REVIEWER 1

Dear Reviewer 1,

We thank you for the time taken to give us considered feedback on our manuscript and your suggestions for improvement which will aid in the readability and credibility of our manuscript.

We have worked diligently to address your feedback in as comprehensive a manner as we could, and addressed each of your concerns in a point by point manner. We have done our best to add to the manuscript, based on your suggestions, where we think appropriate, and have provided extensive rationale for any recommendations we have not directly addressed so you can best understand what we have done with the manuscript.

Overall, we are appreciative of the opportunity you have given us to edit this manuscript in order to meet publication standards and to promote our work to a wider audience.

Please find our point-by-point discussion and response to suggestions below.

1. *The two-cohort design is well thought out, but further clarification on the rationale behind the choice of control conditions would help the reader understand why the specific design was used. Specifically, how do the control conditions relate to the observed results in terms of baseline measurements?*

   Thank you for bringing to our attention that there may be some confusion over our interchangeable use of the terms 'control condition' and baseline. For our purposes, both terms refer to our healthy participants prior to any pain/HS intervention. In this way, each participant acts as their own control when we compare their non-pain state (baseline) to their HS-induced pain interactions. The use of these terms somewhat interchangeably is a somewhat commonplace practice in pain specific journals; however, we concede that the broader readership of IJMS necessitates a consistent and accessible approach when referring to the baseline state a participant arrives in.

   To help with the clarity of this, we have ensured that the terms used to describe a participants' 'pre-pain state' are consistently referred to as baseline throughout the manuscript and notably, have edited Figures 3 and 5 and Table 2. Efforts have also been made to ensure this is reflected throughout the manuscript with the term 'control condition' replaced with baseline where appropriate.

   To directly answer the final part of this query, 'control condition' refers to measurements taken from a healthy participant with no history of musculoskeletal disorders prior to the induction of hypertonic saline (HS) induced pain.

2. *The cytokine data suggests sustained inflammation, but it would be beneficial to explore the temporal dynamics of cytokine expression in more detail. How does the persistence of cytokine alterations correlate with the duration of muscle pain? Authors showed (TRP) channels and inflammatory response were involved in the development of pain. Here are some references: PMID:* ***40362205, PMID: 40195186,***

   Unfortunately, the available budget did not allow us to also examine the cytokines at the T30 timepoint to track their assumed recovery as pain dissipated. Due to funding limitations, it was decided that it was best to be strategic with the cytokine kits that were run and only examine the baseline samples and the samples were participants were feeling either a

dynamic/establishing pain (T10) or stable pain (T20). We have now included a section in our molecular discussion listing in our inability to run additional cytokine kits as a limitation for this work and area for future research.

> *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.* (Lines 295-300)

That being said, we do have passages in the discussion which start to explore this temporal profile of cytokine expression. In the below section we include discussion on FLT3LG, TRAIL, IL17C and CCL2, with only CCL19 not discussed in this section, yet discussed in the subsequent paragraph.

> *Our molecular analysis also revealed markers of increased apoptosis following HS-infusion. There was a significant increase in the expression of TRAIL (TNFSF10 cytokine), responsible for cell apoptosis, potentially acting via the activation of caspase 3, a pathway known to be involved in pain [39,40]. Decreased FLT3LG might lead to less functional dendritic cells and fewer T-cell activation [41], which could in turn lead to a reduction in IL17C since this cytokine is activated T-cell derived [42]. Decreased IL17C, together with decreased CCL2 (responsible for recruitment of monocytes, memory T-cells and dendritic cells [43]), suggest dynamic changes in immune cell recruitment and activation. This may be indicative of a disrupted immune response and tissue homeostasis. These observations could be most pertinent to the dynamic/early stages of pain, with all participants reporting a sustained pain at T20, possibly explained by a dynamic rebalancing of immune clearance and recruitment.*
> (Lines 241-252).

We have also made significant alterations to the presentation of our results as the small number of identified cytokines, as opposed to the large number of identified proteins, facilitates a graphical presentation. Please find that Table 2 has been deleted and replaced by the new Figure 1 which shows the shift in expression of significant cytokines over the time-course we were able to examine. We hope this new graphical presentation, when compared to our existing commentary is sufficient for this reviewers' desire to have a more temporal exploration of the cytokine profile, with additional consideration for our budgetary constraints.

We are unsure on the desired changes the reviewer is requesting in relation to our discussion of TRP channels. We do have discussion on the potential implications of TRP channel sensitization in relation to this work within the discussion and this is referenced within the manuscript already.
We do not believe that the included article from the reviewer (*PMID:* ***40362205***) is necessarily applicable to the findings of our work. The recommended reference uses an animal nerve crush injury model with results from the neuropathic pain model only examined at a molecular level after 21 days. This is reflective of a much longer time-course than our presented work and is a very different injury/pain mechanism, being a nerve crush model as opposed to an intramuscular HS-infusion. Whilst we appreciate the complexity of the provided reference, including its cell culture

work, it is substantially different from the models/pain type examined within this paper and may be seen as a stretch to draw direct comparisons between the work.
The second proposed reference is similar in that it uses a prolonged animal model of neuropathic pain, although more in-vivo work is presented, making it potentially more applicable than the cell work of the prior reference. However, the surgical intervention on the animals and the induction of the pain model in this case, is far more invasive than our acute intra-muscular infusion. Whilst the neuropathic pain model is good and sound, and something which is routinely reproduced for the examination of neuropathic pain, we are not yet sure if the applicable pathways will be preserved between the two vastly different pain models, despite mechanical hyperalgesia culminating as a shared symptomology.
It is for the above reasons that we have elected not to include either of the suggested references in the revised version of the manuscript. This is not a reflection on the robustness of the work required to publish these manuscripts, but rather our own apprehension to compare multiple modalities of pain in an interchangeable way. We would welcome such work in the future and hope that our advances in understanding the pain proteome can be expanded to include multiple pain modalities. With chronic pain representing in such a diverse array for different individuals globally, it is fundamentally important that all aspects of pain are examined both in isolation, and in tandem to best serve the global community.

3. *The pain scale used in the study is valid, but more details on how participants subjectively report pain and how these reports were validated would enhance the transparency of the pain measurement process.*

   Pain is an inherently subjective sensory modality with no readily accessible way that we can truly validate whether a moderate level pain in one participant equates objectively to the pain felt by another participant. A visual analogue scale (VAS) is a commonly used tool in the assessment of human pain which allows participants to rank their subjective sensory experience on a sliding scale between two extremes. A rating of 0 indicates that no pain is felt whilst a rating of 10 is the worst pain the participant can imagine. The continuous recording of the VAS allows us to have a record of the temporal aspects of pain at 4k per second sampling rate which is more than enough to accurately capture the responses of our participants in this experimental setting.

   We have expanded an existing excerpt within the methods section, to include descriptors which state that the participants were in control of the VAS for the duration of the procedure and were able to rate their pain continuously as they perceived it.

   "Pain ratings were continuously recorded using the ADInstruments Response Meter connected to the ADInstruments Powerlab (ADInstruments, Dunedin, New Zealand). The Response Meter had a slide control, and the pain scale was divided into ten equal segments within a range of 0 (no pain) to 10 (worst pain). The participants were free to rate their pain as they felt throughout the duration of the study, and remained in control of the VAS at all times. The infusion in this cohort was subject to strict time controls with the infusion lasting 20 minutes total in this group." (lines 374-380, section 4.1)

   The participant is free to judge the severity of their pain between those 2 parameters and at times we will titre the infusion rate of hypertonic saline to allow for the individual's subjective pain rating to fall within our targeted range of moderate pain (4-6 on VAS). This variability in infusion rate is referred to in section 4.1 and section 4.2.2 and is reflective of our attempts to

control (as best we can) the subjective experience of our participants in order to facilitate the successful completion of the experiment.

4. *The proteomics data analysis is comprehensive, but the manuscript could benefit from a more detailed explanation of how the identified proteins are selected for further analysis. A more robust description of the bioinformatics tools and algorithms used to analyze the proteomic data would add depth.*

We thank the reviewer for bringing to our attention that the methods surrounding proteomics could be better explained and have more depth to them. This was a sentiment echoed by the second reviewer also. In light of this we have expanded the methods section 4.1.2 to add the identification method for peptides and proteins. We utilised Progenesis software to analyse the chromatogram and automatic peak picking and identification. Significant proteins were identified with the parameters outlined in the revised text and then all raw abundance data were exported into GraphPad prism to run any further statistics beyond this first pass automatic discovery.

> *All samples were run in a single batch and as duplicates to quantify variability and reproducibility. All samples were imported into Waters Progenesis QI for proteomics (Waters Corporation, Milford, Massachusetts, USA) to analyse the obtained peptides and retrieved proteins, with chromatograms aligning >87% to a pooled sample (quality control sample). Peptides and proteins identification was done on Progenesis using Ion Accounting identification method searching the human proteome database from UniProt [53] FASTA file. Obtained proteins were filtered using mass error (≤ 30ppm) with at least one unique peptide and confidence score (≥ 50) outputted by the Progenesis software to control for false positive results. To assess the correlation between duplicate, samples were submitted to Spearman test, and all replicates had a score >0.9, showing great consistency between replicates and negligeable batch effect. This was replicated for each fraction.*
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> *The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [54] partner repository with the dataset identifier PXD070131.*

We have also added more detail to the results section in Section 2.1 to allow for full transparency on the number of identified proteins and the way they were filtered for further analysis.

> *"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."*

5. *While the statistical analyses are mostly well-conducted, a clearer explanation of why specific tests (e.g., Tukey's posthoc) were chosen for each comparison would improve the manuscript's methodological transparency.*

In retrospect, our methods surrounding the statistical analysis of our proteomic data was somewhat lacking in detail and we thank the reviewer for bringing this to our attention so that we can provide greater transparency to our work. Once again, this lack of detail was also mentioned by the other reviewer, emphasising our need to address this for the readership of IJMS and the comprehension of the works we undertook.

We have updated this section substantially to include a step-by-step breakdown of our statistical approach in much greater detail than previously and included rationale for test choice were appropriate.

> *4.3.1 Molecular data*
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> *Proteins and cytokines raw abundances were normalized on T0 and obtained fold change were used for further analyses. Comparison of our time points (T0 – baseline, T10 – 10 minutes of infusion, T20 – 20 minutes of infusion, and recovery) was undertaken using GraphPad Prism.*
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> *Distribution of the data was analysed using D'Agostino and Pearson omnibus normality test and re-examined using Shapiro-Wilk normality tests. Once the data was confirmed to not conform to a normal distribution, we applied a two-way ANOVA fitting mixed effects (correcting for missing values). As participants were identical for all the different time points (T0, T10, T20 and recovery), we applied matched values to the dataset and examined multiple comparisons with a simple row effect to compare proteins independently. A Dunnet post-hoc test was applied to the ANOVA results to control for Type I and family-wise error as it is a powerful and efficient test focusing on comparison of several groups to a control.*
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> *Cytokine analysis was undertaken using the same statistical methodology as identified proteins.*