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Two structurally defined Aβ polymorphs promote different pathological changes in susceptible mice

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Dear Dr. Morales,

Thank you for submitting your manuscript for consideration by the EMBO Journal.

Given the referees' reports, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. It is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Thank you for providing an initial overview of your plan to address a few key referee concerns in our pre-discussion. Once you've come up with a plan to address all of the concerns raised by the reviewers, I would be happy to discuss this with you via zoom or email in the coming weeks.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Kelly M Anderson, PhD
Editor
The EMBO Journal
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Attached to this email is a guide for submitting a revised version and further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (9th Oct 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

General summary: The study investigates the biochemical properties and the patterns of spreading of different amyloid beta polymorphs (both synthetic and biologically-derived) in an Alzheimer's mouse model.

General comment: the findings are an interesting extension to the established concept of strains of abeta and other proteopathies. While this concept is now generally accepted, the novelty of their study surrounds the finding with the LCOs and regional differences in the hippocampus after inoculation.

Specific comments:
- A number of grammatical errors throughout the m.s. need correction

Because page #s were not given, I cannot reference these in my comments below:
- "Collected brains were separated in both hemispheres, keeping one frozen for biochemical analyses while the other was preserved for histopathological assessments (Supplementary Figure 3).": are the authors satisfied that both hemispheres exhibited pathology that was uniform enough that each half can be take for separate analyses and be confident that the source material showed the same pathology?
- "Histological analyses displayed deposition in the alveus and dentate gyrus of 2F treated mice, suggesting that accumulation in these areas occurs way before the 300 days old experimental endpoint.": what about the alveus and the dentate of 3F and old brain homogenate controls? We assume it was totally negative? State explicitly.
- "Interestingly, Aβ40 and Aβ42 deposits present in the brains of these mice generate different types of amyloid deposits": different in what way?
- "As expected, the alveus of 2F treated animals displayed a significantly higher increase of GFAP burden compared to all other groups (Figure 3K).": why "as expected"? please elaborate.
- "As expected, the alveus of 2F treated animals displayed a significantly higher increase of GFAP burden compared to all other groups (Figure 3K).": were there any patterns of association of microglia with the amyloid deposits?
- "Both, microglial and astroglial reactivity correlated positively with amyloid burden in the dentate gyrus for 3F fibrils, whereas Tg2576 seeds showed positive correlation with microgliosis and 2F with astroglial reaction.": so does 3f represent a mix of 2f and old mice in terms of their glial reactivity?
- "In addition, the glial response towards naturally versus induced aggregates is different, being more prominent in aged, not seeded subjects": this is an interesting observation. Do the authors have any data on whether this parallels toxic effects to surrounding neurons for example, or differential behavioral effects? Is the naturally-evolving amyloid in this strain more toxic that even the seeded amyloid, even from Tg2576 homogenate?
- The periventricular pathology is also interesting but not discussed: is this pathology spread from the brain parenchyma or via seeds leaking into the CSF? Similarly for leptomeningeal vascular amyloidosis observed with their Tg2576 inocula.
- The analysis of vascular amyloid was restricted to meningeal vessels as there was no

parenchymal cerebral amyloid. Was this well away from the injection tract to ensure this was not artifactual in some way?
- The authors discussed the proteolytic resistance of the 2F and 3F synthetic fibrils, is a similar pattern seen with aggregates derived from the mice?

Referee #2:

In this manuscript, Gomez-Gutierrez and colleagues characterize a set of synthetic Abeta conformers - 2F and 3F - using in vitro and in vivo techniques. They show that 2F and 3F have different biochemical properties (protease digestion, thiophene binding and PMCA amplification). They further injected these fibrils into young Tg2576 mice and show that while brain homogenates from Tg2576 induce robust amyloid deposits, 2F preferentially induces ThioS deposits in CA1 and dentate gyrus. Interesting variations in Iba-1 and GFAP reactivity in alveus and CA1 (but not entire hippocampus) demonstrate the differential outcomes of s2F and 3 seeding in Tg2576 mice. Finally, ssNMR studies using amplified brain materials from these seeded mice show that 2F and 3F induced prions incorporate different species of Abeta. Overall, however, this is an interesting set of data but several questions remain.

1) Information is lacking on what the 2F and 3F conformers are - among the provided references is Tycko 2014 (Protein Sci) which does not specifically mention this nomenclature. Please clarify.
Given that these Abeta conformers have been characterized in earlier publications by the Tycko lab, the novelty of the data presented in this manuscript is in question.

2) The authors mention that 'Our findings provide relevant information on the pathological significance of misfolded Aβ strains in AD'. However, no information has been shown how these 2F and 2F or their in vivo propagons match up with AD-derived Abeta fibrils. Since 2F and 3F are completely synthetic, this statement (and similar ones) is over-reaching. Indeed, the authors also say 'even though molecular structures of synthetic Aβ seeds do not propagate faithfully in mouse brains...'. This brings into question the relevance of this study vis a vis the potential prion properties of human (or even mouse) brain-resident Abeta. Overall, the relevance of this data to AD-derived Abeta is lacking.

3) The authors state that 'An important contribution of this work is to describe the pathological significance of strain-specific replication of Aβ misfolding in vivo.' One of the most important features that define the pathological state of Abeta is whether one strain causes AD (dementia) and others do not. It would be interesting to show whether such pathological differences (codified as learning/memory deficits) exist in 2F vs 3F-seeded Tg2576 mice.

4) The authors state that they provide data towards 2F and 3F's property of differential 'tropism to specific brain regions' - transmission characteristics of these Abeta species away from the area of injection (hippocampus) has not been provided.

5) Some specific concerns about data are summarized below
- in Fig. 1, when authors describe the LCO binding characteristics of 2F and 3F, could they provide the LCO binding characteristics of Abeta from AD and healthy controls to show whether 2F and 3F have any morphological similarities with human physiological Abeta?
- in Fig. 2C, 4G8 and ThioS staining in 2F injected mice shows linear distribution of staining. These do not resemble Abeta deposits (as in Old Tg2576 BH panel, B). Would this staining be intracellular Abeta and/or CTFs inside CA1? Or is this staining in the alveus (see similar staining in GFAP and Iba-1 panels in Fig. 3-5) If so, could this be the result of needle point injury? Have the authors stained for Abeta in 2F or 3F injected APP nontransgenic mice?
- in Fig 4A, data from b2 vs b6 is not consistent with data in Fig. 3K (where the GFAP burden is very similar between old Tg2576 BH and monomeric Abeta40)
- in Fig 5B, the intense red staining (Iba-1) in b6 and b14 does not resemble typical microglial staining. Please clarify.
- in Fig. 6, the ssNMR is performed on the brain derived fibrils, but on brain materials amplified subsequently in vitro. Could this additional in vitro amplification step lead to preferential selection and alter the relative predominance of specific prion folds? How would the ssNMR of AD-derived brain materials amplified using this identical protocol look with respect to data from 2F and 3F?

UTHealth™
The University of Texas
Health Science Center at Houston

Medical School
Mitchell Center for Alzheimer's Disease
and Related Brain Disorders
Department of Neurology

Houston, October 10th, 2022

Dr. Kelly M. Anderson,
Editor
EMBO Journal

Dear Dr. Anderson,

Many thanks for your message with the reviewers' comments for our manuscript entitled "**Structure-defined Aβ polymorphs promote different pathological changes in susceptible mice**". We are very glad that reviewers liked the article and we made several modifications to it following their suggestions. We believe that their constructive comments helped to make this article much stronger. Following is a point-by-point response to the reviewers' comments.

Reviewer #1

1) General comment: the findings are an interesting extension to the established concept of strains of abeta and other proteopathies. While this concept is now generally accepted, the novelty of their study surrounds the finding with the LCOs and regional differences in the hippocampus after inoculation.
Answer: We are glad that the reviewer found novelty in our study. We would like to emphasize that in addition to differential LCOs reactivity and brain tropisms, our data highlight the pathological relevance of different Aβ strains in the brain. The latter is emphasized in the revised version of this manuscript.

2) A number of grammatical errors throughout the m.s. need correction.
Answer: We appreciate this comment. We have revised the manuscript and fixed these errors.

3) "Collected brains were separated in both hemispheres, keeping one frozen for biochemical analyses while the other was preserved for histopathological assessments (Supplementary Figure 3).": are the authors satisfied that both hemispheres exhibited pathology that was uniform enough that each half can be take for separate analyses and be confident that the source material showed the same pathology?
Answer: We understand the reviewer's concern. However, each animal was bilaterally injected with Aβ seeds with the same volume of injectate. Our previous experience with *in vivo* seeding studies in transgenic mice injected with amyloid-containing brain homogenates, was that bilateral injection produces similar pathology in both hemispheres. Considering this, we are confident that pathological induction by seeding was equivalent in both brain hemispheres in this study. This information was previously provided in the "Materials and Methods" section.

4) "Histological analyses displayed deposition in the alveus and dentate gyrus of 2F treated mice, suggesting that accumulation in these areas occurs way before the 300 days old experimental endpoint.": what about the alveus and the dentate of 3F and old brain homogenate controls? We assume it was totally negative? State explicitly.

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Answer: This point is addressed in Supplementary Figure 5 showing that the large majority of the animals injected with 3F do not show pathology in alveus, except for one animal where we observed some thioflavin S staining. Control animals injected with the Tg2576 brain extract did not show any pathology in alveus, expect for one animal displaying diffuse Aβ deposition. This information is now explicitly stated in the manuscript. We can add a Supplementary Figure displaying these outliers in case the Reviewer believe it is necessary.

5) "Interestingly, Aβ40 and Aβ42 deposits present in the brains of these mice generate different types of amyloid deposits": different in what way?
Answer: As mentioned in the text, the aggregates generated by the 2F, 3F and Old Tg2576 brain extracts exhibited different proportions of Aβ40 and Aβ42. This is clearly appreciated in Supplementary Figure 8. In addition, these aggregates are morphologically different: deposits induced by the Old Tg2576 brain homogenate were diffuse and ThS negative, while plaques produced by injection of 2F and 3F aggregates were mostly ThS positive. The main morphological difference between 2F and 3F aggregates were observed at the dentate gyrus, where 3F seeds induced larger aggregates. In addition, these aggregates reacted differently to several luminescent conjugated oligothiophenes (LCOs). These differences are now emphasized in a comparative table (Table 1) to best guide the reader on these differences.

6) "As expected, the alveus of 2F treated animals displayed a significantly higher increase of GFAP burden compared to all other groups (Figure 3K).": why "as expected"? please elaborate.
Answer: We understand the reviewers concern. The words “are expected” are linked to the higher amyloid burden observed in this brain region for the 2F treated mice. However, other brain regions, such as the dentate gyrus, did not display the same pattern. Along this line, we have rephrased this sentence by stating “The alveus of 2F treated mice displayed a significantly increase of GFAP signal compared to other groups (Figure 3K), fact that is likely induced by the specific Aβ aggregates being deposited”.

7) "As expected, the alveus of 2F treated animals displayed a significantly higher increase of GFAP burden compared to all other groups (Figure 3K).": were there any patterns of association of microglia with the amyloid deposits?
Answer: Patterns of association with microglia were extensively studied. They are observed in Figure 3, panels E-F and L-N, Figure 5, and in Supplemental Figure 8. In summary, we observed that the alveus region in 2F treated animals contains increased Iba-1(the microglial marker used in this study) signals compared to other groups. In the dentate gyrus, an increase in Iba-1 burden was observed for 3F treated mice. A closer look to the dentate gyrus suggests that the large plaques induced by 3F in this brain region are responsible for this microglia-specific response. This information was already presented and discussed in the manuscript.

8) "Both, microglial and astroglial reactivity correlated positively with amyloid burden in the dentate gyrus for 3F fibrils, whereas Tg2576 seeds showed positive correlation with microgliosis and 2F with astroglial reaction.": so does 3f represent a mix of 2f and old mice in terms of their glial reactivity?
Answer: Not necessarily. We believe that more than a mix, 3F-seeded aggregates represents a unique population of misfolded proteins with specific properties. This is further suggested by the pathological, structural, and biochemical data presented across the text. This statement has now been added into the revised version of this manuscript.

9) "In addition, the glial response towards naturally versus induced aggregates is different, being more prominent in aged, not seeded subjects": this is an interesting observation. Do the authors have any data on whether this parallels toxic effects to surrounding neurons for example, or

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differential behavioral effects? Is the naturally-evolving amyloid in this strain more toxic that even the seeded amyloid, even from Tg2576 homogenate?
Answer: This is an excellent question that we are currently pursuing in the laboratory. Unfortunately, due to all characterizations we performed, we did not have tissue slices enough to visualize strain-specific damage in neurons or synapses. At present, we are repeating these experiments to specifically focus on the behavioral, synaptic and neurotoxic aspects of seeded pathology with these different materials. Our data shows that Aβ aggregates seeded by the Old Tg2576 brain induces diffuse amyloid plaques that are poorly inducers of glial responses (differently to what is observed for naturally occurring plaques in this animal model). A Supplementary Figure displaying this (Supplemental Figure 14), and one sentence in the text, are now included.

10) The periventricular pathology is also interesting but not discussed: is this pathology spread from the brain parenchyma or via seeds leaking into the CSF? Similarly for leptomeningeal vascular amyloidosis observed with their Tg2576 inocula.
Answer: The periventricular pathology in 2F injected animals, and the leptomeningeal amyloid deposition observed in mice treated with the Old Tg2576 brain extracts, are unlikely to be induced by leaking of the injected seeds due to different reasons. The first one is that the induced pathology among the different groups displayed distinctively reproducible characteristics. As pointed by the reviewer, these pathological traits are interesting, as they support our claim that each Aβ strain induce specific pathological features. At this time, we do not know the origin and mechanisms for the deposition of aggregates in periventricular areas or in leptomeningeal vascular lesions. We have included additional discussion on this matter as suggested by the reviewer.

11) The analysis of vascular amyloid was restricted to meningeal vessels as there was no parenchymal cerebral amyloid. Was this well away from the injection tract to ensure this was not artifactual in some way?
Answer: We understand the reviewer's concern. As described in the text, vascular amyloid in meningeal vessels was only observed with one injectate (Old Tg2576 BH). The injection was far from this area, thus we do not believe this is an artifactual result. In addition, this finding is supported by independent experiments listed in our previous publications (using Tg2576 brain extracts from different mice), as well as publications from other groups. We have added few sentences to justify the specificity of this results in the revised version.

12) The authors discussed the proteolytic resistance of the 2F and 3F synthetic fibrils, is a similar pattern seen with aggregates derived from the mice?
Answer: Unfortunately, we were unable to perform experiments of proteolytic resistance with aggregates derived from injected mice, as most of the brain extracts generated in these experiments were used in the structural characterizations (EM and ssNMR) listed in Figure 6, and Supplementary Figure 13. These techniques require high quantities of brain extracts, and we judged that information at this level was more relevant when inquiring our hypothesis. However, it is important to mention that similarities between parental seeds and seeded aggregates were observed when both entities were stained with different LCOs (Supplementary Figure 9), suggesting common (but not identical) structural patterns. We agree with the reviewer's concern, and we are planning to perform additional biochemical studies in a new experiment (that will be focused on behavioral, electrophysiological, synaptic and toxic properties of seeded aggregates, as mentioned above). We plan to communicate these findings in the near future.

We would also like to note that some volume of the brain extracts was also used for biochemical characterizations of 23 cytokines and chemokines (Supplementary Figure 11). Particularly, the PK resistance assays use a substantial amount of brain material. This is explained below:
5 PK concentrations x 50 µL per assay x 3 replicates = 750 µL of material per brain extract (10% w/v)

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Each brain hemisphere provides approximately 2 mL of 10% w/v brain extract. If all works well, the PK assay consume around 50% of the material generated. If the experiment is focused to the hippocampus, the volume to be obtained will be considerably lower.

Reviewer #2

1) Information is lacking on what the 2F and 3F conformers are - among the provided references is Tycko 2014 (Protein Sci) which does not specifically mention this nomenclature. Please clarify. Given that these Abeta conformers have been characterized in earlier publications by the Tycko lab, the novelty of the data presented in this manuscript is in question.

Answer: The 2F and 3F conformers are two distinct Aβ40 fibril polymorphs, prepared *in vitro* using somewhat different fibril growth conditions (quiescent growth for 3F, gentle agitation during growth for 2F). The preparation and characterization of these fibrils has been previously described (Petkova *et al*., 2005) although the "2F, 3F" nomenclature was not used at that moment. Previous work from the Tycko lab shows that these polymorphs exhibit different morphologies in TEM images and different 13C and 15N chemical shifts in ssNMR spectra. Thus, they contain different molecular structures. Structural models based on ssNMR data were deposited in the Protein Data Bank in 2011, namely PDB files 2LMP and 2LMQ for the 3F fibrils, PDB files 2LMN and 2LMO for the 2F fibrils. For clarification, all this information is now added into the manuscript.

The fact that molecular structures of these conformers were characterized previously does not detract from the significance of the current manuscript. Most importantly, the current manuscript shows that these two conformers have significantly different biological effects, which had not been studied previously. Additionally, data in the current manuscript show that the two conformers have different PK resistance profiles and different signatures in fluorescence measurements, which had not been reported previously and which confirms the structural differences revealed by ssNMR. Also, this is the first study in which differences in amyloid pathology induced by injection of fibrils with known differences in molecular structural properties are reported.

2) The authors mention that 'Our findings provide relevant information on the pathological significance of misfolded Aβ strains in AD'. However, no information has been shown how these 2F and 2F or their in vivo propagons match up with AD-derived Abeta fibrils. Since 2F and 3F are completely synthetic, this statement (and similar ones) is over-reaching. Indeed, the authors also say 'even though molecular structures of synthetic Aβ seeds do not propagate faithfully in mouse brains...'. This brings into question the relevance of this study vis a vis the potential prion properties of human (or even mouse) brain-resident Abeta. Overall, the relevance of this data to AD-derived Abeta is lacking.

Response: At present, it is unknown the number and properties of Aβ strains in AD brains. Compelling evidence suggest that there are, indeed, several strains in the same brain. The heterogeneity and the difficulties to separate and purify conformational strains from AD brains without altering their properties, makes very difficult to study them at a molecular detail. The use of synthetic, highly purified Aβ strains with well-defined structures provides the unique opportunity to study the biological behavior of single conformers *in vivo*. Even though we do not know whether these strains represent those naturally present in the diseased brain, we believe our data provide important information showing that structurally different aggregates behave differently upon propagation in the brain *in vivo*. Specifically, what our results show is that exogenous material that differs only in the details of the molecular structure of the *in vitro* fibrils can induce significantly different pathology. These observations strengthen the connection

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between molecular structural variations and differences in amyloid pathology. In the revised version, we will include this caveat and tone down the discussion of the putative biological relevance of our findings.

In regards to the not faithful propagation of 2F and 3F aggregates in Tg2576 brains, we believe that this is due to the contribution of the intrinsic tendency of these animals to generate a specific type of amyloid aggregates. In other words, the pathology induced in these mice is a contribution of both, the exogenous seeds and the spontaneous Aβ conformations generated in these mice. This and other possible explanations have been discussed in the manuscript, but are now further developed in the revised version.

3) The authors state that 'An important contribution of this work is to describe the pathological significance of strain-specific replication of Aβ misfolding in vivo.' One of the most important features that define the pathological state of Abeta is whether one strain causes AD (dementia) and others do not. It would be interesting to show whether such pathological differences (codified as learning/memory deficits) exist in 2F vs 3F-seeded Tg2576 mice.

Response: We completely agree with the reviewer in this point. Studying the relationship between structure/properties of strains and the clinical outcome will be very interesting. Limitations on mice models preclude a detailed study of whether different strains may lead to slightly distinct clinical signs. Unfortunately, we did not run behavioral tests on these mice (due to COVID-19 restrictions to lab access, among other reasons). We plan to follow up this study with experiments focusing on a detailed analysis of the behavioral/electrophysiological/synaptic changes produced by each strain. Considering the significant time and effort required for these experiments (that will take more than one year to be completed), in addition to the material needed per mice to perform multiple characterizations, results in these topics will be communicated in a future report. We believe that if we wait for these experiments to be completed, the novelty and impact of the results included in the current manuscript may importantly decrease.

4) The authors state that they provide data towards 2F and 3F's property of differential 'tropism to specific brain regions' - transmission characteristics of these Abeta species away from the area of injection (hippocampus) has not been provided.

Response: We carefully analyzed the whole brain of all mice included in this study. Similar as previously reported by us (Morales *et al*., 2021), diffuse amyloid deposition was found in brain regions other than the hippocampus for mice treated with the Old Tg2576 brain extract. Specifically, deposits for this group of mice were found in the cortex and caudate nucleus/putamen. Deposits for mice treated with 2F and 3F seeds were restricted to the hippocampus and the lateral ventricle (Supplementary Figure 5). This statement is now rephrased to emphasize that differences in aggregates seeded by the three injectates were mostly restricted to different structures of the hippocampus and the lateral ventricle. We also added a new Supplementary Figure (Supplementary Figure 4) displaying different brain regions analyzed in all experimental groups.

Some specific concerns about data are summarized below.

6) in Fig. 1, when authors describe the LCO binding characteristics of 2F and 3F, could they provide the LCO binding characteristics of Abeta from AD and healthy controls to show whether 2F and 3F have any morphological similarities with human physiological Abeta?

Answer: We explored the two most relevant LCOs (HS-194 and HS-68) in two human brains: one from an AD patient and one from a non-demented control harboring amyloid pathology. There, we observed reactivity similar to what was observed in experimental mice. As pointed by the reviewer, this data suggests that seeded aggregates in rodents resemble the amyloid deposits present in patients. However, and as displayed in the new Supplementary Figure 15, aggregates in human subjects are heterogenous,

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and differentially bind to these dyes. Specifically, while some parenchymal deposits may be recognized by either one, or both LCOs, others appears to be recognized by a single dye. In addition, the proportion of LCO binding appears to be different, depending in the type (vascular/parenchymal) and morphology of the aggregates. Interestingly, the pattern of LCO binding was different for amyloid deposits in the AD and non-demented brains, suggesting that these clinically diverse cases accumulate different proportions of Aβ strains. Whether the biological properties of aggregates and LCOs reactivity are equivalent in humans and experimental rodents will be investigated in future studies.

7) in Fig. 2C, 4G8 and ThioS staining in 2F injected mice shows linear distribution of staining. These do not resemble Abeta deposits (as in Old Tg2576 BH panel, B). Would this staining be intracellular Abeta and/or CTFs inside CA1? Or is this staining in the alveus (see similar staining in GFAP and Iba-1 panels in Fig. 3-5) If so, could this be the result of needle point injury? Have the authors stained for Abeta in 2F or 3F injected APP nontransgenic mice?

Answer: Unfortunately, we did not inject 2F or 3F fibrils in wild type mice as they are not likely to propagate Aβ misfolding (as human Aβ is required for this to occur). However, we do not believe that accumulation in the alveus is due to the needle track for several reasons, including: i) accumulation in the alveus was specific for 2F treated mice; ii) mice sacrificed at earlier time points (Supplementary Figure 7) do not show this pattern of aggregation; and iii) the needle track lesion looks quite different to the deposition observed in the alveus. We now provide a new Supplementary Figure (Supplementary Figure 5) depicting the long-term lesion generated by the needle track after injecting an Old Tg2576 brain extract in younger mice. As appreciated in the figure, the injury and amyloid deposition associated to the needle track runs horizontally from the cortex to the hippocampus. In addition, it is important to consider that plaques seeded by the Old Tg2576 brain extract are different to the ones naturally observed in this transgenic mouse model at advanced age. This is also depicted in the newly added Supplementary Figure 5 and Supplementary Figure 7.

8) in Fig 4A, data from b2 vs b6 is not consistent with data in Fig. 3K (where the GFAP burden is very similar between old Tg2576 BH and monomeric Abeta40).

Answer: We thank the reviewer for this observation. We have examined all pictures again and confirmed our quantifications. After confirming our previous observations, we replaced the panels for images more representative of the quantification values.

9) in Fig 5B, the intense red staining (Iba-1) in b6 and b14 does not resemble typical microglial staining. Please clarify.

Answer: We apologize for this oversight. We have replaced the panel with a picture not displaying the unspecific staining previously shown.

10) in Fig. 6, the ssNMR is performed on the brain derived fibrils, but on brain materials amplified subsequently in vitro. Could this additional in vitro amplification step lead to preferential selection and alter the relative predominance of specific prion folds? How would the ssNMR of AD-derived brain materials amplified using this identical protocol look with respect to data from 2F and 3F?

Answer: In order to perform ssNMR, isotope-labeled amino acids have to be used and therefore, it is not possible to obtain the structure of the aggregates present in the brain without *in vitro* amplification. As already stated above, Supplementary Figure 13B does compare ssNMR signals for the "*in vivo* propagons" (cross peaks shown as red and blue contours) with signals from fibrils derived from AD brain tissue (green X's). The signals are clearly different. In this comparison, the same protocols were used for AD brain-derived fibrils and Tg2756 brain-derived fibrils, except that experiments with AD brain tissue used cortical tissue homogenates, rather than whole brain homogenates.

The fact that different ssNMR results were obtained with human brain tissue and mouse brain tissue (and different results were obtained with human brain tissue from rapidly-progressing AD cases vs.

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typical long-duration AD cases, as reported by Qiang *et al*., Nature 2017) supports the idea that *in vitro* amplification does not preferentially select a single polymorph (or strain or conformer). It is possible that *in vitro* amplification alters the relative populations of different polymorphs to some extent, but we have designed our amplification protocol to minimize this effect. In particular, we confirm by TEM that abundant long fibrils develop within 4 hours after the initial seeding step, and we avoid multiple rounds of seeded fibril growth in our protocol for preparing solid state NMR samples (see Materials and Methods). This information is now added to the text for clarification.

We are really grateful for the useful comments. In summary, we carefully considered the reviewers' suggestions and addressed their comments, we improved several figures, we modified the text, and added 1 Table and 4 additional Supplementary Figures that further support our conclusions. We believe that all suggestions and comments have made this study much stronger.

I look forward to hear from you.

Sincerely,

Rodrigo Morales, PhD
Associate Professor
Department of Neurology
The University of Texas Medical School at Houston

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Dear Dr. Morales,

Thank you for submitting your manuscript for consideration by the EMBO Journal.

I've shared your plan to address the remaining concerns of Referee 2 and unfortunately they are not convinced this would address the concern that relevance would be demonstrated by these additional experiments. We've also consulted with an expert advisor who agrees with the conclusions of Referee 2 in this case. Given these opinions and the fact that the EMBO Journal can only afford to accept papers which receive enthusiastic support from a majority of referees, I am afraid we can not offer to publish it here.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Kelly M Anderson, PhD
Editor
The EMBO Journal
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Referee #1:

The authors have done a very good job addressing my previous major criticisms. While the concept of abeta strains is now established, I believe their extensive and well-executed dataset provides important additional information on this very important aspect of abeta biology. Other than some errors related to English grammar scattered throughout the revised manuscript, it is now suitable for publication.

Referee #2:

While the data seta are technically sound and the authors have responded to the previous critiques, some major concerns remain as to

1) novelty of the findings as these structural isomorphs have been characterized in multiple earlier published reports
2) functional or biological changes (related to pathology in disease) have not been generated as requested (memory or behavior changes to support the seeding observations; OR, maybe transcriptomic or proteomic changes to support the glial changes). This is critical as it is

stated that these structural isomorphs give the readers biological insights into the disease pathogenesis.
3) biological significance of these synthetic artificial structural polymorphs in Abeta seeding. Given that multiple studies have been already reported on the prion properties of AD and ADRD derived brain lysates (or purified protein materials), this remains a big concern. While biochemical studies such as this current study are important, the esoteric nature of synthetic constructs precludes interpretations of pathogenicity as it occurs in disease. The authors were requested to provide some information on the similarities and differences between these synthetic constructs and brain-resident Abeta which was not provided: "Whether the biological properties of aggregates and LCOs reactivity are equivalent in humans and experimental rodents will be investigated in future studies"

Dear Rodrigo,

Thank you for transferring your revised manuscript for consideration by EMBO reports. I have now read it and discussed it with the other members of our editorial team. We have also taken into consideration the reports of the referees who have previously evaluated your study, your rebuttal letter, as well as informal advice from an expert who is familiar both with the field and with our journal and its scope.

Our conclusion is that we would be interested in publishing your study in EMBO reports if you textually addressed the remaining concerns of the referees in a revised version of the manuscript. In particular:
i. regarding the first concern of referee #2: please revise your abstract, introduction, and discussion sections to emphasize where exactly the novelty of this study lies (along the lines you suggest in your rebuttal letter) and to place the study accurately into the context of the relevant literature;
ii. regarding the second concern of referee #2: please revise your manuscript to clearly present the changes/differences that you have investigated in this study, and make sure that any claims of biological insights into the disease pathogenesis are fully supported by the presented results or toned down or removed, as necessary;
iii. regarding the third concern of referee #3: please interpret carefully your data regarding biological significance/relevance of your findings, taking the criticism of referee #2 (which was also pointed out by our advisor) on board; please remove any over-statements or not fully supported claims, and present fairly the limitations of the present study.
iv. please correct all grammar mistakes throughout the revised manuscript (raised by referee #1).

We would like to invite you to revise your manuscript as detailed above, with the understanding that acceptance of your manuscript will depend on the completeness of your responses to the requested changes in the next, final version of the manuscript. Please make sure that all changes are highlighted to be clearly visible. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

*****
IMPORTANT NOTE:
Your revised manuscript will FAIL the initial quality control and the handling will be DELAYED if the following APPLIES:

1) If a data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that (for more information, please see below).

2) If your manuscript contains statistics and error bars based on n=2. Please use scatter plots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.
*****

- The title should be short (up to 100 characters including spaces), informative, and accurate, and it should not contain any abbreviations.

- The abstract should be a single paragraph describing all key novel findings of the study, written in present tense, and it should not exceed 175 words.

- Please provide up to 5 keywords in your revised manuscript.

- Your Figure legends have been inspected by our data editors for completeness and accuracy. Please see the required changes in the attached Word file and address all comments in your revised manuscript (with tracked changes).

- Please note that a "Data availability" section at the end of Materials and Methods is mandatory. In case you have no data that require deposition in a public database, please state so instead of refereeing to the database: "Our study includes no data deposited in public repositories." under the heading "Data availability".
See also). Please note that the Data availability statement is restricted to new primary data that are part of this study.

- We request authors to consider both actual and perceived competing interests. Please review our policy () and update your competing interests statement. Please name this section 'Disclosure and competing interests statement' and place it after the Acknowledgements section. If you have no competing interests to declare, you can use the statement "The authors declare that they have no conflict of interest.".

- The author contributions statement should be removed from the manuscript file. Instead, we now use CRediT to specify the

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- You are kindly requested to note our reference format (you currently list more than 10 authors in your citations) and update the list of references accordingly:
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- We need a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF (please see below for more information).

- Please upload Individual production quality figure files as .eps, .tif, .jpg (one file per figure). You can download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

- Please make sure that all Figure panels are called out in your revised manuscript, and that the panel callouts are called out alphabetically. We noted that callouts for Fig. 2E and 3K are currently missing.

- Your supplementary materials should be replaced by an Appendix, which should be a single PDF file with a table of contents on its first page and your supplementary figures (together with their legends). Please rename these figures to "Appendix Figure S#) and update their callouts in the manuscript accordingly.

- Your "Scheme 1" and "Scheme 2" in the Materials and Methods should be included as separate main Figures and called out accordingly. Please make sure that all Figure callouts are corrected throughout the manuscript.

- Table 1 should either be included in the manuscript Word file or uploaded as an individual Word file. Please remove the colored text; if you wish the colored text to remain, the table must be uploaded as Table EV1 in a separate file.

- Please remove Supplementary Figure legends from the manuscript, they must be included in the Appendix (see above). Only Expanded View (EV) Figure legends may remain in the manuscript file.

- Please note that EMBO press papers are accompanied online by
A) a short (1-2 sentences) summary of the findings and their significance,
B) 2-4 short bullet points highlighting the key results, and
C) a synopsis image that is exactly 550 pixels wide and 200-600 pixels high (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 your revised manuscript.

Please also note that as part of the EMBO publications' Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a cover.

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

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

McGovern Medical School
Mitchell Center for Alzheimer's Disease
and Related Brain Disorders
Department of Neurology

Dr. Ioannis Papaioannou,
Editor
EMBO Reports Journal

Dear Dr. Papaioannou,

Many thanks for handling our manuscript entitled "**Structure-defined Aβ polymorphs promote different pathological changes in susceptible mice**".

Considering your previous letter, I am including a revised version of this manuscript. In this new version, we followed your advice by:

i) noting the novelty of our findings in the abstract, introduction and discussion.
ii) indicating the changes/differences described in this article and supported by the data (summarized in Table 1)
iii) revising the interpretation of our data, removing over-statements and not fully supported claims.
iv) correcting grammar mistakes.

The above-mentioned changes have been tracked for your reference.

Changes were not extensive, as many limitations of the current study and novel aspects of the same were already mentioned in the previous version. As suggested by you, we highlighted the limitations (by adding additional text) and removed over-statements as indicated above. I hope these changes are satisfactory for you and you fell that the manuscript is now appropriate for publication in EMBO Reports.

Thanks again for your suggestions. We now feel that the article is focused, stronger, and a better contribution to the field.

I look forward to hear from you.

Sincerely,

Rodrigo Morales, PhD
Associate Professor
Department of Neurology
The University of Texas Medical School at Houston

Dear Rodrigo,

Thank you for submitting your revised manuscript for consideration by EMBO reports. I am glad to say that you have sufficiently addressed the previous referee concerns by appropriate textual changes, and we can therefore proceed with processing of your manuscript.

From the editorial side, there are a few remaining things that we need from you before acceptance of the manuscript for publication:

- The panels of Figure 3 are not called out alphabetically in your manuscript. Please make sure that all panels are called out in alphabetical order in your revised manuscript (please use change tracking in Word).

- We would like to ask you to remove any numbering from the scale bars in the figures (Figure 1A and Appendix Figure S13A). The scales should be added to the Figure legends instead.

- Please consider improving your synopsis image by adding necessary annotation or a graphical summary of your main findings. The image should be exactly 550 pixels wide and 300-600 pixels high. Please note that the text needs to be readable at the final size and not distorted.

- We now request publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

- I took the liberty to edit the title, the abstract, the short summary, and the bullet points (please see them below). I would be grateful if you could review the changes and let me know whether you agree with them.

Title:
Two structurally defined Aβ polymorphs promote different pathological changes in mice

Abstract:
Misfolded Aβ is involved in the progression of Alzheimer's disease (AD). However, the role of its polymorphic variants or conformational strains in AD pathogenesis is not fully understood. Here, we study the seeding properties of two structurally defined synthetic misfolded Aβ strains (termed 2F and 3F) using in vitro and in vivo assays. We show that 2F and 3F strains differ in their biochemical properties, including resistance to proteolysis, binding to strain-specific dyes and in vitro seeding. Injection of these strains into a transgenic mouse model produces different pathological features, namely different rates of aggregation, formation of different plaque types, tropism to specific brain regions, differential recruitment of Aβ40/Aβ42 peptides, and induction of microglial and astroglial responses. Importantly, the aggregates induced by 2F and 3F are structurally different as determined by ssNMR. Our study analyzes the biological properties of purified Aβ polymorphs that have been characterized at the atomic resolution level and provides relevant information on the pathological significance of misfolded Aβ strains.

Short summary:
Two structurally defined Aβ conformational variants differ in their biochemical properties in vitro and induce different pathological features in a transgenic mouse model.

Bullet points:
• Amyloid pathology induced by different Aβ strains is characterized by different tropism, morphology and tinctorial properties.
• Synthetic Aβ fibrils (2F and 3F) preferentially seed Aβ40 while in vivo-derived seeds have a preference to recruit Aβ42.
• Neuroinflammation induced in treated mice is Aβ strain-specific.
• Seeded aggregates induced by structurally different Aβ strains in mice display different conformations.

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We would also welcome the submission of cover suggestions or motifs to be used by our Graphics Illustrator in designing a

cover.

We look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

UTHealth
The University of Texas
Health Science Center at Houston

McGovern Medical School
Mitchell Center for Alzheimer's Disease
and Related Brain Disorders
Department of Neurology

Dr. Ioannis Papaioannou,
Editor
EMBO Reports Journal

Dear Dr. Papaioannou,

Many thanks for handling our manuscript entitled "**Structure-defined Aβ polymorphs promote different pathological changes in susceptible mice**".

Considering your previous communication, I am including revised files associated with this manuscript. Please find a point-by-point response to your comments below.

1. The panels of Figure 3 are not called out alphabetically in your manuscript. Please make sure that all panels are called out in alphabetical order in your revised manuscript (please use change tracking in Word).
Answer. We identified one mistake on this Figure ("Figure 3E-F" was corrected and replaced by "Figure 3E-H"). We tracked-changed this change in the attached version.

2. We would like to ask you to remove any numbering from the scale bars in the figures (Figure 1A and Appendix Figure S13A). The scales should be added to the Figure legends instead.
Answer. We corrected this for all Figures involved.

3. Please consider improving your synopsis image by adding necessary annotation or a graphical summary of your main findings. The image should be exactly 550 pixels wide and 300-600 pixels high. Please note that the text needs to be readable at the final size and not distorted.
Answer. We have created a new graphical abstract following the requirements. Please let me know if this is adequate or if it needs additional editing.

4. We now request publication of original source data with the aim of making primary data more accessible and transparent to the reader. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.
Answer. We have included all the requested source data in this submission.

5. I took the liberty to edit the title, the abstract, the short summary, and the bullet points (please see them below). I would be grateful if you could review the changes and let me know whether you agree with them.
Answer. All these has been addressed in the main text, and in the Synopsis file. Please find attached all edited files.

Thanks again for your suggestions. I look forward to hear from you.

6431 Fannin St. MSB 7.218, Houston, TX 77030
(713) 500-7442 • Rodrigo.MoralesLoyola@uth.tmc.edu

Sincerely,

Rodrigo Morales, PhD
Associate Professor
Department of Neurology
The University of Texas Medical School at Houston

6431 Fannin St. MSB 7.218, Houston, TX 77030
(713) 500-7442 • Rodrigo.MoralesLoyola@uth.tmc.edu

Dr. Rodrigo Morales
The University of Texas Health Science Center at Houston
Neurology
Houston 77030
United States

Dear Rodrigo,

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.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

****

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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots 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. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship 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 χ2 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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Not Applicable	

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

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If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

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Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

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Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods, Figures
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Yes	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Material and Methods, Figures

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Yes	Material and Methods
Studies involving **human participants**: 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.	Yes	Material and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Material and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	