EMBO Molecular Medicine

ETS-guided iPSC-endothelial models recapitulate malaria pathogenesis

François Korbmacher, Rory Long, Hannah Fleckenstein, Patryk Poliński, Dennis Crusius, Livia Piatti, Borja López-Gutiérrez, Alina Batzilla, Vikas Trivedi, Miki Ebisuya, and Maria Bernabeu Aznar

Corresponding authors: Maria Bernabeu Aznar (maria.bernabeu@embl.es) , Miki Ebisuya (miki.ebisuya@tu-dresden.de)

Review Timeline:

Submission Date:	7th Aug 25
Editorial Decision:	24th Sep 25
Revision Received:	30th Mar 26
Editorial Decision:	13th May 26
Revision Received:	28th May 26
Accepted:	29th May 26

Editor: Zeljko Durdevic

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

24th Sep 2025

Dear Dr. Bernabeu Aznar,

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now received feedback from the three reviewers who agreed to evaluate your manuscript. All three referees recognize interest of the study but also raise important concerns that should be addressed in a major revision. Particular attention should be given to providing deeper mechanistic insights as suggested by the referee #3. If you would like to discuss further the points raised by the referees, I am available to do so via email or video. Let me know if you are interested in this option.

Further consideration of a revision that addresses reviewers' concerns in full will entail a second round of review. EMBO Molecular Medicine encourages a single round of revision only and therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. For this reason, and to save you from any frustrations in the end, I would strongly advise against returning an incomplete revision.

We would welcome the submission of a revised version within three months for further consideration. Please let us know if you require longer to complete the revision.

I look forward to receiving your revised manuscript.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Senior Editor
EMBO Molecular Medicine

*****

When submitting your revised manuscript, please carefully review the instructions that follow below. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF': (https://www.embopress.org/page/journal/17574684/authorguide#figureformat).

3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#submissionofrevisions). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary

datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public (see https://www.embopress.org/page/journal/17574684/authorguide#dataavailability).

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.). See also 'Figure Legend' guidelines: https://www.embopress.org/page/journal/17574684/authorguide#figureformat

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

See detailed instructions here:

.

11) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting

- the medical issue you are addressing,

- the results obtained and

- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

12) Author contributions: You will be asked to provide CRediT (Contributor Role Taxonomy) terms in the submission system. These replace a narrative author contribution section in the manuscript.

13) A Conflict of Interest statement should be provided in the main text.

14) Every published paper now includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach

these in a separate file or send them by email, we will incorporate them accordingly.

Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300-600 px high.

15) Include a Reagents and Tools Table as part of the Methods section, which can be downloaded from our author guidelines (https://www.embopress.org/page/journal/17574684/authorguide#structuredmethods)

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

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

Referee #1 (Comments on Novelty/Model System for Author):

In this well-designed paper, Korbmacher et al generate and characterize a stable iPSC cell line with inducible expression of three transcription factors that have been shown to promote endothelial identity for the purpose of generating an improved model system to study interactions between the Plasmodium falciparum parasites and blood vessels, which are key to the pathogenesis of cerebral malaria. They employ immunofluorescence, EM and transcriptomics to characterize the induced, differentiated cells (called ETS-iBMECs), as compared to the established iBMEC cell model, and show that the ETS-iBMEC cells have a stronger endothelial identity more similar to primary brain endothelial cells. They also functionally characterize the barrier function of ETS-iBMEC cells using a previously-described 3D microfluidic device, showing that the ETS-iBMEC form microvessels with strong barrier integrity, similar to iBMEC, which is a limitation of primary brain endothelial cells. Binding studies with P. falciparum-infected cells provide further support for this model for studies of malaria pathogenesis, though there appeared to be strain-dependent binding differences that were not fully explored. Parasite material was also shown to alter the ETS-iBMEC at the transcriptional level and disrupt the barrier by compromising endothelial integrity, supporting its use as an improved in vitro model to study cerebral malaria.

Referee #1 (Remarks for Author):

1. The studies measuring the binding activity of P. falciparum-infected red blood cells to the different cell lines in microfluidic channels support the notion that the ETS-iBMEC cells will be useful for the study of malaria pathogenesis, but there appears to be a large difference in the ability of the two strains studied to bind to any of the cell lines. The paper would be strengthened by an enhanced discussion about these different results and their implications for the host vs parasite factors that impact binding. What would be expected to be seen if clinical samples were employed?

2. On a related note, the experiments investigating transcriptional changes induced by P. falciparum-egress products appeared to use a different strain, and the nature of the parasite products that are thought to be altering the host cell transcription and integrity are not discussed. Is the parasite expressing specific factors that are inducing these responses in the cell lines?

3. The authors note that while the ETS-iBMEC cell line has key strengths as a model in terms of barrier integrity, parasite binding, and endothelial identity, transciptional analysis showed that it did not show alteration of the JAK-STAT pathway in response to parasite products, which has been shown to be a key pathogenesis pathway in primary brain ECs. It would be helpful to provide some additional context for this observation and whether it should be considered a limitation of the model e.g. for use in drug screening.

Referee #2 (Comments on Novelty/Model System for Author):

No suggestions to improve the model. As such, the manuscript presents a new method for the generating an iPSC line with inducible and simultaneous expression of ETS transcription factors with improved endothelial identity model has the potential to facilitate studies on malaria pathogenesis and other related disease states.

Referee #2 (Remarks for Author):

Manuscript by Korbmacher et al generated an iPSC line with inducible and simultaneous expression of ETS transcription factors with improved endothelial identity which allows for efficient binding of plasmodium infected RBCs. They have also performed transcriptomic analysis which identified significant endothelial metabolic changes and splicing alterations. This new model has

the potential to facilitate studies on malaria pathogenesis and other related disease states.

This is a beautiful piece of research, the overall study is well presented and conclusions backed by data, I have only minor comments to make.

Minor comments
1. An ethics statement for blood collection is required, if it wasn't collected through a blood bank.
2. In addition to the top 5 pathways (Fig. 2A, B), were there additional pathways- such as those related to cell-cell interaction, attachment, or cytoskeletal dynamics?
3. For the parasite egress media preparation upon treatment with compound # 2, some data on the parasite egress window (such as Giemsa images or flow plots after treatment and upon removal of compound) might be useful, as supporting data.

Referee #3 (Comments on Novelty/Model System for Author):

General Assessment
This is a well-written and carefully executed study. The authors establish an innovative iPSC-derived endothelial model with improved identity and barrier function, and demonstrate its application in studying Plasmodium falciparum sequestration and endothelial disruption in vitro. The methodology is sound, the figures are clear, and the work addresses an important limitation in cerebral malaria research. The manuscript will be of interest to the infection biology and stem cell modeling communities.

However, the study is primarily descriptive. While it successfully documents differentiation improvements, barrier properties, parasite adhesion, and transcriptomic alterations, it stops short of probing mechanistic underpinnings. This limits its impact in terms of uncovering new biology.

Major Comments

Mechanistic depth

The transcriptomic analyses highlight alterations in junctional, cytoskeletal, angiogenic, and metabolic pathways, but the study does not establish causality.

Functional validation of candidate pathways (e.g., through knockdown/overexpression, pharmacological inhibition, or rescue assays) would greatly strengthen the conclusions.

Barrier disruption

The finding that parasite egress products disrupt endothelial integrity is important, but the work does not dissect which specific mediators or pathways drive this effect. Clarifying whether the observed junctional changes are primary or secondary consequences would add mechanistic weight.

Comparisons with primary endothelial cells

ETS-iBMECs outperform primary brain ECs in barrier properties, but the differences in pathogenic responses between the two models are not fully explored. Additional comparative analysis (e.g., pathway-level or functional differences) could help contextualize the utility and limitations of this new system.

Physiological relevance

The model convincingly captures parasite adhesion and barrier disruption. However, incorporating or at least discussing additional physiological components (e.g., leukocyte interactions, brain-specific transcription factors, or immune responses) would strengthen the claim of relevance to cerebral malaria.

Additional clarifications and controls

Nature of the ETS-iBMEC line: The authors refer to ETS-iBMEC as a cell line. Is it truly stable? Once DOX is removed, for how long is transgene expression maintained?

TNFα stimulation and marker expression: When TNFα is added, do ETS-iBMECs still express CD31 while EPCAM remains

suppressed? This would further validate endothelial identity under inflammatory conditions.

Transgene integration: Did the authors check the genomic insertion sites of the piggyBac transposon? This could be relevant to rule out positional effects.

Figure consistency: In Fig. 3F-H, the ETS-iBMEC images are shown at different magnifications compared to controls, making direct comparison difficult. Please harmonize image scales.

TNFα in egress assays: In Fig. 5A, was TNFα pre-treatment included before exposure to parasite egress products? If so, this should be clearly stated.

Antigen presentation: Brain endothelial cells are known to present parasite antigens in mouse models. Did the authors assess expression of MHC class I/II in ETS-iBMECs?

Alternative splicing relevance: The observation of widespread splicing changes (Fig. 5G) is intriguing. Can the authors expand on the potential biological relevance of these splicing events in endothelial function or malaria pathogenesis?

Minor Comments

The discussion could better distinguish which observations are novel mechanistic insights versus confirmation of known processes.

Figures are generally clear, but some legends are dense. Simplification or an added schematic summary would help readability.

A short statement on throughput and reproducibility of this model in routine laboratory settings would be useful for readers considering adoption.

Referee #3 (Remarks for Author):

Recommendation
Major Revision. The manuscript is strong in methodology and descriptive characterization, but its impact would be significantly enhanced by additional mechanistic interrogation and clarification of the points listed above.

Referee #1 (Comments on Novelty/Model System for Author):

In this well-designed paper, Korbmacher et al generate and characterize a stable iPSC cell line with inducible expression of three transcription factors that have been shown to promote endothelial identity for the purpose of generating an improved model system to study interactions between the Plasmodium falciparum parasites and blood vessels, which are key to the pathogenesis of cerebral malaria. They employ immunofluorescence, EM and transcriptomics to characterize the induced, differentiated cells (called ETS-iBMECs), as compared to the established iBMEC cell model, and show that the ETS-iBMEC cells have a stronger endothelial identity more similar to primary brain endothelial cells. They also functionally characterize the barrier function of ETS-iBMEC cells using a previously-described 3D microfluidic device, showing that the ETS-iBMEC form microvessels with strong barrier integrity, similar to iBMEC, which is a limitation of primary brain endothelial cells. Binding studies with P. falciparum-infected cells provide further support for this model for studies of malaria pathogenesis, though there appeared to be strain-dependent binding differences that were not fully explored. Parasite material was also shown to alter the ETS-iBMEC at the transcriptional level and disrupt the barrier by compromising endothelial integrity, supporting its use as an improved in vitro model to study cerebral malaria.

We thank the reviewer for their positive and thoughtful assessment of our work and for summarizing the characterization of the ETS-iBMEC model. We address the reviewer's suggestions point-by-point below.

Referee #1 (Remarks for Author):

1. The studies measuring the binding activity of *P. falciparum*-infected red blood cells to the different cell lines in microfluidic channels support the notion that the ETS-iBMEC cells will be useful for the study of malaria pathogenesis, but there appears to be a large difference in the ability of the two strains studied to bind to any of the cell lines. The paper would be strengthened by an enhanced discussion about these different results and their implications for the host vs parasite factors that impact binding.

We thank the reviewer for this important observation, which highlights the well-known heterogeneity of binding studies with *P. falciparum*-infected RBC. Our microfluidic binding experiments with EPCR- (IT4var19) and ICAM1-binders (ITGICAM1) demonstrate efficient binding and validate that ETS-iBMECs display these two receptors at a conformation that facilitates infected-RBC binding. As highlighted by the reviewer, the binding behavior differs between parasite lines, consistent with prior studies reporting strain-dependent cytoadhesion phenotypes on primary endothelial cells (Avril *et al*, 2016; Lennartz *et al*, 2017). We and others have hypothesised in the past that this heterogeneity benefits the parasite in a changing microvascular environment during disease, or could be a mechanism used by the parasite to target different organotypic blood vessels (e.g. brain vs lung) (Avril *et al*, 2013; Ortolan *et al*, 2022. However, the parasite or endothelial molecular mechanisms that drive this heterogeneity have been linked to biophysical (shear stress, temperature) or biochemical (organotypic endothelial differences, cytokine activation) and probably many others that have not been fully defined. We have including these points in the discussion (see below)

What would be expected to be seen if clinical samples were employed?

We expect that clinical samples from severe and cerebral malaria patients would bind to ETS-iBMEC, as several studies have confirmed that their major endothelial binding phenotype is to EPCR and ICAM-1, the two receptors targeted by the endothelial cells used in this study. While the discussion is of interest, we prefer not to speculate on the manuscript.

To address the reviewer's comment, we extended the discussion section as outlined below. On a second change, we addressed the implications of host vs parasite factors:

lines 600 – 614:

"Although this phenotype cannot be explained through our transcriptomic analysis, other mechanisms may account for the non-significant increase in binding in ETS-iBMEC. As previously reported, *P. falciparum* lines present a wide heterogeneity in binding depending on the parasite or endothelial cell line, endothelial activation status, flow mechanical cues and temperature (Bernabeu *et al*, 2019; Introini *et al*, 2025). Despite this heterogeneity, the pronounced binding of *P. falciparum* to ETS-iBMEC highlights this model's relevance for studying host–parasite interactions in severe malaria and confirms receptor-specific cytoadhesion under flow. Future studies could include additional parasite lines with a dual EPCR–ICAM1 binding phenotype (Bernabeu *et al*, 2019; Reyes *et al*, 2024) or test the binding of clinical isolates from patients with severe and uncomplicated malaria to capture patient-derived sequestration phenotypes under controlled vascular conditions, including inflammatory activation (Storm *et al*, 2019; Tuikue Ndam *et al*, 2017). Moreover, an additional future advantage of iPSC-derived models is the ability to biochemically and functionally dissect ligand-receptor interactions, including targeted knockouts or the introduction of point mutations in receptors such as EPCR or ICAM1."

2. On a related note, the experiments investigating transcriptional changes induced by *P. falciparum*-egress products appeared to use a different strain, and the nature of the parasite products that are thought to be altering the host cell transcription and integrity are not discussed. Is the parasite expressing specific factors that are inducing these responses in the cell lines?

We agree with the reviewer that it would have been ideal to use the same parasite lines as for the binding studies. However, the use of the HB3var03 line was mostly due to logistical reasons. During the time in which we performed these assays, we were also running experiments for two other projects of the group that tested egress products in 3D models (Piatti *et al*., 2025, and Long *et al*., 2025). Given that the generation of egress media is quite complex, we used joint preparations that were split into the appropriate endothelial and vascular cell media. As a secondary advantage, the use of egress products from the same line creates better standards for internal transcriptomic data comparison within projects in our research group (2D vs 3D, primary ECs vs iPSC derived), and we expect that these data on systematic approaches can also be useful for others in the future. In the parasite egress media, we finally capture a broad range of factors, including heme, hemozoin, parasite histones, PfHRP2, and GPI anchors, which are all described to impact endothelial health, as discussed in our previous study (Piatti *et al*, 2025). The induction of the response is most likely an accumulative effect of all these factors. We acknowledge that the different genetic background (HB3 vs IT4) could be responsible for potential changes in endothelial response, nevertheless understanding parasite genetic-induced

differences is beyond the scope of this paper, that was mostly focused on the benchmarking of the generated iPSC-differentiated line ETS-iBMEC.

To clarify this, we added to the discussion the following sentence:

lines 622-629:

“Our previous work, along with others, has shown that *P. falciparum* egress in proximity to endothelial cells is a major driver of barrier breakdown in primary brain EC (Long *et al*, 2025; Piatti *et al*, 2025; Zuniga *et al*, 2022). These studies suggest that parasite-mediated disruption is a consequence of a combination of conserved parasite-egress released factors, such as heme, hemozoin, HRP2 or GPI anchors, rather than a single dominant effector. Previous studies have shown that parasite lines expressing different PfEMP1 induced differential transcriptional changes in endothelial cells, a finding that could be further studied in the future with the ETS-iBMEC model (Othman *et al*, 2023).”

3. The authors note that while the ETS-iBMEC cell line has key strengths as a model in terms of barrier integrity, parasite binding, and endothelial identity, transciptional analysis showed that it did not show alteration of the JAK-STAT pathway in response to parasite products, which has been shown to be a key pathogenesis pathway in primary brain ECs. It would be helpful to provide some additional context for this observation and whether it should be considered a limitation of the model e.g. for use in drug screening.

We agree with the reviewer that the absence of these key pathogenesis pathways is an important limitation that needs to be addressed in future iPSC-based models for infection studies. Therefore, we extended the manuscript with a new expanded view figure (Figure EV5) and further discussions on limitations of ETS-iBMECs in immunomodulatory assays as outlined below:

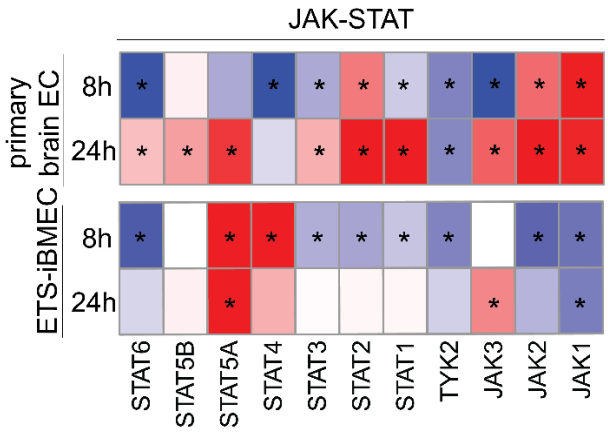
Results:

lines 495-502:

“We then examined inflammatory pathways previously identified in primary endothelial malaria studies (Piatti *et al*, 2025; Gomes *et al*, 2023). Viral response and interferon signalling pathways, including JAK–STAT, were previously reported in a primary 3D blood–brain barrier model in response to parasite egress products (Piatti *et al*, 2025), and were similarly observed in the primary EC transcriptomic analysis in this study. However, ETS-iBMEC did not show overexpression of these pathways (see comparison in Fig EV5E). Similarly, antigen presentation pathways (MHCI and MHCII) were largely restricted to primary brain EC and were not prominent in ETS-iBMEC (Fig EV5F-G).”

Figure EV5E now includes a direct comparison for JAK-STAT, as well as genes from MHCI and MHCII, between ETS-iBMEC and primary brain ECs

E



Furthermore, we extended in the discussion:

lines 680 – 682:

"Another limitation is that ETS-iBMEC do not activate biological processes associated with the response to viruses upon challenge with *P. falciparum*-infected RBC products, including JAK-STAT signalling."

lines 687 – 694:

"Notably, the limited innate immune signalling response in ETS-iBMEC could reflect incomplete maturation of antiviral sensing pathways. Consequently, while ETS-iBMEC are well-suited for studying parasite-endothelium interactions and barrier dysfunction, their use for screening immunomodulatory therapies targeting innate immune pathways should be complemented with primary BBB models. Therefore, future improvements in endothelial differentiation approaches should enhance innate immune responses in iPSC-derived cells, as well as improve applicability to 3D physiological models."

Referee #2 (Comments on Novelty/Model System for Author):

No suggestions to improve the model. As such, the manuscript presents a new method for the generating an iPSC line with inducible and simultaneous expression of ETS transcription factors with improved endothelial identity model has the potential to facilitate studies on malaria pathogenesis and other related disease states.

Referee #2 (Remarks for Author):

Manuscript by Korbmacher et al generated an iPSC line with inducible and simultaneous expression of ETS transcription factors with improved endothelial identity which allows for efficient binding of plasmodium infected RBCs. They have also performed transcriptomic analysis which identified significant endothelial metabolic changes and splicing alterations. This

new model has the potential to facilitate studies on malaria pathogenesis and other related disease states.

This is a beautiful piece of research, the overall study is well presented and conclusions backed by data, I have only minor comments to make.

We thank Referee #2 for their positive evaluation of our manuscript. We appreciate their recognition of the novelty of the model system and its potential relevance for studies of malaria pathogenesis and related disease states.

Minor comments 1. An ethics statement for blood collection is required, if it wasn't collected through a blood bank.

We thank the reviewer for this important observation. The blood was obtained from the Catalan Blood Bank. In the methods section we clarified the origin of used blood samples. Furthermore, the blood origin can be found in the reagents and tools table

lines 753-756:

"*P. falciparum* parasite lines HB3var03 (Claessens *et al*, 2012), IT4var19 (EPCR binding) (Avril *et al*, 2012) and ITGICAM1 (ICAM1 binding) (Janes *et al*, 2011) were cultured in human O+ erythrocytes from anonymised donors, obtained through an agreement with the Catalan Blood Bank (Banc de Sang i Teixits)."

2. In addition to the top 5 pathways (Fig. 2A, B), were there additional pathways- such as those related to cell-cell interaction, attachment, or cytoskeletal dynamics?

We thank the reviewer for this insightful question, and indeed, beyond the top five enriched biological processes in Fig. 2A, B, we observe pathways related to cell-cell interaction being upregulated, as listed in TableS1_GO_enrichment_iBMEClike_vs_ETSiBMEC. These include pathways of cell-cell adhesion via plasma-membrane adhesion (GO:0098742), homophilic cell adhesion via plasma membrane adhesion (GO:0007156), leukocyte cell-cell adhesion (GO:0007159), among others. In contrast, pathways associated with cytoskeletal dynamics cluster in GO-term over-representation analysis categories like muscle contraction, extracellular organization and cell-contact organization within the top 75 most significant downregulated pathways, as illustrated in Fig. 2 D, likely reflecting reduced mesenchymal or migratory features relative to iBMEC-*like* cells. All enriched GO terms, including those related to cell–cell adhesion, attachment, and cytoskeletal organization, are provided in full in source data file 1 (GO_enrichment_iBMEClike_vs_ETSiBMEC). To clarify this point, we have now explicitly referenced these pathways in the Results section:

lines 188-190:

"Gene ontology (GO) over-representation analysis further supports the shift towards an improved endothelial identity in ETS-iBMEC, including upregulation of biological processes involved in angiogenesis, endothelial proliferation, blood circulation, and cell adhesion (Source Data File 1)."

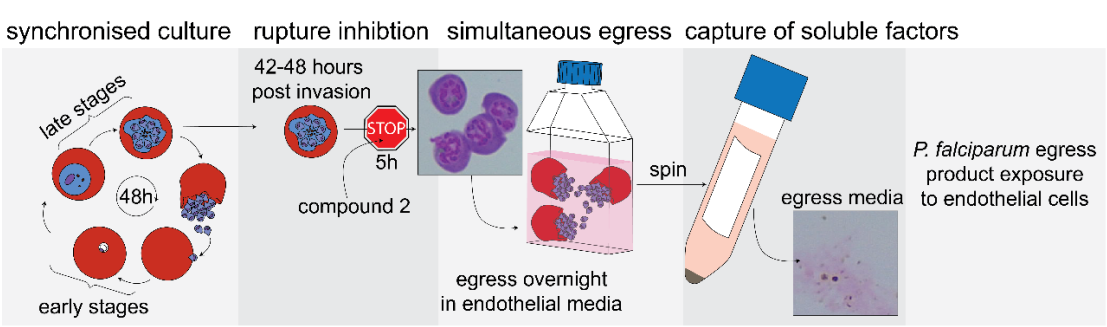
lines 204-207:

"Among these, epithelial developmental processes were found to be downregulated, together with terms associated with cell-contact organisation, extracellular organisation, cell motility, contraction and multicellular organisation (Fig 2D)."

3. For the parasite egress media preparation upon treatment with compound # 2, some data on the parasite egress window (such as Giemsa images or flow plots after treatment and upon removal of compound) might be useful, as supporting data.

We thank the reviewer for this important point. For clarifying egress media preparation in more detail, we added details and Giemsa images into the preparation flow plot of Fig. 5A. In the figures we exemplify the tight synchronization of infected-RBC as well as the lack of intact infected-RBC in the egress media. The figure 5A looks now like this:

A



Referee #3 (Comments on Novelty/Model System for Author):

General Assessment

This is a well-written and carefully executed study. The authors establish an innovative iPSC-derived endothelial model with improved identity and barrier function, and demonstrate its application in studying *Plasmodium falciparum* sequestration and endothelial disruption in vitro. The methodology is sound, the figures are clear, and the work addresses an important limitation in cerebral malaria research. The manuscript will be of interest to the infection biology and stem cell modeling communities.

However, the study is primarily descriptive. While it successfully documents differentiation improvements, barrier properties, parasite adhesion, and transcriptomic alterations, it stops short of probing mechanistic underpinnings. This limits its impact in terms of uncovering new biology.

We thank the reviewer for their positive and thoughtful evaluation of our study and for recognising the technical clarity and relevance of the ETS-iBMEC platform for cerebral malaria research. We agree that the work was primarily descriptive in nature as it was targeted to a "Methods manuscript", but in the current version, we have incorporated suggestions from referee three to make it more mechanistic. We appreciate the suggestion of the reviewer and we believe that the addition of the suggestions has strengthened the biological insight of the manuscript.

Major Comments

Mechanistic depth

The transcriptomic analyses highlight alterations in junctional, cytoskeletal, angiogenic, and metabolic pathways, but the study does not establish causality.

Functional validation of candidate pathways (e.g., through knockdown/overexpression, pharmacological inhibition, or rescue assays) would greatly strengthen the conclusions.

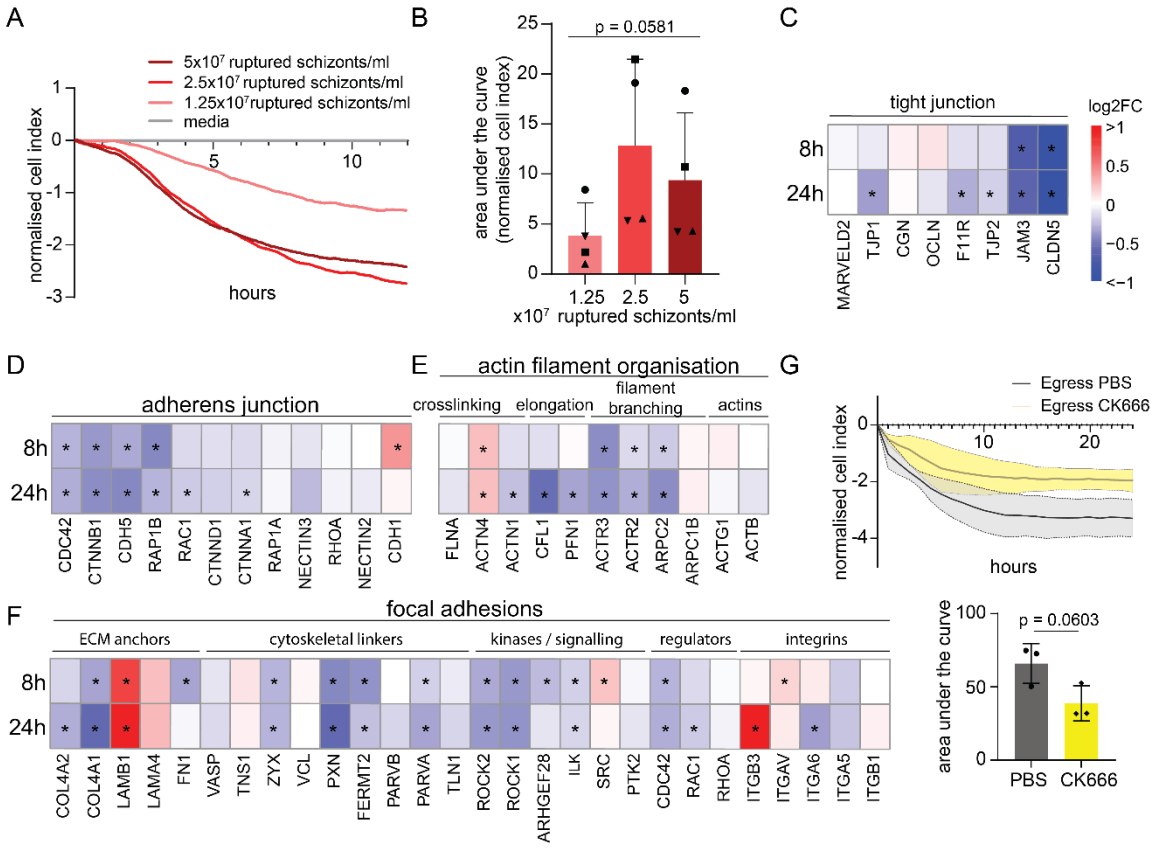
We thank the reviewer for highlighting this important point. In the current version, we extended the manuscript with the addition of 4 new chemical/pharmacological rescue assays that increase the mechanistic depth of the study. Our current and previous studies indicate that endothelial disruption during malaria infections results from the combined impact on multiple pathways. We focus these mechanistic assays in pathways prominently altered by malaria egress product. First, we focused on the well-known Angipoietin-Tie2 axis (AKB-9778), and later in two new disruptive mechanisms, cytoskeletal disruption (CK666) and Notch-VEGF (recombinant DLL4 and ROBO4). These assays allowed us to test whether modulation of these pathways prevents endothelial breakdown during *in vitro* infection, suggesting a prominent role in malaria pathogenesis.

The results of these functional perturbation assays are now included in Figure 6 and the newly added Figure 7. We have integrated transcriptomic analysis with mechanistic assays to improve the readability of the manuscript.

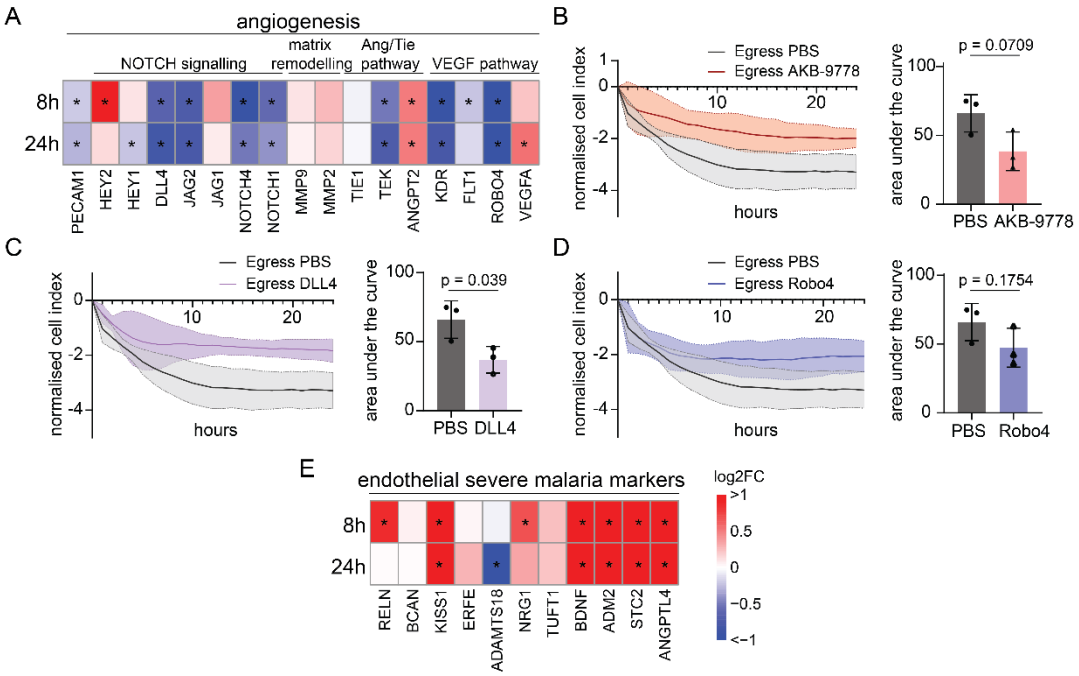
lines 440-442:

“Interestingly, pharmacological interference of the Arp2/3 complex using CK666, an actin polymerisation inhibitor, partially attenuated endothelial barrier disruption under malaria conditions (Fig 6F).”

Updated Figure 6



New Figure 7, containing previous transcriptomic analysis of Fig 6 and new mechanistic rescue experiments:



We furthermore added the following sections to describe the new results:

lines 479-494:

“To determine whether these vascular pathways play a causal role in parasite-induced barrier disruption, we conducted targeted rescue experiments. We first focused on the Angiopoietin-Tie2 axis, because previous pharmacological rescue using AKB-9778, a Tie2 activator, had shown barrier-protective effects in primary 3D brain microvascular models (Long *et al*, 2025). Consistent with these results, a 1-hour AKB-9778 pre-treatment provided a partial protective effect against parasite-mediated barrier disruption (Fig 7B). Next, we examined the newly observed alterations in VEGF and Notch signalling. A 1-hour pre-treatment with soluble recombinant DLL4, the Notch ligand delta-like ligand 4 (Boardman *et al*, 2019), significantly rescued parasite-mediated barrier disruption, providing the first demonstration that DLL4 can modulate endothelial barrier function in the context of malaria (Fig 7C). A 1-hour pre-treatment with recombinant Robo4, a protein that counteracts VEGF signalling (Jones *et al*, 2008), showed a non-significant trend towards barrier protection (Fig 7D). Together, these results confirm the therapeutic value of the Angiopoietin-Tie2 axis in protecting against malaria-mediated barrier disruption and identify VEGF-Notch signalling as an additional potential therapeutic target that partially restores endothelial barrier integrity during *P. falciparum* egress.”

lines 649-669:

“Chemical modulation of this pathway has shown protective effects in animal models (Higgins et al, 2016) and a primary 3D brain microvascular model (Long *et al*, 2025), a finding confirmed in this study through barrier protection after addition of the Tie2 activator AKB-9778. Importantly, the use of ETS-iBMEC revealed for the first-time *in vitro* alteration of the Notch and VEGF pathways. Elevated VEGF has been observed in regions of *P. falciparum*-infected RBC sequestration in the brain of cerebral malaria patients and is strongly associated with patient

outcomes (Furuta *et al*, 2010; Jain *et al*, 2008; Medana *et al*, 2010). Consistent with these reports, we observed a modest rescue of barrier disruption following treatment with Robo4, a molecule that counteracts VEGF signalling. Our study also identified, for the first time, parasite-mediated inhibition of the Notch pathway, a key regulator that counterbalances VEGF angiogenic and barrier-disruptive effects. We showed that activation of the Notch pathway through the addition of the soluble recombinant ligand DLL4 promoted barrier-restorative responses in ETS-iBMEC exposed to parasite products. In addition to these signalling pathways, modulation of actin dynamics also contributed to endothelial barrier function. The partial protection observed upon Arp2/3 inhibition suggests a potential role of endothelial mechanics on endothelial barrier integrity during malaria exposure. Additionally, this study gives novel perspectives towards broad alterations of RNA processing at the transcript level. Our data indicate that exposure to parasite products directly modulates alternative splicing outcomes. Although conclusions on their immediate impact on endothelial function remain limited, the findings highlight the importance of considering post-transcriptional regulation, including alternative splicing, as an additional layer contributing to endothelial dysfunction in malaria."

Barrier disruption

The finding that parasite egress products disrupt endothelial integrity is important, but the work does not dissect which specific mediators or pathways drive this effect. Clarifying whether the observed junctional changes are primary or secondary consequences would add mechanistic weight.

We thank the reviewer for raising this very important point. We fully agree that identifying the precise parasite-derived mediators and the pathways through which they induce barrier disruption would provide very valuable mechanistic insight. Understanding whether the observed junctional changes are direct or secondary is an important point, but this is beyond the scope of the paper. Our group is trying to identify in a different study endothelial disruptive mechanism that appear quickly after exposure to parasite egress products. Our unpublished data supports that malaria parasites cause a quick mechanical change on the endothelial cell cytoskeleton that could induce these changes. These changes have also been proposed before by others through the use of kinase inhibitors (Ortolan *et al*, 2025). The rescue with CK666 observed in this study also point into this direction. Nevertheless, this study is mostly focused on late transcriptomic changes that are quite likely a secondary consequence.

To reflect the reviewer's important point in the discussion of the study, we added the following paragraph:

lines 636-643:

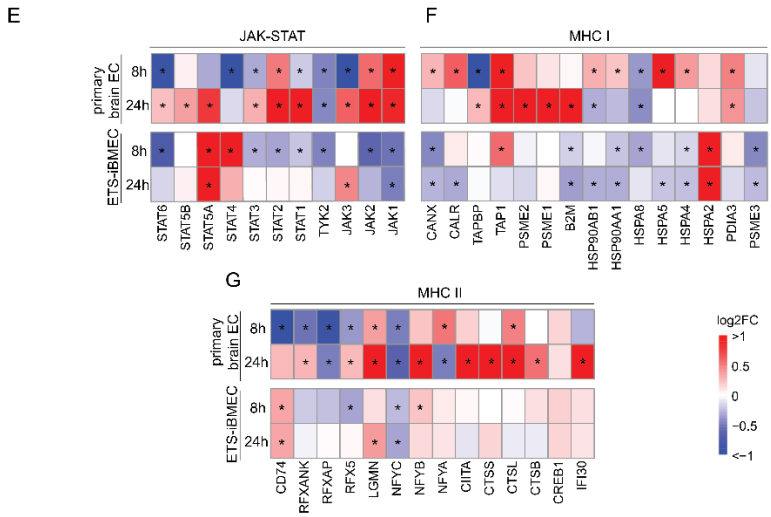
"Nevertheless, it remains unclear whether junctional and barrier transcriptional changes are directly caused by the parasite or secondarily from downstream activation of other endothelial pathways. As previous studies have shown a link between barrier breakdown and alterations in inflammatory pathways or endothelial-pericyte crosstalk, we hypothesise that the observed barrier transcriptional effects in ETS-iBMEC reflect a secondary response to parasite egress products (Long *et al*, 2025; Piatti *et al*, 2025). Nonetheless, direct parasite-mediated endothelial disruption cannot be ruled out, and dissecting the specific parasite mediators and the timeline of the disruptive mechanisms will require further investigation."

Comparisons with primary endothelial cells

ETS-iBMECs outperform primary brain ECs in barrier properties, but the differences in pathogenic responses between the two models are not fully explored. Additional comparative analysis (e.g., pathway-level or functional differences) could help contextualize the utility and limitations of this new system.

We thank the reviewer for this thoughtful comment. We agreed that a direct comparison between primary ECs and ETS-iBMEC, is important for assessing both strengths and limitations of the platform. Accordingly, we performed direct comparison analysis between ETS-iBMEC, primary brain endothelial cells, and iBMEC-*like* cells across multiple dimensions of *P. falciparum* pathogenesis. We have redrafted the manuscript to emphasized sections that included a comparison. These included:

- Cytoadhesion experiments that directly compare the three cell types in Figure 4.
- Transcriptional response events following parasite egress product exposure in all three cell types, including global differential gene expression analyses were already included in Fig. EV4.
- Direct comparison across barrier-associated junctional genes, cytoskeletal organization, and cellular attachment pathways (Fig. 6 for ETS-iBMEC and Fig. EV5 for primary EC). To extend and further contextualize the utility and limitations we provided additional comparative analyses on JAK-STAT, MHC I and MHC II directly comparing primary EC with ETS-iBMEC and highlighted its conclusions in the discussion. Therefore, Figure EV5 was extended with the following panels:



The results were changed accordingly (underlined showing improvements on the text for the comparison":

lines 496-502:

"Viral response and interferon signalling pathways, including JAK–STAT, were previously reported in a primary 3D blood–brain barrier model in response to parasite egress products (Piatti *et al*, 2025), and were similarly observed in the primary EC transcriptomic analysis in this study. However, ETS-iBMEC did not show overexpression of these pathways (see comparison

in Fig EV5E). Similarly, antigen presentation pathways (MHCI and MHCII) were largely restricted to primary brain EC and were not prominent in ETS-iBMEC (Fig EV5F-G)."

lines 4448-455:

"Conversely, primary brain ECs presented a mixed transcriptional response characterised by a general upregulation of cytoskeletal transcripts, alongside the downregulation of several key barrier-associated transcripts, including tight junctional genes (*OCLN*, *TJP1*, *CLDN5*), adherens junction gene *CDH5*, the cytoskeletal regulator ACTB and focal adhesion components such as PXN. Notably, the downregulation of junctional, cytoskeletal and focal adhesion transcripts was more pronounced and robust in ETS-iBMEC than in primary brain ECs (Fig EV5A-C). These results indicate that ETS-iBMEC present a more coordinated and robust transcriptional response than primary brain EC."

A few paragraphs above, we also highlighted the transcriptomic analysis comparison between the three cell types.

Lines 368-375:

"Shared dysregulated GO term processes across primary, iBMEC-*like* and ETS-iBMEC included upregulation of sterol and cholesterol synthesis pathways (Figs 5B-E and EV4C). Other upregulated processes specific to ETS-iBMEC were circulatory regulation and synaptic organisation, with the latter including endothelial transporters important for barrier regulation (AMPAR, NMDAR, KAR, CACN) and angiogenesis-related transcripts (*NTN1, SEMA3, SEMA4, SEMA7, L1CAM*) (Fig 5B, Source Data file 2). A common downregulated process among the three endothelial cell lines with special relevance in ETS-iBMEC was RNA splicing, along with other terms associated with RNA processes and translational regulation (Figs 5C and EV4C)."

In the discussion we extended conclusions on the comparison with primary ECs:

Lines 629-634:

"Consistent with observations in primary endothelial models, ETS-iBMEC exhibited barrier breakdown measured by real-time impedance assays. Endothelial barrier disruption was accompanied by a robust downregulation of nearly all key transcripts encoding tight and adherens junctions, including occludin and ZO-1, which have been previously reported as decreased in histopathological labelling of cerebral malaria from paediatric (Brown *et al*, 2001) and adult cerebral malaria post-mortem samples (Brown *et al*, 1999)."

Lines 680-682:

"Another limitation is that ETS-iBMEC do not activate biological processes associated with the response to viruses upon challenge with *P. falciparum*-infected RBC products, including JAK-STAT signalling."

Lines 687-693:

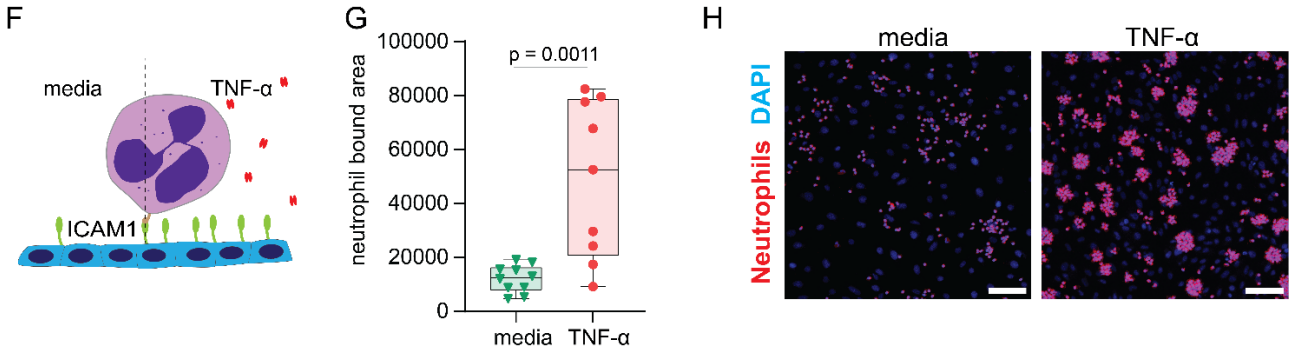
"Notably, the limited innate immune signalling response in ETS-iBMEC could reflect incomplete maturation of antiviral sensing pathways. Consequently, while ETS-iBMEC are well-suited for studying parasite-endothelium interactions and barrier dysfunction, their use for screening immunomodulatory therapies targeting innate immune pathways should be complemented with primary BBB models. Therefore, future improvements in endothelial differentiation approaches

should enhance innate immune responses in iPSC-derived cells, as well as improve applicability to 3D physiological models."

Physiological relevance

The model convincingly captures parasite adhesion and barrier disruption. However, incorporating or at least discussing additional physiological components (e.g., leukocyte interactions, brain-specific transcription factors, or immune responses) would strengthen the claim of relevance to cerebral malaria.

We thank the reviewer for this important point. We agree that incorporating additional physiologically relevant components, particularly inflammatory and immune interactions, is critical for modelling cerebral malaria. For example, previous reports in patients have highlighted the importance of Neutrophil Extracellular Traps (Knackstedt *et al*, 2019). To address neutrophil-endothelial interactions in our system, we performed neutrophil perfusion experiments under flow using resting and TNFα-activated ETS-iBMEC microfluidic channels. As expected, TNF-α stimulation significantly increased neutrophil adhesion to the endothelium, demonstrating that ETS-iBMECs respond to inflammatory cues and recapitulate physiologically relevant immune cell recruitment. We have added the new data to Figure 4:



Furthermore, we added the following sections:

lines 320-328:

"We next evaluated the capacity of ETS-iBMEC to facilitate neutrophil adhesion under inflammatory conditions, given the reported association between neutrophil extracellular traps (NETs) and malaria pathogenesis (Knackstedt *et al*, 2019). Fluorescently labelled neutrophils from healthy donors were perfused using the same protocol applied for *P. falciparum*-infected RBCs (Fig 4F). Neutrophils adhered to ETS-iBMEC under both resting and TNF-α-activated conditions, with endothelial activation inducing a four-fold increase in adhesion (Fig. 4G-H). Together, the cytoadhesion of *P. falciparum*-infected RBCs and neutrophils to ETS-iBMEC supports their use as a physiologically relevant endothelial model to study host-pathogen interactions in malaria and other vascular infectious diseases."

lines 538-541:

"We confirmed ETS-iBMEC as a valid model to study malaria pathogenesis and inflammatory response through binding studies of *P. falciparum*-infected RBCs and neutrophils under flow, transcriptomics analysis, as well as barrier disruption and rescue assays."

Brain specific transcription factors:

Beyond immune interactions, we agree that refining brain-specific lineage specification is an important future direction for closer mimicking brain endothelial identity. In the previous version, we already discuss recent advances incorporating brain-specific transcription factors such as FOXF2 and ZIC3, as well as modulation of Wnt and TGFβ signaling pathways, which have been shown to enhance brain endothelial identity (Cui et al., 2024; Ding et al., 2025).

To further improve clarity, we have slightly expanded this section to more explicitly position these strategies as potential next steps for strengthening the brain specificity of the ETS-iBMEC platform:

lines 574-583:

"Future iterations of the model to further improve the differentiation toward brain-specific lineage, relevant for cerebral malaria studies, could incorporate additional transcriptional programs. For example, inducible expression of brain-enriched transcription factors such as FOXF2 and ZIC3 has been shown to strengthen brain identity and barrier properties (Cui *et al*, 2024). Similarly, SOX18 induction promotes barrier tightening accompanied by increased P-glycoprotein expression, indicative of enhanced BBB maturation (Zhang *et al*, 2023). In parallel, the addition of Wnt activators and TGFβ inhibitors has also been shown to further promote brain endothelial specification (Ding *et al*, 2025). Together, integrating these combinatorial strategies into the ETS-iBMEC platform may further advance the development of physiologically refined, brain-specific endothelial infection models."

Additional clarifications and controls Nature of the ETS-iBMEC line: The authors refer to ETS-iBMEC as a cell line. Is it truly stable? Once DOX is removed, for how long is transgene expression maintained?

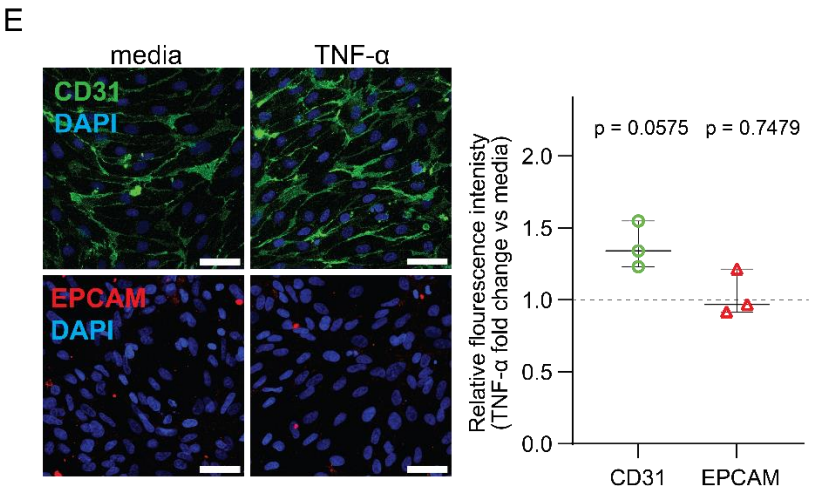
We thank the reviewer for this important clarification. We agree that the term "stable cell line" may be misleading in the context of a DOX-inducible Tet-On system. As demonstrated in previous work using an identical inducible setup, transgene expression declines rapidly after washing out DOX, with degradation of the expressed proteins occurring within hours (Matsuda *et al*, 2020).

To avoid confusion, we have revised the manuscript throughout and now refer to ETS-iBMEC as "cell model".

TNFα stimulation and marker expression: When TNFα is added, do ETS-iBMECs still express CD31 while EPCAM remains suppressed? This would further validate endothelial identity under inflammatory conditions.

We thank the reviewer for this important point, which is highly relevant for validating endothelial phenotypes under inflammatory conditions. To address this, we performed TNF-α stimulation and quantified immunofluorescence intensities of endothelial CD31 and epithelial EPCAM in ETS-iBMECs. TNF-α treatment did not alter the expression of either maker relative to the control intensity, with CD31 remaining robustly expressed and EPCAM staining remaining absent. We only observed a slight increase of CD31 staining, probably as a consequence of

junctional reorganization These results confirm that ETS-iBMECs maintain endothelial identity under inflammatory stimulation. We included this data in Fig. EV1



Furthermore, we added to the results section:

lines 155-159:

"We did not observe changes in the cellular identity under inflammatory conditions following TNF-α treatment, as indicated by the lack of EPCAM induction. However, we observed a moderate increase in CD31 expression after TNF-α stimulation, likely reflecting junctional rearrangement associated with endothelial activation (Fig EV1E)."

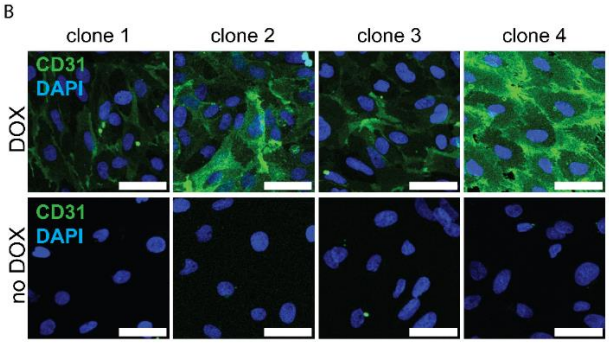
Transgene integration: Did the authors check the genomic insertion sites of the piggyBac transposon? This could be relevant to rule out positional effects.

We thank the reviewer for this important question. While we did not perform genomic insertion site mapping, piggyBac-mediated transposition typically results in multiple genomic integration events, often exceeding more than ten copies per clone (Matsuda *et al*, 2015; Yusa *et al*, 2011);

Importantly, we isolated and characterized several ETS-iBMEC clones. All clones exhibited comparable endothelial recovery profiles following doxycycline induction. The consistency of the phenotype across independent clones supports the interpretation that positional integration effects are minimal.

We added a panel showing endothelial recovery through DOX induction in Fig. EV1 and added the following sections to the manuscript:

Fig EV1:



lines 130-134:

"Stable genomic integration of the three inducible ETS factors was achieved using the piggyBac transposase system (Fig EV1A). All clonal lines isolated presented increased expression of the endothelial marker CD31 after DOX induction (Fig EV1B). We selected clone 1 that showed robust induction of all three factors for further experiments (Fig EV1C)."

We mentioned the use of a single clone as a limitation in the discussion:

lines 677-680:

"Additionally, this study uses a single ETS-iBMEC clone for infection readouts; however, consistent expression of endothelial markers across independent clones and previous reports on ETS-based endothelial programming support our approach (Gong *et al*, 2022; Koyano-Nakagawa & Garry, 2017; Lu *et al*, 2021)."

Figure consistency: In Fig. 3F-H, the ETS-iBMEC images are shown at different magnifications compared to controls, making direct comparison difficult. Please harmonize image scales.

We thank the reviewer for this observation. Image scales are now harmonized for Fig. 3F – H:

TNFα in egress assays: In Fig. 5A, was TNFα pre-treatment included before exposure to parasite egress products? If so, this should be clearly stated.

No, the three cell types were not TNF-α primed before parasite egress product exposure as our study aim to focus on parasite-mediated alterations in endothelial transcription. To clarify this for all readers of the article, we mentioned this important point in the results section.

lines 361-365:

"To understand the molecular mechanisms upon exposure to *P. falciparum*-iRBC products, we performed bulk RNA-seq after 8 and 24 hours of exposure to 5 × 10^7 egressed infected RBCs/ml on resting ETS-iBMEC. As a control, we also sequenced iBMEC-*like* and primary brain EC under the same conditions."

Antigen presentation: Brain endothelial cells are known to present parasite antigens in mouse models. Did the authors assess expression of MHC class I/II in ETS-iBMECs?

This is a very important point raised by the reviewer, we have provided additional data on MHC class I/II expression in both ETS-iBMEC and primary ECs Figure EV6, as already highlighted above in the point "Comparisons with primary endothelial cells".

Alternative splicing relevance: The observation of widespread splicing changes (Fig. 5G) is intriguing. Can the authors expand on the potential biological relevance of these splicing events in endothelial function or malaria pathogenesis?

We thank the reviewer for highlighting the need to discuss these finding. While direct conclusions on endothelial function remain limited, we extended the discussion about alternative splicing as contributor to malaria pathology.

lines 664-669:

"Additionally, this study gives novel perspectives towards broad alterations of RNA processing at the transcript level. Our data indicate that exposure to parasite products directly modulates alternative splicing outcomes. Although conclusions on their immediate impact on endothelial function remain limited, the findings highlight the importance of considering post-transcriptional regulation, including alternative splicing, as an additional layer contributing to endothelial dysfunction in malaria"

Minor Comments

The discussion could better distinguish which observations are novel mechanistic insights versus confirmation of known processes.

We clarified the following sections in the discussion.

lines 600-602:

"As previously reported, *P. falciparum* lines present a wide heterogeneity in binding depending on the parasite or endothelial cell line, endothelial activation status, flow mechanical cues and temperature (Bernabeu *et al*, 2019; Introini *et al*, 2025)."

lines 622-626:

"Our previous work, along with others, has shown that *P. falciparum* egress in proximity to endothelial cells is a major driver of barrier breakdown in primary brain EC (Long *et al*, 2025; Piatti *et al*, 2025; Zuniga *et al*, 2022). These studies suggest that parasite-mediated disruption is a consequence of a combination of conserved parasite-egress released factors, such as heme, hemozoin, HRP2 or GPI anchors, rather than a single dominant effector."

lines 638-641:

"As previous studies have shown a link between barrier breakdown and alterations in inflammatory pathways or endothelial-pericyte crosstalk, we hypothesise that the observed barrier transcriptional effects in ETS-iBMEC reflect a secondary response to parasite egress products (Long *et al*, 2025; Piatti *et al*, 2025)."

lines 644-646:

"Exposure of ETS-iBMEC to parasite egress products led to a transcriptional upregulation of genes implicated in malaria pathogenesis, as initially described in *in vitro* and patient plasma biomarker samples (Gomes *et al*, 2023; Zuniga *et al*, 2022)."

lines 650-653:

"Chemical modulation of this pathway has shown protective effects in animal models (Higgins et al, 2016) and a primary 3D brain microvascular model (Long *et al*, 2025), a finding confirmed in this study through barrier protection after addition of the Tie2 activator AKB-9778."

lines 652-653:

"Importantly, the use of ETS-iBMEC revealed for the first-time *in vitro* alteration of the Notch and VEGF pathways."

lines 657-659:

"Our study also identified, for the first time, parasite-mediated inhibition of the Notch pathway, a key regulator that counterbalances VEGF angiogenic and barrier-disruptive effects."

lines 664-665:

"Additionally, this study gives novel perspectives towards broad alterations of RNA processing at the transcript level."

Figures are generally clear, but some legends are dense. Simplification or an added schematic summary would help readability.

We thank the reviewer for pointing that out and tried to keep the figure legends simpler. Nevertheless, because this article is a Methods paper, the current version still presents still presents extended descriptions in figure legends that we considered important from a methods perspective.

A short statement on throughput and reproducibility of this model in routine laboratory settings would be useful for readers considering adoption.

All the studies shown in the study were done in independent differentiations performed by different members of the group. We extended our statement in the limitations section of the discussion.

lines 674-676:

"The differentiation protocols require specialised training and are laborious, and although ETS-iBMEC can be incorporated in complex 3D microvessel models, the low throughput of these systems limits their use in complex mechanistic infection studies in 3D."

Referee #3 (Remarks for Author):

Recommendation
Major Revision. The manuscript is strong in methodology and descriptive characterization, but its impact would be significantly enhanced by additional mechanistic interrogation and clarification of the points listed above.

Additional references:

Avril M, Bernabeu M, Benjamin M, Brazier AJ, Smith JD (2016) Interaction between Endothelial Protein C Receptor and Intercellular Adhesion Molecule 1 to Mediate Binding of Plasmodium falciparum-Infected Erythrocytes to Endothelial Cells. *mBio* 7

Avril M, Brazier AJ, Melcher M, Sampath S, Smith JD (2013) DC8 and DC13 var genes associated with severe malaria bind avidly to diverse endothelial cells. *PLoS Pathog* 9: e1003430

Bernabeu M, Gunnarsson C, Vishnyakova M, Howard CC, Nagao RJ, Avril M, Taylor TE, Seydel KB, Zheng Y, Smith JD (2019) Binding heterogeneity of Plasmodium falciparum to engineered 3D brain microvessels is mediated by EPCR and ICAM-1. *MBio* 10: 10.1128/mbio. 00420-00419

Knackstedt SL, Georgiadou A, Apel F, Abu-Abed U, Moxon CA, Cunnington AJ, Raupach B, Cunningham D, Langhorne J, Kruger R *et al* (2019) Neutrophil extracellular traps drive inflammatory pathogenesis in malaria. *Sci Immunol* 4

Lennartz F, Adams Y, Bengtsson A, Olsen RW, Turner L, Ndam NT, Ecklu-Mensah G, Moussiliou A, Ofori MF, Gamain B *et al* (2017) Structure-Guided Identification of a Family of Dual Receptor-Binding PfEMP1 that Is Associated with Cerebral Malaria. *Cell Host Microbe* 21: 403-414

Matsuda M, Hayashi H, Garcia-Ojalvo J, Yoshioka-Kobayashi K, Kageyama R, Yamanaka Y, Ikeya M, Toguchida J, Alev C, Ebisuya M (2020) Species-specific segmentation clock periods are due to differential biochemical reaction speeds. *Science* 369: 1450-1455

Matsuda M, Koga M, Woltjen K, Nishida E, Ebisuya M (2015) Synthetic lateral inhibition governs cell-type bifurcation with robust ratios. *Nat Commun* 6: 6195

Ortolan LS, Avril M, Xue J, Seydel KB, Zheng Y, Smith JD. (2022) *Plasmodium falciparum* parasite lines expressing DC8 and Group A PfEMP1 bind to brain, intestinal, and kidney endothelial cells. *Frontiers in Cellular and Infection Microbiology*.12:813011.

Ortolan LS, Bansal P, Primavera VI, Freitas R, Wei L, Epiphanio S, Kaushansky A, Smith JD (2025) Nilotinib attenuates vascular pathology in experimental cerebral malaria. *Blood Adv* 9: 2473-2488

Piatti L, Batzilla A, Nakaki F, Fleckenstein H, Korbmacher F, Long RKM, Schraivogel D, Hawkins JA, Romero-Urunuela T, Lopez-Gutierrez B *et al* (2025) Plasmodium falciparum egress disrupts endothelial junctions and activates JAK-STAT signaling in a microvascular 3D blood-brain barrier model. *Nat Commun* 16: 7262

Yusa K, Zhou L, Li MA, Bradley A, Craig NL (2011) A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci U S A* 108: 1531-1536

13th May 2026

Dear Dr. Bernabeu Aznar,

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

1) Authors: We note name discrepancy in the manuscript and in our submission system: Alina Batzilla vs. Alina Bazilla. Please correct.
2) Figures: We note that some panels/images are reused e.g. Figure 3C, F and G and Figure EV3A-B. Please cite in the respective figure legend every reused panel/image.
3) Source Data Files: The source data files 1 - 3 should be renamed Dataset EV1 - Dataset EV3 and a legend should be added to each file, in a separate tab/worksheet. The file uploaded as source data file 4 should be renmaed Table EV1 and a legend should be added to the top of the page. Please update their callouts in the main text.
4) In the main manuscript file, please do the following:
- Please address all comments suggested by our data editors listed below:
o Data availability statment: Please note that the specific URL for E-MTAB-14937 dataset is not provided.
o Figure legends:
1. Please indicate the statistical test used for data analysis in the legends of figures 2A-D; 5B, C.
2. Please note that the box plots need to be defined in terms of minima, maxima, centre, bounds of box and whiskers, and percentile in the legends of figures 3D, E; 4D, E, G.
3. Please note that information related to n is missing in the legend of figure EV1 C.
4. Please note that the error bars are not defined in the legends of figures 6B, G; EV1 E.
- Remove all figures and leave only their legends at the end of the file.
- Add callouts for Figure EV4.
- In Methods, statistical paragraph should reflect all information that you have filled in the Authors Checklist, especially regarding randomization, blinding, replication.
- Remove Reagents and Tools Table and upload it as a separate .doc file. Please check "Author Guidelines" for more information and to download table templates.
https://www.embopress.org/page/journal/17574684/authorguide#structuredmethods
- Indicate in legends exact n and exact p values, not a range, along with the statistical test used. To keep the figures "clear" some authors found providing an Appendix table Sx with all exact p-values preferable. You are welcome to do this if you want to.
- Rename "Declaration of interests" to "Disclosure and competing interests statement". We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary.
- Author contributions: Please remove it from the manuscript and specify author contributions in our submission system. CRediT has replaced the traditional author contributions section because it offers a systematic machine-readable author contributions format that allows for more effective research assessment. You are encouraged to use the free text boxes beneath each contributing author's name to add specific details on the author's contribution. More information is available in our guide to authors:
https://www.embopress.org/page/journal/17574684/authorguide#authorshipguidelines
- In data availability statement please use the following format to report the accession number of your data:

The datasets produced in this study are available in the following databases:
[data type]: [full name of the resource] [accession number/identifier] ([doi or URL or identifiers.org/DATABASE:ACCESSION])

Please check "Author Guidelines" for more information.
https://www.embopress.org/page/journal/17574684/authorguide#availabilityofpublishedmaterial
5) Appendix: Please upload the Appendix Figure S1 as an EV figure and update its callouts in the text.
6) Funding: Please make sure that information about all sources of funding are complete in both our submission system and in the manuscript. The European Research Council and the Alexander von Humboldt Foundation are currently missing in our submission system.
7) Synopsis:
- Synopsis image: Please resize the image to exactly 550 px-wide x 300 pixels high and upload it as a high-resolution jpeg file.
- Please check your synopsis text and image before submission with your revised manuscript. Please be aware that in the proof stage minor corrections only are allowed (e.g., typos).
8) As part of the EMBO Publications transparent editorial process (see our Editorial at http://embomolmed.embopress.org/content/2/9/329), EMBO Molecular Medicine will publish online a Review Process File (RPF) to accompany accepted manuscripts. This file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. Let us know whether you agree with the publication of the RPF and as here, if you want to remove or not any figures from it prior to publication. Please note that the Authors checklist will be published at the end of the RPF.

9) Please provide a point-by-point letter INCLUDING my comments as well as the reviewer's reports and your detailed responses (as Word file).

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Zeljko Durdevic

Zeljko Durdevic
Senior Editor
EMBO Molecular Medicine

*** Instructions to submit your revised manuscript ***

*** PLEASE NOTE *** As part of the EMBO Publications transparent editorial process initiative (see our Editorial at https://www.embopress.org/doi/pdf/10.1002/emmm.201000094), EMBO Molecular Medicine will publish online a Review Process File to accompany accepted manuscripts.

In the event of acceptance, this file will be published in conjunction with your paper and will include the anonymous referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript. If you do NOT want this file to be published, please inform the editorial office at contact@embomolmed.org.

*****

When preparing your revised manuscript, please refer to our guidelines: https://link.springer.com/journal/44321/submission-guidelines#cms-Revised-submissions. We perform an initial quality control of all revised manuscripts before re-review; failure to include requested items will delay the evaluation of your revision.

We require:

1) A .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) Individual production quality figure files as .eps, .tif, .jpg (one file per figure). For guidance, download the 'Figure Guide PDF': https://media.springernature.com/original/springer-cms/rest/v1/content/27825798/data/v1.

3) A .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) A complete author checklist, which you can download from our author guidelines. Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript.

6) It is mandatory to include a 'Data Availability' section after the Materials and Methods. Before submitting your revision, primary datasets produced in this study need to be deposited in an appropriate public database, and the accession numbers and database listed under 'Data Availability'. Please remember to provide a reviewer password if the datasets are not yet public.

In case you have no data that requires deposition in a public database, please state so in this section. Note that the Data Availability Section is restricted to new primary data that are part of this study.

7) For data quantification: please specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments (specify technical or biological replicates) underlying each data point and the test used to calculate p-values in each figure legend. The figure legends should contain a basic description of n, P and the test applied. Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

8) At EMBO Press we ask authors to provide source data for the main manuscript figures. You will receive a separate email with instructions for providing source data with your revised manuscript, including how to upload and organize the files.

9) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference.

10) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc.

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

11) The paper explained: EMBO Molecular Medicine articles are accompanied by a summary of the articles to emphasize the major findings in the paper and their medical implications for the non-specialist reader. Please provide a draft summary of your article highlighting

- the medical issue you are addressing,

- the results obtained and

- their clinical impact.

This may be edited to ensure that readers understand the significance and context of the research. Please refer to any of our published articles for an example.

12) Author contributions: You will be asked to provide CRediT (Contributor Role Taxonomy) terms in the submission system. These replace a narrative author contribution section in the manuscript.

13) A Conflict of Interest statement should be provided in the main text.

14) Every published paper includes a 'Synopsis' to further enhance discoverability. Synopses are displayed on the journal webpage and are freely accessible to all readers. They include a short stand first (maximum of 300 characters, including space) as well as 2-5 one-sentences bullet points that summarizes the paper. Please write the bullet points to summarize the key NEW findings. They should be designed to be complementary to the abstract - i.e. not repeat the same text. We encourage inclusion of key acronyms and quantitative information (maximum of 30 words / bullet point). Please use the passive voice. Please attach these in a separate file or send them by email, we will incorporate them accordingly.

Please also suggest a striking image or visual abstract to illustrate your article as a PNG file 550 px wide x 300 px high.

15) Include a Reagents and Tools Table as part of the Methods section, which can be downloaded from our author guidelines.

EMBO Molecular Medicine has a "scooping protection" policy, whereby similar findings that are published by others during review or revision are not a criterion for rejection. Should you decide to submit a revised version, I do ask that you get in touch after three months if you have not completed it, to update us on the status.

*Additional important information regarding Figures

Each figure should be given in a separate file and should have the following resolution:

Graphs 800-1,200 DPI
Photos 400-800 DPI
Colour (only CMYK) 300-400 DPI"

Figures are not edited by the production team. All lettering should be the same size and style; figure panels should be indicated by capital letters (A, B, C etc). Gridlines are not allowed except for log plots. Figures should be numbered in the order of their appearance in the text with Arabic numerals. Each Figure must have a separate legend and a caption is needed for each panel.

*Additional important information regarding figures and illustrations can be found at https://media.springernature.com/original/springer-cms/rest/v1/content/27825798/data/v1

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

Referee #1 (Comments on Novelty/Model System for Author):

This manuscript describes the generation and characterization of an improved iPSC-derived model system to study interactions between the Plasmodium falciparum parasites and blood vessels, which are key to the pathogenesis of cerebral malaria. The study is well designed, the analyses are robust and quantitative, and the conclusions are well-supported by the data. This is an important contribution to the literature.

Referee #1 (Remarks for Author):

The authors have adequately responded to the reviewer feedback. The inclusion of the rescue studies in particular highlights the importance of working towards functional validation of transcriptional changes observed in the model, which is a clear future path for this research.

Referee #3 (Remarks for Author):

The authors have made an excellent effort to address the referee's questions and have strengthened the manuscript. Congratulations!!

The authors addressed the remaining editorial issues.

29th May 2026

Dear Dr. Bernabeu Aznar,

We are pleased to inform you that your manuscript is accepted for publication and is now being sent to our publisher to be included in the next available issue of EMBO Molecular Medicine.

Your manuscript will be processed for publication by EMBO Press. It will be copy edited and you will receive page proofs prior to publication. Please note that you will be contacted by Springer Nature Author Services to complete licensing and payment information.

You may qualify for financial assistance for your publication charges - either via a Springer Nature fully open access agreement or an EMBO initiative. Check your eligibility: https://link.springer.com/journal/44321/how-to-publish-with-us

Should you be planning a Press Release on your article, please notify the production team via this form in order to coordinate publication and release dates: https://forms.cloud.microsoft/e/eH4A8JVP6g

If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Molecular Medicine.

Yours sincerely,
Zeljko Durdevic

Zeljko Durdevic
Senior Editor
EMBO Molecular Medicine

>>> Please note that it is EMBO Molecular Medicine policy for the transcript of the editorial process (containing referee reports and your response letter) to be published as an online supplement to each paper. If you do NOT want this, you will need to inform the Editorial Office via email immediately. More information is available here: https://link.springer.com/partners/embo-press/editorial-policies#Peer%20review

EMBO Press Author Checklist

Corresponding Author Name: Maria Bernabeu, Miki Ebisuya	
Journal Submitted to: EMBO Molecular Medicine	
Manuscript Number: EMM-2025-22404	

USEFUL LINKS FOR COMPLETING THIS FORM
The EMBO Journal - Author Guidelines
EMBO Reports - Author Guidelines
Molecular Systems Biology - Author Guidelines
EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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

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

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

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

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Source_data_file_4_Oligonucleotides

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Reagents and Tools Table and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Reagents and Tools Table and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Methods

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

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Yes	Reagents and Tools Table and Methods

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Yes	Methods

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

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

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

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

Reporting

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

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Methods
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	