REVIEWER 1

Dear Reviewer 1,

We once again thank you for the time taken to give us considered feedback on our manuscript and your suggestions for improvement and comments.

Across both rounds of feedback we have worked diligently to ensure we have addressed all of the concerns of this reviewer and amended the manuscript where appropriate to bring it up to a publishable standard.

We will address the remaining queries of this reviewer on a point-by-point basis.

Additionally, regarding the comment surrounding the English language quality, we have ensured that the manuscript has been thoroughly reviewed by multiple members of the author team and minor edits to grammar or word choice have been made where appropriate. As these changes are quite small and spread throughout the manuscript, please see the track-changed version of the paper for these edits.

1. *the sample size in the proteomics cohort (n=16, with successful venepuncture in 13) is relatively small, which may limit the generalizability of the findings*

   We acknowledge that, as this was a discovery proteomics project, our sample size was smaller than that which would be required for more definitive pathway analysis. This was a comment echoed by the other reviewer which led to changes and additions in the manuscript, notably, the inclusion that any such pathway level interpretation needs to be considered as hypothesis generating and still requires further examination. Being the first study to examine the acute pain proteome in humans, we welcome advances in this field/work in the future and hope that this study kickstarts research in this area.

   We have included a section in the revised discussion which identifies the small sample size as a limitation of the generalizability and pathway level interpretation of these results, yet urges future research for the benefit of pain understanding.

> *Altogether, these results suggest an impaired adaptive and anti-inflammatory response following an acute bout of musculoskeletal pain. The vast majority of these molecular alterations resolve following the cessation of HS-induced muscle pain suggesting a causal link between pain induction and a transiently altered immune profile. However, as this represents the first examination of proteomics in an acute experimental pain*

> *setting, and due to the comparatively small sample size for proteomics, mechanistic and pathway implications need to be explored further and with larger datasets. Future expansion of the acute pain proteome will bring greater certainty to the molecular pathways which underpin acute pain sensation in humans. Additionally, more detailed insight into the temporal dynamics of cytokine changes in relation to pain resolution (T30) would be beneficial in future work, as we did not have the resources to explore this within this study.* (Line 289-300)

Additionally, we have expanded the methods section to allow for greater transparency on our use of technical duplicates for all participants at all time points which does assist the statistical power of our findings.

> *2.1. Cohort one – Molecular data (proteomics and cytokines)*
>
> *All 16 participants recruited for this stage of the study were able to achieve a stable moderate baseline pain between 4 and 6 out of 10 in response to intramuscular 5% HS infusion. Of these 16 participants, successful venepuncture was achieved in 13 individuals. For each of the 13 samples at each time point (T0, T10, T20 and T30), technical duplicates were processed.*
>
> *Untargeted plasma-based proteomics identified 1671 quantifiable proteins. By applying filters as per the methods (confidence score ≥50, ≥1 unique peptides and ≤30ppm mass error) 547 proteins were identified for further statistical analysis. Analysis of the proteomics and targeted cytokine/chemokine analysis revealed 29 proteins (Table 1) and 2 cytokines (Figure 1) differentially expressed between T0 and T10. Furthermore, comparisons between T0 and T20 revealed differential expression of 17 proteins (Table 1) and 5 cytokines (Figure 1), and 12 proteins were differentially expressed between T0 and recovery.*

Overall, we agree with the comments of this reviewer that the generalizability of these findings was potentially over-emphasised in our original version of the manuscript. As such, we have walked through the discussion section and toned down any definitive links or mechanistic pathways we have described. We recognise that a degree of caution needs to be observed when making, or appearing to make, definitive statements that are not yet possible within the scope of our study. Track-changes can be found throughout the discussion

where we have elected to be more cautious with our word choice and softened statements where appropriate.

2. *The results clearly present the differentially expressed proteins and cytokines during acute muscle pain. The use of heatmaps and tables effectively visualizes the data. Consider adding more specific information on the fold changes and statistical significance of key proteins to strengthen the findings.*

We thank the reviewer for the kind words regarding our presentation of the results for both the molecular and sensory data.

We are additionally thankful for the original suggestion of this reviewer to change our presentation of the cytokine data to a figure to better depict the temporal changes in cytokine expression. It greatly improved the flow and overall readability of the results and has enhanced the quality of the manuscript overall.

We have amended the table summarising the significant proteins to also include an indication of the level of significance of the observed interaction. Please find these changes throughout table one with a description of the levels of significance also included in the table description.

We consider all the presented proteins as 'key', or possibly key proteins, due to the hypothesis generating and exploratory nature of this work. As our original manuscript may have generated some ambiguity around this we have made extensive amendments to the methods section in addition to the following paragraph in the results which walks readers through the workflow of protein identification.

> *Untargeted plasma-based proteomics identified 1671 quantifiable proteins. By applying filters as per the methods (confidence score ≥50, ≥1 unique peptides and ≤30ppm mass error) 547 proteins were identified for further statistical analysis. Analysis of the proteomics and targeted cytokine/chemokine analysis revealed 29 proteins (Table 1) and 2 cytokines (Figure 1) differentially expressed between T0 and T10. Furthermore, comparisons between T0 and T20 revealed differential expression of 17 proteins (Table 1) and 5 cytokines (Figure 1), and 12 proteins were differentially expressed between T0 and recovery.* (Lines 79-87)