EMBO reports

Blood flow diverts extracellular vesicles from endothelial degradative compartments to promote angiogenesis

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Dear Vincent,

Thank you for the transfer of your research manuscript for consideration by EMBO reports. Your manuscript has previously been reviewed by four experts in the field, who have provided constructive comments and suggestions for the improvement of the work and the manuscript (their reports are included below). We would also like to thank you for providing us with a point-by-point response to those comments (including additional data) and a revision plan, which I have now read and discussed with our editorial team.

We find your plan likely sufficient to address the referees' concerns to a satisfactory extent, and we would therefore like to invite you to revise your manuscript along the lines you suggested, with the understanding that the referee concerns (as detailed in their reports) must be taken on board. Please address all referee concerns in a complete point-by-point response. I would like to note that although we agree with referee #1 that further investigation of the fate of internalized EVs that do not follow the RAB14 path would be desirable, it will not be required for further consideration of your work by EMBO reports. Similarly, we agree with referee #4 that the transcriptional comparison of endothelial cells exposed to tEVs or synthetic nanoparticles of similar size could provide a useful additional control, but we will not require such experiments for the current study.

Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is our policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript. If you have any questions or comments, we can also discuss the revisions in a video chat, if you like.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we usually recommend a revision within 3 months (May 20th). Please discuss with me the revision progress ahead of this time if you require more time to complete the revisions.

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- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

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The following points must be specified in each figure legend:

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- Please also include scale bars in all microscopy images.

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

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have any questions or comments regarding the revision.

Best regards,

Ioannis

Ioannis Papaioannou, PhD
Editor
EMBO reports

Referee #1:

Summary

tEVs are tumour-associated extracellular vesicles that tumours release into the bloodstream and are thought to impact microenvironments favourably for tumour growth and metastasis. Here authors aim to investigate the link between tEV uptake into endothelial cells and the blood flow velocity of the respective vessels. Here authorss propose hemodynamics as a primary factor for tEV uptake and fate. Since endothelial cells are known to be responsive to mechanic stimuli, they suggest that venous blood flow velocities of around 400 m/s promote efficient tEV uptake. These optimal hemodynamics might also be involved in tEV escape from degrading acidic lysosomal pathways via RAB14/ NHE9 positive vesicles, which provide permissive environments for tEV-mediated pro-angiogenic signalling.

Over the past years, tEVs have evolved as important players involved in disease progression promoting survival, growth, pre-metastatic niches (PMNs) and altering biomedical properties of target cells. Previous studies also proposed the prognostic value of tEV plasma levels as they are associated with the metastatic rate in gastric cancer. While the functions of tEVs are diverse

depending on the malignancy and targeted cells, they have been linked several times to neoangiogenesis/ lymphangiogenesis and remodelling of the endothelium when taken up by endothelial cells (2-5). Endothelial cells have been shown to react to mechanical stimuli as shear forces generated by different blood flow velocities. Hemodynamics are likely involved in the alteration of transcriptomics and the capability of adhesion of immune cells as well as circulating tumour cells (CTCs). It was shown that CTCs depend on certain permissive flow mechanisms to transit through the endothelium.

The authors propose a very interesting and novel data investigating the uptake mechanism of tEVs into endothelial cells. The study is well-written, concise, and easy to follow.

Major comments

Overall, the experiments are coherent, and the used models (zebrafish for real-time imaging of circulation and microfluidics systems) were well chosen. The use of 4T1 cell line to acquire tEVs seems a compelling choice, especially for transcriptomic and angiogenesis analysis, as triple-negative breast cancer cells. It would be good to include the reasoning for this model and the expected transferability and possible limitations of the results.

Results section "Blood flow tunes endothelial uptake of circulating tEVs"
Figure 1a
- This part of the figure has a lot of different panels with scarce labelling. It is partly difficult to match the image with its respective description in the figure legend. One should consider either splitting figure 1a into, e.g. 1a-c or labelling each image/ graph clearly with what it is displaying (e.g. iCLAM panel, hemodynamics panel) .

Also, one could consider labelling the mentioned anatomic structures (Injection site (Duct of Cuvier) and dissemination site (caudal plexus via dorsal artery) in the graphic representation of the workflow to increase the explanatory power.

Red arrowhead indicators on iCLAM panel presumably point to labelled tEVs. This and other descriptive information about the iCLAM panel are not included in the figure legend.

Pearson Correlation: This was done with sample size n=3 and a resulting correlation coefficient of -0.65. These results were interpreted as flow profiles significantly influencing tEV uptake (see lines 126-132). However, -0.7 is the usual cut-off value for strong relationships between two variables.
(see: https://www.sciencedirect.com/topics/computer-science/pearson-correlation) Since this is the crucial set-up experiment for this study, it might have been good to try getting a stronger correlation with a bigger sample size: Later, sample sizes are often around n=30. Why such a low sample size was chosen for this experiment remains unclear.

Figure 1b-d
- Lidocaine and IBMX are given to reduce or increase heart rates to reduce or increase blood flow velocities. However, the data on how fast or slow blood flow has become is not provided, only data concerning the heartbeat. As these experiments are the reason for suspecting a tEV uptake optimum at 400 m/s these flow velocities would have been interesting as they could lend validity to the chosen optimal velocity.

In subsequent experiments using microfluidics systems, the velocities chosen are always 400 m/s versus 0 m/s (static/ no flow conditions, see e.g. Fig. 1d). These settings were elected "to confirm these in vivo data suggesting that tEV uptake is most efficient at lower velocity flow profiles of 400 m/s." However, the Licocaine/ IBMX experiments did not provide this concrete number. Previous experiments measuring tEV uptake found the most efficient uptake generally in venous regions (400-450 m/s) but this experiment had its own limitations (see F1a, Pearson Correlation).

Referencing previous studies here that confirm that 400 m/s has permissive effects on tEVs is helping their point. However, it could have been more coherent if data concerning velocities had been included
The reasoning behind static settings in microfluidics systems, opposed to e.g. lower flow settings (<400 m/s) and/ or higher velocities (to mimic arteries), remains undisclosed. No flow setting doesn't seem to allow a lot of physiological transferability. This issue applies to all subsequent microflow experiments.

Result Section "Circulating tEVs are partially re-routed to alternative non-acidic and non-degradative RAB14 positive compartments."

pHluorin is quenched when in an acidic environment and gives a stronger fluorescent signal when in a neutral environment. This information should perhaps be included so readers can immediately understand the meaning of the pHluorin/mScarlet ratio.

Figure 2a
- p-value is not given in the graph. All other graphs provide stars to indicate p-value. Here there are none, and only one p-value is given in the figure legend. Does this count for all presented plots?

Figure 2d

- Data of tEVs shift towards neutral RAB14 positive vesicles in flow settings has a lot of scattering. Only a mean of ~8% of all tEVs were found in RAB14 positive vesicles. So just looking at this data one might question how significant RAB14-positive vesicles are for the trafficking of tEV in general.

A follow-up question that arises here: "We have seen that only a small fraction (in all cases below 40%, mean ~8%) go on the RAB14 path" - Others presumably do not escape and get degraded. Or might there be another mechanism of escape? Is there a threshold of surviving tEVs for there to be biological tEV-effects on the endothelial cell?

Figure 2e
- The displayed graph does not support the point the authors want to make very well as the difference of the ratios depending on blood flow velocities can hardly be seen due to the scale of the graphs. Also, the scale is probably off because there seem to be quite a few extreme outliers and, therefore a lot of scattering. The way the data is presented it cannot be assessed how much the ratio dropped, it seems rather minimal. That very small drop might be highly significant, but it would also mean there is only a small percentage of tEVs that switch compartments which would pose the question if this shift is still biologically significant.

There is seemingly no p-value given for the IBMX experiment. No stars/p value indicators displayed in graph either. Does this experiment have the same p-value as the Lidocaine experiment? Is it non-significant?

Figure 3b/3b
- F3b graph shows "mean lysot (= lysotracking?) area" which is decreased significantly in cells from flow areas to cells from stall areas. However, only in figure legend 3d is an explanation offered of how the reduced mean area can be interpreted. This explanation should maybe be given when the term is introduced in 3b.

Figure 3e
- There seems to be a contradiction of the data shown in the graph versus the interpretation offered in the written results: While >50% of tEVs co-locate with NHE9 there is no significant difference between flow settings and no-flow settings. This is not mentioned in the written results. However, they conclude that flow dynamics could trigger NHE9 positive vesicle formation where tEV accumulate (l 331-334). This is supported by RNAseq data but not necessarily by F3e.

Figure 4a
- F4a second panel: labelling is slightly confusing and not explained in figure legend. The categories "up/ down with flow" and "up/down with EV" are not self-explanatory.

Figure 4b
- Figure legend should include all used abbreviations.
- How high is the biological significance of 2 neo-angiogenic sprouts (embryo with only tumour mass) versus 4 sprouts (Tumour mass + TC + tEV)? It could be interesting to discuss how strong and meaningful the angiogenic effect of tEVs are.

Referee #2:

General Comment:
In this manuscript, Benjamin Mary et al. set out to uncover how blood flow velocity affects the uptake of circulating tumour extravascular vesicles (tEVs) by endothelial cells, and this can affect endothelial cell biology. The authors used both in vitro and in vivo (zebrafish) models. The authors propose that endothelial cells are sensitized to uptake tEVs at a moderate blood flow velocity. Interestingly, this effect is coupled with a rerouting of internalized tEVs towards non-acidic and non-degradative Rab14-positive endosomes. The authors further propose that tEVs promote the expression of pro-angiogenic transcription factors in flow-stimulated endothelial cells which favours tumour angiogenesis in zebrafish.
Overall, this manuscript provides an interesting and novel perspective on how endothelial cells may react to tEVs. However, the manuscript has some weaknesses. The effect of tEVs in establishing the metastic niche and promoting tumour angiogenesis was described previously. Thus, the major novelty resides only on the effect of blood flow in the activity of tEVs. Yet, this aspect remains very unclear and requires additional experiments. In addition, despite the compelling evidences that blood flow may alter the trafficking path and activity of tEVs in endothelial cells, the mechanism causing this remains unidentified.
The reviewer proposes major and minor comments to improve the robustness and scope of the manuscript.

Major comments:
1- Generalization of the conclusions. The authors used tEVs derived from mouse breast 4T1 cancer cells. As the source of EVs may regulate the specificity of EV uptake, the generalization of these findings should be demonstrated by replicating the key experiments using tEVs coming from other (2/3) cancer cell lines.

2- Relationship between flow and tEVs uptake/rerouting.
a) The authors have focused exclusively in blood flow velocity. However, it would be interesting to untangle between blood flow velocity and wall shear stress (WSS) levels. Most of mechanotransduction channels have been associated with WSS rather than flow velocities, as the same flow rate can translate into very different WSS levels at the surface of endothelial cells. For instance, the authors propose that a correlation between blood flow and tEVs accumulation. The authors should express these levels in shear stress levels as well. Moreover, the authors should do a calibration curve of tEVs uptake in vitro, using a wide range of flow rates, and stating clearly the WSS levels of each flow condition.

b) Along the same lines, the authors wrote "Decreasing flow velocities with lidocaine (Fig.1b, left), a sodium channel blocker that reduces pacemaker activity of the heart (Follain et al., 2018), lead to a significant decrease of tEVs uptake by endothelial cells (Fig.1b, right). Conversely, increasing the heart pacemaker activity (Fig.1c, left) using IBMX (3-isobutyl-1-methylxanthine) (Follain et al., 2018) results in a slight albeit non-significant increase in tEVs uptake in the venous endothelium (Fig.1c, right)." What is the flow profile in these experiments? These data points should be added to the correlation graph. The reviewer interpreted these results as there was a bell shape curve of sensitivity for the uptake of tEVs. The authors should also state where these measurements were performed? Only vein, only artery or both vessels?

c) In addition, the authors should demonstrate that blood velocity and not endothelial cell type (artery vs vein) is responsible for such differences (uptake and rerouting). For instance, the authors could to tune flow in artery to 400 to 450 µm/s with lidocaine, or by other means, and observe if this upregulates tEVs uptake/rerouted in arterial regions. In parallel, the authors could use arterial endothelial cells in vitro and test if similar sensitivity is observed. In addition, whilst confirmation in vivo is very positive (fig.2), it is puzzling that the authors associated the effects of different flow velocities on the routes of internalization of tEVs only in vivo and not in vitro, which is a system much more controllable. Thus, the authors should vary flow in the microfluidic setting to correlate levels of flow velocity and trafficking of tEVs.

3- Transcriptional analyses.
a) The reviewer could not find the GEO numbers for the datasets that the authors have produced. These should be deposited for the general public. In addition, in the methods, the authors describe "Up- and down-regulated genes were selected based on adjusted p-values < 0.1 and log2 fold-changes > 0.5 and Functional enrichment analyses were performed using STRING v11 (Szklarczyk et al., 2019) and Gene Ontology (Carbon et al., 2021)." The cut off for adjusted p-values and log2 fold-changes are not the standard ones (adjusted p-values < 0.05 and log2 fold-changes > 1). The authors should explain their rational for selecting these cut off values. Finaly, despite the fact that Fig.4a showcase data from 4 different conditions, the reviewer could not find any information in the supplemental materials related to flow + tEVs. Therefore, the authors should provide a more comprehensive tables for their different gene expression profiles.

b) The authors mentioned "This demonstrates that endothelial cells respond differently to tEVs when subjected to flow. As tEVs escape lysosomal compartments in such conditions, this suggests that such endosomal re-routing allows tEVs to transfer their message that ultimately tunes gene expression. However, their transcriptomic analyses clearly show that tEVs have an effect in static conditions, as exemplified by this statement "While the number of genes dysregulated by tEVs is similar in static and flow, their identity differs: genes whose transcription was impacted when tEVs were internalized by flow-stimulated endothelial cells remain unaltered when tEVs were internalized in static endothelial cells (Fig.4a, right)." These experiments suggest that EVs are able to modulate EC expression in both settings. So, rerouting does not allow tEVS to transfer their message. It is either the same message is interpreted differently because cells are under flow, tEVs release a different content because of the rerouting, or a combination of both. Thus, it becomes unclear the reason for differential expression in combined (flow + tEVs) conditions. It would be important for the authors to clarify their interpretations.

4-

Pro-angiogenic effects of tEVs. The reviewer requires additional analyses in the tumor studies, in particular to answer these specific questions. What is the flow velocity/WSS in the vessels surrounding tumors? What is the identity (vein/artery) of these vessels? Is this pro-angiogenic effect only present in the context of a tumor? If yes, why? Since the fish embryo is developing and many vessels are pro-angiogenic, and that the pro-angiogenic signature is seen in in vitro (tumor free conditions), one would expect that the effects would be more general rather restricted to when exogenous tumor cells are implanted. The requirement of tumor cells also directly contradicts the proposed model/effect on the pre-metastatic niche, which should occur prior to the presence of tumor cells.

Minor comments:

1- Fig1a iCLEM images would benefit from increasing their size, or by providing higher magnifications in a supplementary figure;
2- Some graphs are missing some information related to units.
3- Fig2a. The % presented is confusing as the sum of all is higher than 100%.

4- Fig3d contains a panel with the MagRed area however it is not explained in the text.
5- The text associated with the results of Fig4a should be improved. It is difficult to agree or follow the authors' conclusion with the referred panel. Particularly the following part should be better explained "While the number of genes dysregulated by tEVs is similar in static and flow, their identity differs: genes whose transcription was impacted when tEVs were internalized by flow-stimulated endothelial cells remain unaltered when tEVs were internalized in static endothelial cells (Fig.4a, right)" line348 to line353;
6- Missing explanation for CTCs abbreviation - Circulating tumor cells (CTCs);
7- Fig4c the unit of flow velocity is mm/s but perhaps the authors meant µm/s.
8- These two additional studies may be of interest to discuss within the context of this paper: 10.1016/j.bioactmat.2021.10.038 - this study shows that ECs can capture EVs in low WSS regions; and 10.1101/2022.12.31.522373 - this study (pre-print) that WSS has an impact in the biogenesis and uptake of EVs.

Referee #3:

Here, Mary et al. Investigate the effect of hemodynamics on the interaction of tumor-derived extracellular vesicles (tEVs) with the endothelium and find that vessels or endothelial cells exposed to lower blood flow (denoted venous or capillary-like) but not high flow (arterial) allow uptake of tEVs. After uptake at such sites, tEVs are routed away from lysosomal degradation to the Rab14 recycling compartment which correlates with specific gene regulation and pro-angiogenesis, assumed to favor formation of premetastatic sites. The analyses are carried out in vitro using primary and transformed endothelial cells and in vivo, in the zebrafish. The authors conclude that hemodynamics regulate the pro-tumoral function of tEVs. Certain of the strategies employed to follow the localization of internalized tEVs (such as the use of a pH-sensitive reporter) are elegant. Moreover, although the overall impression is that mechano-regulation of tEVs is very similar to what the Goetz group previously reported for circulating tumor cells using very similar models, the current report presents additional information such as the intracellular routing of tEVs. However, the study is not well controlled and the presentation is immature and leaves the authors wondering which types of hemodynamics (low, high, intermediate) regulates which processes.

1. The authors categorize venous blood flow rate as <450 um/sec and arterial blood flow as >550 um/sec. I would expect there to be a bigger difference between arterial and venous blood flow and for the venous flow to be slower. The blood flow velocities applied here are based on measurements (line scanning of individual RBCs) of the flow in large vessels in the zebrafish embryo, the dorsal aorta and the cardinal vein (Follain et al., Dev Cell 2018). It is likely that zebrafish embryo blood flow rates cannot be compared with flow rates in mice and humans, on which most of the reported flow values have been recorded. However, one can ask whether the zebrafish flow velocities are relevant for tEV-endothelial interactions? Certainly, for the in vitro analyses, it would be preferable if the authors conformed to what now is standard, namely to compare effects of static, low and high flow conditions where 20 dyn cm−2 would to represent arterial flow while 2-3 dyn cm−2 would represents venous flow. See for example the protocol in Vion et al., Front Physiol. 2021 Mar 2;11:623769. doi: 10.3389/fphys.2020.623769.
2. The request above that the in vitro analyses are made in high, low and no-flow conditions stems from the fact that the authors don't seem to have made up their minds on how to consistently describe the flow properties that impact tEV uptake and intracellular routing. In the abstract, the authors describe the flow-properties of vessels that retain and reroute tEV as capillary-like ("demonstrate that capillary-like flow profiles potentiate the pro-tumoral function of circulating tEVs by promoting their uptake and rerouting their trafficking"). What "capillary-like" entails is then not further described in the text. Subsequently in the main text, the authors instead refer to the tEV permissive endothelial flow as (lines 155-159): "We propose that binding and arrest of tEVs at the endothelial surface are prevented when flow velocities exceed the permissive value in the range of 400 µm/s while both uptake and trafficking of tEVs would be impaired, through mechanosensing, at low flow velocities." Then, on lines 254-258, the authors conclude that: "Altogether, our results show that the moderate flow regimes partially switch EVs trafficking toward Rab14 positive nonacidic and non-degradative compartments" but also (lines 273-274) "At moderate or high velocities, internalized tEVs are partially rerouted towards non-acidic compartments...". Finally, in their schematics in Fig 4c, tEVs appear to be retained in vessels with flow rates between 1000-500 uM/sec, which would correspond to arterial flow rates (> 550 uM/sec)? These incompatible statements leave the reader confused. The authors need to be more consistent and not meander between capillary-like, low, moderate or even moderate to high blood flow to define the flow rates that allow tEVs to be taken up and rerouted.
3. The authors examine transcriptional regulation induced by static and flow (400um/sec) -exposed cultures; Fig 3a) and find that lysosomal genes are markedly upregulated by flow. What I find surprising here is that cytoskeletal and anchoring programs are downregulated by flow in comparison to static cultures - it should be the reverse. There is ample documentation in the literature and quite intuitive that flow affects the cytoskeleton and cell-matrix adhesion. The unexpected finding on lack of cytoskeletal regulation with flow calls the data on transcriptional regulation induced by tEVs into question.
4. Seemingly a minor but still important comments relates to the VeravecTM HUVECs. One should not have to go to the company website to find out that these are SV40 immortalized HUVECs. Please describe correctly in the Methods. Moreover, SV40 T transformation will broadly impact cell behavior, for example by constitutive PI3K signaling among other effects (see literature by Sara Courtneidge). The authors must be careful in concluding on the vHUVECs. An important control would be to compare flow effects on primary HUVECs and transformed vHUVECs to make sure they respond similarly to mechanosensing.
5. To ultimately provide proof of tEVs' specific effect on transcriptional regulation in endothelial cells would entail to compare

transcriptional profiles of endothelial cells exposed to tEVs and synthetic nanoparticles of similar size.

Referee #4:

The authors show that 'hemodynamics' promote the uptake of tumor (4T1) secreted EVs, and that this influences their intracellular fate and thus function in endothelial cells. This lead them to conclude that how that the blood flow provides a lysosomal escape mechanism by which they can enhance a pro-angiogenic function. This is a very intriguing hypothesis with potential major implications for EV-based therapy. This is a very intriguing study with carefully conducted experiments that should spur even more interest in in vivo EV biology. Nevertheless, I have several questions that need addressing. In particular their claim of flow induced 'rerouting' of tEVs inside their endothelial cells requires some more experimental scrutiny in my opnion.

Major questions:

1) The authors have developed a highly physiological in vivo model to study the fate of EVs. Specifically, they isolated EVs from mouse breast cancer cells by differential centrifugation, labeled them with a dye and inject them in the circulation of zebrafish embryos and analyzed 4 vascular regions. They observed a significant inverse correlation between the 4 associated flow profiles and endothelial accumulation/uptake of their tEVs (Fig 1). Because their method of EV isolation is rather crude (ultra centrifugation) how can the authors exclude the potential influence of EV aggregates?
2) In addition, while the differences seen with these tEVs appear compelling, does this count for all (tumor) EVs? Indeed, the authors speculate that it is likely that the destination of their circulating mouse tumor EVs relies on the combined effect of hemodynamics and EV-endothelial receptor-ligand interactions. To demonstrate this connivingly a (control) experiment with distinct EVs is needed. In their own words, can the authors show that endothelial adhesion prone 4T1 EVs, behave distinctly from 'non-adhesion prone' tEVs? The authors could find inspiration for such a control in the paper of Hoshino et al which they cite.
3) In general, most non-phagocytic cells are very poor in taking up EVs. Their zebrafish embryo endothelial cells seem to do this quite efficiently in vivo but also their HUVECs in vitro. When comparing static or flow conditions with their clever microfluidic device, applying flow seems to increase the uptake. One could explain this in multiple ways. What is the actual uptake mechanism here? Is it receptor mediated endocytosis? If so, can the authors demonstrate this with inhibitors? Is it possible that endocytosis (or other EV uptake mechanisms) itself is increased in these cells under mechanical stress?
4) The authors data suggests that internalized tEVs are at least partially redirected to non-degradative compartments upon flow mechanosensing. Indeed, while most of the internalized tEVs accumulate in Magic Red positive compartments, flow reduces tEV signal in cathepsin B-positive vesicles. This is a very intriguing observation and the intracellular compartments that are positive for the tEV signal seems to be RAB14 positive/less acidic compartments. Is it possible that tEVs are more prone for internalization directly into RAB14 compartments under flow? In other words how are the authors so sure it's re-directing from degradative compartments? Can the authors turn the flow off and on? If so, what happens if you add tEVs under static conditions and then turn on the flow? Is live-imaging an option?
5) The authors show that as I expected the flow itself effects the physiology of the endothelial cells and focus on the lysosomal pathways based on transcriptomics. Apart from transcriptional regulation one may argue other regulatory mechanisms may also have a role. This is not mentioned. For example phospho-proteomics may lead to different pathways. Can the authors exclude endocytic pathways are not equally affected as well, just not at the transcriptional level?

Point-by-point response for our manuscript entitled: "Blood flow diverts extracellular vesicles from endothelial degradative compartments to promote angiogenesis**"**

by Benjamin Mary*, Nandini Asokan*, Katerina Jerabkova-Roda, Annabel Larnicol, Ignacio Busnelli, Tristan Stemmelen, Angélique Pichot, Anne Molitor, Raphaël Carapito, Olivier Lefebvre, Jacky G. Goetz and Vincent Hyenne.
** Now co-first authors*

First of all, we would like to thank the reviewers for their thoughtful comments, questions and suggestions which improved the quality of our work. We provide here a point-by-point response to the comments they raised. Our responses appear in *blue* in this document and all the changes are highlighted in yellow in the manuscript. The significant amount of additional experiments led to now include Nandini Asokan with shared co-authorship with Benjamin Mary.

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Referee #1:

Summary

tEVs are tumour-associated extracellular vesicles that tumours release into the bloodstream and are thought to impact microenvironments favourably for tumour growth and metastasis. Here authors aim to investigate the link between tEV uptake into endothelial cells and the blood flow velocity of the respective vessels. Here authorss propose hemodynamics as a primary factor for tEV uptake and fate. Since endothelial cells are known to be responsive to mechanic stimuli, they suggest that venous blood flow velocities of around 400 m/s promote efficient tEV uptake. These optimal hemodynamics might also be involved in tEV escape from degrading acidic lysosomal pathways via RAB14/ NHE9 positive vesicles, which provide permissive environments for tEV-mediated pro-angiogenic signalling.

Over the past years, tEVs have evolved as important players involved in disease progression promoting survival, growth, pre-metastatic niches (PMNs) and altering biomedical properties of target cells. Previous studies also proposed the prognostic value of tEV plasma levels as they are associated with the metastatic rate in gastric cancer. While the functions of tEVs are diverse depending on the malignancy and targeted cells, they have been linked several times to neoangiogenesis/ lymphangiogenesis and remodelling of the endothelium when taken up by endothelial cells (2-5). Endothelial cells have been shown to react to mechanical stimuli as shear forces generated by different blood flow velocities. Hemodynamics are likely involved in the alteration of transcriptomics and the capability of adhesion of immune cells as well as circulating tumour cells (CTCs). It was shown that CTCs depend on certain permissive flow mechanisms to transit through the endothelium.

The authors propose a very interesting and novel data investigating the uptake

mechanism of tEVs into endothelial cells. The study is well-written, concise, and easy to follow.

Major comments

Overall, the experiments are coherent, and the used models (zebrafish for real-time imaging of circulation and microfluidics systems) were well chosen. The use of 4T1 cell line to acquire tEVs seems a compelling choice, especially for transcriptomic and angiogenesis analysis, as triple-negative breast cancer cells. It would be good to include the reasoning for this model and the expected transferability and possible limitations of the results.

Response 1.1: we now address this comment in the manuscript at the end of the introduction (a) and in the results section (b)

(a) "We chose to use EVs isolated from a triple negative breast cancer line, 4T1, as we have shown that they prime metastatic niches in secondary organs in a syngeneic model (Ghoroghi et al., 2021b). We documented a central role for the EV bound adhesion molecule CD146 in this priming, notably in the hemodynamic context of zebrafish embryo."

(b) "Such an hypothesis could be tested using 4T1 EVs in a syngeneic model of metastasis."

Results section "Blood flow tunes endothelial uptake of circulating tEVs" Figure 1a - This part of the figure has a lot of different panels with scarce labelling. It is partly difficult to match the image with its respective description in the figure legend. One should consider either splitting figure 1a into, e.g. 1a-c or labelling each image/ graph clearly with what it is displaying (e.g. iCLEM panel, hemodynamics panel).

Re 1.2: we have improved the display and the legends of Figure 1a (see also Re1.4, 1.13-16) in addition to providing additional panels for the iCLEM (new Fig.S1).

Also, one could consider labelling the mentioned anatomic structures (Injection site (Duct of Cuvier) and dissemination site (caudal plexus via dorsal artery) in the graphic representation of the workflow to increase the explanatory power.

Re 1.3: we improved the labelling of Figure 1a by adding the suggested descriptions. We would like however to emphasize that earlier results documenting the behavior of EVs in the ZF embryo have been published in a previous study from our group (Hyenne et al., 2019).

Red arrowhead indicators on iCLEM panel presumably point to labelled tEVs. This and other descriptive information about the iCLEM panel are not included in the figure legend.

Re 1.4: we modified the figure legends accordingly in addition to providing additional panels for the iCLEM (new Fig.S1).

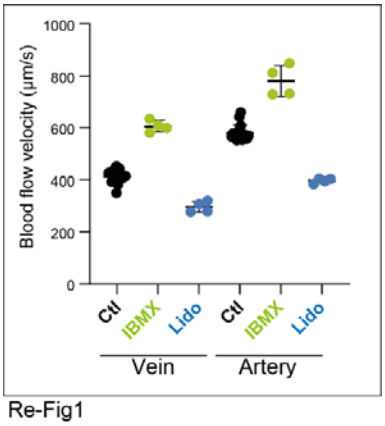
Pearson Correlation: This was done with sample size n=3 and a resulting correlation coefficient of -0.65. These results were interpreted as flow profiles significantly influencing tEV uptake (see lines 126-132). However, -0.7 is the usual cut-off value for strong relationships between two variables. (see: https://www.sciencedirect.com/topics/computer-science/pearson-correlation

) Since this is the crucial set-up experiment for this study, it might have been good to try getting a stronger correlation with a bigger sample size: Later, sample sizes are often around n=30. Why such a low sample size was chosen for this experiment remains unclear.

Re 1.5: Following the reviewer's comment, we reproduced the experiments and thereby doubled the number of embryos analyzed to reach n=6. The Pearson correlation coefficient is now r=-0,813 (p<0,0001), suggesting a strong relationship between the two variables (see new data in Fig.1a). In addition, similar experiments performed with EVs from different origins (AT3, see also Re2.1 and 4.2) or isolated with a different method (SEC, see Re4.1) give similar trend, further consolidating our results (Now presented in Fig S2a). Of note, the low number of embryos analyzed in these experiments (i.e flow velocity correlation with local EV uptake in endothelial cells) is due to the overall complexity of the experimental set-up (combined high-speed imaging of red blood cells (RBCs) and EV uptake) and the time-consuming in-depth image analysis (needed to extract tracking-based RBC velocities for example).

Figure 1b-d - Lidocaine and IBMX are given to reduce or increase heart rates to reduce or increase blood flow velocities. However, the data on how fast or slow blood flow has become is not provided, only data concerning the heartbeat. As these experiments are the reason for suspecting a tEV uptake optimum at 400 m/s these flow velocities would have been interesting as they could lend validity to the chosen optimal velocity.

Re 1.6: We would like to thank the reviewer for this comment (see also Reviewer 2.2), as the additional experiments we performed allowed us to refine our understanding of circulating EV uptake by endothelial cells.
Following the reviewer's suggestion, we precisely analyzed blood flow velocities in aortic and venous regions by tracking RBCs in IBMX/Lido-treated embryos as previously performed and displayed in Fig.1a. As expected, we found that IBMX and Lidocain lead, in average and in all regions analyzed, to a 30% increase or decrease in flow velocity, respectively (see Re-Fig1 and new FigS2c). This is in agreement with previously-published results from our group where these drugs had already been exploited in similar approaches (Follain et al., 2018).



Re-Fig1

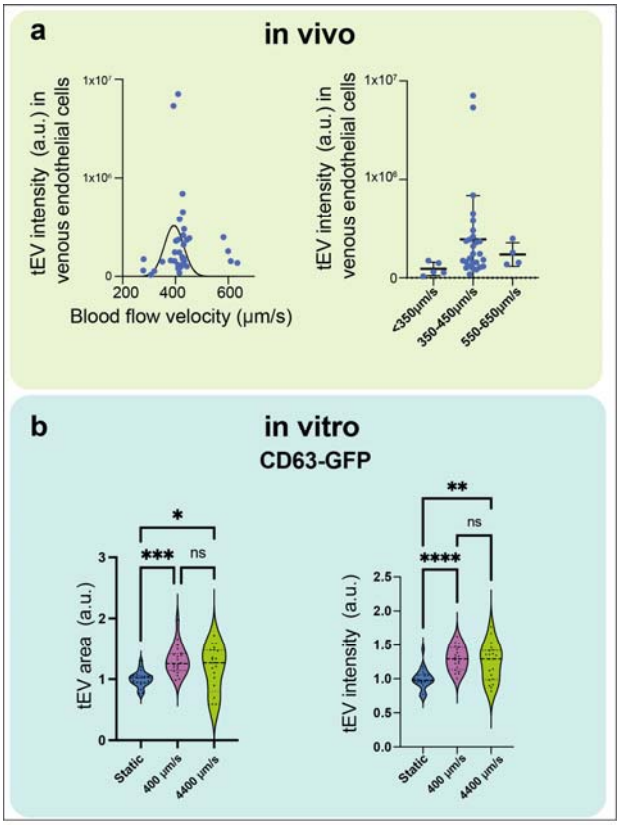
While measuring endothelial EV uptake in these experiments, we observed that i) endothelial EV uptake is increased in IBMX-treated embryos, and decreased in lidocaine-treated ones. This confirms our earlier observations (Fig.1b,c) and further demonstrates the contribution of flow velocity to EV uptake. We further observed that ii) the proportion of EVs internalized in venous vs aortic endothelial cells remains unchanged in control-, IBMX- or Lidocain-treated embryos (see new Fig.S2c). These new results suggest that EV uptake, in addition to depending on hemodynamic profiles, also depends on the arterio-venous identity of endothelial cells. Since endothelial cells in venous regions better internalize EVs, independently of flow speed, we focused our study on venous endothelial cells, using HUVEC- and VERAVEC-based *in vitro* models. We now include the RBC tracking in Fig-S1c and the conclusion in our manuscript, as follows:
"These data suggest a positive impact of blood flow velocity on tEVs uptake by endothelial cells. Nevertheless, a careful analysis of the correlation between EV internalization and blood flow profiles reveals that venous endothelial cells showed superior EV uptake levels and that *the arterio-venous uptake ratio remains*

unchanged (vs controls) in embryos where flow was pharmacologically manipulated (Fig. S2c). This suggests that the EV uptake potential of endothelial cells of the caudal vein exceeds the one of the dorsal aorta and implies that in addition to hemodynamics, the identity of endothelial cells contributes to EV uptake. While this question would require further investigation, we decided to focus the rest of our study on venous endothelial cells, as they internalize most of circulating EVs."

In subsequent experiments using microfluidics systems, the velocities chosen are always 400 m/s versus 0 m/s (static/ no flow conditions, see e.g. Fig. 1d). These settings were elected "to confirm these in vivo data suggesting that tEV uptake is most efficient at lower velocity flow profiles of 400 m/s." However, the Licocaine/ IBMX experiments did not provide this concrete number. Previous experiments measuring tEV uptake found the most efficient uptake generally in venous regions (400-450 m/s) but this experiment had its own limitations (see F1a, Pearson Correlation).

Referencing previous studies here that confirm that 400 m/s has permissive effects on tEVs is helping their point. However, it could have been more coherent if data concerning velocities had been included. The reasoning behind static settings in microfluidics systems, opposed to e.g. lower flow settings (<400 m/s) and/ or higher velocities (to mimic arteries), remains undisclosed. No flow setting doesn't seem to allow a lot of physiological transferability. This issue applies to all subsequent microflow experiments.

Re 1.7: As discussed before, our in vivo results suggest that flow velocity and endothelial cell identity both contribute to EV uptake. When pooling all our flow vs uptake *in vivo* experiments in venous regions (where most EVs are taken up), we observe that EV uptake peaks at velocities approaching 400µm/s (see Re-Fig2a in vivo). Although one cannot ascertain that 400µm/s is the optimal flow velocity for EV uptake and for the sake of simplicity, we decided to use it as our reference flow velocity value for an optimal EV uptake by venous endothelial cells. To determine the optimal flow velocity in vitro, we characterized EVs uptake by HUVEC cells at different flow speeds ((see Re-Fig2b in vitro and see also Re 2.2). Following the reviewer's recommendation, we tested the effect of higher velocities on EV uptake by HUVEC cells in vitro (400µm/s, and 4400µm/s - shear stress respectively 0,43 and 5 Dyn/cm2). We used genetically engineered CD63-GFP EVs to avoid potential confounding effects coming from the labeling dye. First, we confirmed the positive effect of flow at 400µm/s Vs static for CD63-GFP EVs (now added in the manuscript Fig.S2d). Second, at 4400µm/s, we



Re-Fig2

observed a plateau for CD63-GFP EVs uptake (now added in the manuscript Fig.S2d). In addition, we observed that the pattern of uptake varies depending on the flow speed: while EV internalization is very homogenous at 400μm/s, it becomes highly heterogenous at higher flow speed, with most of the cells taking up low amounts of EVs and few cells taking up high amounts.
At this stage, understanding the difference between in vivo and in vitro data or deciphering the mechanisms driving the uptake heterogeneity at high flow speed is difficult and falls beyond the scope of this study. Altogether, our work indicates that there might be an optimal flow speed for EVs uptake by venous endothelial cells. Also this speed might be slightly different *in vitro* and *in vivo*, it falls in the same range.

Altogether, these results also reinforce the relevance of performing experiments throughout the study comparing static and 400μm/s as: 1) endothelial EV uptake is homogenously increased both *in vivo* and *in vitro* when subjected to 400 μm/s velocities, 2) further increasing shear/velocity has a mild effect on EV uptake overall efficiency yet favoring heterogeneity of uptake which requires additional work that, although of interest, falls beyond the scope of the current study.

Result Section "Circulating tEVs are partially re-routed to alternative non-acidic and non-degradative RAB14 positive compartments." pHluorin is quenched when in an acidic environment and gives a stronger fluorescent signal when in a neutral environment. This information should perhaps be included so readers can immediately understand the meaning of the pHluorin/mScarlet ratio.

Re 1.8: we addressed this issue in the manuscript by better explaining the principle of the pHluorin probe as follows:

"Since the degradative activity of lysosomes hydrolases relies on the acidic luminal pH (Perera and Zoncu, 2016), we next probed the pH of tEV-containing compartments by exploiting the pH-sensitive reporter pHluorin. pHluorin is anchored on EVs external membrane via an insertion in the extracellular domain of the tetraspanin CD63, while the pH-insensitive mScarlet is fused to its intracellular C-terminal end (Sung et al., 2020). As pHluorin is fluorescent in a neutral environment but quenched in an acidic environment, it allows to probe the pH of the compartments targeted by tEVs (Fig.2c, left)."

Figure 2a - p-value is not given in the graph. All other graphs provide stars to indicate p-value. Here there are none, and only one p-value is given in the figure legend. Does this count for all presented plots?

Re 1.9: The reviewer is right, the absence of star in Figure 2a implies that the statistics test provided p-values below significance when comparing static vs flow conditions. We have clarified this issue and now indicate "non-significant (ns)" when appropriate.

Figure 2d - Data of tEVs shift towards neutral RAB14 positive vesicles in flow settings has a lot of scattering. Only a mean of ~8% of all tEVs were found in RAB14 positive vesicles. So just looking at this data one might question how significant RAB14-positive vesicles are for the trafficking of tEV in general.

A follow-up question that arises here: "We have seen that only a small fraction (in all cases below 40%, mean ~8%) go on the RAB14 path" - Others presumably do not escape and get degraded. Or might there be another mechanism of escape? Is there a threshold of surviving tEVs for there to be biological tEV-effects on the endothelial cell?

Re 1.10: We thank the reviewer for these outstanding questions, which should indeed pave the way for exciting lines of research in the future. We nevertheless humbly believe that these cannot, at this stage and unfortunately, be easily addressed experimentally. We now acknowledge these questions in our manuscript and state:

"Characterizing the proportion of internalized EVs following each of these fates will be essential in the future and would require the development of innovative *in vivo* compatible tools. "

Figure 2e - The displayed graph does not support the point the authors want to make very well as the difference of the ratios depending on blood flow velocities can hardly be seen due to the scale of the graphs. Also, the scale is probably off because there seem to be quite a few extreme outliers and, therefore a lot of scattering. The way the data is presented it cannot be assessed how much the ratio dropped, it seems rather minimal. That very small drop might be highly significant, but it would also mean there is only a small percentage of tEVs that switch compartments which would pose the question if this shift is still biologically significant.

Re 1.11: Following the reviewer's comment, we re-analyzed the data presented Fig.2e. In the two graphs, we originally had plotted the pHluorin/mScarlet ratio per z plan. In our new analysis, we now present the pHluorin/mScarlet ratio per embryo (by averaging all the individual Z plans). This new analysis gained in accuracy and allows to reduce the variability of the data which can be, in part, explained by the heterogeneity of uptake observed *in vivo*. Importantly, we focused our analysis on the venous part of the endothelium (caudal vein) in these experiments and now document statistical differences in the uptake efficiency when flwo is pharmacologically perturbed. The new analysis is shown as a revised panel Fig.2e and the respective legend have been modified accordingly.

There is seemingly no p-value given for the IBMX experiment. No stars/p value indicators displayed in graph either. Does this experiment have the same p-value as the Lidocaine experiment? Is it non-significant?

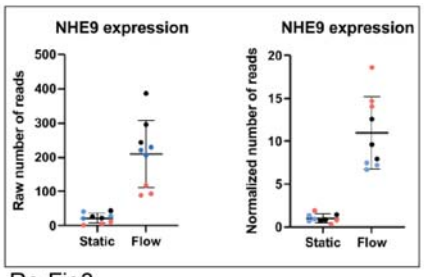
Re 1.12: The absence of a star in this experiment was a mistake as the difference was highly significant (with a p-value of p<0,0001). These results however, have been replaced by our new analysis (also statistically significant, see above).

Figure 3b/3b - F3b graph shows "mean lysot (= lysotracking?) area" which is decreased significantly in cells from flow areas to cells from stall areas. However, only in figure legend 3d is an explanation offered of how the reduced mean area can be interpreted. This explanation should maybe be given when the term is introduced in 3b.

Re 1.13: we corrected this in the legend and used the explanation from Fig3d as suggested.

Figure 3e - There seems to be a contradiction of the data shown in the graph versus the interpretation offered in the written results: While >50% of tEVs co-locate with NHE9 there is no significant difference between flow settings and no-flow settings. This is not mentioned in the written results. However, they conclude that flow dynamics could trigger NHE9 positive vesicle formation where tEV accumulate (l 331-334). This is supported by RNAseq data but not necessarily by F3e.

Re 1.14: While reading this reviewer's comment, we acknowledge that Fig3e might have been misleading. In this panel NHE9-Cherry is over-expressed (via lentivirus-



Re-Fig3

based cell engineering) in endothelial cells, in both static and flow conditions. We believe that this forced over-expression (using the strong sFFV promoter) of exogenous NHE9 explains the important colocalization with EVs in both conditions. In native HUVECs however, endogenous NHE9 expression is barely detected in static condition and strongly increased in flow condition (see the attached figure Re-Fig3 showing analysis of three different transcriptomic datasets). While we worked hard in providing a clear analysis of endogenous expression levels using immunofluorescence that is compatible to a parallel assessment of EV uptake, we unfortunately failed in identifying a good antibody allowing to track endogenous NHE9 (3 different anti-NHE9 antibodies were tested (Abcam ab167157; Thermo Fisher PA5-101895 and Proteintech #66577-1-Ig). Altogether, our data show a flow-dependent induction of NHE9 expression in addition to demonstrating the internalized EVs do colocalize with NHE9 upon forced expression of a fluorescently-tagged version in endothelial cells (the later being previously described in the literature (Beydoun et al., 2017). To clarify this issue in the manuscript, we now better introduce the need to use a fluorescently-tagged version of NHE9 that, although it allows to track NHE9, does not allow to track its endogenous version and thus the flow-dependent control of its expression levels.

Figure 4a - F4a second panel: labelling is slightly confusing and not explained in figure legend. The categories "up/ down with flow" and "up/down with EV" are not self-explanatory.

Re 1.15: We have improved the legends of Figure 4a. We now state:
"The right graph shows differentially expressed genes, with increased (=Up) or decreased (=Down) expression with flow and/or tEVs (pAdj<0,1)."

Figure 4b - Figure legend should include all used abbreviations.

Re 1.16: we addressed this point throughout the manuscript by including all abbreviations in the figure legends.

- How high is the biological significance of 2 neo-angiogenic sprouts (embryo with only tumour mass) versus 4 sprouts (Tumour mass + TC + tEV)? It could be interesting to discuss how strong and meaningful the angiogenic effect of tEVs are.

Re 1.17: We agree with this reviewer and have now updated the extensive discussion on that topic in the final section of the Ms (See also Reviewer 2, comment 4).
"When embryos are injected with tEVs, the tumor-induced sprouting is potentiated with an increased number of neo-vascular sprouts per embryo (Fig.4b). Importantly, tEVs also increase the percentage of embryos bearing tumor-induced endothelial sprouts when compared to embryos injected with PBS. While it remains unclear whether and how the positive impact of tEVs on sprouting efficacy of the endothelium impacts metastasis progression, we speculate that it acts as an initial trigger of the priming of metastatic niches or of the vascularization of metastatic foci. Indeed, these results confirm that circulating tEVs promote tumor-induced neo-angiogenesis *in vivo*, a scenario

that is likely to happen when tEVs shape pre-metastatic niches (Peinado et al., 2017; Ghoroghi et al., 2021a).

Referee#2:

General comment: In this manuscript, Benjamin Mary et al. set out to uncover how blood flow velocity affects the uptake of circulating tumour extravascular vesicles (tEVs) by endothelial cells, and this can affect endothelial cell biology. The authors used both in vitro and in vivo (zebrafish) models. The authors propose that endothelial cells are sensitized to uptake tEVs at a moderate blood flow velocity. Interestingly, this effect is coupled with a rerouting of internalized tEVs towards non-acidic and non-degradative Rab14-positive endosomes. The authors further propose that tEVs promote the expression of pro-angiogenic transcription factors in flow-stimulated endothelial cells which favours tumour angiogenesis in zebrafish. Overall, this manuscript provides an interesting and novel perspective on how endothelial cells may react to tEVs. However, the manuscript has some weaknesses. The effect of tEVs in establishing the metastic niche and promoting tumour angiogenesis was described previously. Thus, the major novelty resides only on the effect of blood flow in the activity of tEVs. Yet, this aspect remains very unclear and requires additional experiments. In addition, despite the compelling evidences that blood flow may alter the trafficking path and activity of tEVs in endothelial cells, the mechanism causing this remains unidentified.
The reviewer proposes major and minor comments to improve the robustness and scope of the manuscript.

Major comments:

1- Generalization of the conclusions. The authors used tEVs derived from mouse breast 4T1 cancer cells. As the source of EVs may regulate the specificity of EV uptake, the generalization of these findings should be demonstrated by replicating the key experiments using tEVs coming from other (2/3) cancer cell lines.

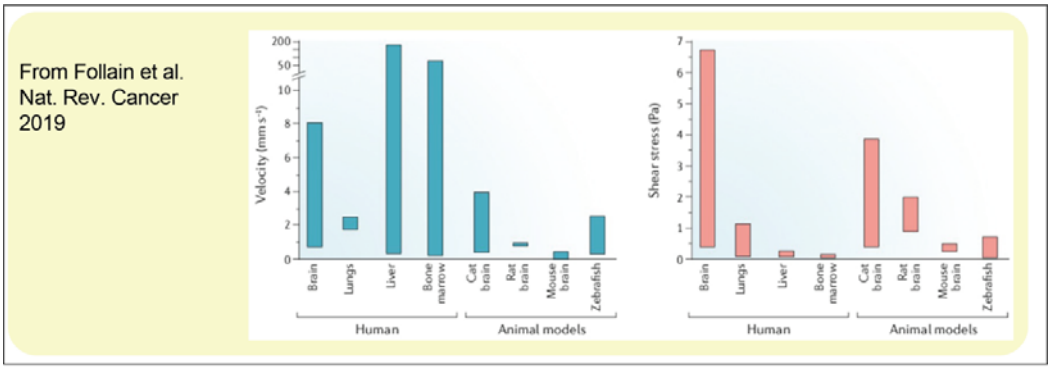
Re 2.1: While we appreciate the relevance of this reviewer's comment, we would like to point to our previous studies where we have shown that EVs from different origin, namely zebrafish melanoma (Zmel1) and fibroblasts (AB9), accumulate mostly in regions of low flow velocity in zebrafish (Hyenne et al., 2019)(see Re-Fig4A). Similarly, EVs from mouse 4T1 cells treated with a CD146 blocking antibody, or derived from a cell line knock-down for the GTPases RalA or RalB mostly end up in low flow regions (Ghoroghi et al., 2021b). Finally, in a collaborative study spearheaded by colleagues from Institut Curie (Team of G. Van Niel, Paris), we observed a similar accumulation of endogenous zebrafish EVs in the venous plexus (Verweij et al., 2019). Therefore, we believe that our main finding on the role of flow on EV uptake is not restricted to 4T1 EVs and are tempted to generalize these conclusions as the previously-cited studies show a similar behavior for EVs independently of the cell type (when exogenously administrated) and independently of the species (endogenous ZF EVs).
That being said, in order to further strengthen this observation and comply with this reviewer's request, we determined precisely the flow-dependent accumulation of EVs isolated from another tumor cell line (AT3, a murine breast carcinoma cell line, MMTV-PyMT), in zebrafish, following the experiments presented on Figure 1a (see also Re4.2). As shown in Re-Fig4B, we found that AT3 EVs follow a pattern similar to 4T1 EVs, with a preferential internalization around 400um/s. We have now included these data in our manuscript, now Fig-S2b. This further demonstrates that different types of EVs follow similar flow-dependent internalization rules. Nevertheless, as we believe that specific ligands/receptors present at the surface of EVs and endothelial

cells also contribute to EV uptake, we have now added a sentence related to EV identity in the discussion of our manuscript.
"Expression of these receptors at the surface of subtypes of EVs or on EVs from different origins could account for different uptake patterns. In addition, while the identity of the ligands of such receptors remains obscure, but their differential expression in arterio-venous endothelial cells or in different organs could contribute to EV biodistribution."

Figure for referees not shown.

2- Relationship between flow and tEVs uptake/rerouting.
a) The authors have focused exclusively in blood flow velocity. However, it would be interesting to untangle between blood flow velocity and wall shear stress (WSS) levels. Most of mechanotransduction channels have been associated with WSS rather than flow velocities, as the same flow rate can translate into very different WSS levels at the surface of endothelial cells. For instance, the authors propose that a correlation between blood flow and tEVs accumulation. The authors should express these levels in shear stress levels as well. Moreover, the authors should do a calibration curve of tEVs uptake in vitro, using a wide range of flow rates, and stating clearly the WSS levels of each flow condition.

Re 2.2a: We agree with this reviewer that the relationship between shear stress and flow velocity deserves a better explanation. The velocity of 400µm/s used in our *in vitro* model aims at reproducing the average blood flow velocity observed in the venous region of zebrafish embryo plexus (Follain et al., 2018) (Re-Fig5, and the measurements presented Fig.1a of our manuscript) where estimation of concomitant shear values remains difficult and fall in the low shear range (as previously observed in early-stage embryos (Goetz et al., 2014; Freund et al., 2012)). Nevertheless, such velocity profiles correspond to an approximate shear stress of 0,5 (+/- 0,2) dyn/cm^2 on the endothelial monolayer cultured in the microfluidic channel. Importantly, this value is in the range of the shear stress reported on the endothelial walls of caudal ventral vein in zebrafish larvae (Gomez-Garcia et al., 2018). Therefore, our *in vitro* microfluidic system accurately reproduces the (low) shear and flow parameters observed in early zebrafish embryo circulation. Importantly, these values also match the velocities and shear described in human lungs or mouse brains (See figure attached, from our review, (Follain et al., 2020)). Following the reviewer's suggestion, we now precise the shear stress value corresponding to 400µm/s in the manuscript and in the figure legends (see also Re 3.1).
Regarding the last point of the reviewer, please refer to Re1.7.



Re-Fig5

b) Along the same lines, the authors wrote "Decreasing flow velocities with lidocaine (Fig.1b, left), a sodium channel blocker that reduces pacemaker activity of the heart (Follain et al., 2018), lead to a significant decrease of tEVs uptake by endothelial cells (Fig.1b, right). Conversely, increasing the heart pacemaker activity (Fig.1c, left) using IBMX (3-isobutyl-1-methylxanthine) (Follain et al., 2018) results in a slight albeit non-significant increase in tEVs uptake in the venous endothelium (Fig.1c, right)." What is the flow profile in these experiments? These data points should be added to the correlation graph. The reviewer interpreted these results as there was a bell shape curve of sensitivity for the uptake of tEVs. The authors should also state where these measurements were performed? Only vein, only artery or both vessels?

Re 2.2b: As requested by this reviewer and reviewer #1, we measured the corresponding flow profiles in embryos treated with IBMX and lidocaine (see Re1.7 and Re-Fig2) and have now added the results to Fig1d and FigS2c of our manuscript. Those measurements were performed in both arterial and venous compartments. For the sake of simplicity of comparison, however, results displayed in Fig1b and 1c come from experiments performed only in venous regions (We have paid attention to accurately indicate which regions have been measured along the Ms).
Importantly, and as explained in Re1.6, the arterio-venous ratio of tEVs uptake remains similar in control, IBMX and lidocaine treated fish, suggesting that, in addition to hemodynamics, the arterio-venous identity of endothelial cells affects tEVs uptake. We have now included this observation in our manuscript and discussed it further in response to reviewer 1's comments (and the next one).

c) In addition, the authors should demonstrate that blood velocity and not endothelial cell type (artery vs vein) is responsible for such differences (uptake and rerouting). For instance, the authors could to tune flow in artery to 400 to 450 µm/s with lidocaine, or by other means, and observe if this upregulates tEVs uptake/rerouted in arterial regions. In parallel, the authors could use arterial endothelial cells in vitro and test if similar sensitivity is observed. In addition, whilst confirmation in vivo is very positive (fig.2), it is puzzling that the authors associated the effects of different flow velocities on the routes of internalization of tEVs only in vivo and not in vitro, which is a system much more controllable. Thus, the authors should vary flow in the microfluidic setting to correlate levels of flow velocity and trafficking of tEVs.

Re 2.2c: As discussed above and in response to reviewer 1 (Re1.6), decreasing the flow velocity to 400µm/s in arterial regions does not increase tEVs uptake compared to venous regions, suggesting that endothelial identity indeed actually contributes to the efficiency of EV uptake, that remains flow-dependent. This is now further discussed in our manuscript and we invite this reviewer to refer to Re1.6 for more

details. While we provide now a better demonstration of the tight link between flow regimes, EV uptake and potentially endothelial identity, we would like to point out that endothelial identity of early ZF embryo vasculature remains rather plastic at this stage, especially in the caudal plexus region. We thank the two reviewers for raising that possibility and agree that a dedicated independent study aiming at comparing the impact of endothelial cell fate on EV uptake would provide additional insights.
As for the second point, we did assess the re-routing of EVs in vitro mostly, as it is indeed much more controllable, as stated by the reviewer. And confirmed that the re-routing of EVs toward less acidic compartments is flow-dependent in vivo as well.

3- Transcriptional analyses.
a) The reviewer could not find the GEO numbers for the datasets that the authors have produced. These should be deposited for the general public. In addition, in the methods, the authors describe "Up- and down-regulated genes were selected based on adjusted p-values < 0.1 and log2 fold-changes > 0.5 and Functional enrichment analyses were performed using STRING v11 (Szklarczyk et al., 2019) and Gene Ontology (Carbon et al., 2021)." The cut off for adjusted p-values and log2 fold-changes are not the standard ones (adjusted p-values < 0.05 and log2 fold-changes > 1). The authors should explain their rational for selecting these cut off values. Finaly, despite the fact that Fig.4a showcase data from 4 different conditions, the reviewer could not find any information in the supplemental materials related to flow + tEVs. Therefore, the authors should provide a more comprehensive tables for their different gene expression profiles.

Re 2.3a: We thank the reviewer for these requests :
1. We have now deposited our data in public datasets (ArrayExpress accession E-MTAB-13156) and added the accession number to the manuscript.
2. In the case of the transcriptomic analysis presented in Fig4a, we applied less stringent cut offs to be able to compare multiple conditions (static, flow +/-EVs). Doing so, we could highlight genes whose regulation is clearly significant in one comparison (flow vs static for instance) and only borderline in another (EVs vs no EVs). This is the case for instance of SMAD6, which has an adjusted p-value <0,05 in all comparisons, a log2Fc>1 in flow vs Static and a log2Fc=0,81 in EVs vs no EVs. Besides, the genes highlighted in Figure 4a and discussed in the text (pro-angiogenic transcription factors and TGFb/BMP genes) are all highly significant they all have an adjusted p-value lower than 0.05. The only exception was HES1, which we have finally removed from the analysis.
3. Since we used these unconventional thresholds for the RNAseq presented Figure 4a, we had initially decided to keep the same parameters to analyze the RNAseq presented Figure 3a (Flow vs No Flow). Because this analysis is less complex, we increased stringency and correct the adjusted p-value and log2Fc thresholds to the classical ones (0.05 and 1 respectively). We ran over the analysis with this new threshold and found mostly the same deregulated pathways as the ones initially presented in Figure 3a.We adjusted our figures and manuscript accordingly.
4. Finally and to address the last comment, we have decided, for the sake of clarity, to establish a table (Table 2) that emphasizes and thus displays the two comparison (flow vs static) and (EVs vs no EVs), which are presented side by side, and does not show the raw sequencing data. We believe that this offers a better and more simple display of the resulting differences.

b) The authors mentioned "This demonstrates that endothelial cells respond differently to tEVs when subjected to flow. As tEVs escape lysosomal compartments in such conditions, this suggests that such endosomal re-routing allows tEVs to transfer their message that ultimately tunes gene expression. However, their transcriptomic analyses clearly show that tEVs have an effect in static conditions, as

exemplified by this statement "While the number of genes dysregulated by tEVs is similar in static and flow, their identity differs: genes whose transcription was impacted when tEVs were internalized by flow-stimulated endothelial cells remain unaltered when tEVs were internalized in static endothelial cells (Fig.4a, right)." These experiments suggest that EVs are able to modulate EC expression in both settings. So, rerouting does not allow tEVS to transfer their message. It is either the same message is interpreted differently because cells are under flow, tEVs release a different content because of the rerouting, or a combination of both. Thus, it becomes unclear the reason for differential expression in combined (flow + tEVs) conditions. It would be important for the authors to clarify their interpretations.

Re 2.3b: We fully agree with the comment from the reviewer, as EVs have an impact in the static and in the flow condition. However, as shown in Figure 4a, the genes whose expression is modulated by EVs are different in static and in flow condition. We attribute this difference to a different capacity to transfer their message, as discussed in the manuscript. Although the mechanism of EV cargo transfer are not well understood, it is plausible that different messages will be transferred from when EVs are stored in different compartments. However, we do not exclude that this difference could result from a different interpretation of the message due to the fact that cells are cultured under flow. We have now clarified this point in the manuscript as follows: *"This demonstrates that endothelial cells respond differently to tEVs when subjected to flow. This could originate from different cargo transfer in static and flow conditions or from a different interpretation of EV signaling by endothelial cells."*

4- Pro-angiogenic effects of tEVs. The reviewer requires additional analyses in the tumor studies, in particular to answer these specific questions. What is the flow velocity/WSS in the vessels surrounding tumors? What is the identity (vein/artery) of these vessels? Is this pro-angiogenic effect only present in the context of a tumor? If yes, why? Since the fish embryo is developing and many vessels are pro-angiogenic, and that the pro-angiogenic signature is seen in in vitro (tumor free conditions), one would expect that the effects would be more general rather restricted to when exogenous tumor cells are implanted. The requirement of tumor cells also directly contradicts the proposed model/effect on the pre-metastatic niche, which should occur prior to the presence of tumor cells.

Re 2.4: We thank the reviewer for raising issues that we faced when trying to provide an *in vivo* demonstration that EVs, in conjunction with flow, have a pro-angiogeneic function. The ZF has indeed a highly angiogenic and remodeling vasculature, mostly governed by biomechanical and developmental cues that made the investigation of exogenously-injected EVs challenging. Thus, as a first answer, such difficulty motivated us to find an experimental set-up allowing us to see significant differences in angiogenic behaviors.

The SIV model, although similarly challenging and not necessarily the most representative of a pre-metastatic niche priming, stood out by its capacity to detect angiogenic sprouts, from flow-perfused and tEVs stimulated vessels, unequivocally. We thus picked this model, with all the caution that it required. That being said, we feel that such tumor cell-induced angiogenesis model allows to recapitulated metastasis-induced angiogenesis that allows secondary foci to perform metastatic outgrowth.

Having in mind to establish a model that better mimicks a situation where tEVs would prime the vascular system and to comply with this reviewer's request, we exploited a novel assay developed in the lab in the frame of another project, which aims at documenting the early and protrusive endothelial response to arrested circulating tumor cell (as per our previous studies (Follain et al., 2021, 2018; Osmani et al., 2019; Karreman et al.)). Using this model, we went on testing the capacity of tEVs stimulate

Figure for referees not shown.

an early protrusive response of the endothelium using a zebrafish transgenic line allowing to visualize micro-scale protrusions at the inner and external side of the endothelial cells (based on LifeAct). These protrusions are essential in stabilizing the arrest of CTCs ((Follain et al., 2018) + unpublished) which is an early, yet essential step of metastatic seeding. When probing the protrusive response of the endothelial, we found that they significantly increase when subjected to tEVs (Re-Fig6). While we believe that these protrusions are involved in the arrest of circulating tumor cells prior to their extravasation, we also think that this further demonstrates the pro-metastatic priming potential of EVs (in flow-stimulated vessels) with a direct effect on endothelial response that is pivotal for favoring the subsequent arrested of CTCs. However, and as you have probably understood, the nature, regulation and importance of these intraluminal endothelial protrusions are part of another ongoing study on the laboratory, and more work is needed to provide a thorough characterization which we wish to remain confidential at this stage. For all the reasons and hoping that this reviewer will be understanding, we wish to exclude these data from this manuscript while providing them here as an additional rebuttal point.

Minor comments:

1- Fig1a iCLEM images would benefit from increasing their size, or by providing higher magnifications in a supplementary figure;
2- Some graphs are missing some information related to units.
3- Fig2a. The % presented is confusing as the sum of all is higher than 100%.
4- Fig3d contains a panel with the MagRed area however it is not explained in the text.
5- The text associated with the results of Fig4a should be improved. It is difficult to agree or follow the authors' conclusion with the referred panel. Particularly the following part should be better explained "While the number of genes dysregulated by tEVs is similar in static and flow, their identity differs: genes whose transcription was impacted when tEVs were internalized by flow-stimulated endothelial cells remain unaltered when tEVs were internalized in static endothelial cells (Fig.4a, right)" line348 to line353;
6- Missing explanation for CTCs abbreviation - Circulating tumor cells (CTCs);
7- Fig4c the unit of flow velocity is mm/s but perhaps the authors meant µm/s.
8- These two additional studies may be of interest to discuss within the context of this paper: 10.1016/j.bioactmat.2021.10.038 - this study shows that ECs can capture EVs in low WSS regions; and 10.1101/2022.12.31.522373 - this study (pre-print) that WSS has an impact in the biogenesis and uptake of EVs.

Re 2.5:

Minor comments #1, 2, 4, 5 and 6 have been addressed in the manuscript (leading to the creation of a new Fig.S1 and text changes)
Minor comment #3: LAMP1 and RAB7 are known to display and important overlap in late endosomes, explaining why the total is higher than 100% in Fig2a.
Minor comment #7: the unit is correct in mm/s, we corrected the color display in the figure to better match the data.
Minor comment #8: we now cite the mentioned paper as follows : "Two recent studies confirm that such regions of low flow speed and low shear stress preferentially accumulate RBC or endothelial EVs, confirming that flow-dependent EV uptake by endothelial cells is not restricted to tumor EVs (Qin et al., 2022; Coly et al., 2023)."

Referee #3:

Here, Mary et al. Investigate the effect of hemodynamics on the interaction of tumor-derived extracellular vesicles (tEVs) with the endothelium and find that vessels or endothelial cells exposed to lower blood flow (denoted venous or capillary-like) but not high flow (arterial) allow uptake of tEVs. After uptake at such sites, tEVs are routed away from lysosomal degradation to the Rab14 recycling compartment which correlates with specific gene regulation and pro-angiogenesis, assumed to favor formation of premetastatic sites. The analyses are carried out in vitro using primary and transformed endothelial cells and in vivo, in the zebrafish. The authors conclude that hemodynamics regulate the pro-tumoral function of tEVs. Certain of the strategies employed to follow the localization of internalized tEVs (such as the use of a pH-sensitive reporter) are elegant. Moreover, although the overall impression is that mechano-regulation of tEVs is very similar to what the Goetz group previously reported for circulating tumor cells using very similar models, the current report presents additional information such as the intracellular routing of tEVs. However, the study is not well controlled and the presentation is immature and leaves the authors wondering which types of hemodynamics (low, high, intermediate) regulates which processes.

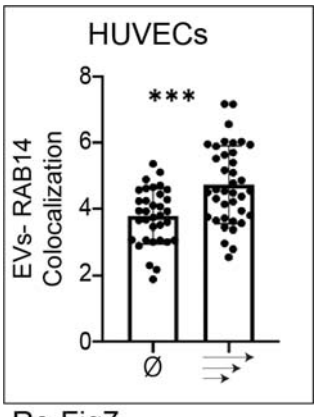
1. The authors categorize venous blood flow rate as <450 um/sec and arterial blood flow as >550 um/sec. I would expect there to be a bigger difference between arterial and venous blood flow and for the venous flow to be slower. The blood flow velocities applied here are based on measurements (line scanning of individual RBCs) of the flow in large vessels in the zebrafish embryo, the dorsal aorta and the cardinal vein (Follain et al., Dev Cell 2018). It is likely that zebrafish embryo blood flow rates cannot be compared with flow rates in mice and humans, on which most of the reported flow values have been recorded. However, one can ask whether the zebrafish flow velocities are relevant for tEV-endothelial interactions? Certainly, for the in vitro analyses, it would be preferable if the authors conformed to what now is standard, namely to compare effects of static, low and high flow conditions where 20 dyn cm−2 would to represent arterial flow while 2-3 dyn cm−2 would represents venous flow. See for example the protocol in Vion et al., Front Physiol. 2021 Mar 2;11:623769. doi: 10.3389/fphys.2020.623769.

Re 3.1: As pointed out in reply to Referee#2 (Re2.2a) and also to Referee#1, we have indeed very accurately characterized hemodynamics in the caudal plexus of zebrafish embryo in the past (Follain et al., 2018) (Re-Fig1) using a combination of RBC tracking and accurate PIV (Particle Image Velocimetry) analysis, and showed that velocities and shear stand in the lower range of what has been described in human lungs or mouse brains (please refer to (Follain et al., 2020) Re-Fig5). Therefore, we believe that the EV behavior reported here is relevant for what could

happen in some mammalian organs, especially since some of our recent findings regarding EV behavior has been mirrored both in ZF and mouse models (i.e. CD146 enriched EVs, Fig 5 of (Ghoroghi et al., 2021b)). In addition, we have now extended the range of shear/velocity (see point 1.6) to further confirm that our ZF findings are relevant.

2. The request above that the in vitro analyses are made in high, low and no-flow conditions stems from the fact that the authors don't seem to have made up their minds on how to consistently describe the flow properties that impact tEV uptake and intracellular routing. In the abstract, the authors describe the flow-properties of vessels that retain and reroute tEV as capillary-like ("demonstrate that capillary-like flow profiles potentiate the pro-tumoral function of circulating tEVs by promoting their uptake and rerouting their trafficking"). What "capillary-like" entails is then not further described in the text. Subsequently in the main text, the authors instead refer to the tEV permissive endothelial flow as (lines 155-159): "We propose that binding and arrest of tEVs at the endothelial surface are prevented when flow velocities exceed the permissive value in the range of 400 µm/s while both uptake and trafficking of tEVs would be impaired, through mechanosensing, at low flow velocities." Then, on lines 254-258, the authors conclude that: "Altogether, our results show that the moderate flow regimes partially switch EVs trafficking toward Rab14 positive nonacidic and non-degradative compartments" but also (lines 273-274) "At moderate or high velocities, internalized tEVs are partially rerouted towards non-acidic compartments...". Finally, in their schematics in Fig 4c, tEVs appear to be retained in vessels with flow rates between 1000-500 uM/sec, which would correspond to arterial flow rates (> 550 uM/sec)? These incompatible statements leave the reader confused. The authors need to be more consistent and not meander between capillary-like, low, moderate or even moderate to high blood flow to define the flow rates that allow tEVs to be taken up and rerouted.

Re 3.2: We thank the reviewer for raising this issue and would like to point out that, indeed, such accurate definition isn't trivial. Yet, thanks to this reviewer's suggestions, we have now improved it by i) explaining that that the previously identified flow velocities (around 400 µm/s, in addition to endothelial identity) are indeed "permissive" for tEVs uptake while ii) an accurate flow regime or velocity can't be concluded. Thus, and following the reviewer's recommendation, we have now improved such consistency and now use the term "low velocity flow" throughout the manuscript. We will revise our model Fig4c accordingly.



Re-Fig7

3. The authors examine transcriptional regulation induced by static and flow (400um/sec) -exposed cultures; Fig 3a) and find that lysosomal genes are markedly upregulated by flow. What I find surprising here is that cytoskeletal and anchoring programs are downregulated by flow in comparison to static cultures - it should be the reverse. There is ample documentation in the literature and quite intuitive that flow affects the cytoskeleton and cell-matrix adhesion. The unexpected finding on lack of cytoskeletal regulation with flow calls the data on transcriptional regulation induced by tEVs into question.

Re 3.3: First, we would like to mention that, as discussed above (Re 2.2a), the flow velocity (regime) used here in our *in vitro* microfluidic chip correspond to low shear stress values (0,5 (+/- 0,2) dyn/cm^2), which has, to our knowledge, only rarely been studied and thus cannot be necessarily compared to existing transcriptomic data. This

further implies that endothelial response might differ significantly from when subjected to high(er) shear stress such as the ones found in (10 dyn/cm^2, García-Cardeña et al., 2001). We therefore believe that classical transcriptomics analysis published so far cannot be directly compared to our results, where endothelial cells are subjected to rather low flow/shear regimes. That being said, we believe (and observed) that even low flow regimes are likely to change the endothelial response. Indeed and as examples, among the GO terms increased by flow (in absence of EVs), we find the terms “angiogenesis”, “blood vessel morphogenesis”, “blood vessel development”. Furthermore, the GO term “Cytoskeleton” noted by the reviewer could be misleading. Indeed, genes in this group are mostly linked to the microtubule cytoskeleton that is at play in cell division mechanisms. We believe that such decrease is linked to the anti-mitotic response commonly observed when endothelial cells are cultured under flow. In particular, the following GO terms were highlighted in parallel to the cytoskeleton one: “chromosome segregation”, “spindle” and “mitotic cell cycle”. Finally, the GO term “microtubule cytoskeleton” has similar fold enrichment, raw p-value and FDR than the GO term “cytoskeleton”. For more clarity, we will thus replace “cytoskeleton” by “microtubule cytoskeleton” and add the GO terms related to angiogenesis in Fig 3a. We now discuss these questions in our manuscript :

“While earlier work had provided transcriptomic analysis of endothelial cells subjected to high-shear flow regimes (for example 10 dynes/cm2 (García-Cardeña et al., 2001), we provide here an original documentation of the impact of low velocity flow regimes on endothelial transcriptional programs.”

4. Seemingly a minor but still important comments relates to the VeravecTM HUVECs. One should not have to go to the company website to find out that these are SV40 immortalized HUVECs. Please describe correctly in the Methods. Moreover, SV40 T transformation will broadly impact cell behavior, for example by constitutive PI3K signaling among other effects (see literature by Sara Courtneidge). The authors must be careful in concluding on the vHUVECs. An important control would be to compare flow effects on primary HUVECs and transformed vHUVECs to make sure they respond similarly to mechanosensing.

Re 3.4: vHUVECs were purchased from Angiocrine Biosciences. They were originally generated using a single protein from Adenovirus (E4ORF1) to adapt endothelial cell culture for multiple passages. As a result, vHUVECs have increased long term survival and preserved angiogenic potential, yet they are not immortal(ized). In addition, it has been reported that they respond to pro-angiogenic factors such as VEGF-A or FGF similarly to HUVEC(Seandel et al., 2008). While we agree that they differ from primary conventional HUVECs, in our study, we used vHUVECs only to generate stable endothelial cell lines and facilitate live imaging of endosomal compartments (Fig.2a, 2d and 3e only). All other results from our study were actually obtained with primary HUVECs, and we have now made this clear in the Ms. To confirm our main findings obtained with vHUVECs (namely EV storage in RAB14 compartments in flow condition), we investigated EV trafficking in RAB14 compartments in primary HUVECs. We found that similary to what was observed in vHUVECs, tEVs are partially re-routed to RAB14 compartments in flow conditions (Re-Fig7). In addition, and according to the Reviewer’s request, we now properly describe the vHUVECs in the method section as follows:
“Veravec^{TM} are HUVECs human endothelial cells adapted to longer term culture using a single adenovirus protein, E4ORF1 (Angiocrine Biosciences).”

5. To ultimately provide proof of tEVs' specific effect on transcriptional regulation in endothelial cells would entail to compare transcriptional profiles of endothelial cells exposed to tEVs and synthetic nanoparticles of similar size.

Re 3.5: Although we acknowledge that such analysis would increase the level of control over the exact impact of tEVs on transcriptional regulation of endothelial cells, we humbly believe that such experiments go beyond the scope of our study that is dedicated to the dissection of blood flow regimes on tEVs-mediated endothelial response. In addition, uptake of nanoparticles are likely to tune endothelial response (incidentally, in another project, we are currently interested in such effect where synthetic NPs are subjected to human immune cells: they do lead to transcriptional effects even at very low concentrations (i.e. below the range of concentration used in the clinics, data not shown)). For all these reasons and because this level of control might raise additional issues inherent to NPs uptake biology, we sticked to simple comparison where flow is the only tunable parameter which should avoid further confusion. Of note, in a previous study, we have shown that injecting 100nm synthetic beads in zebrafish embryo does not have the same functional impact on pre-metastatic niche priming than zebrafish melanoma EVs(Hyenne et al., 2019).

Referee #4:

The authors show that 'hemodynamics' promote the uptake of tumor (4T1) secreted EVs, and that this influences their intracellular fate and thus function in endothelial cells. This lead them to conclude that how that the blood flow provides a lysosomal escape mechanism by which they can enhance a pro-angiogenic function. This is a very intriguing hypothesis with potential major implications for EV-based therapy. This is a very intriguing study with carefully conducted experiments that should spur even more interest in in vivo EV biology. Nevertheless, I have several questions that need addressing. In particular their claim of flow induced 'rerouting' of tEVs inside their endothelial cells requires some more experimental scrutiny in my opnion.

Major questions:

Figure for referees not shown.

1) The authors have developed a highly physiological in vivo model to study the fate of EVs. Specifically, they isolated EVs from mouse breast cancer cells by differential centrifugation, labeled them with a dye and inject them in the circulation of zebrafish embryos and analyzed 4 vascular regions. They observed a significant inverse correlation between the 4 associated flow profiles and endothelial accumulation/uptake of their tEVs (Fig 1). Because their method of EV isolation is rather crude (ultra centrifugation) how can the authors exclude the potential influence of EV aggregates?

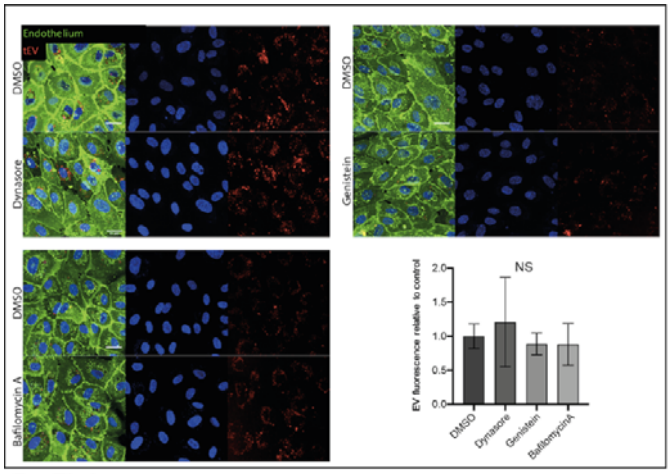
Re 4.1: Using Nanoparticle Tracking Analysis (NTA), we systematically measure the size distribution of EVs before injection or further manipulation. Doing so, we could not observe major size distribution differences when EVs are isolated by ultracentrifuge or by size exclusion chromatography (SEC). The latter is a rather gentle method that prevents potential EV aggregation. To fully exclude this possibility, we reproduced the experiment displayed in figure 1a using EVs isolated with SEC. We found that SEC and UC isolated EVs show comparable uptake patterns by endothelial cells, with higher uptake at low velocity (Re-Fig8 and now Fig-S2a in the manuscript). We added this information to the manuscript. Altogether, we thus believe

that our initial results were relevant and provide additional evidence that the methods of isolation has little impact on tEVs uptake in our model.

2) In addition, while the differences seen with these tEVs appear compelling, does this count for all (tumor) EVs? Indeed, the authors speculate that it is likely that the destination of their circulating mouse tumor EVs relies on the combined effect of hemodynamics and EV-endothelial receptor-ligand interactions. To demonstrate this connivingly a (control) experiment with distinct EVs is needed. In their own words, can the authors show that endothelial adhesion prone 4T1 EVs, behave distinctly from 'non-adhesion prone' tEVs? The authors could find inspiration for such a control in the paper of Hoshino et al which they cite.

Re 4.2: To address the first point raised by this reviewer, we performed additional experiments using EVs from a different cell line, the AT3 mammary tumor cells (Stewart and Abrams, 2007), as discussed earlier (see Re 2.1 and Re-Fig4). These data are now included in the manuscript as a new Fig.S2b. We also invite this reviewer to consult additional results published in our previous study (Hyenne et al., 2019), where other EV population had been tested in the context of metastatic niche priming in zebrafish, but also the ones published in (Verweij et al., 2019)(same issue) where similar results were obtained for endogenous EVs. Altogether, this shows that the overall EV uptake behavior is rather homogenous and not drastically impacted by either their source, methods of isolation of whether they were exogenously administrated.
Regarding the Reviewer's second point, we have shown in the past that CD146/MCAM, an adhesion protein present at the surface of 4T1 EVs contributes to their internalization by endothelial cells in zebrafish embryos (Ghoroghi et al., 2021b). More precisely, inhibition of CD146, decrease the uptake of circulating EVs in zebrafish endothelial cells. This demonstrates the contribution of adhesion receptors to EV internalization by endothelial cells in a dynamic environment and furthers adds to the demonstration that flow, and adhesive potential of EVs, control EV arrest and uptake.

3) In general, most non-phagocytic cells are very poor in taking up EVs. Their zebrafish embryo endothelial cells seem to do this quite efficiently in vivo but also their HUVECs in vitro. When comparing static or flow conditions with their clever microfluidic device, applying flow seems to increase the uptake. One could explain this in multiple ways. What is the actual uptake mechanism here? Is it receptor mediated endocytosis? If so, can the authors demonstrate this with inhibitors? Is it possible that endocytosis (or other EV uptake mechanisms) itself is increased in these cells under mechanical stress?



Re-Fig9

Re 4.3: We thank the reviewer for this comment, which matches our own interrogations. To address this question, we first analyzed the transcriptomics of endothelial cells, which did not reveal any specific endocytic signature that would be impacted by flow (data not shown). We then pharmacologically impaired classical endocytic pathways and assessed EV

uptake in flow condition (400µm/s). We used Dynasore to inhibit clathrin-mediated endocytosis, genistein to inhibit clathrin-independent endocytosis and Bafilomycin A to target micropinocytosis, which were all shown to decrease EV uptake in various cell types in static conditions (Fitzner et al., 2011; Costa Verdera et al., 2017). None of these drugs decreased EV uptake in our microfluidics system (See Re-Fig9). We thus concluded that there could be several endocytic mechanisms operating in parallel in flow-stimulated endothelial cells. With this in mind and the original observation that flow could impact endosomal/lysosomal pathways during EV uptake, we have decided to focus this study on the latter as we think that it potentially, yet partly, solves a long-standing question in the field.

4) The authors data suggests that internalized tEVs are at least partially redirected to non-degradative compartments upon flow mechanosensing. Indeed, while most of the internalized tEVs accumulate in Magic Red positive compartments, flow reduces tEV signal in cathepsin B-positive vesicles. This is a very intriguing observation and the intracellular compartments that are positive for the tEV signal seems to be RAB14 positive/less acidic compartments. Is it possible that tEVs are more prone for internalization directly into RAB14 compartments under flow? In other words how are the authors so sure it's re-directing from degradative compartments? Can the authors turn the flow off and on? If so, what happens if you add tEVs under static conditions and then turn on the flow? Is live-imaging an option?

Re 4.4: At this stage, we do not know whether internalized tEVs first transit trough acidic compartments or whether they are directly targeted to RAB14 compartments in endothelial cells cultured under flow. The precise tracking of individual EVs between different compartments would require complex experimental set-up. We now take into account the existence of different trafficking possibilities in our manuscript:
“Altogether, our results show that the moderate flow regimes partially switch EVs trafficking, either directly or not, toward Rab14 positive non-acidic and non-degradative compartments suggesting that endothelial mechanosensing of flow would prevent some tEVs from degradation.”

5) The authors show that as I expected the flow itself effects the physiology of the endothelial cells and focus on the lysosomal pathways based on transcriptomics. Apart from transcriptional regulation one may argue other regulatory mechanisms may also have a role. This is not mentioned. For example phospho-proteomics may lead to different pathways. Can the authors exclude endocytic pathways are not equally affected as well, just not at the transcriptional level?
Re 4.5: We agree with the referee that additional levels of regulation might exist. While we believed that this work require an additional study, we now broaden the discussion in our manuscript as follows:
“Therefore, although we cannot exclude that flow impacts additional pathways whose fast signaling could be probed with techniques such as phospho-proteomics, flow not only strengthens the lysosomal pathway in endothelial cells, through transcriptional control, it also triggers the formation of NHE9 compartments where EVs accumulate.”

Rebuttal references

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Dear Dr. Hyenne

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, the referees note that the revised study has been significantly strengthened but they also have a few remaining concerns:

1) Unclear mechanism of tEV uptake.
2) Different flow regimes do not affect the uptake of tEVs, it is rather the identity (venous) that determines uptake.

Upon further discussing these points with the referees we suggest addressing these in an extended discussion. Referee #2 confirmed that s/he considers the data strong and important but also indicated that rewriting specific sections of the manuscript is required "[...] since all evidence points towards endothelial cell identity rather than shear stress levels controlling tEVs uptake." A reanalysis of the data at hand and a display of the data in a different format as outlined in "Example 2" from referee #2 should also be performed.

Referee #2 and #3 suggested to separate Results from the Discussion to give enough room for the discussion and to make it easier to read and follow all arguments. This suggestion is OK from our side. We can change the status of your manuscript to a full article instead of a report.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Source data: Please add a link to the source data at biostudies in the manuscript.

- Please upload the source data in our submission system as 1 folder per figure with subfolders for each figure panel.

- Please address some additional comments in the figure legends (see attached file). Please do not calculate statistics based on 2 independent experiments.

- Please reduce the number of keywords to 5.

- Please add a 'Disclosure and competing interests statement'. For more information see https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest

- Please remove the Author Contributions from the manuscript file and make sure that the author contributions in our online submission system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article. You can enter additional information in the free text box provided, if you wish.

- The legends for Dataset EV1 and EV2 are currently provided as a separate Word file. Please paste the legends into a separate tab of each Dataset .xls file instead.

- "Supplementary Material:" in the manuscript file should be changed to "EV Figure Legends" and each figure legend needs to have corrected figure names: e.g. "Figure EV1" instead of "Supplementary Figure 1", etc.

- Figure callouts: please change "Movie 1" to "Movie EV1"; callouts for EV figures need to be corrected: "Fig. EV1" instead of "Fig. S1"

- Finally, EMBO Reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

- On a different note, I would like to alert you that EMBO Press offers a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:

https://www.embopress.org/video_synopses
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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

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

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

****

Referee #1:

The authors have addressed all my comments and I have no further comments.

Referee #2:

The reviewer highly appreciates the rigorous and compelling revision of the manuscript made by the authors. The new results and experiments have addressed all the major concerns, with the exception of one of utmost importance.
The reviewer acknowledges that the presence of flow has a positive effect on EV internalization and rerouting of intracellular vesicles. Yet, all the evidence presented in the manuscript points to the fact that identity (venous) rather than flow regime explains patterns of EV uptake.
For instance, in figure S2c (right-hand side panel), venous cells uptake similar levels of EVs at 300, 400 or 600 µm/s. In addition, in vitro assays further confirm that a very high flow regime (4400 µm/s) does not significantly change the uptake of EVs in HUVECs. Overall, there is no evidence for the influence of different flow regimes in the regulation of EV internalization.
Thus, the authors should acknowledge this fact and change the manuscript accordingly. This implies removing all sentences inferring an optimal range of flow rates for EV accumulation/internalization by venous cells. Otherwise, it may lead to the wrong conclusions/interpretations from readers.

A few examples where text should be changed:
Example 1 (Abstract)
"We experimentally tuned flow profiles in vitro (microfluidics) and in vivo (zebrafish) and demonstrated that efficient uptake of tEVs occurs in venous endothelial cells subjected to specific hemodynamics." This is incorrect as venous cells uptake EVs at the same rate in any flow regime.

Example 2 (Results)
"We analyzed four vascular regions (Fig.1a, hemodynamics panel) that are representatives of the flow profiles found in the caudal plexus and plotted the amount of tEVs internalized by endothelial cells with respect to the flow velocities (Fig.1a and Movie 1). We observed a significant inverse correlation between flow profiles and endothelial accumulation of tEVs (Pearson correlation coefficient r=-0,81; p<0,0001): while accumulation of tEVs is maximal in venous regions averaging 400 to 450 µm/s flow velocity, it is significantly reduced in arterial regions where flow ranges from 575 to 650 µm/s."
This correlation only exists because of the identity of cells rather than flow regime. If the authors would plot the correlation between flow profiles and endothelial accumulation of tEVs per endothelial cell identity (one plot for arterial cells and another for venous cells), the correlation is expected to be much weaker, and likely non-significant.

Example 3 (Results)
"Altogether, our in vitro and in vivo experiments demonstrate that venous endothelial cells most efficiently accumulate tEVs when subjected to low flow velocities of 400 µm/s."
Here, the authors use ambiguous language that suggests an optimal range of flow rates for accumulation of tEVs.

Referee #3:

The authors have undertaken a very ambitious review and done a good job in responding to the reviewers' criticisms. Possibly, the conclusions would have been even clearer if the results had been separated from the discussion, but this is not a demand. I have no further comments.

Referee #4:

The authors have dealt with most of my prior concerns accept one.

The mechanism of tEV uptake remained unclear, which is a major limitation of this study. The authors looked at a few endocytosis inhibitors (i presume under flow conditions) but none of them restricted uptake. How does this look like under static conditions can the authors explain?

If uptake is not mediated by endocytosis, this reviewer, and im sure many readers will be left wondering; how do the tEVs end up in the RAB compartments? If the authors choose not delve further into this conundrum, the reader should be made fully aware that further experimentation is needed one to properly judge the physiological relevance of the current findings.

Point-by-point response for our manuscript entitled: "Blood flow diverts extracellular vesicles from endothelial degradative compartments to promote angiogenesis**"**

by Benjamin Mary, Nandini Asokan, Katerina Jerabkova-Roda, Annabel Larnicol, Ignacio Busnelli, Tristan Stemmelen, Angélique Pichot, Anne Molitor, Raphaël Carapito, Olivier Lefebvre, Jacky G. Goetz, Vincent Hyenne.

First of all, we would like to thank the reviewers for their thoughtful comments, questions and suggestions which improved the quality of our work. We provide here a point-by-point response to the comments they raised. Our responses appear in *blue* in this document. You will find all our changes highlighted in green in the manuscript.

Dear Dr. Hyenne

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, the referees note that the revised study has been significantly strengthened but they also have a few remaining concerns:

1) Unclear mechanism of tEV uptake.

2) Different flow regimes do not affect the uptake of tEVs, it is rather the identity (venous) that determines uptake.

Upon further discussing these points with the referees we suggest addressing these in an extended discussion. Referee #2 confirmed that s/he considers the data strong and important but also indicated that rewriting specific sections of the manuscript is required "[...] since all evidence points towards endothelial cell identity rather than shear stress levels controlling tEVs uptake." A reanalysis of the data at hand and a display of the data in a different format as outlined in "Example 2" from referee #2 should also be performed.

Referee #2 and #3 suggested to separate Results from the Discussion to give enough room for the discussion and to make it easier to read and follow all arguments. This suggestion is OK from our side. We can change the status of your manuscript to a full article instead of a report.

Re: We would like to thank the reviewers for their positive comments. We have now modified our manuscript according to their recommendations, with a special emphasis on the two points highlighted above :
- we have clarified the questions of flow regimes (versus cell identity) pointed out by Referee #2 and made significant corrections in figure 1A and supplementary Figure 2a, 2b and 2c (ex: we removed the correlation curves and placed more emphasis on the contribution of endothelial cell identity to EV uptake).
- we have switched our paper to an article format and have added an independent discussion.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Source data: Please add a link to the source data at biostudies in the manuscript.
Re: This has been done.

- Please upload the source data in our submission system as 1 folder per figure with subfolders for each figure panel.
Re : this has been done.

- Please address some additional comments in the figure legends (see attached file). Please do not calculate statistics based on 2 independent experiments.
Re: On this particular point, we respectfully disagree. The statistics in these panels (Fig3c, d and e) are calculated by comparing the density of objects per field of view (or the average size of the studied compartments), not by comparing averages per experiment. One field of view represents one independent cell. Therefore, we believe it is legit to compare the quantifications of densities as we collected a significant number of fields of view (especially in the case of the electron microscopy experiments, which is tedious and rarely performed in triplicate). We would like to keep the statistical analysis, which is informative to the reader, and we now mention in the legends how the comparisons are performed.

- Please reduce the number of keywords to 5.
Re: This has been done.

- Please add a 'Disclosure and competing interests statement'. For more information see https://www.embopress.org/page/journal/14693178/authorguide#conflictsofinterest
Re: This has been done.

- Please remove the Author Contributions from the manuscript file and make sure that the author contributions in our online submission system are correct and up-to-date. The information you specified in the system will be automatically retrieved and typeset into the article. You can enter additional information in the free text box provided, if you wish.
Re: This has been done.

- The legends for Dataset EV1 and EV2 are currently provided as a separate Word file. Please paste the legends into a separate tab of each Dataset .xls file instead.
Re: This has been done.

- "Supplementary Material:" in the manuscript file should be changed to "EV Figure Legends" and each figure legend needs to have corrected figure names: e.g. "Figure EV1" instead of "Supplementary Figure 1", etc.
Re: This has been done.

- Figure callouts: please change "Movie 1" to "Movie EV1"; callouts for EV figures need to be corrected: "Fig. EV1" instead of "Fig. S1"
Re: This has been done.

- Finally, EMBO Reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x300-600 pixels large (width x height) in PNG for JPG format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.
Re: This has been done.

- On a different note, I would like to alert you that EMBO Press offers a new format for a video-synopsis of work published with us, which essentially is a short, author-generated film explaining the core findings in hand drawings, and, as we believe, can be very useful to increase visibility of the work. This has proven to offer a nice opportunity for exposure i.p. for the first author(s) of the study. Please see the following link for representative examples and their integration into the article web page:

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Please let me know, should you be interested to engage in commissioning a similar video synopsis for your work. According operation instructions are available and intuitive.

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

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

2nd Revision - Editorial Decision **27th Oct 2023**

Dear Dr. Hyenne,

Thank you for submitting your revised manuscript. I have now looked at everything and all is fine. Therefore, I am very pleased to accept your manuscript for publication in EMBO Reports.

Congratulations on a nice work!

Kind regards,

Deniz Senyilmaz Tiebe
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Deniz Senyilmaz Tiebe, PhD
Editor
EMBO Reports

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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 χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
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Please complete ALL of the questions below.
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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.	Not Applicable	

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	**Yes**	Figures 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.	Not Applicable	
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**	Figures legends, Material 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**	Figures legends
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figures 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 approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	**Yes**	Material and Methods
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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**	Material and 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	