Dear Dr. Lotvall,

We sincerely appreciate your detailed comments on our manuscript and the speed with which you were able to respond, especially given the manuscript was read by multiple senior editors. We have rewritten the manuscript to address your concerns in a new submission. Below we indicate how we responded and if we had additional context to provide:

1) It is not clear which material is put into the size exclusion columns (pellets of 2x2000 g centrifugation or remaining supernatant?)

The supernatant was used, we have clarified the methods section accordingly.

2) The starting volume of the CSF is not clarified.

The starting volume per sample was 1mL of CSF, we have clarified the methods section accordingly.

3) The claim that up to fraction 6 is "dead volume", but fraction 6 has CD63 according to the data.

The unit for the Olink data output is normalized protein expression values (NPX) which is a relative value based on controls. There is not a calibration curve for each protein measured. Therefore, there is no limit of detection in the Olink HD platform, and all values represent relative signal. The values in fraction six and seven of the CD63 graph likely represent the assay background. The language in the methods section has been updated to make this point clear.

4) The abstract is exceptionally brief and insufficiently describing the methods and findings.

We have expanded the abstract to describe our methods and findings more clearly.

5) To really claim that identified transmembrane proteins are NOT associated to EVs, JEV would require additional evidence. Only pursuing one method, based on a single company commercial assay, would not suffice. I am sure the co-authors would agree that this single assay would not be enough to make these claims.

Our goal here is not to claim that proteins that do not meet our criteria are not associated with EVs. Rather, we think that if something *does* peak in the EV fraction it is likely to be a good candidate for EV analysis. Beyond what we stated in the text of the manuscript, we strongly believe that for proteins that have substantial presence in the soluble protein fractions and do not have a definable EV peak, skepticism is merited and extensive validation of these proteins is needed for them to be considered as associated with EVs. Nonetheless, for the purposes of only making substantiated claims in this manuscript, we carefully revised the conclusions section to clearly state that we cannot rule out EV association for targets without an “EV pattern”. To that end we state: “Our analysis is useful for identifying potential EV-associated proteins but cannot rule out EV association for proteins that do not meet our criteria or that are not included in the Olink HT platform.”

6) the manuscript is unfortunately not very easy to follow, and lacks clarity.

We have substantially edited the manuscript to clarify our methodology and claims. We look forward to further clarifying with additional feedback if sent for review.

In conclusion, we believe that this dataset will be of high value to the readership of JEV, both because of the targets suggested, and because of the targets that we believe should be reassessed and further evaluated based on their predominantly late elution pattern in SEC. More importantly, because of the difficulty of assessing the free protein fractions of size exclusion chromatography and density gradient density chromatography with mass spectrometry, this is to our knowledge the largest unbiased database of EV association ever compiled, and therefore a valuable resource for the community. We hope you will consider sending the manuscript for review.

Sincerely,

David R. Walt