an important regulator of immune

escape through the induction of T cell apoptosis [42]" (p8, lines 192-197).

Question 9. Please expand on this. It reads as an incomplete story.

Response: As suggested, the original sentence has been phrased to "Loskog et al. demonstrated that CD40L-transduced MB49 cells suppressed the production of IL-10 and TGF-β, which promoted the maturation and activation of DCs, and induced a Th1-type response and the activation of CTLs in the tumor area. These results suggested that immunosuppressive signaling molecules were the possible candidates for the treatment of UBC." (p9, lines 221-226).

Question 10. This is not a complete sentence. I think you are referring back to the section header, but this should be an independent sentence.

Response: This sentence has been phrased to "There are many kinds of immunosuppressive cells in tumor microenvironment, and myeloid-derived suppressor cells (MDSCs), T regulatory cells (Tregs) and M2-type tumor associated macrophages (TAMs) were reported to be functional in the microenvironment of UBC.", which has been revised into **2.1 Recruitment of Immunosuppressive Cells in Tumor Microenvironment** section (p6, lines 119-122).

Question 11. how does this promote chemo resistance? No mechanism described.

Response: The phrase "and promotion of chemotherapy resistance" has been deleted.

Question 12. And suggest adding it is the first checkpoint inhibitor approved for metastatic BC treatment.

Response: This sentence has been phrased to "Moreover, the first programmed cell death 1 (PD-1) ligands (PD-L1) inhibitor atezolizumab was approved for the treatment of metastatic UBC in 2016 [13]." (p5, lines 80-81).

Question 13. This does not in any way discuss the potential immune basis for why BCG is effective, which is purported to be the goal of this article. While the exact mechanisms of BCG remain somewhat elusive and uncertain, a lot of research has attempted to ask this question and should be discussed here.

Response: Thank you very much for your advice. The mechanisms of BCG in the treatment of bladder cancer have been added in the revised manuscript as follows:

Mechanically, BCG could generate oxidative stress in UBC cells, lead to cell apoptosis and necrosis of UBC cells, and induce the immune response in the host [57]. Firstly, BCG may

activate the TLR7 and the following caspase 8 signaling pathway in UBC cells, which initiated the extrinsic apoptosis pathway of UBC cells [58]. Another study demonstrated that BCG could also increase the expression of lysosomal hydrolase cathepsin B and activate pro-apoptotic protein BID and pro-caspase 9 in UBC cells, which initiated the intrinsic apoptosis pathway of UBC cells [59]. Besides apoptosis, BCG led to the caspase-independent cell membrane integrity damage, ultrastructural changes, and the release of necrosis associated chemokine high molecular group box protein 1 (HMGB1) [60]. Secondly, BCG induced the generation of nitric oxide synthase (iNOS) [61, 62] or reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) [63], which both produced nitric oxide (NO). This process led to the damage of DNA and proteins in UBC cells, causing cell apoptosis and autophagy ultimately [64]. Finally, BCG could activate nuclear factor kappa-B (NF-κB) and promote the transcription of cytokines, which participated in the immune response [65, 66]. BCG and the released cytokines could also activate $CD8^+$ CTLs, macrophages, neutrophils, NK cells, and others effector cells to kill tumor cells in distinct manners [67, 68]. (p11, lines 260-277)

Question 14. This is not true. BCG absolutely has toxicity.

Response: This sentence has been phrased to "Regarding safety, BCG is well tolerated and no grade 3 or 4 toxicity has been reported [54]." (p10, lines 254-255).

Question 15. All your discussion is in animals or cell models, not sure that makes it that promising

Response: "promising" has been revised to "encouraging". (p19, line 524)

Question 16. I have not heard this term. Please consider elucidating.

Response: "splenocyte-mediated cytotoxicity" has been revised to "complement--mediated cytotoxicity" (p19, line 530).

Question 17. I don't think it necessary to review the original data for these drugs that are now approved with updated data and results from larger, follow-up trials. The goal here should be to put the current knowledge in context

Response: As suggested, the data and results from larger, follow-up trials have been updated in the revised manuscript.

For example, Ref 77 (J Clin Oncol, 2018. 36(16): p. 1579-1587) has been updated with Ann Oncol. 2019 Jun 1;30(6):970-976. (p12-13, lines 310-322)

In another randomized phase III KEYNOTE-045 trial (NCT02256436) [76, 77], 542 participants who had locally advanced. metastatic or unresectable UBC that recurred or progressed after a platinum-based therapy were randomized to receive pembrolizumab 200 mg IV every three weeks or chemotherapy (paclitaxel, docetaxel, or vinflunine). The median 1- and 2-year OS rates of pembrolizumab group were 44.2% and 26.9%, respectively, which were higher than those in chemotherapy group (29.8% and 14.3%, respectively). Additionally, the ORR was also higher in pembrolizumab group (21.1%) compared to chemotherapy group (11.0%). Moreover, pembrolizumab prolonged the OS of patients with advanced UBC to 10.3 months, compared with 7.4 months in those who received chemotherapy. As a second-line therapy for platinum-refractory advanced UBC, pembrolizumab also exhibited a lower rate of treatment-related adverse events than chemotherapy [76].

Additionally, Ref 83 (Lancet Oncol, 2017. 18(3): p. 312-322.) has been updated with Clin Cancer Res. 2020 Oct 1;26(19):5120-5128. (p13-14, lines 349-360)

Nivolumab is another monoclonal antibody directed against PD-1. In a phase II, single-arm, open-label CheckMate 275 study (NCT02387996) [83], 270 patients with metastatic or unresectable locally advanced UBC were enrolled to receive nivolumab 3 mg/kg IV every 2 weeks until measured disease progression, clinical deterioration, or unacceptable toxicity. Tumor PD-L1 expression was also quantified as ≥5 percent or ≥1 percent. With the minimum follow-up of 33.7 months, the ORR, median PFS, and median OS (95% CI) were 20.7% (16.1-26.1), 1.9 months (1.9-2.3), and 8.6 months (6.1-11.3) in all patients, respectively. Additionally, the higher tumor mutational burden (TMB) was associated ($P < 0.05$) with improved ORR [OR (95% CI): 2.13 (1.26-3.60)], PFS [HR: 0.75 (0.61-0.92)], and OS [HR: 0.73 (0.58-0.91)]. These results indicated that nivolumab was clinical benefit with satisfactory safety profile and was initially approved by the FDA in February of 2017 [14].

Question 18. This is not true. Tumor cells do not necessarily "ubiquitously express PD-L1." There is actually a wide range, differing by tumor type, in a specific tumor histology, and even with heterogeneity in a single individual tumor. Not sure this means what you think it means.

Response: As suggested, this sentence has been phrased to "In cancer, PD-L1 expressing tumor cells could bind to the PD-1 receptors expressed in T cells.**"** (p12, lines 290-291)

Question 19. While Dr Plimack was the first author on one of the early reports of this arm of the trial, this was a very large multi-armed, Pharma sponsored trial that Dr.Plimack was not in

charge of.

Response: "Plimack et al." has been revised to "Merck Sharp & Dohme Corp." (p12, line 303)

Question 20. Please consider including the role and data for maintenance avelumab in metastatic disease after platinum-based chemo.

Response: According to the reviewers' and your advice, we have restructured **3.2 Checkpoint Inhibitors** section and supplemented more denials about different approved checkpoint inhibitors. (p11-19, lines 279-511). The data about avelumab also has been updated as follows (p16-17, lines 432-457):

3.2.1.5 Avelumab

Avelumab is another anti-PD-L1 IgG1 monoclonal antibody. In order to assess the safety and antitumor activity of avelumab, forty-four patients with refractory metastatic UBC were recruited in Multicenter, Phase I Study (NCT01772004) [90]. All the patients received avelumab 10 mg/kg intravenously every 2 weeks after platinum-based chemotherapy and unselected for PD-L1 expression. In the analysis process, PD-L1 positivity was defined as expression by immunohistochemistry on ≥ 5% of tumor cells. In a median of 16.5 months of follow-up, the ORR was 18.2% (95% CI, 8.2% to 32.7%; five complete responses and three partial responses), and seven of eight responding patients had PD-L1-positive tumors. Additionally, the responses were ongoing in six patients (75.0%). The median PFS was 11.6 weeks (95% CI, 6.1 to 17.4 weeks), the median OS was 13.7 months (95% CI, 8.5 months to not estimable), and the 12-month OS rate of 54.3% (95% CI, 37.9% to 68.1%). Furthermore, the most frequent treatment-related adverse events of any grade were fatigue/asthenia (31.8%), infusion-related reaction (20.5%), and nausea (11.4%). Grades 3 to 4 treatment-related adverse events occurred in three patients (6.8%) including asthenia, AST elevation, creatine phosphokinase elevation, and decreased appetite.

In 2020, a phase III clinical trial (NCT02603432) demonstrated that avelumab significantly improved survival in patients who developed the most common type of UBC. In that program, treatment with avelumab resulted in a 31% reduction in the risk of death and a median OS of 21.4 months, compared to 14.3 months for patients not treated with the drug [91]. Overall, avelumab showed strong antitumor activity with an acceptable safety profile and prolonged survival in patients with platinum-refractory metastatic UBC, with greater activity noted in PD-L1 positive tumors. These results accelerated the FDA approval for this indication.

Question 21. What does this mean?

Response: This sentence has been phrased to "However, some issues including side-effects and curative effect need to be addressed [101]." (p19, lines 508-509).

Question 22. There is no proof of this for which I am aware.

Response: This sentence "a combination of chemotherapy and CTLA4 blockade can increase overall response and survival in metastatic BC patients." has been deleted.

Question 23. the metinel nodes

Response: "the metinel nodes" has been revised to "the sentinel nodes". (p20, line 538).

Question 24. Worthy of further study. This is not an approved therapy

Response: This sentence has been phrased to "However, UBC specific DCs based cancer immunity still needs to be further explored." (p21, lines 571-572).

Responses to Re viewer #1's comments

Question 1. While the manuscript is clearly thoroughly researched, I think it could be improved by taking a bit of a narrower focus. Starting from the title, it is not clear what the true goal or focus of the paper is, and that theme persists throughout.

The title suggests discussions of immune escape/resistance mechanisms, but that is not really addressed in any great detail in the paper.

Response: This manuscript focuses on the recent progress in **immune escape mechanisms and immunotherapy of urothelial bladder cancer** (UBC). As suggested, we have thoroughly restructured and revised the manuscript with more details and evidence to meet the requirement of the journal.

1. Introduction

2. Immune Escape Mechanisms of UBC

2.1 Recruitment of Immunosuppressive Cells in Tumor Microenvironment

2.1.1 Myeloid-Derived Suppressor Cells

2.1.2 Regulatory T Cells

2.1.3 Tumor-Associated Macrophages

2.2 Upregulation of Immunosuppressive Molecules

2.3 Secretion of Immunosuppressive Signaling Molecules

2.4 Dendritic Cells

3. Immunotherapy of UBC

3.1 Bacillus Calmette-Guerin

3.2 Checkpoint Inhibitors

3.2.1 PD-1/PD-L1

3.2.1.1 Pembrolizumab

3.2.1.2 Nivolumab

3.2.1.3 Atezolizumab

3.2.1.4 Durvalumab

3.2.1.5 Avelumab

3.2.2 CTLA-4

3.3 Cytokines

3.4 Adoptive T Cell Immunotherapy

3.5 Dendritic Cells

3.6 Macrophages

4. Discussion

Specifically, the immune escape mechanisms of UBC were elaborated in the **2. Immune escape mechanisms of UBC** section in the revised manuscript, which was supplemented with more references and details. (p6-10, lines 110-234)

Question 2. The introduction talks about bladder cancer in general, and then immunotherapy with checkpoint blockade in general, but does not clearly let the reader know what the paper will be about.

Response: Sorry for this confusion. The introduction has been restructured thoroughly as follows:

Paragraph (Para) 1 introduced the epidemiology and pathogenic factors of UBC, Para 2 described the type and clinical characteristics of UBC, Para 3 demonstrated the development of immunotherapies for UBC, Para 4 described the relationship between the microenvironment and the immunotherapy of UBC and the focus of this review. Thus, the revised introduction section was consistent with the focus of the whole manuscript.

Question 3. Then there is a section on various immune microenvironmental pathways involved in tumor immunity, but each is 1-2 sentences, so they do not provide a very in depth understanding of any of these pathways, and it is not clear that these connect to the discussions on individual therapies.

Response: This manuscript focuses on the recent progress in immune escape mechanisms and immunotherapy of UBC. As suggested, we have thoroughly restructured and revised the manuscript with more details and evidence to meet the requirement of the journal. Specifically, the 2. Immune suppression in UBC microenvironment section has been revised to **2. Immune escape mechanisms of UBC** section in the revised manuscript, which elaborated the immune escape mechanisms of UBC with more references and details. (p6-10, lines 110-234)

Question 4. The next section describes some history of therapies for bladder cancer, as well as some of the history of immunotherapy, but other than the part about BCG, I'm not sure it fits the rest of the paper.

Response: As suggested, we have restructured and revised the manuscript thoroughly to make the manuscript more focused and logical. For example, Para 2 demonstrated the development of traditional UBC treatments and Para 3 demonstrated the development of immunotherapies for UBC including BCG. (p4-5, lines 66-96)

However, the history of immunotherapy has been deleted.

Question 5. Much of the history section is just a repeat of Figure 1 from line form to sentence form. And I'm not sure all the important highlights of the Hx of immunotherapy are truly addressed.

Response: To make the immunotherapy to be the focus of this manuscript, the traditional methods section has been revised to the Para 2 of introduction as suggested (p4, lines 66-71). Furthermore, Para 3 of introduction described the development of immunotherapies for UBC based on checkpoint inhibitors (p4-5, lines 72-96).

More importantly, Figure 1 has been modified to focus the development of immunotherapies for UBC and the section **3. Immunotherapy of UBC** has been supplemented with more denials. (p10-22, lines 236-603).

Question 6. Next the paper moves to systemic tx for bladder cancer. It includes approved immunotherapies (aPD1, BCG) as well as investigational therapies. Again, I think the decision to be inclusive is to the detriment of new understanding of any of these therapies. Particularly in the anti-PD1 section, the authors spend time reviewing results from the initial phase I studies, yet do not comment on larger more contemporary Phase 3 single agent or combo studies, including leaving out data on maintenance avelumab which is now the standard of care. Following this, they move on to more investigational therapies, which are exciting, but details about where these therapies stand in context to the landscape of trials in BC is given short shrift.

Response: As suggested, we have restructured and revised the manuscript thoroughly to make the manuscript more focused and logical, and the data and results from larger, follow-up trials have been updated in the revised manuscript. (p11-19, 279-511)

For example, Ref 77 (J Clin Oncol, 2018. 36(16): p. 1579-1587) has been updated with Ann Oncol. 2019 Jun 1;30(6):970-976. (p12-13, lines 310-322)

In another randomized phase III KEYNOTE-045 trial (NCT02256436) [76, 77], 542 participants who had locally advanced. metastatic or unresectable UBC that recurred or progressed after a platinum-based therapy were randomized to receive pembrolizumab 200 mg IV every three weeks or chemotherapy (paclitaxel, docetaxel, or vinflunine). The median 1- and 2-year OS rates of pembrolizumab group were 44.2% and 26.9%, respectively, which were higher than those in chemotherapy group (29.8% and 14.3%, respectively). Additionally, the ORR was also higher in pembrolizumab group (21.1%) compared to chemotherapy group

(11.0%). Moreover, pembrolizumab prolonged the OS of patients with advanced UBC to 10.3 months, compared with 7.4 months in those who received chemotherapy. As a second-line therapy for platinum-refractory advanced UBC, pembrolizumab also exhibited a lower rate of treatment-related adverse events than chemotherapy [76].

Additionally, Ref 83 (Lancet Oncol, 2017. 18(3): p. 312-322.) has been updated with Clin Cancer Res. 2020 Oct 1;26(19):5120-5128. (p13-14, lines 349-360)

Nivolumab is another monoclonal antibody directed against PD-1. In a phase II, single-arm, open-label CheckMate 275 study (NCT02387996) [83], 270 patients with metastatic or unresectable locally advanced UBC were enrolled to receive nivolumab 3 mg/kg IV every 2 weeks until measured disease progression, clinical deterioration, or unacceptable toxicity. Tumor PD-L1 expression was also quantified as ≥5 percent or ≥1 percent. With the minimum follow-up of 33.7 months, the ORR, median PFS, and median OS (95% CI) were 20.7% (16.1-26.1), 1.9 months (1.9-2.3), and 8.6 months (6.1-11.3) in all patients, respectively. Additionally, the higher tumor mutational burden (TMB) was associated ($P < 0.05$) with improved ORR [OR (95% CI): 2.13 (1.26-3.60)], PFS [HR: 0.75 (0.61-0.92)], and OS [HR: 0.73 (0.58-0.91)]. These results indicated that nivolumab was clinical benefit with satisfactory safety profile and was initially approved by the FDA in February of 2017 [14].

Moreover, more investigational therapies have been supplemented.

In 2017, Shenzhen Geno-Immune Medical Institute carried out a Phase I/II and multicenter study including 20 locally advanced or metastatic UBC patients who have no further treatment available, to evaluate the efficacy and safety of 4SCAR-T cells (Fig.1 and Fig. 2, NCT03185468). (p20, lines 548-551)

Question 7. I recommend to the authors deciding what they would like to most focus on. Is this a review article about current immunotherapies for bladder cancer? If so, more detail about the history of BCG and checkpoint blockade might be helpful, and then I would suggest more updated data including phase 3 studies and combo treatments would be needed.

Then one might choose to speculate at how combinations will move in the future, and/or maybe include some info about pathways and discuss potential strategies to overcome resistance as the title implied.

Response: This manuscript focuses on the recent progress in immune escape mechanisms and immunotherapy of UBC.

1) As suggested, more detail about the history of BCG and checkpoint blockade have

been supplemented and updated with more details and cohorts. For example,

3.1 Bacillus Calmette-Guerin

BCG is a live, slow-growing, attenuated form of *Mycobacterium bovis*, which was discovered by French scientists Albert Léon Charles Calmette and Camille Guérin. Previously, it was used as a vaccine for newborns to prevent tuberculosis [50]. Currently, BCG is the gold-standard intravesical immunotherapy for the treatment of NMIBC. The potential of BCG to treat UBC was discovered by Morales et al., who successfully injected BCG into the bladder for the treatment of recurrent superficial UBC in 1976 [9] (Fig. 1) .

Encouraging data suggest that BCG treatment reduces long-term tumor relapse rate, tumor progression, tumor metastasis, and mortality of UBC patients. For example, a meta-analysis showed that intravesical instillation of BCG combined with TURBT could reduce the risk of UBC recurrence compared with TURBT alone [51]. In patients with medium- or high-risk Stage Ta and T1 UBC, combined TURBT and BCG treatment led to a reduction of approximately 56% in recurrence rate compared with TURBT alone [51]. Furthermore, clinical studies have shown that the recurrence rate with BCG perfusion and continuous treatment was about 32% lower than that achieved with anti-tumor antibiotic mitomycin C [52]. Finally, BCG also reduced the risk of progression from NMIBC to MIBC [53]. Regarding safety, BCG is well tolerated and no grade 3 or 4 toxicity has been reported [54]. Based on these findings, the FDA approved intravesical BCG instillation for treatment of NMIBC in 1990 [43, 55] (Fig. 1). Currently, the European Association of Urology, American Urological Association, and Urological Guidelines of China provide comprehensive guidance for BCG as a standard treatment for intermediate- and high-risk NMIBC patients [56].

Mechanically, BCG could generate oxidative stress in UBC cells, lead to cell apoptosis and necrosis of UBC cells, and induce the immune response in the host [57]. Firstly, BCG may activate the TLR7 and the following caspase 8 signaling pathway in UBC cells, which initiated the extrinsic apoptosis pathway of UBC cells [58]. Another study demonstrated that BCG could also increase the expression of lysosomal hydrolase cathepsin B and activate pro-apoptotic protein BID and pro-caspase 9 in UBC cells, which initiated the intrinsic apoptosis pathway of UBC cells [59]. Besides apoptosis, BCG led to the caspase-independent cell membrane integrity damage, ultrastructural changes, and the release of necrosis associated chemokine high molecular group box protein 1 (HMGB1) [60]. Secondly, BCG induced the generation of nitric oxide synthase (iNOS) [61, 62] or reactive oxygen species (ROS) such as hydrogen peroxide

(H_2O_2) [63], which both produced nitric oxide (NO). This process led to the damage of DNA and proteins in UBC cells, causing cell apoptosis and autophagy ultimately [64]. Finally, BCG could activate nuclear factor kappa-B (NF-κB) and promote the transcription of cytokines, which participated in the immune response [65, 66]. BCG and the released cytokines could also activate $CD8^+$ CTLs, macrophages, neutrophils, NK cells, and others effector cells to kill tumor cells in distinct manners [67, 68]. (p10-11, lines 237-277).

3.2.1.1 Pembrolizumab

Pembrolizumab is a monoclonal antibody targeting the PD-1 receptor initially approved for advanced melanoma, which could prolong OS with less toxicity and improved quality of life compared to additional lines of chemotherapy. In 2013, Merck Sharp & Dohme Corp. first carried out a non-randomized, open-label, phase Ib clinical study KEYNOTE-012 (NCT01848834, Fig. 1) to assess the efficacy and safety of pembrolizumab [75]. After a median follow-up of 13 months, seven of 27 assessable patients showed significant overall response (OR). The median progression-free survival (PFS) and OS were 2.0 and 12.7 months, respectively. However, 53% of UBC patients experienced drug-related adverse reactions, and three patients experienced five serious treatment-related adverse events.

In another randomized phase III KEYNOTE-045 trial (NCT02256436) [76, 77], 542 participants who had locally advanced. metastatic or unresectable UBC that recurred or progressed after a platinum-based therapy were randomized to receive pembrolizumab 200 milligrams (mg) intravenous (IV) every three weeks or chemotherapy (paclitaxel, docetaxel, or vinflunine). The median 1- and 2-year OS rates of pembrolizumab group were 44.2% and 26.9%, respectively, which were higher than those in chemotherapy group (29.8% and 14.3%, respectively). Additionally, the ORR was also higher in pembrolizumab group (21.1%) compared to chemotherapy group (11.0%). Moreover, pembrolizumab prolonged the OS of patients with advanced UBC to 10.3 months, compared with 7.4 months in those who received chemotherapy. As a second-line therapy for platinum-refractory advanced UBC, pembrolizumab also exhibited a lower rate of treatment-related adverse events than chemotherapy [76].

The effect of pembrolizumab was also examined in the first-line therapy. For example, the phase II KEYNOTE-052 (NCT02335424) study recruited 370 advanced UBC patients who were not suitable for cisplatin-based therapy and treated with 200 mg pembrolizumab every three weeks for up two years [78]. The ORR was 29% for the entire cohort, including 9% complete response and 20% partial response. The median duration of response was 30 months,

with a median OS of 11.3 months [78, 79]. In another large phase III trial, KEYNOTE 361 trial (NCT02853305) [80], the effect of pembrolizumab as a monotherapy was compared with chemotherapy with gemcitabine and cisplatin or carboplatin versus chemotherapy with pembrolizumab followed by maintenance pembrolizumab [81]. Approximately 1010 patients with advanced UBC were recruited and randomized in 1:1:1 fashion. The ORR of the combination group was 54.7%, which was better than those of chemotherapy group (44.9%) or pembrolizumab group (30.3%). Moreover, the median PFS of the combination group was 8.3 months, which was better than those of chemotherapy group (7.1 months) or pembrolizumab group (3.9 months). Additionally, the median OS of the combination group was 17.0 months, which was also better than those of chemotherapy group (14.3 months) or pembrolizumab group (15.6 months).

Although the results of KEYNOTE 361 has dampened the enthusiasm regarding the pembrolizumab as a first line therapy solely, the combination with chemotherapy of other agents provided a promising direction. Recently, the effect of another agent with antibody drug conjugate (ADC) enfortumab and pembrolizumab were examined in EV-103 study including first-line cisplatin-ineligible cohort of 45 patients [82]. The ORR was 73.3% (95% confidence interval (CI), 58.1, 85.4) and seven patients with liver metastasis had a response rate of 53.3%, showing this potent combination. (p12-13, lines 299-346).

2) Additionally, phase 3 studies and combo treatments have also been updated. For example.

In a phase III trial IMvigor211 (NCT02302807), 931 patients with metastatic UBC who have previously failed platinum-based chemotherapy were enrolled and were randomly assigned to either atezolizumab or chemotherapy (vinflunine, paclitaxel, or docetaxel) [87]. In the IC2/3 population (n=234), the OS and ORR were similar between the atezolizumab group and the chemotherapy group. However, the duration of response was remarkably longer in the atezolizumab group than in the chemotherapy group (median 15·9 months [95% CI 10·4 to not estimable] vs 8.3 months [5·6-13·2]; HR 0.57, 95% CI 0·26-1·26). Safety analysis also favored atezolizumab with lower high-grade toxicities (20 versus 43 percent) and lower incidence of treatment discontinuation (7 versus 18 percent). Additionally, 195 patients were classified into luminal (n=73) and basal (n=122) subtypes as according to the gene expression profile defined by TCGA. Surprisingly, the ORR in the luminal cluster II subtype (34%) was significantly higher than those in luminal cluster subtype I (10%), basal cluster subtype III (16%), and basal cluster subtype IV (20%). Furthermore, the median mutation load was significantly increased

in responders (12.4 per megabase [Mb]) compared with non-responders (6.4 per Mb). Taken together, atezolizumab was approved for patients with locally advanced or metastatic UBC who progressed on or after platinum-based chemotherapy (Fig. 1). (p15, lines 392-409).

3) Moreover, the pathways, combinations and potential strategies to overcome resistance have been added in the revised manuscript. For example,

Although the results of KEYNOTE 361 has dampened the enthusiasm regarding the pembrolizumab as a first line therapy solely, the combination with chemotherapy of other agents provided a promising direction. Recently, the effect of another agent with antibody drug conjugate (ADC) enfortumab and pembrolizumab were examined in EV-103 study including first-line cisplatin-ineligible cohort of 45 patients [82]. The ORR was 73.3% (95% confidence interval (CI), 58.1, 85.4) and seven patients with liver metastasis had a response rate of 53.3%, showing this potent combination. (p13, lines 340-346)

Therefore, combination therapy could be investigated to improve treatment outcomes, such as the dual immune combination regimen of PD-1/PDL-1 inhibitors with CTLA-4 inhibitors. For example, P. Sharma, et al. improved the ORR of patients with nivolumab plus ipilimumab combination therapy [98]. CTLA-4 inhibitors go to the source and increase the anti-cancer T-cell numbers, while PD-1 inhibitors act in peripheral blood or tumors, allowing the binding process of PD-1 to PD-L1 to be blocked in these viable agents, thus freeing ICs to kill and launch a strike against the tumor [126]. Another combination strategy is composed of immune checkpoint blockade and chemotherapy. For example, the combination of pembrolizumab, and gemcitabine and cisplatin or carboplatin enhanced the ORR, median PFS and median OS of UBC patients compared to those of chemotherapy or pembrolizumab [81]. (p23-24, lines 650-660)

Question 8. Another route would be to focus on novel therapies for bladder cancer, with more time given to NK cells, TAMs, DCs, etc.

Response: The novel therapies for UBC have been revised to **3.5 Dendritic cells and 3.6 Macrophages sections**. However, the NK cells section lack of necessary evidence supporting our manuscript, and has been deleted.

Question 9. Thus, more detail about the preclinical and clinical work that has been done would be informative, and then where things stand currently in the clinic and how the authors see

things moving forward.

Response: 1) As suggested, more detail about the preclinical and clinical work, and the future directions have been added in the revised manuscript as follows:

In 2017, Shenzhen Geno-Immune Medical Institute carried out a Phase I/II and multicenter study including 20 locally advanced or metastatic UBC patients who have no further treatment available, to evaluate the efficacy and safety of 4SCAR-T cells (Fig.1 and Fig. 2, NCT03185468). (p20, lines 548-551)

2) Our opinions about the specific treatments or future development of the immunotherapies of UBC have been supplemented in the end of each section or the discuss section, respectively. For example,

"Currently, five checkpoint inhibitors, atezolizumab, avelumab, durvalumab, nivolumab, and pembrolizumab, are available for the treatment of UBC [100]. However, some issues including side-effects and curative effect need to be addressed [101]. Therefore, further research and consideration of interventions such as combinational therapies are warranted to improve the clinical activities of PD-1/PD-L1 inhibitors in the future." (p19, lines 506-511)

"However, despite these anti-tumor effects, cytokines did not show great advantage over BCG therapy." (p19, lines 530-531)

"Therefore, combination therapy could be investigated to improve treatment outcomes, such as the dual immune combination regimen of PD-1/PDL-1 inhibitors with CTLA-4 inhibitors. For example, P. Sharma, et al. improved the ORR of patients with nivolumab plus ipilimumab combination therapy [98]. CTLA-4 inhibitors go to the source and increase the anti-cancer T-cell numbers, while PD-1 inhibitors act in peripheral blood or tumors, allowing the binding process of PD-1 to PD-L1 to be blocked in these viable agents, thus freeing ICs to kill and launch a strike against the tumor [126]. Another combination strategy is composed of immune checkpoint blockade and chemotherapy. For example, the combination of pembrolizumab, and gemcitabine and cisplatin or carboplatin enhanced the ORR, median PFS and median OS of UBC patients compared to those of chemotherapy or pembrolizumab [81]." (p23-24, lines 650-660)

Question 10. As is, I cannot recommend the paper for publication. Please see my attached specific edits for more detailed comments.

Response: Sorry for the faultiness of the previous manuscript. We appreciated very much for those valuable comments and helpful suggestions from you, which have guided us to significantly improve the quality of our manuscript. We have thoroughly revised the manuscript accordingly, including the reconstruction of the introduction section, the supplement of more

clinical data and necessary discussion. We hope that the revised manuscript could meet the requirement of the journal. Thank you again for your valuable suggestions.

Responses to Reviewer #3's comments

Major comments:

Question 1. Rather than speaking about bladder cancer, it is better to speak about urothelial carcinoma, from bladder cancer or upper urinary tract.

Response: As suggested, "bladder cancer" has been revised to "urothelial bladder carcinoma (UBC)" thought the manuscript. (p 1, line 1, p 3, line 31, p 4, line 49, etc.).

Question 2. Line 38: the level of evidence of neo adjuvant chemo prior to radical cystectomy is really higher than adjuvant chemo. This sentence has to be more accurate and rephrased.

Response: The original sentence has been deleted.

Question 3. Immune therapies such as BCG therapy and immune checkpoint inhibitors (in urothelial carcinoma) need to be more developed in introduction in order to highlight their importance compared to other approaches.

Response: As suggested, Para 3 of introduction has been revised to demonstrate the development of immunotherapies for UBC including BCG and immune checkpoint inhibitors.

Immunotherapy has revolutionized cancer treatment in recent years, which enhances or inhibits the immune function of the body to achieve the purpose of the treatment of diseases. The development of immunotherapy for UBC is shown in Figure 1. In 1976, Morales et al. first reported the treatment of UBC with BCG [9]. Furthermore, Lamm et al. confirmed the effect of BCG in the treatment of UBC in 1980 [10]. Additionally, more evidence proved that BCG is an effective biological immunotherapy in treating carcinoma in situ, preventing tumor progression and postoperative recurrence, and improving survival rate of UBC patients [11, 12]. Moreover, the first programmed cell death 1 (PD-1) ligands (PD-L1) inhibitor atezolizumab was approved for the treatment of metastatic UBC in 2016 [13]. Then in 2017, the US Food and Drug Administration (FDA) approved additionally four immune checkpoint drugs for the treatment of UBC. Specifically, nivolumab and avelumab were approved for the treatment of locally advanced or metastatic UBC on February 2 [14] and May 9 [15], respectively. Furthermore, durvalumab developed by AstraZeneca received accelerated approval from the FDA for the treatment of patients with locally advanced or metastatic UBC after failure of a platinum-containing regimen on May 1 [16]. Based on the results of the KEYNOTE-045 test, the FDA also approved pembrolizumab for certain locally advanced or metastatic UBC patients on May 18 [17]. Furthermore, pembrolizumab was also approved by the FDA on January 8, 2020 for the treatment of NMIBC patients, which is the first PD-1 inhibitor approved for the treatment of

specific high-risk NMIBC patients [18]. Finally, the European Commission (EC) has approved the anti-PD-L1 therapy avelumab as a monotherapy for the first-line maintenance treatment of adult patients with locally advanced or metastatic UBC who have not progressed after receiving first-line platinum-containing chemotherapy in 2021 [19]. (p4-5, lines 72-96).

Question 4. Line 93: this sentence has to be rephrased, and it is supposed to introduce this paragraph.

Response: This sentence has been phrased to "There are many kinds of immunosuppressive cells in tumor microenvironment, and myeloid-derived suppressor cells (MDSCs), regulatory T cells (Tregs) and M2-type tumor associated macrophages (TAMs) were reported to be functional in the microenvironment of UBC." (p6, lines 119-122).

Question 5. Paragraph 4: Results of studies have to be more accurately reported; it is necessary to understand why BCG therapy was approved. Which results based on?

Response: As suggested, the specific results of studies about the effect of BCG therapy in UBC have been supplemented in the 3.1 Bacillus Calmette-Guerin section.

3.1 Bacillus Calmette-Guerin

BCG is a live, slow-growing, attenuated form of *Mycobacterium bovis*, which was discovered by French scientists Albert Léon Charles Calmette and Camille Guérin. Previously, it was used as a vaccine for newborns to prevent tuberculosis [50]. Currently, BCG is the gold-standard intravesical immunotherapy for the treatment of NMIBC. The potential of BCG to treat UBC was discovered by Morales et al., who successfully injected BCG into the bladder for the treatment of recurrent superficial UBC in 1976 [9] (Fig. 1) .

Encouraging data suggest that BCG treatment reduces long-term tumor relapse rate, tumor progression, tumor metastasis, and mortality of UBC patients. For example, a meta-analysis showed that intravesical instillation of BCG combined with TURBT could reduce the risk of UBC recurrence compared with TURBT alone [51]. In patients with medium- or high-risk Stage Ta and T1 UBC, combined TURBT and BCG treatment led to a reduction of approximately 56% in recurrence rate compared with TURBT alone [51]. Furthermore, clinical studies have shown that the recurrence rate with BCG perfusion and continuous treatment was about 32% lower than that achieved with anti-tumor antibiotic mitomycin C [52]. Finally, BCG also reduced the risk of progression from NMIBC to MIBC [53]. Regarding safety, BCG is well tolerated and no

grade 3 or 4 toxicity has been reported [54]. Based on these findings, the FDA approved intravesical BCG instillation for treatment of NMIBC in 1990 [43, 55] (Fig. 1). Currently, the European Association of Urology, American Urological Association, and Urological Guidelines of China provide comprehensive guidance for BCG as a standard treatment for intermediate- and high-risk NMIBC patients [56].

Mechanically, BCG could generate oxidative stress in UBC cells, lead to cell apoptosis and necrosis of UBC cells, and induce the immune response in the host [57]. Firstly, BCG may activate the TLR7 and the following caspase 8 signaling pathway in UBC cells, which initiated the extrinsic apoptosis pathway of UBC cells [58]. Another study demonstrated that BCG could also increase the expression of lysosomal hydrolase cathepsin B and activate pro-apoptotic protein BID and pro-caspase 9 in UBC cells, which initiated the intrinsic apoptosis pathway of UBC cells [59]. Besides apoptosis, BCG led to the caspase-independent cell membrane integrity damage, ultrastructural changes, and the release of necrosis associated chemokine high molecular group box protein 1 (HMGB1) [60]. Secondly, BCG induced the generation of nitric oxide synthase (iNOS) [61, 62] or reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) [63], which both produced nitric oxide (NO). This process led to the damage of DNA and proteins in UBC cells, causing cell apoptosis and autophagy ultimately [64]. Finally, BCG could activate nuclear factor kappa-B (NF-κB) and promote the transcription of cytokines, which participated in the immune response [65, 66]. BCG and the released cytokines could also activate $CD8^+$ CTLs, macrophages, neutrophils, NK cells, and others effector cells to kill tumor cells in distinct manners [67, 68]. (p10-11, lines 237-277).

Question 6. Paragraph 5 may be shortened, since there is no major advance in this lead. Add a ref to the last sentence lines 182-183. I am not sure about this statement.

Response: As suggested, Paragraph 5 has been shortened and revised into **3.3 Cytokines** section, in which the original last sentence has been deleted. (p19 line 513-531).

Question 7. Paragraph 6 Line 197: the adverb ubiquitously has to be verified.

Response: This sentence has been phrased to "In cancer, PD-L1 expressing tumor cells could bind to the PD-1 receptors expressed in T cells." (p12, lines 290-291).

Question 8. Paragraph 6.1 has to be lengthened! This is the major milestone reached in urothelial carcinoma!

1- Discuss results in fit and unfit patients (to cisplatin). Mut load and response (Balar Lancet 2017)

2- Discuss in 2nd line, the link between objective response and duration of response (Fradet Ann Oncol 2019).

3- Speak about the rationale of treatment of maintenance, and cite and discuss Javelin Bladder 100 (Powles NEJM 2020). mOS is 15Mo in standard arm, 21 Mo in exp arm (benefit ever seen before!)

4- MSI phenotype +/- in Lynch sd (especially in upper urinary tract carcinoma) and efficacy of ICI

Response: As suggested, **3.2 Checkpoint inhibitors** section has been elaborated described in the revised manuscript. **(**p11-19, lines 279-511).

All above references have been cited in the revised manuscript.

1.1 Balar Lancet 2017 has been cited as Ref 85

In Cohort 1, 119 cisplatin-ineligible patients with locally advanced and metastatic UBC patients were enrolled to assess the efficacy of atezolizumab as a first-line treatment [85]. For a median follow-up of 17.2 months, the objective response rate was 23% (95% CI 16–31) and the complete response rate was 9%. Additionally, the median PFS was 2.7 months and median OS was 15.9 months. In Cohort 2, 310 UBC patients who had disease progression during or following a prior platinum-based chemotherapy regimen were enrolled [86]. PD-L1 expression on tumor-infiltrating ICs were prospectively determined by immunohistochemistry, and all the patients were categorized into in three different groups based on percentage of PD-L1-positive immune cells: IC0 (<1 percent expression), IC1 (≥1 percent, but ≤5 percent expression), and IC2/3 (≥5 percent expression). For patients with a minimum of 6 weeks of follow-up, the objective response rates were 26% (95% CI 18 to 36) in the IC2/3 group, 18% (95% CI 13 to 24) in the IC1/2/3 group and 15% (95% CI 11 to 19) in all patients. Additionally, the median OS were 11.4 months (95% CI 9.0 to not estimable) in the IC2/3 group, 8.8 months (95% CI 7.1 to 10.6) in the IC1/2/3, and 7.9 months (95% CI 6.6 to 9.3) in all patients. The results firstly demonstrated that atezolizumab was active in UBC. These results indicated that atezolizumab demonstrated encouraging durable response rates, survival, and tolerability, supporting its therapeutic use in untreated UBC. **(**p14-15, lines 373-391).

1.2 The relationship between mutation load and response have also been added as follows:

Additionally, the higher tumor mutational burden (TMB) was associated (P < 0.05) with improved ORR [OR (95% CI): 2.13 (1.26-3.60)], PFS [HR: 0.75 (0.61-0.92)], and OS [HR: 0.73 (0.58-0.91)]. (p14, lines 356-358).

Furthermore, the median mutation load was significantly increased in responders (12.4 per megabase [Mb]) compared with non-responders (6.4 per Mb). (p15, lines 406-407).

2.1 Fradet Ann Oncol 2019 has been cited as Ref 77

In another randomized phase III KEYNOTE-045 trial (NCT02256436) [76, 77], 542 participants who had locally advanced. metastatic or unresectable UBC that recurred or progressed after a platinum-based therapy were randomized to receive pembrolizumab 200 mg IV every three weeks or chemotherapy (paclitaxel, docetaxel, or vinflunine). The median 1- and 2-year OS rates of pembrolizumab group were 44.2% and 26.9%, respectively, which were higher than those in chemotherapy group (29.8% and 14.3%, respectively). Additionally, the ORR was also higher in pembrolizumab group (21.1%) compared to chemotherapy group (11.0%). Moreover, pembrolizumab prolonged the OS of patients with advanced UBC to 10.3 months, compared with 7.4 months in those who received chemotherapy. As a second-line therapy for platinum-refractory advanced UBC, pembrolizumab also exhibited a lower rate of treatment-related adverse events than chemotherapy [76]. (p12-13, lines 310-322).

2.2 The link between objective response and duration of response have also been added as follows:

The median duration of response was 30 months, with a median OS of 11.3 months [78, 79]. (p13, lines 327-328).

However, the duration of response was remarkably longer in the atezolizumab group than in the chemotherapy group (median 15.9 months [95% CI 10.4 to not estimable] vs 8.3 months [5.6-13.2]; HR 0.57, 95% CI 0.26-1.26). (p15, lines 396-399).

3.1 Powles NEJM 2020 has been cited as Ref 91

In 2020, a phase III clinical trial (NCT02603432) demonstrated that avelumab significantly improved survival in patients who developed the most common type of UBC. In that program, treatment with avelumab resulted in a 31% reduction in the risk of death and a median OS of 21.4 months, compared to 14.3 months for patients not treated with the drug [91]. (p17, lines 450-454).

3.2 The rationale of treatment of maintenance have also been added as follows:

In another large phase III trial, KEYNOTE 361 trial (NCT02853305) [80], the effect of pembrolizumab as a monotherapy was compared with chemotherapy with gemcitabine and cisplatin or carboplatin versus chemotherapy with pembrolizumab followed by maintenance pembrolizumab [81]. Approximately 1010 patients with advanced UBC were recruited and randomized in 1:1:1 fashion. The ORR of the combination group was 54.7%, which was better than those of chemotherapy group (44.9%) or pembrolizumab group (30.3%). Moreover, the median PFS of the combination group was 8.3 months, which was better than those of chemotherapy group (7.1 months) or pembrolizumab group (3.9 months). Additionally, the median OS of the combination group was 17.0 months, which was also better than those of chemotherapy group (14.3 months) or pembrolizumab group (15.6 months). **(**p13, lines 328-339).

Combination immunotherapy with nivolumab and ipilimumab for locally advanced or metastatic UBC is under intensive investigation, which has proven to be effective in other forms of malignancy with potentiated cancer immune response with the dual-agent approach, followed by nivolumab maintenance therapy [97]. In an open-label phase II study CheckMate 032 (NCT01928394) [98], 274 patients with advanced or metastatic UBC previously treated with platinum-based chemotherapy were enrolled to investigated this regimen. Patients were randomly assigned to receive single-agent nivolumab 3mg/kg (N group) or nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (NI group) or nivolumab 1mg/kg plus ipilimumab 3 mg/kg, with the combinations followed by nivolumab 3 mg/kg maintenance therapy (NIN group). After a follow-up of eight months, the ORR of NIN group, NI group and N group was 38%, 27% and 26%, respectively. The expression of PD-L1 did not influence the ORR. Furthermore, there was no statistically significant improvement in PFS or OS between groups [97]. **(**p18, lines 482-495).

In another phase III trial (DANUBE, NCT02516241), durvalumab was combined with tremelimumab as a first line treatment for metastatic UBC patients [39, 99]. 1032 patients were randomized in a 1:1:1 fashion to durvalumab monotherapy at 1.5 g IV every 4 weeks (D group) or durvalumab with tremelimumab at 75 mg IV every 4 weeks induction as 4 doses followed by maintenance durvalumab at 1.5g IV every 4 weeks (DTD group) versus chemotherapy with gemcitabine and cisplatin or carboplatin for up to 6 cycles (C group), until disease progression or unacceptable toxicity. The median OS was not significantly different among D group, DTD group and C group. The overall results of DANUBE were therefore considered negative for its primary endpoint. **(**p18-19, lines 496-505).

4.1 About the efficacy of ICI (Ref 87)

For patients with a minimum of 6 weeks of follow-up, the objective response rates were 26% (95% CI 18 to 36) in the IC2/3 group, 18% (95% CI 13 to 24) in the IC1/2/3 group and 15% (95% CI 11 to 19) in all patients. Additionally, the median OS were 11.4 months (95% CI 9.0 to not estimable) in the IC2/3 group, 8.8 months (95% CI 7.1 to 10.6) in the IC1/2/3, and 7.9 months (95% CI 6.6 to 9.3) in all patients. **(**p15, lines 383-386).

Question 9. Paragraphs 7, 8 and 9 are interesting but need clarification/ synthesis at the end. They may be shortened.

Response: As suggested, the original Paragraphs 8 and 9 have been revised and shorted to **3.5 Dendritic cells and 3.6 Macrophages** sections. However, the NK cells section (Paragraphs 7) lacks of necessary evidence supporting our manuscript, and has been deleted.

Question 10. Add a paragraph of combination: rationales and results. BCG therapy and ICI; ICI – ICI; ICI chemo (phases III have already given some results)

Response: As suggested, the combination therapy strategies including checkpoint inhibitors, chemotherapy or BCG have been added.

In another large phase III trial, KEYNOTE 361 trial (NCT02853305) [80], the effect of pembrolizumab as a monotherapy was compared with chemotherapy with gemcitabine and cisplatin or carboplatin versus chemotherapy with pembrolizumab followed by maintenance pembrolizumab [81]. Approximately 1010 patients with advanced UBC were recruited and randomized in 1:1:1 fashion. The ORR of the combination group was 54.7%, which was better than those of chemotherapy group (44.9%) or pembrolizumab group (30.3%). Moreover, the median PFS of the combination group was 8.3 months, which was better than those of chemotherapy group (7.1 months) or pembrolizumab group (3.9 months). The median OS of the combination group was 17.0 months, which was also better than those of chemotherapy group (14.3 months) or pembrolizumab group (15.6 months). **(**p13, lines 328-339).

Although the results of KEYNOTE 361 has dampened the enthusiasm regarding the pembrolizumab as a first line therapy solely, the combination with chemotherapy of other agents provided a promising direction. Recently, the effect of another agent with antibody drug conjugate (ADC) enfortumab and pembrolizumab were examined in EV-103 study including first-line cisplatin-ineligible cohort of 45 patients [82]. The ORR was 73.3% (95% CI, 58.1, 85.4)

and even patients with liver metastasis had a response rate of 53.3%, showing this potent combination. (p13, lines 340-346).

In an open-label phase II study CheckMate 032 (NCT01928394) [98], 274 patients with advanced or metastatic UBC previously treated with platinum-based chemotherapy were enrolled to investigated this regimen. Patients were randomly assigned to receive single-agent nivolumab 3mg/kg (N group) or nivolumab 3 mg/kg plus ipilimumab 1 mg/kg (NI group) or nivolumab 1mg/kg plus ipilimumab 3 mg/kg, with the combinations followed by nivolumab 3 mg/kg maintenance therapy (NIN group). After a follow-up of eight months, the ORR of NIN group, NI group and N group was 38%, 27% and 26%, respectively. The expression of PD-L1 did not influence the ORR. Furthermore, there was no statistically significant improvement in PFS or OS between groups [97]. (p18, lines 486-495).

Question 11. Add a paragraph on bladder cancer and molecular subtypes: what is the most responsive? Basal? Neuronal subgroup TCGA 2017 and response to atezo (Kim Eur Urol 2019)
Response: As suggested, the relationship between molecular subtypes and response has been added.
Additionally, 195 patients were classified into luminal (n=73) and basal (n=122) subtypes as according to the gene expression profile defined by TCGA. Surprisingly, the ORR in the luminal cluster II subtype (34%) was significantly higher than those in luminal cluster subtype I (10%), basal cluster subtype III (16%), and basal cluster subtype IV (20%). (p15, lines 401-405).

Question 12. Discussion has to be developed : discuss BCGtherapies, rationale of combination with immune checkpoint, why maintenance immune therapies are > combo (chem + immune checkpoints).
Response: As suggested, the discussion section has been revised thoroughly, including the BCG therapies, the combination therapy strategies including checkpoint inhibitors and chemotherapy. For example,

Therefore, combination therapy could be investigated to improve treatment outcomes, such as the dual immune combination regimen of PD-1/PDL-1 inhibitors with CTLA-4 inhibitors. For example, P. Sharma, et al. improved the ORR of patients with nivolumab plus ipilimumab combination therapy [98] .CTLA-4 inhibitors go to the source and increase the anti-cancer T-cell

numbers, while PD-1 inhibitors act in peripheral blood or tumors, allowing the binding process of PD-1 to PD-L1 to be blocked in these viable agents, thus freeing them to kill and launch a strike against the tumor [126]. Another combination strategy is composed of immune checkpoint blockade and chemotherapy. For example, the combination of pembrolizumab, and gemcitabine and cisplatin or carboplatin enhanced the ORR, median PFS and median OS of UBC patients compared to those of chemotherapy or pembrolizumab [81]. (p23-24, lines 650-660).

Minor comments:

Question 1. Not necessary to repeat each time Fig1. To add once at the beginning of the paragraph.

Response: As suggested, Fig.1 was added once in each paragraph. (p4, line 75, p10, line 239, p10, line 251, p12, line 298, p15, line 399, p17, line 454, p20, line 538).

Question 2. Line 376: Platinum based chemo rather than cisplatin because roughly 40% of patients are unfit for cisplatin.

Response: This sentence has been phrased to "Cisplatin-based chemotherapy has been the standard of care for UBC for the past 30 years. The emergence of anti-PD-1/PD-L1 treatment in UBC clinical settings suggests that immunotherapy is a promising therapeutic approach for this disease" (p24, lines 661-663).

2nd Editorial decision
27-May-2021

Ref.: Ms. No. JCTRes-D-20-00138R1
Immune Escape Mechanisms and Immunotherapy of Urothelial Bladder Cancer
Journal of Clinical and Translational Research

Dear Dr. Yang,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.
Please note that the reviewer is a top expert in the field and that the editorial board agrees with the reviewer's assessment. There is lack of cohesion between the different segments of the manuscript. Nevertheless, the editorial board does deem the topic to be of great importance, and would like to extend the authors one more chance for revision. Please follow the reviewer's advise to the very detail. By doing so you will greatly augment the value of this

manuscript.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you submit the revised manuscript. Also, please ensure that the track changes function is switched on when implementing the revisions. This enables the reviewers to rapidly verify all changes made.

Your revision is due by Jun 26, 2021.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: The authors have submitted an updated manuscript regarding systemic therapy for urothelial carcinoma, with a stated focus on immunotherapy and potential mechanisms of immune escape. Unfortunately, though the authors have clearly put a lot of effort into making changes, I do not think the focus of the manuscript has become more clear or been improved. The stated goal is to discuss mechanisms of immune escape to immunotherapy for bladder cancer, but that point does not come across in the current iteration. Instead, it reads as several separate articles. One looks at various immune cell types and how they may act to affect immune recognition at the TME. The next section is a review article on current immunotherapy for bladder cancer, including BCG. aPD1, and aCTLA4, but does not make the connection between these drugs and the previously reviewed immune cell populations and how they may interrelate. The last section talks about new types of therapies, but does not clearly connect how these might improve upon the limitations of current immune therapies, or how they might capitalize on the immune cell subsets from the first section. This paper really needs to hone in on what the focus is and present a unified story. I do not see that happening and thus I can not recommend this paper for publication.

There is additional documentation related to this decision letter. To access the file(s), please click the link below. You may also login to the system and click the 'View Attachments' link in the Action column.

Authors' response

Response to Editor and Reviewer Comments

Responses to Editor's comments

Question 1. There is lack of cohesion between the different segments of the manuscript.

Response: This manuscript focuses on the recent progress in **immune escape mechanisms and immunotherapy of urothelial bladder cancer** (UBC) and the different segments of the manuscript **are closely related.**

Regarding BCG, In 1976, Morales et al. first reported the treatment of UBC with BCG [9]. Furthermore, Lamm et al. confirmed the effect of BCG in the treatment of UBC in 1980 [10]. Additionally, more evidence proved that BCG is an effective biological immunotherapy in treating carcinoma in situ, preventing tumor progression and postoperative recurrence, and improving survival rate of UBC patients [11, 12]. (p4-5, lines 75-79). **Based on the function of BCG in UBC**, researches found that "Furthermore, **augmented infiltration of TAMs in UBC patients correlated with the resistance to BCG immunotherapy [39] and poor prognosis after intravesical instillation of BCG [30]**." (p8, lines 179-181). This review also demonstrated **the effect and possible mechanisms of BCG in the immunotherapy of UBC**. (p10-11, lines 245-277).

Additionally, **2. Immune Escape Mechanisms of UBC section contained four parts: 2.1 Recruitment of Immunosuppressive Cells in Tumor Microenvironment including** MDSCs, Tregs and TAMs, **2.2 Upregulation of Immunosuppressive Molecules including** PD-L1 and FasL, **2.3 Secretion of Immunosuppressive Signaling Molecules including** PGE2, **IL-10** and TGF-β, and **2.4 Dendritic Cells. 3. Immunotherapy of UBC section included six parts: 3.1 Bacillus Calmette-Guerin, 3.2 Checkpoint Inhibitors including PD-1/PD-L1 Inhibitors and CTLA-4 Inhibitors, 3.3 Cytokines including IFN-α, IFN-γ, IL-2, and IL-12, 3.4 Adoptive T Cell Immunotherapy, 3.5 Dendritic Cells, and 3.6 Macrophages. Obviously, 2. Immune Escape Mechanisms of UBC section and 3. Immunotherapy of UBC section are closely related. For example,**

TAMs may induce angiogenesis by secreting pro-angiogenic molecules [31], eliminate $CD8^+$ T cells [32, 33], support the induction and transportation of Tregs [34-36] via secreting immunosuppressive cytokines and bioactive lipids [37, 38]. (p8, lines 176-179). Moreover, Yang et al. established an orthotopic urinary UBC model by intravesical injection of MBT-2 cells [119]. They found that TAMs are closely related to lymph angiogenesis and lymphatic metastasis of UBC. (p22, lines 599-601). **Based on the function of TAMs in UBC**, researchers demonstrated that *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin (PA-MSHA) could also promote apoptosis and inhibit proliferation, invasion, and migration of mouse UBC cells **by inducing M1 polarization and inhibiting M2 polarization of TAMs via downregulating**

expression of M2-related genes such as IL-4, IL-10, and TGF-β [117]. Furthermore, OK-432 could inhibit proliferation, migration, and metastasis and induce apoptosis of UBC cells *in vitro* **via decreasing the expression of IL-10 and increasing the expression of TNF-α in TAMs** [118]. (p21-22, lines 588-599). Thus, macrophages serve as potential targets in the immune landscape of the UBC tumor microenvironment (Fig. 2). (p22, lines 601-603).

Tumor cells evade immunosurveillance through the elevation of co-inhibitory/stimulatory **PD-L1 or B7 ligands**, which binds to inhibitory **PD-1 or CTLA4 receptors** on T cells and resulted in the inhibition of anti-tumor immunity and exhaustion of T cells [40]. (p8, lines 186-188). Moreover, as tumor cells could evade host immunity via expression of immune checkpoint molecules, notably PD-L1 and B7-1/2, immune checkpoint blockade using monoclonal antibodies is a potential therapeutic strategy to prevent immune escape by UBC cells, thereby reactivating T cells and impeding tumor growth [69]. (p11, lines 281-285). **Based on the function of Immunosuppressive Molecules in UBC**, this review introduced the recent progress of **3.2 Checkpoint Inhibitors in UBC therapy**. (p11-19, lines 288-511).

DCs also contributed significantly to the tumorigenesis of UBC. Troy et al. reported that tumor-infiltrating DCs in UBC tissue were mainly immature and significantly fewer in number compared with those in normal bladder tissue. The low infiltration and functional deficiency of DCs resulted in non-effective antigen presentation, and the expression of costimulatory and adhesion molecules were too low to induce a specific CTLs response, which eventually led to immune escape of UBC [49]. (p10, lines 229-234). **Based on the function of DCs in UBC**, tumor-specific antigen-sensitized DCs are also used in tumor immunotherapy. In general, tumor-specific antigens or antigenic peptides are used to sensitize DCs *in vitro* and they are then reinfused back into patients to stimulate the production of antigen-specific CTLs, resulting in a protective immune response and elimination of tumor cells [109]. Nishiyama et al. showed that **a combination of MAGE-3 (melanoma antigens-3) antigen peptide (IMPKAGLLI) and HLA-A24-sensitized DCs possessed significantly elevated ability to induce a MAGE-3$^+$ cell-specific CTLs response compared with MAGE-3-expressing UBC cells or non-pulsed DCs *in vitro*** [110]. Four HLA-A24$^+$ patients with advanced MAGE-3$^+$ UBC were treated with injections of sensitized DCs every 2 weeks, a minimum of six and a maximum of 18 times. Three of the four patients showed significant reductions in size of lymph node metastases and/or liver metastases, with no significant untoward side-effects (Fig. 2). (p20-21, lines 560-571).

Taken together, the different segments of the manuscript **are closely related.**

Responses to Re viewer #1's comments

Question 1. the stated goal is to discuss mechanisms of immune escape to immunotherapy for bladder cancer, but that point does not come across in the current iteration.

Response: This manuscript focuses on the recent progress in **immune escape mechanisms and immunotherapy of urothelial bladder cancer** (UBC), not the mechanisms of immune escape to immunotherapy for bladder cancer.

"The research on the mechanisms of immune escape in UBC is helpful to design new approaches for the immunotherapy." (p6, lines 112-114). we introduced the immune escape mechanism of urothelial bladder cancer to pave the way for the introduction of immunotherapy for urothelial bladder cancer. For example,

2. Immune Escape Mechanisms of UBC section contained four parts: 2.1 Recruitment of Immunosuppressive Cells in Tumor Microenvironment including MDSCs, Tregs and TAMs, **2.2 Upregulation of Immunosuppressive Molecules including** PD-L1 and FasL, **2.3 Secretion of Immunosuppressive Signaling Molecules including** PGE2, **IL-10** and TGF-β, and **2.4 Dendritic Cells. 3. Immunotherapy of UBC section included six parts: 3.1 Bacillus Calmette-Guerin, 3.2 Checkpoint Inhibitors including PD-1/PD-L1 Inhibitors and CTLA-4 Inhibitors, 3.3 Cytokines including IFN-α, IFN-γ, IL-2, and IL-12, 3.4 Adoptive T Cell Immunotherapy, 3.5 Dendritic Cells, and 3.6 Macrophages. Obviously, 2. Immune Escape Mechanisms of UBC section and 3. Immunotherapy of UBC section are closely related.**

Question 2. it reads as several separate articles. One looks at various immune cell types and how they may act to affect immune recognition at the TME. The next section is a review article on current immunotherapy for bladder cancer, including BCG. aPD1, and aCTLA4, but does not make the connection between these drugs and the previously reviewed immune cell populations and how they may interrelate.

Response: This manuscript focuses on the recent progress in **immune escape mechanisms and immunotherapy of urothelial bladder cancer** (UBC) and the different segments of the manuscript **are closely related.**

Regarding BCG, In 1976, Morales et al. first reported the treatment of UBC with BCG [9]. Furthermore, Lamm et al. confirmed the effect of BCG in the treatment of UBC in 1980 [10]. Additionally, more evidence proved that BCG is an effective biological immunotherapy in treating carcinoma in situ, preventing tumor progression and postoperative recurrence, and improving survival rate of UBC patients [11, 12]. (p4-5, lines 75-79). **Based on the function of BCG in UBC**, researches found that "Furthermore, **augmented infiltration of TAMs in UBC**

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Taken together, the **Immune Escape Mechanisms of UBC, such as the Recruitment of Immunosuppressive Cells in Tumor Microenvironment** and **Immunotherapy of UBC are closely related with Immunotherapy of UBC, such as Checkpoint Inhibitors.**

Question 3. The last section talks about new types of therapies, but does not clearly connect how these might improve upon the limitations of current immune therapies, or how they might capitalize on the immune cell subsets from the first section.

Response: Within the scope of our knowledge, the new types of therapies you mentioned refers to parts **3.3 Cytokines** to **3.6 Macrophages** (p19-22, lines 513-603).

However, **3.3 Cytokines, 3.4 Adoptive T Cell Immunotherapy, 3.5 Dendritic Cells, and 3.6 Macrophages mentioned in the manuscript are not proposed as** new types of immune therapies.

Currently, BCG and immune checkpoint blockades have been approved by the FDA for the treatment of UBC patients. **Cytokines, Dendritic Cells and Macrophages based therapies, and Adoptive T Cell Immunotherapy were under investigated, which did not show the improvement upon** BCG and immune checkpoint blockades and still needs to be further explored.

Regarding the relationship between above therapies and immune cell subsets,

TAMs may induce angiogenesis by secreting pro-angiogenic molecules [31], eliminate $CD8^+$ T cells [32, 33], support the induction and transportation of Tregs [34-36] via secreting immunosuppressive cytokines and bioactive lipids [37, 38]. (p8, lines 176-179). Moreover, Yang et al. established an orthotopic urinary UBC model by intravesical injection of MBT-2 cells [119]. They found that TAMs are closely related to lymph angiogenesis and lymphatic metastasis of UBC. (p22, lines 599-601). **Based on the function of TAMs in UBC**, researchers demonstrated that *Pseudomonas aeruginosa*-mannose-sensitive hemagglutinin (PA-MSHA) could also promote apoptosis and inhibit proliferation, invasion, and migration of mouse UBC cells **by inducing M1 polarization and inhibiting M2 polarization of TAMs via downregulating expression of M2-related genes such as IL-4, IL-10, and TGF-β** [117]. Furthermore, OK-432 could inhibit proliferation, migration, and metastasis and induce apoptosis of UBC cells *in vitro* **via decreasing the expression of IL-10 and increasing the expression of TNF-α in TAMs** [118]. (p21-22, lines 588-599). Thus, macrophages serve as potential targets in the immune landscape of the UBC tumor microenvironment (Fig. 2). (p22, lines 601-603).

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Taken together, 3.3 Cytokines to **3.6 Macrophages sections are closely related with** the **Immune Escape Mechanisms of UBC, such as the Recruitment of Immunosuppressive Cells in Tumor Microenvironment.**

3rd Editorial decision
25-Jun-2021

Ref.: Ms. No. JCTRes-D-20-00138R2
Immune Escape Mechanisms and Immunotherapy of Urothelial Bladder Cancer
Journal of Clinical and Translational Research

Dear authors,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly review for any errors.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers: