Author's response to reviews

Title: Transient spurious intrathecal immunoglobulin synthesis in neurological patients after therapeutic apheresis

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Version: 6 Date: 22 October 2015

Author's response to reviews: see over
Dear Dr. Lou,

thank you for considering our article for publication. The authors wish to thank the Editor and the Reviewers for their very helpful and detailed comments and suggestions, which helped to improve our manuscript. The comments show that spurious immunological disturbances in CSF after therapeutic apheresis are a topic of intense interest and controversy in neurological disorders. We tried to include most of these hints in our revised manuscript.

In the following we provide a point-by-point-revision of changes that have been made to the manuscript according to the reviewers' comments.

I. Reviewer #1

Abstract

1. The abstract would benefit from providing more relevant background and objective. It is not clear who were 12 patients? What tests were performed and what were the timings? What were the neurological disorders? Conclusion should be provided before recommendations.

Response: We expanded the background information of the abstract and gave more information on the 12 patients.
Since there was no extra test necessary, but data had been acquired during routine work-up and the study consisted of a retrospective analysis of these data, we added the phrase “...that had been raised during routine diagnostic work-up...” as well as the range of therapeutic treatment cycles applied before CSF analysis (“...after a varying number of treatments of therapeutic apheresis).
Furthermore, the neurological disorders were added as background information. Since the
patients included had a considerable variety of neurological diagnosis, we included the two most frequent diagnoses in the abstract (“...predominantly Guillain-Barré syndrome and autoimmune encephalitis...”).

The 1st phrase in the conclusions part of the abstract is a summary of our conclusions and preceding their relevance (second phrase) and finally recommendations (last phrase of the abstract) how to address the mentioned risk of misinterpretations.

**Background**

2. Name the neurological disorders on page 3

**Response:** We added examples of disorders potentially leading to intrathecal immunoglobulin synthesis in parenthesis: “…(e.g., neuroborreliosis, other viral and bacterial infections, and multiple sclerosis),…”

3. References are required for text given in para 2 and 3 of page 3

**Response:** In paragraph 2 of page 3 we added two references indicating the necessity of CSF analysis in the diagnosis of Guillain-Barré syndrome and autoimmune encephalitis (van den Berg B et al., Nat Rev Neurol 2014; 10(8): 469-82; Dalmau J et al., Lancet Neurol Review 2011).

In paragraph 3 of page 3, we describe the pathophysiologic concept underlying “spurious intrathecal immunoglobulin synthesis”. Further referencing is impossible, since the case report cited at the end by Madzar et al. (2011) is the only published work on this issue so far.

4. Please explain briefly what information is already available to create a rationale for the study

**Response:** The published information available on this issue is sparse, comprising merely one single case report that had been cited in the manuscript at the end of paragraph 3 in the Background section (Madžar et al., J Neural Transm 2011; 118: 219–22). The rationale is based primarily on the concept of intrathecal Ig calculation from CSF and serum Ig concentrations. For this, we modified the 3rd paragraph on page 3 accordingly:

“In particular, the relative CSF immunoglobulin fractions and the respective IgG index are expected to increase, based on the current mathematical concepts used to describe CSF
protein composition and dynamics (Reiber et al. J Neurol Sci 2001). This could misleadingly suggest an intrathecal antibody synthesis, and hence prompting unnecessary extra diagnostics for search of an infectious or autoimmune disease (e.g., multiple sclerosis). Although this phenomenon is acknowledged by CSF experts, systematic data proving the existence of this “spurious quantitative intrathecal synthesis” are not available, and, to our knowledge, there is to date only one case report describing spurious intrathecal immunoglobulin synthesis after PE [5]."

5. Objective should indicate the diseases studied. The objective of the study was to quantify Ig in serum and CSF before and after apheresis in......disorders. Please delete vague phrases like occurrence of spurious .....

Response: We modified the last paragraph of the introductory part: “The objective of the present study was to retrospectively determine intrathecal Ig fractions from patients with various inflammatory neurological disorders (predominantly Guillain-Barré syndrome, and autoimmune encephalitis), and compare these before and after PE or IADS treatment to estimate the influence of therapeutic apheresis.”

Method

5. How the cohort of 12 patients selected? What was their clinical diagnosis? Ages?

Response: The patients of the Berlin cohort were selected non-randomized, just by their occurring CSF data during clinical routine. We acknowledged this by adding the word “unsystematically”. To our knowledge, it is more usual to put baseline data like demographics and clinical data (diagnosis, age) into the Results section. But if it is according to the journals recommendations to put these baseline data into the Methods section, we would be happy to make the respective changes.

6. First two lines of page #5 are ambiguous.

Response: We suppose that the part “…CSF and serum samples not taken on the same date…” was meant and changed the last phrase of the inclusions criteria part into “Further inclusion criteria required subjects to be aged ≥ 18 years and to have had at least one LP (including a CSF and serum sample taken on the same date) done within two months after PE or IADS”.
7. Analysis is done between two groups? What were these groups? This should be mentioned in respondents.

**Response:** The groups were the *Freiburg study cohort* before as compared to after therapeutic apheresis, which is now acknowledged in the Methods section: “…The two-tailed Mann-Whitney U test was used for statistical analyses comparing CSF results before and after therapeutic apheresis within the Freiburg study group.”

8. Exclusion criteria are not clear. What do authors mean by …unless they showed no….apheresis?

**Response:** For clarification we modified the respective phrase:

*Former version:* “We excluded all patients with neurological diseases (e.g., patients with multiple sclerosis or autoimmune encephalitis), which commonly cause intrathecal Ig synthesis, unless they showed no quantitative intrathecal Ig synthesis in another CSF analysis before or later in the disease course after therapeutic apheresis.”

*Revised version:* “We excluded all patients with neurological diseases (e.g., patients with multiple sclerosis or autoimmune encephalitis), which commonly cause intrathecal Ig synthesis. Exceptions were patients with these conditions who had no intrathecal Ig synthesis in a CSF analysis before or more than two months after therapeutic apheresis.”

9. Was methodology of CSF and serum analysis same at both centers?

**Response:** Yes, it was. For clarification we added the following phrase:

“The methodology of CSF and serum analysis was equivalent in both centers and according to the German Society for Cerebrospinal Fluid Diagnostics and Clinical Neurochemistry.”
10. Details of the participants should go into the method section

Response: Please see our response to comment 5.

11. It is not clear if six patients in Berlins study had elevated Ig in CSF or not because of contradictory statements given.

Response: For clarification that six patients of the Berlin cohort had elevated Ig fractions in the CSF, whereas the remaining six had not we modified the respective 1st paragraph in the Results section:

Former version: “…All six patients who had an LP one day after PE showed increased quantitative intrathecal immunoglobulin fractions, supporting the hypothesis of spurious intrathecal Ig synthesis: one of all three classes, two of IgG and IgA, and three of IgG. However, one patient with spurious IgG and IgA synthesis had persistence of an increased IgA fraction in the further course, whereas the other five had a CSF analysis before PE without increased intrathecal immunoglobulin fractions. The remaining six patients who had an LP more than one day after PE had a median time interval to CSF analysis of 18.0 days (range 3–26 days) and did not demonstrate increased intrathecal immunoglobulin fractions.”

Revised version: “…Six of the 12 patients had an LP one day after PE that showed increased quantitative intrathecal immunoglobulin fractions (one of all three classes, two of IgG and IgA, and three of IgG), supporting the hypothesis of spurious intrathecal Ig synthesis. By contrast, the remaining six patients who had an LP more than one day after PE with a median time interval to CSF analysis of 18.0 days (range 3–26 days) did not demonstrate increased intrathecal immunoglobulin fractions. Of the six patients with spurious intrathecal Ig synthesis, one patient with autoimmune encephalitis and IgG as well as IgA synthesis had persistence of an increased IgA fraction in the further course, whereas the other five (two with autoimmune encephalitis, two with neuromyelitis optica, and one with myelitis) had a CSF analysis before PE without increased intrathecal immunoglobulin fractions.”
12. What was the diagnosis of patients who had spurious results? In which cycle of apheresis, LP was performed?

Response: Concerning the Berlin study cohort we added the diagnoses of the six patients with spurious Ig synthesis as can be seen in the response to comment 11. With respect to the number of cycles in the patients with spurious intrathecal Ig fractions we added the phrase: “In these six patients LP showing increased intrathecal Ig fractions was performed after a median number of three apheresis treatment cycles (range 1-6 cycles).”

Concerning the Freiburg study group we added the phrase: “Of these 13 patients nine had Guillain-Barré syndrome and one chronic relapsing inflammatory optic neuropathy (CRION), multiple sclerosis, paraneoplastic cerebellar degeneration and neuromyelitis optica each. A median number of four treatment cycles (range 1-5 cycles) were performed before increased intrathecal Ig fractions occurred.”

13. It would be interesting to study patients with specific neurological disorders irrespective of placement of patients.

Response: We agree that it would be interesting to confirm the observed effects in larger, prospective multicenter studies, focusing also on subgroup analyses of patients with specific neurological disorders. However, this is beyond the scope of the present retrospective study.

14. Table 1 should give characteristics of 19 patients only as they were of interest only.

Response: We deleted several rows (“n”, “demographics”, “therapeutic apheresis procedure”, and “previous immunotherapies”) in order to avoid duplication with the text and to sort out “unnecessary” information that was of no further relevance for the study.

15. Table 2 can be merged to avoid duplication of rows by adding three more columns. Statistical analysis should be provided here. What was achieved through fisher exact and Mann-Whitney tests?

Response: Table 2 was merged according to the comment. The table contains no statistical analysis; these are included in Figure 2.
16. Sera results were not provided.

Response: That is true, but we do not think it would be worth showing. To keep the manuscript not too long we decided to provide only intrathecal Immunoglobulin synthesis (which is calculated from serum and CSF Ig concentrations) of all patients of the Freiburg group in table 2. Additionally, we added detailed CSF and serum results of one representative patient in table 3.

17. Table 3 is not cited in text. What was its rationale?

Response: Table 3 is cited in the 4th paragraph of the Discussion section. Its purpose was to demonstrate the “pathophysiologic” concept of “spurious intrathecal Ig synthesis” in a representative patient: reduction of serum Ig levels by apheresis in combination with unchanged CSF Ig levels leads to a “spurious” increase of the intrathecal Ig fractions.

However, we additionally put a reference to this table into the respective paragraph of the Results section (end of 1st paragraph of the Freiburg study group): “…Table 3 showing CSF and serum data of a representative patient before and one day after PE, demonstrates that reduced serum Ig levels were accompanied by hardly altered CSF results, therefore leading to an increase of the respective quotients and intrathecal immunoglobulin fractions.”

Discussion

18. The discussion should begin with important findings observed in the study.... Spurious results were observed in ---of---patients. Since only 12 patients in Berlin and 19 patients in Freiburg were studied. % should be followed by absolute numbers and population denominator for clarity to the readers.

Response: According to the reviewer’s comment, we modified the 1st paragraph of the Discussion:

Former version: "In this study, we describe spurious intrathecal immunoglobulin (Ig) synthesis … occurring one day after therapeutic apheresis in 12 selected patients, as well as in a well-defined, independent, confirmatory, cohort of patients with various neurological disorders. According to the data from the Freiburg study group, elevated intrathecal Ig fractions were found in 68.4% of the patients who had LPs performed within one day after therapeutic
apheresis. More significantly, 94.7% of these patients had spuriously elevated IgG indexes. While being well-acknowledged among CSF experts, to our knowledge this is the first systematic study on this spurious phenomenon. In fact, only one case of this issue has been reported to date [5].”

Revised version: “In this study, we describe spurious intrathecal immunoglobulin (Ig) synthesis occurring one day after therapeutic apheresis in 6 of 12 selected patients (50.0%, the Berlin study cohort), as well as in 13 of 19 patients (68.4%) from a well-defined, independent, confirmatory cohort of patients with various neurological disorders (the Freiburg study group). Additionally, 18 of these 19 patients (94.7%) had spuriously elevated IgG indexes. While being well-acknowledged among CSF experts, to our knowledge this is the first systematic study on this spurious phenomenon.”

19. Only two new references were used in discussion. Discussion entailed the description of study results without comparative analysis. Thorough research review was not provided.

Response: After adding new references (4th paragraph: Correale et al. J Neurol 2002; 249(4): 375-89; 5th paragraph: Mühlhausen J, Kitze B et al., Atheroscler Suppl. 2015; 18: 251-6), there are two more new references in the discussion part. As mentioned, there is only a single case report published addressing this issue. This underlines the novelty of our findings, but makes further comparison with published literature impossible.

20. Strengths/ limitations were not given. Only 19 of 346 were studied; number was wrongly stated as 50.

Response: As stated in the Results section 50 is not the number of participants of the study and also not the complete number of excluded patients, but of a proportion of the 346 patients that had to be excluded due to incomplete data:

“At University Medical Center Freiburg, 346 neurological patients who were treated with either PE or IADS between 2005 and 2014 were identified. The vast majority (234 cases) had to be excluded since no CSF analysis had been performed within two months after therapeutic apheresis; an additional 50 had incomplete CSF data (e.g., no serum taken).”

We used this 1st paragraph of the Freiburg study group in Results to clarify how many patients were identified by our search algorithm and how many had to be excluded for various reasons.
as cited above.

Strengths of the study are stated in the 1st paragraph of the discussion:
“...a well-defined, independent, confirmatory, cohort of patients with various neurological disorders (the Freiburg study group).”
“...this is the first systematic study on this spurious phenomenon.”

The main limitation of the study is stated in the last paragraph of the Discussion:
“A limitation of this study is its retrospective character and the small sample size of only 41 systematically selected patients in the Freiburg study group.”

According to reviewer #2 we additionally added a short comparison of plasmapheresis and immunoadsorption in this last paragraph of the Discussion (see reviewer #2, comment 4).

21. What is the generalizability of this study?

Response: All general conclusions (“generalizability”) are mentioned in the Conclusions section at the end of the manuscript: “Transiently elevated intrathecal Ig fractions and increased IgG index (“spurious intrathecal immunoglobulin synthesis”), ... are frequent phenomena ... following therapeutic apheresis. To avoid unnecessary diagnostic and therapeutic procedures, clinicians should be aware of this spurious phenomenon. Recognition of “spurious intrathecal synthesis” is possible when taking into account the absolute concentrations of CSF and serum Ig. In addition, OCBs should be considered to distinguish between “real” and “spurious” Ig synthesis, .... If CSF analysis of intrathecal Ig synthesis is indicated after therapeutic apheresis, we recommend a minimum time interval of three days between apheresis and LP.”

General/Minor Issues

22. The paper would benefit from a thorough proof reading to ensure clarity etc. There are several sections where it is unclear what the authors are trying to communicate. In addition, the authors should ensure that all relevant references are included

Response: All authors did proof reading. Additionally, we used the professional proofreading service of Scribendi Inc (Chatham, Ontario, Canada). We revised vague phrases in the revised version. To our knowledge, all relevant references are now mentioned in the revised version, since we added some new citations (Reviewer #1, comments 3 and 19).
Reviewer #2

The authors analyzed the effects of plasmapheresis on CSF proteins. The authors show intrathecal synthesis of immunoglobulins after apheresis as detected on Reiber diagrams. Although not surprising the results are worth to share with the scientific community. The manuscript would benefit from some additional attention. I have only some minor points:

1. In results of the abstract the authors describe spurious immunoglobulin synthesis of all subclasses in 68% patients. One might suggest that all three subclasses were elevated. Please re-write the sentence.

Response: We added “…of at least one subclass (IgG, IgA and/or IgM)…” for clarification.

2. Authors describe that LP was performed after apheresis. Was LP performed after the end of a treatment cycle or within a treatment cycle? This should be incorporated. However, I did not find.

Response: We agree with the reviewer that the term “cycle” can be confusing in this context. For clarification, we added the following phrase in the Methods section: “In some patients LP was performed within, in others after a complete apheresis cycle consisting of up to six treatments”. Additionally, we replaced the term “cycle” (consisting of up to six apheresis treatments) by “treatments” (a single apheresis treatment) in the entire manuscript.

We also refer to our response to comment 12 of reviewer #1:
Concerning the **Berlin study cohort** with respect to the number of treatments in the patients with spurious intrathecal Ig fractions we added the phrase: “...In these six patients LP showing increased intrathecal Ig fractions was performed after a median number of three apheresis treatments (range 1-6 treatments).”
Concerning the **Freiburg study group** we added the phrase: “...A median number of four apheresis treatments (range 1-5 treatments) were performed before increased intrathecal Ig fractions were detected.”

3. There is some discrepancy. In the abstract the authors conclude that Ig synthesis did not occur when LP was performed two or more days after apheresis. In the conclusion of the discussion the authors write that this phenomena was found within the first two days after...
apheresis. In results it is stated that in one patient an elevated IgG index was found two days after apheresis.

Response: The seeming discrepancy results from the distinction between quantitative Ig synthesis and elevated IgG index. Quantitative Ig synthesis (“phenomenon A”) did not occur when LP was performed two or more days after apheresis, but elevated IgG index (“phenomenon B”) was found until two days after apheresis. Consequently, the correct phrase is: “Transiently elevated intrathecal Ig fractions and increased IgG index … are frequent phenomena (“A + B”) occurring in the first two days following therapeutic apheresis.” We thank the reviewer for this suggestion and corrected the respective passage in the Abstract: “…In one patient, an elevated IgG index was noticed even two days after plasmapheresis. Neither quantitative Ig synthesis, nor elevated IgG index was observed when the LP was performed three or more days after therapeutic apheresis.”

4. The authors combine the effects of plasmapheresis and immunoadsorption. The limitation should be discussed. Furthermore, the authors should explain both procedures and especially the difference.

Response: We added the respective information in the last paragraph of the Discussion: “...Additionally, we included both patient groups receiving plasmapheresis or immunoadsorption, which are different therapeutic measures (Mühlhausen J, Kitze B et al., Atheroscler Suppl. 2015; 18: 251-6). Briefly, therapeutic plasmapheresis removes and replaces the complete plasma, containing among others immunoglobulins, immune complexes and coagulation factors. By contrast, immunoadsorption exclusively removes immunoglobulins and immune complexes from the plasma by a special “absorber”. However, the relevant “pathophysiologic principle” important for this study, namely reducing serum immunoglobulin levels while leaving CSF levels unchanged, is equivalent in both.”

5. The authors discuss that OCB are less susceptible to apheresis. In fact, OCB are not susceptible to such procedures.

Response: We agree with this comment. Therefore, the respective paragraph was modified for clarification (Correale et al. J Neurol 2002; 249(4): 375-89):
**Former version:** "..., OCBs measured in Western blots provide qualitative and highly sensitive evidence for intrathecal IgG synthesis and are thus less susceptible to spurious alterations due to non-steady-state serum/CSF conditions."

**Revised version:** "..., OCBs measured in Western blots provide qualitative and highly sensitive evidence for intrathecal IgG synthesis and are not susceptible to spurious alterations due to non-steady-state serum/CSF conditions."

6. Results: Change 45.8 years 46 years and 58.3 to 58.

**Response:** We made the respective changes.

7. Line 213-215: the two sentences can be deleted since already mentioned in the introduction.

**Response:** As suggested by the reviewer, we deleted the second sentence. However, we kept the first sentence since we found it important to emphasize that this is the first systematic study on this issue and since reviewer #1 encouraged us to state this strength of the study (see reviewer #1, comment 20) at the beginning of the discussion.

Yours sincerely,

Benjamin Berger, Tilman Hottenrott, Jonas Leubner, Rick Dersch, Sebastian Rauer, Oliver Stich, and Harald Prüss