nature portfolio

Peer Review File

To boldly go where no microRNAs have gone before: Spaceflight impact on risk for small-for-gestational-age infants

Corresponding Author: Professor Afshin Beheshti

This file contains all editorial decision letters in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
1. Brief summary of the manuscript
This study examines the enduring effects of space environment on female pregnancy-related risks, specifically SGA births. The authors have used published database to identify miRNAs associated with SGA and the space environment, then explored potential mitigatory effects of current FDA approved drugs on miRNAs. Total of 13 miRNAs were identified to be associated with SGA and exposure to space environment, and in silico analyses have shown potential biological role of these miRNAs in relation to SGA. The authors then used machine learning technique to identify two potential drugs to mitigate miRNA dysregulation.
2. Overall impression of the work
The manuscript is well written and the research topic is very interesting and novel. There is great potential to take the findings from this study to further validation work. The authors have reported some of the limitations of the study and it is especially important to highlight that the findings in this study have focused on in silico approach and so any conclusions should be taken cautiously until validated. Any overreaching statements should be omitted.
Without confirming the functional role of the 13 miRNAs, it is questionable how significant it is to find drugs to countermeasure miRNA changes, however, the methodology of identifying such drugs is interesting and have potential to be applied in many other miRNA studies that have progressed to confirm functional roles.
3. Specific comments, with recommendations for addressing each comment
1) The authors have used data from a single publication (reference 8) to identify plasma miRNAs associated with SGA births. Could the authors clarify whether raw data was used or normalised data? Often the data from nCounter platform undergo normalisation using their built-in positive/negative controls, endogenous controls or binding efficiencies. It would be important to know what dataset was used for analysis here.
2) The comparison between miRNA expression in SGA patients and mice exposed to simulated space conditions have been justified by the fact that the 13 identified miRNAs are highly conserved. However, this is a comparison between samples taken from pregnant women vs non-pregnant mice. If this is correct, are there any information about the 13 identified miRNA pre- vs post-conception? This should be discussed as you may have missed some miRNA targets due to this.
Eg. Human plasma pregnancy-associated miRNAs and their temporal variation within the first trimester of pregnancy (2022, Reprod Biol Endocrinol)
By Cécilia Légaré et al. have looked at approx. 2000miRNAs where almost 200 of them were expressed differently in pregnant women compared to non-pregnant.
3) Line 157-159: Should this be referring to Fig S2?
4) Fig 1b&f: for timepoint independent analysis, could you clarify whether all tps were included in the analysis? If so, did you account for multiple samples originating from a single patient? It would be helpful to see n numbers in all figure legends.
5) For OSD-336, how were the samples processed for plasma? Was risk of haemolysis considered? Various factors during plasma processing have been shown to affect miRNA levels, including type of anticoagulant, haemolysis, time to processing, storage conditions etc. This should be included in methods and discussed if not considered.
6) Discussion should also include whether the 13 identified miRNAs would target same genes/pathways in pregnant vs non-pregnant women
7) Without any data showing that the actual miRNA level changes following exposure to space stressors is maintained for prolonged period of time (ie. Until first trimester of pregnancy), it is difficult to say these miRNAs can be used to assess risk of SGA in future pregnancies after return to Earth. Is there any evidence in literature that any of the 13 miRNAs are affected by environmental/other factors such as exposure to pollution, poor diet, lack of exercise? Including such details in discussion

could help strengthen the statement that these miRNAs have potential to predict future SGA births following space travel.
8) Line 273-275: The authors state that the 13 miRNAs identified in the study can have long lasting effects on children's development as specific functions are related to organs associated with defects in SGA infants. Is there any evidence that these 13 miRNA changes are also seen in SGA babies? Or whether these miRNAs are able to cross the placenta from maternal circulation? It would be important to include these details as without any evidence that these miRNA changes on maternal side are reflected on the fetal side, this statement would be overreaching.
9) Without confirming the functional role of the 13 miRNAs, it is questionable how significant it is to find drugs to countermeasure miRNA changes.

Reviewer #2

(Remarks to the Author)
What are the major claims of the paper?
Using published miRNA databases and computational methods, the authors discuss a shared miRNA signature between SGA and space environment conserved between humans and mice. Then they use machine learning to identify potential FDA- approved drugs to mitigate the risk of SGA in female astronauts.

Overall impression and specific points
It is a well written paper with interesting concept and 'catchy' title. It uses novel methodology but relies on several assumptions. One of them is that women astronauts with prolonged exposure to the space environment may have an elevated risk of SGA. The authors then use this hypothesis (and without any data to support an increased risk of SGA) they then computationally compare real life data of human SGA pregnant signature to mice simulated spaceflight biology experiments particularly focusing on female non pregnant C57BL/6 mice. In their previous study (published a couple of years ago) on spaceflight-associated miRNA signature, that was also defined computationally, based on transcriptomic analysis, and not measured experimentally (Ref 27).

Although whole body mice weights were significantly reduced in GCR and SPE irradiation groups in combination with simulated microgravity (hindlimb unloading, HU), I cannot find literature suggesting that mice exposed in simulated spaceflight conditions have an increased risk of SGA. Therefore, it is difficult to assess the value of the 13 common miRNAs between SGA vs control human pregnancies and spaceflight vs terrestrial mice.

Are any of these miRNAs change in humans exposed to space conditions?

The authors then explore or the various biological functions that can be implicated on children's development. The methods again are appropriate but the concept and basis for the investigation is debatable. How do these gene pathways change after space travel?

The authors then aim to confirm the key SGA-related molecular changes in astronauts, through using data published for scRNA-seq from 2 human males and 2 non pregnant females who participated in the I4 mission occurring in the high altitude of 590km. Can the authors provide further explanation as to how they come to the conclusion that a T cell population suppression demonstrated by suppressed CD4 and CD8 T cells within 24 hours of 0.5 Gy GCR irradiation supports the heightened risk of SGA after space flight?

The authors then are investigating potential FDA-approved small molecule drugs to target SGA miRNAs. Although the results is an interesting read, I am uncertain about the value of defining triamcinolone and perfluorodecalin as possible drug molecules. Traimcinolone is a class C drug and has not been associated with increased risk of spontaneous abortion or SGA in humans. Are there any studies suggesting a beneficial growth profile through its use in animal models? Also what is the placental transfer of triamcinolone and perfluorodecalin?
In a 2023 Swedish study of maternal serum concentrations of perfluoroalkyl substances poor growth was observed in women with higher concentrations of per- and polyfluoralkyl substance (PFOA and PFHxS) as compared to the controls. Are these different to perfluorodecalin?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Thank you. I agree with the provided responses.

Reviewer #2

(Remarks to the Author)
I am happy with the changes of the manuscript and with the reply

Dear Editor and Reviewers,

We thank both the editor and reviewers for the comments. We believe based on these revisions we have made that this manuscript is now stronger and much improved. We have addressed the comments by the reviewers and our responses appear in red font below the original reviewer comment. We look forward to the next steps.

On behalf of the authors,

Afshin Beheshti, PhD

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

1. Brief summary of the manuscript
This study examines the enduring effects of space environment on female pregnancy-related risks, specifically SGA births. The authors have used published database to identify miRNAs associated with SGA and the space environment, then explored potential mitigatory effects of current FDA approved drugs on miRNAs. Total of 13 miRNAs were identified to be associated with SGA and exposure to space environment, and in silico analyses have shown potential biological role of these miRNAs in relation to SGA. The authors then used machine learning technique to identify two potential drugs to mitigate miRNA dysregulation.

2. Overall impression of the work
The manuscript is well written and the research topic is very interesting and novel. There is great potential to take the findings from this study to further validation work. The authors have reported some of the limitations of the study and it is especially important to highlight that the findings in this study have focused on in silico approach and so any conclusions should be taken cautiously until validated. Any overreaching statements should be omitted.

We have revised the manuscript and made edits to omit the overreaching statements. In the Discussion section, we have clearly stated that our current findings are based on *in-silico* analysis, and that further experimental validations are needed in the future.

Without confirming the functional role of the 13 miRNAs, it is questionable how significant it is to find drugs to countermeasure miRNA changes, however, the methodology of identifying such drugs is interesting and have potential to be applied in many other miRNA studies that have progressed to confirm functional roles.

We appreciate the reviewer's comment and agree that determining the functional role of the 13 miRNAs is crucial to fully understanding and validating our findings. We have addressed this point in our reply to your comment #9 and provided further discussion in the manuscript. Briefly, our methodology to predict drugs for miRNAs has now been published, and we have cited the reference in the paper and here: Galeano, D. et al. sChemNET: A deep learning framework for predicting small molecules targeting microRNAs. Nat. Commun. (in press) (2024). In this publication, we provided functional validation for our drug predictions related to the miRNAs, which gives us confidence that this model can offer reasonable and valid predictions. Of course, as with any model, validation and functional analysis are necessary, as you mention. However, this is beyond the scope of the current manuscript and will be addressed in future work. With this manuscript, we aim to introduce a new hypothesis and potential health risk for the community to further investigate. In space biology research, it is essential to uncover health risks before they occur during long-term deep space missions. As we state in the manuscript, there are significant gaps in knowledge regarding how the space environment impacts the female reproductive system. Our goal with this manuscript is to generate the hypotheses needed for future work to validate our predictions, including potential countermeasures to mitigate the health risks.

3. Specific comments, with recommendations for addressing each comment
1) The authors have used data from a single publication (reference 8) to identify plasma miRNAs associated with SGA births. Could the authors clarify whether raw data was used or normalised data? Often the data from nCounter platform undergo normalisation using their built-in positive/negative controls, endogenous controls or binding efficiencies. It would be important to know what dataset was used for analysis here.

We thank the reviewer for the comment. We utilized the normalized data that was available on the ImmPort database. We had indicated this in the methods by stating (lines 738-739):

"*Normalized miRNA count data, available on ImmPort, underwent analysis using the R package DESeq2 (ver.1.36.0)*"

2) The comparison between miRNA expression in SGA patients and mice exposed to simulated space conditions have been justified by the fact that the 13 identified miRNAs are highly conserved. However, this is a comparison between samples taken from pregnant women vs non-pregnant mice. If this is correct, are there any information about the 13 identified miRNA pre- vs post-conception? This should be discussed as you may have missed some miRNA targets due to this.
Eg. Human plasma pregnancy-associated miRNAs and their temporal variation within the first trimester of pregnancy (2022, Reprod Biol Endocrinol)
By Cécilia Légaré et al. have looked at approx. 2000 miRNAs where almost 200 of them were expressed differently in pregnant women compared to non-pregnant.

We appreciate the reviewer's comment. As we discussed in our paper, if the miRNAs associated with SGA remain elevated during spaceflight and persist when women become

pregnant, this could potentially increase the risk of SGA during pregnancy. Kamity et al. describe in a comprehensive review of miRNAs related to inflammation and immunity that certain miRNAs expressed in circulation in women prior to pregnancy can persist during pregnancy, potentially increasing the risk of complications for both the pregnancy and the fetus (*Kamity R, Sharma S, Hanna N. MicroRNA-Mediated Control of Inflammation and Tolerance in Pregnancy. Front Immunol. 2019 Apr 5;10:718. doi: 10.3389/fimmu.2019.00718. PMID: 31024550; PMCID: PMC6460512*).

In the paper suggested by the reviewer, as noted, nearly 200 miRNAs were found to exhibit differential expression between pregnant and nonpregnant women. Among these, only two of our 13 miRNAs show differential expression: hsa-miR-22-3p and hsa-miR-378a-3p, which are less abundant in pregnant women compared to non-pregnant women (*Légaré, C. et al. Human plasma pregnancy-associated miRNAs and their temporal variation within the first trimester of pregnancy. Reprod Biol Endocrinol 20, 14 (2022*)). This study does not specifically investigate the potential risk of SGA, so it is noteworthy that these miRNAs we have identified as associated with SGA are less abundant or don't exist in circulation during normal pregnancy.

Currently, pregnant women are not permitted to participate in spaceflight missions. The 13 miRNAs identified in our study overlap with those observed in women who experience SGA births and in non-pregnant mice subjected to spaceflight, as noted by the reviewer. These miRNAs are overexpressed due to SGA, and in non-pregnant women, they typically would not be overexpressed unless other health conditions are present. Individually, these miRNAs may also be differentially expressed for various reasons, including their association with diseases such as cancer, as detailed in our paper. When this specific signature of miRNAs is expressed, as demonstrated in our study, it correlates with an increased risk of SGA and overlaps with miRNAs affected by spaceflight-related conditions. We have further discussed this comment and addressed some of the points raised here in the following section of the discussion:

The correction is in the discussion: lines 565-578:
"*During pregnancy, miRNAs have been shown to play dual roles, contributing to both healthy stages of pregnancy and potential complications for the fetus. For instance, Kamity et al. discuss in a comprehensive review of miRNAs related to inflammation and immunity how certain miRNAs expressed in circulation prior to pregnancy can persist during pregnancy, potentially increasing the risk of complications for both the pregnancy and the fetus*[104]*. In contrast, a study by Cécilia Légaré et al. identified 191 miRNAs commonly expressed between pregnant and nonpregnant women*[105.] *Among these, only two of our 13 miRNAs show differential expression: hsa-miR-22-3p and hsa-miR-378a-3p, which are less abundant in pregnant women compared to nonpregnant women. Although this study does not specifically investigate the potential risk of SGA, it is noteworthy that these miRNAs we have identified as associated with SGA are less abundant during normal pregnancy. The 13 miRNAs we identified as elevated during spaceflight in nonpregnant female mice may potentially contribute to the risk of SGA upon women's return to Earth, as there was no overlap or increase of these miRNAs observed in healthy pregnant women.*"

3) Line 157-159: Should this be referring to Fig S2?

We thank the reviewer for the comment and catching this mistake. Yes, it should be referring to **Fig. S2** and we have made the appropriate change to the manuscript.

4) Fig 1b&f: for timepoint independent analysis, could you clarify whether all tps were included in the analysis? If so, did you account for multiple samples originating from a single patient? It would be helpful to see n numbers in all figure legends.

We thank the reviewer for this comment. We did include all the time points for the PCA plots in figures 1b & f. We thought it would be best to include all time points for those plots regardless if it originated from a single patient. This is due to the fact each time point can be treated as an individual data point.

As stated in the methods section there were a total of n = 29 patients included in the original study with n =16 with normal birth outcomes and n = 13 with SGA births. For each time point these replicates were also present. We have included this N number in the figure captions in addition to what is stated in the methods.

5) For OSD-336, how were the samples processed for plasma? Was risk of haemolysis considered? Various factors during plasma processing have been shown to affect miRNA levels, including type of anticoagulant, haemolysis, time to processing, storage conditions etc. This should be included in methods and discussed if not considered.

Unfortunately, at the time of the experiments, no hemolysis data was measured on the samples before the isolation of the miRNAs. This is a valid point to consider since, as you are aware, hemolysis can potentially impact some miRNA signals. In our original paper (Malkani et al., Cell Reports 2020), we discussed the potential impact of hemolysis and provided indications of why it might not have strongly affected our results for this dataset. We have referenced this and provided additional discussion regarding hemolysis in the methods section under “Analysis of miRNA sequencing from murine samples” (lines 835-849):

“*It is important to also consider the issue of hemolysis with our analysis. Although hemolysis is unavoidable for blood/plasma samples obtained from in vivo studies similar to those described in OSD-336, there are ways to account for this in the analysis when hemolysis isn’t measured. Numerous papers discuss the impact of hemolysis on circulating miRNAs, concluding that while hemolysis in in vivo samples cannot be entirely avoided, procedures can significantly reduce its effects*[142,143,144]*. Given the constraints of space biology experiments, the primary step to reduce hemolysis variations for miRNAs is by handling all samples identically. Both the controls and experimental samples were handled under the exact same conditions throughout the study. Thus, if miRNA levels had been altered due to hemolysis, all miRNA levels would have been altered similarly across our comparisons. For the miRNA-seq data, the variation across the biological replicates was minimal, and the miRNAs that showed high variation were either not significant or filtered out during the preprocessing step. This suggests that while hemolysis can*

alter miRNA levels[143], the levels of the remaining miRNAs after data processing should have been altered identically in our controls and experimental conditions, reducing variability between results."

6) Discussion should also include whether the 13 identified miRNAs would target same genes/pathways in pregnant vs non-pregnant women

Fundamentally, miRNAs target the same genes and pathways regardless of the context. What can change between different conditions are the miRNA expression levels and the specific miRNAs involved in a disease versus healthy controls. In response to this comment, we conducted a literature search on how different circulating miRNAs and their specific genes/pathways are involved in pregnant versus non-pregnant women. For most miRNAs, there is limited literature on their behavior. This aligns with our findings that 12 of the 13 miRNAs are overexpressed in SGA females. Most studies profiling circulating plasma miRNAs in normal pregnancy focus on upregulated miRNAs. Therefore, we expanded our search to include how these miRNAs, in general, are impacted during pregnancy. From this search, we created **Table S6**, which provides a comprehensive literature review and summary of how these miRNAs are regulated in the circulation during pregnancy. We found literature indicating that miR-22-3p and miR-378a-5p are downregulated in healthy pregnant women compared to non-pregnant women, which were the only two miRNAs we found relevant to the original comment. Although we did not find literature on the behavior of the remaining 11 miRNAs when comparing pregnant to non-pregnant women, we believe that summarizing how these miRNAs change during pregnancy adds valuable context to the manuscript. We have added the following brief discussion around **Table S6** in the discussion (lines: 578-583):

"*When conducting a comprehensive literature review of the remaining 11 miRNAs, we discovered that these miRNAs are heavily involved in various pregnancy complications, including preeclampsia, gestational diabetes mellitus (GDM), preterm birth, and miscarriages. These complications are associated with specific pathways such as the TGF-β pathway, metabolic pathways, and immune-related pathways (see **Table S6** for a complete list and related references).*"

7) Without any data showing that the actual miRNA level changes following exposure to space stressors is maintained for prolonged period of time (ie. Until first trimester of pregnancy), it is difficult to say these miRNAs can be used to assess risk of SGA in future pregnancies after return to Earth. Is there any evidence in literature that any of the 13 miRNAs are affected by environmental/other factors such as exposure to pollution, poor diet, lack of exercise? Including such details in discussion could help strengthen the statement that these miRNAs have potential to predict future SGA births following space travel.

We thank the reviewer for the comment. Different studies demonstrate how miRNA can be altered by environmental factors (*Tammen SA, Friso S, Choi SW. Epigenetics: the link between nature and nurture. Mol Aspects Med. 2013 Jul-Aug;34(4):753-64. doi: 10.1016/j.mam.2012.07.018. Epub 2012 Aug 10. PMID: 22906839; PMCID: PMC3515707*).

Concentrating on the common factors, such as pollution, diet,and exercise, we found that all the 13 miRNAs can be influenced by environmental factors. We added a supplementary table (i.e. **Table S2)** to show the effects of these categories on the 13 miRNAs and added the following additional discussion in the results section, pg 9 lines 385 - 396 (we thought it would be appropriate to discuss this in this part of the paper rather than the discussion):

"*As the NASA Twin Study suggests, there is potential for these miRNAs to remain overexpressed for a period after returning to Earth. Although there is no evidence indicating whether these miRNAs will remain dysregulated in females or in a larger cohort of astronauts, potentially increasing the risk of SGA, we observed a long-lasting impact on the dysregulation of these 13 miRNAs due to factors such as the environment, diet, or exercise (**Table S2**). A comprehensive literature search revealed that a high-fat diet causes long-lasting dysregulation in miRNAs similar to the signature observed with spaceflight and SGA (**Table S2**). Additionally, certain pollutants and toxic environments, such as exposure to polychlorinated biphenyls[64] or specific hydrocarbons[65,66], can cause long-lasting miRNA upregulation. While this is not direct evidence that these miRNAs will persist long-term after astronauts return to Earth, it indicates that long-term dysfunction associated with these 13 miRNAs can significantly impact human health, influencing downstream pathways and genes."*

8) Line 273-275: The authors state that the 13 miRNAs identified in the study can have long lasting effects on children's development as specific functions are related to organs associated with defects in SGA infants. Is there any evidence that these 13 miRNA changes are also seen in SGA babies? Or whether these miRNAs are able to cross the placenta from maternal circulation? It would be important to include these details as without any evidence that these miRNA changes on maternal side are reflected on the fetal side, this statement would be overreaching.

We thank the reviewer for the comment. From the current available literature, miRNAs of SGA children were compared to appropriate for gestational age (AGA) children: 22 miRNAs were found differentially expressed in the two groups. It's interesting to notice that two of the 13 miRNAs individuated by our analysis, hsa-miR-29b-3p and hsa-miR-29c-3p, are upregulated in SGA children. In particular, the authors suggest that hsa-miR-29c-3p is part of the miRNA group responsible for the catch-up growth in SGA children (*Jeong HR, Han JA, Kim H, Lee HJ, Shim YS, Kang MJ, Yoon JS, Ryu S, Hwang IT. Exosomal miRNA Profile in Small-for-Gestational-Age Children: A Potential Biomarker for Catch-Up Growth. Genes (Basel). 2022 May 24;13(6):938. doi: 10.3390/genes13060938. PMID: 35741700; PMCID: PMC9223036*).

The correction is at the lines 281-286:
"*Jeong et al.[54] demonstrated from miRNAs of SGA children compared to appropriate for gestational age children that 22 miRNAs were found differentially expressed in the two groups. It's interesting to note that two of the 13 miRNAs from our analysis, hsa-miR-29b-3p and hsa-miR-29c-3p, are upregulated in SGA children. In particular, the authors suggest that hsa-miR-29c-3p is responsible for the recovery in growth in SGA children*."

9) Without confirming the functional role of the 13 miRNAs, it is questionable how significant it is to find drugs to countermeasure miRNA changes.

We thank the reviewer for the comment. The tool we used to predict the drugs targeting the miRNAs is called sChemNet, which is currently in press at Nature Communications. In that publication, we provide a detailed discussion of this model and perform additional functional assays on key predicted drugs targeting specific miRNAs. We experimentally validated sChemNet predictions for miR-451 in a zebrafish model under oxidative stress and for miR-181 in breast cancer cell lines. This previous work, currently in press, gives us confidence in the accuracy and functional relevance of our predictions for targeting miRNAs and improving disease outcomes. However, for new predicted drug and miRNA interactions, further functional assays are necessary to validate the predictions, as is common with any in silico work. These assays were beyond the scope of this manuscript and will be pursued in future research. As the reviewer might acknowledge, it is not feasible to address everything in a single manuscript.

We have already acknowledged this when addressing the limitations of our study in the discussion by providing this statement (lines 709-8711):

"*The predicted countermeasures show promise but warrant further experimental validation. Given the challenges of spaceflight-related experiments, leveraging resources such as ImmPort and GeneLab becomes essential for expediting the discovery of biological health risks. Timely dissemination of these findings within the scientific community is crucial, facilitating further experiments and validation for the development of SGA-related biomarkers and countermeasures in the future.*"

Additionally, we have revised the last sentence of the results section to (lines 544-548):

"*Overall, our machine learning prediction identified drugs that may prove useful for targeting miRNA that are associated with both SGA and spaceflight, but extensive preclinical and clinical testing would be needed to assess whether they are effective and safe in women and provide the functional impact on the miRNAs as predicted.*"

Lastly, in the discussion section we have added the following text to further acknowledge the fact that we will need further functional assays to assess the validity of these drugs as potential countermeasures (lines 678-687):

"*As stated in the results, it is important to note that both perfluorodecalin and triamcinolone are predictions made by our sChemNet ML model. In the original manuscript for sChemNet[77], we experimentally validated the model's predictions and provided functional validations for a few miRNAs as proof of principle. For instance, we demonstrated that a few predicted drugs for miR-451 can rescue oxidative stress in a zebrafish animal model, and predicted vitamin D targeting miR-181 can reduce cell proliferation in breast cancer cell lines. Although this provides some proof that our model can produce drug predictions with functional relevance to the miRNAs of interest, further functional experimental assays are necessary to validate their*

impact on the 13 miRNAs and their potential to mitigate the progression of SGA. This validation is crucial to assess their effectiveness as countermeasures for spaceflight."

Reviewer #2 (Remarks to the Author):

What are the major claims of the paper?
Using published miRNA databases and computational methods, the authors discuss a shared miRNA signature between SGA and space environment conserved between humans and mice. Then they use machine learning to identify potential FDA- approved drugs to mitigate the risk of SGA in female astronauts.

Overall impression and specific points
It is a well written paper with interesting concept and 'catchy' title. It uses novel methodology but relies on several assumptions. One of them is that women astronauts with prolonged exposure to the space environment may have an elevated risk of SGA. The authors then use this hypothesis (and without any data to support an increased risk of SGA) they then computationally compare real life data of human SGA pregnant signature to mice simulated spaceflight biology experiments particularly focusing on female non pregnant C57BL/6 mice. In their previous study (published a couple of years ago) on spaceflight-associated miRNA signature, that was also defined computationally, based on transcriptomic analysis, and not measured experimentally (Ref 27).

We appreciate the reviewer's comment. The primary focus of our study is to investigate whether miRNAs associated with SGA might become elevated during spaceflight, as you suggested. Currently, there is no literature addressing the risk of SGA in female astronauts who return to Earth and subsequently become pregnant. This *in silico* study aims to introduce a novel hypothesis, which can later be tested experimentally to determine if this risk is significant during prolonged space travel. As you can imagine, there are also no existing experiments involving rodents at risk of developing SGA after exposure to the space environment. This gap highlights an important area for future research. Given the dawn of the second space age, with increased commercial flights, more young women are likely to travel to space. Therefore, it is essential to consider new health risks that have not been previously explored.

Additionally, the reviewer correctly noted that we defined the spaceflight-associated miRNA signature computationally. However, as shown in Reference 47, we conducted extensive experimental validation. This included quantifying the miRNAs in several mouse and rat models exposed to the space environment. We also confirmed that these miRNAs were impacted by the NASA Twin Study. Furthermore, we inhibited three miRNAs in this signature associated with cardiovascular effects, which resulted in the mitigation of space radiation effects. We have also recently published a follow-up study on countermeasures, demonstrating rescue and improvement of several functions following the inhibition of these three miRNAs (see references 47 and 48 in the manuscript and the following recent publication: *McDonald, J.T., Kim, J.,*

Farmerie, L. et al. Space radiation damage rescued by inhibition of key spaceflight associated miRNAs. Nat Commun 15, 4825 (2024). https://doi.org/10.1038/s41467-024-48920-y).

Although whole body mice weights were significantly reduced in GCR and SPE irradiation groups in combination with simulated microgravity (hindlimb unloading, HU), I cannot find literature suggesting that mice exposed in simulated spaceflight conditions have an increased risk of SGA. Therefore, it is difficult to assess the value of the 13 common miRNAs between SGA vs control human pregnancies and spaceflight vs terrestrial mice.

This is the central focus of our publication. By utilizing data from different public databases (i.e., ImmPort and GeneLab) and creatively combining these datasets, we are able to generate a new hypothesis regarding the potential risk of SGA in females due to spaceflight. Currently, there are no publications addressing the potential risk of SGA due to spaceflight. Historically, the literature and research on spaceflight have been sparse regarding the impact of the space environment on the female reproductive system. This means there are significant gaps in research specifically addressing many potential female-specific health risks, especially those associated with the female reproductive system. Fortunately, as we state in the introduction, there is now an equal distribution of women being trained as astronauts compared to men, a shift from over a decade ago. We specifically emphasize this point in the introduction (lines 69-74).

"*Most prior spaceflight health studies are biased toward male participants. Women comprised just 15% of NASA's astronaut corps until a decade ago. Nowadays, the representation of women in astronaut training has increased to nearly 50%[3]. Emerging studies are beginning to shed light on sex-specific health risks associated with spaceflight, supplementing the traditional sex-independent spaceflight research[4,5]. Yet, our understanding of how space travel can affect women's reproductive systems remains limited*."

To address this specific comment, our work generates a specific hypothesis and identifies a potential gap in knowledge that needs to be further explored in future research. Although it is currently challenging to assess the potential SGA risk due to spaceflight, we have provided some evidence that this avenue of research, which could impact the female reproductive system, should be investigated further. In space research, it is crucial to address potential risks that might arise in the future rather than waiting for problems to occur during long-term deep space missions, by which time the biological damage may be more difficult to remedy. Prevention is key in space biology research, and identifying potential novel health risks for future investigation is essential. To further emphasize this point, we have modified the last paragraph of the discussion to the following (lines 715-729):

"*Our findings pave the way for future studies that can offer clinical therapeutics, extending the boundaries of science into space and accelerating our understanding of various diseases. This discussion underscores the critical need for further investigation into the female reproductive system in space. Female reproductive health is currently understudied but essential for understanding and mitigating health risks associated with space travel. In space research, it is crucial to address potential health risks before they manifest during long-term missions, as they*

can cause long-term health issues that will be difficult to treat at that point. Our work generates a specific hypothesis and identifies a potential gap in knowledge that needs to be further explored in future research. Although it is currently challenging to assess the potential SGA risk due to spaceflight, we have provided some evidence that this research avenue, which could impact the female reproductive system, should be investigated further. Prevention is key in space biology research, and identifying potential novel health risks for future investigation is essential. Finally, the use of space biology databases has been instrumental in this research, offering a unique perspective on biological processes under extreme environmental stress."

Are any of these miRNAs change in humans exposed to space conditions?

This is an important question, but currently, there is no miRNA-sequenced or quantified data from female astronauts exposed to the space environment. We aim to obtain such data in future space missions. However, access to astronaut data and the astronauts themselves is currently limited. The available miRNA-sequence data from the NASA Twin Study is from one male astronaut, which does not represent female astronaut responses.

Despite this limitation, we observed that 7 out of the 13 miRNAs were overexpressed during spaceflight in the NASA Twin Study, suggesting dysregulation of these miRNAs in space (**Fig. S10b**). Although this data is from a male astronaut, it indicates that these miRNAs might be overexpressed during spaceflight in a sex-independent manner, potentially posing long-term risks if upregulated in females. We have provided a plot of the miRNAs for the NASA Twin Study in **Figure S10b** and additional text discussing this in the manuscript (lines 370-384):

"*We also utilized the NASA Twin Study miRNA-sequence data[47,64] to determine if these miRNAs are present in humans during spaceflight (**Fig. S10b**). The study involved male twins, with one twin spending 340 days on the ISS while the other remained on Earth. Blood samples from both were analyzed using miRNA sequencing across different sorted cell populations, in addition to other assays. As this is the only available miRNA-seq data from humans in space, we aimed to see if these miRNAs are expressed during spaceflight regardless of sex. Our analysis showed that 7 out of 13 miRNAs were overexpressed during spaceflight in one of the cell types. Upon return to Earth the miRNAs start to decrease back to control levels, but four of the miRNAs still remain elevated above control levels after flight (**Fig. S10b**). Although this data is from a male astronaut, it indicates that these miRNAs might be overexpressed during spaceflight in a sex-independent manner, potentially posing long-term risks if upregulated in females. This highlights the need for more sex-dependent studies on miRNA changes in astronauts and additional confirmation from further human studies. Overall, the gene targets of these miRNAs may pose a risk of increased birth defects if inhibited over time, as indicated by our findings related to spaceflight.*"

The authors then explore or the various biological functions that can be implicated on children's development. The methods again are appropriate but the concept and basis for the investigation is debatable. How do these gene pathways change after space travel?

Unfortunately, there is limited data available from space travel experiments in humans or animals that study the long-term changes after returning to Earth. In our paper, we present data from the Inspiration 4 mission, as shown in **Figures 6** and **7**. These figures illustrate the changes in the 45 genes identified in Figure 5 for both male and female astronauts after returning to Earth. The longest time point we have is 82 days post-return. Our findings indicate long-term inhibition of these genes in certain cell types, even 82 days after returning to Earth, with more pronounced changes observed in female astronauts. To address your point regarding pathways, we conducted additional analyses on the Inspiration 4 data to show how the related pathways discussed in **Figures 4** and **5**, associated with the 13 miRNAs, are changing in both female and male Inspiration 4 astronauts. These figures and analysis are included as **Figures S12-S15** and **Table S4**.

We also included additional analyses and figures based on simulated space radiation experiments performed on both female and male mice exposed to 50 cGy GCR irradiation, which approximates the total dose of a deep space mission to Mars and back, lasting about a year. Blood samples were collected from these mice 14 days post-exposure, followed by RNA sequencing. We performed pathway analysis (using Gene Set Enrichment Analysis) on the data, focusing on the pathways regulated by the 13 miRNAs shown in **Figures 4** and **5**. These figures are included in the supplemental material as **Figures S7** and **S9**. Although this data is not from human subjects, it demonstrates the long-term impact of space radiation exposure on specific miRNA-related pathways. We show that most pathways in these mice are dysregulated in females, with some pathways similarly affected in both sexes, and a few unique to males. Interestingly, pathways related to the immune system, mitochondria, and RNA metabolism are suppressed long-term exclusively in female mice. This long-term suppression may contribute to the potential risk of SGA, as discussed in the paper.

We have also added the text to the results to discuss these additions to the paper:
In line 298-309:
"*To determine if these pathways and functions are impacted in females long-term after exposure to the space environment, we included RNA-seq analysis of blood samples from male and female C57BL/6J mice exposed to 50 cGy GCR simulated irradiation*[57]*. The blood samples were collected 14 days post-irradiation to assess any long-term biological impact. Although this data is not from human subjects, it demonstrates the long-term impact of space radiation exposure on specific miRNA-related pathways. Our results show that the majority of the pathways related to the 13 miRNAs (**Fig. 4**) are dysregulated in the female mice, with some pathways similarly affected in both sexes and a few unique to males when compared to the non-irradiated control mice (**Fig. S7** and **Table S1**). Interestingly, pathways related to the immune system and mitochondria are suppressed long-term exclusively in female mice, raising further concerns about the health risks associated with these 13 miRNAs.*"

And in lines 341-348:
"*To further determine the long-term impact of these pathways after exposure to the space environment, we analyzed the mice blood data collected 14 days post-exposure to 50 cGy GCR irradiation (described above). The pathway analysis revealed that certain pathways were*

*suppressed exclusively in female mice over the long term, including immune-related pathways associated with both the adaptive and innate immune systems and RNA metabolism (**Fig. S9**). Interestingly, signal transduction pathways were upregulated only in male mice, indicating that these pathways might not be as critical in females for contributing to the long-term risk associated with SGA.*"

For the sex specific Inspiration4 astronaut data analysis we added the following text in lines 439 - 455:
*"Lastly, we aimed to determine if the key pathways impacted by the 13 miRNAs and their gene targets are also dysregulated in astronauts, specifically the I4 astronauts. Utilizing the I4 scRNA-seq data, we performed pathway analysis and identified key pathways similarly regulated by the miRNAs (**Figs. 4** and **5**). Since the I4 crew included two males and two females, we were able to perform sex-dependent analysis and observe the key pathways regulated immediately upon return to Earth and the long-term post-flight effects (**Figs. S12 - S15, Table S4**). The I4 astronaut data showed similar results to the simulated space radiation experiments performed on mice (**Figs. S7 and S9**). Specifically, female astronauts exhibited similar suppression of the majority of pathways observed for the 13 miRNAs (**Fig. S12**). A similar pattern was observed in male I4 astronauts (**Fig. S13**). Although there were overlapping pathways between males and females, distinct pathway patterns specific to female astronauts were not present in males. Additionally, we observed similar suppression of pathways as in the mouse study (**Fig. S9)** in both female and male astronauts for the 45 gene targets (**Figs. S14 and S15**). Interestingly, there was suppression of the RNA metabolism pathway across all cell types. Through this analysis, we provide indirect confirmation that miRNAs potentially influence key pathways long-term, which may increase health risks associated with SGA."*

Lastly, we conducted additional pathway analysis on the 45 gene targets shared by 10 or more of the 13 miRNAs (**Fig. S8**) using tools such as Ingenuity Pathway Analysis. This analysis offers further information and insights into the functional impacts of these genes. Although this is an indirect comparison of functional impacts via pathway analysis, it enhances our understanding of how these genes are related to the female reproductive system. This, in turn, provides additional confidence in the impact of these miRNAs during spaceflight. The following text has been added to the results section to reflect this additional data (lines 324-332):

"*Unbiased analysis of these 45 gene targets using Ingenuity Pathway Analysis (IPA) tool identified that estrogen signaling receptor (ESR)-mediated signaling was the third most significant canonical pathway (p value = 6.78E-05) (**Fig. S8a**). Four candidate genes identified to be participating were AGO1, POU2F1, TNRC6B, and YY1. All of these genes were found to be associated with reproductive system diseases including female genital tract adenocarcinoma, endometrioid endometrial adenocarcinoma and adenosquamous ovarian carcinoma (IPA, p value range 1.43E-3 to 2.6 E-2) (**Fig. S8b**). Some of the key Subsequent analysis using upstream analysis tool of IPA predicted 10 genes in different cellular compartment to be regulated by ESR1 (**Fig. S8c**).*"

The authors then aim to confirm the key SGA-related molecular changes in astronauts, through using data published for scRNA-seq from 2 human males and 2 non pregnant females who participated in the I4 mission occurring in the high altitude of 590km. Can the authors provide further explanation as to how they come to the conclusion that a T cell population suppression demonstrated by suppressed CD4 and CD8 T cells within 24 hours of 0.5 Gy GCR irradiation supports the heightened risk of SGA after space flight?

We have provided additional information in the results to further explain how dysfunction in the T cell population can significantly heighten the risk of SGA. From the I4 data, we determined that the most impacted cell population for the gene targets of the 13 miRNAs was the T cell population, as discussed in the paper and shown in **Figures 7** and **S10**. This data demonstrates that there is a lasting impact on the T cell population up to 82 days after returning to Earth. These changes in the T cell population can influence potential birth defect risks, as discussed in the results.

Additionally, research by Gomez-Lopez et al. has shown that suppression or reduction in regulatory T cells (Tregs) can cause serious issues during pregnancy, leading to preterm labor, birth, and other complications for the fetus. We have included the following statement in the results to further discuss the impact that T cell population suppression will have during pregnancy (lines 428-431):

"*CD4 Treg cells at the maternal-fetal interface are important for establishing tolerance in early pregnancy. A reduction in CD4 Tregs within the overall population suggests suppressed tolerance and elevated inflammation, which could contribute to numerous labor and birth issues, including potentially SGA*[69]*.*"

The authors then are investigating potential FDA-approved small molecule drugs to target SGA miRNAs. Although the results is an interesting read, I am uncertain about the value of defining triamcinolone and perfluorodecalin as possible drug molecules. Traimcinolone is a class C drug and has not been associated with increased risk of spontaneous abortion or SGA in humans. Are there any studies suggesting a beneficial growth profile through its use in animal models?

We appreciate the reviewer's comment. The use of triamcinolone in animal models can show potential benefits, such as protective effects in retinopathy of prematurity (*Chung IY, Kim YH, Park JM, Seo SW, Choi WS, Cho GJ, Yoo JM. Protective effects of triamcinolone acetonide upon the upregulation and phosphorylation of GAP 43 in an animal model of retinopathy of prematurity. Acta Ophthalmol. 2010 Sep;88(6):e217-21. doi: 10.1111/j.1755-3768.2010.01951.x. PMID: 20560891*) and reductions in neovascularization and capillary density (*Hartnett ME, Martiniuk DJ, Saito Y, Geisen P, Peterson LJ, McColm JR. Triamcinolone reduces neovascularization, capillary density and IGF-1 receptor phosphorylation in a model of oxygen-induced retinopathy. Invest Ophthalmol Vis Sci. 2006 Nov;47(11):4975-82. doi: 10.1167/iovs.06-0450. PMID: 17065516; PMCID: PMC1828044*). Others have highlighted negative effects on skeletal growth and structure, including growth retardation and osteoporosis (*Wood CL, Soucek O, Wong SC, Zaman F, Farquharson C, Savendahl L, Ahmed SF. Animal*

models to explore the effects of glucocorticoids on skeletal growth and structure. J Endocrinol. 2018 Jan;236(1):R69-R91. doi: 10.1530/JOE-17-0361. Epub 2017 Oct 19. PMID: 29051192). A study on neonatal mice found that triamcinolone significantly reduced chondrocyte growth in the mandibular condyle, inhibiting the normal process of endochondral bone formation (*Silbermann M, Weiss A, Raz E. Retardative effects of a corticosteroid hormone upon chondrocyte growth in the mandibular condyle of neonatal mice. J Craniofac Genet Dev Biol. 1981;1(1):109-22. PMID: 7341638*). These findings suggest that the effects of triamcinolone on growth profiles in animal models are complex and may vary depending on the specific context and dosage.

It is important to emphasize the point that we are not suggesting to give any of the potentially predicted countermeasures during pregnancy. As we state throughout the paper, we suggest that these predicted countermeasures will be given during spaceflight, when the astronauts will not be pregnant. This will potentially act as a way to revert the circulating miRNAs levels back to control levels on Earth. Then upon returning to Earth the predicted countermeasures will not be given. That said, there is an interesting prospective clinical study that indicates that triamcinolone when given intranasally to pregnant women was not associated with risk of "SGA/spontaneous abortions/overall malformations." They did however show that intranasal triamcinolone will increase the risk of respiratory system development causing potential defects. (*Bérard A, Sheehy O, Kurzinger ML, Juhaeri J. Intranasal triamcinolone use during pregnancy and the risk of adverse pregnancy outcomes. J Allergy Clin Immunol. 2016 Jul;138(1):97-104.e7. doi: 10.1016/j.jaci.2016.01.021. Epub 2016 Apr 1. PMID: 27045580*). We have added the following statement to the paper to further address this point (664-668):

"*Although we are not recommending that this countermeasure should be given to pregnant women, Bérard et al.*[114] *demonstrated that the administration of triamcinolone intranasally to pregnant women does not increase the risk of SGA, malformation or spontaneous abortion, but is associated with potential risk of respiratory system defects.*"

Also what is the placental transfer of triamcinolone and perfluorodecalin?

We thank the reviewer for the comment. It is a valid concern that taking these drugs could pose risks to the fetus, particularly for triamcinolone since it is a steroid. This is why we did not suggest administering these drugs to pregnant women. These drugs are intended as countermeasures, and we propose in the manuscript that they be administered to women during spaceflight. Currently, pregnant women are not authorized for spaceflight, and this assumption underlies the statements made throughout the manuscript. In discussing the results of our predictions in the results section, we have already included the following statement (lines 477-486):

"*To identify any countermeasures that currently exist for targeting the SGA-spaceflight miRNA signature, we leveraged our well-established machine learning tool, sChemNET*[84]*. Using sChemNET, we predicted potential FDA-approved small molecule drugs that could target these miRNAs and be further developed as countermeasures. The goal of this analysis was to find small molecule drugs that could potentially inhibit spaceflight-associated SGA miRNAs both*

during spaceflight and before pregnancy. Any potential toxicity concerns related to these treatments during pregnancy are alleviated, as the drugs will not be administered during pregnancy. Theoretically, the associated miRNAs should be suppressed due to implemented countermeasures."

We have further modified the above paragraph to address your comment to the following:

"*To identify any countermeasures that currently exist for targeting the SGA-spaceflight miRNA signature, we leveraged our well-established machine learning tool, sChemNET[77]. Using sChemNET, we predicted potential FDA-approved small molecule drugs that could target these miRNAs and be further developed as countermeasures. The goal of this analysis was to find small molecule drugs that could potentially inhibit spaceflight-associated SGA miRNAs both during spaceflight and before pregnancy. Any potential toxicity concerns related to these treatments during pregnancy and potential placental transfer of the drugs to the fetus are alleviated, as the drugs will not be administered during pregnancy, given that currently, pregnant women do not participate in spaceflight.Theoretically, the associated miRNAs should be suppressed due to implemented countermeasures.*"

In addition, we modified the following statement in the discussion to further address this comment (lines 653-655):

"To mitigate the effects of spaceflight on future SGA risk, we explored potential countermeasures for nonpregnant women on future spaceflight missions. Using our machine learning framework, sChemNET[77], we aim to provide a basis for future clinical studies."

We underline the fact that these drugs are hypothesized to prevent SGA, so they would be given to nonpregnant women in spaceflight.

In a 2023 Swedish study of maternal serum concentrations of perfluoroalkyl substances poor growth was observed in women with higher concentrations of per- and polyfluoralkyl substance (PFOA and PFHxS) as compared to the controls. Are these different to perfluorodecalin?

Per- and polyfluoroalkyl substances (PFAS) are synthetic chemicals [32388293], in the carbon chain the hydrogen atoms are substituted by fluorine atoms [35405506]. Human exposure to PFAS mainly occurs through drinking water sources near fluorochemical manufacturing locations and the consumption of contaminated food [36740157]. PFAS has been reported to disrupt thyroid function in pregnancies, causing circulating free THs to increase [32388293]. There is no statistical evidence that the increase of PFAs concentration in maternal serum can enhance the risk of SGA birth [maternal serum concentration]. Perfluorodecalin can be considered a PFAS, because it is a synthetic organic chemical with the hydrogen substituted with fluorine atoms. However, compared to the other PFAS substances, perfluorodecalin has not been considered dangerous for human health (*Céline C, Catherine B, Romane C, Laurence C. Per- and polyfluoroalkyls used as cosmetic ingredients - Qualitative study of 765 cosmetic*

products. Food Chem Toxicol. 2024 May;187:114625. doi: 10.1016/j.fct.2024.114625. Epub 2024 Apr 4. PMID: 38582342).

We addressed the comment adding the following text:
"*Although perfluorodecalin is considered as part of per- and poly-fluoroalkyl substances, which are classified as pollutants*[91] *and might be toxic*[92]*, perfluorodecalin has been declared safe for human health*[93]." (lines 542-544)