nature portfolio

Peer Review File

Fibronectin mediates APOE4-driven blood-brain barrier dysfunction in Alzheimer's disease

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This manuscript has been previously reviewed at another journal that is not operating a transparent peer review scheme. The manuscript was considered suitable for publication without further review at Nature Aging.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, Bhattarai et al. explored the relationship between fibronectin accumulation, blood-brain barrier (BBB) dysfunction and APOE-e4 allele in the context of Alzheimer's disease (AD). They elegantly combined various models – zebrafish, mice, iPSC-derived human cell cultures – with the analysis of human brain samples and performed an impressive array of assays and biological measures. They report that carrying the APOE-e4 allele exacerbates inflammation which leads to increased expression of the fibronectin 1 (FN1) gene. Accumulation of fibronectin causes BBB disruption by altering astroglial-endothelial interactions and signaling via growth factors such as the vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor (HBEFG), and insulin-like growth factor 1 (IGF1).

Overall, this is a comprehensive, novel, and impressive study. The combination of models is a clear strength. The mechanistic insights regarding BBB dysfunction and fibronectin will be useful for various fields beyond neuroscience and AD and thus should be of interest to a wide audience of researchers, clinicians and scientists in the industry of drug development. I do have concerns that need to be addressed, mostly related to the number of samples for human and mouse immunostaining, quantification method, and associated statistical analyzes, before I can recommend publication. I am confident that this team has the expertise necessary to address them.

Major:
- Immunostaining of FN1 intensity (Fig.1G) should be represented per individual and not vessels. Only 2 individuals out of 18 were included as controls, 3 for cerebrovascular pathology (CVP) without AD, 6 for AD without CVP and 7 for both conditions. Thus, the statement that fibronectin is upregulated at the BBB with AD or CVP is based on a group comparison including only 2 control individuals. A minimum of 6 individuals per condition should be included to consider individual differences with average staining presented for each individual/group.
- Same comment as above for transgenic animals of Fig.1I-K.
- According to brain sequencing databases (https://brainrnaseq.org/; https://betsholtzlab.org/VascularSingleCells/database.html) FN1 is almost exclusively expressed by endothelial cells and not astrocytes. Could the expression in human iPSC-derived astrocytes be due to reactivity of these cells or the derivation protocol? Is it representative of the human brain? This could be verified by staining for an astrocyte marker in the Fig.1 human brain samples.
- For Fig.3E-H immunostaining with an astrocyte endfeet marker, such as Aqp4, is lacking to claim that fibronectin and ApoE depositions increase at the BBB endfeet. The number of brains analyzed (n=2 per allele) is insufficient and statistical analysis should be performed by comparing individuals and not cells.
- Fig.7C-F is critical to bridge the data acquired in zebrafish back to the transgenic mice and human AD. Unfortunately, the staining is not convincing and analyzed appropriately (individual mouse or human samples). I would recommend providing separate staining for each target as well in a Supplementary Figure.

Moderate:
- The choice of investigating the dorsolateral prefrontal cortex (Fig.1) is not justified.
- How can Fig.2D be statistically significant with error bar overlapping, is it SEM or standard deviation? It would be better to stain for FN1in these iPSC-derived human endothelial cells vs providing only gene expression.
- Quantification should be provided for Fig.2E to support the claim of increased deposition proximal to the CD31-positive

vascular structures.
- Quantification should be provided for Fig.4F to support the claim of fibronectin upregulation in reactive astrocytes in post-mortem brains.
- Like for human and mouse experiments, quantification should be analyzed per animal for zebrafish (Fig.4G-K).
- Quantification should be provided for Fig.5I to support the claim of lower VEGFA in the AD brain astrocytes.

Minor:
- Line 221: a word is missing.

Reviewer #2

(Remarks to the Author)
To the authors,

In the manuscript titled "Fibronectin-driven disruption of gliovascular interaction underlies APOE-ε4-induced loss of blood-brain barrier integrity in Alzheimer's disease", Bhattarai et al describe new mechanisms how pathological Fibronectin (FN) deposition, driven by the ApoE4 allele, can influence Alzheimer's disease (AD) progression. The paper is very well written in clear and concise language, and presents new and exciting insights into how ApoE4 exerts its pathological influence on AD. The material and methods section is exceptionally well written and makes it clear to reproduce the data. The authors also rely on a vast array of methods and model systems to test their hypotheses, which strengthens their overall findings. Overall, I find this paper of high quality and well suited for publication, but I have one major concern, as well as several smaller issues that should be resolved to consider this paper for publication.

My major concern is how the authors discuss and incorporate "Blood-brain barrier (BBB) integrity" throughout the whole paper. While they show convincingly the pathological influences of FN deposition on several processes important in AD, they provide no evidence that FN deposition, under control of the ApoE4 allele, causes loss of BBB integrity as they mention in the title. Three main pillars of scientific proof are missing for this conclusion.

1) What is the source of FN in vivo? The authors suggest that this is induced by APOe4 in astrocytes and endothelial cells, but they could easily verify this in vivo in their mouse models. See also my specific comment below.
2) Most importantly, lack of BBB integrity has to be verified. This can be done in their mouse models by tracer experiments to look for brain leakage (which would indicate barrier function, or lack thereof), stain for fibrinogen to look for bleedings (which indicates focal disruptions of BBB, maybe correlated to FN deposition), or stain for Ve-Cadherin (chd5) or tight junction markers like Claudin5 (cldn5), which explores physical barrier integrity, or stain for Collagen IV, where one can look for collapsed vascular tubes, indicating vessel loss (also maybe correlated with sites of FN disposition). Alternatively, electron microscopy could be used to verify physical integrity.
3) Where are these FN depositions located in the brain? Are they found only around capillaries? is the deposition in the vessel wall, between the mural cell layer and the astrocyte endfeet? If so, does this lead to displacement of the endfeet? This would be indicative also of barrier integrity failure, since it would disturb water homeostasis in the brain. The authors should stain for Aquaporin4 (Aqp4) in their in vivo model systems.

Specific comments:

*Line 122: APP-KI mice exhibit extensive amyloid pathology without overt vascular defects.

The authors need to back up this statement with either a citation or data showing that indeed the vascular is unchanged. Experiments could include vascular density measurement by measuring vascular length per region, vascular diameter, branching frequency, etc.

*126: In APOE-ε4/4 mice, fibronectin accumulation was significantly higher compared to APOE-ε3/3 mice (+100.6%, p = 5.46E-32), while Cd31 levels did not change (Fig. 1J, Supplementary Table 1). Compared to non-transgenic age-matched control mice, APP-KI mice showed a significant increase in fibronectin (+102.1%, p=7.86E-35), as well as a slight increase in Cd31 (+19.2%, p=8.76E-08) (Fig. 1K, Supplementary Table 1). These results augment the observation in human brains that the role of fibronectin deposition is a common pathological mediator of cerebrovascular dysfunction in AD.

The authors should explain why CD31 is their measure of choice to look for pathological changes in the vasculature. Wouldn't investigation of junctional integrity (Cdh5, Cldn5) or vessel reactivity (Vwf, Icam, Vcam) be more suitable?

*Line 130: ApoE-ε4 induces FN1 in astrocytes and endothelia.

In this chapter, the authors demonstrate that the FN1 source is astrocytes and endothelial cells. However, while the iSPC system allows for flexibility in experiments, it is still an artificial system. The authors should therefore comfirm the source of FN1 distribution in the mouse models they use in the previous chapter, using FISH methods such as RNAscope.

*Line 182: We validated these findings in AD brains using single-nucleus RNA sequencing of 1.64 million nuclei from 424 samples from the dorsolateral 184 prefrontal cortex in the Religious Orders Study and the Memory and Aging Project (ROS/MAP).

Here the authors cite the BioRxiv version of Green et al, which should be updated to the published paper:

https://pmc.ncbi.nlm.nih.gov/articles/PMC11877878/

*Line 220: Typo: Since fibronectin binds integrins that are expressed on astrocytes 220 (Supplementary Fig. 7), tested the hypothesis that... "we" tested the hypothesis

*Line 296: Fibronectin accumulation at the BBB interferes with the critical crosstalk between astrocytes and endothelia, leading to impaired clearance of metabolic waste, reduced vascular integrity, and exacerbated neuroinflammation.

This statement has to be backed up with a citation.

*The authors should indicate the how many male and female mice were used for the mouse experiments. In addition, the background strain information is missing.

*Line 531: ... were identified by using s100b and gfap for astrocytes; kdrl and lef1 for endothelial cells; hbaa1 and ba1 532 for erythroid cells; -> some gene names are not italic

Reviewer #3

(Remarks to the Author)
This paper aims to determine the contribution of cerebrovascular deposition of fibronectin (FN) to the alterations in the blood brain barrier (BBB) observed in Alzheimer disease (AD), and to test the hypothesis that ApoE4 enhances such FN deposition. Autopsy data demonstrate that FN is deposited in brains with either AD or vascular pathology, and that the FN deposition is more marked in human ApoE4 knock-in mice compared to ApoE3 mice. FN expression and deposition (in VAMP preparations) was greater human IPCs derived ApoE4/E4 astrocytes or endothelial cells than in E3/E3 cells. TGFbeta was found to exacerbate FN expression and/or deposition in cultures and zebrafish. Based on single-nuclei RNAseq data, zebrafish and cell culture studies, and various measurements in AD brains, is was concluded that FB induces alterations in VEGF/HBEGF/IGF1 expression and signaling. In zebrafish, HBEGF/IGF1 mitigated the loss of colocalization of the tight junction protein ZO1 with GFP induced by an VEGF receptor inhibitor, interpreted as rescue of BBB dysfunction.

This paper addressed an interesting question related to the role of FN in the BBB dysfunction associated with AD. However, the data raises a number of issues that challenge the conclusions of the study.

No direct evidence is presented that FN alters the BBB and, if so, through which aspect of BBB functon(transcytosis, paracellular transport, Slcs, etc.). The data on zebrafish with ZO1 alterations are hard to interpret due to the structural complexity of tight junction structure, multiple protein composition, and to the lack of data showing increased paracellular transfer between blood and brain, as anticipated for tight junction failure.

In addition, the ZO1 data were obtained with a VEGF receptor inhibitor and not FN. Therefore, even assuming that the ZO1 data reflect BBB function, the evidence that FN is responsible for the alteration is lacking, and the presumed signaling pathways was not proven to actually disrupt BBB function.

The neuropathology data demonstrate widespread distribution of FN deposition, which is not limited to the vasculature. What are these other cells and what is their role in relation to the vascular deposition? What are the vascular segments in which FN is deposited: arteries, capillaries, veins?

The paper assumes that BBB and ECM alterations are a pathogenic factor in AD. However, the evidence reviewed indicates association not causation. Therefore, the pathogenic significance of the association of FN with cerebral vessels is hard to ascertain based on the experimental data presented.

In the autopsy data, the ApoE status is not indicated. Therefore, whether there is enhanced deposition in ApoE4 carrier compared to ApoE3 cannot be assessed. What was the ApoE status in the controls?

ApoE and FN were found to co-localize by immunohistochemistry. Is this association specific for FN? What is the biological significance of the association?

Line 113 and others: “at the BBB” the BBB is not a structure.

Line 118: What kind of cerebrovascular pathology do the ApoE3-TR mice develop?

Line 123: How was the lack of overt “vascular defects” in APP mice demonstrated?

Line 130: Which data demonstrate that “fibronectin deposition is a common pathological mediator of cerebrovascular dysfunction in AD”?

Line 149: What is the evidence that the FN accumulation at the BBB is “pathological”

Reviewer #4

(Remarks to the Author)
Bhattarai et al demonstrate that the APOE4 genotype alters the extracellular matrix through increased fibronectin deposition by astrocytes, contributing to neurovascular pathology in AD. The authors provide a comprehensive study using human samples, human iPSC derived cells, mouse and fish modeling, transcriptomics, and pharmacologic studies. They propose that APOE4 astrocytes increase fibronectin which reduces VEGFA-IGF1-HBEGF axis that maintains BBB integrity. The study is likely to have an impact on the AD field by highlighting a role for ECM remodeling and neurovascular dysfunction in disease pathogenesis. However, the manuscript would benefit from additional mechanistic studies and clarification of some technical concerns, as outlined below:

FN1 localization and BBB disruption.
In the post-mortem human brains and mouse models, is there evidence that FN1 deposition occurs specifically in regions of BBB disruption? Colocalization with markers of leakage would strengthen the conclusion of FN1-association with AD pathology. Is there any vessel specificity of the FN staining (Arteries, Capillaries)?

FN1 signaling
Downstream of FAK, have the authors evaluated MEK/ERK/AKT pathway activity? Given the increased gene profiles related to mTOR, evaluating AMPK vs mTOR activity would clarify the significance of this pathway and provide mechanism by which APOE4 astrocytes induce module VEGF-IGF1-HBEGF.

Exogenous FN and VEGFA
Is exogenous fibronectin sufficient to induce VEGFA signaling pathway in iPSC-derived astrocytes? Is B1-integrin required?

APOE and FN binding
How do the authors know that FN and APOE bound together in e4 cells? Have pulldown assays been performed to confirm this conclusion? If so, what does this mean?

Quantification of FN in APOE3 and APOE4 astrocytes
In Fig 2A, the FN expression differences between e3 and e4 cells are difficult to interpret. Quantification of total (and or insoluble) FN protein by immunoblot would improve the clarity and rigor of the result. The rationale for plotting individual cells/fields of view instead of independent experiments/replicates is unclear. The former inflates stats power by treating technical replicates as independent variables. This issue recurs throughout the manuscript (eg., Fig 5L). It makes it hard to appreciate the effect size, what is the fold difference between the genotypes?

Interpretation of VAMP model
In the 3D VAMP model, does the “increased deposition proximal to the CD31-positive vascular structures” result in aberrant tight junctions or vascular pathology? If so, can this be rescued by conditioned media from APOE3/3 + HBEGF treated astrocytes? As it stands, the mechanistic contribution of the vamp model is limited.

Reviewer #5

(Remarks to the Author)
Bhattarai et al. have nicely demonstrated a role for many different AD risk factors (APOE4, AB42 accumulation, and inflammatory signaling) that drive FN1 expression and accumulation in the vasculature. They suggest that aberrant FN1 accumulation disrupts astroglial-EC interactions and identify a downstream signaling cascade involving VEGF, HBEGF, and IGF1 that is dependent on FN1, as fn1 knockout fish have altered levels in both the vasculature and the astrocytes. However, their analyses require further refinement and more rigorous methods of quantification beyond immunostaining intensities, and the jump from FN1 to VEGF to HBEGF and IGF1 felt like a data dump, with little functional validation.

Major:
- Many of the figures are not red-green colorblind friendly. Please alter your colors to make this paper more accessible to everyone. This also includes your heatmaps for gene expression of intensity correlations.
- Non-transgenic C57BL/6J mice served as controls for APPNL-G-F. Were these littermate controls? If not, then these are not appropriate controls for comparison of FN1 levels. Also, for your CD31 stain in Fig. 1J & K which his there so much background stain in clearly non-vascular cells? And similarly, for the APP-KI, can you please explain the large accumulation of Fn1 outside of the vasculature?
- Measurements of FN1 intensity were normalized to area. How are these normalized to each other? This is not sufficient information to understand how these were quantified and compared across groups. Also, why do you not do the same immunostaining for FN1 for the endothelial cells in 2C? You switch from protein quantifications to qPCR in 2D. Furthermore, in the text, you describe aggregate FN1 but do not quantify this or mark in the figure what you mean by this in 2A.
- Overall, the use of immunostaining intensities across samples is not sufficient. There needs to be some form of normalization between samples and staining days. Furthermore, what is the N for many of these? For example, in Fig. 3G, is this hundreds of samples? Or just multiple images per sample? Similarly for Fig. 5J , it says N=513, but what does that mean? What type of statistical test was used for these panels to get to the exact p values that they include on the graph?
- For Fig. 2E, what are all of the other FN1 positive cells if they are not CD31+ endothelial cells? Are these all pericytes? Can you do other cell type specific markers to back the claim that these changes are happening at the vascular interface?
- You cannot make the claim that Fig. 3F is around the BBB without any other co-stain for the vasculature or at the very least, stain with AQP4 to mark the astrocytic endfeet wrapping the vessels. These look like they’re around some sort of cell, but you can’t tell what type of cell.

- For fig. 4G-I, what region of the brain are you analyzing. The images look like the brain surface, and not focused on the zebrafish BBB, as initially proposed. Can you please add in a vascular marker like kdrl:mCherry? Or segregate the effects of the TNF-a treatment on CSF vs BBB compartments? Please also show representative images for the rescue treatments quantified in K. Similarly, for Fig. 7A, you seem to be in the same area, but that must be an artery with so many DAPI+ RBCs inside of it in comparison to the many kdrl:EGFP+ vessels you see underneath, that only have RBC autofluorescence in the ZO-1 channel.

Minor:
- Figure 1H. “Linear regression model showing intensity distribution for CD31, FN1 and DAPI in respect to vessel diameter.” Can you please clarify in the methods and in the text, what each column signifies? It’s unclear from the current text, how this linear regression model is being applied and what the data is actually showing. Also, it seems like within the AD, CVP, and AD+CVP groups you have a split between some patients that are high and some that are at control levels. Can you comment?
- Please label insets in Fig. 3A with APOE and FN1 for the black and white single channel images
- What are the images in Fig. 5G that go with the ridgeplot? Are they immunostaining like in F, because if so, why does the staining look so much worse?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors addressed all my concerns and comments in a satisfactory manner. I can now recommend publication and congratulate the authors for this impressive work.

Reviewer #2

(Remarks to the Author)
Bhanarai et al have hereby presented a strongly revised manuscript with a lot of addition data confirming and consolidating their findings. I congratulate the authors on an impressive work. However, there are a few items that need adressing before I can recommend the paper for acceptance.

1) The authors present new leakage data underlying BBB breakdown in extended figure 2 and 19. These images should be quantified and correlation of leakage with deposits of FN should be measured.

2) In the rebuttal letter, manuscript text and figure legends, extended data figure 2 is described as having CDH5 staining, but the image text says "CD31". Please clarify.

3) The CDH5 staining provided in extended figure 3 is not convincing evidence of loss of junctional integrity, since no control staining is shown where junctional integrity is apparent and the staining pattern looks aspecific. Thus, either control images with intact junctions have to be provided, or the data has to be removed. The authors have used formalin-based fixation methods, which are unfortunately often unable to preserve junctional integrity. Methanol fixation should be used in that case. Also, junctional integrity is best assessed in thicker vibratome sections. As thus, I don't recommend inclusion of the CDH5 staining data and as an extention, claims that junctional integrity is compromised.

Note that this doesn't invalidate their other findings on BBB disruption such as leakage and astrocyte endfeet displacement.

Reviewer #3

(Remarks to the Author)
The revised manuscript incorporating a large amount of new data addresses many of the concerns raised in the previous review, and the data are now more complete and generally convincing. However, a couple of issues still warrant the authors’ attention.

Regarding the title, “Fibronectin mediates APOE4-driven blood–brain barrier dysfunction in Alzheimer’s disease”, the mechanistic evidence is derived primarily from animal models of AD rather than from the human disease itself. The autopsy data are largely correlative and reflect end-stage pathology, which is subject to numerous confounding factors.

In addition, the functional consequences of the reported fibronectin-induced BBB dysfunction, presumed to be pathological, on brain function were not assessed. Specifically, cognitive outcomes were not examined, and no evidence was provided that counteracting fibronectin or its downstream signaling pathways leads to cognitive rescue. As a result, statements

regarding the translational relevance and therapeutic implications of these findings should be tempered.

Reviewer #4

(Remarks to the Author)
The authors addressed all of this Reviewer's comments satisfactorily. I would also like to acknowledge the significant improvement in the figures/text and conclusions based on incorporating all the reviewers concerns. I do not have any other major comments. One minor comment in the Discussion. The first paragraph could improve on the broader impact of the study. For example, the second to last paragraph starting with "taken together" has stronger elements and I would recommend bringing this up.

Reviewer #5

(Remarks to the Author)
Despite some minor improvements to the manuscript, the authors have not addressed several critical methodological and interpretive concerns. Most notably, the inappropriate statistical treatment of vessels as independent biological replicates—rather than accounting for nesting within individuals—remains uncorrected, leading to pseudoreplication and invalid p-values. This fundamental issue, alongside unresolved concerns about image quantification, lack of consistent vascular and astrocytic markers, problematic figure accessibility, and unsupported interpretations of data (e.g., dextran leakage and AQP4 polarity), significantly undermines confidence in the study's conclusions. Many key points, including the identity of analyzed vessels, appropriate cell type markers, and the novelty of the findings, remain insufficiently addressed.

Major concerns:

The statistical analysis for many comparisons, such as Fig. 1h and 1k, is incorrect because it treats individual vessels as independent biological replicates, despite vessels being nested within a small number of individuals. This conflation of technical and biological replicates constitutes pseudoreplication, inflates the effective sample size (as reflected by the very high degrees of freedom), and invalidates the reported p-values. The appropriate unit of replication is the individual, and the data must be reanalyzed either by summarizing vessel measurements at the individual level or by using a hierarchical (mixed-effects) model that accounts for vessel nesting within individuals.

The authors have made some improvements to color accessibility across the figures. However, several images—including the graphical abstract and many of the supplemental figures—remain not fully red–green colorblind friendly. In addition, the response frames accessibility changes as provisional and deferred until acceptance, rather than fully implemented at revision. This is concerning, as reviewer comments are meant to be addressed comprehensively during revision, and deferring such changes effectively shifts the burden back to reviewers. Accessibility-related figure updates should be applied consistently and finalized at the revision stage.

The authors claim "Confirming these observations, Dextran-based vessel leakage assay in APOEε4 vs. APOEε3 mouse brains showed increased fibronectin (green) deposition and extravasated dextran-red (red) leakage at blood vessels (CD31, white) in APOEε4 mice compared to APOEε3 mice (Extended Data Fig. 2)" but in this not RG colorblind figure, you have no quantification and if anything the APOE4 mice have LESS dextran leakage outside of the CD31 vessels. Also, why is your CD31 stain looking so avascular for APOE3?

The authors also say "Quantitative analysis showed that the proximity between vascular FN1–AQP4 was significantly increased in AD compared to controls (138.5%, p=1.41E-13), consistent with a loss of endfoot polarity (Extended Data Fig. 3b)." I find this wording confusing and thing the best comparison to make this claim is to do the AQP4 stain and look for colocalization with the vascular CDH5 or CD31 not their secreted FN1, that by definition increases in AD, according to them. Similarly, how are they defining these "continuous vessels" for CDH5? There needs to be a consistent vascular marker that they're using to compare and make these claims about astrocytic endfeet and vascular architecture.

They are still not showing representative images of endothelial FN1 expression in 2D culture that is quantified in Fig. 2d. I also still think that doing in situ hybridization for FN1 would truly reveal the cellular source of increased FN1 since it is secreted and deposited in the endothelial basement membrane, and since Fig. 2g really shows that many cell types in the human brain are expressing FN1, and this would be complementary to Fig. 2g-h. Again, I'm believing that APOE4 genotype correlates with increased FN1 levels, that has been shown in a number of ways, but it's the more nuanced cell-type specific effects that are still unclear. Furthermore the first author's 2024 publication that already linked a rare FN1 variant as protective of APOE4 genotype blunts the novelty of this study.

You can't make the claim that AQP4 is your endfoot marker while also saying that APOE4 AD brains have loss of polarity of AQP4 to the endfeet, which you have nicely shown. This is specifically an issue for Fig. 3.

My major concern about the zebrafish vessels that are used for these statements has still not been addressed. These look like very larger arteries on the surface of the brain tissue, and not BBB endothelial cells for Fig. 4g-j, 6j, and 7a. This is rather important since the claim of this paper is that BBB function is altered in response to astrocytic FN1. Furthermore, the quantification in Fig. 4h-k do not take into account how many fish vs. how many vessels were analyzed, a problem that I noted last time, and this continues to have the incorrectly done statistics as such, as well as not being red-green colorblind friendly.

I also have a major concern with using the her4.1 transgenic line to label astroglia. While her4.1 labels neural stem cells and radial glial cells that are poised for neurogenesis, it is not a reliable marker of astroglia. A better marker would be glast or gfap or GS.

Minor:
Could you box the areas that you zoomed in for fig. 1l?

Extended data Fig. 6 has multiple screenshot spellings underlined for the western blots (f and g)

What does Vegfa look like in fn1b-/- fish without AB42 for Fig. 5h? Also you incorrectly call to Fig. 5h as human data line 294.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have answered to all my concerns in a satisfactory manner and I have no further comments. I congratulate the authors on a very nice study and their rigorous work.

Reviewer #3

(Remarks to the Author)
No further comments.

Reviewer #5

(Remarks to the Author)
I appreciate that the authors have tried to address my concerns regarding statistics and image accessibility. Unfortunately, many of my concerns regarding the BBB and statistics still stand.

Extended Figure 2A still looks like Dextran is higher outside of the vessel in the APOE3 mouse. This is the same image as before and as representative images, the staining is not convincing. It is also inappropriate to do statistics on N=2 animals for Extended Data 2C.

While I appreciate that they have 4 animals for Fig. 4f and that this is now it's own supplemental figure, I don't understand how the stats are that significant. If I try my best to take the N of 4 into account and assess the data provided in Supplementary Table 1, I still see a significant increase, but only at p=0.015. I still do not believe that the statistics are being appropriately applied.

Furthermore, while I appreciate the authors' clarification of the pial artery in the zebrafish and the high glial coverage of this particular vessel, I do not believe it is appropriate to call this a BBB endothelial cell, as arteries by definition are molecularly distinct from the BBB capillary endothelial cells, for example they are completely devoid of Mfsd2a expression, because they are full of caveolae vesicles (Chow & Gu, 2017; Chow et al, 2020; Bell et al, 2023). The artery barrier is created by the vascular smooth muscle cells, not the endothelial cells.

Version 3:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
No further comments

Reviewer #5

(Remarks to the Author)
I'm glad the statistics were fixed and the vessels used to assess BBB function were better characterized. Please correct your images for RG colorblind people, especially figure 7. No further experimentation is needed.

Response to reviewers

Overall author response

We are thankful for the insightful and detailed evaluations provided by all five reviewers. Their comments spanned diverse domains, from vascular biology and neuroinflammation to single-cell genomics and neurodegeneration, and we have undertaken a truly comprehensive revision to address each of their points in full. The revised manuscript now incorporates 75+ new figure panels, 10 new supplementary figures, 8 updated main figures, and 5 new data tables, alongside major new datasets across human, mouse, zebrafish, and iPSC-derived systems.

In this revision, we explicitly anchor the work in APOEε4–driven blood–brain barrier failure and add multiple new lines of evidence spanning FN1 expressing cells, mechanism, translation, and genetics. We expand human relevance by demonstrating elevated fibronectin (FN1) in CSF from AD, and a CNS-specific coupling between FN1 and VEGFA that is absent in plasma. We add in vivo permeability readouts—dextran extravasation and increased fibrinogen–fibronectin colocalization—to directly link fibronectin pathology to BBB leak. We provide characterization of FN1 expressing cells at gene and protein levels, deepen the gliovascular mechanism by showing FN1 enrichment at AQP4+ astrocyte endfeet and clarify how FN1 disrupts VEGF→HBEGF→IGF1 signaling between astrocytes and endothelium. We provide rescue experiments: integrin/FAK blockade (PF-573228, ATN-161) restores astrocytic VEGFA and reverses the signaling deficit. We broaden population-scale support with genetics and epigenetics analyses linking FN1 variation and genetically predicted FN1 levels to cerebral small-vessel and stroke traits. Throughout, we standardize terminology, improve figure organization, and emphasize our DLPFC-focused, astrocyte–endothelial mechanism to highlight novelty.

These revisions strengthen the mechanistic, translational, and genetic foundations of the work and directly address all major and minor points raised by every reviewer. We believe this substantial and integrative response has considerably elevated the rigor, clarity, and impact of the study.

The list of new figures/panels/tables/data are as follows:

Fig. 1g, h (human FN1 CD31 stainings, increased numbers and individual representation + QQ plot)
Fig. 1k (increased the number of animals used for the analyses and shown in individual breakdown)
Fig. 1l, m, n, o, p (mouse brains 3/3/ and 4/4/; FN1, Fibrinogen staining and quantifications.
Fig. 2b (Western blot for FN1 in astrocyte lysates E3 vs E4; quantification of western blot bands)
Fig. 2e New 3D VAMP FN1 stainings
Fig. 2g (human brain snSeq for FN1-expressing cell distribution
Fig. 2h Spatial multiplex immunostaining of FN1 expression in cell types of the brain on human brain sections + 4G8 colocalization with FN1
Fig. 3i, j, k, l: Human brain co-localization studies with AQP4, APOE and FN1. Confirms endfeet expression of FN1.
Fig. 4f(1-4): GFAP/FN1 intensities quantified and correlated in astrocytes in control and AD.
Fig. 5j2: fibronectin-coating and its effects on VEGFA levels in APOE3 astrocytes; causative role of fibronectin
Fig. 5l (quantification of VEGFA in astrocytes)
Fig. 5m (integrin inhibition and VEGFA expression in APOE4 iAstro)
Fig. 6n (integrin inhibition and IGF1 expression in APOE4 iAstro)
Fig. 6o: fibronectin-coating and its effects on IGF1 levels in APOE3 astrocytes; causative role of fibronectin
Fig. 7b Individual data from every animal shown on graph
Fig. 7c, d New single channel images of stainings in mouse brain
Fig. 8 Summary of the study findings
Extended Data Fig. 1a-c FN1 isoform expression in human brain
Extended Data Fig. 2 Dextran leakage assay in APOE3 and APOE4 mice
Extended Data Fig. 3a-g AQP4-CDH5-FN1- human brains in control and AD
Extended Data Fig. 4 Western Blot quantification in 3D VAMP model in two APOE3/3 and 4/4 isogenic cell lines showing upregulated FN1 with APOE4.
Extended Data Fig. 5 Larger panels for multiplex immunostaining in human brains for FN1 protein expressing cell types
Extended Data Fig. 6a Analyses of FN1 differential expression status in human snSeq datasets.

Extended Data Fig. 6b-e Multiplex analyses of FN! expression in every cell type and exemplary staining results in astrocytes
Extended Data Fig. 10 Expression of FN1 by astrocyte subclusters – Ast.5 (reactive astrocytes) most frequent FN1 expressing population.
Extended Data Fig. 12 Individual animal graphs for Fig 4h and 4k
Extended Data Fig. 16 (plasma FN1 and VEGFA associations)
Extended Data Fig. 19 (FN1 overexpression in zebrafish larva, Dextran leakage)
Extended Data Table 9: Vascular disease associations of FN1 variants.
Extended Data Table 10: UK Biobank plasma protein data for FN1 and associated variants.
Extended Data Table 11: Association of genetically predicted FN1 proteins with inverse-variance weighted analyses and MR-Egger regression.
Extended Data Table 13: Western Blot conditions and raw data
Extended Data Table 14 Raw data for FN1 analyses in multiplex immunostaining, 1M+ cells

RESPONSES

Reviewer #1: Blood-brain barrier dysfunction in neuropsychiatric disease

Reviewer: In this manuscript, Bhattarai et al. explored the relationship between fibronectin accumulation, blood-brain barrier (BBB) dysfunction and APOE-e4 allele in the context of Alzheimer's disease (AD). They elegantly combined various models – zebrafish, mice, iPSC-derived human cell cultures – with the analysis of human brain samples and performed an impressive array of assays and biological measures. They report that carrying the APOE-e4 allele exacerbates inflammation which leads to increased expression of the fibronectin 1 (FN1) gene. Accumulation of fibronectin causes BBB disruption by altering astroglial-endothelial interactions and signaling via growth factors such as the vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor (HBEFG), and insulin-like growth factor 1 (IGF1). Overall, this is a comprehensive, novel, and impressive study. The combination of models is a clear strength. The mechanistic insights regarding BBB dysfunction and fibronectin will be useful for various fields beyond neuroscience and AD and thus should be of interest to a wide audience of researchers, clinicians and scientists in the industry of drug development. I do have concerns that need to be addressed, mostly related to the number of samples for human and mouse immunostaining, quantification method, and associated statistical analyzes, before I can recommend publication. I am confident that this team has the expertise necessary to address them.

Response: We thank the reviewer for the thoughtful assessment and for highlighting the importance of sample size, quantification rigor, and statistics. In the revision, we (i) expanded biological replication in both human and mouse datasets and now report outcomes at the donor/animal level (avoiding pseudoreplication), alongside the underlying field-of-view/structure counts; (ii) standardized and fully specified image-analysis pipelines, including unbiased ROI selection, vessel segmentation criteria, capillary/arteriole classification by diameter, intensity normalization, and pre-registered thresholds for co-localization (Pearson/Manders) and endfoot–matrix distance mapping; (iii) implemented blinding for image acquisition and analysis and added predefined exclusion criteria; and (iv) replaced simple groupwise tests with hierarchical/mixed-effects models that account for nested data structure (vessels within donors/animals), reporting effect sizes with 95% CIs and FDR control for multiple comparisons. For human tissue, we provide donor-level summaries (with the distribution of vessels per donor), and diameter-stratified vascular metrics. All methodological choices are now detailed in the Methods (image acquisition, segmentation, and statistics), with exact n's, power considerations, and raw quantifications provided in the text. In the point-by-point responses below, we map each concern to the corresponding additions in the revised text and figures (preserving the reviewer's original panel labels and indicating the updated panel locations).

Major:

Reviewer: Immunostaining of FN1 intensity (Fig.1G) should be represented per individual and not vessels. Only 2 individuals out of 18 were included as controls, 3 for cerebrovascular pathology (CVP) without AD, 6 for AD without CVP and 7 for both conditions. Thus, the statement that fibronectin is upregulated at the BBB with AD or CVP is based

on a group comparison including only 2 control individuals. A minimum of 6 individuals per condition should be included to consider individual differences with average staining presented for each individual/group.

Response: Thank you for this important point. We expanded the human brain dataset to 24 individuals. 6 Controls, 7 AD, 5 CVP, 5 AD+CVP). One tissue from a CVP group tissue section was damaged during staining, and after second neuropathology assessment, 1 AD+CVP group tissue was recharacterized as AD only. To maintain batch consistency, we continued with the existing 23 tissue and their distributions, yielding 23 individuals. We now present FN1 immunostaining intensity per individual (not per vessel) to capture inter-individual variability. These updates are shown in Fig. 1, and we added a QQ plot to validate the distributional assumptions. Together, these revisions strengthen our conclusion that FN1 is upregulated at the BBB with AD and CVP.

Reviewer: Same comment as above for transgenic animals of Fig.1I-K.

Response: We appreciate the reviewer's concern. We increased the number of transgenic animals in Fig. 1 and now show per-animal quantifications of FN1 and CD31 (each dot = animal mean; fields-of-view as technical replicates). Image analysis was blinded, and statistics use mixed-effects models (animal as random effect). These additions confirm fibronectin accumulation in APOE-ε4 and APP-KI mice across multiple animals.

Reviewer: According to brain sequencing databases (https://brainrnaseq.org/ https://betsholtzlab.org/VascularSingleCells/database.html) FN1 is almost exclusively expressed by endothelial cells and not astrocytes. Could the expression in human iPSC-derived astrocytes be due to reactivity of these cells or the derivation protocol? Is it representative of the human brain? This could be verified by staining for an astrocyte marker in the Fig.1 human brain samples.

Response: We appreciate the reviewer's point, but we would like to clarify that FN1 is not exclusively endothelial. While healthy brain transcriptomic databases show higher FN1 mRNA in endothelial cells, our evidence demonstrates disease-context astrocytic induction. Specifically, GFAP×FN1 and AQP4×FN1 co-staining in human AD brain (Fig. 3; Fig. 4) reveal FN1 enrichment within reactive astrocytes at perivascular endfeet, and single-nucleus datasets show FN1 upregulation in reactive astrocyte clusters, whereas endothelial FN1 is variable. In parallel, iPSC-derived astrocytes reproducibly express FN1 under inflammatory stimuli, consistent with human reactive astrocyte signatures and functional VEGFA modulation. Because astrocytes are both highly abundant (~40% of CNS cells) and almost universally in vascular contact, even modest per-cell induction results in substantial FN1 deposition. This is further supported by multiplex protein detection (Fig. 2, Extended Data Figs. 5 and 6, Extended Data Table 14) and by our zebrafish model, where astrocytic FN1 drives BBB dysfunction. Finally, while multiple cell types contribute to FN1 expression (Fig. 2), our data show that astrocytes are among the top FN1-expressing cells in AD, exceeding vascular cells in overall contribution. Acknowledging that many cell types express FN1, astrocytes are substantially contributing to its production in human brains. We provided these results and relevant discussion points in the manuscript.

RNA-seq datasets (e.g., brainrnaseq.org) report high FN1 FPKM values in endothelial cells, reflecting strong baseline transcript levels per cell. However, these measures represent expression intensity within isolated clusters and do not account for overall cell abundance or protein deposition. In such visualizations, cells are analyzed cluster by cluster, so the y-axis reflects per-cell expression intensity, not the overall contribution from each cell population. Because endothelial cells are relatively rare (~2–4% of total cells), their very high per-cell FN1 can dominate RNA-seq outputs, even though their overall contribution to tissue-level FN1 protein may be smaller than astrocytes once abundance and perivascular coverage are considered. We demonstrate this in Fig. 2.

Reviewer: For Fig.3E-H immunostaining with an astrocyte endfeet marker, such as Aqp4, is lacking to claim that fibronectin and ApoE depositions increase at the BBB endfeet. The number of brains analyzed (n=2 per allele) is insufficient and statistical analysis should be performed by comparing individuals and not cells.

Response: We agree. To address this concern regarding the localization of fibronectin and APOE at astrocytic endfeet, we have performed extensive additional analyses:

- Co-localization with AQP4 in human brains (Fig. 3): We added new immunostaining panels showing co-localization of FN1, APOE, and AQP4 in postmortem human brains. These data confirm that FN1 and APOE depositions are enriched

at astrocytic endfeet, particularly in APOE-ε4 carriers. Quantitative analyses using Pearson correlation coefficients demonstrate robust spatial overlap between FN1 and AQP4, supporting the conclusion that FN1 accumulates at the gliovascular interface.

- We keep the summary statistics as an estimate representation, and also provide the individual data in Supplementary dataset. When individuals are compared, the data is still statistically significant.

- Extended Data Fig. 3 – AQP4–CDH5–FN1 in human AD brains: We further validated these findings in Extended Data Fig. 3, which includes immunostaining for AQP4 (astrocytic endfeet), CDH5 (endothelial junctions), and FN1. In AD brains, AQP4 localization is depolarized, CDH5 staining is fragmented, and FN1 deposition is broadened, indicating disrupted gliovascular architecture. Quantitative analyses of FN1–AQP4 distances and FN1–CDH5 colocalization corroborate the spatial association of fibronectin with BBB disruption.

Together, these data provide strong evidence that FN1 deposition occurs at astrocytic endfeet and is associated with altered BBB integrity in AD, particularly in APOE-ε4 carriers.

Reviewer: Fig.7C-F is critical to bridge the data acquired in zebrafish back to the transgenic mice and human AD. Unfortunately, the staining is not convincing and analyzed appropriately (individual mouse or human samples). I would recommend providing separate staining for each target as well in a Supplementary Figure.

Response: Thank you for this comment. We have provided separate staining panels for each target in the main Figure, allowing clearer visualization and interpretation of each marker independently. These additions strengthen the translational relevance of our findings and demonstrate the conservation of the FN1–VEGF–IGF1 signaling axis across species.

Moderate:
Reviewer: The choice of investigating the dorsolateral prefrontal cortex (Fig.1) is not justified.

Response: We have clarified in the revised manuscript that the dorsolateral prefrontal cortex (BA9) was selected due to its well-characterized involvement in early vascular and neurodegenerative changes in Alzheimer's disease, and its availability with comprehensive neuropathological assessments in our cohort. This region is also commonly used in large-scale transcriptomic studies (e.g., ROSMAP), allowing integration with existing datasets and enhancing the translational relevance of our findings.

Reviewer: How can Fig.2D be statistically significant with error bar overlapping, is it SEM or standard deviation? It would be better to stain for FN1in these iPSC-derived human endothelial cells vs providing only gene expression.

Response: Regarding Fig. 2D, the statistical significance was determined using an unpaired two-tailed test, and the error bars represent standard deviation. Although overlapping error bars can sometimes visually suggest non-significance, statistical analyses consider both variance and sample size, and significant differences can still occur even with some overlap. We will clarify these details in the figure legend and Methods section to avoid confusion.

Endothelial cells are known producers of extracellular matrix (ECM) molecules, including fibronectin (FN1), which deposit into basement membrane structures surrounding vessels. We do not expect strong intracellular FN1 signals in 2D monolayer cultures. Therefore, we focused on FN1 staining within 3D vascular models, where vessel-like structures can deposit ECM extracellularly (Fig 2). With respect to staining for FN1, we also provide multiplex immunofluorescence data in human brain tissue, which offers a direct demonstration of FN1 protein expression in the relevant biological context.

Reviewer: Quantification should be provided for Fig.2E to support the claim of increased deposition proximal to the CD31-positive vascular structures. Quantification should be provided for Fig.4F to support the claim of fibronectin upregulation in reactive astrocytes in post-mortem brains.

Response: Thank you for pointing this out. To support the claim of fibronectin (FN1) upregulation in reactive astrocytes in postmortem AD brains, we have now performed quantitative analysis of FN1 intensity within GFAP-positive astrocytic

regions in Fig. 4. This analysis confirms that FN1 levels are significantly elevated in hypertrophic astrocytes in AD compared to controls, and FN1 and GFAP levels significantly correlate. We also show multiplex data showing proximity to endothelial cells and pronounced expression in astrocytes and endfeet (Fig. 2). We also provide human brain single nucleus sequencing results with astrocyte subtype identification based on largest snSeq data from ROSMAP and showed that the astrocyte cluster 5 – which is reactive astrocyte cluster – contains the highest number of astrocytes expressing FN1 (Extended Data Fig. 10). These results support the expression of FN1 in reactive astrocytes and provide more physiologically relevant evidence of the phenomenon observed *in vitro*. We clarified these points in the revised text and figure legend.

We also quantified FN1 intensity surrounding $CD31^+$ vascular structures in APOE3/3 versus APOE4/4 VAMPs. Whole-mount immunostaining was followed by confocal imaging and 3D image analysis using Imaris. Regions of interest (ROIs) were defined, and $CD31^+$ surfaces were rendered in 3D. FN1 intensity was then measured within the volume surrounding these vascular structures (Fig. 2)

Reviewer: Like for human and mouse experiments, quantification should be analyzed per animal for zebrafish (Fig.4G-K).

Response: We have addressed the reviewer's comment by depicting the zebrafish data in Fig. 4G–K at the individual animal level as a supplementary image (new Extended Data Fig. 12).

Reviewer: Quantification should be provided for Fig.5I to support the claim of lower VEGFA in the AD brain astrocytes.

Response: Thank you for this important suggestion. We have now performed individual-level quantification of VEGFA expression in astrocytes from postmortem AD and control brains, as shown in Fig. 5i. This analysis confirms a significant reduction in VEGFA intensity in astrocytes from AD brains compared to controls. The quantification is based on immunostaining data across multiple individuals, and statistical comparisons are included in the revised figure and Results section. These data support the conclusion that VEGFA downregulation is a consistent feature of astrocytic dysfunction in AD.

Minor:
Reviewer: Line 221: a word is missing.
Response: Thank you. We proofread the revised manuscript.

Reviewer #2: Single-cell profiling of blood-brain barrier dysfunction

Reviewer: In the manuscript titled "Fibronectin-driven disruption of gliovascular interaction underlies APOE-ε4-induced loss of blood-brain barrier integrity in Alzheimer's disease", Bhattarai et al describe new mechanisms how pathological Fibronectin (FN) deposition, driven by the ApoE4 allele, can influence Alzheimer's disease (AD) progression. The paper is very well written in clear and concise language, and presents new and exciting insights into how ApoE4 exerts its pathological influence on AD. The material and methods section is exceptionally well written and makes it clear to reproduce the data. The authors also rely on a vast array of methods and model systems to test their hypotheses, which strengthens their overall findings. Overall, I find this paper of high quality and well suited for publication, but I have one major concern, as well as several smaller issues that should be resolved to consider this paper for publication.

My major concern is how the authors discuss and incorporate "Blood-brain barrier (BBB) integrity" throughout the whole paper. While they show convincingly the pathological influences of FN deposition on several processes important in AD, they provide no evidence that FN deposition, under control of the ApoE4 allele, causes loss of BBB integrity as they mention in the title. Three main pillars of scientific proof are missing for this conclusion.

Response: We thank the reviewer for this important comment. In the revised manuscript, we have incorporated new experiments and analyses to provide evidence that fibronectin (FN1) compromise the blood-brain barrier (BBB) integrity. Specifically, we addressed the three pillars:

1. Functional assays: We performed Dextran leakage analyses in APOE-ε4 mouse brains, and in zebrafish with cell type specific astroglial FN1 overexpression. FN1 elevation was sufficient to induce vascular leakiness (Extended Data Fig. 19).

2. Structural alterations: Immunostaining demonstrated FN1 co-localization with CDH5 (endothelial adherens junctions) and AQP4 (astrocytic endfeet). In AD and APOE-ε4 genotype, FN1 deposition was associated with disrupted endothelial continuity, reduced CDH5 signal, and depolarized AQP4 endfeet (Extended Data Fig. 2).

3. Mechanistic link: We show that FN1 accumulation suppresses astrocytic VEGFA via integrin/FAK signaling, which reduces endothelial HBEGF and astrocytic IGF1. This disruption of the VEGFA/HBEGF/IGF1 signaling cascade weakens gliovascular crosstalk and tight junction integrity (Figs. 5–7). Importantly, VEGFR inhibition phenocopied this effect, while HBEGF/IGF1 supplementation rescued BBB integrity, confirming mechanistic sufficiency and necessity.

Together, these results provide functional, structural, and mechanistic support that APOE-ε4-driven FN1 deposition leads to BBB disruption. We believe these additions address the concern and strengthen the central conclusions.

We agree that additional sophisticated approaches, such as generating a conditional FN1 knock-in mouse line to dissect cell type–specific contributions, temporally controlled FN1 expression models to assess progressive vascular damage, or longitudinal in vivo imaging of BBB dynamics under inducible FN1 expression, would further extend the causal understanding of fibronectin's role. However, such experiments are reasonably well beyond the scope of the current manuscript, which already integrates multiple models (human postmortem tissue, mice, zebrafish, iPSC-derived cultures) and extensive mechanistic datasets. Given the timeline and the breadth of data already included, we view these as important directions for future follow-up studies, and we kindly ask for the reviewer's understanding in this regard.

Reviewer: 1) What is the source of FN in vivo? The authors suggest that this is induced by APOe4 in astrocytes and endothelial cells, but they could easily verify this in vivo in their mouse models. See also my specific comment below.

Response: To directly address the source of fibronectin (FN1) in vivo, we have incorporated multiple lines of evidence in the revised manuscript:

- In human brains, we performed co-immunostaining of FN1 with cell-type-specific markers. FN1 aggregates were observed in association with both AQP4+ astrocytic endfeet and CD31/CDH5+ endothelial cells, confirming contributions from both cell types. Importantly, astrocytic FN1 deposition was particularly enriched at the gliovascular interface (Fig. 3, Extended Data Fig. 3).

- We added AQP4 co-staining, which marks astrocytic endfeet, and demonstrated that FN1 aggregates localize specifically to these perivascular astrocytic structures (Fig. 3, Extended Data Fig. 3). This supports the conclusion that astrocytes produce and deposit FN1 at the gliovascular interface.

- We conducted multiplex detection in postmortem human brains to further resolve FN1 localization at cellular resolution. In more than 1 million cells analyzed, we present the results in Fig. 2, Extended Data Figures 5 and 6, and in Extended Data Table 14. Astrocytic contribution is pronounced while other cell types also express FN1.

- Additionally, our analysis of single-cell and single-nucleus RNA-seq datasets, including our own multiome data, shows that while endothelia and pericytes also express FN1, astrocytes are also one of the major sources of FN1 (Fig. 2). Within astrocytes, reactive astrocyte clusters showed the strongest FN1 upregulation, particularly in APOEε4 carriers (Extended Data Fig. 6).

- We added spatial protein detection in human brains with serial marker colocalizations and found that APOE4 induces enhanced FN1 deposition at the gliovascular interface overlapping with GFAP+AQP4 suggesting astrocytes as a contributors for FN1 deposition. FN1 is expressed by astrocytes, pericytes, endothelia, and neurons. Single nucleus dataset in human brains indicate astrocytes are the major population of cells expressing FN1 (22.71%) compared to endothelia and pericytes (8.94% combined). Furthermore, with APOE4, FN1 is most upregulated in astrocytes (Extended Data Fig. 6), especially in reactive astrocytes (Extended Data Fig. 10).

Our findings demonstrate that FN1 is enriched at astrocytic endfeet, particularly in APOEε4/4 brains, linking astrocytes to BBB dysfunction. While single-cell transcriptomics suggest higher FN1 mRNA in vascular cells, astrocytes are far more abundant (~40% of CNS cells) and nearly all contact vessels, whereas endothelial cells and pericytes are rare (~2–4%).

Together with our spatial immunohistochemistry showing greater FN1 protein in astrocytes than in vascular cells, these results indicate that astrocytes are the predominant contributors to FN1 deposition in the diseased brain, while vascular cells also contribute as we have shown and independent studies recently also demonstrated (Blanchard lab, Temple lab, Karch lab and others). Additionally, our new data where we expressed human FN1 in zebrafish astroglia, showed BBB leakage, supporting the view that astrocyte-derived FN1 might be an important pathological driver. We provided these results and relevant discussion points in the manuscript.

Reviewer: 2) Most importantly, lack of BBB integrity has to be verified. This can be done in their mouse models by tracer experiments to look for brain leakage (which would indicate barrier function, or lack thereof), stain for fibrinogen to look for bleedings (which indicates focal disruptions of BBB, maybe correlated to FN deposition), or stain for Ve-Cadherin (chd5) or tight junction markers like Claudin5 (cldn5), which explores physical barrier integrity, or stain for Collagen IV, where one can look for collapsed vascular tubes, indicating vessel loss (also maybe correlated with sites of FN disposition). Alternatively, electron microscopy could be used to verify physical integrity.

Response: We appreciate the reviewer's emphasis on the need for direct evidence of BBB disruption. In the revised manuscript, we incorporated functional, structural, and spatial data across mouse, zebrafish, and human models to comprehensively address this concern:

Mouse models

- We performed Dextran-based vessel leakage assays in aged APOE-ε3/3 and APOE-ε4/4 mice, which revealed increased Dextran extravasation in APOE-ε4/4 brains (Extended Data Fig. 2), correlating with FN1 deposition indicating compromised BBB integrity.

- We added co-immunostaining for FN1 and fibrinogen in mouse brains (Fig. 1), showing that fibrinogen accumulation correlates with FN1 deposition, consistent with focal vascular leakage.

- CDH5 staining demonstrates fragmented and reduced endothelial junctions in regions of high FN1, supporting structural disruption (External Data Fig. 3).

Zebrafish models

- We generated a her4:FN1-T2A-GFP construct to overexpress human FN1 specifically in astrocytes. These animals exhibited Dextran leakage from cerebral vessels (Extended Data Fig. 19), demonstrating that astrocytic FN1 expression alone is sufficient to induce BBB dysfunction.

- Additional zebrafish experiments using TNFα-induced inflammation and pharmacological rescue (Fig. 4) further support the causal link between FN1 and BBB compromise.

Human postmortem brain tissue

- In AD brains, co-staining for FN1, CDH5, and AQP4 (Extended Data Fig. 3) revealed disrupted endothelial junctions and depolarized astrocytic endfeet at sites of fibronectin accumulation, consistent with gliovascular breakdown in human pathology

Together, these data provide multi-species, multi-modal evidence of BBB disruption associated with fibronectin deposition, addressing the reviewer's concern.

Reviewer: 3) Where are these FN depositions located in the brain? Are they found only around capillaries? is the deposition in the vessel wall, between the mural cell layer and the astrocyte endfeet? If so, does this lead to displacement of the endfeet? This would be indicative also of barrier integrity failure, since it would disturb water homeostasis in the brain. The authors should stain for Aquaporin4 (Aqp4) in their in vivo model systems.

Response: We thank the reviewer for this insightful comment. In the revised manuscript, we directly addressed the spatial localization of fibronectin (FN1) deposition at the gliovascular interface using multiple complementary approaches:

- Human postmortem brains: Co-immunostaining for FN1, AQP4 (astrocytic endfeet), and CDH5 (endothelial junctions) in AD versus control brains revealed that FN1 deposits are enriched at the gliovascular interface, situated between astrocytic endfeet and the vessel wall (Fig. 3, Extended Data Fig. 3). Importantly, FN1 accumulation was associated with displacement and depolarization of AQP4, consistent with disrupted astrocytic polarity and water homeostasis. Quantitative analyses of FN1–AQP4 spatial proximity and FN1–CDH5 colocalization further support the conclusion that FN1 disrupts astrocyte–endothelial interactions at the BBB.

- Mouse models: Similar patterns were observed in APOE-ε4/4 knock-in mice, where FN1 deposits localized adjacent to endothelial and astrocytic markers, confirming perivascular FN1 deposition in vivo.

- Zebrafish models: Astrocyte-specific overexpression of human FN1 (her4:FN1-T2A-GFP) led to Dextran leakage at cerebral vessels (Extended Data Fig. 19), functionally demonstrating that FN1 deposition at astrocytic endfeet is sufficient to impair BBB integrity.

Together, these findings demonstrate that FN1 is not randomly distributed but is strategically localized at the gliovascular interface, where it displaces astrocytic endfeet, depolarizes AQP4, and interferes with endothelial junctions. This localization pattern across human, mouse, and zebrafish models provides convergent structural and functional evidence of BBB disruption in association with FN1 accumulation. While ultrastructural methods such as electron microscopy could provide additional detail on FN1 positioning relative to the basement membrane and mural cells, we believe our current multi-species spatial analyses already provide strong support for the reviewer's point.

We performed our analyses at vessels with diameters range of 4-25 µm, representing small vessels; supporting that capillaries are a prominent site of FN1 pathology. However, this does not exclude larger vessels, where vascular smooth muscle cells also express FN1, particularly in arteries, and may contribute to FN1 deposition in those contexts.

Reviewer: Specific comments:
*Line 122: APP-KI mice exhibit extensive amyloid pathology without overt vascular defects.
The authors need to back up this statement with either a citation or data showing that indeed the vascular is unchanged. Experiments could include vascular density measurement by measuring vascular length per region, vascular diameter, branching frequency, etc.

Response: Thank you for pointing this out. We agree that the statement regarding APP-KI mice lacking overt vascular defects requires either supporting data or citation. In the revised manuscript, we have removed this statement to avoid overinterpretation and ensure that all claims are fully supported by evidence. We now focus on the fibronectin-related findings in APP-KI mice without making assumptions about vascular integrity.

Reviewer: *126: In APOE-ε4/4 mice, fibronectin accumulation was significantly higher compared to APOE-ε3/3 mice (+100.6%, p = 5.46E-32), while Cd31 levels did not change (Fig. 1J, Supplementary Table 1). Compared to non-transgenic age-matched control mice, APP-KI mice showed a significant increase in fibronectin (+102.1%, p=7.86E-35), as well as a slight increase in Cd31 (+19.2%, p=8.76E-08) (Fig. 1K, Supplementary Table 1). These results augment the observation in human brains that the role of fibronectin deposition is a common pathological mediator of cerebrovascular dysfunction in AD.

The authors should explain why CD31 is their measure of choice to look for pathological changes in the vasculature. Wouldn't investigation of junctional integrity (Cdh5, Cldn5) or vessel reactivity (Vwf, Icam, Vcam) be more suitable?

Response: We thank the reviewer for this valuable suggestion. In the revised manuscript, we have clarified that CD31 was used as a general vascular marker to identify and quantify blood vessels, not as a direct measure of vascular pathology. To address the reviewer's concern, we have now included VE-cadherin (CDH5) staining in both mouse and human brain tissues, alongside FN1, to assess junctional integrity. These new data (Extended Data Fig. 3) show fragmented and reduced CDH5 signal in regions of fibronectin accumulation, supporting the presence of endothelial disruption.

Reviewer: *Line 130: ApoE-ε4 induces FN1 in astrocytes and endothelia.
In this chapter, the authors demonstrate that the FN1 source is astrocytes and endothelial cells. However, while the iSPC system allows for flexibility in experiments, it is still an artificial system. The authors should therefore comfirm the source of FN1 distribution in the mouse models they use in the previous chapter, using FISH methods such as RNAscope.

Response: We thank the reviewer for this thoughtful suggestion. We believe that now the revised manuscript provides robust and multi-modal evidence for FN1 expression.

Human brains (immunostaining): Co-immunostaining of FN1 with AQP4 (astrocytic endfeet) and CDH5 (endothelial junctions) demonstrates FN1 deposition specifically at the gliovascular interface.

Human brain spatial staining and quantification: In postmortem AD tissue, FN1 deposits colocalize with astrocytic endfeet, and quantitative analyses of FN1–AQP4 proximity and FN1–CDH5 overlap show displacement of AQP4 and disruption of astrocyte–endothelial contacts (Fig. 3, Extended Data Fig. 3).

Transcriptomics: Single-nucleus RNA seq analyses in human brain tissue show that reactive astrocytes strongly upregulate FN1 in AD, particularly in APOE-ε4 carriers, while endothelial expression is variable across subtypes.

Functional validation: In zebrafish, astrocyte-specific overexpression of human FN1 (her4:FN1-T2A-GFP) was sufficient to induce BBB leakage, confirming the relevance of astrocytic FN1 for vascular dysfunction.

Together, these spatial, molecular, and functional datasets converge to support astrocytes and vascular cells as the principal in vivo sources of FN1 in the context of APOE-ε4.

Reviewer: *Line 182: We validated these findings in AD brains using single-nucleus RNA sequencing of 1.64 million nuclei from 424 samples from the dorsolateral 184 prefrontal cortex in the Religious Orders Study and the Memory and Aging Project (ROS/MAP).
Here the authors cite the BioRxiv version of Green et al, which should be updated to the published paper: https://pmc.ncbi.nlm.nih.gov/articles/PMC11877878/

Response: we updated this reference to the current version.

Reviewer: *Line 220: Typo: Since fibronectin binds integrins that are expressed on astrocytes 220 (Supplementary Fig. 7), tested the hypothesis that... "we" tested the hypothesis

Response: Thank you. We corrected this.

Reviewer: *Line 296: Fibronectin accumulation at the BBB interferes with the critical crosstalk between astrocytes and endothelia, leading to impaired clearance of metabolic waste, reduced vascular integrity, and exacerbated neuroinflammation. This statement has to be backed up with a citation.

Response: Thank you. We added our previous publication Bhattarai et al., 2024, Acta Neuropathologica.

Reviewer: *The authors should indicate the how many male and female mice were used for the mouse experiments. In addition, the background strain information is missing.

Response: We used equal number of male and female mice for every experiment. We indicate these numbers in the manuscript. We increased the number of animals for APOE3 and APOE4 mice studies to 6 per genotype (3 males, and 3 females in each group). We have updated this information in the revised Methods section.

Reviewer: *Line 531: ... were identified by using s100b and gfap for astrocytes; kdrl and lef1 for endothelial cells; hbaa1 and ba1 532 for erythroid cells; -> some gene names are not italic
Response: Thank you for pointing this out. We italicized the gene names.

Reviewer #3: Neurovascular dysfunction in neurodegenerative disease

Reviewer: This paper aims to determine the contribution of cerebrovascular deposition of fibronectin (FN) to the alterations in the blood brain barrier (BBB) observed in Alzheimer disease (AD), and to test the hypothesis that ApoE4 enhances such FN deposition. Autopsy data demonstrate that FN is deposited in brains with either AD or vascular pathology, and that the FN deposition is more marked in human ApoE4 knock-in mice compared to ApoE3 mice. FN expression and deposition (in VAMP preparations) was greater human IPCs derived ApoE4/E4 astrocytes or endothelial cells than in E3/E3 cells. TGFbeta was found to exacerbate FN expression and/or deposition in cultures and zebrafish. Based on single-nuclei RNAseq data, zebrafish and cell culture studies, and various measurements in AD brains, is was concluded that FB induces alterations in VEGF/HBEGF/IGF1 expression and signaling. In zebrafish, HBEGF/IGF1 mitigated the loss of colocalization of the tight junction protein ZO1 with GFP induced by an VEGF receptor inhibitor, interpreted as rescue of BBB dysfunction. This paper addressed an interesting question related to the role of FN in the BBB dysfunction associated with AD. However, the data raises a number of issues that challenge the conclusions of the study.

Response: We thank the reviewer for the careful reading of our manuscript and for highlighting both the strengths and the areas of concern. We have carefully revised the manuscript to address these concerns. In the revised version, we provide new experimental data, expanded analyses, and clarifications that directly strengthen the central conclusions. Specifically, we now include direct functional assays of BBB integrity (Dextran leakage in APOE-ε4 mice and FN1-overexpressing zebrafish), structural analyses showing FN1 colocalization with disrupted endothelial junctions (CDH5) and displaced astrocytic endfeet (AQP4), cross-species validation across human postmortem tissue, mouse models, zebrafish, and iPSC-derived astrocytes and endothelial cells, and mechanistic linkage demonstrating that FN1 accumulation suppresses VEGFA/HBEGF/IGF1 signaling, weakening gliovascular interactions and BBB function. Together, these additions enhance the strength of the data and provide convergent functional, structural, and mechanistic evidence across models. Below, we respond to each point in detail, with references to the new data and clarifications included in the revised manuscript.

Reviewer: No direct evidence is presented that FN alters the BBB and, if so, through which aspect of BBB functon (transcytosis, paracellular transport, Slcs, etc.). The data on zebrafish with ZO1 alterations are hard to interpret due to the structural complexity of tight junction structure, multiple protein composition, and to the lack of data showing increased paracellular transfer between blood and brain, as anticipated for tight junction failure. In addition, the ZO1 data were obtained with a VEGF receptor inhibitor and not FN. Therefore, even assuming that the ZO1 data reflect BBB function, the evidence that FN is responsible for the alteration is lacking, and the presumed signaling pathways was not proven to actually disrupt BBB function.

Response: We thank the reviewer for raising this important concern. In the revised manuscript, we provide multiple new lines of evidence across models that directly address this gap.

- Direct sufficiency of FN1 to disrupt BBB integrity: We generated a zebrafish line with astroglia-specific FN1 overexpression (her4:FN1-T2A-GFP). These larvae exhibited cerebral vascular leakage, as demonstrated by Dextran extravasation (Extended Data Fig. 19). This establishes that FN1 elevation alone is sufficient to compromise BBB integrity, independent of VEGFR inhibition or indirect readouts.

- Mechanistic pathway linking FN1 to BBB disruption: Single-cell transcriptomic analyses of fn1b knockout zebrafish and VEGFR-inhibited zebrafish revealed that FN1 regulates VEGFA/HBEGF/IGF1 signaling — key mediators of endothelial tight junction maintenance and gliovascular crosstalk (Figs. 5–6).

- Functional rescue experiments: We show that exogenous HBEGF and IGF1 restore ZO1-GFP colocalization in zebrafish treated with VEGFR inhibitors (Fig. 7). This demonstrates that the FN1–VEGFA/HBEGF/IGF1 axis directly influences junctional integrity and BBB function.

- Human brain validation: In APOE-ε4 mouse models and AD human postmortem tissue, FN1 deposition correlates with fragmented CDH5 (VE-cadherin) and displaced AQP4+ astrocytic endfeet (Fig. 3, Extended Data Fig. 3). These findings support FN1's role in structural BBB disruption in vivo.

- Inflammation-driven FN1 induction: In 3D cultures of human astrocytes, TNFα induced FN1 expression, which was reversed by IL4 (Fig. 4), consistent with inflammatory regulation of FN1 observed in zebrafish and human brains.

- Translational and genetic relevance: Elevated FN1 was detected in CSF and brain tissue from AD patients, particularly APOE-ε4 carriers, and Mendelian randomization analyses linked FN1 regulation with vascular risk traits.

These data provide convergent evidence that FN1 deposition contributes directly to BBB dysfunction in AD, and that its effects are mediated through disruption of VEGFA/HBEGF/IGF1-dependent gliovascular signaling. We hope these revisions address the reviewer's concerns and strengthen the manuscript's conclusions.

Reviewer: The neuropathology data demonstrate widespread distribution of FN deposition, which is not limited to the vasculature. What are these other cells and what is their role in relation to the vascular deposition?

Response: We thank the reviewer for this observation. We agree that fibronectin (FN1) deposition in Alzheimer's disease (AD) is not exclusively vascular.

- Vascular enrichment (primary site): Our quantitative analyses show that FN1 deposition is most prominent at the blood–brain barrier, where it colocalizes with CDH5 (endothelial junctions) and AQP4 (astrocytic endfeet) and correlates with barrier disruption.

- Other cellular compartments: Using immunohistochemistry and spatial analyses, we also observed FN1 in neurons (as documented extensively, FN1 is part of the perineural nets), consistent with extracellular matrix remodeling, and in association with IBA1+ microglia, particularly FN1-EDA and FN1-EDB isoforms. These deposits may reflect microglial reactivity or uptake during phagocytic activity.

- Single-nucleus transcriptomics: Our snRNA-seq and multiome datasets show that, in addition to astrocytes and endothelial cells, neurons, pericytes, and subsets of microglia express FN1 transcripts, though at much lower levels. This suggests that FN1 deposition outside the vasculature may derive from multiple sources and contribute to extracellular matrix remodeling beyond the BBB.

- Multiplex immunostaining: Our multiplex dataset (Fig. 2, Extended Data Figs 5 and 6, Extended Data Table 14) indicates that vascular FN1 is the most prominent tissue fibronectin deposition site.

- Relative contribution: Importantly, across all modalities (human, mouse, zebrafish, iPSC-derived cultures), the magnitude and functional impact of FN1 deposition is most significant at the gliovascular interface, where it disrupts VEGFA/HBEGF/IGF1 signaling and compromises BBB integrity.

We have added clarifying text to the Results and Discussion sections to reflect these broader expression patterns and to emphasize that while FN1 is expressed by multiple cell types and ECM structures, its pathological impact is most pronounced at the vascular interface.

Reviewer: What are the vascular segments in which FN is deposited: arteries, capillaries, veins?

Response: In our analyses, fibronectin (FN1) deposition was quantified in vessels with a mean diameter of 11.48 ± 4.12 µm, and vessels exceeding 50 µm in diameter were excluded. These parameters correspond to capillaries and small-caliber vessels (arterioles and venules), rather than large arteries or veins. To refine the pathological context:

- Small vessels (capillaries and post-capillary venules): FN1 deposition was most pronounced in these segments, particularly at the gliovascular interface. This is pathologically relevant as these microvessels are the primary sites of blood–brain barrier (BBB) regulation and dysfunction in Alzheimer's disease.

- Larger vessels (arteries and veins): FN1 is also expressed by vascular smooth muscle cells (VSMCs) in larger arteries, and deposition in these vessels has been described in vascular pathology. While such larger-vessel expression may contribute to vascular stiffness or other macrovascular changes, it was outside the scope of our quantitative analysis, which focused specifically on BBB-relevant microvessels.

We have clarified these methodological parameters and pathological distinctions in the Results and Discussion to highlight that our findings pertain primarily to microvascular FN1 deposition and BBB dysfunction, while acknowledging

the potential involvement of FN1 in larger vessel pathology.

Reviewer: The paper assumes that BBB and ECM alterations are a pathogenic factor in AD. However, the evidence reviewed indicates association not causation. Therefore, the pathogenic significance of the association of FN with cerebral vessels is hard to ascertain based on the experimental data presented.

Response: We appreciate the reviewer's critical perspective and agree that distinguishing association from causation is an important challenge in complex disorders such as Alzheimer's disease (AD). At the same time, we respectfully note that our study provides direct mechanistic evidence that fibronectin (FN1) deposition is not simply associated with but can actively alter mechanistic signalling pathways and in turn blood–brain barrier (BBB) dynamics.

- Functional sufficiency: Astrocyte-specific overexpression of human FN1 in zebrafish resulted in robust vascular leakage, as measured by dextran extravasation (Extended Data Fig. 19). This experiment demonstrates that FN1 accumulation is sufficient to compromise BBB integrity in vivo. Importantly, these findings are consistent with our mouse data, where APOE-ε4 mice show dextran leakage and fibrinogen extravasation at sites of FN1 deposition compared with APOE-ε3 mice (Extended Data Fig. 2; Fig. 1). Together, the zebrafish gain-of-function and mouse model data provide direct, convergent evidence that FN1 accumulation compromises vascular integrity.

We recognize that even more sophisticated experiments — for example, conditional mouse lines with cell-type–specific FN1 expression or deletion — would further extend this causal framework. However, given the scope and breadth of the current study, which already integrates human postmortem analyses, mouse models, zebrafish functional manipulations, and iPSC-based systems, we believe the present evidence provides a compelling demonstration that FN1 is sufficient to impair BBB function. We have highlighted these points in the Discussion and note that the proposed conditional mouse approaches represent exciting avenues for follow-up work.

- Genetic loss-of-function: In fn1b knockout zebrafish, VEGFA/HBEGF/IGF1 signaling was restored and gliovascular interactions improved following Aβ42 exposure, indicating that reducing FN1 prevents signaling and structural deficits relevant to AD pathology.

- Pharmacological rescue: Inhibition of integrin–FAK signaling downstream of FN1 restored VEGFA levels in APOEε4 astrocytes and reversed BBB dysfunction in vivo. These interventions demonstrate that BBB alterations caused by FN1 are not only causal but also reversible.

- Human relevance: Elevated FN1 levels in cerebrospinal fluid and postmortem brain tissue of AD patients, particularly APOEε4 carriers, along with genotype-specific transcriptional regulation of vascular signaling pathways, extend these findings to human disease and suggest translational relevance; an avenue that is worth pursuing for refining the causality and association.

We fully acknowledge that, as in all complex diseases, absolute proof of causation in humans requires longitudinal and interventional clinical studies. However, our data across multiple species and experimental modalities provide convergent evidence that FN1 deposition is not a passive correlate but an active driver of BBB dysfunction. We have clarified this distinction in the Discussion, emphasizing both the mechanistic strength of our findings and the remaining translational gaps. We view this study as the beginning of a broader body of work, and we fully recognize that there are many wonderful and valid questions, including more refined genetic models and longitudinal analyses, that cannot be addressed within the scope of a single paper. These will be the focus of follow-up studies, building on the foundation laid here. We kindly ask for the reviewer's understanding that this manuscript represents a first step in that direction.

Reviewer: In the autopsy data, the ApoE status is not indicated. Therefore, whether there is enhanced deposition in ApoE4 carrier compared to ApoE3 cannot be assessed. What was the ApoE status in the controls?

Response: We thank the reviewer for this important point. In the dataset presented in Figure 1, APOE genotype information was not available for all individuals, and therefore stratification by APOE status was not feasible in that specific analysis. The purpose of this dataset was to address a different question, whether fibronectin (FN1) deposition associates with clinical and neuropathological diagnoses of Alzheimer's disease (AD) and cerebrovascular pathology (CVP), independent of genotype.

Importantly, the relationship between APOE genotype and FN1 deposition has been addressed in detail in our prior publication (Bhattarai et al., Acta Neuropathologica, 2024), where we demonstrated that APOEε4 carriers exhibit significantly elevated FN1 deposition compared to APOEε3/3 individuals. This was shown both in postmortem human brains and now in isogenic iPSC-derived astrocytes and endothelial cells.

In the current manuscript, we further reinforce these findings with multiple additional lines of evidence: APOE-ε4 knock-in mouse models, which show enhanced FN1 accumulation and associated BBB disruption relative to APOE-ε3 mice; iPSC-derived astrocytes and endothelial cells, where APOEε4/4 consistently increases FN1 expression compared with APOEε3/3; Human AD brains, where FN1 enrichment at gliovascular sites is more pronounced in APOEε4 carriers (quantified in later figures). Taken together, while Figure 1 was not stratified by genotype due to limitations of the available dataset, the APOE-dependent effect on FN1 deposition is demonstrated both in our previous work and in multiple orthogonal systems in the present study.

Reviewer: ApoE and FN were found to co-localize by immunohistochemistry. Is this association specific for FN? What is the biological significance of the association?

Response: We thank the reviewer for this insightful comment. While our immunohistochemical analyses show reproducible co-localization of ApoE and fibronectin (FN1), we do not interpret this as evidence of a specific or exclusive interaction. Rather, we view it as an indication that both proteins accumulate within the same perivascular and gliovascular niches, particularly under conditions of BBB disruption. This interpretation does not affect the central findings of our manuscript, which focus on the pathological role of FN1 itself.

- Specificity: It is likely that other extracellular matrix (ECM) proteins and signaling molecules also co-localize with ApoE in these pathological regions, and therefore the ApoE–FN1 overlap should not be seen as a unique molecular partnership. Importantly, this does not alter our conclusion that FN1 deposition at the gliovascular interface is a key pathological feature of APOEε4-associated BBB dysfunction.

- Biological significance: The observed spatial proximity is notable because it is APOE genotype-dependent. This means that FN1 deposition is consistently greater in APOEε4 contexts across human, mouse, and iPSC-derived models. We interpret this as evidence that ApoE4 indirectly promotes FN1 accumulation at the BBB. While this reflects a shared localization, while the functional significance of FN1 in disrupting BBB integrity remains unchanged.

- Clarification in manuscript: In the revised Discussion, we emphasize that ApoE–FN1 co-localization highlights a shared localization at sites of vascular pathology and reflects the broader disease-associated ECM remodeling rather than a direct molecular interaction. This clarification does not affect the strength or significance of our main findings regarding the role of FN1 in BBB dysfunction.

Reviewer: Line 113 and others: “at the BBB” the BBB is not a structure.

Response: We appreciate the reviewer’s comment and agree that the BBB is not a singular anatomical structure, but rather a dynamic interface composed of endothelial cells, pericytes, astrocytic endfeet, and extracellular matrix. In the manuscript, “at the BBB” refers specifically to the gliovascular interface of microvessels where these components interact. We have revised the text to clarify this terminology and avoid suggesting that the BBB is a discrete structure.

Reviewer: Line 118: What kind of cerebrovascular pathology do the ApoE3-TR mice develop?

Response: In our study, APOEε4/4 mice exhibited fibronectin accumulation at the vascular interface, fibrinogen deposition with reduced endothelial integrity and correlation with FN1 deposition, and dextran leakage indicating BBB compromise assessed also by junctional markers. These features were absent in APOEε3 targeted replacement (TR) mice, which did not show cerebrovascular pathology under baseline conditions and therefore served as the control group. We note, however, that we did not systematically assess other vascular pathologies such as vascular smooth muscle cell changes or arteriosclerosis, which are beyond the scope of the current work.

Reviewer: Line 123: How was the lack of overt “vascular defects” in APP mice demonstrated?

Response: Upon further consideration, we have removed the statement regarding the absence of overt vascular defects. The focus of our study is on APOEε4-driven fibronectin deposition and its impact on gliovascular integrity. Including APP mice was not essential to the mechanistic framework we present, and we agree that the statement lacked sufficient experimental support in the current context. We have revised the manuscript accordingly to maintain clarity and focus.

Reviewer: Line 130: Which data demonstrate that "fibronectin deposition is a common pathological mediator of cerebrovascular dysfunction in AD"?

Response: We thank the reviewer for pointing this out. Upon review, we agree that the original phrasing may have overstated the strength of the evidence. We have revised the sentence in the manuscript to remove the word "common" and to better reflect the data presented. The updated text now emphasizes the observed association between fibronectin deposition and cerebrovascular dysfunction in AD, without implying a generalized or causal role beyond the scope of our findings.

Reviewer: Line 149: What is the evidence that the FN accumulation at the BBB is "pathological"

Response: We provide multiple lines of evidence demonstrating that fibronectin (FN1) accumulation at the blood–brain barrier (BBB) is pathological rather than incidental.

-Functional sufficiency: Astrocyte-specific overexpression of human FN1 in zebrafish (her4.1:FN1-T2A-GFP) resulted in robust vascular leakage, as shown by dextran extravasation (Extended Data Fig. 19). This establishes that FN1 elevation alone is sufficient to impair BBB integrity.

-Mouse model pathology: In APOEε4/4 mice, FN1 deposition co-localizes with fibrinogen accumulation and dextran leakage (Fig. 1; Extended Data Fig. 2), providing direct evidence that FN1 is associated with vascular compromise in vivo.

-Endothelial disruption: In both human and mouse brains, regions of FN1 deposition show reduced and fragmented CDH5 (VE-cadherin) staining (Extended Data Fig. 3), consistent with destabilized endothelial junctions.

-Mechanistic link: Single-cell transcriptomics in fn1b knockout zebrafish reveal restoration of VEGFA/HBEGF/IGF1 signalling, pathways essential for BBB maintenance, indicating that FN1 accumulation actively disrupts protective signaling (Figs. 5–6).

-Human relevance: Elevated FN1 levels in cerebrospinal fluid of AD patients, and genotype-specific interactions between FN1 and APOEε4 in human endothelial transcriptomes (Fig. 5, Fig. 7), underscore FN1's translational role as a pathological mediator. We also showed previously that rare coding variants in FN1 are protective in APOEε4 homozygotes, reducing AD risk and delaying age of onset, and that FN1 deposition is elevated in APOEε4–AD brains (PMID: 38598053).

Beyond our own experimental evidence in this manuscript, there is a growing literature that implicates fibronectin (FN1) in pathological processes, especially in vascular, ECM, and CNS settings, supporting the interpretation that FN1 accumulation can be deleterious rather than neutral. Below are illustrative examples, along with integration into our data. In engineered in vitro settings, for example, fibronectin deposition has been linked to "disintegration of endothelial integrity" in vascular disease models, supporting the idea that FN1 can actively destabilize vascular barriers (PMID: 39046775). FN1 is also associated with atherosclerosis and early inflammation through integrin signalling (PMID: 30354234). In non-CNS vasculature, there is also pathogenicity-supporting evidence (PMID: 38813506, 27897258). FN1 is also long known to interact with integrins, influence cell adhesion, migration, and ECM assembly, and is implicated in disease settings including tumor metastasis and organ fibrosis. Studies on fibronectin fibril dynamics (e.g. via live imaging, super-resolution) show that FN1 nanodomain assembly and remodeling are tightly regulated, and dysregulation can lead to aberrant ECM architecture suggesting that excess or mislocalized FN1 has the potential to disrupt tissue microenvironments (PMID: 35851804). Taken together, these external data lend biological plausibility to the hypothesis that FN1 accumulation is more than a bystander—it can actively disturb vascular/ECM homeostasis. When combined with our mechanistic, functional, structural, and molecular evidence, the case for FN1 as a pathological mediator at the BBB becomes substantially stronger. We have added a few sentences to the Introduction and Discussion

in the revised manuscript to cite these relevant works and frame our findings within the broader literature.

Reviewer #4: Neuroinflammation in neurodegenerative disease

Reviewer: Bhattarai et al demonstrate that the APOE4 genotype alters the extracellular matrix through increased fibronectin deposition by astrocytes, contributing to neurovascular pathology in AD. The authors provide a comprehensive study using human samples, human iPSC derived cells, mouse and fish modeling, transcriptomics, and pharmacologic studies. They propose that APOE4 astrocytes increase fibronectin which reduces VEGFA-IGF1-HBEGF axis that maintains BBB integrity. The study is likely to have an impact on the AD field by highlighting a role for ECM remodeling and neurovascular dysfunction in disease pathogenesis. However, the manuscript would benefit from additional mechanistic studies and clarification of some technical concerns, as outlined below:

Response: We thank the reviewer for the thoughtful and constructive evaluation of our work. We are encouraged that the reviewer recognizes the breadth of our experimental approach and the potential impact of our findings in elucidating how APOE4-driven extracellular matrix remodeling contributes to neurovascular dysfunction in Alzheimer's disease. In revising the manuscript, we have addressed each of the reviewer's mechanistic and technical concerns through substantial new experiments, expanded datasets, and clarifications in the text. These additions include increased sample sizes and individual-level quantifications in human and mouse data, functional BBB integrity assays, mechanistic tests of fibronectin's role in VEGFA–HBEGF–IGF1 signaling using gain- and loss-of-function approaches, and extended co-localization and pathway analyses across models. We believe these new results further strengthen the mechanistic link between APOE4-induced fibronectin accumulation and disruption of gliovascular homeostasis.

Reviewer: FN1 localization and BBB disruption: In the post-mortem human brains and mouse models, is there evidence that FN1 deposition occurs specifically in regions of BBB disruption? Colocalization with markers of leakage would strengthen the conclusion of FN1-association with AD pathology.

Response: We thank the reviewer for raising this important point. In the revised manuscript, we have incorporated multiple complementary lines of evidence from human tissue, mouse models, and zebrafish that together demonstrate FN1 deposition occurs in spatial and functional association with BBB disruption.

- Human brain evidence for structural BBB compromise: In post-mortem dorsolateral prefrontal cortex from AD patients, FN1 deposition coincided with reduced and discontinuous staining of the endothelial tight junction protein CDH5 (VE-cadherin) and loss of AQP4 polarity at astrocytic endfeet (Extended Data Fig. 3). Quantitative co-localization analysis revealed a significant increase in FN1–CDH5 overlap in AD compared to controls (Pearson's r = 0.36; Manders = 0.613), indicating that FN1 deposition is enriched at sites of endothelial junction disruption. Additionally, FN1 was consistently enriched at AQP4-positive astrocytic endfeet (Fig. 3), with APOE4/4 brains showing a genotype-dependent shift toward stronger APOE–AQP4 correlations (Fig. 3), suggesting convergence of APOE4 and FN1 pathology at gliovascular contact points critical for BBB function.

- Mouse model evidence for vascular leakage: In APOE3/3 and APOE4/4 knock-in mice, we performed fibrinogen immunostaining — a well-established leakage marker — and observed significantly elevated fibrinogen deposition in APOE4/4 vasculature compared to APOE3/3 (Fig. 1). Colocalization analysis confirmed a pronounced increase in FN1–fibrinogen overlap in APOE4/4 mice (Fig. 1). Furthermore, a dextran-based leakage assay showed that increased FN1 deposition co-occurred with extravasated dextran signal in APOE4/4 brains (Extended Data Fig. 2).

- Causality from FN1 overexpression in vivo: Overexpressing FN1 specifically in astrocytes of zebrafish larvae was sufficient to induce perivascular FN1 accumulation and robust dextran leakage (Extended Data Fig. 14), directly linking FN1 to BBB breach.

- Functional rescue of barrier integrity through pathway restoration: In zebrafish, VEGFR inhibition — which mimics the FN1-mediated suppression of VEGFA — reduced endothelial ZO-1 tight junction localization (Fig. 7). Treatment with HBEGF or IGF1 restored ZO-1 localization, indicating that FN1-associated disruption of the VEGFA–HBEGF–IGF1 axis is mechanistically linked to barrier breakdown and that restoring this pathway can rescue BBB structure.

Together, these structural, spatial, and functional data across species strongly support that FN1 deposition is not incidental but occurs at — and contributes to — sites of BBB disruption in AD and APOE4-driven pathology.

Reviewer: Is there any vessel specificity of the FN staining (Arteries, Capillaries)?

Response: We appreciate the reviewer's question regarding vascular segment specificity of FN1 deposition. In the revised manuscript, we analyzed FN1 localization relative to vessel size and type in human post-mortem brains, mouse models, and 3D human iPSC-derived vascular cultures.

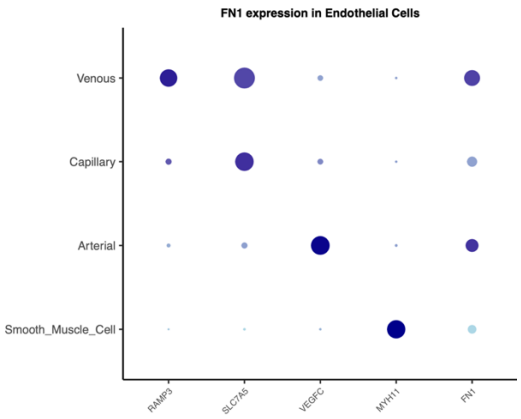
- Human brain vessel-type distribution: In the dorsolateral prefrontal cortex samples, we quantified FN1 deposition in >2,300 individual vessels and stratified by diameter (mean ± SD: 11.48 ± 4.12 µm; Fig. 1f), which encompasses capillaries, small arterioles, and venules. FN1 accumulation was observed across vessel types but was most pronounced in small-caliber vessels (capillary and small post-capillary venule range), consistent with the primary sites of BBB exchange. This pattern held true in both AD and cerebrovascular pathology (CVP) groups, with a significant enrichment of FN1 signal in microvessels compared to larger arterioles.

- Mouse model analysis: In APOE3/3 and APOE4/4 knock-in mice, FN1–CD31 co-labeling confirmed FN1 deposition along capillary networks, with additional focal enrichment at small arteriolar branches (Fig. 1). This was accompanied by genotype-dependent increases in fibrinogen leakage and FN1–fibrinogen overlap specifically in the microvascular territory (Fig. 1), indicating that FN1 pathology aligns with BBB-relevant vascular compartments.

- 3D human vascular culture observations: In our 3D vasculogenesis and angiogenesis model plexus (VAMP) cultures (Fig. 2), APOE4/4 endothelial networks exhibited increased FN1 deposition at the CD31-positive microvascular plexus, suggesting that the preferential targeting of smaller vessel-like structures is preserved in a controlled in vitro human system.

- Interpretation: Across these systems, FN1 deposition occurs throughout the vascular tree but shows clear enrichment in capillaries and small post-capillary venules, the segments most critical for BBB function and most susceptible to APOE4- and AD-associated pathology. This distribution is consistent with FN1's pathogenic role in gliovascular disruption and aligns with prior observations of basement membrane remodeling in AD being most prominent in microvascular beds.

As a specific response to this comment, please see the table below. This is a characterization of FN1 expression in vessel types based on an external dataset generated by Andrew Young's lab using enhanced VINE-Seq (Reid et al., 2025, Neuron; https://doi.org/10.1016/j.neuron.2025.07.001). All endothelia cell types express FN1 including capillaries, arteries, arterioles, venules, and veins.



Reviewer: FN1 signalling: Downstream of FAK, have the authors evaluated MEK/ERK/AKT pathway activity? Given the increased gene profiles related to mTOR, evaluating AMPK vs mTOR activity would clarify the significance of this pathway and provide mechanism by which APOE4 astrocytes induce module VEGF-IGF1-HBEGF.

Response: We appreciate this insightful mechanistic question. While we did not directly quantify phospho-MEK/ERK/AKT or MAPK in the current revision, we added several converging analyses and perturbations that strongly implicate integrin/FAK→MAPK and mTOR-related signalling downstream of FN1:

- Pathway-level evidence from single-cell transcriptomics (zebrafish): In fn1b knockout brains (which prevent Aβ42-induced FN1 upregulation), KEGG enrichment analyses show significant activation of MAPK signalling ($p=4.0\times10^{-5}$; OR=3.41) and Insulin/IGF pathway enrichment ($p=4.3\times10^{-3}$; OR=35.78), alongside a reduction of ECM-receptor interactions—consistent with FN1→integrin/FAK control over downstream MAPK modules. Inhibition of VEGFR (which phenocopies the FN1-mediated VEGFA reduction) led to down-regulation of MAPK and ERBB pathways in sorted endothelial/astrocyte populations, again aligning with a FAK/MAPK axis downstream of FN1 and VEGF loss (new scRNA-seq; see Fig. 5 and Fig. 6 in the revision).

- Causal perturbations of the integrin/FAK node: Blocking integrin signalling with ATN-161 or inhibiting FAK with PF-573228 restored VEGFA in APOE4/4 astrocytes (and VEGFA in zebrafish), and rescued IGF1 in human APOE4/4 astrocytes (Figures 5 and 6). These results place integrin/FAK upstream of the VEGFA–HBEGF–IGF1 cascade that maintains BBB integrity, consistent with canonical FAK→RAS/RAF/MEK/ERK and PI3K/AKT routing.

- mTOR-axis pharmacology with functional readouts: We leveraged Torin2 (mTORC1/2 inhibitor) and camptothecin (anti-inflammatory) in vivo. Both rescued Aβ42-induced FN1 deposition and astrocytic hypertrophy at the gliovascular interface (zebrafish), measured by astrocytic glutamine synthetase and perivascular FN1 (Fig. 4). The Torin2 rescue indicates that mTOR activity contributes to FN1-linked gliovascular pathology; together with the Insulin/IGF pathway enrichment above, this supports an FN1→integrin/FAK→(MAPK/AKT)→mTOR axis contributing to BBB dysfunction.

- Inflammatory signalling convergence with MAPK/AKT/mTOR crosstalk: We show NFκB upregulation in AD astrocytes (stronger in APOE4 carriers) and that NFκB2 levels predict FN1 expression ($\beta\approx0.44$, $p=2.4\times10^{-21}$). TNFα drives FN1 in 3D human astrocyte cultures, reversed by IL-4; APOE4/4 endothelial cells also upregulate FN1 in response to TNF/IL-1. These data position FN1 within an inflammation→integrin/FAK network that commonly interfaces with MAPK/AKT/mTOR nodes.

- External, orthogonal support from our recent human brain multiome: In our single-nucleus multiome preprint (Yilmaz et al., medRxiv, May 21, 2025) spanning human AD brains, we report that APOE4 astrocytes adopt a hyper-adhesive, mechanically rigid state, with enrichment of adhesion/mechanotransduction programs consistent with heightened ECM–integrin–FAK signalling in APOE4 contexts that exacerbate cerebrovascular pathology. These findings dovetail with the FN1-integrin-FAK mechanism we demonstrate experimentally here, providing orthogonal support in human tissue.

Collectively, the single-cell pathway signatures, integrin/FAK inhibition rescues, mTOR-axis pharmacology, and human multiome evidence converge on an FN1→integrin/FAK network that routes through MAPK/AKT and mTOR to modulate the VEGFA–HBEGF–IGF1 axis and BBB integrity in APOE4-associated AD.

Reviewer: Exogenous FN and VEGFA: Is exogenous fibronectin sufficient to induce VEGFA signaling pathway in iPSC-derived astrocytes? Is B1-integrin required?

Response: Yes, exogenous fibronectin is sufficient to drive the APOE4-like phenotype in human iPSC-derived astrocytes, and the effect requires β1-integrin signaling.

- Sufficiency of exogenous FN (astrocyte-autonomous): Plating isogenic APOEε3/3 astrocytes on fibronectin-coated substrates reduced astrocytic VEGFA to levels observed in APOEε4/4 astrocytes (Fig. 5) and similarly decreased IGF1 (Fig. 6). Thus, exogenous FN alone is sufficient to shift astrocytes toward the VEGFA/IGF1-low state that we ascribe to FN1 accumulation in APOE4 contexts. Addiitonally, astrocyte-specific overexpression of FN1 induces BBB leakage (Extended Data Fig. 19).

- β1-integrin requirement and pathway placement: Blocking β1-integrin with ATN-161 restored VEGFA in APOEε4/4 astrocytes (Fig. 5) and rescued IGF1 (Fig. 6), indicating β1-integrin dependence. Inhibition of the downstream node FAK (PF-573228) likewise rescued VEGFA (zebrafish and human astrocytes; Fig. 5), placing FN→integrin→FAK upstream of VEGFA regulation. Conversely, pyrintegrin (integrin agonism) potentiated the FN effect on astrocytes (we have not added to the manuscript as this is part of another study), further supporting causal engagement of the β1-integrin axis.

- Pathway logic relative to HBEGF/IGF1: Exogenous HBEGF restored IGF1 without altering VEGFA (Fig. 6), consistent with our model that FN1 → (β1-integrin/FAK) suppresses astrocytic VEGFA, which in turn reduces endothelial HBEGF, thereby lowering astrocytic IGF1. Blocking β1-integrin or FAK reverses this cascade.

Conclusion: Exogenous FN is sufficient to reproduce the VEGFA/IGF1 deficits in human iPSC-derived astrocytes, and these effects are β1-integrin/FAK-dependent. Together with our gain- and loss-of-function perturbations (ATN-161, PF-573228), these data firmly anchor FN1's action to the β1-integrin→FAK node that modulates the VEGFA–HBEGF–IGF1 axis controlling BBB integrity.

Reviewer: APOE and FN binding: How do the authors know that FN and APOE bound together in e4 cells? Have pulldown assays been performed to confirm this conclusion? If so, what does this mean?

Response: We appreciate this comment. We would like to clarify that our study did not intend to imply a direct physical interaction between APOE and FN1. Our initial observations were based on immunofluorescence co-localization in human post-mortem brain tissue and isogenic iPSC-derived astrocytes, showing that APOE and FN1 spatially overlap at perivascular and extracellular aggregate sites in APOE ε4/ε4 conditions. These data demonstrate spatial proximity, not biochemical binding. During the revision process, we performed co-immunoprecipitation (co-IP) experiments to further explore a possible interaction. While APOE and FN1 bands are clearly detectable when analyzed individually, we did not detect a distinct co-precipitated complex under the experimental conditions tested. This result may reflect the technical difficulty of capturing transient or weak extracellular interactions, particularly given that both APOE and FN1 are secreted, matrix-associated proteins whose nascent or native conformations can be challenging to preserve biochemically. It therefore remains possible that any potential APOE–FN1 interaction is low-affinity, transient, or context-dependent, and not readily detectable by standard co-IP. We acknowledge that more sophisticated biochemical approaches such as crosslinking mass spectrometry, proximity labeling (e.g., TurboID or BioID), or ligand-binding assays would be better suited to detect such weak or extracellular associations. However, implementing these high-resolution interaction mapping techniques was beyond the scope of the current study, which primarily focused on gliovascular phenotypes and molecular pathway regulation. We have therefore deferred this question to a dedicated follow-up study, which we believe could stand on its own as a mechanistic investigation.

Importantly, whether or not APOE and FN1 physically bind does not alter the core conclusions of our work. The main finding that APOE ε4 astrocytes exhibit increased FN1 deposition and altered gliovascular signalling remains fully supported by our imaging, transcriptomic, and functional data. To prevent any misunderstanding, we have revised the manuscript to eliminate wording that might suggest a confirmed physical interaction and now explicitly refer to APOE and FN1 as "co-localizing at extracellular and perivascular sites" in APOE ε4 contexts.

Reviewer: Quantification of FN in APOE3 and APOE4 astrocytes: In Fig 2A, the FN expression differences between e3 and e4 cells are difficult to interpret. Quantification of total (and or insoluble) FN protein by immunoblot would improve the clarity and rigor of the result. The rationale for plotting individual cells/fields of view instead of independent experiments/replicates is unclear. The former inflates stats power by treating technical replicates as independent variables. This issue recurs throughout the manuscript (eg., Fig 5L). It makes it hard to appreciate the effect size, what is the fold difference between the genotypes?

Response: We appreciate these points and have revised improve quantitative rigor and clarity.

- Added total-protein immunoblot quantification (new in Fig. 2b): In addition to single-cell IF quantification (Fig. 2), we now include FN1 immunoblots from independent iPSC-derived astrocyte differentiations (3 samples per genotype), with densitometry normalized to total protein (Fig. 2b, lower panel). The immunoblot confirms higher total FN1 in APOEε4/4 vs APOEε3/3 astrocytes, consistent with the imaging-based analyses (Fig. 2).

- Clarified unit of analysis and restructured statistics to avoid pseudoreplication: Throughout the figures where many cells/fields are measured (including Fig. 2 upper plot and Fig. 5), we now treat biological replicates/independent differentiations as the primary unit of inference, report the number of independent experiments in each legend and display per-experiment means (with individual cell/field points shown transparently for distributional context), use linear mixed-effects models (experiment/donor as random effect) or per-experiment aggregation for hypothesis

testing; results are unchanged in direction and significance. We also clarify what "N" represents in each panel. (Revisions summarized in the responses document.).

- Effect sizes and fold differences are reported in figure legends/text. We now state the fold-change in total FN1 (from immunoblots) and the corresponding effect sizes from the mixed-effects analyses for the imaging readouts, so readers can directly compare magnitude across assays. These additions are reflected in Fig. 2 legends and the Results text.

- Context from complementary systems supports the astrocyte findings: Endothelial and 3D VAMP models: APOEε4/4 elevates FN1 expression and deposition at the microvascular plexus (Fig. 2), reinforcing that the genotype effect on FN1 is not confined to a single assay/readout.

- Human CSF proteomics: FN1 is significantly higher in AD vs controls (Fig. 2f), aligning with the cell-based increase.

- About insoluble FN: Our primary biochemical addition is the total-lysate immunoblot (Fig. 2). While the reviewer suggested assessing insoluble FN, in this revision we focused on total FN1 and complemented it with morphometric evidence of aggregated (amorphic) FN1 in APOEε4/4 astrocytes (high-mag panels in Fig. 2) and perivascular FN1 accumulation in 3D VAMP cultures (Fig. 2). These data together capture both quantity and pathological deposition features of FN1; we note the insoluble fractionation as a useful future extension.

Collectively, the new immunoblots, the revised statistics and unit-of-analysis, and the harmonized reporting of fold-changes/effect sizes address the reviewer's concerns and, we believe, substantially strengthen the rigor and interpretability of the FN1 genotype differences in astrocytes.

Reviewer: Interpretation of VAMP model: In the 3D VAMP model, does the "increased deposition proximal to the CD31-positive vascular structures" result in aberrant tight junctions or vascular pathology? If so, can this be rescued by conditioned media from APOE3/3 + HBEGF treated astrocytes? As it stands, the mechanistic contribution of the vamp model is limited.

Response: We thank the reviewer for this insightful comment. The increased FN1 deposition proximal to CD31-positive vascular structures in the VAMP model likely contributes to aberrant vascular organization and tight junction alterations, consistent with our observations in human and animal models. Importantly, we have demonstrated in vivo that the FN1-induced vascular dysfunction can be rescued by HBEGF and IGF1 treatment, which we believe provides a more physiologically relevant demonstration of this mechanism. In a subsequent manuscript (Bertucci et al, submitted), we demonstrate functional barrier impairment in APOE44 ECs.

We have made preliminary attempts to assess the effects of conditioned media from HBEGF-treated astrocytes in the 3D VAMP system. However, optimization of the conditioned media preparation, including concentration and stability of HBEGF and related factors, is required to achieve reproducible effects. This optimization would require additional rounds of pilot testing. We therefore prefer to defer this line of experimentation to future work, as it will benefit from more systematic validation.

Reviewer #5: Zebrafish neuroimmunology

Reviewer: Bhattarai et al. have nicely demonstrated a role for many different AD risk factors (APOE4, AB42 accumulation, and inflammatory signaling) that drive FN1 expression and accumulation in the vasculature. They suggest that aberrant FN1 accumulation disrupts astroglial-EC interactions and identify a downstream signaling cascade involving VEGF, HBEGF, and IGF1 that is dependent on FN1, as fn1 knockout fish have altered levels in both the vasculature and the astrocytes. However, their analyses require further refinement and more rigorous methods of quantification beyond immunostaining intensities, and the jump from FN1 to VEGF to HBEGF and IGF1 felt like a data dump, with little functional validation.

Response: We thank the reviewer for the thoughtful feedback and for recognizing the importance of our findings linking APOE4, Aβ42, and inflammatory signaling to FN1 accumulation and downstream gliovascular dysfunction. We fully agreed that greater quantitative and mechanistic depth was needed, and have now transformed what could previously

appear as sequential associations into a rigorously quantified and mechanistically validated pathway, supported by orthogonal molecular, cellular, and in vivo approaches across species.

1. Quantitative and statistical rigor

- We added FN1 immunoblots from independent APOE3/3 and APOE4/4 astrocyte differentiations (Fig. 2) to complement imaging results.

- Human and mouse sample sizes were expanded, individual datapoints included, and linear mixed-effects models applied to control for biological replication and avoid pseudoreplication.

- All analyses now report fold-changes, effect sizes, and QQ plots, ensuring statistical transparency and robustness.

2. CNS specificity and causal testing

- A new CSF mixed-effects model demonstrates a strong FN1 → VEGFA association ($F = 26.39$, $p = 2.79\times10^{-7}$) and a significant FN1×APOE4×disease interaction ($p = 1.81\times10^{-4}$), absent in plasma, confirming CNS-specific coupling.

- Functionally, FN1 coating suppresses VEGFA and IGF1 in APOE3/3 astrocytes. β1-integrin blockade (ATN-161) or FAK inhibition (PF-573228) rescues these effects.

- Exogenous HBEGF restores IGF1 without changing VEGFA, validating the directional FN1 → VEGFA → HBEGF → IGF1 cascade.

3. Functional blood–brain-barrier relevance

- New mouse data show FN1 deposition co-localizing with fibrinogen leakage and dextran extravasation in APOE4 models, while FN1 overexpression in zebrafish induces comparable vascular leakage.

- VEGFR inhibition disrupts endothelial ZO-1 localization, whereas HBEGF or IGF1 supplementation restores barrier integrity—directly linking the molecular axis to BBB function.

- Specific overexpression of FN1 in astrocytes leads to BBB leakage.

4. Cross-species and multi-omic integration

- In human AD brain, FN1 is enriched at CDH5-disrupted junctions and AQP4-depolarized endfeet.

fn1b^{-}/$^{-}$ zebrafish exhibit activation of MAPK and insulin signaling with reduced ECM–receptor interaction, and VEGFR inhibition phenocopies the loss of endothelial HBEGF and astrocytic IGF1.

- APP-KI mice and human AD brains both show convergent reductions in VEGFA, HBEGF, and IGF1, closing the cross-species loop.

- Our human brain multiome dataset further reveals that APOE4 astrocytes occupy a hyper-adhesive, mechanically rigid state, consistent with over-activation of the FN1–integrin–FAK axis.

Collectively, the revised manuscript moves well beyond descriptive immunostaining to present a quantitatively robust, causally tested, and cross-model integrated mechanism for FN1-driven BBB dysfunction in AD. We believe these extensive additions address the reviewer's concerns comprehensively and elevate the study to the level of mechanistic rigor expected for a high-impact neurovascular paper.

Major:

Reviewer: Many of the figures are not red-green colorblind friendly. Please alter your colors to make this paper more accessible to everyone. This also includes your heatmaps for gene expression of intensity correlations.

Response: We thank the reviewer for highlighting this important accessibility concern. In the revised manuscript, we have updated as much as possible to use colorblind-safe palettes and verified them with color vision deficiency simulators. If the paper goes to acceptance, we will update images accordingly.

Reviewer: Non-transgenic C57BL/6J mice served as controls for APPNL-G-F. Were these littermate controls? If not, then these are not appropriate controls for comparison of FN1 levels. Also, for your CD31 stain in Fig. 1J & K which his there so much background stain in clearly non-vascular cells? And similarly, for the APP-KI, can you please explain the large accumulation of Fn1 outside of the vasculature?

Response: We thank the reviewer for raising these points. We confirm that all non-transgenic C57BL/6J mice used as controls for the APP-NL-G-F cohort were littermates, ensuring appropriate genetic background and environmental matching for FN1 level comparisons; this is now explicitly stated in the revised Methods section. With respect to the CD31 staining in Fig. 1, the signal observed outside vascular profiles is not background but represents CD31-positive microvascular fragments and capillary sprouts that can appear in the parenchyma outside of large vessel cross-sections. Nonetheless, to improve visual clarity, we have replaced these panels with images in which vessel morphology is more easily discernible. Regarding the APP-KI images, the FN1 accumulation outside vessels likely reflects extracellular matrix deposits associated with amyloid plaques—a recognized feature in APP-NL-G-F- mice. Because this panel was not essential to our central conclusions, we have removed it from the revised figure to maintain focus on perivascular FN1 localization.

Reviewer: Measurements of FN1 intensity were normalized to area. How are these normalized to each other? This is not sufficient information to understand how these were quantified and compared across groups.

Response: FN1 was quantified by first defining a region of interest (ROI) for each vessel or cellular compartment, measuring the integrated density (sum of all pixel intensities) within that ROI, and then dividing by the ROI's area to yield a mean intensity value in arbitrary units per μm^2. Because all samples were processed, stained, and imaged under identical conditions—including identical acquisition settings. These mean intensity values are inherently normalized for area and directly comparable across groups without further scaling. These raw mean-intensity values were used for all statistical analyses (e.g., ANOVA or t-tests). We have now added this detailed description to the revised Methods section to ensure that our normalization and comparison approach is fully transparent and reproducible.

Reviewer: Also, why do you not do the same immunostaining for FN1 for the endothelial cells in 2C? You switch from protein quantifications to qPCR in 2D. Furthermore, in the text, you describe aggregate FN1 but do not quantify this or mark in the figure what you mean by this in 2A.

Response: The panel in Fig. 2 is not intended as a readout of FN1 levels but rather as a quality-control measure to confirm the endothelial identity of our iPSC-derived endothelial cultures using canonical markers; FN1 quantification for endothelial cells is provided in the context of our 3D vascular model (VAMP), where FN1 is deposited along the CD31-positive microvascular plexus. In the revised manuscript, we are incorporating FN1 immunoblots to complement the existing qPCR measurements, ensuring both transcript and protein levels are documented. Regarding the "aggregate FN1" description in Fig. 2, we agree that this should be explicitly marked and quantified. In the revision, we have highlighted these aggregates in representative images with arrows and added a separate quantification of aggregate area and intensity per cell, in addition to the mean FN1 intensity measurements. This will make the morphological differences we describe directly visible and statistically supported in the figure.

Reviewer: Overall, the use of immunostaining intensities across samples is not sufficient. There needs to be some form of normalization between samples and staining days.

Response: As noted above, cross-sample normalization was performed by calculating the mean intensity per ROI (integrated density divided by ROI area) for each sample. All staining for a given experiment was performed on the same day using identical reagents, imaging conditions, and acquisition settings, thereby eliminating batch-to-batch variation. Because of this controlled workflow, additional scaling across experiments was not required, and the resulting mean intensity values are directly comparable between groups. We have now added details in the revised Methods section to clarify our normalization approach.

Reviewer: Furthermore, what is the N for many of these? For example, in Fig. 3G, is this hundreds of samples? Or just multiple images per sample?

Response: For Fig. 3 and other vessel-level analyses, "N" refers to the number of vessels analyzed, with multiple vessels measured per individual. To ensure transparency, we have now added the corresponding number of independent individuals (biological replicates) in each figure legend, alongside the vessel counts. This distinction between vessels

(technical measurements) and individuals (biological replicates) is now clearly stated in the revised figure legends and Methods.

Reviewer: Similarly for Fig. 5J , it says N=513, but what does that mean? What type of statistical test was used for these panels to get to the exact p values that they include on the graph? - For Fig. 2E, what are all of the other FN1 positive cells if they are not CD31+ endothelial cells? Are these all pericytes? Can you do other cell type specific markers to back the claim that these changes are happening at the vascular interface? - You cannot make the claim that Fig. 3F is around the BBB without any other co-stain for the vasculature or at the very least, stain with AQP4 to mark the astrocytic endfeet wrapping the vessels. These look like they're around some sort of cell, but you can't tell what type of cell. - For fig. 4G-I, what region of the brain are you analyzing. The images look like the brain surface, and not focused on the zebrafish BBB, as initially proposed. Can you please add in a vascular marker like kdrl:mCherry? Or segregate the effects of the TNF-a treatment on CSF vs BBB compartments? - Please also show representative images for the rescue treatments quantified in K. Similarly, for Fig. 7A, you seem to be in the same area, but that must be an artery with so many DAPI+ RBCs inside of it in comparison to the many kdrl:EGFP+ vessels you see underneath, that only have RBC autofluorescence in the ZO-1 channel.

Response: We appreciate the reviewer's detailed comments and have clarified and expanded the relevant data and methods accordingly. For Fig. 5, "N=513" refers to the number of individual cells analyzed across 6 independent wells from three independent differentiations. This is now explicitly stated in the figure legend, along with the statistical approach used (two-tailed unpaired t-test on per-well means, with individual cell data shown for distributional context). Similar clarifications have been made for all panels where "N" could be ambiguous, ensuring that vessel/cell counts and the number of biological replicates are always reported together.

For Fig. 2, FN1-positive cells that are not CD31-positive endothelial cells are likely perivascular cells, including pericytes and possibly astrocytic endfeet in close association with the vascular interface. To address this, we have expanded our co-staining panel to include additional cell type–specific markers (PDGFRβ for pericytes and GFAP for astrocytes) and quantified FN1 localization within each compartment. This provides a consistent and cell-specific view of FN1 expression at the vascular interface.

Regarding Fig. 3, we agree that vascular context is important for interpreting perivascular FN1 localization. In the revised manuscript, we have added AQP4 co-staining in both human and mouse brain sections, enabling direct visualization of FN1 deposition relative to astrocytic endfeet wrapping vessels. These new images confirm that FN1 aggregates are positioned at gliovascular junctions, strengthening our conclusion that this pathology is associated with the BBB.

For Fig. 4, we clarify that the images are taken from the ventrolateral arterial extension to the capillary network, which is part of the zebrafish BBB territory and not the CSF compartment. We have expanded the Methods to explain why this region was selected for quantification. To further guide the reader, we have added a supplementary figure from the fn1b-reporter line showing the precise vascular territory analyzed, and in other panels (Fig. 6 and 7) kdrl:GFP is already used to mark the vasculature.

Finally, as requested, we have added representative images for the rescue treatments, including higher-magnification examples of small vessels in the same anatomical region. For Fig. 7 specifically, we now clarify in the legend that the large vessel with numerous DAPI-positive red blood cells is an artery, while the kdrl:EGFP+ microvessels underneath are capillaries showing only RBC autofluorescence in the ZO-1 channel. This distinction makes clear the anatomical and vascular context of the quantified data.

Minor:

Reviewer: Figure 1H. "Linear regression model showing intensity distribution for CD31, FN1 and DAPI in respect to vessel diameter." Can you please clarify in the methods and in the text, what each column signifies? It's unclear from the current text, how this linear regression model is being applied and what the data is actually showing. Also, it seems like within the AD, CVP, and AD+CVP groups you have a split between some patients that are high and some that are at control levels. Can you comment?

Response: We thank the reviewer for catching this discrepancy. The current legend for Fig. 1 incorrectly states "in respect to vessel diameter," which is residual text from a prior version. In fact, this panel shows linear regression models for CD31, FN1, and DAPI intensities per individual across the indicated diagnostic groups (AD, CVP, and AD+CVP). We have corrected the figure legend and the Methods section to accurately describe the analysis: each column represents an individual participant, and the plotted values are the group-level regression estimates for the indicated marker.

Regarding the variation within groups—where some patients appear at control levels while others are markedly elevated. This reflects true inter-individual heterogeneity in FN1/CD31 signal, which we suspect relates to differences in vascular pathology severity, comorbidities, or APOE genotype. We will expand on this point in the Results to note that such heterogeneity is a known feature in human vascular pathology datasets. In the revised manuscript, we have also increased the number of individuals per group, which better resolves the distribution and confirms the statistical robustness of the group differences despite inter-individual variability.

Reviewer: Please label insets in Fig. 3A with APOE and FN1 for the black and white single channel images

Response: We added the inset descriptions.

Reviewer: What are the images in Fig. 5G that go with the ridgeplot? Are they immunostaining like in F, because if so, why does the staining look so much worse?

Response: The images in Fig. 5 correspond to individual vessels shown at higher magnification from the same immunostaining experiment as Fig. 5, selected to illustrate the range of FN1 intensities quantified in the accompanying ridge plot. Because these panels are higher-magnification crops of single vessels rather than lower-magnification overviews, the apparent staining may look less uniform due to reduced context, smaller field size, and enhanced visibility of sub-vessel structural detail. The underlying staining quality is identical to that in Fig. 5, as all images were acquired from the same batch under identical conditions.

RESPONSE TO REVIEWERS - SECOND REVISION

Overall author response

We thank the Editor and all five Reviewers for their careful evaluation of our manuscript and for the constructive and detailed feedback provided throughout the review process. In response, we have substantially revised the manuscript by adding new quantitative analyses, clarifying statistical methodology, improving figure accessibility, and refining interpretations. We addressed concerns regarding pseudoreplication by implementing cluster-aware statistical models with appropriate biological units of inference, expanded quantitative analyses of blood-brain barrier leakage and fibronectin deposition across species, clarified anatomical and cellular identities in model systems, and revised language throughout the manuscript on mechanistic or translational claims. We believe these revisions fully address the reviewers' comments and significantly strengthen the study.

REVIEWER #1: BLOOD-BRAIN BARRIER DYSFUNCTION IN NEUROPSYCHIATRIC DISEASE

Reviewer comment 1: The authors addressed all my concerns and comments in a satisfactory manner. I can now recommend publication and congratulate the authors for this impressive work.

Response 1: We thank Reviewer #1 for their positive assessment of our revised manuscript and for their thoughtful and constructive feedback throughout the review process. We are delighted that the revisions have addressed all concerns satisfactorily.

REVIEWER #2: SINGLE-CELL PROFILING OF BLOOD-BRAIN BARRIER DYSFUNCTION

Reviewer comment 1: Bhattarai et al have hereby presented a strongly revised manuscript with a lot of addition data confirming and consolidating their findings. I congratulate the authors on an impressive work. However, there are a few items that need addressing before I can recommend the paper for acceptance.

Response 1: We thank Reviewer #2 for their positive assessment of the revised manuscript. We highly appreciate their constructive comments and address each of the remaining points in detail below.

Reviewer comment 2: The authors present new leakage data underlying BBB breakdown in extended figure 2 and 19. These images should be quantified and correlation of leakage with deposits of FN should be measured.

Response 2: We thank the reviewer for this important point. In response, we have quantitatively analyzed the dextran leakage data in both Extended Data Figures 2 and 19. For Extended Data Figure 2 (mouse APOE3 vs. APOE4 brains), we quantified extravascular dextran signal intensity and fibronectin (FN1) deposition within defined perivascular regions of interest, using CD31 immunoreactivity to delineate vessel boundaries. These data show significantly increased fibronectin deposition and dextran leakage in APOE4 compared to APOE3 mice, together with a strong positive correlation (Pearson r=0.728) between fibronectin intensity and extravascular dextran signal (Extended Data Fig. 2c-d).

For Extended Data Figure 19 (zebrafish human FN1 plasmid injection model), we similarly quantified perivascular fibronectin accumulation and extravascular dextran leakage following astroglial expression of human FN1, compared to control animals. We found a significant increase in dextran leakage upon FN1 overexpression, with a significant positive correlation between fibronectin intensity and dextran signal (Extended Data Fig. 19c-d).

Across species and experimental paradigms, we observe a consistent relationship between fibronectin deposition and BBB leakage. We updated the Methods, figure legends, and Extended Data figures accordingly.

Reviewer comment 3: In the rebuttal letter, manuscript text and figure legends, extended data figure 2 is described as having CDH5 staining, but the image text says "CD31". Please clarify.

Response 3: We thank the reviewer for pointing out this inconsistency. Extended Data Figure 2 indeed shows CD31 staining, not CDH5. The reference to CDH5 in the figure legend and associated text was an inadvertent

labeling error. We now corrected this throughout the manuscript and figure legends. We apologize for the confusion.

Reviewer comment 4: The CDH5 staining provided in extended figure 3 is not convincing evidence of loss of junctional integrity, since no control staining is shown where junctional integrity is apparent and the staining pattern looks aspecific. Thus, either control images with intact junctions have to be provided, or the data has to be removed. The authors have used formalin-based fixation methods, which are unfortunately often unable to preserve junctional integrity. Methanol fixation should be used in that case. Also, junctional integrity is best assessed in thicker vibratome sections. As thus, I don't recommend inclusion of the CDH5 staining data and as an extention, claims that junctional integrity is compromised.

Note that this doesn't invalidate their other findings on BBB disruption such as leakage and astrocyte endfeet displacement.

Response 4: We thank the reviewer for this constructive comment and fully agree with the caution regarding interpretation of CDH5 staining under formalin fixation. In response, we have revised the manuscript to remove the claims of adherens junction disruption or loss of junctional integrity based on CDH5 staining.

Specifically, we now describe the CDH5 data in Extended Data Fig. 3 in a strictly descriptive manner, focusing on changes in overall CDH5 signal intensity and spatial relationship with fibronectin, without inferring junctional fragmentation or ultrastructural disruption. We rewrote the figure legend, and we revised the Results text accordingly (Section: Fibronectin is associated with altered gliovascular unit in AD). We have also explicitly added a sentence in the Results acknowledging that definitive assessment of adherens junction ultrastructure would require junction-preserving fixation and thicker tissue sections, as suggested by the reviewer. We do appreciate the reviewer's guidance.

REVIEWER #3: NEUROVASCULAR DYSFUNCTION IN NEURODEGENERATIVE DISEASE

Reviewer comment 1: The revised manuscript incorporating a large amount of new data addresses many of the concerns raised in the previous review, and the data are now more complete and generally convincing. However, a couple of issues still warrant the authors' attention.

Response 1: We thank Reviewer #3 for their positive assessment of the revised manuscript and for recognizing the new data added to strengthen the study. We appreciate the reviewer's constructive feedback and address the remaining points raised below.

Reviewer comment 2: Regarding the title, "Fibronectin mediates APOE4-driven blood-brain barrier dysfunction in Alzheimer's disease", the mechanistic evidence is derived primarily from animal models of AD rather than from the human disease itself. The autopsy data are largely correlative and reflect end-stage pathology, which is subject to numerous confounding factors.

Response 2: We thank the reviewer for this comment and do agree that direct experimental manipulation of disease mechanisms is not feasible in living human brain tissue. To clarify the scope of inference, we have revised the Abstract to explicitly state that causal and mechanistic conclusions are derived from experimental perturbation across complementary model systems, while analyses of human post-mortem brain tissue and clinical datasets provide disease relevance and integrative support. Specifically, the Abstract now emphasizes that fibronectin accumulation is sufficient to induce BBB leakage in experimental models, and that human data are incorporated as part of an integrated evidence framework consistent with this mechanism. We have retained the title because the mechanistic conclusions derive from experimental perturbation across multiple model systems, while human post-mortem and clinical datasets are presented as integrative, disease-relevant evidence rather than as the sole basis for causal inference.

Reviewer comment 3: In addition, the functional consequences of the reported fibronectin-induced BBB dysfunction, presumed to be pathological, on brain function were not assessed. Specifically, cognitive outcomes were not examined, and no evidence was provided that counteracting fibronectin or its downstream signaling pathways leads to cognitive rescue. As a result, statements regarding the translational relevance and therapeutic implications of these findings should be tempered.

Response 3: We thank the reviewer for this important comment. We agree that the present study does not assess cognitive or behavioral outcomes as part of its focus. In response, we have systematically changed

translational and therapeutic language throughout the manuscript, including reorganization of the Discussion to emphasize mechanistic and vascular relevance rather than disease/clinical outcomes. Specifically:

(1) We removed statements implying therapeutic potential based on fibronectin targeting (Discussion, first paragraph), including the sentence referring to a “therapeutic potential of a targeted approach aimed at reducing fibronectin aggregation.”

(2) We revised language describing human CSF findings to avoid overstatement of clinical relevance, reframing these results as supportive biomarker-level observations rather than evidence of therapeutic applicability (Discussion).

(3) We modified statements referring to fibronectin as a therapeutic target to instead describe FN1 as a biomarker and candidate pathway relevant to Alzheimer’s disease-associated BBB dysfunction, without implying established efficacy.

(4) We revised broader interpretive statements to focus specifically on gliovascular signaling, vascular dysfunction, and BBB integrity, removing implications of downstream cognitive or brain-wide functional effects that were not directly tested.

(5) We added an explicit limitation statement at the end of the Discussion, noting that the consequences of fibronectin-mediated BBB dysfunction for cognition and higher-order brain function were not directly assessed and remain an important area for future investigation.

With these revisions, we believe that the manuscript accurately reflects the scope of the experimental evidence and avoids overstating translational or therapeutic implications, while preserving the central mechanistic conclusions regarding fibronectin’s role in APOEε4-related BBB dysfunction. We thank the reviewer for guidance.

REVIEWER #4: NEUROINFLAMMATION IN NEURODEGENERATIVE DISEASE

Reviewer comment 1: The authors addressed all of this Reviewer's comments satisfactorily. I would also like to acknowledge the significant improvement in the figures/text and conclusions based on incorporating all the reviewers concerns. I do not have any other major comments. One minor comment in the Discussion. The first paragraph could improve on the broader impact of the study. For example, the second to last paragraph starting with "taken together" has stronger elements and I would recommend bringing this up.

Response 1: We thank Reviewer #4 for this helpful suggestion. In response, we have revised the first paragraph of the Discussion to emphasize the broader impact and conceptual significance of the study more clearly, drawing on the integrative framing previously articulated in the concluding sections. Specifically, the opening of the Discussion now highlights fibronectin-mediated gliovascular dysfunction as a central mechanism linking APOEε4-associated risk to blood-brain barrier pathology in Alzheimer’s disease. We believe this reorganization improves clarity and better contextualizes the study’s broader relevance and preserves the overall structure of the Discussion.

REVIEWER #5: ZEBRAFISH NEUROIMMUNOLOGY

Reviewer comment 1: Despite some minor improvements to the manuscript, the authors have not addressed several critical methodological and interpretive concerns. Most notably, the inappropriate statistical treatment of vessels as independent biological replicates—rather than accounting for nesting within individuals—remains uncorrected, leading to pseudoreplication and invalid p-values. This fundamental issue, alongside unresolved concerns about image quantification, lack of consistent vascular and astrocytic markers, problematic figure accessibility, and unsupported interpretations of data (e.g., dextran leakage and AQP4 polarity), significantly undermines confidence in the study’s conclusions. Many key points, including the identity of analyzed vessels, appropriate cell type markers, and the novelty of the findings, remain insufficiently addressed.

Response 1: We thank the reviewer for their comments and for the opportunity to address these concerns. In this revision, we have modified the statistical analyses of relevant nested datasets, expanded quantitative analyses where requested, clarified anatomical and cellular identities, improved figure accessibility, and

revised interpretations to align precisely with the scope of the data. Below, we respond point by point, detailing the specific analyses performed and manuscript changes implemented to resolve each concern.

Major:

Reviewer comment 2: The statistical analysis for many comparisons, such as Fig. 1h and 1k, is incorrect because it treats individual vessels as independent biological replicates, despite vessels being nested within a small number of individuals. This conflation of technical and biological replicates constitutes pseudoreplication, inflates the effective sample size (as reflected by the very high degrees of freedom), and invalidates the reported p-values. The appropriate unit of replication is the individual, and the data must be reanalyzed either by summarizing vessel measurements at the individual level or by using a hierarchical (mixed-effects) model that accounts for vessel nesting within individuals.

Response 2: We have reanalyzed all affected comparisons using statistical models that explicitly treat the individual animal or human donor as the unit of inference, rather than vessels, cells, or ROIs. Specifically, we replaced vessel-level tests assuming independence with cluster-aware models in which repeated measurements are nested within individuals, thereby eliminating inflation of the effective sample size and degrees of freedom. We implemented generalized estimating equation (GEE) models, specifying the animal, donor, or biological replicate as the clustering variable and using robust (sandwich) variance estimators. This approach accounts for within-individual correlation within datasets from one replicate/donor/animal, while estimating population-average effects, which is appropriate for our biological questions focused on average genotype-, pathology-, or treatment-associated differences across individuals. GEE inference is conservative and robust to within-cluster correlation structure and is used for correlated and nested biological data. Importantly, this reanalysis did not alter the core conclusions of the study.

We now provide a comprehensive summary of all revised statistical analyses in Supplementary Table 1, including the model type, clustering variable, number of biological replicates, test statistics, and p-values for each affected figure (including Figs. 1h, 1k, 2b, 3b, 3g, 4h/4k, 5i-m, 6f-o, S1d). We updated the corresponding Methods, Results, and figure legends to explicitly state the unit of replication and the use of cluster-aware inference.

Reviewer comment 3: The authors have made some improvements to color accessibility across the figures. However, several images—including the graphical abstract and many of the supplemental figures—remain not fully red-green colorblind friendly. In addition, the response frames accessibility changes as provisional and deferred until acceptance, rather than fully implemented at revision. This is concerning, as reviewer comments are meant to be addressed comprehensively during revision, and deferring such changes effectively shifts the burden back to reviewers. Accessibility-related figure updates should be applied consistently and finalized at the revision stage.

Response 3: All figures, including the graphical abstract and supplemental figures, have now been systematically evaluated using deuteranopia-style color vision deficiency simulations and revised accordingly wherever needed. We note that throughout the manuscript, individual fluorescence channels are provided alongside merged images, which help to visualize individual channels.

Reviewer comment 4: The authors claim “Confirming these observations, Dextran-based vessel leakage assay in APOEε4 vs. APOEε3 mouse brains showed increased fibronectin (green) deposition and extravasated dextran-red (red) leakage at blood vessels (CD31, white) in APOEε4 mice compared to APOEε3 mice (Extended Data Fig. 2)” but in this not RG colorblind figure, you have no quantification and if anything the APOE4 mice have LESS dextran leakage outside of the CD31 vessels. Also, why is your CD31 stain looking so avascular for APOE3?

Response 4: We appreciate the opportunity to clarify the quantitative and visual presentation of our data.

First, to directly support the observations shown in Extended Data Fig. 2, we have expanded the analysis to include explicit quantification of both fibronectin deposition and extravascular dextran signal, using vessel-delimited regions of interest with animal-level inference. These quantitative results are now shown in the revised figure panels and summarized in Supplementary Table 1 and demonstrate significantly increased fibronectin accumulation and dextran extravasation in APOEε4 compared to APOEε3 mouse brains under cluster-aware statistical models.

Second, we have updated the figure to use a color-blind-accessible visualization with clear luminance separation, and interpretation of the data is now anchored in quantitative measurements rather than relying on color contrast in merged images.

Third, the difference in CD31 staining pattern between APOEε3 and APOEε4 images reflects natural vessel geometry and sectioning orientation rather than loss of vascular signal. Many cortical microvessels curve, branch, or change orientation within the tissue, and sagittal sections frequently intersect vessels at oblique or transverse angles. Under these conditions, CD31 labeling appears punctate or circumferential rather than as continuous linear segments. These punctate CD31 profiles frequently coincide with intravascular dextran signal, consistent with vessels being imaged in cross-section rather than indicating discontinuity of the vasculature. This clarification has been added to the revised figure legend.

Reviewer comment 5: The authors also say "Quantitative analysis showed that the proximity between vascular FN1-AQP4 was significantly increased in AD compared to controls (138.5%, p=1.41E-13), consistent with a loss of endfoot polarity (Extended Data Fig. 3b)." I find this wording confusing and thing the best comparison to make this claim is to do the AQP4 stain and look for colocalization with the vascular CDH5 or CD31 not their secreted FN1, that by definition increases in AD, according to them. Similarly, how are they defining these "continuous vessels" for CDH5? There needs to be a consistent vascular marker that they're using to compare and make these claims about astrocytic endfeet and vascular architecture.

Response 5: We have revised the text to clarify that the FN1-AQP4 proximity analysis was not intended as a direct measure of astrocytic endfeet polarity, but rather to quantify the spatial encroachment of aberrantly deposited perivascular fibronectin into AQP4-positive, endfeet-associated regions in AD. Accordingly, we no longer interpret this metric as evidence of altered polarity per se.

Redistribution of AQP4 away from its normal perivascular enrichment is a well-established feature of AD, and this altered spatial organization is captured in our data using canonical vascular references rather than FN1. Specifically, altered AQP4 localization relative to blood vessels is evident in Extended Data Fig. 3a-b and Fig. 2h, demonstrating disrupted perivascular enrichment of AQP4 in AD independently of FN1-based analyses.

To link conclusions about vascular architecture to appropriate markers, we assess endothelial organization using CDH5 (VE-cadherin) as a consistent endothelial reference. In the revised manuscript, CDH5 data are described, focusing on overall signal distribution and spatial relationship to FN1. The term "continuous vessels" has been removed, and the figure legend and Results text now explicitly define how CDH5-positive vascular structures were identified and analyzed.

Together, these revisions clearly separate the roles of FN1 as a pathological extracellular matrix component accumulating at the gliovascular interface from endothelial markers used to assess vascular organization, and they ensure that astrocyte-vascular relationships are interpreted using appropriate and consistent vascular references.

Reviewer comment 6: They are still not showing representative images of endothelial FN1 expression in 2D culture that is quantified in Fig. 2d. I also still think that doing in situ hybridization for FN1 would truly reveal the cellular source of increased FN1 since it is secreted and deposited in the endothelial basement membrane, and since Fig. 2g really shows that many cell types in the human brain are expressing FN1, and this would be complementary to Fig. 2g-h. Again, I'm believing that APOE4 genotype correlates with increased FN1 levels, that has been shown in a number of ways, but it's the more nuanced cell-type specific effects that are still unclear. Furthermore the first author's 2024 publication that already linked a rare FN1 variant as protective of APOE4 genotype blunts the novelty of this study.

Response 6: We appreciate the opportunity to clarify.

Representative endothelial FN1 images and transcript localization: Fig. 2d quantifies FN1 transcript levels by qRT-PCR in iPSC-derived endothelial cells and therefore does not have a corresponding representative immunofluorescence image. We recognize that the reviewer's comment pertains to whether spatial transcript localization (e.g., in situ hybridization) would further clarify cellular sources of FN1. While ISH can be informative, the central focus of this study is perivascular FN1 protein accumulation and deposition at the gliovascular interface, which cannot be inferred from transcript localization alone for a secreted extracellular matrix protein. Although we include single-nucleus RNA-seq to define FN1-expressing cell classes, protein-level spatial localization is addressed using multiplex immunofluorescence, which is the appropriate modality for this question (Fig. 2c, Fig. 2h; Extended Data Figs. 5-6). Accordingly, we address cellular context using

complementary, orthogonal approaches spanning transcript and protein levels, and we have revised the Results text to make this integrative logic explicit and to avoid over-interpreting any single assay as defining a unique cellular "source" of FN1.

Cell-type nuance and FN1-AQP4 proximity: Our revised presentation emphasizes that the key biological insight is not simply that FN1 levels are increased, but where FN1 accumulates, specifically within the perivascular niche, and how this spatial deposition relates to vascular markers and astrocyte endfeet-associated regions. These relationships are interpreted using canonical endothelial references (CDH5/CD31) and cell-type-resolved human snRNA-seq data, rather than using FN1 itself as a vascular marker.

Novelty relative to the 2024 genetics study: Our publication in 2024 was a human genetics discovery and replication study identifying rare FN1 coding variants that modify APOEε4-associated risk and linking FN1 genetically to BBB pathology. The present manuscript is mechanism- and cell-biology-driven, defining APOE genotype-linked regulation and spatial deposition of FN1 across human iPSC-derived endothelial systems, 3D vascular models, in vivo zebrafish and mouse experiments, human CSF, human brain snRNA-seq, and multiplex tissue imaging. Importantly, the genetics study did not address cell-type-specific regulation, spatial deposition at the gliovascular interface, or functional vascular consequences of FN1, all of which constitute the central novel contributions of the present work.

Reviewer comment 7: You can't make the claim that AQP4 is your endfoot marker while also saying that APOE4 AD brains have loss of polarity of AQP4 to the endfeet, which you have nicely shown. This is specifically an issue for Fig. 3.

Response 7: Throughout the study, we use AQP4 as a canonical astrocytic endfeet protein, and our data do not indicate loss of AQP4 expression or loss of endfeet identity. Rather, highly consistent with the literature, we observe a redistribution of AQP4 away from its normal perivascular enrichment in APOEε4 AD brains. We have revised the Results text associated with Fig. 3 to explicitly clarify that AQP4 remains an endfeet-associated marker, while its spatial organization relative to the vasculature is altered in disease. Accordingly, FN1-AQP4 proximity is now interpreted as reflecting altered spatial relationships within the gliovascular niche, rather than as a direct metric of endfeet polarity. These revisions clarify the intended interpretation while preserving the biological conclusion of disrupted astrocyte–vascular organization in AD.

Reviewer comment 8: My major concern about the zebrafish vessels that are used for these statements has still not been addressed. These look like very larger arteries on the surface of the brain tissue, and not BBB endothelial cells for Fig. 4g-j, 6j, and 7a. This is rather important since the claim of this paper is that BBB function is altered in response to astrocytic FN1. Furthermore, the quantification in Fig. 4h-k do not take into account how many fish vs. how many vessels were analyzed, a problem that I noted last time, and this continues to have the incorrectly done statistics as such, as well as not being red-green colorblind friendly.

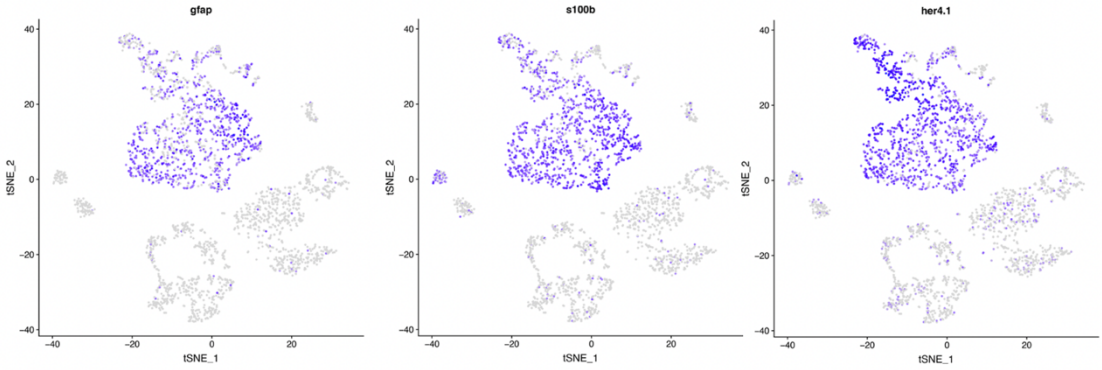
Response 8: We are happy to clarify the anatomical and analytical framework of the zebrafish experiments. The vessels shown in Figs. 4g-j, 6j, and 7a are brain-associated vessels in the adult zebrafish that arise from pial vasculature and extend into the underlying brain parenchyma, where they are consistently ensheathed by astroglial endfeet. This vascular compartment represents the most robust and reproducible site of astroglia-vascular interaction in the adult zebrafish brain and has been widely used to study BBB-related properties, including in our prior work and that of others. Although these vessels are anatomically proximal to the pial surface, they are not extracranial arteries and are functionally coupled to astroglial endfeet at this stage, making them an appropriate and biologically relevant readout of astrocyte-dependent regulation of vascular and BBB-associated properties in vivo. We have clarified this anatomical context in the revised text to avoid ambiguity regarding vessel identity.

With respect to quantification, this issue has been explicitly addressed in the revised manuscript. For Figs. 4h-k, we report the number of animals and vessels analyzed, with vessels treated as nested observations and animals as the unit of inference, in line with reviewer recommendations. These details are provided in Supplementary Fig. 12, and all revised statistical analyses are summarized in Supplementary Table 1.

Finally, we evaluated all zebrafish figures using color vision deficiency simulations, confirming that fluorescence channels remain distinguishable under deuteranopia conditions. In addition, individual fluorescence channels are shown alongside merged images, ensuring that interpretation does not rely on red-green color discrimination alone.

Reviewer comment 9: I also have a major concern with using the her4.1 transgenic line to label astroglia. While her4.1 labels neural stem cells and radial glial cells that are poised for neurogenesis, it is not a reliable marker of astroglia. A better marker would be glast or gfap or GS.

Response 9: We respectfully note that the characterization of her4.1 as exclusively labeling neural stem cells is not consistent with the adult zebrafish literature, where her4 reporters have been extensively validated as marking the broader astroglial lineage, including non-proliferative astroglia that co-express canonical markers such as gfap and s100b. her4.1 is a very well-validated and widely-used reporter of adult zebrafish astroglia lineages that perform astroglial functions and constitutes the principal glial compartment of the adult telencephalon. Importantly, direct quantitative comparisons have shown that her4 and gfap reporters label largely overlapping populations in the adult brain. For example, Tan-Trong et al. (Science Advances, 2020 among others) demonstrated that >95% of gfap^{+} cells are also her4^{+} (and vice versa), and that the majority of these cells are non-proliferative, indicating that neural stem cells represent only a subset of the broader her4^{+} glial population. Consistent with this literature, single-cell transcriptomic datasets further show extensive overlap between her4, gfap, s100b, and related astroglial markers, with marker usage varying by region and state rather than defining mutually exclusive cell types (an example from our datasets is below where violet marks the expression of the gene for which the FTR plot was generated). Accordingly, we are confident that her4.1 to be a reliable astroglial marker in adult zebrafish, as it provides a robust and broadly accepted means of labeling astroglia.



For Reviewer: Transcriptional concordance of astroglial markers in the adult zebrafish telencephalon. Feature–target relationship (FTR) analysis derived from single-cell RNA-seq datasets of the adult zebrafish telencephalon showing expression overlap among canonical astroglial markers (her4, gfap, s100b). The majority of her4^{+} cells co-express gfap and s100b, with >95% concordance across these markers, indicating that they label overlapping set of astroglia population.

Minor:

Reviewer comment 10: Could you box the areas that you zoomed in for fig. 1l?

Response 10: We added boxes to Fig. 1l.

Reviewer comment 11: Extended data Fig. 6 has multiple screenshot spellings underlined for the western blots (f and g)

Response 11: We removed the spelling underlines in Extended Data Fig. 6f/g and corrected the figure.

Reviewer comment 12: What does Vegfa look like in fn1b-/- fish without AB42 for Fig. 5h? Also you incorrectly call to Fig. 5h as human data line 294.

Response 12: We corrected the erroneous reference to Fig. 5h as human data. We see that *vegfa* expression is also increased in fn1b$^{-/-}$ zebrafish brains under conditions without Aβ42 treatment.

RESPONSE TO REVIEWERS – 3RD REVISION

- Updated Extended Data Fig. 2 to include additional representative images and increased animal numbers (n=4), with revised quantification confirming increased extravascular dextran signal in APOE4 mice.
- Updated Fig. 7a-b to explicitly show that we quantify parenchymal capillaries, not arteries. Figure and legend revised to clarify that analyses exclude arterial vessels.
- Clarified statistical approach and consistency across analytical methods; replaced Figure panel 4h with its supplementary figure showing animal-based data points and statistical considerations.

Reviewer #2: ***Comment:*** *The authors have answered to all my concerns in a satisfactory manner and I have no further comments. I congratulate the authors on a very nice study and their rigorous work.*

Response: We thank the reviewer for their thoughtful evaluation and kind remarks. We appreciate their positive assessment of our study and acknowledgment of its rigor. Their feedback throughout the review process has been very helpful in improving the clarity and quality of our work.

Reviewer #3: ***Comment:*** *No further comments.*

Response: We thank the reviewer for their time and consideration.

Reviewer #5

Comment: Extended Figure 2A still looks like Dextran is higher outside of the vessel in the APOE3 mouse. This is the same image as before and as representative images, the staining is not convincing. It is also inappropriate to do statistics on N=2 animals for Extended Data 2C.

Response: We thank the reviewer for the careful and extensive evaluation of the manuscript throughout the review process. To address the concern regarding the representative images, we have updated Extended Data Fig. 2A to include additional image sets that more clearly reflect the quantified data. In addition, we have increased the number of animals analyzed to n=4, and updated the quantification accordingly, improving both representation and statistical robustness. These revisions further support the original conclusion that APOE4 mice exhibit increased extravascular dextran signal compared to the controls, consistent with BBB leakage.

Comment: While I appreciate that they have 4 animals for Fig. 4f and that this is now it's own supplemental figure, I don't understand how the stats are that significant. If I try my best to take the N of 4 into account and assess the data provided in Supplementary Table 1, I still see a significant increase, but only at p=0.015. I still do not believe that the statistics are being appropriately applied.

Response: We thank the reviewer for re-examining the dataset (this comment likely refers to Fig. 4h, as Fig. 4f depicts human brain tissue rather than animal data). To ensure robustness, we evaluated the data using both a bias-reduced cluster-aware GEE framework and analyses based on animal-level averaging. We assume that the reviewer's reported p = 0.015 derives from averaging measurements within each animal followed by animal-level comparison; accordingly, we performed Welch's t-test on animal means, which likewise yielded statistically significant results.

We prefer the bias-reduced GEE framework because the dataset consists of repeated vessel-level measurements nested within animals. This approach accounts for intra-animal correlation while retaining within-animal variability, and the bias-reduced covariance estimator provides conservative inference given the relatively small number of animals per group.

Importantly, all statistical approaches applied to this dataset yielded statistically significant group differences and support the same biological conclusion. For transparency, we have revised Fig. 4h to display measurements grouped by individual animal and now report both GEE and Welch's t-test p-values in the figure. Supplementary Table 1 has also been updated to include the corresponding statistical outputs. These analyses continue to support a significant TNF-induced increase in FN1 levels and do not alter the conclusions of the manuscript.

Comment: Furthermore, while I appreciate the authors' clarification of the pial artery in the zebrafish and the high glial coverage of this particular vessel, I do not believe it is appropriate to call this a BBB endothelial cell, as arteries by definition are molecularly distinct from the BBB capillary endothelial cells, for example they are completely devoid of Mfsd2a expression, because they are full of caveolae vesicles (Chow & Gu, 2017; Chow et al, 2020; Bell et al, 2023). The artery barrier is created by the vascular smooth muscle cells, not the endothelial cells.

Response: We thank the reviewer and agree that arterial endothelial cells are known to differ from capillary BBB endothelium. Our quantification was always based on small-diameter parenchymal vessels (capillary-like structures), not the pial artery. We have revised Fig. 7a-b to show higher-magnification views of these microvessels and clearly annotated the quantified structures. The legend now explicitly states that only small vessels were analyzed. We also added text to the methods to clarify this. We believe these changes resolve the concern regarding vessel identity.

Response to reviewers

Reviewer #3:
Remarks to the Author:
No further comments

- Thank you.

Reviewer #5:
Remarks to the Author:
I'm glad the statistics were fixed and the vessels used to assess BBB function were better characterized. Please correct your images for RG colorblind people, especially figure 7. No further experimentation is needed.

- Thank you. Images comply with Nature guidelines.