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Manuscript EMBO-2016-43519

Posttranslational modification impact on the mechanism by which amyloid- β induces synaptic dysfunction

Katarzyna M. Grochowska, PingAn Yuanxiang, Julia Bär, Rajeev Raman, Gemma Brugal, Giriraj Sahu, Michaela Schweizer, Arthur Bikbaev, Stephan Schilling, Hans-Ulrich Demuth, and Michael R. Kreutz

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Editor: Esther Schnapp

Transaction Report:

(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.)

1st Editorial Decision 08 November 2016

Thank you for the transfer of your research manuscript to EMBO reports. We have now received the enclosed reports on it. As you will see, the referees acknowledge that the results are potentially intersting and novel. However, they also suggest a few more experiments to strengthen the study, and given the overall number and nature of the concerns, I think that all of them can and should be addressed.

We would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

REFEREE REPORTS

Referee #1:

The manuscript by Grochowska et al. investigates the role of the Aβ3(pE)-42 isoform on synaptic dysfunction. They found that Aβ3(pE)-42, unlike Ab1-42, induces TNFa in astrocytes associated with synaptic dysfunction. This synaptic dysfunction was independent of NMDAR signaling. The manuscript includes important results, which show that Aβ3(pE)-42 plaus an important role in

neuroinflammatory processes and pathogenesis of AD. However there are several points the authors should address to improve their work.

Major comments:

1. It is not clear whether the experiment for LTP recording of the effect of Ab1-42 and Ab3(pE)-42 shown in Fig. 3B,C was performed in the same experiment with D1/D5 receptor agonist shown in Fig. 3G,H, as both shown in separate panels. The experimental groups should be performed and shown in the same experiment/panels similarly to Fig. 3I. Otherwise, the authors should clearly state it.
2. Did the authors check the presence of microglia in their primary culture? What is the role of Aβ3(pE)-42 on microglia and their contribution to synaptic dysfunction if the primary culture is contaminated and microglia proliferate in cultures treated with Ab3(pE)-42?
3. The authors show significant increase in Iba+ microglial signal in organotypic hippocampal slices treated with Ab3(pE)-42. From Figure S3B its not clear whether Ab3(pE)-42 induced microglia activation or proliferation. Nuclear (DAPI) quantification should be provided. The authors should assess the effect of Ab3(pE)-42 on microglia proliferation (i.e. BrdU incorporation).
4. The authors stated that hippocampal primary cultures revealed negative Iba1+ signal. Thus, they concluded that astrocytes were the source for TNFa. This is unlikely, as microglia present in the hippocampus.
5. The major discovery of this study is the role of Aβ3(pE)-42, unlike Ab1-42, on induction of TNFa in astrocytes associated with synaptic dysfunction. The authors should carefully examine the effect of Ab3(pE)-42 on astrocytes and microglia. They can implement several widely used today approaches to pharmacologically or genetically deplete microglia in primary cells or hippocampal cultures to address whether by Ab3(pE)-42-induced synaptic dysfunctions is mediated by astrocytes or microglia or both. Moreover, the authors can specifically deplete TNFa in microglia using recently described technique (CX3CR1(cre) /TNFα(fl/fl): PMID: 27145902) or use global TNFa-KO mice donors for primary culture or OHSCs.
6. The authors used anti-TNFa neutralizing antibody. The authors did not show the specificity and efficacy of these antibodies i.e. competition assay.

Minor comments:

1. Results section should describe the results in the figures, other references should be for the discussion section
2. Methods: Authors should state n numbers, sex and ages in the methods section to clarify for readers.
3. Figure 1: Legend is mismatch for I and K letters of the correspondent panels.
4. Figure 2: This figure needs to be re-organised. Change location of E,F to G,H to reflect it in the result section. Legend needs to be re-organised: instead listing A, C, E, and G, describe each panel in consecutive order. Authors should take care when describing the figure legends so as it corresponds to what is in the figure.
5. Avert to use "stark contrast", just say "in contrast".
6. Authors should carefully scan text for grammatical and spelling errors. There are several mistakes within the manuscript.

Referee #2:

In the manuscript entitled « Posttranslational modification impact on the mechanism by which amyloid-β induces synaptic dysfunction » investigate the molecular mechanisms involved in the synaptic dysfunction. For this purpose they studied multiples parameters of synaptic activity exposed to the same concentration of oligomers of Human Aβ 1-42 and Aβ 3(pE)-42. Overall, they described that if both species triggered synaptic dysfunction, they exert their synaptotoxic effect through a completely different molecular mechanisms. These results are interesting and raise questions that should be addressed:

Page 6 result section: How long the cultures have been exposed to the different oligomers species to induce synaptic loss? (It seems to be 3 days as mentioned in the method section). This point should be mentioned clearly in the result section. Is the difference in synapse loss observed after treatment

with the different species of oligomers really drastically different (as mentioned in the text) since the difference (in fig 1) between these two conditions seems to be rather small? Is the drastic reduction in synapse density reversed by TNF-α antibody or mimicked by TNF-α?
These points should be clarified.

Figure 1: The Scholl analysis reveals an important reduction in dendritic complexity induced by both Aβ 1-42 and Aβ 3(pE)-42. How do the authors explain this effect? Does the same common effector P38 trigger this effect? Is it mimicked by TNF-α? This is a very drastic effect that may have an important role in the physiopathology of both oligomeric species.

Page 10: The decrease in spontaneous neuronal network activity is obtained after a 40 min exposure to both oligomeric species and retraction of synaptic contact induced by Aβ 1-42 are observed the same time frame (3 days). Is these two important synaptotoxic features related ? is the dendritic retraction observable is reversed by Ifenprodyl incubation?

To further validate that these different species of oligomers are disrupting synaptic function by both activating P38, it will be necessary to show a western blot with the activation pattern of P38 at the key time points of the experiments presented (i.e. 40 min and 3 days)?
This would be a great additional figure to further validate P38 as a nodal signaling pathway for both oligomers species.

In the final scheme that summarizes the observations presented in the paper, the authors added phosphorylated tau in the scheme. Do they have specific information on the status of tau after exposure to Aβ 1-42 and Aβ 3(pE)-42?

Referee #3:

While there is widespread, but less than universal agreement that amyloid-β oligomers (AβOs) are potently toxic agents in Alzheimer's disease (AD), relatively scant attention has been paid to the diversity of Aβ species, and the homotypic and heterotypic oligomers that they may form. The present study provides compelling evidence that oligomers made from Aβ1-42 and AβpE3-42 harm neurons by distinct mechanisms. Most notably, in contrast to Aβ1-42 oligomers, Aβ3(pE)-42 oligomers induce early neuronal dysfunction neither by activation of GluN2B-containing NMDA receptors nor by a PrP-dependent mechanism, and their toxicity is mediated by TNFα that is secreted by astrocytes following their apparent uptake of the pyroglutamylated AβOs.

Taken as a whole, the authors make an impressive case that biochemical differences between different Aβ species lead to the formation of funtionally distinct AβOs. This study is arguably one of the most comprehensive demonstrations in support of that idea, and should attract wide interest within the AD research community.

Further attention should be paid to the following significant issues as a prelude to publication. 1) A previous report from several of the present authors showed that AβOs made from AβpE3-42 or co-incubated mixtures of AβpE3-42 and Aβ1-42 were potently cytotoxic to neurons at submicromolar concentrations within hours (Nussbaum, et al. Nature 2012). In contrast, the current report states on page 22 (Materials and Methods) that neurons were exposed for up to 3 days to AβpE3-42 oligomers at 500 nM total peptide, but no mention of neuron death under these conditions was mentioned. 2) Figure 5 is somewhat problematic. Most importantly, the claim that astrocytes take up oligomers made from AβpE3-42 or mixtures of AβpE3-42 and Aβ1-42 is not convincing. Although the claim is based on 3D reconstructions of confocal images, the Z-resolution in individual images is ~500 nm, which is likely very similar to the thickness of the astrocyte cytoplasm in the reconstructed images that were provided. In other words, it it possible that the oligomers in question were actually resting on top of the astrocytes, instead if having been internalized. Figure 5 would also be improved by showing original fluorescence micrographs of astrocytes labeled for AβpE3-42 and Aβ1-42.

Finally, several minor issues warrant further attention as well. 1) A related study from a different group showed that AβOs made from Aβ1-42 provoke microglia to secrete TNFα that then drives post-mitotic neurons back into the cell cycle, which leads them to die eventually (Bhaskar, et al.

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2014. Neurobiology of Disease 62: 273-285). This paper should be acknowledged. 2) On the top of page 6 the authors describe Thioflavin T as a reagent that detects amyloid fibrils. This statement is correct, but misleading. ThT actually detects β-sheet structure, irrespective of fibril formation. 3) Please include catalog numbers for antibodies and other biological reagents. 4) In figure 4, anti-PrP is mistakenly labeled as anti-PrnP. 5) Fig S6 is mistakenly listed in the text on page 18 as Fig S8. 6) I do not understand Fig 4A.

1st Revision - authors' response 08 February 2017

Please find enclosed the revised version of the manuscript entitled 'Posttranslational modification impact on the mechanism by which amyloid- induces synaptic dysfunction' (EMBOR-2016-43519V2). We think that we could fully address the criticism of the reviewers and we hope that the paper is now suitable for publication in EMBO Reports. If any questions arise please do not hesitate to contact me.

Point by point response:

We would like to thank the referees for their constructive criticism and their positive comments on the manuscript. In the revised version of the manuscript, we have included several novel experiments to address the suggestions of all three reviewers and we think that the additional data strengthen our observations and conclusions further. We hope that the improved version of the manuscript is suitable for publication in EMBO Reports. Please find below our detailed responses to the raised concerns.

Referee #1:

The manuscript by Grochowska et al. investigates the role of the Ab3(pE)-42 isoform on synaptic dysfunction. They found that Ab3(pE)-42, unlike Ab1-42, induces TNFa in astrocytes associated with synaptic dysfunction. This synaptic dysfunction was independent of NMDAR signaling. The manuscript includes important results, which show that Ab3(pE)-42 plaus an important role in neuroinflammatory processes and pathogenesis of AD. However there are several points the authors should address to improve their work.

Major comments:

1. It is not clear whether the experiment for LTP recording of the effect of Ab1-42 and Ab3(pE)-42 shown in Fig. 3B,C was performed in the same experiment with D1/D5 receptor agonist shown in Fig. 3G,H, as both shown in separate panels. The experimental groups should be performed and shown in the same experiment/panels similarly to Fig. 3I. Otherwise, the authors should clearly state it.

Reply: The data presented in the Fig. 3 come from the same experiments – i.e. the slices from C57/B6J mice littermates were treated with the same Aβ preparations with or without the presence of the D1/D5 agonist. For the sake of clarity we have presented the different groups in separate panels since a combined panel would have been overcrowded. We have included a sentence in the figure legend to clarify this issue.

2. Did the authors check the presence of microglia in their primary culture? What is the role of Ab3(pE)-42 on microglia and their contribution to synaptic dysfunction if the primary culture is contaminated and microglia proliferate in cultures treated with Ab3(pE)-42?

Reply: The preparation protocol for primary cultures includes several steps that are not in favor of microglia attachment. But to address the question we have checked now for the presence of microglia in hippocampal primary cultures before and after treatment with oligomers and we could neither detect microglia by means of immunocytochemistry nor by immunoblotting using Iba1 as a marker. We have included this information in two additional panels in the Figure S2C,D.

3. The authors show significant increase in Iba+ microglial signal in organotypic hippocampal slices treated with Ab3(pE)-42. From Figure S3B its not clear whether Ab3(pE)-42 induced microglia activation or proliferation. Nuclear (DAPI) quantification should be provided. The authors should assess the effect of Ab3(pE)-42 on microglia proliferation (i.e. BrdU incorporation).

Reply: In the original manuscript we have already counted the number of Iba-1 positive cells (see EV3). We have also performed a BrdU experiment that did not provide conclusive evidence for an increased proliferation (see picture below). Collectively the results of our experiments point to microglia activation.

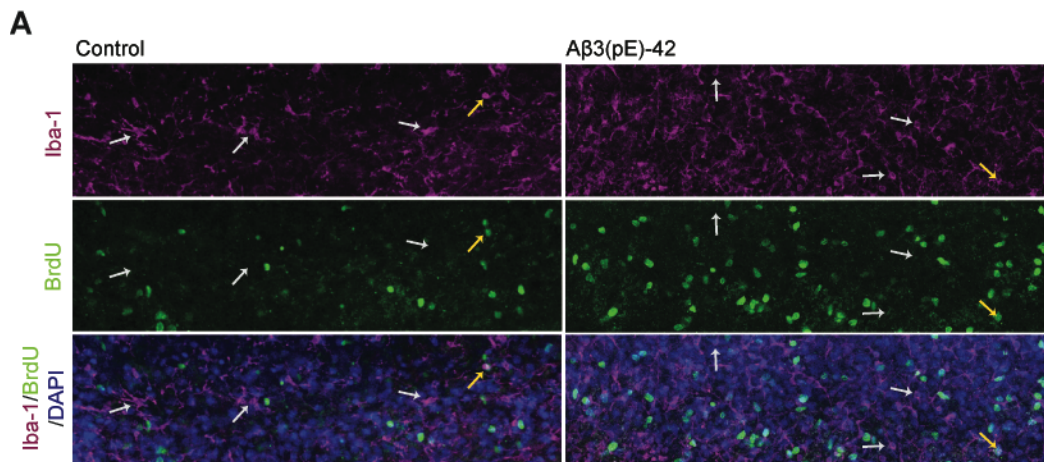

Figure legend: Organotypic hippocampal slices were treated with Ab3(pE)-42 like it is indicated in the manuscript. Yellow arrow – Iba+ and BrdU+ cell; white arrow, BrdU negative microglia. The modest increase in the BrdU signal does not significantly co-localie with IBA1 positive microglia cells.

4. The authors stated that hippocampal primary cultures revealed negative Iba1+ signal. Thus, they concluded that astrocytes were the source for TNFa. This is unlikely, as microglia present in the hippocampus.

Reply: We have indeed proven that there is no microglia in our culture preparation (see our reply to point 2). Nonetheless the reviewer is obviously right and many reports have clearly shown the importance of microglia-derived TNFα in AD pathology. **It is important to note that we don't believe that Aβ3(pE)-42 exclusively induces its synaptotoxic effect via TNFα released from astrocytes. We want to stress that our results do not exclude and actually point to the possibility that microglia as well as astrocytes are important in Ab3(pE)-42-induced synaptic pathology.** In our initial experimental design, we used pure, primary astrocytic cultures (the preparation protocol includes a shaking step to remove microglia). In line with previous reports we could show that astrocytes can release TNFα upon treatment with oligomeric preparations (Fig. 7A). Furthermore, the effect of conditioned media from these cultures treated with Aβ3(pE)-42 can be rescued by prior depletion with TNFα neutralizing antibody (Fig. 7B-E) as well as with a TNFR1 antagonist (Fig. EV4). Finally, we could show that Aβ3(pE)-42 activates not only astrocytes but also microglia in hippocampal organotypic slices. These data, together with the previously mentioned reports, indicate that both astrocytes and microglia contribute to neuroinflammation at the onset of AD. The study how Aβ3(pE)-42 contributes to microglia activation and the consequences of the activation is beyond the scope of the manuscript. We have now included a paragraph clarifying this issue in the Discussion section of the manuscript.

5. The major discovery of this study is the role of Ab3(pE)-42, unlike Ab1-42, on induction of TNFa in astrocytes associated with synaptic dysfunction. The authors should carefully examine the effect of Ab3(pE)-42 on astrocytes and microglia. They can implement several widely used today approaches to pharmacologically or genetically deplete microglia in primary cells or hippocampal

cultures to address whether by Ab3(pE)-42-induced synaptic dysfunctions is mediated by astrocytes or microglia or both. Moreover, the authors can specifically deplete TNFa in microglia using recently described technique (CX3CR1(cre) /TNFα(fl/fl): PMID: 27145902) or use global TNFa-KO mice donors for primary culture or OHSCs.

Reply: As outlined above there is a major misunderstanding. **We don't believe that the effect is specific for astrocytes (and have not stated this in the previous version of the manuscript) and we have included experimental evidence that also microglia is involved in the effects of Aβ3(pE)-42.** The main scope of the manuscript is to compare the differential signaling pathways evoked by Aβ1-42 and Aβ3(pE)-42 and we agree that the interplay between microglia and astrocytes is an interesting topic. However, the detailed investigation of the microglia, astrocytic, and neuronal interactions in Aβ1-42- or Aβ3(pE)-42-induced pathology is not within the scope of the manuscript and does not add much to the story at the present stage. The important finding is Aβ3(pE)-42-induced synaptic pathology relies on glial release of TNF-alpha. We think that we could answer the question of the reviewer raised and emphasize now in the Discussion that Ab3(pE)-42-induced synaptic dysfunctions is likely mediated by both astrocytes and microglia.

6. The authors used anti-TNFa neutralizing antibody. The authors did not show the specificity and efficacy of these antibodies i.e. competition assay.

Reply: The utilized anti-TNFα antibody was characterized previously in other reports (Colston et al., 2007, PMID: 17616748; Nadeau and Rivest, 2003, PMID: 12843254). In addition we have included below here the results of a competition assay requested by this reviewer. To further support the antibody-based experiments we have performed a second set of experiments using a TNFR1 peptide antagonist blocking the interaction site between TNFα and TNFR1 (Bachem, N-1685; Takasaki et al., 1997; DOI:10.1038/nbt1197-1266; Xu et al., 2015 DOI: 10.1159/000430823). Like the neutralizing antibody this inhibitor also prevented Ab3(pE)-42- but not Aβ1-42 induced CREB shut-off. These data are now included in EV4 in the revised version of the manuscript.

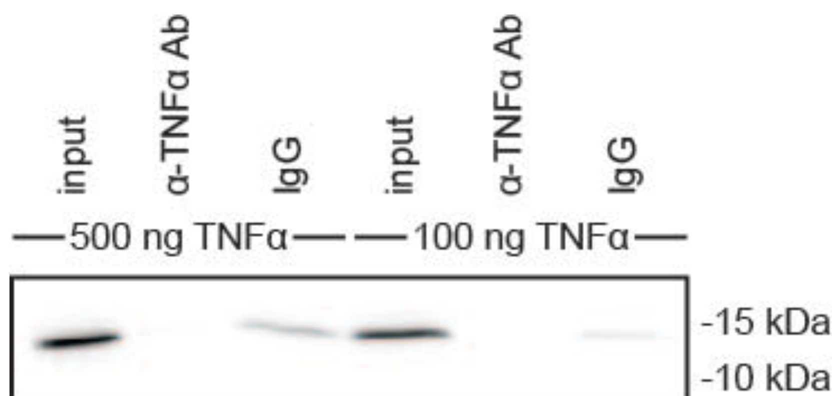

Figure legend: 500 or 100 ng of TNFα was diluted in TBS buffer with protease inhibitor. Part of the sample was kept as an input and the rest was split in 2 and 0,4 µg/ml of neutralizing antibody or IgG was added to samples and incubated over night in 4°C. Next, the previously blocked protein G Dyneabeads (Invitrogen, Cat. No. 10004D) were added and after the pull down the supernatants were diluted with sample buffer and visualized by SDS-PAGE. For immunodetection ms anti-rTNFα antibody was used (R&D, Cat. No. MAB510).

<u>*Minor comments:*</u>

1. Results section should describe the results in the figures, other references should be for the discussion section

We have used the references exclusively to introduce the rationale of the experiments, not to discuss the results. We feel that it is easier for the reader to follow for the experimental workflow and would therefore suggest to this reviewer to keep the references in the Reference section.

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2. Methods: Authors should state n numbers, sex and ages in the methods section to clarify for readers.

We have followed the instructions for authors of the Journal and included this information in the Figure legends. Furthermore, the sex and ages of mice used for LTP experiments were already included in materials and methods section .

3. Figure 1: Legend is mismatch for I and K letters of the correspondent panels.

4. Figure 2: This figure needs to be re-organised. Change location of E,F to G,H to reflect it in the result section. Legend needs to be re-organised: instead listing A, C, E, and G, describe each panel in consecutive order. Authors should take care when describing the figure legends so as it corresponds to what is in the figure.

5. Avert to use "stark contrast", just say "in contrast".

6. Authors should carefully scan text for grammatical and spelling errors. There are several mistakes within the manuscript.

Reply to minor comments 3-6: We have corrected the indicated mistakes and provided the missing information.

Referee #2:

In the manuscript entitled "Posttranslational modification impact on the mechanism by which amyloid-β induces synaptic dysfunction" investigate the molecular mechanisms involved in the synaptic dysfunction. For this purpose they studied multiples parameters of synaptic activity exposed to the same concentration of oligomers of Human Aβ 1-42 and Aβ 3(pE)-42. Overall, they described that if both species triggered synaptic dysfunction, they exert their synaptotoxic effect through a completely different molecular mechanisms. These results are interesting and raise questions that should be addressed:

Page 6 result section: How long the cultures have been exposed to the different oligomers species to induce synaptic loss? (It seems to be 3 days as mentioned in the method section). This point should be mentioned clearly in the result section. Is the difference in synapse loss observed after treatment with the different species of oligomers really drastically different (as mentioned in the text) since the difference (in fig 1) between these two conditions seems to be rather small?

Reply: The cultures were exposed to the oligomers for 3 days. This information is now included in the results section as well. The p-value by one-way ANOVA between Aβ1-42- and Aβ3(pE)-42-treated group is 0.0617. We have removed the sentence including 'drastically'.

Is the drastic reduction in synapse density reversed by TNF-α antibody or mimicked by TNF-α? These points should be clarified:

Reply: Yes the reduction in synapse-density was prevented by the TNFα neutralizing antibody which was co-applied together with Aβ1-42 or Aβ3(pE)-42 (Fig. 8). Furthermore, we now performed several experiments demonstrating that application of TNFα causes reductions in synapse density like Aβ3(pE)-42 (EV5A,B). Furthermore, the anti-TNFα antibody was used at a concentration that does not change the number of synapses (Fig. 8A,B), the levels of pCREB (Fig. 8C,D), and LTP (Fig. 9B, C). In conjunction with the results of novel experiments using a TNFR1 peptide antagonist (EV4 A,B) these data demonstrate that Aβ3(pE)-42 induced neuronal dysfunction essentially requires TNFα signaling in neurons

Figure 1: The Scholl analysis reveals an important reduction in dendritic complexity induced by both Aβ 1-42 and Aβ 3(pE)-42. How do the authors explain this effect? Does the same common effector P38 trigger this effect? Is it mimicked by TNF-α? This is a very drastic effect that may have an important role in the physiopathology of both oligomeric species.

Reply: A new set of experiments indicates that the effect is indeed mimicked by TNF-alpha (EV5C). A rescue experiment with a p38 inhibitor rescued both phenotypes, dendrite retraction and synapse loss after the treatment with TNFα, Aβ1-42 or Aβ3(pE)-42 (EV5 and Fig. S4A-C).

Page 10: The decrease in spontaneous neuronal network activity is obtained after a 40 min exposure to both oligomeric species and retraction of synaptic contact induced by Aβ 1-42 are observed the same time frame (3 days). Is these two important synaptotoxic features related? is the dendritic retraction observable is reversed by Ifenprodyl incubation?

Reply: We have clarified this in the revised version of the manuscript. The Ca2+ imaging was done 3 days after application. We indeed believe that synaptic retraction might precede dendritic retraction and we indicate this now more clearly in the revised version of the manuscript. We have no conclusive data whether also the dendrite retraction is reversed by ifenprodil.

To further validate that these different species of oligomers are disrupting synaptic function by both activating P38, it will be necessary to show a western blot with the activation pattern of P38 at the key time points of the experiments presented (i.e. 40 min and 3 days)? This would be a great additional figure to further validate P38 as a nodal signaling pathway for both oligomers species.

Reply: We have included in the revised version of the manuscript a western blot showing that administration of both Aβ species in cortical primary neurons induces enhanced pP38 levels (Fig. S4F-I).

In the final scheme that summarizes the observations presented in the paper, the authors added phosphorylated tau in the scheme. Do they have specific information on the status of tau after exposure to Aβ 1-42 and Aβ 3(pE)-42?

Reply: We have included phosphorylated Tau in the scheme by reference to previously published work (e.g. Ittner et al., 2010, PMID:20655099). But we have not investigated phospho-Tau in the present study. To prevent misunderstandings we have removed Tau from the scheme in the revised version of the manuscript.

Referee #3:

While there is widespread, but less than universal agreement that amyloid-β oligomers (AβOs) are potently toxic agents in Alzheimer's disease (AD), relatively scant attention has been paid to the diversity of Aβ species, and the homotypic and heterotypic oligomers that they may form. The present study provides compelling evidence that oligomers made from Aβ1-42 and AβpE3-42 harm neurons by distinct mechanisms. Most notably, in contrast to Aβ1-42 oligomers, Aβ3(pE)-42 oligomers induce early neuronal dysfunction neither by activation of GluN2B-containing NMDA receptors nor by a PrP-dependent mechanism, and their toxicity is mediated by TNFα that is secreted by astrocytes following their apparent uptake of the pyroglutamylated AβOs.

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Further attention should be paid to the following significant issues as a prelude to publication.

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Reply: In the cited paper by Nussbaum et al, Nature 2012, authors observed significant cell death of neurons but not glia following an 12 h exposure to 500 nM Aβ3(pE)-42 as estimated by a calcein-AM assay. In addition, Nussbaum et al. reported visible degeneration and detachment of neurons. In our work, we have not noticed visible signs of neuronal degeneration even after 3 days of treatment with 500 nM Aβ3(pE)-42. In addition, we have performed a LDH release assay for the revised version of the manuscript to address this issue (see below the graph that we included for the reviewer) and also this experiment did not indicate any sign of neurodegeneration in organotypic slices and in primary cell cultures. It is likely that the discrepancies stem from the different culture

model used in the cited work. In our manuscript we have used primary, rat, hippocampal cultures that were kept in culture min. 17 days prior to treatment with oligomers. In contrast, Nussbaum et al., used murine, forebrain cultures that were kept in culture 7 to 8 days prior to oligomer treatment. The difference in age of culture as well as neuronal subtypes could contribute to the different effects observed. Thus, mouse primary neurons kept under these conditions might be more susceptible to Aβ3(pE)-42 toxicity.

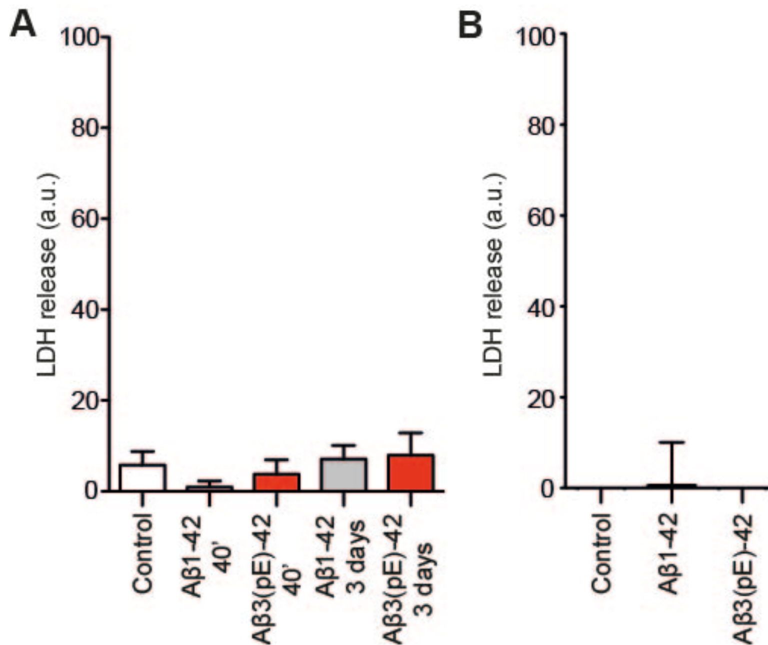

Figure legend: LDH release was monitored after application of Aβ3(pE)-42 or Aβ31-42 and at a concentration of 500 nM for 3 days in rat hippocampal primary neurons (A) or slices (B). The values were normalized to the positive control (lysed cells). The media come from 3 (A) or 2 (B) independent experiments where each group was done at least in duplicate.

2) Figure 5 is somewhat problematic. Most importantly, the claim that astrocytes take up oligomers made from AβpE3-42 or mixtures of AβpE3-42 and Aβ1-42 is not convincing. Although the claim is based on 3D reconstructions of confocal images, the Z-resolution in individual images is ~500 nm, which is likely very similar to the thickness of the astrocyte cytoplasm in the reconstructed images that were provided. In other words, it it possible that the oligomers in question were actually resting on top of the astrocytes, instead if having been internalized. Figure 5 would also be improved by showing original fluorescence micrographs of astrocytes labeled for AβpE3-42 and Aβ1-42.

Reply: In the original micrograph the z resolution was ~250 nm. However, we agree with the reviewer that it is indeed difficult to make this judgement since astrocytes are quite flat. We have included for this reviewer original fluorescence micrographs for this Figure at the end of the rebuttal (see below). For the revision we have also transfected astrocytes with a membrane marker and repeated the experiment (see Fig. 5E). The original fluorescence micrographs can be also found below. Collectively the data suggest that AβpE3-42 is indeed taken up by astrocytes.

Finally, several minor issues warrant further attention as well. 1) A related study from a different group showed that AβOs made from Aβ1-42 provoke microglia to secrete TNFα that then drives post-mitotic neurons back into the cell cycle, which leads them to die eventually (Bhaskar, et al. 2014. Neurobiology of Disease 62: 273-285). This paper should be acknowledged.

Reply: We have included the mentioned publication and we have also stressed more clearly in the discussion the microglia-related role in AβpE3-42 pathology. This is in line with published work (see for instance Alexandru et al., 2011).

2) On the top of page 6 the authors describe Thioflavin T as a reagent that detects amyloid fibrils. This statement is correct, but misleading. ThT actually detects β-sheet structure, irrespective of fibril formation.

Reply: We have corrected this statement.

3) Please include catalog numbers for antibodies and other biological reagents.

Reply: We have included the requested catalog numbers.

4) In figure 4, anti-PrP is mistakenly labeled as anti-PrnP.

Reply: We have corrected this mistake.

5) Fig S6 is mistakenly listed in the text on page 18 as Fig S8.

Reply: We have corrected this mistake.

6) I do not understand Fig 4A.

Reply: Figure 4A represents images from confocal microscope of HEK293T cells transfected with Prp-GFP or GPI-GFP constructs. The cells were treated for 40 min with oligomers, fixed and stained with antibodies detecting oligomers. We observed that there is a significant amount of Aβ1-42 on the surface of cells expressing Prp-GFP but not GPI-GFP. This could not be observed for Aβ3(pE)-42. In addition, we decided to visualize the outline of the cells (marked with a yellow line) based on GFP expression. We have tried to clarify this in the Figure legend.

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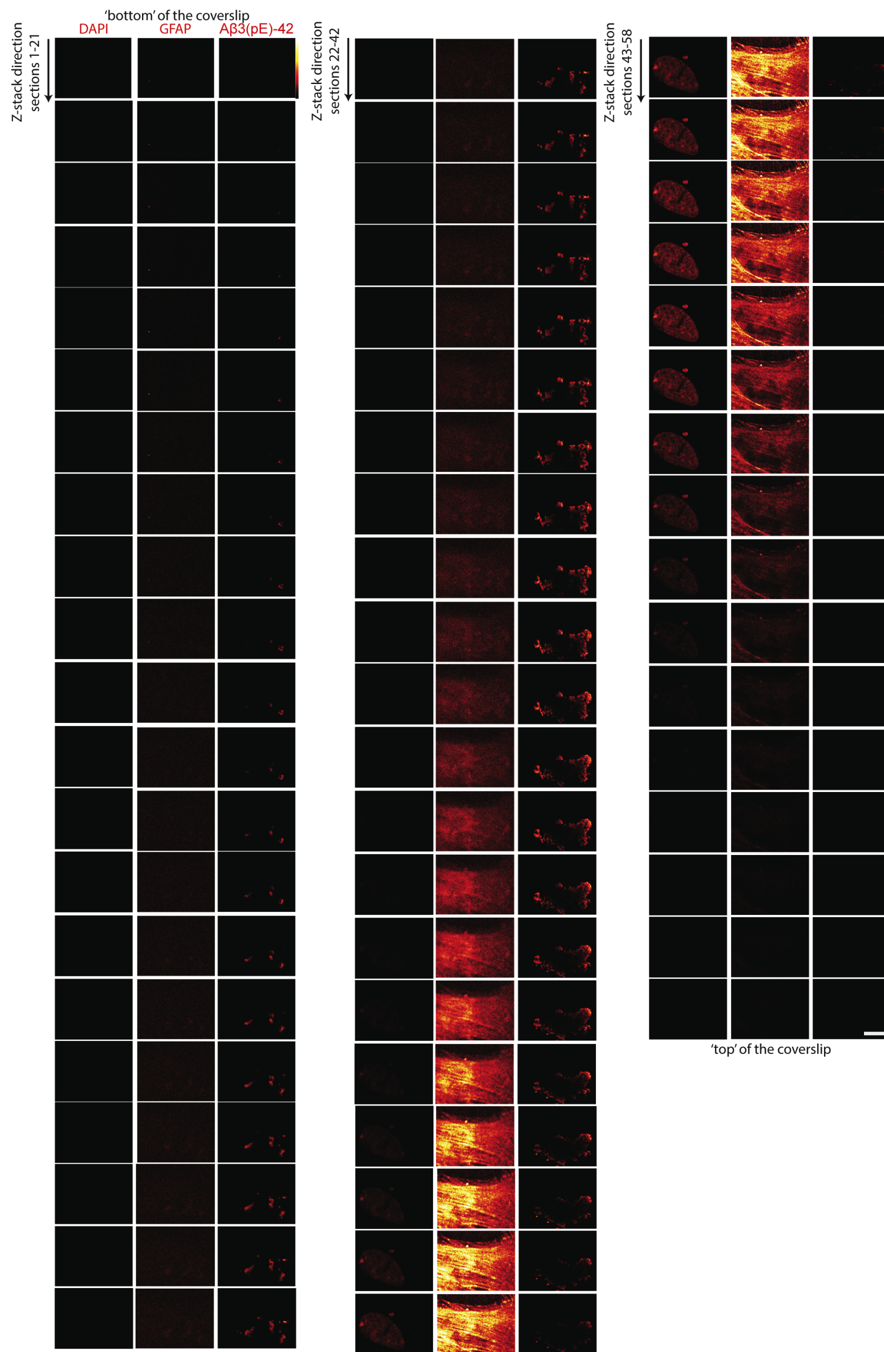
Fluorescence micrographs for Figure 5D



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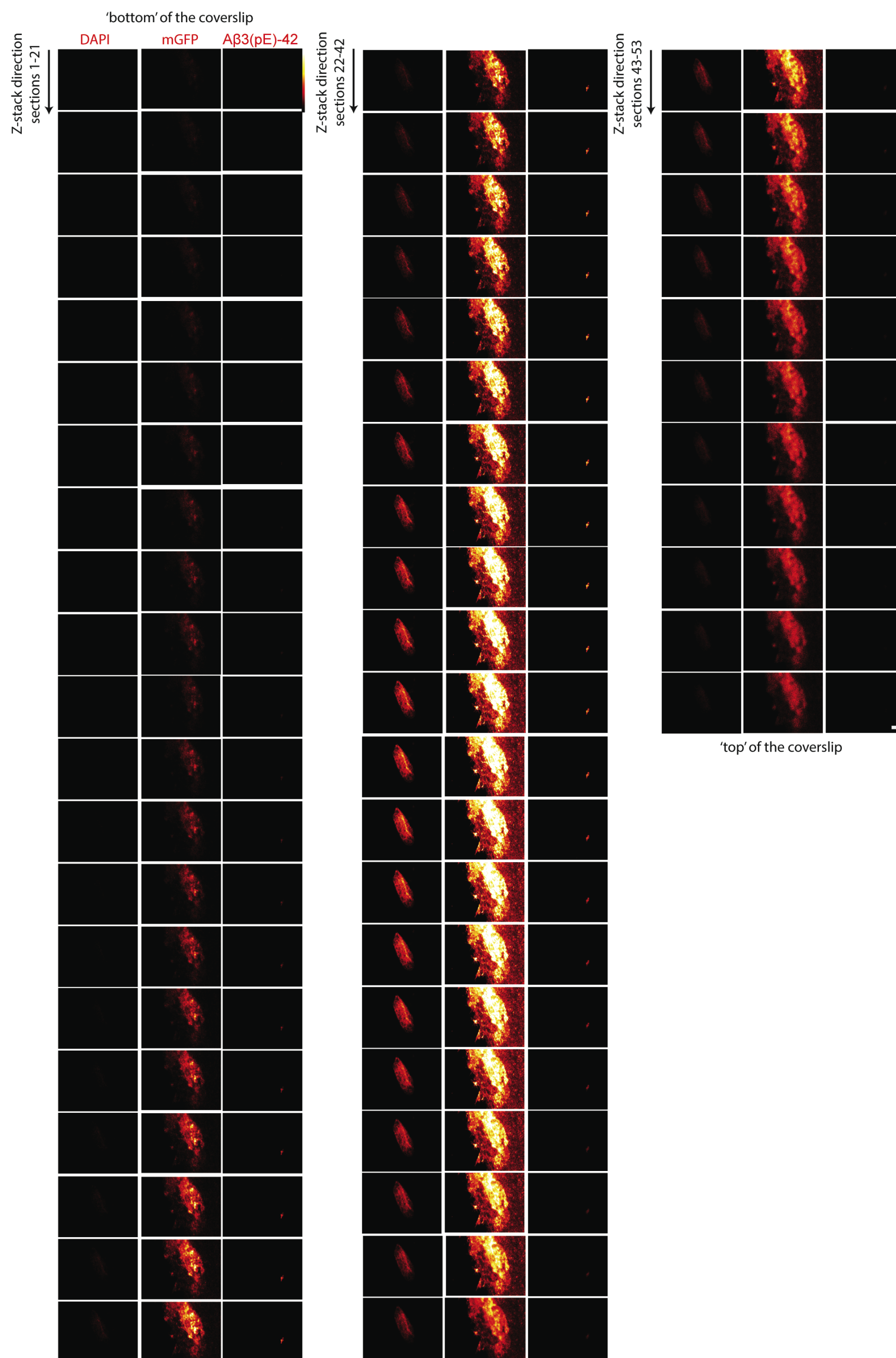
Fluorescence micrographs for figure 5D



2nd Editorial Decision 02 March 2017

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. Both still have a few more minor comments that I would like you to address before we can proceed with the official acceptance of your manuscript. A few other changes are also needed:

Please move figures S1-S5 with their legends to an Appendix file, and upload this as a single Appendix file.

Although the EMBO reports reference style is numbered, the current format in your manuscript has too many author names. The EMBO reports style is also part of EndNote. Please correct.

Figures 9 & 10 and EV figures 1,2,4 & 5 need to be changed to portrait format.
Figures 2 & 6 and S4 are a bit too big. All our figures need to fit on a single page, so these need to be modified or rearranged.

Please delete the open access statement and paper explained section from the manuscript file.

EMBO press papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-400 pixels large (the height is variable). You can either show a model or key data in the synopsis image. Please note that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

In the revised version, the authors addressed all reviewer's comments.
The manscript is written in a clear and logical way, and the authors used several interesting techniques to support and proof their data.

Minor comment to address:

Referee#1 question 3/ Figure EV3:
The authors describe an increase in Iba+1 cells, however as they state in the rebuttal letter they could not detect an increase in BrdU+ cells. This should be clarified.

Referee #3:

As reviewer #3 of the prior version of this paper, I am satisfied with the authors' responses to my suggestions for how to improve the manuscript, and I believe that this study will be of substantial interest to Alzheimer's disease researchers. In the course of reading the revised version, however, I did notice the following minor points that deserve further attention and can be adjudicated by the monitoring editor without the need for further input from reviewers.

1) In Fig. 1F , the gray (control) line is barely visible.

2) Similarly, in Fig. 3G, it is difficult to discriminate the clear triangles from the gray triangles.

3) There are numerous minor grammatical errors scattered throughout the text, especially extraneous indefinite articles and prepositions.

2nd Revision - authors' response 13 March 2017

Please find enclosed a revised version of manuscript EMBOR-2016-43519. We made all requested changes and hope that the manuscript is now acceptable for publication in EMBO Reports. Reply to Reviewers' comments:

Referee #1:

In the revised version, the authors addressed all reviewer's comments. The manscript is written in a clear and logical way, and the authors used several interesting techniques to support and proof their data.

Minor comment to address: Referee#1 question 3/ Figure EV3: The authors describe an increase in

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Iba+1 cells, however as they state in the rebuttal letter they could not detect an increase in BrdU+ cells. This should be clarified.

Reply: We state in the manuscript that the increase in IBA1 immunofluorescence might indicate microglia activation but not proliferation.

Referee #3:

As reviewer #3 of the prior version of this paper, I am satisfied with the authors' responses to my suggestions for how to improve the manuscript, and I believe that this study will be of substantial interest to Alzheimer's disease researchers. In the course of reading the revised version, however, I did notice the following minor points that deserve further attention and can be adjudicated by the monitoring editor without the need for further input from reviewers.

1) In Fig. 1F , the gray (control) line is barely visible.

Reply: We have adjusted the gray line.

2) Similarly, in Fig. 3G, it is difficult to discriminate the clear triangles from the gray triangles.

Reply: We have changed the triangles in Fig. 3G

3) There are numerous minor grammatical errors scattered throughout the text, especially extraneous indefinite articles and prepositions.

Reply: The manuscript has been cross-checked for grammatical errors.

3rd Editorial Decision 17 March 2017

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Michel R. Kreutz
Journal Submitted to:
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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).

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We chose a smaple size thatt allows for group comparison with the indicated parametric tests.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable
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.	No subjective assessment of data was made.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
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.	Samples were assigned randomly and treated equally. Experiments with treatment were performed without bias and run in parallel with different treatment conditions.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes.
Is there an estimate of variation within each group of data?	In many cases we have performed an analysis of variance (One or two-way ANOVA).
Is the variance similar between the groups that are being statistically compared?	Yes (see above)

C- Reagents

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).	The information is included in the manuscript
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cell lines (HEK-293T and COS7 cells) were ontained from commercial suppliers from commercial suppliers and are regularly tested for contamination.

* 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.	This information is included in the manuscript.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	N.A.
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.	We have followed these guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N.A.
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.	N.A.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N.A.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N.A.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N.A.
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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.	N.A.

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	N.A.
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).	N.A.
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).	N.A.
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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.	N.A.

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.	Not applicable