

sTREM2 cerebrospinal fluid levels are a potential biomarker for microglia activity in early-stage Alzheimer's disease and associate with neuronal injury markers

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Céline Carret

1st Editorial Decision

21 January 2016

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the three referees who were asked to assess it. As you will see the reviewers are globally supportive and only request a minor revision to discuss a few points and rewrite maybe the discussion section. I am pleased to inform you that we will be able to accept your manuscript pending editorial final amendments.

1) please address the reviewers concerns and provide a letter INCLUDING the reviewer's reports and your detailed responses to their comments (as Word file).

Please submit your revised manuscript as soon as possible but within 3-months.

I look forward to receiving the revised article.

***** Reviewer's comments *****

Referee #1 (Remarks):

The authors present a large cross-sectional study with samples collected at multiple sites to examine the CSF levels of soluble TREM2 (sTREM2) from controls, pre-AD, MCI-AD, AD with dementia, MCI-non-AD (mild cognitive impairment without AD CSF biomarker changes), and SNAP (suspected non-AD pathology; cognitively normal individuals with tau biomarker changes in CSF). Soluble TREM2 is released upon cleavage of TREM2 on microglia and may reflect microglial activation. CSF sTREM2 increased significantly with age but was not influenced by gender or ApoE E4 status within each group. CSF sTREM2 levels were significantly elevated in MCI-AD vs. controls and AD with dementia. CSF sTREM2 levels were higher in MCI-AD vs. pre-AD but statistical analysis revealed a strong trend. No such increase was observed in MCI-non-AD CSF. Unlike the previous report from the authors in which AD patients had lower CSF sTREM2, there was no significant difference in sTREM2 CSF levels between AD and controls in this study. In fact, CSF sTREM2 levels were non-significantly higher in AD CSF vs. controls (adjusted for gender, age and site). This result may be due to the high degree of variability in the CSF sTREM2 levels in samples collected at different sites. CSF sTREM2 levels positively correlated with CSF total tau and p-tau in MCI-AD, suggesting that sTREM2 levels may reflect an early microglial response to neuronal injury. Interestingly, CSF sTREM2 levels were significantly increased in SNAP CSF, again suggesting a link to neurodegenerative processes.

The paper provides an excellent, first-ever evaluation of CSF soluble TREM2 in the AD continuum and in SNAP. The data are in agreement with other studies that suggest that early microglial activation plays a role in AD pathogenesis and cognitive impairment. This study offers an important contribution to the field of neurodegeneration in general, and AD, in particular, and will undoubtedly lead to further research into mechanisms underlying the connection between early microglial activation and neurodegeneration.

Comments:

1. Is the TREM2 genotype available for the subjects in this study? Is it possible that the difference in the AD results (from the previous study) might be due to fewer patients with a TREM2 variant, which would be predicted to have reduced TREM2 expression? As the authors noted in the Introduction, TREM2 variants are fairly common in AD. Do the authors predict that the CSF sTREM2 levels would be different in an MCI-AD patient with a TREM2 variant vs. an MCI-AD patient without a TREM2 variant? Please discuss.
2. The authors suggest that increased CSF sTREM2 levels reflect a response to neurodegeneration. Is it possible that microglial activation precedes (and drives) neurodegeneration?
3. Optional: How do these results line up with the differences in the TREM2 KO mouse publications? For example, could the disease stage in the mice have influenced the results? Or, is it possible that we need to wait for the Tau Tg/TREM2 KO to see an effect?
4. Please check the references for accuracy of spelling (e.g. authors' names and initials).

Referee #2 (Remarks):

The authors performed an ambitious, multicenter study examining whether CSF sTREM2 levels were different in individuals representing the spectrum of Alzheimer's disease, from controls to preclinical AD to mild cognitive impairment (MCI) to dementia. This study faced some significant challenges but accounted for these well in their analyses. Part of the difficulty of a multicenter study on CSF biomarkers is that each center has different criteria for controls, CSF collections protocols, CSF biomarker cut-offs, etc. Although there were important differences between centers, this group did a good job of documenting the relevant center-specific information and controlled for a center effect by adding it into their models.

The authors found that CSF sTREM2 levels increased with age and this effect was found in both controls and individuals on the AD spectrum. Levels of CSF sTREM2 were highest in the MCI-AD and AD dementia groups. Interestingly, levels of CSF sTREM2 were lower in the AD dementia

group than in the MCI-AD group, suggesting that sTREM2 peaks with the onset of cognitive symptoms and then later declines. If this is true, it would be important because high sTREM2 levels might identify individuals with incipient dementia. The study had a potential weakness because their control group was a decade younger than the other groups. Although they included age in their models, they further evaluated this potential confound by repeating their analysis in age-matched groups and convincingly found that the MCI-AD group still had higher sTREM2 levels than controls. The authors also found correlations between CSF sTREM2 and CSF Tau, pTau and A 42. As might be expected, CSF sTREM2 levels correlated better with CSF Tau and pTau levels, again suggesting that elevated sTREM2 levels occur later in the course of AD pathology.

The authors also evaluated the CSF sTREM2 levels of individuals with evidence of neurological dysfunction not related to Alzheimer's disease. These analyses included either cognitively normal individuals with normal CSF A 42 but high CSF tau or ptau (classified as Suspected Non-Alzheimer's Pathology [SNAP]) or with MCI but CSF biomarker not consistent with AD (MCI-no AD). Interestingly, individuals classified as SNAP had elevated sTREM2. Individuals with MCI-AD had higher levels of sTREM2 than individuals with MCI-no AD. The authors should include a control group in this MCI-AD and MCI-no AD analysis (Fig. 4B), because it is not clear whether levels of sTREM2 in MCI-no AD are normal.

The authors do need to significantly change their discussion (and some parts of their introduction) because two papers have come out in the past month that examine CSF sTREM2 in AD and have highly relevant and supportive results to this work. The first is by Piccio et al. in *Acta Neuropathologica* and the second is by Heslegrave in *Molecular Neurodegeneration*.

Piccio L, et al.. Cerebrospinal fluid soluble TREM2 is higher in Alzheimer disease and associated with mutation status. *Acta Neuropathol.* 2016 Jan 11. [Epub ahead of print]

Heslegrave A, Heywood W, Paterson R, Magdalinou N, Svensson J, Johansson P, +hrfelt A, Blennow K, Hardy J, Schott J, Mills K, Zetterberg H. Increased cerebrospinal fluid soluble TREM2 concentration in Alzheimer's disease. *Mol Neurodegener.* 2016 Jan 12;11(1):3. doi: 10.1186/s13024-016-0071-x

Despite the overlap between this manuscript and the Piccio/Heslegrave papers, this topic is highly impactful and replication advances the field. Further, this study represents a larger sample than either the Piccio/Heslegrave papers and has the unique and important finding that sTREM2 levels may change along the AD spectrum, peaking in MCI-AD and then declining.

Referee #3 (Remarks):

This is a very well done study looking at Soluble Trem2 levels in CSF among individuals with AD, MCI, SNAP and preclinical AD. CSF was obtained from multiple sites and the assay shows excellent performance. While, I find the data compelling with the exception of one small quirk (noted explicitly below) I do find the discussion both a little long and a little overreaching in terms of possible implications of this data.

Indeed, how sTREM2 relates to microglial activation states is not clearly established. It is shed but what regulates that shedding Especially in isolation it is unlikely that sTREM2 will be that informative as microglial cells produce hundreds of secreted proteins. Data describing how sTREM2 levels are altered by various immune stimuli might make this an article of broader impact. Additionally looking at other innate immune markers might be very important to put this data in context.

The data from Saint PAs cohort is somewhat problematic as it is almost the opposite of the other data. Lower levels in the AD continuum subjects. If there is this kind of site to site variability this may be a very hard finding to reproduce. Some discussion of this is warranted and perhaps some investigation into possible confounds from various sites

Please find attached the revised version of the manuscript EMM-2015-06123. We have now carefully addressed all points raised by the reviewers and specifically re-written the discussion with a special emphasis on the new data presented in the communications published while our paper was under consideration. In detail we addressed the points of the reviewers as follows:

Reviewer 1:

1) Is the TREM2 genotype available for the subjects in this study?

We have not screened the individuals included in the study for *TREM2* mutations. However, the prevalence of *TREM2* mutations is rare (minor allele frequency <1%) (Guerreiro et al 2013; Jonsson et al. 2013). Therefore, it is extremely unlikely that our sample contains a significant number of *TREM2* mutant carriers that may affect our results. Moreover, the main analyses have been confirmed by Bootstrapping in order to control for the effect of any potential outlier. However, we agree with the reviewer that this is a limitation of the study and, therefore, we have mentioned it in the discussion section. The reviewer stated that we mentioned in the introduction that “*TREM2* variants are fairly common in AD”. We like to clarify that we noted in the introduction that *TREM2* mutations are associated with increased risk of AD. However, this does not imply that the mutations are prevalent in AD dementia.

2) The authors suggest that increased CSF sTREM2 levels reflect a response to neurodegeneration. Is it possible that microglial activation precedes (and drives) neurodegeneration? Optional: How do these results line up with the differences in the TREM2 KO mouse publications?

Both points are now specifically discussed on page 10 and 11 of our revised manuscript. Please note that we have re-written and streamlined the entire discussion as requested by reviewers 2 and 3.

3) Please check the references for accuracy

All references were checked for accuracy.

Reviewer 2:

1) Individuals with MCI-AD had higher levels of sTREM2 than individuals with MCI-no AD. The authors should include a control group in this MCI-AD and MCI-no AD analysis (Fig. 4B), because it is not clear whether levels of sTREM2 in MCI-no AD are normal.

We have now added the control group to the new Fig. 4B as requested.

2) The authors do need to significantly change their discussion (and some parts of their introduction) because two papers have come out in the past month that examine CSF sTREM2 in AD and have highly relevant and supportive results to this work.

We have now extensively re-written the discussion also in accord to reviewer's 3 comments (see below). This includes a detailed comparison of our concept to investigate the AD continuum versus the rather simple comparison of AD (a mixture of all stages) with controls. Moreover, reviewer 3 found our discussion a bit long and overreaching. In accordance with that we reduced the discussion by 50% and streamlined it to the most important points (please refer to the completely new discussion section).

Reviewer 3:

1) I do find the discussion both a little long and a little overreaching in terms of possible implications of this data.

Please refer to the similar point raised by reviewer 2 (and also reviewer 1) and the completely new discussion section.

2) The data from Saint PAs cohort is somewhat problematic as it is almost the opposite of the other data.

We agree with the reviewer that there is significant variability between centers and it needs to be addressed in the future which pre-analytical issues may influence CSF sTREM2 measurement, as it has been done with other CSF biomarkers. This is a limitation of the study and we have clearly highlighted that in the discussion section. In order to control for the center effect, we used a linear mixed effects model with center as a random effect. Noteworthy, the highest levels of CSF sTREM2 in all centers occur in the MCI-AD group (also in Sant Pau center) as depicted in Appendix Table S4, with the only exception of the Bonn center in which the highest levels occurs in the preclinical stage.

Taken together we believe that we have carefully addressed all points raised by the reviewers. We are now looking forward to the publication of our findings in EMBO Mol Med. Again, many thanks for considering our manuscript.

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: CHRISTIAAN HAASS / MICHAEL EWERS
 Journal Submitted to: EMBO MOLECULAR MEDICINE
 Manuscript Number:

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if $n < 5$, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Material and Methods section, subheading "Study design and participants"
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Material and Methods section, subheading "Study design and participants"
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Measurements of sTREM2 in ELISA were performed blinded for diagnosis.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	YES. All figures contain a description of the statistical test used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	The data was log-transformed to approach the assumption of normality (see statistics sections in Material and Methods)
Is there an estimate of variation within each group of data?	The 95% confidence intervals of all groups compared are showed in the graphs.
Is the variance similar between the groups that are being statistically compared?	The data was log-transformed to approach the assumption of homoscedasticity. In order to confirm the robustness of our results, we calculated the 95% confidence intervals of each of the compared groups by bootstrapping

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

<http://www.antibodypedia.com>
<http://1degreebio.org>
<http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo>

<http://grants.nih.gov/grants/olaw/olaw.htm>
<http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm>
<http://ClinicalTrials.gov>
<http://www.consort-statement.org>
<http://www.consort-statement.org/checklists/view/32-consort/66-title>

<http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun>

<http://datadryad.org>
<http://figshare.com>

<http://www.ncbi.nlm.nih.gov/gap>

<http://www.ebi.ac.uk/ega>

<http://biomodels.net/>

<http://biomodels.net/miriam/>
<http://jij.biochem.sun.ac.za>
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
<http://www.selectagents.gov/>

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia [see link list at top right] , 1DegreeBio [see link list at top right] .	In methods section (p.15) and in the Appendix Supplementary Methods there is a detailed description of the antibodies used and the characteristics of the ELISA.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines [see link list at top right] (PLOS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH [see link list at top right] and MRC [see link list at top right] recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Ethics committee at each center approved the study.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	All participants or their relatives gave their written consent and was in accordance with the declaration of Helsinki.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram [see link list at top right] and submit the CONSORT checklist [see link list at top right] with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines [see link list at top right] . See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad [see link list at top right] or Figshare [see link list at top right]).	Measurements of CSF sTREM2 can be provided. However, the confidentiality of the participants must be protected and hence the demographic and clinical data that could identify a participant can not be provided.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP [see link list at top right] or EGA [see link list at top right] .	NA
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22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines [see link list at top right] and deposit their model in a public database such as Biomodels [see link list at top right] or JWS Online [see link list at top right] . If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents [see link list at top right] and list of select agents and toxins (APHIS/CDC) [see link list at top right] . According to our biosecurity guidelines, provide a statement only if it could.	NA
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