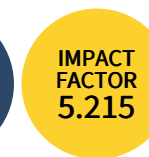




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Analgesics Induce Alterations in the Expression of SARS-CoV-2 Entry and Arachidonic-Acid-Metabolizing Genes  
in the Mouse Lungs

Authored by:

Fatima Khirfan; Yazun Jarrar; Tariq Al-Qirim; Khang Wen Goh; Qais Jarrar; Chrismawan Ardianto;  
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Abstract	<p>Paracetamol and nonsteroidal anti-inflammatory drugs are widely used in the management of respiratory viral infections. This study aimed to determine the effects of the most commonly used analgesics (paracetamol, ibuprofen, and diclofenac) on the mRNA expression of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry and arachidonic-acid-metabolizing genes in mouse lungs. A total of twenty eight Balb/c mice were divided into four groups and treated separately with vehicle, paracetamol, ibuprofen, and diclofenac in clinically equivalent doses for 14 days. Then, the expressions of SARS-CoV-2 entry, <i>ACE2</i>, <i>TMPRSS2</i>, and <i>Ctsl</i> genes, in addition to the arachidonic-acid-metabolizing <i>cyp450</i>, <i>cox</i>, and <i>alox</i> genes, were analyzed using real-time PCR. Paracetamol increased the expressions of <i>TMPRSS2</i> and <i>Ctsl</i> genes by 8.5 and 5.6 folds, respectively, while ibuprofen and diclofenac significantly decreased the expression of the <i>ACE2</i> gene by more than 2.5 folds. In addition, all tested drugs downregulated (<math>p &lt; 0.05</math>) <i>cox2</i> gene expression, and paracetamol reduced the mRNA levels of <i>cyp4a12</i> and <i>2j5</i>. These molecular alterations in diclofenac and ibuprofen were associated with pathohistological alterations, where both analgesics induced the infiltration of inflammatory cells and airway wall thickening. It is concluded that analgesics such as paracetamol, ibuprofen, and diclofenac alter the expression of SARS-CoV-2 entry and arachidonic-acid-metabolizing genes in mouse lungs.</p>
Keywords	COVID-19; lung disease; chronic respiratory disease; acute respiratory distress syndrome; gene expression



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Han, G.; Sinjab, A.; Hara, K.; Treekitkarnmongkol, W.; Brennan, P.; Chang, K.; Bogatenkova, E.; Sanchez-Espiridon, B.; Behrens, C.; Solis, L.M.; Gao, B.; Girard, L.; Zhang, J.; Sepesi, B.; Cascone, T.; Byers, L.A.; Gibbons, D.L.; Chen, J.; Moghaddam, S.J.; Ostrin, E.J.; Scheet, P.; Fujimoto, J.; Shay, J.; Heymach, J.V.; Minna, J.D.; Dubinett, S.; Wistuba, I.I.; Stevenson, C.S.; Spira, A.E.; Wang, L.; Kadara, H. Single-Cell Expression Landscape of SARS-CoV-2 Receptor ACE2 and Host Proteases in Normal and Malignant Lung Tissues from Pulmonary Adenocarcinoma Patients. *Cancers* **2021**, *13*, 1250. doi: 10.3390/cancers13061250 (<https://doi.org/10.3390/cancers13061250>)

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Title Analgesics Induce Alterations in the Expression of SARS-CoV-2 Entry and Arachidonic-Acid-Metabolizing Genes in the Mouse Lungs (https://www.mdpi.com/1424-8247/15/6/696)

Authors Fatima Khirfan , Yazun Jarrar \* , Tariq Al-Qirim , Khang Wen Goh , Qais Jarrar , Chrismawan Ardianto \* , Mohammad Awad , Hamzeh J. Al-Ameer , Wajdy Al-Awaida , Said Moshawih , Long Chiau Ming

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Special Issue COVID-19 in Pharmaceuticals (https://www.mdpi.com/journal/pharmaceuticals/special\_issues/COVID-19\_ph)

Abstract Background: Paracetamol and nonsteroidal anti-inflammatory drugs are widely used in the management of respiratory viral infections. Aims: This study aimed to determine the effect of the most commonly used analgesics, paracetamol, ibuprofen, and diclofenac on the mRNA expression of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry and arachidonic acid metabolizing genes in the mouse lungs. Methods: Twenty eight balb/c mice were divided into 4 groups and were treated separately with vehicle, paracetamol, ibuprofen, and diclofenac in clinically equivalent doses for 14 days. Then, the expression of SARS-cov2 entry, ace2, tmprss, and cathepsin I genes, in addition to the arachidonic acid metabolizing cyp450, cox, and alox genes were analyzed using real-time PCR. Results: It is found that paracetamol downregulated significantly (P < 0.05) the expression of tmprss and cathepsin I genes by 8.5 and 5.6 folds, respectively, while ibuprofen and diclofenac upregulated significantly (P < 0.05) the expression of ace2 gene by more than 2.5 folds. In addition, all tested drugs downregulated (P < 0.05) cox2 gene expression, and paracetamol reduced the mRNA levels of cyp4a12 and 2j5 genes. These molecular alterations of diclofenac and ibuprofen were associated with pathohistological alterations, where both analgesics induced infiltration of inflammatory cells and airway wall thickening. Conclusion: It can be concluded that analgesics alter the expression of SARS-cov2 entry and arachidonic acid metabolizing genes in the mouse lungs.

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Authors' Responses to Reviewer's Comments (Reviewer 1)

Author's Notes REVIEWER 1

Thank you very much for your constructive comments and suggestions. We replied to all your comments and revised the manuscript accordingly. Hoping you find our manuscript is currently suitable for publication.

Dear Author

I read with great interest your paper entitled "Analgesics Induced Alterations in the Expression of Angiotensin-Converting Enzyme 2 Receptor, Cathepsin L, Transmembrane Serine Protease and Arachidonic Acid Metabolizing Genes in the Mouse Lungs". The topic was interesting. However, here I reported my issues, especially on statistical methods.

1) you should mention the software used to compute statistics;

Reply: First, thanks for the reviewer for these valuable comments. In response to this comment, the software used in the statically analysis is added in the revised manuscript, in the method part, as followings:

" Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA) version 23 for Windows."

2) you used one-way ANOVA to compare differences among groups, but you did not report if assumptions for ANOVA were respected, in detail the normal distribution and homogeneous variance. The issue was not secondary because the respect or not of these assumptions requires further correction about the statistical tests;



**Reply:** Thank you for your comment. We used ANOVA test after we analysed the normality of data in each group using Kolmogorov–Smirnov test. The data of gene expression in each group was normally distributed and accordingly we used ANOVA test, either one- (for gene expression) or two- way (for animal weight with time) analysis. In response to the reviewer comment, the following is added in the statistical part of the revised manuscript, as following:

" The mRNA expression of the tested genes, in each group, was normally distributed according to the Kolmogorov–Smirnov test. The comparison between the control and other groups was done using two-way, for the body weight, and one-way, for the gene expression, analysis of variance (ANOVA) test and Tukey's HSD post-hoc test."

**3) for post-hoc analysis, you should report which methods for p-value correction had been used (i.e. Bonferroni or other methods);**

**Reply:** Thank you for your comment. The type of post-hoc analysis used is added in the revised manuscript, in the method part, as following:

" The comparison between the control and other groups was done using one-way analysis of variance (ANOVA) test and Tukey's HSD *post-hoc* test."

**4) Please you should indicate the correct value for p-value.**

**Reply:** Thank you for pointing it out. The correct values of P-value is added in the revised manuscript.

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 Dear Author  
 I read with great interest your paper entitled "Analgesics Induced Alterations in the Expression of Angiotensin-Converting Enzyme 2 Receptor, Cathepsin L, Transmembrane Serine Protease and Arachidonic Acid Metabolizing Genes in the Mouse Lungs". The topic was interesting. However, here I reported my issues, especially on statistical methods.  
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 According to my evaluation, the paper requires major revision.



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Journal	Pharmaceuticals (https://www.mdpi.com/journal/pharmaceuticals) (ISSN 1424-8247)
Manuscript ID	pharmaceuticals-1697590
Type	Article
Title	Analgesics Induce Alterations in the Expression of SARS-CoV-2 Entry and Arachidonic-Acid-Metabolizing Genes in the Mouse Lungs (https://www.mdpi.com/1424-8247/15/6/696)
Authors	Fatima Khirfan , Yazun Jarrar * , Tariq Al-Qirim , Khang Wen Goh , Qais Jarrar , Chrismawan Ardianto * , Mohammad Awad , Hamzeh J. Al-Ameer , Wajdy Al-Awaida , Said Moshawih , Long Chiau Ming
Section	Medicinal Chemistry (https://www.mdpi.com/journal/pharmaceuticals/sections/pharm_medicinal_chemistry)
Special Issue	COVID-19 in Pharmaceuticals (https://www.mdpi.com/journal/pharmaceuticals/special_issues/COVID-19_ph)
Abstract	Background: Paracetamol and nonsteroidal anti-inflammatory drugs are widely used in the management of respiratory viral infections. Aims: This study aimed to determine the effect of the most commonly used analgesics, paracetamol, ibuprofen, and diclofenac on the mRNA expression of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry and arachidonic acid metabolizing genes in the mouse lungs. Methods: Twenty eight balb/c mice were divided into 4 groups and were treated separately with vehicle, paracetamol, ibuprofen, and diclofenac in clinically equivalent doses for 14 days. Then, the expression of SARS-cov2 entry, ace2, tmprss, and cathepsin I genes, in addition to the arachidonic acid metabolizing cyp450, cox, and alox genes were analyzed using real-time PCR. Results: It is found that paracetamol downregulated significantly (P < 0.05) the expression of tmprss and cathepsin I genes by 8.5 and 5.6 folds, respectively, while ibuprofen and diclofenac upregulated significantly (P < 0.05) the expression of ace2 gene by more than 2.5 folds. In addition, all tested drugs downregulated (P < 0.05) cox2 gene expression, and paracetamol reduced the mRNA levels of cyp4a12 and 2j5 genes. These molecular alterations of diclofenac and ibuprofen were associated with pathohistological alterations, where both analgesics induced infiltration of inflammatory cells and airway wall thickening. Conclusion: It can be concluded that analgesics alter the expression of SARS-cov2 entry and arachidonic acid metabolizing genes in the mouse lungs.

Authors' Responses to Reviewer's Comments (Reviewer 2)

**Author's Notes** Thank you very much for your constructive comments and suggestions. We replied to all your comments and revised the manuscript accordingly. Hoping you find our manuscript is currently suitable for publication.

**The present study used a molecular approach to evaluate the effect of analgesics on the expression of genes involved in SARS-cov2 entry and arachidonic acid metabolism. The study is relevant, timely, well written, however I suggest that it be reviewed before publication.**

- 1. The title is very long and can be simplified. Additionally the current title is very generic. Which analgesics studied? The entire introduction focuses on the role of some genes for SARS-coV2 entry. However, this scope of the research is not suggested in the title. In this context, the text should be more precise and concise.**

**Reply:** This is a very valid point, thank you. In response to the reviewer comment, the title of the manuscript is changed in the revised manuscript to " Analgesics Induced Alterations in the Expression of SARS-COV2 Entry and Arachidonic acid metabolizing Genes in the Mouse Lungs.

- 1. Why was an n= 7 (animals) used for each group? What statistical analysis supports this decision? Did the authors consider the 3R principle in the experimental design?**

**Reply:** We understand your concern. Yes, **the 3R principle in the experimental design was applied. Meanwhile, we have added such info in the revised text.** According to the guidelines of using animals in researches, including Canadian guideline of animal handling and caring, it is strongly recommended to use minimal number of animals in the researches





with study design including animal euthanasia. Hence, the recommended 7 mice in each group were used in this study. Of note, current literature also used 7 or even a smaller number of mice in each group.

1. Hassan et al (2020). A SARS-CoV-2 infection model in mice demonstrates protection by neutralizing antibodies. *Cell*, 182(3), 744-753.
2. Winkler et al. "SARS-CoV-2 causes lung infection without severe disease in human ACE2 knock-in mice." *Journal of Virology*96, no. 1 (2021): e01511-21.

**1. Line 88: What is the drug used? What is the form of administration?**

**Reply:** The drug vehicle and form of administration in line 88 were defined in the revised manuscript as following:

"1) Control group which received a once-daily intraperitoneal dose of 50% polyethylene glycol 400, the vehicle used for solubilization of analgesic drugs."

**1. Line 99-100: Avoid sentence paragraphs. This information can be added to the previous paragraph.**

**Reply:** In response to the reviewer comment, the sentence paragraph (line 99-100) was added to the previous paragraph, in the revised manuscript, as followings:

" The drugs were administrated to the animals for a continuous 14 days. The used doses of NSAIDs were corresponding to the daily dose for humans, which depends on the surface area of the animal body [17]. This period of analgesic treated mimics the period of disease symptoms that patients administrate the analgesic in it and were reported to alter the expression of arachidonic acid-metabolizing enzyme genes [12,18]."

**1. What euthanasia method was used for the endpoint of the experiment? Can this method affect gene expression?**

**Reply:** Thank you for raising this question. Since central anesthesia of the animals before isolating the biological samples could affect gene expression, we anesthetized the animals using cervical dislocation as suggested by Canadian Council on Animal Care. The following research also have used the cervical dislocation to sacrifice the animals:

1. Mout, L., van Royen, M. E., de Ridder, C., Stuurman, D., van de Geer, W. S., Marques, R., ... & van Weerden, W. M. (2021). Continued androgen signalling inhibition improves cabazitaxel efficacy in prostate cancer. *EBioMedicine*, 73, 103681.
2. Bilinska K, Jakubowska P, Von Bartheld CS, Butowt R. Expression of the SARS-CoV-2 Entry Proteins, ACE2 and TMPRSS2, in Cells of the Olfactory Epithelium: Identification of Cell Types and Trends with Age. *ACS Chem Neurosci*. 2020;11(11):1555-1562. doi:10.1021/acschemneuro.0c00210
3. Bilinska, K., Jakubowska, P., Von Bartheld, C. S., & Butowt, R. (2020). Expression of the SARS-CoV-2 entry proteins, ACE2 and TMPRSS2, in cells of the olfactory epithelium: identification of cell types and trends with age. *ACS chemical neuroscience*, 11(11), 1555-1562.

**In response to the reviewer comment, the following is added into the revised manuscript:**

" The euthanasia of the mice was done by cervical dislocation as suggested by Canadian Council on Animal Care [16].

It is good to say that The euthanasia of the mice was done by cervical dislocation as suggested by Canadian Council on Animal Care since the that was the method used. However, in my opinion, it is too far assuming that central overdose anesthesia will generally affect the expression mRNA in certain organs. We should be careful if we deal with brain or spinal tissue. However, I am sure that the lung is not affected by 1 minute exposure of overdosed central anesthetic drugs. Moreover, Reference no 18 seems not suitable to support our answer.

**1. Why did the authors not consider evaluating the expression of these genes before starting treatment?**

**Reply:** Thanks to the reviewer for this comment. Analyzing of the gene expression, in this study, is done after isolation of the lung samples which causes death to the mice. Therefore, it is technically impossible to evaluate the expression of these genes before and after treatment. Instead, we have the negative control group where the mice were healthy and did not receive any drug. We compared the expression of these genes in the lungs of mice treated with analgesics with the expression in the lungs of the negative control group. The



results of gene expression after analgesics treatment are relative to the negative control group. Therefore, the difference in gene expression found in this study is due to analgesic treatment.

We made it clear in the revised manuscript that the control group is a negative groups and did not receive any drug. In addition, the results of gene expression in the lung of treated mice were relative to the expression in the lungs of the negative control mice.

**1. Figure 1. It is not clear why the authors assess the weight of animals during treatment. Has any previous study already reported this change in studies with these analgesics? The authors reported no differences during treatment. But is there a significant difference between the groups studied? Could this affect the analysis of gene expression?**

**Reply:** Thank you for the very useful comment. Toxicological studies used body weight and pathohistological examination as mark-ers of drug-induced toxicity on the animals and organs. We evaluated the weight of the mice to indicate if the difference in the expression of *Ace2*, *Tmprss2*, *Ctsl*, and Arachidonic acid-metabolizing genes were associated with toxicological influence of NSAIDs on the lungs. Although the body weight of the mice was not significantly decreased by NSAID administration, the histological examination showed that NSAIDs caused pathological effects on the lungs. Therefore, we could conclude that the alterations in the gene expression of tested genes, in this study, was associated with toxicological influence of NSAIDs on the lungs.

In response to the reviewer comment, the following paragraph is made clearer in the revised manuscript:

" Toxicological studies used body weight and pathohistological examination as mark-ers of drug-induced toxicity on the animals and organs [22]. In this study, we found that 14 days of treatment with ibuprofen and diclofenac caused toxicological changes as rep-resented by the histological examination of the mouse lungs, where both NSAIDs caused infiltration of inflammatory cells and increased the thickness of the wall of the bronchioles. It was reported that NSAIDs have the capacity to induce oxidative stress on the cells [23]. Accordingly, the molecular alterations in the mRNA expression of arachidonic ac-id-metabolizing genes, *Ctsl*, *Tmprss2*, and *Ace2* were associated with the toxicological effects of NSAIDs on the mouse lungs.

**1. The authors should indicate in the images with an arrow the inflammatory response suggested in the histological analysis.**

**Reply:** In response to the reviewer comment, the arrow refer to the inflammatory response were added on the histology Figure in the revised manuscript. Thank you for the suggestion.

**1. Figure 2: Were all images acquired with the same magnification?**

**Reply:** All images were captured under the same magnification power of 400X. In addition, each image has a scale bar of 100 µm. These information were added in the revised manuscript, in red colour. Thank you.

**1. Quality of figure 4 should be improved.**

**Reply:** The quality of Figure 4 is much improved in the revised manuscript, as suggested by the reviewer. Thank you.

**1. The presentation of figures must be standardized., for example, review figures 3, 4 and 5. Review font format and size.**

**Reply:** In response to the reviewer comment, all Figures were reviewed regarding the font format and size and they are uniformed in the revised manuscript. Thank you.

**1. Authors should broaden the discussion of limitations and perspectives of the work. Despite the relevant findings, the study presents only a transcriptomic approach. Proteomics and signaling cascade studies should strengthen the hypothesis and conclusion presented.**

**Reply:** Thank you for this very useful insight. In response to the reviewer comment, the limitations of this study were expanded and revised.



- Quality of English Language
- English very difficult to understand/incomprehensible
  - Extensive editing of English language and style required
  - Moderate English changes required
  - English language and style are fine/minor spell check required
  - I am not qualified to assess the quality of English in this paper

	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Are all the cited references relevant to the research?	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Is the research design appropriate?	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Are the methods adequately described?	<input type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Are the results clearly presented?	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Are the conclusions supported by the results?	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Comments and Suggestions for Authors

The present study used a molecular approach to evaluate the effect of analgesics on the expression of genes involved in SARS-cov2 entry and arachidonic acid metabolism. The study is relevant, timely, well written, however I suggest that it be reviewed before publication.

1. The title is very long and can be simplified. Additionally the current title is very generic. Which analgesics studied? The entire introduction focuses on the role of some genes for SARS-coV2 entry. However, this scope of the research is not suggested in the title. In this context, the text should be more precise and concise.
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6. Why did the authors not consider evaluating the expression of these genes before starting treatment?
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9. Figure 2: Were all images acquired with the same magnification?
10. Quality of figure 4 should be improved.
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12. Authors should broaden the discussion of limitations and perspectives of the work. Despite the relevant findings, the study presents only a transcriptomic approach. Proteomics and signaling cascade studies should strengthen the hypothesis and conclusion presented.

Submission Date 09 April 2022

Date of this review 06 May 2022 15:35:07



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Journal Pharmaceuticals (https://www.mdpi.com/journal/pharmaceuticals) (ISSN 1424-8247)

Manuscript ID pharmaceuticals-1697590

Type Article

Title Analgesics Induce Alterations in the Expression of SARS-CoV-2 Entry and Arachidonic-Acid-Metabolizing Genes in the Mouse Lungs (https://www.mdpi.com/1424-8247/15/6/696)

Authors Fatima Khirfan , Yazun Jarrar \* , Tariq Al-Qirim , Khang Wen Goh , Qais Jarrar , Chrismawan Ardianto \* , Mohammad Awad , Hamzeh J. Al-Ameer , Wajdy Al-Awaida , Said Moshawih , Long Chiau Ming

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Section Medicinal Chemistry (https://www.mdpi.com/journal/pharmaceuticals/sections/pharm\_medicinal\_chemistry)

Special Issue COVID-19 in Pharmaceuticals (https://www.mdpi.com/journal/pharmaceuticals/special\_issues/COVID-19\_ph)

Abstract Background: Paracetamol and nonsteroidal anti-inflammatory drugs are widely used in the management of respiratory viral infections. Aims: This study aimed to determine the effect of the most commonly used analgesics, paracetamol, ibuprofen, and diclofenac on the mRNA expression of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) entry and arachidonic acid metabolizing genes in the mouse lungs. Methods: Twenty eight balb/c mice were divided into 4 groups and were treated separately with vehicle, paracetamol, ibuprofen, and diclofenac in clinically equivalent doses for 14 days. Then, the expression of SARS-cov2 entry, ace2, tmprss, and cathepsin I genes, in addition to the arachidonic acid metabolizing cyp450, cox, and alox genes were analyzed using real-time PCR. Results: It is found that paracetamol downregulated significantly (P < 0.05) the expression of tmprss and cathepsin I genes by 8.5 and 5.6 folds, respectively, while ibuprofen and diclofenac upregulated significantly (P < 0.05) the expression of ace2 gene by more than 2.5 folds. In addition, all tested drugs downregulated (P < 0.05) cox2 gene expression, and paracetamol reduced the mRNA levels of cyp4a12 and 2j5 genes. These molecular alterations of diclofenac and ibuprofen were associated with pathohistological alterations, where both analgesics induced infiltration of inflammatory cells and airway wall thickening. Conclusion: It can be concluded that analgesics alter the expression of SARS-cov2 entry and arachidonic acid metabolizing genes in the mouse lungs.

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Quality of English Language

English very difficult to understand/incomprehensible

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Moderate English changes required

English language and style are fine/minor spell check required

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	Yes	Can be improved	Must be improved	Not applicable
Does the introduction provide sufficient background and include all relevant references?	<input checked="" type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Are all the cited references relevant to the research?	<input checked="" type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Is the research design appropriate?	<input checked="" type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Are the methods adequately described?	<input checked="" type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>
Are the results clearly presented?	<input type="radio"/>	<input checked="" type="radio"/>	<input type="radio"/>	<input type="radio"/>
Are the conclusions supported by the results?	<input checked="" type="radio"/>	<input type="radio"/>	<input type="radio"/>	<input type="radio"/>

Comments and Suggestions for Authors  
The authors addressed most of my concerns. During proofreading I recommend improving the quality of the figures. In general terms, this manuscript can be accepted for publication in its current version.

Submission Date 09 April 2022

Date of this review 22 May 2022 17:16:59

