nature portfolio

Peer Review File

Integrative single-cell analysis reveals endothelial diversity in the vasa vasorum of human atherosclerosis

Corresponding Author: Professor Donghai Wang

This manuscript has been previously submitted at another journal. This document only contains information relating to versions considered at Communications Biology.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

A version of this paper was originally rejected for publication by Communications Biology, however that decision was reconsidered after appeal by the authors.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The study from Wu and colleagues presents a detailed single-cell transcriptomic atlas of endothelial cells in human atherosclerotic plaques, integrating data from multiple vascular beds.
The manuscript offers an overview of the complexity and heterogeneity of endothelial cell populations and their roles in atherosclerosis progression.
However, there are several aspects that require revision to enhance clarity and coherence and that would strongly improve the current submission.

Major:
The rationale behind the study could benefit from greater clarity. The authors start describing vasa vasorum in the context of plaque stability. Further, in the scRNA-seq analysis, they perform an analysis of multiple publicly available datasets that are not specifically related to intra plaque hemorrage or plaque instability.
As an example, the dataset from Alsaigh et al. pertains to scRNA-seq analysis comparing atherosclerotic plaques with healthy tissue. In contrast, the data from Quertermous were generated from scRNA-seq of transplanted human hearts. Although some limitations are acknowledged in the Discussion, the rationale for selecting these specific datasets should be clearly articulated. Moreover, the selection criteria for these datasets should be justified, and incorporating datasets more directly linked to plaque instability would strengthen the study.

Figure 1 presents an H&E staining of what appear to be human tissue samples, possibly carotid arteries. The authors should clarify this in the Methods section by specifying the exact source of the human samples, including details on ethical approval. Additionally, the staining procedure should be described in the Methods, along with clinical information that informed the classification of plaques as stable or unstable, since the expansion and proliferation of the vasa vasorum can occur, especially in unstable plaques. Moreover, given that vasa vasorum are challenging to visualize and characterize with H&E staining alone, the manuscript would benefit from additional immunostaining to specifically identify vasa vasorum structures: at a minimum, including markers for endothelial cells and vascular smooth muscle cells. Incorporating other markers associated with plaque instability would also strengthen the study and enhance the clarity and impact of the findings.

In Figures 2A, C, and D, the analyses appear to reflect the differing cellular composition of the samples, across the various datasets. The manuscript would benefit from a clearer discussion and explicit mention of these differences and their potential impact on the results.

In Figure 3, the authors describe a weighted gene co-expression network analysis aimed at identifying co-expression networks across cellular and spatial hierarchies. However, the concept of a "Module" is not clearly defined in the Methods. It would be helpful for the authors to clarify what is meant by a Module in this context, including the criteria and parameters

used in the analysis.

Figure 4C present an immunofluorescence staining to highlight ACKR1 and NR2F2 expression. No additional information are given on the sample's origin or tissue composition. This should be included in the Methods and properly described in the specific result paragraph as well as in the figure legend.

Figure 5 is intended to illustrate endothelial cell (EC) sprouting and angiogenic activity within the tissue. While the authors mention an angiogenesis score, no details are provided on how this score was calculated or which criteria were used. Including this information would enhance the clarity and rigor of the analysis and provide important context for the subsequent identification of cellular trajectories and genes involved in angiogenesis.

In the assessment of EndMT in Figure 6 and in the discussion, the authors could benefit from the work from Markova et al. It describes how VSMC markers (i.e., ACTA2 and MYH11) provide a higher signal-to-noise ratio compared to EC markers when staining the vasa vasorum. This data should be included in the manuscript and discussed properly in the text.

The authors should improve the Figure legends by including more detailed information on statistical analyses, such as statistical power and clear references to the specific sample populations represented in each graph.

Minor
The Methods section should clearly state the source of the datasets used (e.g., GSE159677 for the dataset from Alsaigh et al.) and describe in detail the procedure used to generate the data.
The authors may consider briefly summarizing key results without generic statements such as "Following stringent quality control (Methods)," to improve clarity and readability.

Reviewer #2

(Remarks to the Author)
This study uncovered novel endothelial heterogeneity with distinct transcriptional and functional characteristics in the complex landscape of endothelial adaptation in the atherosclerotic microenvironment through integrating multiple scRNA-seq data. The topic is interesting; however, the manuscript has several critical limitations, as outlined below.

1. Fig. 1, In Fig1, it is best to demonstrate the presence of endothelial cells within the plaque and on the lumen CD31/vWF immunofluorescence

2. The authors conducted a very detailed data processing for the quality control, integration, and batch correction of scRNA-seq data. It is suggested that a corresponding workflow diagram be provided to illustrate this process, which would help readers better understand the procedure.

3. Line 108 "LECs were mainly derived from Hu's cohort9 108 (Extended Data Fig. 2G)." I cannot identify LECs among E1-E11, did LECs were excluded before further refine classification? Why LECs were excluded?

4. Line 128 "Module1 was predominantly enriched in ArtECs and E01-veins-SELE1", however, the dotplot in extended Data Fig.4 D revealed that Module 1 was rarely enriched in E01-veins-SELE1?

5. Angiogenesis is a process related to the proliferation, differentiation and migration of endothelial cells. How do these abilities change in endothelial cell subclusters, especially E05-E07?

6. The figure legend of Fig. 6 is incorrect? And y-axis of the Fig 6A should be RM/E? And line 275 "ArtECs and the inflammatory endothelial subcluster E01-veins-SELE were among the top-ranked clusters based on RM/E (Fig. 6A)." Did E01-veins-SELE also had experienced EndMT?

7. In the plaques, the differences in spatial distribution among the arterial (ArtECs), venous (VenECs), and capillary-like (CapECs) subtypes were not identified?

Reviewer #3

(Remarks to the Author)
The authors present a comprehensive single cell atlas of ECs from human atherosclerotic lesions. They integrated five data sets from previous studies and identified 11 distinct EC subpopulations. Special attention is paid to inflammatory signatures from venule endothelial cells, angiogenesis and endothelial to mesenchymal transition. These processes are detected by using pseudotime analysis, hdWGCNA, gene expression analysis and supported by immunofluorescence staining.
The results substantially expand current knowledge, as they systematically characterize novel functional EC subtypes and their disease-relevant dynamics in EndMT, angiogenesis, and inflammation for the first time based on single-cell sequencing.

The manuscript reveals a comprehensive and well integrated single-cell analysis of endothelial heterogeneity in

atherosclerosis, with robust bioinformatics and valuable insights into EndMT and angiogenesis. However, it remains purely descriptive and lacks experimental validation (e.g. quantitative evaluation of the immunofluorescence images would further strengthen the findings).
Further limitations may arise from the anatomical heterogeneity of sampled arteries/tissues and the sex imbalance.

Major:
1. The authors should consider to include QC comparison of the used data sets

2. The authors should consider to validate the major finding using FACS

Minor points:
1. Figure 1 is only supportive, the authors may consider integrating it into another figure e.g. Fig.2
2. Several key methods are missing details for reproducibility, including the calculation of the R-T/S and R-M/E scores, specific parameters used in Monocle3 and in hdWGCNA. A more transparent and parameterized methods section would enhance reproducibility and allow critical evaluation of the analyses.
3. Fig. 3G improve conclusion
4. Extended Fig. 3D: Ambiguous labeling
5. review reference in line 133
6. Fig6.: review figure legend title
7. Fig. 4D: review x-axis labeling
8. Extended Fig. 6: review reference in figure legend (B/C)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed the points raised in my previous review and I think this revision benefit for those improvements.

I still have a few minor comments:

Some conceptual issues remain in the way results are introduced. For example, the phrase “Following quality control (Methods),” should be removed, as it is redundant. The method can be briefly mentioned briefly, since it is already described in the Methods section.

The H&E section in the Methods still requires additional detail. The source of the samples and information on processing are missing and should be added.

Reviewer #2

(Remarks to the Author)
The authors have solved my concerns, except for a few mistakes.
Why are the legends of extended Data Figure 2 and Extended Data Figure 3 the same?

Reviewer #3

(Remarks to the Author)
Unfortunately, this is a very unfortunate revision in which the authors resort to incomprehensible methods and, sadly, do not address the reviewers' questions. I have requested validation of the major results, which clearly involve the identification of different cell types in the plaque area, using FACS. Unfortunately, the authors provide a FACS analysis of in vitro cells from a different source (HUVEC). In addition, each reviewer is given a different answer using the same data/figures. To prevent this from being noticed, the answers to each reviewer are stored in a separate file. My other points were also addressed inadequately.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
All points are adressed

Jinan, Nov 12, 2025

Dear Reviewer 1,

We sincerely thank you for your constructive and insightful comments. Below we provide a point-by-point response.

Yours sincerely,

On behalf of the corresponding authors,

Donghai Wang, MD, PhD

Professor & Team Leader, Department of Neurosurgery Qilu Hospital, Cheeloo College of Medicine and Institute of Brain and Brain-Inspired Science, Shandong University, Jinan, China

Anton Gisterå, MD, PhD

Assistant Professor & Team Leader, Atherosclerosis Immunology Laboratory

Department of Medicine, Solna, Karolinska Institutet, Sweden

Response to Reviewers' comments:

Re: Reviewer #1

The study from Wu and colleagues presents a detailed single-cell transcriptomic atlas of endothelial cells in human atherosclerotic plaques, integrating data from multiple vascular beds. The manuscript offers an overview of the complexity and heterogeneity of endothelial cell populations and their roles in atherosclerosis progression. However, there are several aspects that require revision to enhance clarity and coherence and that would strongly improve the current submission.

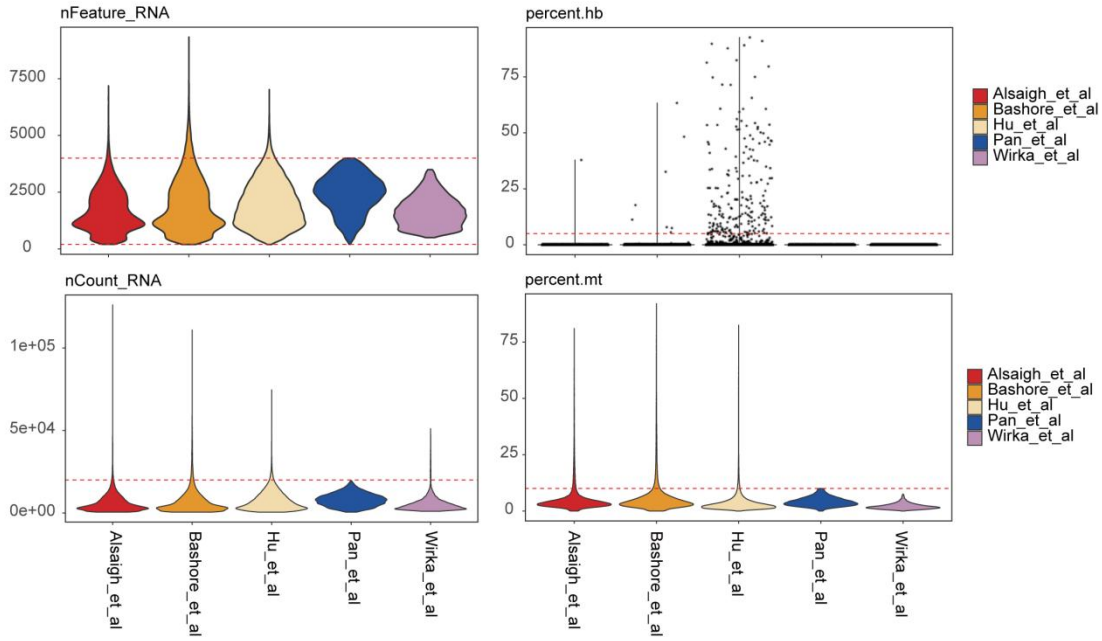
Response: We sincerely thank the Reviewer for the constructive comments and for recognizing the value of our endothelial cell atlas in human atherosclerotic plaques. We fully agree with the Reviewer's suggestions and have carefully revised the manuscript to enhance clarity and coherence.

1. Comments： The rationale behind the study could benefit from greater clarity. The authors start describing in the context of plaque stability. Further, in the scRNA-seq analysis, they perform an analysis of multiple publicly available datasets that are not specifically related to intra plaque hemorrage or plaque instability.As an example, the dataset from Alsaigh et al. pertains to scRNA-seq analysis comparing atherosclerotic plaques with healthy tissue. In contrast, the data from Quertermous were generated from scRNA-seq of transplanted human hearts. Although some limitations are acknowledged in the Discussion, the rationale for selecting these specific datasets should be clearly articulated. Moreover, the selection criteria for these datasets should be justified, and incorporating datasets more directly linked to plaque instability would strengthen the study.

Response： In our initial study design, we intended to investigate the potential role of the vasa vasorum by focusing on plaque stability. However, given the functional heterogeneity and complexity of the vasa vasorum, their widespread

expansion and proliferation within atherosclerotic plaques, and the current limited understanding regarding the heterogeneity among endothelial cell clusters, we chose not to emphasize the distinction between stable and unstable plaques. Instead, our primary objective was to broadly integrate multiple publicly available vascular single-cell RNA sequencing datasets related to atherosclerosis, aiming to assess the functional heterogeneity of endothelial cell clusters. The selected datasets, including those from Alsaigh et al. (comparing atherosclerotic plaques with adjacent tissues) and the Quertermous group (vascular tissues from transplanted human hearts), met the following selection criteria: (1) clear tissue origins relevant to atherosclerosis; (2) well-defined cell types and high data quality (Response Figure 1) enabling accurate identification of vasa vasorum-related endothelial subpopulations and (3) public availability. These datasets are also widely used for integrating single-cell atherosclerosis data[1-3]. This has been addressed in lines 333 – 337. Although these datasets do not explicitly distinguish plaque stability states, their broad coverage of vascular cell heterogeneity within the atherosclerotic microenvironment offers valuable support for exploring the potential functional diversity of the vasa vasorum. We agree with the reviewer's suggestion that future studies should incorporate datasets directly related to plaque instability to further investigate the role of the vasa vasorum under specific pathological conditions. In the present study, our dataset selection strategy was intended to explore the functional diversity of vasa vasorum endothelial cells in the context of atherosclerosis.

Response Figure 1

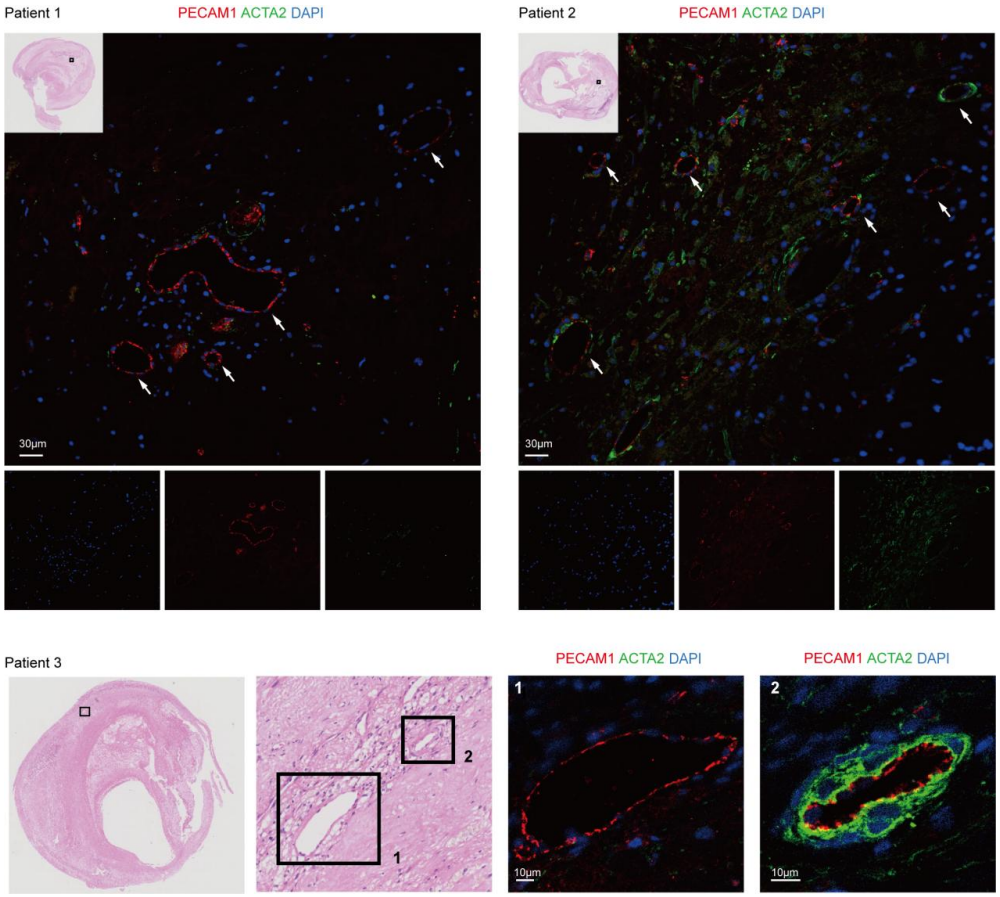


2. Comments： Figure 1 presents an H&E staining of what appear to be human tissue samples, possibly carotid arteries. The authors should clarify this in the Methods section by specifying the exact source of the human samples, including details on ethical approval. Additionally, the staining procedure should be described in the Methods, along with clinical information that informed the classification of plaques as stable or unstable, since the expansion and proliferation of the vasa vasorum can occur, especially in unstable plaques. Moreover, given that vasa vasorum are challenging to visualize and characterize with H&E staining alone, the manuscript would benefit from additional immunostaining to specifically identify vasa vasorum structures: at a minimum, including markers for endothelial cells and vascular smooth muscle cells. Incorporating other markers associated with plaque instability would also strengthen the study and enhance the clarity and impact of the findings.

Response： Thanks very much for your insightful comments and suggestions. We have clarified in the Methods section that the tissue samples presented in

Figure 1 are indeed human carotid artery specimens. We have now specified the precise source of these samples, included details regarding ethical approval, and described the H&E staining procedure in greater detail. This has been addressed in lines 412–420. Due to the difficulty in directly comparing endothelial cell heterogeneity within stable and unstable plaques using integrated single-cell data, we no longer emphasized the distinction between stable and unstable plaques. To address your recommendation about the limitations of H&E staining for visualizing vasa vasorum, we agree that additional immunostaining would enhance clarity and rigor. Accordingly, we conducted immunostaining using endothelial (PECAM1) and vascular smooth muscle (ACTA2) markers and present these data as a new Figure 1 in the revised manuscript (Response Figure 2). The Methods, Results, and figure legend have been updated accordingly, and all revisions were highlighted in red for ease of review.

Response Figure 2



3. Comments： In Figures 2A, C, and D, the analyses appear to reflect the differing cellular composition of the samples, across the various datasets. The manuscript would benefit from a clearer discussion and explicit mention of these differences and their potential impact on the results.

Response： As indicated by the UMAP clustering (Fig. 2A, 2C) and the absolute cell counts per dataset (Fig. 2D), our integrated analysis reflected cell-type composition between datasets. We applied a batch integration approach to mitigate technical batch. However, this procedure preserved differences in cell-type proportions across samples. We focused more on the commonalities in cellular composition across different cohorts, as the major cell clusters could be observed across all datasets. To reduce potential bias, we analyzed multiple cohorts to study endothelial cell function using single-cell transcriptomics. We acknowledged that this aspect represented a challenge inherent to single-cell data integration and that further studies are needed to

clarify its impact on downstream analyses[1,4].

4. Comments: In Figure 3, the authors describe a weighted gene co-expression network analysis aimed at identifying co-expression networks across cellular and spatial hierarchies. However, the concept of a "Module" is not clearly defined in the Methods. It would be helpful for the authors to clarify what is meant by a Module in this context, including the criteria and parameters used in the analysis.

Response： In the methods sections “Computing co-expression networks” and “Computing module eigengenes” of Morabito et al., the authors comprehensively describe the procedures used for module construction and gene identification. Briefly, hdWGCNA aggregates similar cells into metacells to mitigate data sparsity, computes a signed gene-gene adjacency matrix, and subsequently constructs a topological overlap matrix (TOM). Modules are then identified using hierarchical clustering with the Dynamic Tree Cut algorithm and summarized by module eigengenes (MEs) derived via singular value decomposition (SVD). In our implementation of the hdWGCNA workflow, we respectfully adopted customized parameter settings for several key analytical steps, as detailed explicitly in the Methods section. Following preparation of the Seurat object for WGCNA, metacells were generated with the following parameters: k = 10, assay = 'SCT', max_shared = 5, min_cells = 300, reduction = 'umap', and slot = 'data'. Additionally, as illustrated in Extended Data Figures 4A and 4B, we carefully evaluated the selection of the soft-thresholding power and hierarchical clustering approach. The corresponding analysis code to reproduce steps of the hdWGCNA workflow is provided (see Code availability statement).

5. Comments: Figure 4C present an immunofluorescence staining to highlight ACKR1 and NR2F2 expression. No additional information are given on the sample’s origin or tissue composition. This should be included in the Methods and properly described in the specific result paragraph as well as in

the figure legend.

Response: Thank you for the Reviewer's suggestion. We have clarified the patient cohort as adults (≥18 years) who underwent carotid endarterectomy (CEA) for carotid artery stenosis or occlusion due to atherosclerosis between December 2020 and January 2023. We have updated the descriptions of patient information in both the Results and Methods sections. Detailed demographics, clinical characteristics, and procedural information are summarized in Table S2.

6. Comments: Figure 5 is intended to illustrate endothelial cell (EC) sprouting and angiogenic activity within the tissue. While the authors mention an angiogenesis score, no details are provided on how this score was calculated or which criteria were used. Including this information would enhance the clarity and rigor of the analysis and provide important context for the subsequent identification of cellular trajectories and genes involved in angiogenesis.

Response: We used the GO:0002040(sprouting angiogenesis) gene set (http://amigo.geneontology.org/amigo/term/GO:0002040) to calculate AUCell scores via the AUCell_run function (https://github.com/aertslab/AUCell). The mean and variance of AUCell scores across different cell types were computed and visualized using bar plots. A higher AUCell score indicates that the corresponding cell type is more likely to be involved in sprouting angiogenesis . Differences were assessed using the Kruskal-Wallis test. We updated the scoring code; details and access were provided in the code availability statement.

7. Comments: In the assessment of EndMT in Figure 6 and in the discussion, the authors could benefit from the work from Markova et al. It describes how VSMC markers (i.e., ACTA2 and MYH11) provide a higher signal-to-noise ratio compared to EC markers when staining the vasa vasorum. This data should

be included in the manuscript and discussed properly in the text.

Response: We incorporated insights from Markova et al. and related studies to refine marker selection and performed dual immunofluorescence staining for PECAM1 and ACTA2 on carotid plaque specimens. The updated data are presented in the new Figure 1 and demonstrate an improved signal-to-noise ratio for the identification of vasa vasorum within atherosclerotic tissue.

8. Comments: The authors should improve the Figure legends by including more detailed information on statistical analyses, such as statistical power and clear references to the specific sample populations represented in each graph.

Response: We sincerely thank the reviewer for this valuable suggestion. We have revised the manuscript to include descriptions of the sample characteristics and ethics approvals. Statistical analyses were performed in R with commonly used packages, as detailed in the Methods. As an example, enrichment analysis was conducted with RunEnrichr (hdWGCNA)[5]. We evaluated AUCell scores using the Kruskal–Wallis test, following the approach of Li et al[6]. We have clarified this in the figure legend and the changes have been highlighted in red.

9. Comments: The Methods section should clearly state the source of the datasets used (e.g., GSE159677 for the dataset from Alsaigh et al.) and describe in detail the procedure used to generate the data.
The authors may consider briefly summarizing key results without generic statements such as “Following stringent quality control (Methods),” to improve clarity and readability.

Response: We thank the reviewer for the suggestion. We have provided more detailed sample information in the Methods and revised the wording to remove generic statements for clarity and readability. Edits were highlighted in red in the revised manuscript.

References

1 Mosquera, J. V. *et al.* Integrative single-cell meta-analysis reveals disease-relevant vascular cell states and markers in human atherosclerosis. *Cell Rep* **42**, 113380 (2023). https://doi.org/10.1016/j.celrep.2023.113380

2 Traeuble, K. *et al.* Integrated single-cell atlas of human atherosclerotic plaques. *Nat Commun* **16**, 8255 (2025). https://doi.org/10.1038/s41467-025-63202-x

3 Bleckwehl, T. *et al.* Encompassing view of spatial and single-cell RNA sequencing renews the role of the microvasculature in human atherosclerosis. *Nat Cardiovasc Res* **4**, 26-44 (2025). https://doi.org/10.1038/s44161-024-00582-1

4 Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* **36**, 411-420 (2018). https://doi.org/10.1038/nbt.4096

5 Morabito, S., Reese, F., Rahimzadeh, N., Miyoshi, E. & Swarup, V. hdWGCNA identifies co-expression networks in high-dimensional transcriptomics data. *Cell Rep Methods* **3**, 100498 (2023). https://doi.org/10.1016/j.crmeth.2023.100498

6 Li, J. *et al.* Pan-cancer integrative analyses dissect the remodeling of endothelial cells in human cancers. *Natl Sci Rev* **11**, nwae231 (2024). https://doi.org/10.1093/nsr/nwae231

Jinan, Nov 12, 2025

Dear Reviewer 2,

We sincerely thank you for your constructive and insightful comments. Below we provide a point-by-point response.

Yours sincerely,

On behalf of the corresponding authors,

Donghai Wang, MD, PhD

Professor & Team Leader, Department of Neurosurgery Qilu Hospital, Cheeloo College of Medicine and Institute of Brain and Brain-Inspired Science, Shandong University, Jinan, China

Anton Gisterå, MD, PhD

Assistant Professor & Team Leader, Atherosclerosis Immunology Laboratory

Department of Medicine, Solna, Karolinska Institutet, Sweden

Response to Reviewers' comments:

Re: Reviewer #2

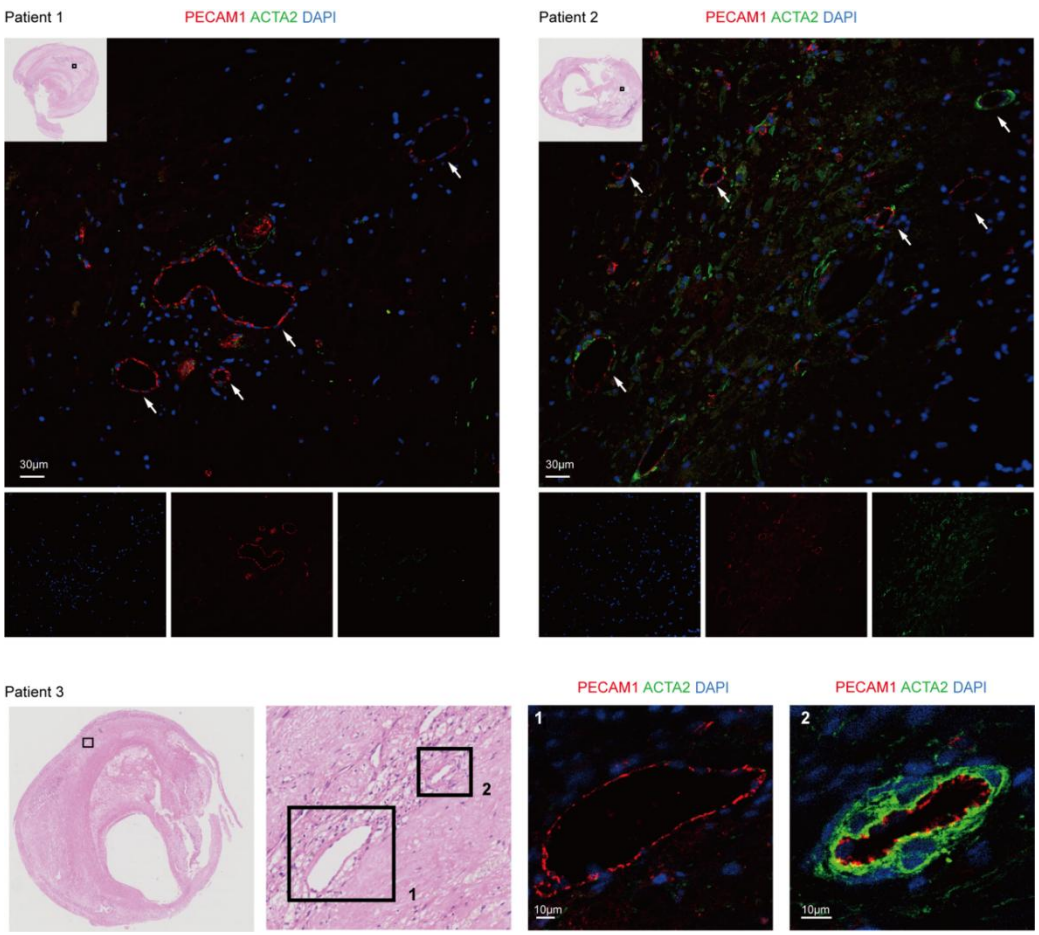
This study uncovered novel endothelial heterogeneity with distinct transcriptional and functional characteristics in the complex landscape of endothelial adaptation in the atherosclerotic microenvironment through integrating multiple scRNA-seq data. The topic is interesting; however, the manuscript has several critical limitations, as outlined below.

Response: We sincerely thank the reviewer for the constructive and insightful comments. We are grateful that the reviewer found our study on endothelial heterogeneity in the atherosclerotic microenvironment to be interesting. We acknowledge the concerns raised regarding the limitations of the manuscript. In the revised version, we have carefully addressed each of the critical points, as detailed below. We appreciate the opportunity to clarify and strengthen our work accordingly.

1. Comments: In Fig1, it is best to demonstrate the presence of endothelial cells within the plaque and on the lumen CD31/vWF immunofluorescence.

Response: We appreciate the reviewer's helpful suggestion to demonstrate the presence of endothelial cells within the plaque and on the lumen using CD31/vWF immunofluorescence. In our study, we conducted immunofluorescence staining using CD31 together with ACTA2 to better delineate endothelial cells in the context of vascular structure[1]. In addition, we have supplemented and clarified the specimen information regarding the carotid atherosclerotic plaques (TableS2). Response figure 3 showed endothelial cells of the vasa vasorum within the carotid atherosclerotic plaques.

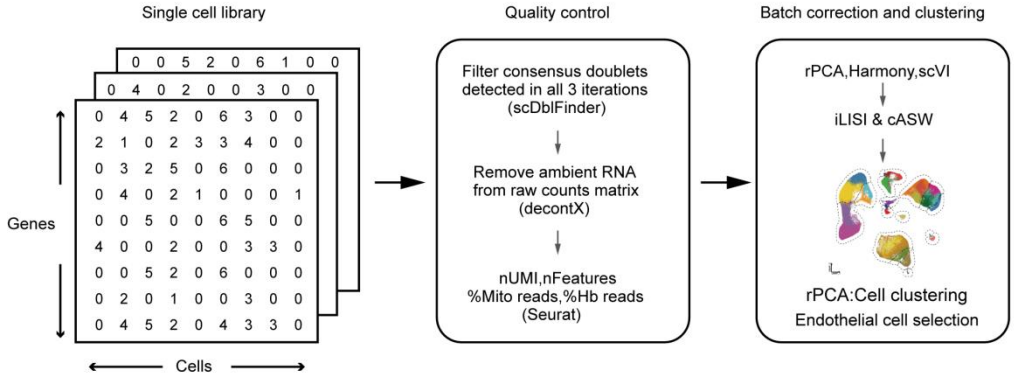
Response Figure 3



2. Comments: The authors conducted a very detailed data processing for the quality control, integration, and batch correction of scRNA-seq data. It is suggested that a corresponding workflow diagram be provided to illustrate this process, which would help readers better understand the procedure.

Response: We sincerely thank the reviewer for the valuable suggestion. To improve the clarity and reproducibility of our scRNA-seq data processing, we have added a schematic workflow diagram outlining the steps of quality control, integration, and batch correction (Response Figure 4). The updated version of the figure is now shown in Extended Data Fig. 1A.

Response Figure 4



3. Comments: Line 108 “LECs were mainly derived from Hu’s cohort 108 (Extended Data Fig. 2G).” I cannot identify LECs among E1-E11, did LECs were excluded before further refine classification? Why LECs were excluded?

Response: Lymphatic endothelial cells (LECs) were not included in the refined subclustering (E1–E11) presented in the downstream analyses. Given the functional differences between lymphatic endothelial cells (LECs) and vascular endothelial cells (VECs), LECs were excluded from downstream analyses in order to reduce noise and better characterize the functional heterogeneity within VECs.

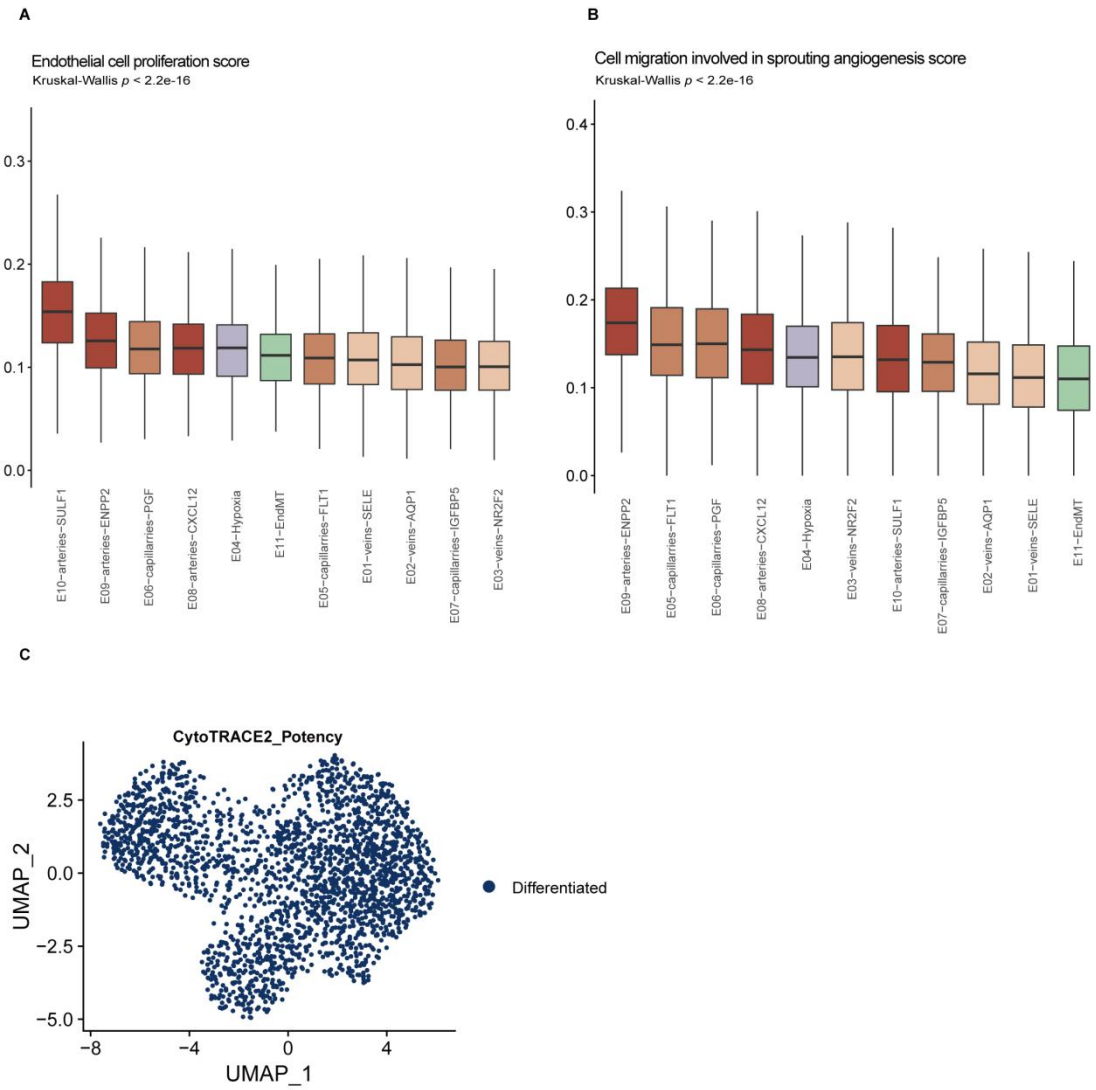
4. Comments: Line 128 “Module1 was predominantly enriched in ArtECs and E01-veins-SELE1”, however, the dotplot in extended Data Fig.4 D revealed that Module 1 was rarely enriched in E01-veins-SELE1?

Response: We thank the reviewer for pointing out this inconsistency. Upon re-evaluation of Module 1 enrichment based on the updated dot plot (Extended Data Fig. 5D), we confirm that Module 1 is predominantly enriched in ArtECs and E11-EndMT, but not in E01-veins-SELE1 as previously stated. We apologize for the oversight and have revised the manuscript accordingly to correct this statement (Line 126). We appreciate the reviewer’s careful observation, which helped us improve the accuracy of our data interpretation.

5. Comments: Angiogenesis is a process related to the proliferation, differentiation and migration of endothelial cells. How do these abilities change in endothelial cell subclusters, especially E05-E07?

Response: To evaluate the proliferative and migratory capacities of endothelial subpopulations, we applied the AUCell_run function (https://github.com/aertslab/AUCell) using two gene sets: GO:0001935 (endothelial cell proliferation; https://amigo.geneontology.org/amigo/term/GO:0001935) and GO:0002042 (cell migration involved in sprouting angiogenesis; https://amigo.geneontology.org/amigo/term/GO:0002042). Due to the limitations of high-throughput data, we were unable to obtain reliable results for differentiation potential. In terms of proliferative capacity, E06 exhibited a relatively high proliferation score (Response Figure 5A). Among the other endothelial subtypes, arterial endothelial cells also showed elevated proliferation activity (Response Figure 5A). Regarding migratory capacity, E05 and E06 scored markedly higher than E07 (Response Figure 5B). Consistent with previous results, E05 and E06 represent active endothelial populations involved in sprouting angiogenesis. We used CytoTRACE2 to assess the differentiation potential of capillary endothelial cells (Response Figure 5C).

Response Figure 5

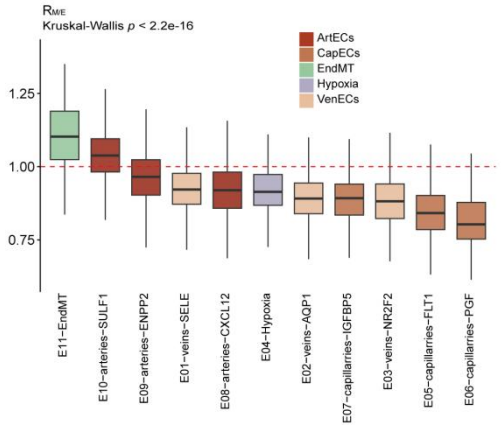


6. Comments: The figure legend of Fig. 6 is incorrect? And y-axis of the Fig 6A should be RM/E? And line 275 “ArtECs and the inflammatory endothelial subcluster E01-veins-SELE were among the top-ranked clusters based on RM/E (Fig. 6A).” Did E01-veins-SELE also had experienced EndMT?

Response: We appreciate the reviewer’s comments. In the revision, we corrected the Fig. 6 legend and updated the y-axis label in Fig. 6A to RM/E. EndMT is a complex and reversible process. To evaluate the cellular state of endothelial cells in high-throughput data, we used the ratio of mesenchymal score to endothelial score as a metric to assess whether endothelial cells underwent mesenchymal transition. We replaced bar charts with box plots (Response Figure 6). A ratio greater than 1 was considered indicative of

EndMT. Based on this analysis, we identified the SULF1+endothelial clusters as a potentially important group undergoing mesenchymal transition. In contrast, the E01-veins-SELE cluster exhibited a lower mesenchymal score relative to the endothelial score, suggesting that these cells retain predominant endothelial characteristics under our evaluation criteria.

Response Figure 6



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7. Comments: In the plaques, the differences in spatial distribution among the arterial (ArtECs), venous (VenECs), and capillary-like (CapECs) subtypes were not identified?

Response: Identifying the spatial distribution of distinct endothelial subtypes (ArtECs, VenECs, and CapECs) within atherosclerotic plaques remains technically challenging. Although single-cell transcriptomic data and immunostaining provide valuable information on cellular identities, they are limited in spatial resolution and may not be sufficient to resolve the precise localization of closely related endothelial subtypes. We agree that spatial transcriptomics holds great potential for addressing this question. In future studies, pending availability of resources and funding, we plan to employ high-resolution spatial transcriptomic approaches to further investigate the spatial distribution of endothelial subtypes within the plaque microenvironment.

References

1 Markova, V. *et al.* Endothelial Cell Markers Are Inferior to Vascular Smooth Muscle Cells Markers in Staining Vasa Vasorum and Are Non-Specific for Distinct Endothelial Cell Lineages in Clinical Samples. *Int J Mol Sci* **24** (2023). https://doi.org/10.3390/ijms24031959

Jinan, Nov 12, 2025

Dear Reviewer 3,

We sincerely thank you for your constructive and insightful comments. Below we provide a point-by-point response.

Yours sincerely,

On behalf of the corresponding authors,

Donghai Wang, MD, PhD

Professor & Team Leader, Department of Neurosurgery Qilu Hospital, Cheeloo College of Medicine and Institute of Brain and Brain-Inspired Science, Shandong University, Jinan, China

Anton Gisterå, MD, PhD

Assistant Professor & Team Leader, Atherosclerosis Immunology Laboratory

Department of Medicine, Solna, Karolinska Institutet, Sweden

Response to Reviewers' comments:

Re: Reviewer #3

The authors present a comprehensive single cell atlas of ECs from human atherosclerotic lesions. They integrated five data sets from previous studies and identified 11 distinct EC subpopulations. Special attention is paid to inflammatory signatures from venule endothelial cells, angiogenesis and endothelial to mesenchymal transition. These processes are detected by using pseudotime analysis, hdWGCNA, gene expression analysis and supported by immunofluorescence staining.The results substantially expand current knowledge, as they systematically characterize novel functional EC subtypes and their disease-relevant dynamics in EndMT, angiogenesis, and inflammation for the first time based on single-cell sequencing.

The manuscript reveals a comprehensive and well integrated single-cell analysis of endothelial heterogeneity in atherosclerosis, with robust bioinformatics and valuable insights into EndMT and angiogenesis. However, it remains purely descriptive and lacks experimental validation (e.g. quantitative evaluation of the immunofluorescence images would further strengthen the findings).Further limitations may arise from the anatomical heterogeneity of sampled arteries/tissues and the sex imbalance.

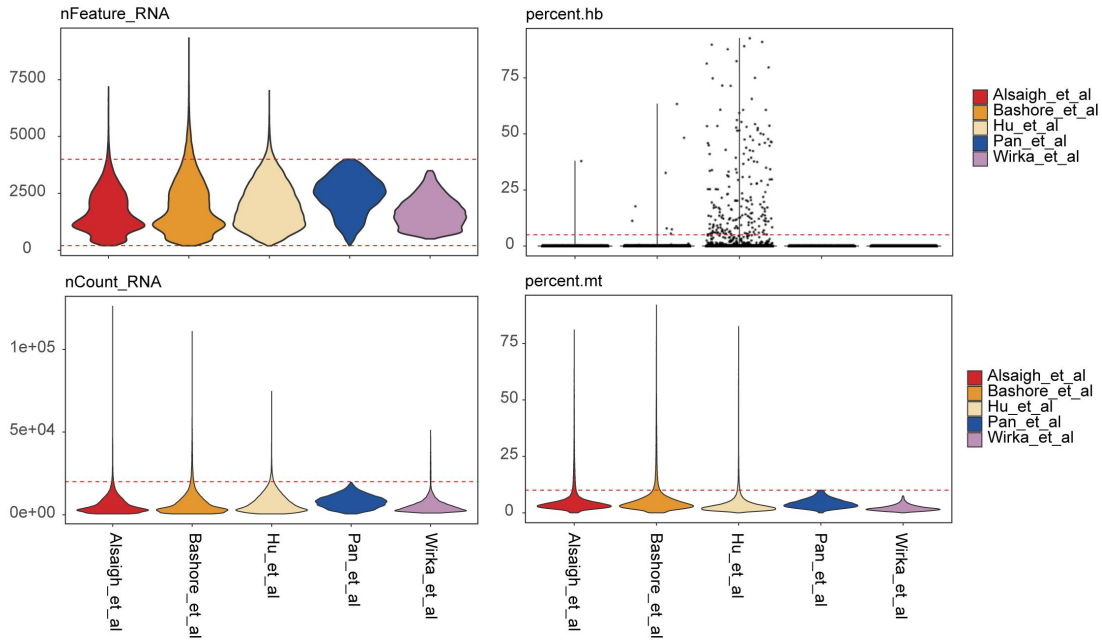
Response: We are grateful to the reviewer for the thoughtful comments, and we are encouraged that our analysis of endothelial heterogeneity was received. We acknowledge the concerns regarding the manuscript's limitations and have carefully revised the text to address each major point, detailed below. We appreciate the opportunity to improve the clarity and rigor of the study.

1. Comments: The authors should consider to include QC comparison of the used data sets.

Response: Thanks for this constructive suggestion. In the revised manuscript, we included a cross-dataset quality-control (QC) comparison performed prior

to integration and downstream analyses (Response Figure 7); using clearly defined filtering thresholds, we retained the vast majority of high-quality cells and excluded a small subset of cells likely influenced by confounders. The updated version of the figure is now shown in Extended Data Fig. 1B

Response Figure 7

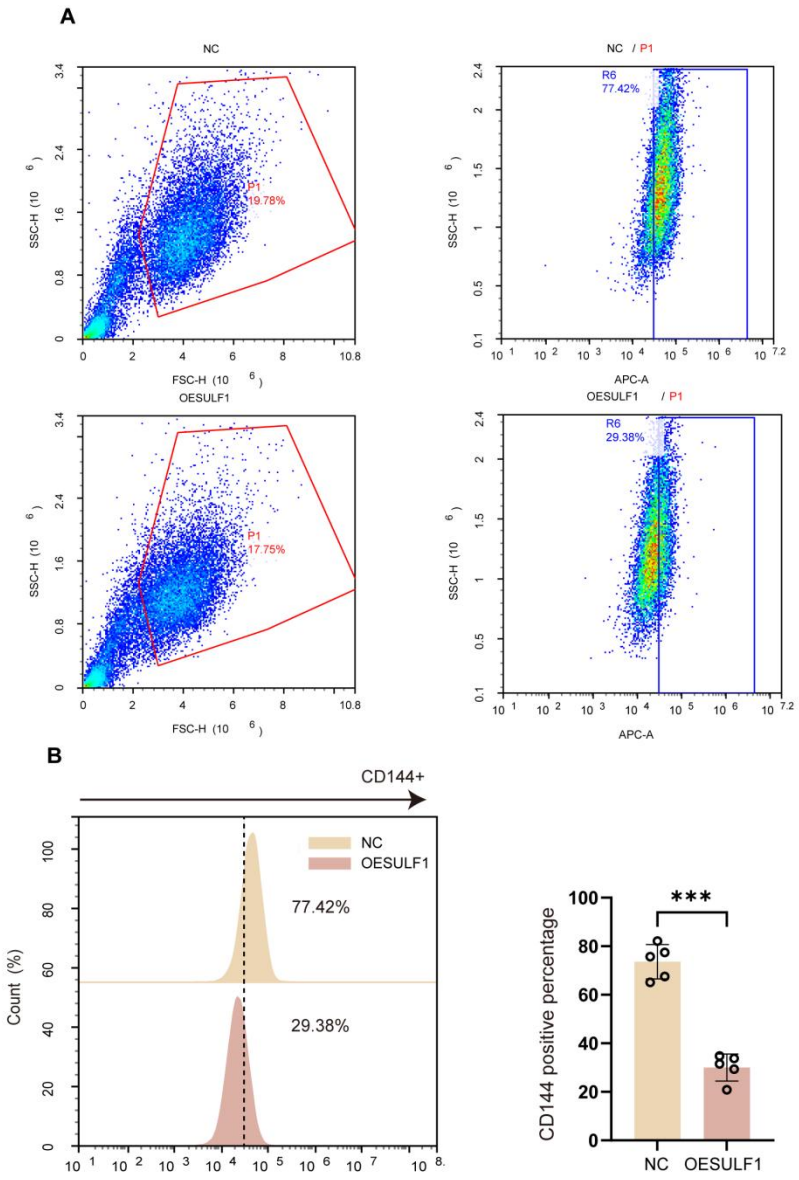


2. Comments: The authors should consider to validate the major finding using FACS.

Response: We thank the reviewer for the suggestion and validated our key finding by flow cytometry. HUVECs overexpressing SULF1 (OESULF1) showed reduced CD144[1] signal relative to empty-vector controls (NC). For cytometry, we gated live singlets and defined the $CD144^{+}$ gate using an FMO control; single-stain controls were used for compensation and an isotype control confirmed specificity (Response Figure 8A). Across n=5 independent samples per group, the percentage of CD144-positive cells (anti-CD144-APC, APC-FcA98071; Proteintech) was markedly lower in OESULF1 than NC (mean±SEM: 30.06% vs 73.60%; Response Figure 8B). An unpaired Welch's

t-test showed a marked reduction in the CD144-positive percentage in OESULF1 versus NC (p<0.001; 95% CI), indicating that SULF1 overexpression reduced endothelial CD144 surface signal. Response Figure 7B can be found in Extended Data Fig. 8A. We have updated the Methods and Results and highlighted the changes in red.

Response Figure 8

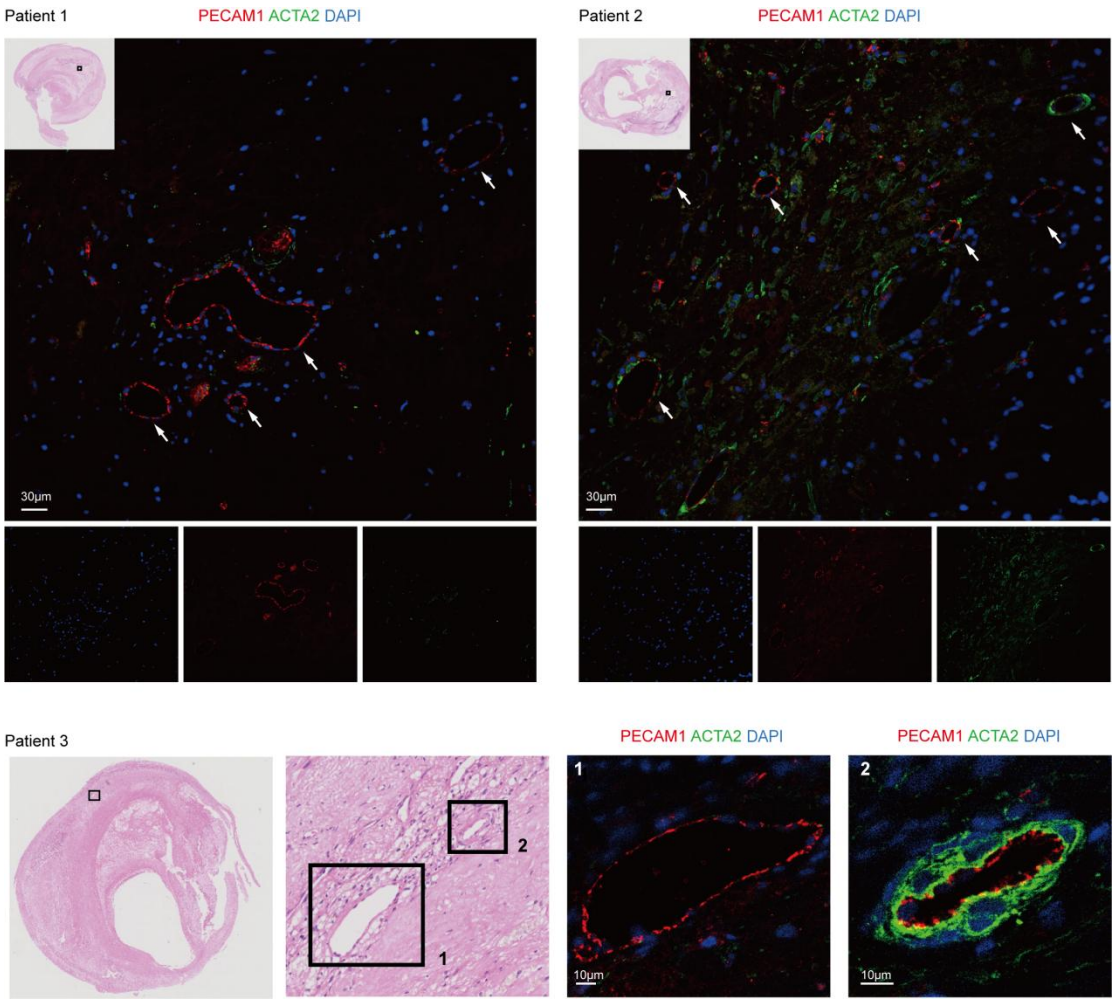


3. Comments: Figure 1 is only supportive, the authors may consider

integrating it into another figure e.g. Fig.2.

Response: We appreciate the suggestion. In the revision, we reconstructed Figure 1 to provide essential biological context: using immunofluorescence staining of atherosclerotic plaques (CD31 and ACTA2, DAPI), we demonstrate the abundant and spatially organized endothelial cells within lesions. The tissue samples presented in Figure 1 are indeed human carotid artery specimens (TableS2).

Response Figure 9



4. Comments: Several key methods are missing details for reproducibility, including the calculation of the R-T/S and R-M/E scores, specific parameters used in Monocle3 and in hdWGCNA. A more transparent and parameterized methods section would enhance reproducibility and allow critical evaluation of

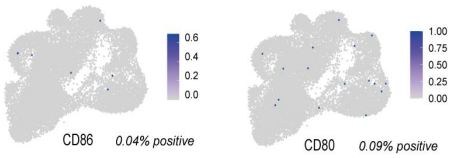
the analyses.

Response: We thank the reviewer for emphasizing reproducibility. To facilitate replication, we provide the R scripts in code availability statement.

5. Comments: Fig. 3G improve conclusion.

Response: We appreciate the reviewer's suggestion. To substantiate the limited expression of costimulatory molecules, we have added the proportion of marker-positive cells to the results and legend. Specifically, we quantified the percentage of cells expressing the designated costimulatory markers (e.g., CD80/CD86) using a predefined positivity threshold (expression > 0 after SCTransform), and report % positive. We replaced the figure with an updated version.

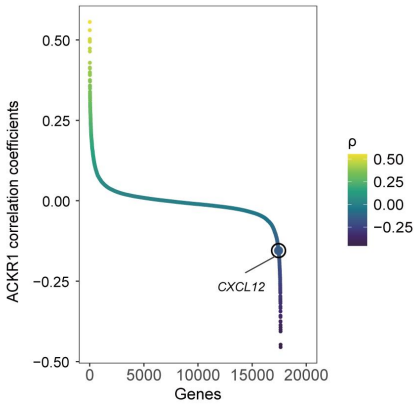
Response Figure 10



6. Comments: Extended Fig. 3D: Ambiguous labeling.

Response: We enhanced the visibility of CXCL12 by enlarging its point marker in Extended Fig. 3D, which clarifies the label without altering the underlying data or conclusions (Response Figure 11). We replaced the figure with an updated version.

Response Figure 11



7. Comments: review reference in line 133.

Response: We have added the relevant references as suggested and updated both the in-text citations and the bibliography (Line 131).

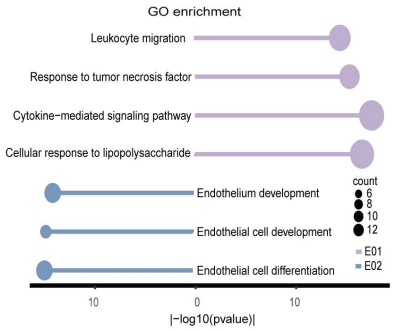
8. Comments: Fig6: review figure legend title.

Response: We appreciate the reviewer's careful correction. We have revised the Figure 6 legend title to "Endothelial-to-mesenchymal transition in atherosclerotic plaques."

9. Comments: Fig. 4D: review x-axis labeling.

Response: We thank the reviewer for the helpful suggestion. In Fig. 4D, the x-axis denotes the magnitude of –log10(P); values are displayed as positive on both sides to present enrichment for the two endothelial subsets in opposite directions, thereby enabling visual comparison. To improve clarity, we have relabeled the axis as "|–log10(P)|". We replaced the figure with an updated version.

Response Figure 12



10. Comments: Extended Fig. 6: review reference in figure legend (B/C).

Response: We thank the reviewer for the careful check. We have corrected the figure legend and updated the references accordingly. In particular, panel C is now explicitly described as a Spearman correlation analysis. The corresponding citations have been revised to match the sources cited in the main text and Methods.

References

1 Harris, E. S. & Nelson, W. J. VE-cadherin: at the front, center, and sides of endothelial cell organization and function. *Curr Opin Cell Biol* **22**, 651-658 (2010). https://doi.org/10.1016/j.ceb.2010.07.006

Jinan, January 7, 2026

Dear Reviewers,

We sincerely thank you for your careful evaluation of our revised manuscript and for your constructive comments. Below we provide a point-by-point response.

On behalf of the corresponding authors,

Donghai Wang, MD, PhD

Professor & Team Leader, Department of Neurosurgery Qilu Hospital, Cheeloo College of Medicine and Institute of Brain and Brain-Inspired Science, Shandong University, Jinan, China

Anton Gisterå, MD, PhD

Assistant Professor & Team Leader, Atherosclerosis Immunology Laboratory

Department of Medicine, Solna, Karolinska Institutet, Sweden

Response to Reviewers' comments:

Re: Reviewer #1

Thank you very much for your careful re-evaluation of our revised manuscript and for acknowledging the improvements made in response to your previous comments.

Comments: Some conceptual issues remain in the way results are introduced. For example, the phrase "Following quality control (Methods)," should be removed, as it is redundant. The method can be briefly mentioned briefly, since it is already described in the Methods section.

Response: We thank the reviewer for this helpful suggestion. We agree that "Following quality control (Methods)," is redundant. We have removed this phrase and revised.

Comments: The H&E section in the Methods still requires additional detail. The source of the samples and information on processing are missing and should be added.

Response: We thank the reviewer for this suggestion. We have revised the H&E Methods to include the sample source and detailed processing steps (Lines 412-424).

Re: Reviewer #2

Thank you for re-assessing our revised manuscript. We appreciate your constructive feedback and your recognition that the main concerns have been resolved.

Comments: Why are the legends of extended Data Figure 2 and Extended Data Figure 3 the same?

Response: We thank the reviewer for noting this. We have corrected the legend of Extended Data Fig. 3 to accurately reflect its content (benchmarking

integration strategies and annotating endothelial subclusters) and to be distinct from Extended Data Fig. 2 (Lines 979-980).

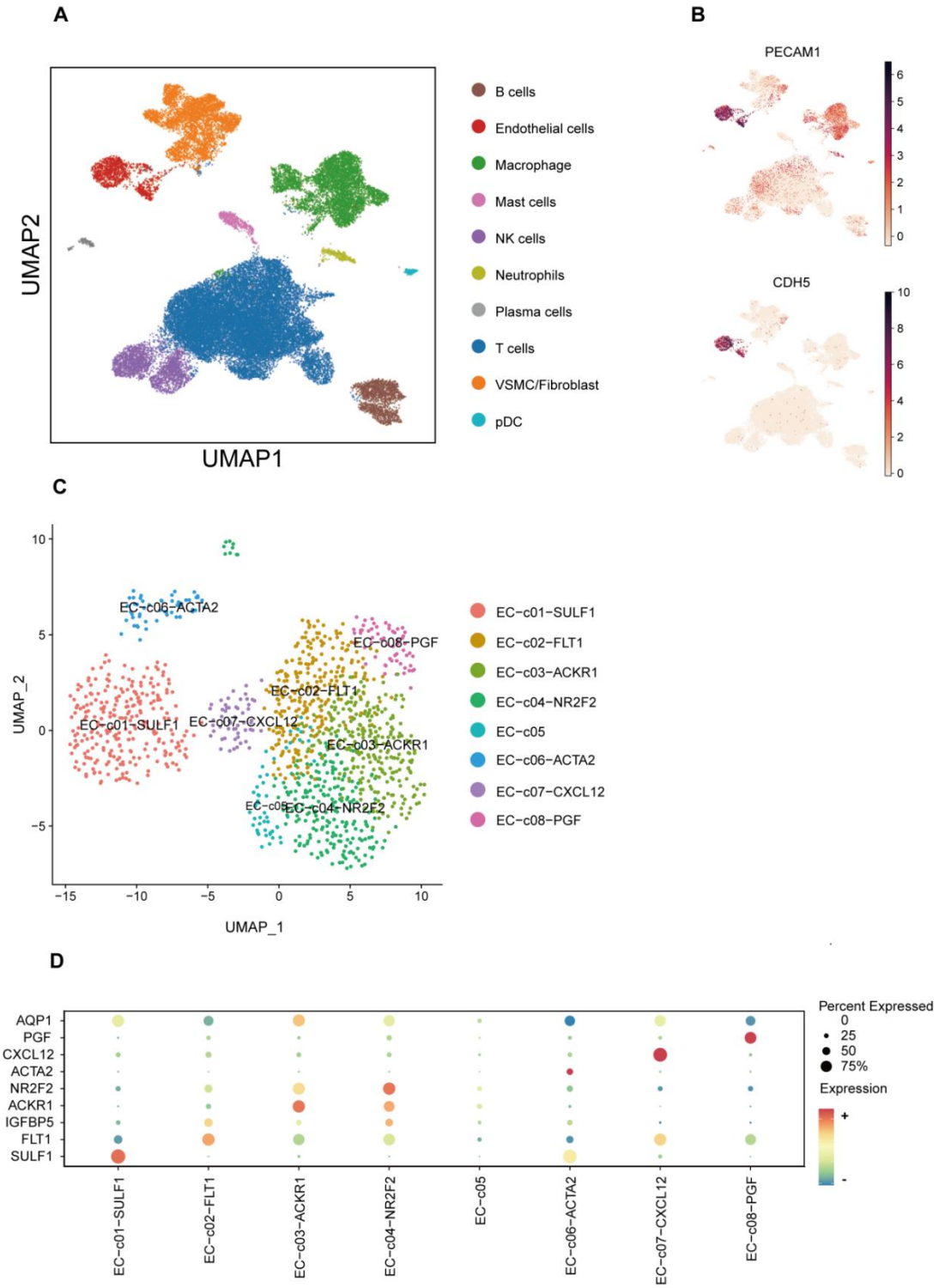
Re: Reviewer #3

We thank the reviewer for the time and effort devoted to evaluating our revision. We take all comments very seriously and regret that our previous responses did not sufficiently address the reviewer's concerns.

Comments: The authors should consider to validate the major finding using FACS.

Response: We initially planned to validate our findings by flow cytometry on endothelial cells isolated from fresh carotid atherosclerotic plaques collected after carotid endarterectomy (CEA). In practice, it was difficult to obtain enough fresh specimens within a consistent time window, and each plaque yielded too few endothelial cells for reliable downstream experiments.We also considered mouse models, but commonly used atherosclerosis strains (e.g., *ApoE*$^{-/-}$ and *LDLR*$^{-/-}$) typically show limited intraplaque neovascularization, making them less suitable for validating vasa vasorum-related endothelial populations[1,2]. To address these limitations, we collected five additional human carotid plaque samples over an extended period and performed scRNA-seq, which reproduced key subpopulations consistent with our main findings. We first performed an overall single-cell analysis of the five patients and extracted endothelial cells based on PECAM1 and CDH5 expression for further subclustering (Response Fig. 1A,B). Using the gene sets we defined previously, these genes showed distinct expression patterns across the different endothelial subclusters (Response Fig. 1C,D). ACKR1 and NR2F2 showed distinct expression distributions, and we could also identify endothelial populations with high expression of FTH1 and PGF, as well as SULF1-high endothelial cells (Response Fig. 1C,D).

Response Fig. 1



Reference

1 Parma, L., Baganha, F., Quax, P. H. A. & de Vries, M. R. Plaque angiogenesis and intraplaque hemorrhage in atherosclerosis. *Eur J Pharmacol* **816**, 107-115 (2017). https://doi.org/10.1016/j.ejphar.2017.04.028

2 Moulton, K. S. *et al.* Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation* **99**, 1726-1732 (1999). https://doi.org/10.1161/01.cir.99.13.1726

Response to Reviewers' comments:

Re: Reviewer #1

The study from Wu and colleagues presents a detailed single-cell transcriptomic atlas of endothelial cells in human atherosclerotic plaques, integrating data from multiple vascular beds. The manuscript offers an overview of the complexity and heterogeneity of endothelial cell populations and their roles in atherosclerosis progression. However, there are several aspects that require revision to enhance clarity and coherence and that would strongly improve the current submission.

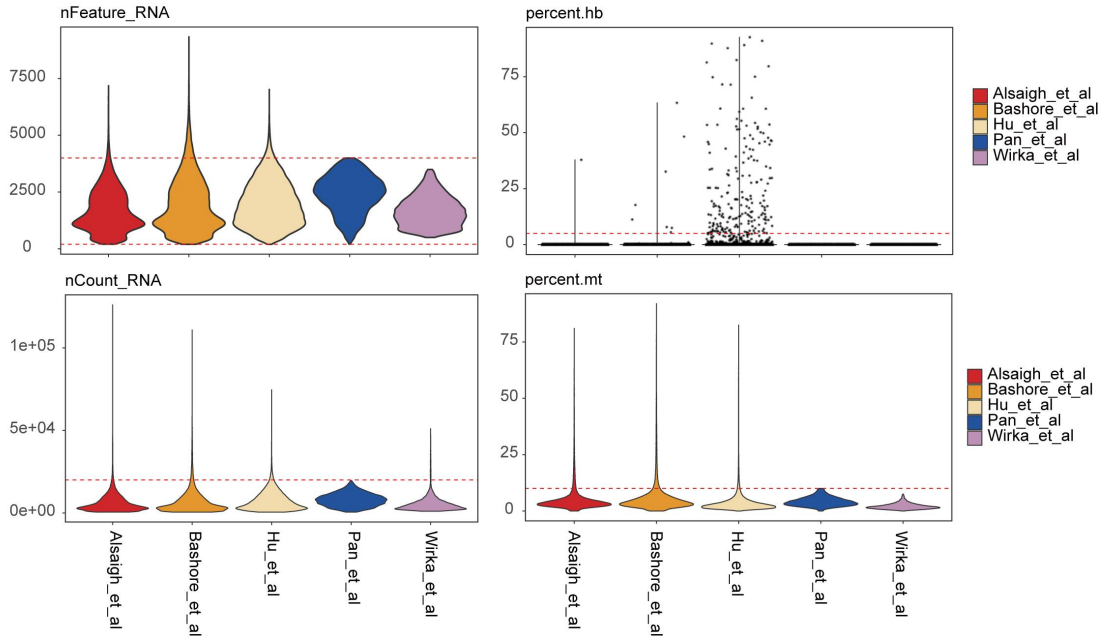
Response: We sincerely thank the Reviewer for the constructive comments and for recognizing the value of our endothelial cell atlas in human atherosclerotic plaques. We fully agree with the Reviewer's suggestions and have carefully revised the manuscript to enhance clarity and coherence.

1. Comments： The rationale behind the study could benefit from greater clarity. The authors start describing in the context of plaque stability. Further, in the scRNA-seq analysis, they perform an analysis of multiple publicly available datasets that are not specifically related to intra plaque hemorrage or plaque instability.As an example, the dataset from Alsaigh et al. pertains to scRNA-seq analysis comparing atherosclerotic plaques with healthy tissue. In contrast, the data from Quertermous were generated from scRNA-seq of transplanted human hearts. Although some limitations are acknowledged in the Discussion, the rationale for selecting these specific datasets should be clearly articulated. Moreover, the selection criteria for these datasets should be justified, and incorporating datasets more directly linked to plaque instability would strengthen the study.

Response： In our initial study design, we intended to investigate the potential role of the vasa vasorum by focusing on plaque stability. However, given the functional heterogeneity and complexity of the vasa vasorum, their widespread

expansion and proliferation within atherosclerotic plaques, and the current limited understanding regarding the heterogeneity among endothelial cell clusters, we chose not to emphasize the distinction between stable and unstable plaques. Instead, our primary objective was to broadly integrate multiple publicly available vascular single-cell RNA sequencing datasets related to atherosclerosis, aiming to assess the functional heterogeneity of endothelial cell clusters. The selected datasets, including those from Alsaigh et al. (comparing atherosclerotic plaques with adjacent tissues) and the Quertermous group (vascular tissues from transplanted human hearts), met the following selection criteria: (1) clear tissue origins relevant to atherosclerosis; (2) well-defined cell types and high data quality (Response Figure 1) enabling accurate identification of vasa vasorum-related endothelial subpopulations and (3) public availability. These datasets are also widely used for integrating single-cell atherosclerosis data[1-3]. This has been addressed in lines 333 – 337. Although these datasets do not explicitly distinguish plaque stability states, their broad coverage of vascular cell heterogeneity within the atherosclerotic microenvironment offers valuable support for exploring the potential functional diversity of the vasa vasorum. We agree with the reviewer's suggestion that future studies should incorporate datasets directly related to plaque instability to further investigate the role of the vasa vasorum under specific pathological conditions. In the present study, our dataset selection strategy was intended to explore the functional diversity of vasa vasorum endothelial cells in the context of atherosclerosis.

Response Figure 1

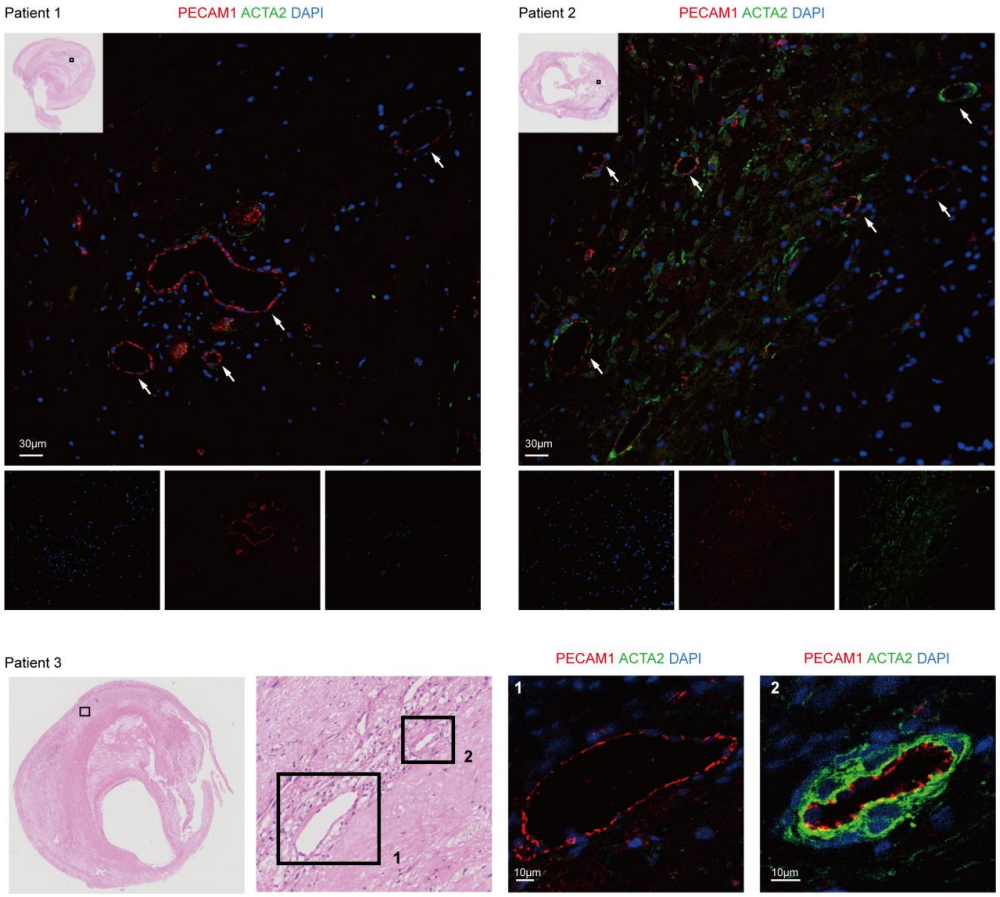


2. Comments： Figure 1 presents an H&E staining of what appear to be human tissue samples, possibly carotid arteries. The authors should clarify this in the Methods section by specifying the exact source of the human samples, including details on ethical approval. Additionally, the staining procedure should be described in the Methods, along with clinical information that informed the classification of plaques as stable or unstable, since the expansion and proliferation of the vasa vasorum can occur, especially in unstable plaques. Moreover, given that vasa vasorum are challenging to visualize and characterize with H&E staining alone, the manuscript would benefit from additional immunostaining to specifically identify vasa vasorum structures: at a minimum, including markers for endothelial cells and vascular smooth muscle cells. Incorporating other markers associated with plaque instability would also strengthen the study and enhance the clarity and impact of the findings.

Response： Thanks very much for your insightful comments and suggestions. We have clarified in the Methods section that the tissue samples presented in

Figure 1 are indeed human carotid artery specimens. We have now specified the precise source of these samples, included details regarding ethical approval, and described the H&E staining procedure in greater detail. This has been addressed in lines 412–420. Due to the difficulty in directly comparing endothelial cell heterogeneity within stable and unstable plaques using integrated single-cell data, we no longer emphasized the distinction between stable and unstable plaques. To address your recommendation about the limitations of H&E staining for visualizing vasa vasorum, we agree that additional immunostaining would enhance clarity and rigor. Accordingly, we conducted immunostaining using endothelial (PECAM1) and vascular smooth muscle (ACTA2) markers and present these data as a new Figure 1 in the revised manuscript (Response Figure 2). The Methods, Results, and figure legend have been updated accordingly, and all revisions were highlighted in red for ease of review.

Response Figure 2



3. Comments： In Figures 2A, C, and D, the analyses appear to reflect the differing cellular composition of the samples, across the various datasets. The manuscript would benefit from a clearer discussion and explicit mention of these differences and their potential impact on the results.

Response： As indicated by the UMAP clustering (Fig. 2A, 2C) and the absolute cell counts per dataset (Fig. 2D), our integrated analysis reflected cell-type composition between datasets. We applied a batch integration approach to mitigate technical batch. However, this procedure preserved differences in cell-type proportions across samples. We focused more on the commonalities in cellular composition across different cohorts, as the major cell clusters could be observed across all datasets. To reduce potential bias, we analyzed multiple cohorts to study endothelial cell function using single-cell transcriptomics. We acknowledged that this aspect represented a challenge inherent to single-cell data integration and that further studies are needed to

clarify its impact on downstream analyses[1,4].

4. Comments: In Figure 3, the authors describe a weighted gene co-expression network analysis aimed at identifying co-expression networks across cellular and spatial hierarchies. However, the concept of a "Module" is not clearly defined in the Methods. It would be helpful for the authors to clarify what is meant by a Module in this context, including the criteria and parameters used in the analysis.

Response： In the methods sections “Computing co-expression networks” and “Computing module eigengenes” of Morabito et al., the authors comprehensively describe the procedures used for module construction and gene identification. Briefly, hdWGCNA aggregates similar cells into metacells to mitigate data sparsity, computes a signed gene-gene adjacency matrix, and subsequently constructs a topological overlap matrix (TOM). Modules are then identified using hierarchical clustering with the Dynamic Tree Cut algorithm and summarized by module eigengenes (MEs) derived via singular value decomposition (SVD). In our implementation of the hdWGCNA workflow, we respectfully adopted customized parameter settings for several key analytical steps, as detailed explicitly in the Methods section. Following preparation of the Seurat object for WGCNA, metacells were generated with the following parameters: k = 10, assay = 'SCT', max_shared = 5, min_cells = 300, reduction = 'umap', and slot = 'data'. Additionally, as illustrated in Extended Data Figures 4A and 4B, we carefully evaluated the selection of the soft-thresholding power and hierarchical clustering approach. The corresponding analysis code to reproduce steps of the hdWGCNA workflow is provided (see Code availability statement).

5. Comments: Figure 4C present an immunofluorescence staining to highlight ACKR1 and NR2F2 expression. No additional information are given on the sample’s origin or tissue composition. This should be included in the Methods and properly described in the specific result paragraph as well as in

the figure legend.

Response: Thank you for the Reviewer's suggestion. We have clarified the patient cohort as adults (≥18 years) who underwent carotid endarterectomy (CEA) for carotid artery stenosis or occlusion due to atherosclerosis between December 2020 and January 2023. We have updated the descriptions of patient information in both the Results and Methods sections. Detailed demographics, clinical characteristics, and procedural information are summarized in Table S2.

6. Comments: Figure 5 is intended to illustrate endothelial cell (EC) sprouting and angiogenic activity within the tissue. While the authors mention an angiogenesis score, no details are provided on how this score was calculated or which criteria were used. Including this information would enhance the clarity and rigor of the analysis and provide important context for the subsequent identification of cellular trajectories and genes involved in angiogenesis.

Response: We used the GO:0002040(sprouting angiogenesis) gene set (http://amigo.geneontology.org/amigo/term/GO:0002040) to calculate AUCell scores via the AUCell_run function (https://github.com/aertslab/AUCell). The mean and variance of AUCell scores across different cell types were computed and visualized using bar plots. A higher AUCell score indicates that the corresponding cell type is more likely to be involved in sprouting angiogenesis . Differences were assessed using the Kruskal-Wallis test. We updated the scoring code; details and access were provided in the code availability statement.

7. Comments: In the assessment of EndMT in Figure 6 and in the discussion, the authors could benefit from the work from Markova et al. It describes how VSMC markers (i.e., ACTA2 and MYH11) provide a higher signal-to-noise ratio compared to EC markers when staining the vasa vasorum. This data should

be included in the manuscript and discussed properly in the text.

Response: We incorporated insights from Markova et al. and related studies to refine marker selection and performed dual immunofluorescence staining for PECAM1 and ACTA2 on carotid plaque specimens. The updated data are presented in the new Figure 1 and demonstrate an improved signal-to-noise ratio for the identification of vasa vasorum within atherosclerotic tissue.

8. Comments: The authors should improve the Figure legends by including more detailed information on statistical analyses, such as statistical power and clear references to the specific sample populations represented in each graph.

Response: We sincerely thank the reviewer for this valuable suggestion. We have revised the manuscript to include descriptions of the sample characteristics and ethics approvals. Statistical analyses were performed in R with commonly used packages, as detailed in the Methods. As an example, enrichment analysis was conducted with RunEnrichr (hdWGCNA)[5]. We evaluated AUCell scores using the Kruskal–Wallis test, following the approach of Li et al[6]. We have clarified this in the figure legend and the changes have been highlighted in red.

9. Comments: The Methods section should clearly state the source of the datasets used (e.g., GSE159677 for the dataset from Alsaigh et al.) and describe in detail the procedure used to generate the data.
The authors may consider briefly summarizing key results without generic statements such as “Following stringent quality control (Methods),” to improve clarity and readability.

Response: We thank the reviewer for the suggestion. We have provided more detailed sample information in the Methods and revised the wording to remove generic statements for clarity and readability. Edits were highlighted in red in the revised manuscript.

Response to Reviewers' comments:

Re: Reviewer #2

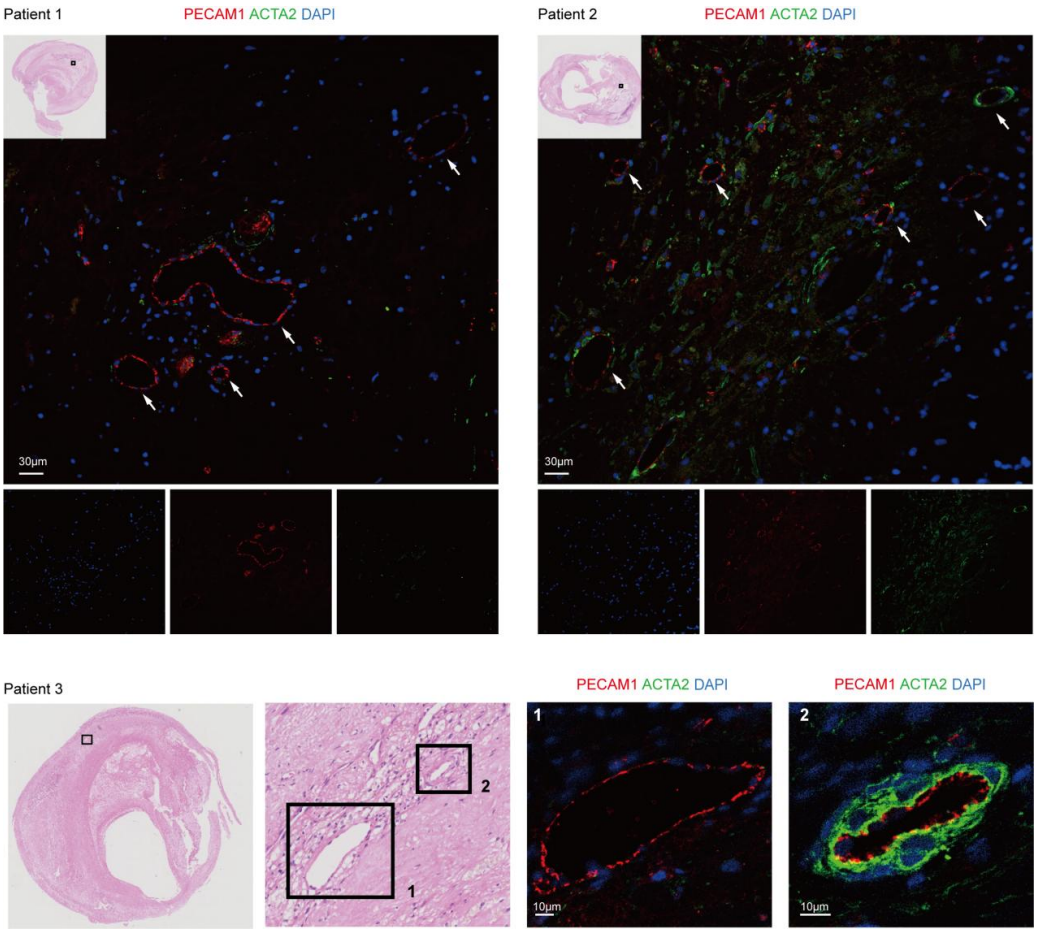
This study uncovered novel endothelial heterogeneity with distinct transcriptional and functional characteristics in the complex landscape of endothelial adaptation in the atherosclerotic microenvironment through integrating multiple scRNA-seq data. The topic is interesting; however, the manuscript has several critical limitations, as outlined below.

Response: We sincerely thank the reviewer for the constructive and insightful comments. We are grateful that the reviewer found our study on endothelial heterogeneity in the atherosclerotic microenvironment to be interesting. We acknowledge the concerns raised regarding the limitations of the manuscript. In the revised version, we have carefully addressed each of the critical points, as detailed below. We appreciate the opportunity to clarify and strengthen our work accordingly.

1. Comments: In Fig1, it is best to demonstrate the presence of endothelial cells within the plaque and on the lumen CD31/vWF immunofluorescence.

Response: We appreciate the reviewer's helpful suggestion to demonstrate the presence of endothelial cells within the plaque and on the lumen using CD31/vWF immunofluorescence. In our study, we conducted immunofluorescence staining using CD31 together with ACTA2 to better delineate endothelial cells in the context of vascular structure[7]. In addition, we have supplemented and clarified the specimen information regarding the carotid atherosclerotic plaques (TableS2). Response figure 3 showed endothelial cells of the vasa vasorum within the carotid atherosclerotic plaques.

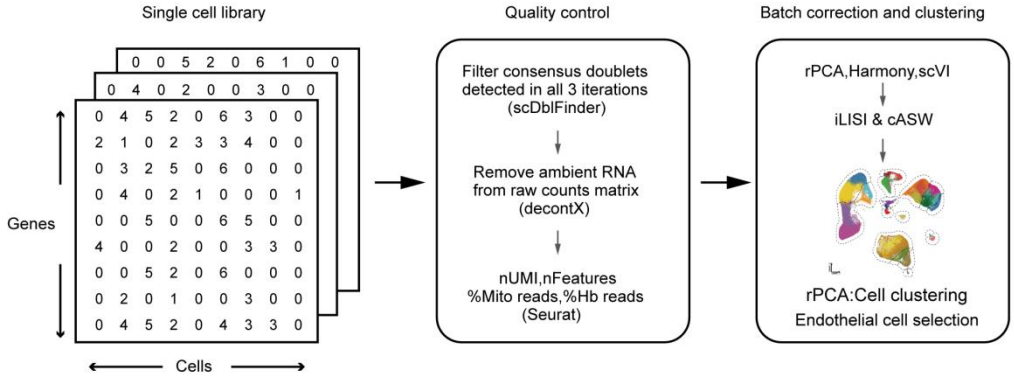
Response Figure 3



2. Comments: The authors conducted a very detailed data processing for the quality control, integration, and batch correction of scRNA-seq data. It is suggested that a corresponding workflow diagram be provided to illustrate this process, which would help readers better understand the procedure.

Response: We sincerely thank the reviewer for the valuable suggestion. To improve the clarity and reproducibility of our scRNA-seq data processing, we have added a schematic workflow diagram outlining the steps of quality control, integration, and batch correction (Response Figure 4). The updated version of the figure is now shown in Extended Data Fig. 1A.

Response Figure 4



3. Comments: Line 108 “LECs were mainly derived from Hu’s cohort 108 (Extended Data Fig. 2G).” I cannot identify LECs among E1-E11, did LECs were excluded before further refine classification? Why LECs were excluded?

Response: Lymphatic endothelial cells (LECs) were not included in the refined subclustering (E1–E11) presented in the downstream analyses. Given the functional differences between lymphatic endothelial cells (LECs) and vascular endothelial cells (VECs), LECs were excluded from downstream analyses in order to reduce noise and better characterize the functional heterogeneity within VECs.

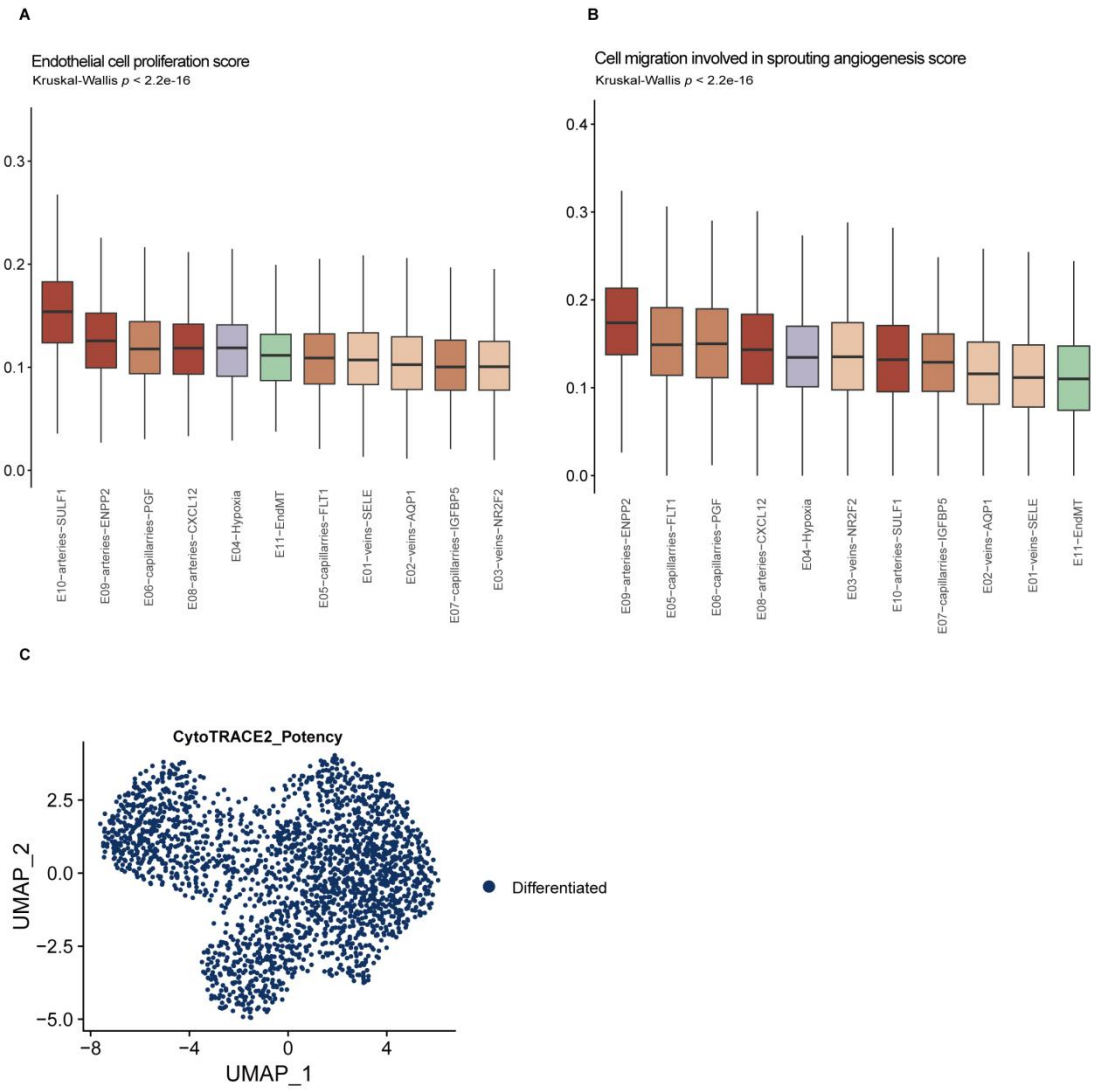
4. Comments: Line 128 “Module1 was predominantly enriched in ArtECs and E01-veins-SELE1”, however, the dotplot in extended Data Fig.4 D revealed that Module 1 was rarely enriched in E01-veins-SELE1?

Response: We thank the reviewer for pointing out this inconsistency. Upon re-evaluation of Module 1 enrichment based on the updated dot plot (Extended Data Fig. 5D), we confirm that Module 1 is predominantly enriched in ArtECs and E11-EndMT, but not in E01-veins-SELE1 as previously stated. We apologize for the oversight and have revised the manuscript accordingly to correct this statement (Line 126). We appreciate the reviewer’s careful observation, which helped us improve the accuracy of our data interpretation.

5. Comments: Angiogenesis is a process related to the proliferation, differentiation and migration of endothelial cells. How do these abilities change in endothelial cell subclusters, especially E05-E07?

Response: To evaluate the proliferative and migratory capacities of endothelial subpopulations, we applied the AUCell_run function (https://github.com/aertslab/AUCell) using two gene sets: GO:0001935 (endothelial cell proliferation; https://amigo.geneontology.org/amigo/term/GO:0001935) and GO:0002042 (cell migration involved in sprouting angiogenesis; https://amigo.geneontology.org/amigo/term/GO:0002042). Due to the limitations of high-throughput data, we were unable to obtain reliable results for differentiation potential. In terms of proliferative capacity, E06 exhibited a relatively high proliferation score (Response Figure 5A). Among the other endothelial subtypes, arterial endothelial cells also showed elevated proliferation activity (Response Figure 5A). Regarding migratory capacity, E05 and E06 scored markedly higher than E07 (Response Figure 5B). Consistent with previous results, E05 and E06 represent active endothelial populations involved in sprouting angiogenesis. We used CytoTRACE2 to assess the differentiation potential of capillary endothelial cells (Response Figure 5C).

Response Figure 5

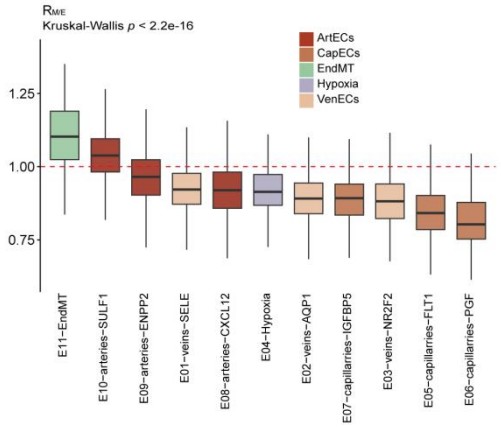


6. Comments: The figure legend of Fig. 6 is incorrect? And y-axis of the Fig 6A should be RM/E? And line 275 “ArtECs and the inflammatory endothelial subcluster E01-veins-SELE were among the top-ranked clusters based on RM/E (Fig. 6A).” Did E01-veins-SELE also had experienced EndMT?

Response: We appreciate the reviewer’s comments. In the revision, we corrected the Fig. 6 legend and updated the y-axis label in Fig. 6A to RM/E. EndMT is a complex and reversible process. To evaluate the cellular state of endothelial cells in high-throughput data, we used the ratio of mesenchymal score to endothelial score as a metric to assess whether endothelial cells underwent mesenchymal transition. We replaced bar charts with box plots (Response Figure 6). A ratio greater than 1 was considered indicative of

EndMT. Based on this analysis, we identified the SULF1+endothelial clusters as a potentially important group undergoing mesenchymal transition. In contrast, the E01-veins-SELE cluster exhibited a lower mesenchymal score relative to the endothelial score, suggesting that these cells retain predominant endothelial characteristics under our evaluation criteria.

Response Figure 6



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7. Comments: In the plaques, the differences in spatial distribution among the arterial (ArtECs), venous (VenECs), and capillary-like (CapECs) subtypes were not identified?

Response: Identifying the spatial distribution of distinct endothelial subtypes (ArtECs, VenECs, and CapECs) within atherosclerotic plaques remains technically challenging. Although single-cell transcriptomic data and immunostaining provide valuable information on cellular identities, they are limited in spatial resolution and may not be sufficient to resolve the precise localization of closely related endothelial subtypes. We agree that spatial transcriptomics holds great potential for addressing this question. In future studies, pending availability of resources and funding, we plan to employ high-resolution spatial transcriptomic approaches to further investigate the spatial distribution of endothelial subtypes within the plaque microenvironment.

Response to Reviewers' comments:

Re: Reviewer #3

The authors present a comprehensive single cell atlas of ECs from human atherosclerotic lesions. They integrated five data sets from previous studies and identified 11 distinct EC subpopulations. Special attention is paid to inflammatory signatures from venule endothelial cells, angiogenesis and endothelial to mesenchymal transition. These processes are detected by using pseudotime analysis, hdWGCNA, gene expression analysis and supported by immunofluorescence staining.The results substantially expand current knowledge, as they systematically characterize novel functional EC subtypes and their disease-relevant dynamics in EndMT, angiogenesis, and inflammation for the first time based on single-cell sequencing.

The manuscript reveals a comprehensive and well integrated single-cell analysis of endothelial heterogeneity in atherosclerosis, with robust bioinformatics and valuable insights into EndMT and angiogenesis. However, it remains purely descriptive and lacks experimental validation (e.g. quantitative evaluation of the immunofluorescence images would further strengthen the findings).Further limitations may arise from the anatomical heterogeneity of sampled arteries/tissues and the sex imbalance.

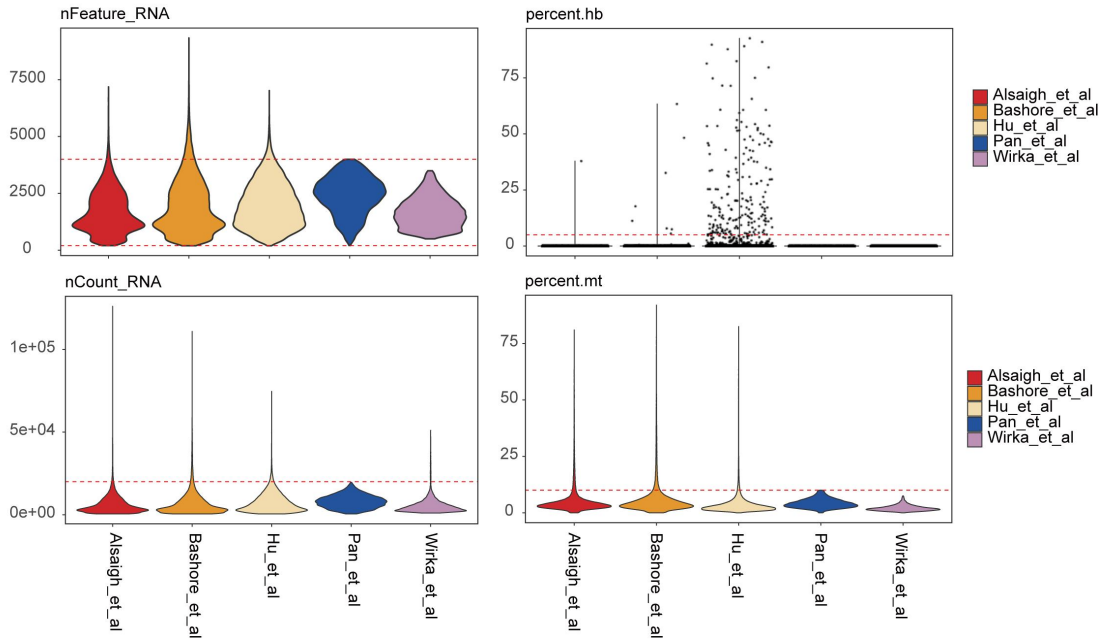
Response: We are grateful to the reviewer for the thoughtful comments, and we are encouraged that our analysis of endothelial heterogeneity was received. We acknowledge the concerns regarding the manuscript's limitations and have carefully revised the text to address each major point, detailed below. We appreciate the opportunity to improve the clarity and rigor of the study.

1. Comments: The authors should consider to include QC comparison of the used data sets.

Response: Thanks for this constructive suggestion. In the revised manuscript, we included a cross-dataset quality-control (QC) comparison performed prior

to integration and downstream analyses (Response Figure 7); using clearly defined filtering thresholds, we retained the vast majority of high-quality cells and excluded a small subset of cells likely influenced by confounders. The updated version of the figure is now shown in Extended Data Fig. 1B

Response Figure 7

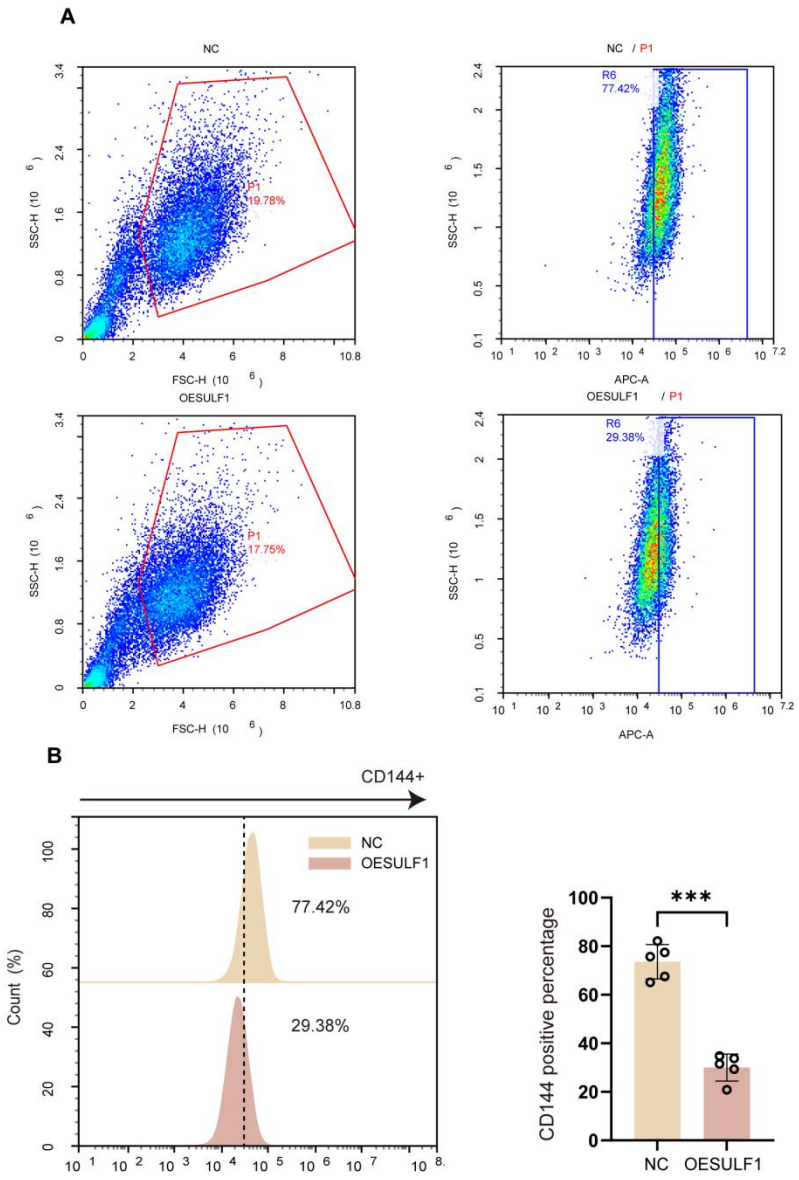


2. Comments: The authors should consider to validate the major finding using FACS.

Response: We thank the reviewer for the suggestion and validated our key finding by flow cytometry. HUVECs overexpressing SULF1 (OESULF1) showed reduced CD144[8] signal relative to empty-vector controls (NC). For cytometry, we gated live singlets and defined the $CD144^{+}$ gate using an FMO control; single-stain controls were used for compensation and an isotype control confirmed specificity (Response Figure 8A). Across n=5 independent samples per group, the percentage of CD144-positive cells (anti-CD144-APC, APC-FcA98071; Proteintech) was markedly lower in OESULF1 than NC (mean±SEM: 30.06% vs 73.60%; Response Figure 8B). An unpaired Welch's

t-test showed a marked reduction in the CD144-positive percentage in OESULF1 versus NC (p<0.001; 95% CI), indicating that SULF1 overexpression reduced endothelial CD144 surface signal. Response Figure 7B can be found in Extended Data Fig. 8A. We have updated the Methods and Results and highlighted the changes in red.

Response Figure 8

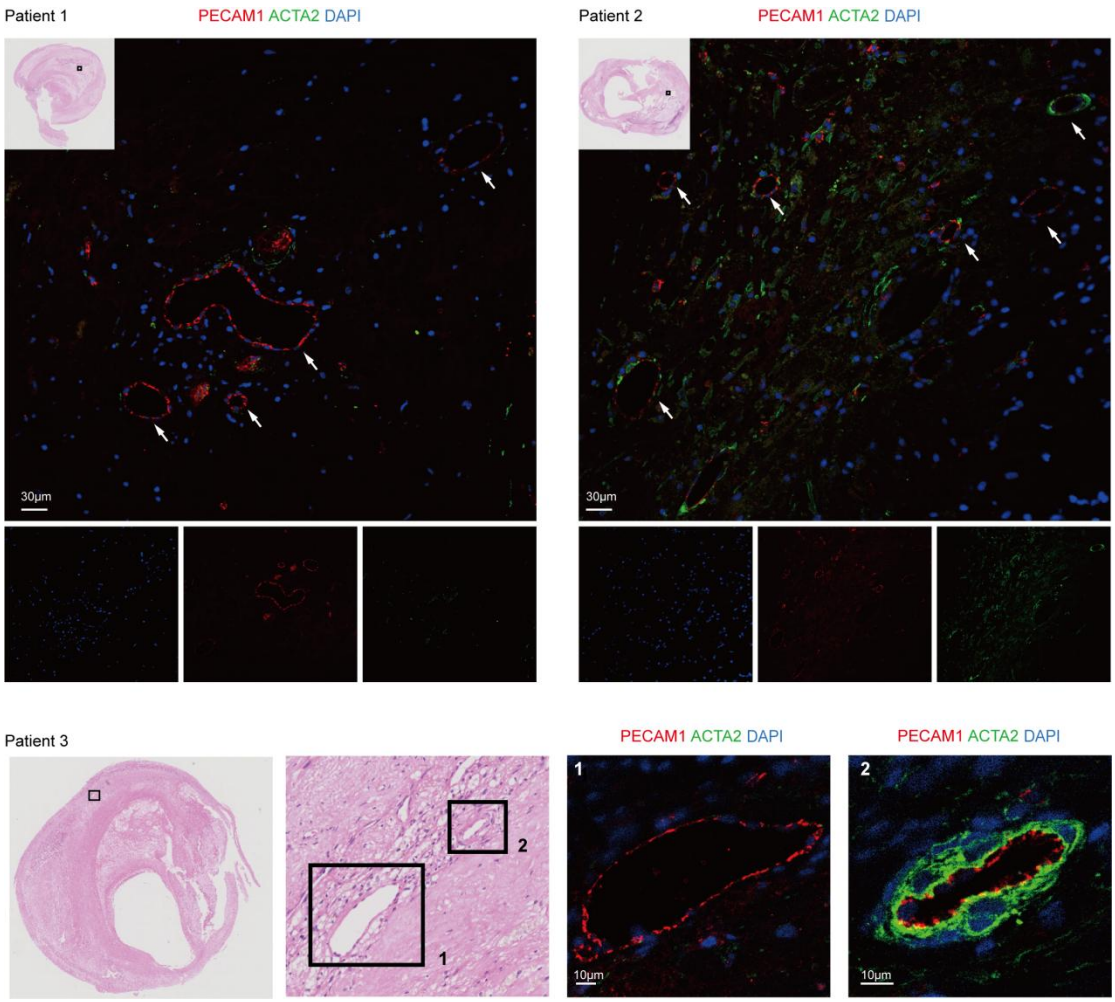


3. Comments: Figure 1 is only supportive, the authors may consider

integrating it into another figure e.g. Fig.2.

Response: We appreciate the suggestion. In the revision, we reconstructed Figure 1 to provide essential biological context: using immunofluorescence staining of atherosclerotic plaques (CD31 and ACTA2, DAPI), we demonstrate the abundant and spatially organized endothelial cells within lesions. The tissue samples presented in Figure 1 are indeed human carotid artery specimens (TableS2).

Response Figure 9



4. Comments: Several key methods are missing details for reproducibility, including the calculation of the R-T/S and R-M/E scores, specific parameters used in Monocle3 and in hdWGCNA. A more transparent and parameterized methods section would enhance reproducibility and allow critical evaluation of

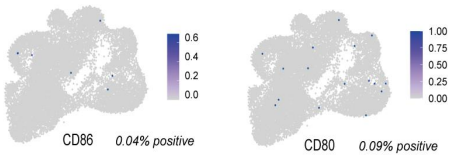
the analyses.

Response: We thank the reviewer for emphasizing reproducibility. To facilitate replication, we provide the R scripts in code availability statement.

5. Comments: Fig. 3G improve conclusion.

Response: We appreciate the reviewer's suggestion. To substantiate the limited expression of costimulatory molecules, we have added the proportion of marker-positive cells to the results and legend. Specifically, we quantified the percentage of cells expressing the designated costimulatory markers (e.g., CD80/CD86) using a predefined positivity threshold (expression > 0 after SCTransform), and report % positive. We replaced the figure with an updated version.

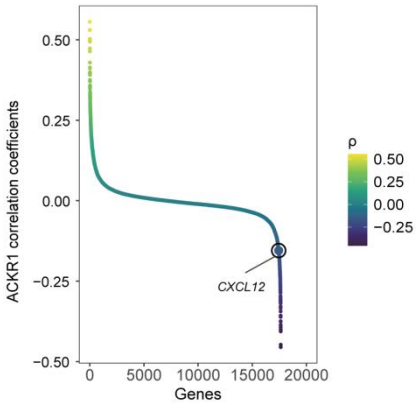
Response Figure 10



6. Comments: Extended Fig. 3D: Ambiguous labeling.

Response: We enhanced the visibility of CXCL12 by enlarging its point marker in Extended Fig. 3D, which clarifies the label without altering the underlying data or conclusions (Response Figure 11). We replaced the figure with an updated version.

Response Figure 11



7. Comments: review reference in line 133.

Response: We have added the relevant references as suggested and updated both the in-text citations and the bibliography (Line 131).

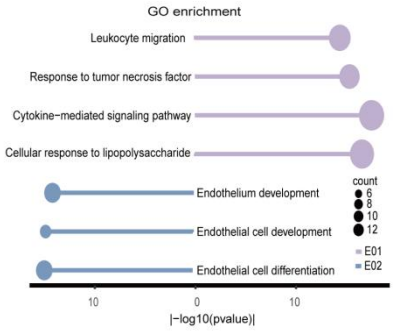
8. Comments: Fig6: review figure legend title.

Response: We appreciate the reviewer's careful correction. We have revised the Figure 6 legend title to "Endothelial-to-mesenchymal transition in atherosclerotic plaques."

9. Comments: Fig. 4D: review x-axis labeling.

Response: We thank the reviewer for the helpful suggestion. In Fig. 4D, the x-axis denotes the magnitude of –log10(P); values are displayed as positive on both sides to present enrichment for the two endothelial subsets in opposite directions, thereby enabling visual comparison. To improve clarity, we have relabeled the axis as "|–log10(P)|". We replaced the figure with an updated version.

Response Figure 12



10. Comments: Extended Fig. 6: review reference in figure legend (B/C).

Response: We thank the reviewer for the careful check. We have corrected the figure legend and updated the references accordingly. In particular, panel C is now explicitly described as a Spearman correlation analysis. The corresponding citations have been revised to match the sources cited in the main text and Methods.

References

1 Mosquera, J. V. *et al.* Integrative single-cell meta-analysis reveals disease-relevant vascular cell states and markers in human atherosclerosis. *Cell Rep* **42**, 113380 (2023). https://doi.org/10.1016/j.celrep.2023.113380

2 Traeuble, K. *et al.* Integrated single-cell atlas of human atherosclerotic plaques. *Nat Commun* **16**, 8255 (2025). https://doi.org/10.1038/s41467-025-63202-x

3 Bleckwehl, T. *et al.* Encompassing view of spatial and single-cell RNA sequencing renews the role of the microvasculature in human atherosclerosis. *Nat Cardiovasc Res* **4**, 26-44 (2025). https://doi.org/10.1038/s44161-024-00582-1

4 Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. *Nat Biotechnol* **36**, 411-420 (2018). https://doi.org/10.1038/nbt.4096

5 Morabito, S., Reese, F., Rahimzadeh, N., Miyoshi, E. & Swarup, V. hdWGCNA identifies co-expression networks in high-dimensional transcriptomics data. *Cell Rep Methods* **3**, 100498 (2023). https://doi.org/10.1016/j.crmeth.2023.100498

6 Li, J. *et al.* Pan-cancer integrative analyses dissect the remodeling of endothelial cells in human cancers. *Natl Sci Rev* **11**, nwae231 (2024). https://doi.org/10.1093/nsr/nwae231

7 Markova, V. *et al.* Endothelial Cell Markers Are Inferior to Vascular Smooth Muscle Cells Markers in Staining Vasa Vasorum and Are Non-Specific for Distinct Endothelial Cell Lineages in Clinical Samples. *Int J Mol Sci* **24** (2023). https://doi.org/10.3390/ijms24031959

8 Harris, E. S. & Nelson, W. J. VE-cadherin: at the front, center, and sides of endothelial cell organization and function. *Curr Opin Cell Biol* **22**, 651-658 (2010). https://doi.org/10.1016/j.ceb.2010.07.006

Response to Reviewers' comments:

Re: Reviewer #1

Thank you very much for your careful re-evaluation of our revised manuscript and for acknowledging the improvements made in response to your previous comments.

Comments: Some conceptual issues remain in the way results are introduced. For example, the phrase "Following quality control (Methods)," should be removed, as it is redundant. The method can be briefly mentioned briefly, since it is already described in the Methods section.

Response: We thank the reviewer for this helpful suggestion. We agree that "Following quality control (Methods)," is redundant. We have removed this phrase and revised.

Comments: The H&E section in the Methods still requires additional detail. The source of the samples and information on processing are missing and should be added.

Response: We thank the reviewer for this suggestion. We have revised the H&E Methods to include the sample source and detailed processing steps (Lines 412-424).

Re: Reviewer #2

Thank you for re-assessing our revised manuscript. We appreciate your constructive feedback and your recognition that the main concerns have been resolved.

Comments: Why are the legends of extended Data Figure 2 and Extended Data Figure 3 the same?

Response: We thank the reviewer for noting this. We have corrected the legend of Extended Data Fig. 3 to accurately reflect its content (benchmarking

integration strategies and annotating endothelial subclusters) and to be distinct from Extended Data Fig. 2 (Lines 979-980).

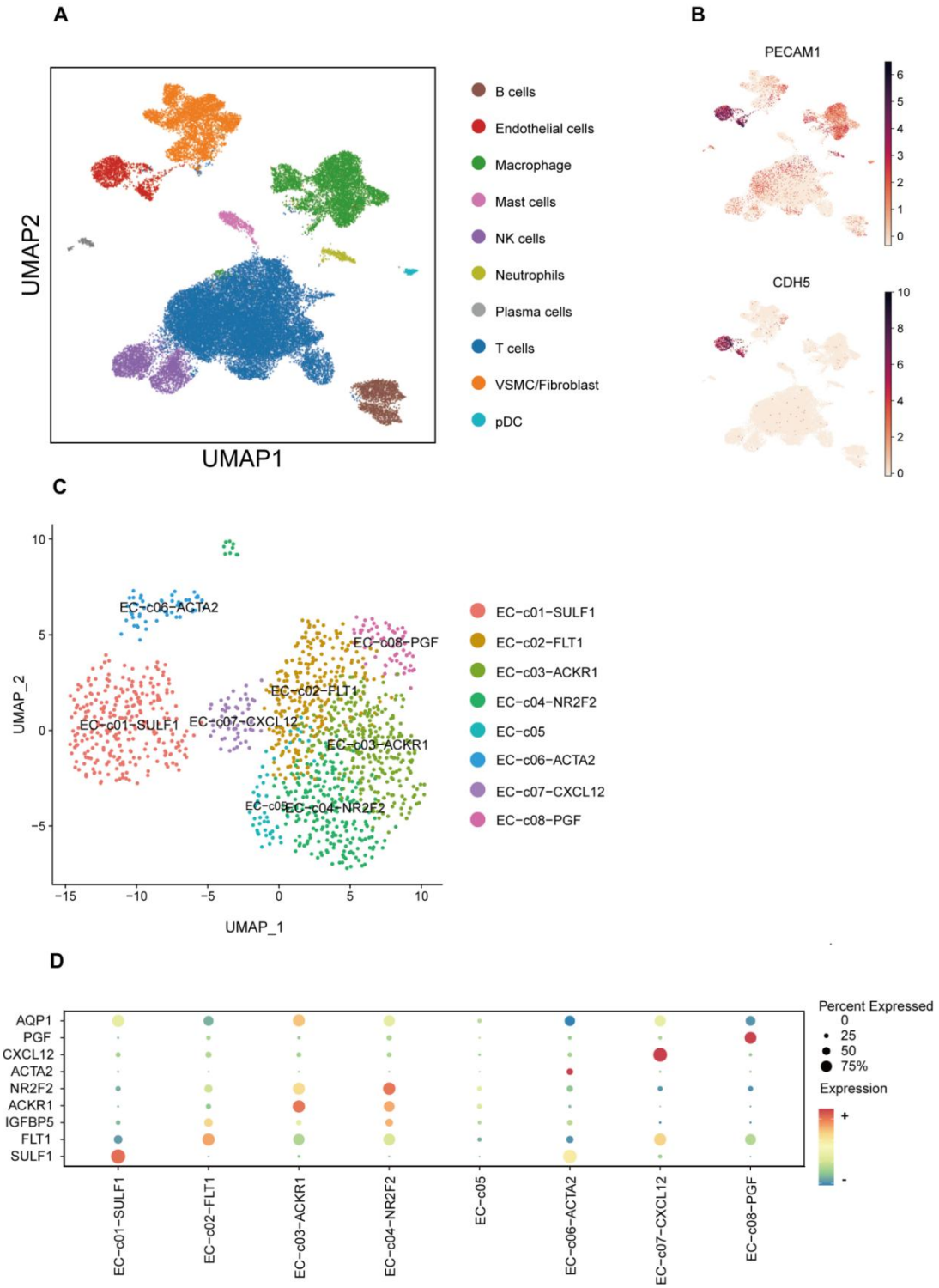
Re: Reviewer #3

We thank the reviewer for the time and effort devoted to evaluating our revision. We take all comments very seriously and regret that our previous responses did not sufficiently address the reviewer's concerns.

Comments: The authors should consider to validate the major finding using FACS.

Response: We initially planned to validate our findings by flow cytometry on endothelial cells isolated from fresh carotid atherosclerotic plaques collected after carotid endarterectomy (CEA). In practice, it was difficult to obtain enough fresh specimens within a consistent time window, and each plaque yielded too few endothelial cells for reliable downstream experiments.We also considered mouse models, but commonly used atherosclerosis strains (e.g., *ApoE*$^{-/-}$ and *LDLR*$^{-/-}$) typically show limited intraplaque neovascularization, making them less suitable for validating vasa vasorum-related endothelial populations[1,2]. To address these limitations, we collected five additional human carotid plaque samples over an extended period and performed scRNA-seq, which reproduced key subpopulations consistent with our main findings. We first performed an overall single-cell analysis of the five patients and extracted endothelial cells based on PECAM1 and CDH5 expression for further subclustering (Response Fig. 1A,B). Using the gene sets we defined previously, these genes showed distinct expression patterns across the different endothelial subclusters (Response Fig. 1C,D). ACKR1 and NR2F2 showed distinct expression distributions, and we could also identify endothelial populations with high expression of FTH1 and PGF, as well as SULF1-high endothelial cells (Response Fig. 1C,D).

Response Fig. 1



Reference

1 Parma, L., Baganha, F., Quax, P. H. A. & de Vries, M. R. Plaque angiogenesis and intraplaque hemorrhage in atherosclerosis. *Eur J Pharmacol* **816**, 107-115 (2017). https://doi.org/10.1016/j.ejphar.2017.04.028

2 Moulton, K. S. *et al.* Angiogenesis inhibitors endostatin or TNP-470 reduce intimal neovascularization and plaque growth in apolipoprotein E-deficient mice. *Circulation* **99**, 1726-1732 (1999). https://doi.org/10.1161/01.cir.99.13.1726