**Author Response:**

**We addressed and modified the following editorial comments in the revised manuscript. Thank you for the direct instruction.**

**Editorial comments:**  
Changes to be made by the author(s) regarding the manuscript:  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.  
2. Please check either the Standard Access or Open Access checkbox in the Author License Agreement (ALA). Please then scan and upload the signed ALA to your Editorial Manager account.  
3. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”  
4. Figure 1: Please include a space between all numerical values and their corresponding time units (0 h, 2 h, 24 h).  
5. Please upload each Table individually to your Editorial Manager account as an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.  
6. Please revise the title to avoid the use of the colon.  
7. Please include a space between all numerical values and their corresponding units: 24 h, 37 °C, 60 s, 40 ft; etc.  
8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: JUGS machine, JPS Sports, SIM-G; Triax Technologies, Inc., etc.  
9. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution’s human research ethics committee.  
10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).  
11. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.  
12. Discussion: Please also discuss the critical steps within the protocol.  
13. References: Please do not abbreviate journal titles.  
14. Table of Materials: Please remove trademark (™) and registered (®) symbols.  
  
**Reviewers' comments:**  
  
Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.  
  
  
Reviewer #1:  
  
The Authors of this manuscript use an automated machinery to induce control head impacts in players. A cohort of "kickers" is used as a control. The manuscript is concisely written and reports results that were already published. The main and perhaps fatal flaw of the design is to use a marker (NF-L) which has been rarely studied in clinical context, while ignoring S`100B and GFAP which are better known. The reason for this comment is not this Reviewer's preference of one marker over another, but rather a concern in the space of general diagnostics. An elevation is meaningless unless a value for controls vs. injured is available. In a previous study with S100B in football players, subconcussive head hits were often associated with an increase of S100B above the published and validated control values. In other words, an elevation of a marker (e.g., blood glucose) within a normal clinical range is meaningless, and does not suggest a diabetic pathology.

**Author Response:**

We thank the reviewer for his/her comments on the use of NF-L as a marker for brain injury. We would like to clarify the rationale of using the marker in this manuscript. As mentioned, extensive research has been conducted to better understand S100B and GFAP (along with UCH-L1 and Tau) in clinical studies, and indeed, NF-L has lesser volumes of evidence to date. This is exactly why our SSHM can be of paramount use in validating the clinical results. Shahim et al.1 and Oliver et al.2,3 have reported that blood levels of NF-L raise in concert with subconcussive head impacts in boxers and football players. However, as elaborated in our discussion section, these studies do not control any extraneous factors, and thus the effects of subconcussive head impact on NF-L is suggestive at best. In this manuscript, we demonstrated that using the laboratory subconcussion model we were able to validate their clinical findings by controlling for extraneous factors that are inherent to field studies (temperature change, exercise effect, body hit, and orthopedic stress). It is also important to emphasize that this manuscript is a methodological paper submitted to the JoVE (which introduces innovative methodologies). While it is best to evaluate other markers (S100B, GFAP, Tau, UCH-L1, NF-H, NSE, SNTF: see our review papers4,5 on their strength and clinical significance), that is not the intent of this manuscript. Also, the sentence on “an elevation is meaningless unless a value for controls vs. injured is available” is confusing to us. We demonstrate the elevation in NF-L which was significantly elevated within-group (heading) and between group (heading vs. kicking control). The clinical significance of such increase is yet to be delineated by follow-up studies, as with any other blood biomarkers. The reviewer’s logic is not clear to us. Could you please elaborate?

An additional factor relates to use of human subjects as experimental tools. If NF-L increases are in your opinion clinically important, were the volunteers aware of the risk of CTE, or brain/axonal damage? It is a hard for me to understand how your IRB committee approved this study. Was the risk of long-term consequences deemed acceptable?

**Author Response:**   
Our apologies for not including an IRB statement. The study has been approved by Indiana University IRB and Temple University IRB (where Dr. Kawata currently and previous affiliate). Subconcussion poses a public health concern and it is important to understand the mechanism of head impact and physiological/biological response. Therefore, our protocol of 10 soccer headers (which is regularly performed in soccer players, as well as players in American football, ice hockey, rugby, and boxing incurring similar levels of head impacts per practice/game), is considered safe and advantageous means to study the subconcussive effect. Subjects were also told regarding risks of head impacts and signed informed consent. The ethical statement was added to the revised manuscript. Thank you for pointing this out.

Methods: "frontal lobe" is not a synonym of forehead and a forehead hit will affect other regions of the brain as well.

**Author Response:**

It is corrected in the revised manuscript.

REFERENCES

1. Shahim P, Zetterberg H, Tegner Y, Blennow K. Serum neurofilament light as a biomarker for mild traumatic brain injury in contact sports. *Neurology.* 2017;88(19):1788-1794.

2. Oliver JM, Jones MT, Kirk KM, et al. Serum Neurofilament Light in American Football Athletes over the Course of a Season. *Journal of neurotrauma.* 2016;33(19):1784-1789.

3. Oliver JM, Jones MT, Kirk KM, et al. Effect of Docosahexaenoic Acid on a Biomarker of Head Trauma in American Football. *Med Sci Sports Exerc.* 2016;48(6):974-982.

4. Kawata K, Tierney R, Langford D. Blood and cerebrospinal fluid biomarkers. *Handbook of clinical neurology.* 2018;158:217-233.

5. Kawata K, Liu CY, Merkel SF, Ramirez SH, Tierney RT, Langford D. Blood biomarkers for brain injury: What are we measuring? *Neuroscience and biobehavioral reviews.* 2016;68:460-473.

Reviewer #2:  
  
Manuscript Summary:  
In this manuscript, the authors describe a soccer heading protocol that provides a means to generate subconcussive head impacts under controlled circumstances. In particular, in a pre/post repeated measures design, participants headed a soccer ball 10 times in a 10 minute period. For the proof of concept purposes of this methods paper, the authors measured a blood biomarker (neurofilament light) which is associated with axonal injury. This was done immediately prior to and after the bout of soccer heading and then at 2 hours and 24 hours after the protocol. For comparison, a control group of participants did all the same procedures except instead of heading the ball they kicked it. Ball speed and trajectory were controlled by a JUGS soccer machine. The results showed a significant increase in NF-L in the heading but not the kicking group.  
  
The manuscript is clearly written and addresses a new experimental manipulation that has been published on only a small number of times. All of the relevant methodological details are present in the manuscript that would allow another scientist to replicate the procedures. I only have a couple of suggestions for the authors.  
  
Major Concerns:  
  
1. Some discussion of the safety and ethics associated with the protocol appears warranted. Researchers wanting to implement this protocol will need to convince their local ethics panel that the injuries that are induced by the head impacts are temporary in nature. One approach is to have the participants come back for additional testing (e.g., by serving as their own controls) to demonstrate that the relevant dependent variable has returned to baseline.

**Author Response:**

Thank you for the pointer and our apologies for not including the ethical statement. We have included an ”ethics statement” outlining our approval and compliance with IRB.

2. An additional paper by our group (Wallace et al., 2018. BMJ Open Sport and Exercise Science) examining NF-L changes following a more intense bout of heading has recently been published and may be worth including in the discussion regarding dose-response effects.

**Author Response:**

We included Wallace et al. paper in the discussion section.

3. The NF-L results are only different at the 24 hour time point between the heading and kicking groups. Although this is not the primary purpose of the manuscript, it might be worth discussing.

**Author Response:**

Although the reviewer’s recommendation is important, we feel that elaborating upon NF-L specifically would detract from the primary goal of the manuscript, which is to showcase the SSHM, and not the biomarkers it can be used with. We believe that the NF-L finding has been discussed sufficiently in the discussion section; therefore, we respectfully choose not to further elaborate on the NF-L result. Readers should refer to Wirsching et al.

Minor Concerns:  
  
1. The Alfonsi (2018) reference appears to be incomplete.

**Author Response:**

This was corrected.