Author's response to reviews

Title: Antibodies against phosphorylcholine are not altered in plasma of patients with Alzheimer's disease

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Author's response to reviews: see over
Dear Editor,

We thank you for giving us the opportunity to respond to reviewer comments and to re-submit our manuscript “Antibodies against phosphorylcholine are not altered in plasma of patients with Alzheimer’s disease”.

Please find our response to reviewer comments below.

Thank you for your consideration and we look forward to hearing from you.

Sincerely,

Edina Silajdžić (on behalf of Oskar Hansson and Maria Björkqvist)
Referee 1
Reviewer’s report:
Silajdžić et al measured levels of anti-PC in plasma from 125 patients with AD, 176 control subjects, 19 patients with VaD, and 63 patients with other types of dementia. The anti-PC levels were not found to differ between these groups. The manuscript is generally well written and easy to understand. The study aimed to replicate a previous report on different serum anti-PC levels in AD patients compared to controls, but no significant association was found here. As such, the study reports a negative result, which is often considered not publishable. However, replication of findings relating to biomarker discovery in independent cohorts is crucial and results failing to replicate other studies should be reported. The authors clearly and concisely report their findings without overstating their results. But the manuscript could be improved by adding more detail and revisiting the statistical analysis of the data.

Major compulsory revisions:
1. The statistical analysis seems quite basic. Please consider the following suggestions:
   a. If any additional relevant information such as ApoE #4 allele status were available for the participants, it should be incorporated into the linear regression analysis.

   We thank the reviewer for this helpful suggestion. We only have information about ApoE4 allele status in a subset of control and AD samples (n=36 and n=31, respectively). For these samples, partial correlation has been carried out, correcting for age, gender, BMI and ApoE4 allele status.

   The methods section now states:
   “In addition, we performed multivariable linear regression with plasma anti-PC as the dependent variable and CSF Aβ42, total tau, and phosphorylated tau concentrations or MMSE as independent variables, adjusting for presence of the ApoE4 allele, age, gender and body mass index (BMI).”

   b. Line 12, page 5 states that regression analyses were only carried out on all clinical groups. The analysis should also be performed on the control group to assess potential associations with MMSE, age, gender, etc within this group.

   Regression analysis has now been carried out on all groups, including control.

   The methods section now states:
   “Linear regression analyses were used to investigate associations between plasma anti-PC and Mini-Mental-State Examination (MMSE), age and gender in all groups, whereas correlation between plasma anti-PC and cerebrospinal fluid (CSF)/serum albumin ratio was only performed in the AD group. Linear regression analyses between plasma anti-PC and CSF biomarkers Aβ42, total tau and phosphorylated tau were only performed in a subset of 36 control and 31 AD samples.”

   The results section now states:
We also examined a possible correlation between plasma anti-PC and MMSE, a measure of global cognition, in each individual group (control, AD, VaD and other dementias), however, there was no correlation between anti-PC levels and MMSE scores in any group tested. Additionally, we examined whether a correlation could be seen between plasma anti-PC and CSF biomarkers Aβ42, total tau and phosphorylated tau in a subset of control and AD samples (n=36 and n=31, respectively). The only significant association found was between CSF total tau and plasma anti-PC in the control group only: CSF total tau levels statistically significantly predicted plasma anti-PC \( F(1, 34) = 7.114 \ p < 0.012, \ R^2 = .173 \). The association between plasma anti-PC and CSF total tau levels in the control group remained significant after controlling for age, gender, BMI and presence of the ApoE4 allele (\( p < 0.008 \)).

c. Please give details of the variables CSF/serum albumin ratio, Aβ42, total tau and phosphorylated tau (mentioned in lines 12-15, p. 5) used for correlation in Table 1. Please explain how these were measured or provide reference if these data were published previously.

The methods section now states:

"The CSF samples were collected into polypropylene tubes at the memory clinics in Malmö according to routine clinical procedures, and the procedure and the analysis followed the Alzheimer’s Association Flow Chart for lumbar puncture (4). After centrifugation, the CSF samples obtained were frozen at -80°C. The samples were analyzed using commercially available enzyme-linked immunosorbent assays (ELISAs) (Innogenetics, Ghent, Belgium) to determine the levels of total tau, Aβ42 and tau phosphorylated at Thr181 (P-tau) (INNOTEST® hTAU Ag, β-AMYLOID(1-42) and PHOSPHO-TAU(181P), respectively). All analyses were performed by board-certified laboratory technicians using procedures accredited by the Swedish Board for Accreditation and Conformity Assessment (SWEDAC). CSF and plasma albumin levels were analysed on a Cobas C501 analyser (Roche Diagnostics, Mannheim, Germany)."

2. Please provide sample numbers for each of the “other” dementia types either within the brackets in line 18, p. 5, or in Table 1. Were plasma levels of anti-PC compared across these different types? Grouping them together into one “other dementia” group could obscure potential differences in anti-PC levels between these types of dementia.

Since we were trying to replicate the study by Eriksson et al, where they grouped all dementias with VaD, we also grouped all the “other” dementias, with the exception that we left VaD as a separate group.

We have added the sample number for each of the “other” dementia types in Table 1 and in the results section. The results section now states:

“We measured plasma levels of anti-PC in subjects with AD, vascular dementia, “other” dementias (including 34 dementia with Lewy Bodies, 17 frontotemporal dementia, and 12 Parkinson’s disease with dementia subjects) and in age matched controls.”
Dividing the plasma anti-PC levels into each individual group yielded a significant result (ANOVA p 0.026), however, post-hoc pairwise comparisons using Bonferroni correction did not yield any significant pairwise comparisons.

Below is the mean and SD of all the individual groups:

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean IgM anti-phosphorylcholine (U/ml)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>FTD</td>
<td>17</td>
<td>59</td>
<td>28</td>
</tr>
<tr>
<td>DLB</td>
<td>34</td>
<td>62</td>
<td>25</td>
</tr>
<tr>
<td>VaD</td>
<td>19</td>
<td>65</td>
<td>27</td>
</tr>
<tr>
<td>Control</td>
<td>176</td>
<td>73</td>
<td>30</td>
</tr>
<tr>
<td>AD</td>
<td>125</td>
<td>73</td>
<td>25</td>
</tr>
<tr>
<td>PDD</td>
<td>12</td>
<td>87</td>
<td>37</td>
</tr>
</tbody>
</table>

3. The discussion of why the results from Eriksson et al 2010 did not replicate in this study (lines 6-11, p 7) should be expanded. What are the differences and limitations of the study here and Eriksson et al 2010? Could the different type of sample (plasma vs serum) or other factors such as fasting/non-fasting play a role? What about other technical issues besides the type of assay, such as sample preparation, storage, freeze thaw cycles, person performing the assay etc? Line 11, p 7: either be more specific or delete the “laboratories”, as currently this is contradictory to line 7, p. 7 “not due to differences in laboratory technique”. Also, the same kit was used but presumably by different operators so there could be differences in laboratory technique. It would be more accurate to say that there are no differences in kit manufacture.

Following the suggestions of the reviewer, we have now expanded the discussion:

"Since we used the same assay as was used in the Eriksson et al study, the contrasting results are not due to differences in kit manufacture. ... Moreover, the gender distributions in the control and AD cohorts are very similar in our study and in the Eriksson et al paper. One of the reasons why we could not replicate the findings of Eriksson et al may be because the cohorts used in the two studies are at different disease stages. The mean MMSE score in our AD and mixed dementia group was 21 ± 5, however, since this information is not available in the Eriksson et al paper, it is difficult to directly compare the disease stage in these two studies. Our cohort is on average younger than that in the Eriksson et al study (76±7 and 82±5 years, respectively, for the AD group), thus, it may be possible that our cohort is at an earlier disease stage. The fasting status of the samples is sometimes the reason for a discrepancy in results, however, in the Eriksson et al study the association between antiPC and dementia was not found to be affected by the fasting status, thus, the fasting status is unlikely to be the reason why we could not replicate the findings of Eriksson et al. In the present study IgM anti-PC was quantified in plasma samples, whereas in the Eriksson et al study, it was quantified
in serum. The levels of some analytes vary significantly between serum and plasma. The higher levels of anti-PC in plasma in this study compared to those in serum in the Eriksson et al study suggest that this may be a case for anti-PC. However, a previous study found similar IgM anti-PC levels in serum and plasma and showed that repeated freeze-thawing had no effect on IgM anti-PC levels [23]. It is possible that anti-PC levels may be different in different clinical populations since differences may exist between laboratories and cohorts with respect to sample handling and storage, diet, use of medication, BMI, blood pressure and other unknown factors.”

4. In methods paragraph “analysis of plasma anti-PC” (line 3, p. 5), please provide more detail on the CVDefine assay used to quantitate anti-PC, such as a very brief overview of assay principle and method including amount of plasma required, the limit of detection, the CV etc. This information is still of interest here even though it was published previously.

We have now added more details about the assay: “Anti-PC levels were quantified in plasma diluted 1:101 in accordance with the manufacturer’s recommendations using CVDefine (Athera Biotechnologies, Stockholm, Sweden), an indirect, noncompetitive, enzyme immunoassay for quantitative determination of anti-phosphorylcholine IgM antibodies in human serum or plasma. The assay is based on PC antigen covalently linked to bovine serum albumin coated onto 96-well microtiter plates and PC-specific IgM antibodies present in the plasma sample bind to the antigen. The detection limit of CV Define is 0.5 U/ml and the inter-assay coefficient of variation is below 8%.”

Minor essential revisions
1. There is a relatively small number of samples in the VaD group. Please state the power calculation used in the study design or how the number of samples for all groups was arrived at?

Since we were trying to replicate the study by Eriksson et al we used their data for the power analysis. The power analysis suggested that only one sample was required to replicate the result, however, we aimed to use a higher number of samples than was used in that study. Since Eriksson et al grouped VaD with “other dementias”, we have done the same in the table below for ease of comparison:

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>AD</th>
<th>VaD + Mixed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Silajdzic</td>
<td>Eriksson</td>
<td>Silajdzic</td>
</tr>
<tr>
<td>n</td>
<td>176</td>
<td>205</td>
<td>125</td>
</tr>
<tr>
<td>Mean age, y (SD)</td>
<td>74 (6)</td>
<td>81 (6)</td>
<td>76 (7)</td>
</tr>
<tr>
<td>% of females</td>
<td>63</td>
<td>62</td>
<td>72</td>
</tr>
<tr>
<td>Mean anti-PC, U/ml [SD]</td>
<td>73.3 [29.9]</td>
<td>49.5 [2.2]</td>
<td>73.4 [25.4]</td>
</tr>
</tbody>
</table>
In the Eriksson et al study, there were 26 VaD + Mixed Dementia patients. In our study there were 82 VaD and Mixed Dementia patients and of those 82, nineteen were VaD patients.

2. What does the asterisk in Figure 1 above control group represent? Please explain in the figure legend.

The asterisk simply denotes an outlier. We have now replaced it with a circle to avoid confusion.

3. Please rephrase the sentence starting in line 18, p.3.

We have re-phrased the sentence in question:
“To determine whether the anti-PC levels in plasma are reduced in individuals with AD and dementia, we measured plasma levels of anti-PC in a cohort comprising of 176 controls, 125 patients with AD, 82 patients with other dementias.”

4. Please include CVD in the list of abbreviations.

CVD has now been added to the list of abbreviations.
1. Was a sensitivity test (exclude outliers and rerun ANOVA) performed to ensure the outliers do not affect the outcome? From the box plot in Figure 1, this seems highly unlikely as the number of outliers in control and AD group is very small compared to the number of samples, but the authors might still want to check for peace of mind.

Excluding the outliers did not affect the outcome of the study.

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**Referee 2**
Previously, it has been shown that patients with Alzheimer’s disease (AD) had lower serum levels of IgM antibodies against phosphorylcholine (anti-PC) than those of controls. Furthermore, individuals with low levels of anti-PC are at risk for dementia or AD. In this study, the authors would like to verify these findings in plasma specimens.

Comments:
1. This is a cross-sectional study examining the levels of anti-PC in the plasma of AD patients. The findings are contradictory to a previous study which showed that the mean anti-PC levels were reduced in the serum of AD patients. In addition to the potential factors that proposed by the authors (e.g. different populations, laboratory processing procedures, or other unknown factors), the authors should consider the differences between serum and plasma. It is well known that protein levels in the serum can be very different from that in the plasma. The higher levels of anti-PC in the plasma (this study) than those in the serum (previous study) support such possibility.

We have now addressed this issue in the discussion section:

“The levels of some analytes vary significantly between serum and plasma. The higher levels of anti-PC in plasma in this study compared to those in the serum in the Eriksson et al study suggest that this may be a case for anti-PC. However, a previous study found similar IgM anti-PC levels in serum and plasma and showed that repeated freeze-thawing had no effect on IgM anti-PC levels [23].”

2. The star marked in figure 1 is not specified.

The asterisk simply denotes an outlier. We have now replaced it with a circle to avoid confusion.

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**Referee 3**
In this manuscript the authors tested whether IgM antibodies against phosphorylcholine would be different among normal controls and patients with Alzheimer’s disease. No significant differences were observed effectively excluding this antibody measurement as a biomarker. The paper can be shortened prior to publication.

In order to respond to the other reviewers’ comments, we have in fact made the paper longer, however, that was unavoidable.
Referee 4
I suggest a major revision. The author should answer below questions

Major concerns:
Authors present a study on levels of plasma antibodies against phosphorycholine, an epitope on OxLDL, in 125 AD patients, 19 VaD patients, 63 other dementia patients and 176 controls. They observed similar plasma antibodies (anti-PC) levels in controls, patients with AD, and other dementias, suggesting that anti-PC is not a useful biomarker for AD. The result of the present paper is different from the observation of Eriksson et al (Low levels of antibodies against phosphorylcholine in Alzheimer’s disease. Journal of Alzheimer’s Disease, Volume 21, Number 2 / 2010: 577-84), using the same ELISA assay for quantitating the levels of anti-PC.

* This paper cited the reports, however, it did not discuss thoroughly the discrepancies in the two results and analyze factors causing the difference.

We have now expanded the discussion to address the discrepancies in the two results:

“Since we used the same assay as was used in the Eriksson et al study, the contrasting results are not due to differences in kit manufacture. ... Moreover, the gender distributions in the control and AD cohorts are very similar in our study and in the Eriksson et al paper. One of the reasons why we could not replicate the findings of Eriksson et al may be because the cohorts used in the two studies are at different disease stages. The mean MMSE score in our AD and mixed dementia group was 21 ± 5, however, since this information is not available in the Eriksson et al paper, it is difficult to directly compare the disease stage in these two studies. Our cohort is on average younger than that in the Eriksson et al study (76±7 and 82±5 years, respectively, for the AD group), thus, it may be possible that our cohort is at an earlier disease stage. The fasting status of the samples is sometimes the reason for a discrepancy in results, however, in the Eriksson et al study the association between antiPC and dementia was not found to be affected by the fasting status, thus, the fasting status is unlikely to be the reason why we could not replicate the findings of Eriksson et al. In the present study IgM anti-PC was quantified in plasma samples, whereas in the Eriksson et al study, it was quantified in serum. The levels of some analytes vary significantly between serum and plasma. The higher levels of anti-PC in plasma in this study compared to those in serum in the Eriksson et al study suggest that this may be a case for anti-PC. However, a previous study found similar IgM anti-PC levels in serum and plasma and showed that repeated freeze-thawing had no effect on IgM anti-PC levels [23]. It is possible that anti-PC levels may be different in different clinical populations since differences may exist between laboratories and cohorts with respect to sample handling and storage, diet, use of medication, BMI, blood pressure and other unknown factors.”
Authors should add positive controls to demonstrate that the observation is reliable and credible, or enlarge the sample size and conduct stratified analysis.

The aim of our study was not to conduct a stratified analysis but to replicate a finding of reduced anti-PC levels in AD patients compared to controls. Since we were trying to replicate the study by Eriksson et al. we aimed to use a higher number of patient samples than was used in the original study. We, thus, used 82 VaD and mixed dementia samples (compared with 26 in the Eriksson et al. study) and 125 AD samples (compared with 71 in the Eriksson et al. study).

In addition, the criteria of inclusion and exclusion in this work is not clear and rigid.

We believe that the inclusion and the exclusion criteria are already clearly outlined in the methods section. We have outlined the diagnostic criteria for each of the dementia groups and we have excluded anyone with neurological diseases, memory complaints or other cognitive symptoms from the control group:

“All patients diagnosed with AD had to meet the DSM-III-R criteria of dementia [1] and the criteria of probable AD defined by NINCDS-ADRDA [20]. Patients diagnosed with VaD fulfilled the DSM-III-R criteria of dementia and the requirements for probable VaD by NINDS-AIREN [24] or the recommendations by Erkinjuntti et al. for VaD of the subcortical type [9]. For the diagnosis of dementia with Lewy Bodies or frontotemporal dementia, the consensus criteria by McKeith et al. [19] and McKhann et al. were used [21], respectively. The healthy volunteers had no memory complaints or other cognitive symptoms, and no active neurological diseases.”

Minor concern:
* I suggest that authors revise the use of abbreviations.

We are unsure as to how exactly the reviewer would like us to revise the use of abbreviations and, as such, we been unable to address this particular point.