Re: Manuscript ID: 819685a0-f30d-4305-a427-29679d73d750

Dear Dr. Sun,

We thank you and the three reviewers for carefully reading our manuscript entitled “Structural Elucidation of Cerebrospinal Fluid Leak Diagnostic Marker β₂-Transferrin Using High-Resolution Mass Spectrometry” and offering us suggestions for improvements. We copied below the comments of you and the reviewers and indicated our responses.

In addition, we would like to mention that the manuscript title has been updated to “Analysis of β₁-Transferrin and β₂-Transferrin Using Microprobe-Capture In-Emitter Elution and High-Resolution Mass Spectrometry to Elucidate Primary Structures” in regards to Comment 1 of Reviewer 1. The Negative 5 sample in Table 1 was removed (reducing the total number of samples from 12 to 11), and it was added to Supplementary Figure S2 as an example of altered Tf glycosylation. This is related to Comment 3 of Reviewer 2. Regarding this particular sample, after careful review of the MPIE-ESI-MS and IFE results as well as the patient information, it was found that the patient likely had altered Tf glycoforms due to alcoholism. The corresponding content in the last paragraph of the Discussion section has been updated.

Reviewer 1’s Comments

Comment: This manuscript elucidated the β₂-Tf proteoforms in the Cerebrospinal fluid via the integration of microprobe-capture in-emitter elution (MPIE) and high-resolution mass spectrometry. The publication of this manuscript can be considered after major revision. Some suggestions are as follows:

1) The title “structural” is not accuracy. “Structural” is generally refers to the three-dimensional structure of proteins. Such word is suggested to be changed in the title and in the text.

Response: We appreciate the reviewer’s comments and value this advice. We changed “structure” to “primary structure” in the entire text to accurately specify the meaning of “structure”. We have also updated the title to “Analysis of β₁-Transferrin and β₂-Transferrin Using Microprobe-Capture In-Emitter Elution and High-Resolution Mass Spectrometry to Elucidate Primary Structures”.

Comment: 2) “serum-type Tf” and “brain-type Tf” made the readers confused.

Response: The “serum-type Tf” and “brain-type Tf” refer to the groups of Tf glycoforms originated in serum and CSF, as named by the neurobiologists in references 13, 15-20. We called the “major serum-type Tf” and “major brain-type Tf” are two specific predominant Tf glycoforms in the groups. Presumably the “major serum-type Tf” and “major brain-type Tf” were actually “β₁-Tf” and “β₂-Tf” named in clinical laboratories, and
this hypothesis was proved in this article. However, we could not merge these names until the Results section where supporting experiment results were shown. To increase the clarity of the statements, we updated “serum-type Tf” and “brain-type Tf” to “serum-type Tf glycoforms” and “brain-type Tf glycoforms” to emphasize these group names and differentiate them from the specific “major serum-type Tf” and “major brain-type Tf”. Please let us know if it meets the requirements and we will be happy to further revise it if needed.

Comment: 3) In Figure S2, the author said “The molecular masses of the putative β1-Tf and β2-Tf were different from those of β1-Tf and β2-Tf in normal CSF samples”. The reason should be discussed.

Response: The corresponding sentences in the figure caption have been updated: “The molecular masses of the putative β1-Tf and β2-Tf shifted a same value from those of β1-Tf and β2-Tf in normal CSF samples, while the mass difference between the two Tf glycoforms retained as 1546 Da, indicating that the mass shift was caused by amino acid variation in the Tf molecule.” The relevant sentence in the main text has also been updated: “In theory, amino acid variation in the Tf molecule can change the molecular masses of β1-Tf and β2-Tf but not the molecular mass difference between the two Tf glycoforms (1546 Da), provided the two glycosylation sites are not modified. This hypothesis is supported by the MPIE-ESI-MS results of a few Tf variant-containing CSF samples, as shown in Supplementary Figure S2.”

Comment: 4) In Figure 2, the gel electrophoresis are suggested to be shown.

Response: Figure 2 has been updated to include the gel images. The figure caption has been updated to include an illustration: “The image of an agarose gel after gel electrophoresis, as well as that of a reference agarose gel after a complete IFE test displaying the positions of β1-Tf and β2-Tf bands, are posted next to the mass spectra.” A statement has been added to the Sample Preparation section: “The image of an agarose gel after gel electrophoresis is shown in Figure 2.”

Comment: 5) The authors just used full MS to elucidate the Tf proteoforms. The MS/MS identification is suggested to confirm the Tf proteoforms. Tf proteoforms might have other PTMs except glycosylation, so only using mass to identify proteoforms is not accuracy.

Response: In fact, the MS² analysis of intact Tf was attempted but good fragmentation could not be achieved to confirm the proteoform structures, probably due to the presence of 19 disulfide bonds in a Tf molecule. On the other hand, bottom-up analysis of the N-glycan containing peptides digested from Tf proteoforms in CSF was reported in references 17 and 18. As discussed in the second paragraph in the Discussion section, the bottom-up analysis laid a strong foundation for this study to figure out the structure of β1-Tf and β2-Tf glycoforms. In addition, bottom-up analysis of the Tf proteoforms in CSF was repeated in our lab to confirm the results reported in the references.

Reviewer 2’s Comments
Comment: The manuscript by Luo et al. described the application of an affinity capture technique coupled with high-resolution mass spectrometry to detect the glycoforms of transferrin in serum, cerebrospinal fluid, and patient secretion samples for potential diagnosis of CSF leak. Microprobe-capture in-emitter elution (MIPE) was coupled with a label-free sensing technique to monitor the capture of analyte transferrin to the probe, and then directly inserted into an electrospray emitter for HR-MS. The authors successfully detected two glycoforms of transferrin in samples, assigned the beta1- and beta2-Tf to different glycoforms, and the results in patient samples mostly matched the diagnosis. This technology has the potential to be implemented for fast diagnosis in clinical settings. Meanwhile, the manuscript seems more like an application note instead of a full article with some key questions unanswered.

Major comments:

1. Without tandem MS, how were the positions and type of N-glycans assigned for all glycoforms detected? The authors cited many previous studies to infer the structure, but without tandem MS on their own samples there is no confirmation of the structure. This weakens the authors claim about structural elucidation.

Response: We appreciate the reviewer’s comments and value the advice. In fact, the MS² analysis of intact Tf was attempted but good fragmentation could not be achieved to confirm the proteoform structures, probably due to the presence of 19 disulfide bonds in a Tf molecule. On the other hand, bottom-up analysis of the N-glycan containing peptides digested from Tf proteoforms in CSF was reported in references 17 and 18. As discussed in the second paragraph in the Discussion section, the bottom-up analysis laid a strong foundation for this study to figure out the structure of β₁-Tf and β₂-Tf glycoforms. In addition, bottom-up analysis of the Tf proteoforms in CSF was repeated in our lab to confirm the results reported in the references.

In other words, the tandem MS-based bottom-up analysis of Tf glycoforms has been accomplished in the previous reports. However, given the diversity of Tf glycoforms in CSF, an intact proteoform analysis is needed to confirm the structure of intact β₁-Tf and β₂-Tf. This is particularly important for laboratory medicine because only the intact proteoform of β₂-Tf can be used as the CSF leak diagnostic marker. This is the rationale for this study.

Comment: 2. The important application of this method is for the diagnosis of CSF leak. It would be the best if authors could run the current diagnosis method and the HR-MS on the same sample and match the results side-by-side.

Response: We apologize for the confusion. All the secretion samples in Table 1 were tested by the conventional agarose gel immunofixation electrophoresis (IFE) method to determine the positive/negative CSF leak results. The statement has been added to clarify this point: “As shown in Table 1, the MS peak at 78008 Da was observed in the MPIE-ESI-MS results of the 5 positive samples but not found in those of the 6 negative samples, which confirmed the consistency between the MPIE-ESI-MS method and the conventional IFE test.”

The IFE test result is based on the presence of a β₂-Tf band in the IFE gel. It might be a redundancy to show all the gel images in Table 1. On the other hand, we placed an IFE gel image of a CSF sample in Figure 2 to illustrate the sample preparation procedure and show an example of IFE results.

Comment: 3. In Table 1, there are two samples (Negative 4 and 5) in which none of the glycoforms were detected. Does that mean the MIPE-ESI-MS method failed on these two samples? I’m having some hard time
thinking about the robustness of the application for clinical diagnosis. A better way to test may be having a testing set of samples to build your method and then a blindly assigned validating set to see how accurately you can assign the positive/negative diagnosis.

Response: We appreciate this question very much as it reminded us to double-check all the results reported in Table 1. Regarding the samples Negative 4 and 5, both were re-tested by MPIE-ESI-MS and the repeated results were identical. Regarding Negative 4, no transferrin was detected, and this result was consistent with the conventional IFE test. It is not uncommon to have secretion samples showing no bands because sometimes a body fluid containing very little transferrin or a saline-wash sample can be sent for CSF leak test. Regarding Negative 5, after careful review of the MPIE-ESI-MS and IFE results as well as the patient information, it was found that the patient likely had altered Tf glycoforms due to alcoholism. It is known that aberrant Tf glycoforms are a result of carbohydrate-deficient syndromes and alcohol abuse, and this point was discussed in the last paragraph of the Discussion section. Therefore, this sample was removed from Table 1 (reducing the total number of samples from 12 to 11), and it was added to Supplementary Figure S2 as an example of altered Tf glycosylation. The corresponding content in the last paragraph of the Discussion section has been updated. In addition, this sample was negative because $\beta_2$-Tf glycoform was neither found in the mass spectra nor observed in the IFE test.

In addition, it should be noted that both the conventional IFE test and the MPIE-ESI-MS method have limits of detection. The limit of detection of the MPIE-ESI-MS method was explored and described in the last paragraph of the Results section.

Comment: 4. In all deconvoluted spectra in Figure 1, there is always a peak around 79700 Da. In Figure S2, both spectra have a peak around 79650. Can authors comment on these peaks?

Response: Besides $\beta_1$-Tf (major serum-type Tf), there are minor serum-type Tf glycoforms, as reported in previous literature (references in the article). Based on the molecular mass, the MS peak around 79700 Da might be the fucosylated $\beta_1$-Tf, and we are not sure about the MS peak around 79650 Da (there are some possibilities). However, this is beyond the scope of this study and further investigation is needed to confirm the structure of these minor Tf glycoforms.

Comment: 5. How quantitative the MIPE-ESI-MS is? Can this method quantitatively determine the extent of CSF leak?

Response: At the current stage the MPIE-ESI-MS technique is positioned as a qualitative analysis tool rather than a solution for quantitative analysis.

There are a few reasons: (1) Accurate quantitation in mass spectrometry requires the use of internal standards, i.e., preferably stable isotope-labeled analytes; however, they are currently unavailable for most protein targets. Without the use of internal standards, quantitation performance is restricted by ion suppression and detector response variation. (2) In MPIE-ESI-MS analysis, quantitation performance is also affected by variation in capture agent loading and analyte binding on a BLI microprobe. During manufacturing, the wall surface adjacent to the end of a microprobe is pre-coated with streptavidin together with the end surface. Although only the microprobe end surface is used to generate BLI responses, the adjacent wall surface can also load the capture agent and then bind the analyte, increasing the total amount of analyte.
captured on a microprobe. The amount of analyte captured on the adjacent wall surface is dependent on the length of a microprobe submerged into the reagent and sample solutions.

In the future, when internal standards become more available for protein analytes, MPIE-ESI-MS may be used in quantitation applications. Moreover, automation of the setup procedure can significantly increase the throughput of MPIE-ESI-MS analysis and facilitate batch processing of samples.

**Comment: Minor comments:**

1. *What are the secretion samples from patients?*

**Response:** Most secretion samples are rhinorrhea or otorrhea samples, but they can also be other types of body fluids. Actually there is significant diversity in the specimens. It was briefly mentioned in the first paragraph in the Introduction section and more details are in references 1-6.

2. *For the samples shown in Figure S2, what are these samples corresponding to in Table 1?*

**Response:** The two samples shown in Figure S2 were not in Table 1. As stated in the article, we need to figure out a method to confirm the molecular masses of $\beta_1$-Tf and $\beta_2$-Tf in Tf variant samples.

3. *For Table S1, a calibration curve should be shown. It doesn’t seem very linear to me though.*

**Response:** As explained above, at the current stage the MPIE-ESI-MS technique is positioned as a qualitative analysis tool rather than a solution for quantitative analysis. This is why a calibration curve was not shown and linearity was not expected.

4. *Figure S1, a blank sample and a negative control (e.g., BSA) should be included to show baseline responses and specificity.*

**Response:** Two sensorgrams of negative controls (PBST-B and PBST) have been added to Figure S1 to show baseline responses and minimized non-specific binding.

5. *For sample prep, why was CSF diluted in PBST while serum samples were diluted in PBST-B?*

**Response:** PBST-B was employed to minimize non-specific binding to BLI microprobes, as serum samples have much higher total protein concentrations than CSF samples.

Again, we appreciate the feedback from the reviewers, and hope that this manuscript is now ready for publication.

Sincerely,

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