nature portfolio

Peer Review File

Integrated Multi-Omics Profiling Reveals Neutrophil Extracellular Traps Potentiate Aortic Dissection Progression

Corresponding Author: Professor Lixin wang

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, the authors employed Multi-Omics analysis to examine the differences in aorta and plasma between patients with aorta dissection (AD) and healthy individuals. They provided clinically applicable biomarkers (NETosis-CitH3) to predict the prognosis after thoracic endovascular aortic repair (TEVAR) for AD. Simultaneously, they validated the inhibitory effect of NETosis on the progression of aortic dissection in a mouse AD model, suggesting a potential targeted therapeutic significance. This research demonstrates innovativeness by identifying clinically promising biomarkers that could be utilized. To a certain extent, this study holds clinical translational value. While the quantity of the manuscript is quite impressive, there are multiple factors the limit my enthusiasm for this manuscript in its present form. These will be outlined in detailed below.

Major concerns:
1. Compared to the previously reported single-cell sequencing results on human AD tissues, is there any difference in this study. A comparative discussion is needed in the DISCUSSION section.
(Circulation, 2023. DOI: 10.1161/CIRCULATIONAHA.123.063332.)
(Circulation, 2020. DOI: 10.1161/CIRCULATIONAHA.120.046528.)
2. In figure 5E, could authors provide Kaplan-Meier curves of patients with combining high CitH3 and high IL-1β, compared to patients with other conditions?
3. In figure 6D, for the BAPN-induced aortic dissection model, mice should have a statistical graph of the incidence of aortic dissection and elastic fiber fracture grades to reflect the severity of the dissection.
Elastic fiber fracture grades: I: 0~25%; II: 26%~50%; III: 51%~75%; IV: 76%~100%
4. In figure 6D, the aorta of AD and DNase I groups seems to be relatively normal from EVG. In theory, the false lumen should be surrounded by elastic fibers, yet the EVG staining displayed a relatively intact aorta and the elastic fibers of outer side of “false lumen” called by authors was not observed. Is there a more suitable representative diagram?
(Refer to Circulation. 2022. DOI: 10.1161/CIRCULATIONAHA.121.056640, Figure 3)
5. In this study, elevated NETosis was observed in patients with AD, and proved to be predictive of the post-TEVAR outcomes. The BAPN animal model reflects the incidence and development of AD. Therefore, in the presentation of results, mouse model should correspond to the findings in patients with AD. This section (mouse model) can be placed following the result "Elevated NETosis occurs in aortic lesions and peripheral blood from patients with AD." Additionally, the prediction of TEVAR outcomes should be discussed in the last paragraph of the results section.
6. The patient tissue used in the study should provide basic information about the patient and healthy controls (such as age, base height, weight, underlying disease, etc.)
Minor concerns:
1. For page 28 line 776, the content in “()” is missing.
2. For page 5 line 143, 149 and page 8 line 226, supplementary figures need to be marked accurately.
3. For page 5 line 152, cluster 13 does not appear to be macrophage_TCR mentioned behind. Is the cluster 4?
4. In figure 6D, if possible, could you provide a suitable representative diagram of the aorta? Additionally, all aorta diagram for every group should be presented in the supplement figure.
5. The role of NETosis also contributes to autoimmune diseases, the initiation and facilitation of metastasis of malignant tumors. Therefore, under these conditions, does the predictive capacity of biomarker NETosis decrease?
6. Please specify the statistical testing approach for each figure in the corresponding legend, avoiding general descriptions.

Wei Guo
Department of Vascular and Endovascular Surgery, Chinese PLA General Hospital, FuXing Road No.28, Beijing 100089, China. E-mail: guoweiplagh@sina.com

Reviewer #2

(Remarks to the Author)
Key results:

Yufei Zhao et al. have applied a multiomics approach to investigate the cell type composition and gene expression profile in blood and tissue samples of patients with aortic dissection (AD) and have deduced an interplay of macrophages and neutrophils, including the formation of neutrophil extracellular traps (NETs). Furthermore, the biomarker potential of neutrophil- and NET-associated parameters was evaluated in a diagnostic and prognostic setting for adverse events after thoracic endovascular aortic repair (TEVAR). In more detail:

Proteomic analysis from plasma samples of 30 AD patients and 30 matched healthy individuals was based on mass spectrometry and revealed an accumulation of proteins related to protein-DNA complex formation and NET formation in blood of AD patients. Overall, inflammatory mediators of leukocyte chemotaxis and activation were enriched.

Single-cell RNA sequencing (scRNAseq) was performed for cells isolated from 3 resected healthy aortas versus 3 AD samples. Data were compared to a publicly available bulk sequencing set from 7 AD patients and 5 healthy controls to delineate cell populations which were predominantly associated with the AD phenotype. While smooth muscle cells (SMCs) and fibroblasts dominated in aorta samples of healthy individuals, about 50-80% of cells isolated from AD samples were identified as macrophages which were further divided into 5 subsets. Two of these subsets (macrophage_FCGR3A and macrophage_OLR1) constituted the majority of cells associated with the AD phenotype. Among various proinflammatory and immunoregulatory pathways, these macrophage subtypes showed gene expression profiles of neutrophil chemotaxis and activation.

The authors then investigated putative cell-cell communication pathways based on matching ligand-receptor pairs in scRNAseq analysis. While the two macrophage subtypes were found to have the highest potential for interactions with other macrophages, fibroblasts and endothelial cells, they also presented putative cross-talk with neutrophils (based on the expression of CXCL2, -3, and -8 and the pertaining receptors CXCR1 and -2 on neutrophils).

The study then focused on the detection of NETs in blood and tissue samples of AD patients and found gene expression modules (in scRNAseq) associated with extracellular trap formation to be elevated in the 3 AD patients versus 3 healthy controls – not only in neutrophils but also in all 5 macrophage subsets. By immunofluorescence staining of FFPE sections, neutrophil/NET markers were further detected in 4 AD tissues but not in 4 healthy aortas of transplant donors. Additionally, neutrophil and NET parameters (citrullinated histone H3 = citH3, cell-free DNA and neutrophil elastase) were measured by ELISA in the plasma of 187 AD patients and were significantly elevated compared to 58 healthy controls.

Based on this diagnostic biomarker assessment, the authors subsequently explored the prognostic potential of these plasma molecules in 115 AD patients. Among the 31 patients who developed adverse events after endovascular repair, 70% had citH3 levels exceeding the defined cut-off level, and plasma citH3 was found to significantly predict adverse events in ROC analysis with an area under the curve of 0.751 (sensitivity of 60% and specificity of 90% at the defined cut-off). In addition to citH3 also IL-1β was identified as independent predictor for post-TEVAR complications.

To investigate the relevance of NETs in vivo, aorta dissection and concomitant aneurysm formation were induced in male wildtype Black6 mice over a time course of 6 weeks by administration of an inhibitor of lysyl-oxidase (BAPN). Anti-NET therapy by either DNase I or Cl-amidine was given for 2 weeks (in week 2 and 3 of BAPN treatment). While BAPN led to high rates of aortic ruptures (63%), anti-NET therapy reduced animal death to 13-33%. Furthermore, aortic dilatation (aneurysm formation) was significantly decreased by the combined anti-NET treatment. Histological stainings and immunofluorescence imaging were conducted to document aorta degeneration and NET accumulation in the experimental groups.

While this is a well-written, comprehensive study based on multiomics of AD specimen which has resulted in a variety of interesting observations, there is no clear evidence for the importance of the proposed macrophage-NET link in AD patients. The omics data offer cues to potential cellular functions and interactions. The biomarker analyses are clearly of interest, yet need to be put into the context of multivariable analysis with potential confounders. Finally, the data of the conducted mouse model do not resolve the impact of anti-NET therapy on aneurysm formation versus aortic dissection, thus making it difficult to support the authors´ conclusion that NETs promote AD.

Originality and significance to the field/related fields:

What is already known:
• scRNAseq has previously been conducted to characterize the cellular composition and gene expression profile of thoracic AD [1] and has resulted in the identification of macrophage subsets distinct from the results in this study.
• Various blood parameters for diagnosis of acute AD have previously been proposed, among them D-dimer with a sensitivity and specificity of 96% and 70%, respectively (AUC 0.940) as calculated in a recent meta-analysis [2]. The authors themselves have recently developed a systemic immune inflammation index to predict adverse events after TEVAR of AD

[3].
• Anti-NET therapy has been shown to prevent the combined AD+aneurysm development in mouse models similar to the model conducted by the authors. Thus, angiotensin II administration to ApoE k.o. mice results in aortic dissection and thoracic as well as abdominal aortic aneurysms. The formation and progression of aortic disease is reportedly reduced by anti-NET therapy [4, 5].

What this study adds:
• The identification of novel AD-specific macrophage subsets by scRNAseq.
• The detection of NETs (NET associated molecules) in tissue and blood of AD patients.
• The biomarker potential (diagnostic and prognostic after TEVAR) of the plasma NET parameter citH3 for AD patients – although multivariable analysis with potential confounders is missing.

What is not resolved/addressed by this study:
• The impact (and therapeutic potential) of the proposed macrophage-NET link on disease development in AD patients. Of note, the mouse model does not differentiate between aneurysm formation and AD – main readouts are aorta rupture and aortic dilatation.

Conclusions and claims:

1. It is not entirely clear to me how the data shown in Suppl. Figure 1 support the conclusion that neutrophil activation is increased (rather than other components of inflammation) in the blood of AD patients, as indicated by the title of the Figure. Please specify the processes or components recognized in these analyses to conclusively identify neutrophils and their activation.

2. In Figure 3C/D the authors have conducted a direct comparison of gene expression in cell clusters predominantly found in AD aorta samples (i.e. mainly macrophages) with the prevailing cell cluster associated with healthy aortas (i.e. mostly SMCs and ECs). I don´t see the benefit of this analysis, since it is to be expected – as found by the authors – that macrophages have more hallmarks of inflammation, including neutrophil chemotaxis and activation among many other immune pathways, yet a lack of SMC/EC differentiation markers (when compared to SMCs/ECs). So the authors have essentially based the main conclusion of their scRNAseq analyses (“An AD phenotype associated macrophage subpopulation promotes NETosis within the aortic microenvironment.”) on the comparison of macrophages with SMCs/ECs which does, in fact, tell us that macrophages are more potent regulators of neutrophil recruitment and activation than the stromal cells.

3. The authors have attempted to illustrate the presence of CXCL3 expressing macrophages in human AD tissue by immunofluorescence staining, but only show a single cell with co-staining of CD68 and CXCL3 (in – what seems to be the adventitia – of the aortic wall). This illustration is neither suited to document the accumulation of macrophages in the AD sample nor their abundant/frequent expression of CXCL3. A whole-tissue scan (as for H&E stainings and as performed for mouse aorta sections in Figure 6) is required in comparison with the healthy control aorta staining to provide convincing evidence.

4. Similarly, Figure 4B documents the presence of citH3 and MPO staining in an AD tissue sample (while these markers are not detected in the healthy control). To be able to follow the conclusion that NETs are more abundant in AD than in healthy aortas, whole-tissue scans of both, histology slides (H&E or MTC) and the immunofluorescence staining of citH3 and MPO should be provided – indicating the zoom-in regions seen in Figure 4B.

5. In line 275, the authors mention that IL-1β is significantly higher in plasma of AD patients than healthy controls. Yet, the data presented in Figure 4C do not support this conclusion.

6. The mouse study is based on a model of combined AD and aneurysm formation. The authors document the efficacy of anti-NET treatment to reduce aortic rupture/animal death and aorta dilatation as well as wall thickness, but it do not provide sufficient evidence for the impact on aorta dissection. Only 1 histological sample/section is given per treatment group showing AD and thrombus formation in the untreated animal as opposed to no dissection in the Cl-amidine treated mice. The frequency of AD occurrence should be calculated and appropriately illustrated for each experimental group (investigating the entire aorta) and statistically evaluated – which might necessitate higher n numbers per group.

7. Since the authors interpret the effects of anti-NET therapy to “ameliorate dissection progression”, it would be essential to additionally document the rate of dissection and max. aortic diameter dilatation at treatment start (i.e. 1 week after AD induction by BAPN, immediately before therapy initiation). Without this comparison, it is difficult to deduce the extent of reduction/blockade of disease progression.

Data and methodology:

8. Proteomic analysis was conducted for plasma samples of AD patients and healthy controls. Please give more information as to when the blood samples were retrieved from AD patients with respect to the occurrence of acute AD and treatment interventions. Please also provide the basic demographics of the two collectives (sex and age distribution, co-morbidities, medication, etc.) to document how well the two groups were matched. Observed differences might be due to confounders, if groups were not well matched. Furthermore, if routine blood parameters (such as D-dimer, CRP, blood lipids etc.) were measured (as indicated in Fig. 1A), a supplementary table with the additional information – including p-values - would be helpful in assessing group differences.

9. The same holds true for the 187 plasma samples of AD patients and 58 samples of healthy individuals which were used for evaluation of inflammation, neutrophil and NET parameters by ELISA: Please provide a comparison of demographic/routine variables of the study collectives and specify the time point of blood withdrawal for the AD group. A multivariable analysis would strengthen the authors´ conclusion that the elevated neutrophil/NET parameters are indeed associated with AD rather than potential confounders.

10. The multivariable Cox regression model calculated in the prognostic study (for prediction of adverse events after AD repair by TEVAR) included all investigated plasma proteins of inflammation (IL-1β, IL-6) and neutrophil activation (elastase, MPO) or NET formation (citH3, cell-free DNA) – even though the majority of these factors did not differ significantly between patients with and without adverse events. Analyzing the two significant parameters (citH3 and MPO) with previously reported risk factors (such as identified in [6]) and prognostic variables for complications post-TEVAR would provide more information as to the usefulness of the investigated neutrophil/NET parameters. The authors have recently published on a systemic immune inflammation index to predict adverse events after AD-TEVAR [3]. How do NET parameters perform in comparison to this predictive index?

11. In accordance with the journal guidelines, please include the sex/gender distribution of your human samples and possibly explain the focus on male mice in your experimental model.

12. Please specify whether tissues were processed immediately after retrieval from surgical intervention or were stored (for how long? under which conditions?) prior to cell isolation. Was the time between resection and processing consistent or did it vary (and to which extent)?

13. Please add CXCL3 as a gene expression – projected UMAP plot to Suppl. Fig. 2B, since it would be of interest to see whether the two identified AD-associated macrophage clusters are the main producers.

14. In our experience, most CTAD plasma samples of healthy individuals range close to the detection limit of the applied commercial citH3 ELISA. Please provide the information, how many samples of the AD and healthy control groups were within the detection range of the ELISA (and at which sample dilution).

15. BAPN is frequently administered in drinking water. Please specify the mode and frequency of BAPN “infusion” mentioned in the methods section – and the applied drug concentration.

16. The information on the time frame of BAPN treatment is inconclusive. It is mentioned that BAPN was administered for 4 weeks (line 586) but a 6-week period is indicated in Figure 6A and in the Methods (line 591) and Results section (line 319). It is also unclear to me why anti-NET therapy was only given for 2 weeks (according to Fig. 6A in week 2 and 3 of BAPN administration) and was apparently discontinued in experimental weeks 4-6 prior to animal sacrifice!?

17. Considering the high death rate in murine AD induction, is the group size of n=8 per experimental group referring to surviving animals to the experimental endpoint after 6 weeks?

18. Regarding “Immunofluorescence analysis of NETs in tissue sections” the authors describe the application of a mouse antihuman Ly6G antibody (ab238132, Abcam). To the best of my knowledge, anti-Ly6G antibodies can only be used to detect neutrophils in murine but not in human tissues as there is no human orthologue for Ly6G [7]. According to Abcam, ab238132 is a rabbit anti-mouse Ly6G antibody. Hence, I assume that the description relates to the separate IHC stainings for citH3 or Ly6G in mouse AD sections (Suppl. Figure 5B). However, since these two antibodies are rabbit-derived, they should not be combined for IF co-stainings as shown in Figure 6E. Therefore, the data shown in Figure 6E are difficult to interpret. Especially the citH3 staining of all nuclei in the Cl-amidine treated mouse is highly unexpected and looks more like a staining/autofluorescence artefact. Please give more details for the mouse IF co-stainings of Figure 6E – and also provide information on the IF staining method of human sections for citH3 and MPO (Figure 4B). Please also indicate in Figure 6 and Suppl. Fig. 5B from which part of the murine aorta the sections were taken, and what you consider the true versus false lumen. Were measurements of maximum diameter focused on the thoracic part of the aorta (ascending/arch/descending)?

Minor issues:

19. Methods/Proteomic Analysis: The word “entially” is used twice. Please explain. Or is it erroneously shortened and derived from “differentially”?

20. Results: Paragraphs in the Results section contain background information (e.g. page 4, line 113-119) which is normally given in the Introduction. Furthermore, conclusions (e.g. page 4, line 120-123) should be moved from the Results to the Discussion section.

21. Supplementary Figure 2: Mast cells seem to be included in the heatmap but are not mentioned in the color code for cell clusters (as in Figure 2).

22. Legend to Supplementary Figure 3G: Please indicate more clearly that these data refer to the publically available bulk RNAseq data set, while all other parts of Suppl. Fig. 3 seem to relate to the self-conducted scRNAseq analysis of aortic tissue from 3 AD patients vs 3 healthy controls.

23. Supplementary Figure 1A-D: Please indicate more clearly, whether a positive or negative enrichment score indicates association with AD. It seems that the negative score indicates upregulation in AD patients.

24. Under Methods (line 566) it is mentioned that the clinical data of AD patients with endovascular repair were retrospectively analyzed in the prognostic study, while it is defined as a prospective cohort study (line 288) in the Results section. Please specify.

Reference to previous literature:

25. The entire introduction is lacking references, i.e. please support your statements by the relevant literature citations on AD and TEVAR.

26. Conversely, I did not find the in-text citations for references 1-3 on single cell omics.

27. The discussion is very well written but predominantly focusses on the diversity and role of macrophage subsets and neutrophils/NETs in atherosclerosis, while comparably little is discussed on what is known for aneurysms. Since aneurysm formation is closely linked to AD, the pertaining literature may be more relevant to compare.

28. Reference 16 is incomplete, i.e. is lacking the article title and further publication details.

Citations:

[1] Liu X, Chen W, Zhu G, Yang H, Li W, Luo M, Shu C, Zhou Z: Single-cell RNA sequencing identifies an Il1rn(+)/Trem1(+) macrophage subpopulation as a cellular target for mitigating the progression of thoracic aortic aneurysm and dissection. Cell Discov 2022, 8(1):11.
[2] Yao J, Bai T, Yang B, Sun L: The diagnostic value of D-dimer in acute aortic dissection: a meta-analysis. J Cardiothorac Surg 2021, 16(1):343.
[3] Zhao Y, Jiang J, Yuan Y, Shu X, Wang E, Fu W, Wang L: Prognostic Value of the Systemic Immune Inflammation Index after Thoracic Endovascular Aortic Repair in Patients with Type B Aortic Dissection. Dis Markers 2023, 2023:2126882.
[4] Eilenberg W, Zagrapan B, Bleichert S, Ibrahim N, Knobl V, Brandau A, Martelanz L, Grasl MT, Hayden H, Nawrozi P et al: Histone citrullination as a novel biomarker and target to inhibit progression of abdominal aortic aneurysms. Transl Res 2021, 233:32-46.
[5] Wei M, Wang X, Song Y, Zhu D, Qi D, Jiao S, Xie G, Liu Y, Yu B, Du J et al: Inhibition of Peptidyl Arginine Deiminase 4-Dependent Neutrophil Extracellular Trap Formation Reduces Angiotensin II-Induced Abdominal Aortic Aneurysm Rupture in Mice. Front Cardiovasc Med 2021, 8:676612.
[6] Wang S, Jia H, Xi Y, Yuan P, Wu M, Ren L, Guo W, Xiong J: Risk Factors Associated with Poor Prognosis in Patients with Stanford Type B Aortic Dissection after Thoracic Endovascular Aortic Repair. Ann Vasc Surg 2023, 93:122-127.
[7] Loughner CL, Bruford EA, McAndrews MS, Delp EE, Swamynathan S, Swamynathan SK: Organization, evolution and functions of the human and mouse Ly6/uPAR family genes. Hum Genomics 2016, 10:10.

Reviewer #3

(Remarks to the Author)
The paper presents potentially valuable and clinically relevant research data in the field of aorta related pathologies and risk assessment. However, below suggested revisions may significantly increase visibility and overall quality of the publication.

1. The title should be reconsidered to attract attention of multi-professional groups to this very important topic and proposed technological solutions such as "The multi-omics profiling is instrumental to facilitate risk assessment, patient stratification and targeted treatments in patients undergoing aortic dissection".

2. As presented, the paper is rather technology-driven, although "the identification of individuals at elevated risk for postoperative AAEs, and therapeutic targets to optimize the efficacy of TEVAR for patients with AD" is envisaged as stated in the Abstract. This evident "gap" has to be closed by the throtoughly performed revision - the authors are kindly requested to provide clear explanatory paragraphs on how the presented achievements may improve healthcare quality in the area benefiting affected individuals and improving individual outcomes. These statements have to be presented in Abstract together with clear rationale why an application of proteomics and transcriptomics has been chosen to reach the goals. Clinical relevance of the proposed approach and achievements has to be detailed in "Conclusions with Outlook".

3. "Phenotyping" is correctly mentioned in the paper as being crucial for risk assessment and targeted treatments". However, too little information is provided on the phenotyping criteria with clinically relevant examples. To this end, below listed references might be supportive for the authors to discuss "phenotyping", "innovative technologies in disease prediction" "risk assessment", "individualised patient profile", "predictive and preventive approach", "targeted treatments" which are highly relevant to the presented study and the field in general:
- https://pubmed.ncbi.nlm.nih.gov/36505894/
- https://pubmed.ncbi.nlm.nih.gov/36061830/

- https://pubmed.ncbi.nlm.nih.gov/36415625/
- https://pubmed.ncbi.nlm.nih.gov/34876936/

4. Introduction is too short and does not provide sufficient information to support the study rationale and working hypothesis. This section has to be extended providing sub-titles as messages from corresponding paragraphs such as
- Unmet patient needs in AD
- Risk assessment tools to be developed
- Technological solutions
- Working hypothesis and expectations

Sufficient references have to be provided which are currently missing in the "Introduction".

5. Discussion has to be presented in a reader-friendly manner providing sub-titles as messages from corresponding paragraphs (see explanation under the above point 4).

6. References' numbering has to be started with 1 in Introduction instead of 4 in Results. Further, references have to be updated: no any references originated from 2023 are presented.

7. Overall terminology has to be rechecked utilising an appropriate wording such as "fracture" instead of "tear".

With my best wishes
Prof. Dr. Olga Golubnitschaja
President
EPMA, Brussels
www.epmanet.eu

Head of 3P Medicine
University of Bonn
Germany

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have effectively addressed the issues we raised in their revised manuscript. Throughout the study, the authors utilized multi-omics approaches to examine the cellular composition and gene expression profiles in blood and tissue samples from aortic dissection (AD) patients. They inferred the interactions between macrophages and neutrophils, including the formation of neutrophil extracellular traps (NETs). Additionally, the biomarker potential of neutrophil and NET-related parameters was assessed in the context of diagnosing and prognosticating adverse events post-thoracic endovascular aortic repair (TEVAR). Furthermore, the authors validated in animal models that inhibiting NETs provides protective effects against AD in mice, and supplemented these findings by demonstrating the protective effects of CXCL3/CXCR2 monoclonal antibody inhibition on mice AD, further illustrating the involvement of the CXCL3/CXCR2 axis in NETs formation and the promotion of AD progression. The study combines clinical, animal, and bioinformatics screenings to identify biomarkers that potentially exacerbate AD progression. The article is fairly complete in its logical structure, I have no further questions to raise.

Reviewer #2

(Remarks to the Author)
The authors have comprehensively addressed my major points of critique by additional experiments and by manuscript changes – thank you and congratulations on the impressive amount of work! Of note, the newly introduced parts have raised some additional (minor) issues in the following manuscript parts:

1. AD is marked by systemic inflammation and neutrophil activation in peripheral blood (Fig. 1, Suppl. Fig. 1, Suppl. Table 1).

Suppl. Table 1: Categorical variables such as gender or occurrence of co-morbidities seem to be listed as mean±SD values, e.g. gender of AD patients: 0.9±0.3. It seems rather strange how gender could have a standard deviation? Please convert the presentation of categorical variables to the usual number of patients and percent frequency – as e.g. given in Suppl. Table 2. Also, how were the p-values calculated for these categorical variables – by Chi square/Fisher´s exact test?
While plasma proteomics analysis identified CRP as significantly upregulated in AD patients vs. controls (Fig. 1B), Suppl. Table 1 lists a lower CRP value for AD patients than controls. Please address this discrepancy.

2. A phenotype-associated macrophage subset orchestrates neutrophils within the aortic microenvironment (Fig. 2, Suppl. Fig. 2 and 3).

Please discuss the finding that “Scissor-positive cells” did not include neutrophils, yet the highlighted enriched pathways were neutrophil chemotaxis, activation, degranulation and NET formation. Why did the scRNAseq analyses not reflect the accumulation of activated neutrophil cell clusters in AD?

3. Neutrophil heterogeneity and differentiation trajectories (Suppl. Fig. 4).

This new chapter is solely based on scRNAseq data, it documents heterogeneity among neutrophils in AD and shows that only 2 of 5 neutrophil subsets express the CXCR1/2 receptors. Since these subsets are proposed to be the target of macrophage regulation by CXCL3 leading to NET formation, it would be of interest whether these subsets are characterized by processes of NET formation (elevated “NET score”) according to the expression data – see point 4.

4. Elevated NETosis occurs in aortic lesions and peripheral blood from patients with AD (Fig. 3, Suppl. Table 2 and 3).

Please specify the determinants of the NET score and whether the NET score was indeed significantly higher in the CXCR1/2-positive neutrophil subsets of AD patients versus controls.

Plasma measurements of CitH3 are briefly mentioned in the Methods section - with reference to the publication by Thalin et al. 2020. In this report Thalin et al. introduced the “nucleosomal CitH3” ELISA detecting complexes of CitH3 and DNA in human plasma (with healthy reference values around 30 ng/ml). Since the authors consistently mention measurements of CitH3 plasma levels (which ranged at 2 ng/ml in healthy individuals and 4 ng/ml in AD patients), please specify whether indeed CitH3-DNA complexes were detected or whether the previously published CitH3 protein ELISA by Thalin et al. (2017) was applied – and at which detection limits?

Please note that the entire chapter cites Figure 4 while the respective data is displayed in Figure 3. The legend to Fig. 3D, E, F seems wrong (E/F do not exist and D is duplicated with a second text). Also, H&E stainings of longitudinal aorta sections are mentioned to be displayed in Suppl. Fig. 5A. Yet, it seems they are actually shown in Suppl. Fig. 6D?
The statistics section mentions that continuous variables were either presented as mean and SD or as median and IQR. Please specify what holds true in the legends to the various Tables. In Suppl. Table 2, please attribute male and female gender to the respective lines. In the text, 58 controls are mentioned while Suppl. Table 2 lists 59 healthy controls – what is correct? As mentioned for Suppl. Table 1, please give the number of affected patients (or controls) and calculated percent frequency for the occurrence of co-morbidities listed in Suppl. Table 2 and verify that the correct statistical test for categorical variables has been applied.

5. Inhibition of NETosis ameliorates dissection progression in AD mice (Fig. 4, Suppl. Fig. 6).

Please acknowledge that a substantial fraction of CitH3 signal is detected without co-localization of neutrophil markers (in both the human – Fig. 3B – and mouse AD sections – Fig. 4K, also seen in Fig. 6J) which would point to additional essential sources of extracellular trap formation in AD.
Several of the results are mentioned twice within this chapter. Please try to streamline and shorten the chapter.

With respect to the Methods applied, please specify whether aortic diameter assessment was based on US analysis or ex vivo measurements, and explain how wall thickness and media thickness were defined. While in the AD mouse model, wall thickness and media thickness are inversely regulated, the results text suggests that both are reduced by the applied inhibitors (lines 350 and 401).
Importantly, Figures 4, 5 and 6 do not include significance levels. It seems that letters (a-d) were used instead but are not explained in any of the figure legends. In Figure 4, the labels of G and H are reversed in the legend, and in 4K the merged image for the DNase I treated aorta is missing (i.e. is replaced by another DAPI image). Suppl. Fig. 6F is labeled by “Ly6G” but shows MPO staining according to the legend. Please show single mice values in Suppl. Fig. 6B and H (as in Figure 4F), since it seems that at treatment start, only 2/48 animals had a detectable aorta dissection.

6. Macrophages foster NET formation via the CXCL3/CXCR2 axis (Fig. 5 and 6, Suppl. Fig. 5).

The communication matrix in Suppl. Fig. 5B would indicate that the proposed interactions between macrophages and neutrophils were rather comparable in AD patients and controls, while communication between macrophages and myofibroblasts were highly increased in AD. Please discuss whether CXCR1/2 positive (myo)fibroblasts could be an additional target of macrophage-derived CXCL3 in AD pathogenesis.

In Figure 6, the labels of E and F are reversed in the legend, and there is no scale bar of 100 µm in 6I.

7. NET-associated markers serve as prognostic risk factors (Fig. 7, Suppl. Fig. 6, Suppl. Table 4 and 5).

Several parameters in Suppl. Table 4 (AAEs, gender, phase) have 3 categories. Please specify the third category – presumably “unknown”? Also, the title of Suppl. Table 4 is incorrect and the title for Suppl. Table 5 should mention AAE prediction. The “numbers at risk” given for Kaplan-Meier curves in Figure 7E specify time in days which is likely months and which should also be labeled in Suppl. Fig. J and K.

Additional minor issues:
• The Abstract could be improved by more precise wording, e.g. “Inhibiting NET formation through the blockade of histone citrullination or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The plasma level of

citrullinated histone H3 (CitH3) could serve as a risk factor for AAEs following endovascular therapy for patients with AD."

• While mostly well-written, the manuscript could profit from another scientific language check, since several sentences are difficult to understand or appear incomplete (e.g. lines 101, 249, 262, 499-507).

• Reference #33 has no title or publication details.

• Methods: The IF chapter accidentally mentions 5 mm rather than 5 µm sections.

Reviewer #3

(Remarks to the Author)
The proposed round of revisions has been accurately performed. The revised manuscript is acceptable for publishing as is.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have conducted a comprehensive second round of revisions to address all raised concerns. In view of their explanation on the underrepresentation of neutrophils in the applied omics technique, I was surprised that an entire chapter is devoted to the characterization of neutrophil subsets and their differentiation trajectories. Yet, based on my limited experience with omics technology, I do not feel sufficiently qualified to evaluate the aptness of these analyses and interpretations.
After carefully reading the manuscript once more, I do not suggest any further major revisions. Yet, there were several minor errors and issues of scientific phrasing which have remained and which are listed below. Pending journal policy and Editor ´s assessment, I would recommend to possibly include these corrections at the type setting (page proof) stage rather than going through another lengthy round of revisions and review.
Please note: The manuscript text was inserted twice in the merged article file (with slightly distinct line numbering). The line numbers listed below refer to the first inserted manuscript version.

Abstract:
An error was accidentally introduced by misinterpreting my statement of the last review, i.e. an entire sentence was duplicated and is now shown twice in results as well as conclusions of the Abstract (rather than improving the phrasing of the results text).
Text now reads:
Results: Integrated multi-omics profiling identified highly phenotype-associated macrophages orchestrate neutrophil extracellular traps (NETs) through CXCL3/CXCR2 axis in fueling the development of AD. Increased NETs formation is a defining feature of systemic immunity and aortic microenvironment of AD. Inhibiting NETs formation through the blockade of CitH3 or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The level of citrullinated histone H3 (CitH3) could serve as a risk factor for AAEs following endovascular therapy for patients with AD.
Conclusions: The multi-omics profiling reveals NETs formation features in the development of AD. Inhibiting NET formation through the blockade of histone citrullination or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The plasma level of citrullinated histone H3 (CitH3) could serve as a risk factor for AAEs following endovascular therapy for patients with AD.
Text suggested by me:
Results: Integrated multi-omics profiling identified highly phenotype-associated macrophages to orchestrate neutrophil extracellular traps (NETs) through the CXCL3/CXCR2 axis in fueling the development of AD. Increased NET formation is a defining feature of systemic immunity and aortic microenvironment of AD. Inhibiting NET formation through the blockade of histone citrullination or the CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The plasma level of citrullinated histone H3 (CitH3) predicted AAEs following endovascular therapy for patients with AD.
Conclusions: The multi-omics profiling revealed NET formation features in the development of AD. NET-associated markers could facilitate the risk stratification and prognostic evaluation, and might serve as potential therapeutic targets of patients with AD.

Apparent errors:
Line 301: Figure 4A not 5A
Line 354: Supplementary Figures 5D-E not 4D-E
Line 372: The BAPN + IgG group (not the BAPN group) shows 50% aortic rupture according to Figure 6B
Line 375: Figure 6C not 6F
Line 936: n=59 not n=58 (based on Suppl. Table 2)
Line 1046: "sequenced mesenchymal lineage cells" should be neutrophil subsets?
Line 1112: "in prognostic cohort" was not replaced by "in predicting AAEs"
Methods – IF analysis of NETs in tissue sections: The authors state that "All aortic tissue samples were formalin fixed, paraffin embedded, and 5 µm sections were subjected to antigen retrieval in citrate buffer, then permeabilized and stained with mouse anti-human." Yet, all listed IF antibodies were apparently of rabbit origin!?

Errors in English spelling/grammar: in particular
Lines 229-247 (chapter on neutrophil heterogeneity)
Lines 474-484 (Discussion)

Cases to improve the accuracy of scientific phrasing:
Legend to Figures 4 and 6: (C) Mice survival due to aortic rupture of each indicated group.
(It is mouse death rather than survival that is due to aorta rupture.)

Lines 286-288: We detected NETs-associated markers in the plasma from 187 patients with AD and 59 healthy individuals with balanced baseline characteristics (Supplementary Table 2).
(Suppl. Table 2 shows that the two groups were not balanced, i.e. differed significantly in almost all baseline characteristics – including sex and age distribution. Yet, these potentially confounding factors were partly included in the multivariable analysis in Suppl. Table 3.)

Lines 333-335: Furthermore, both Cl-amidine-treated and DNase I-treated group (Combo) demonstrated a significant reduction in CitH3 and Ly6G or MPO positive areas when compared to BAPN-induced AD mice (Figure 4K).
(There is no quantitation but representative confocal images of these immunofluorescence tissue stainings, i.e. the term "significant reduction" is somewhat misleading and should rather read "substantial reduction".)

Lines 336-338: Taken together, our findings suggested that alleviating the progression and rupture of AD in mice can be achieved by inhibiting NETs formation through the blockade of CitH3, while inhibiting NETs function via DNase I treatment is not as effective.
(Cl-amidine blocks citrullination of histones and other proteins; CitH3 is the chosen read-out parameter i.e. is not the sole target and not the only citrullinated histone in NET formation. Hence, "blockade of histone citrullination" would seem more appropriate.)

Lines 382-384: These findings suggested that the CXCL3/CXCR2 axis plays an indispensable role in the macrophage-neutrophil interaction, thereby contributing to the progression and rupture of AD.
(The incidence of AD and aorta rupture are reduced but not abrogated by CXCL3/CXCR2 blockade, i.e. the word "indispensable" seems too strong.)

Line 376: The authors forgot to substitute the word "reduction" by "reversal" to indicate that the various investigated disease parameters were either decreased or increased by therapy.

Line 399 and legend to Figure 7B specify a cut-off level at 6.91 ng/ml but do not mention the parameter, i.e. 6.91 ng/ml CitH3.

Results chapter on "Inhibition of NETosis ameliorates dissection progression in AD mice": data are presented twice (lines 301-309 and lines 318-323)

Reviewer #3

(Remarks to the Author)
The authors have adequately revised manuscript in accordance with recommendations provided by the reviewers.

Reviewer #1

In this study, the authors employed multi-omics analysis to examine the differences in aorta and plasma between patients with aorta dissection (AD) and healthy individuals. They provided clinically applicable biomarkers (NETosis-CitH3) to predict the prognosis after thoracic endovascular aortic repair (TEVAR) for AD. Simultaneously, they validated the inhibitory effect of NETosis on the progression of aortic dissection in a mouse AD model, suggesting a potential targeted therapeutic significance. This research demonstrates innovativeness by identifying clinically promising biomarkers that could be utilized. To a certain extent, this study holds clinical translational value. While the quantity of the manuscript is quite impressive, there are multiple factors the limit my enthusiasm for this manuscript in its present form. These will be outlined in detailed below.

Major concerns:

1. Compared to the previously reported single-cell sequencing results on human AD tissues, is there any difference in this study. A comparative discussion is needed in the DISCUSSION section.

(Circulation, 2023. DOI: 10.1161/CIRCULATIONAHA.123.063332.)

(Circulation, 2020. DOI: 10.1161/CIRCULATIONAHA.120.046528.)

Response: Thank you for the comment. The first paper entitled "Epigenetic Induction of Smooth Muscle Cell Phenotypic Alterations in Aortic Aneurysms and Dissections" mainly depicts the phenotype switching of SMCs which is not investigated in this study. And the second one is almost the first single-cell RNA sequencing analysis research published on cardiovascular top journal. We have read and made a discussion about these two papers in the revised manuscript.

Revision:

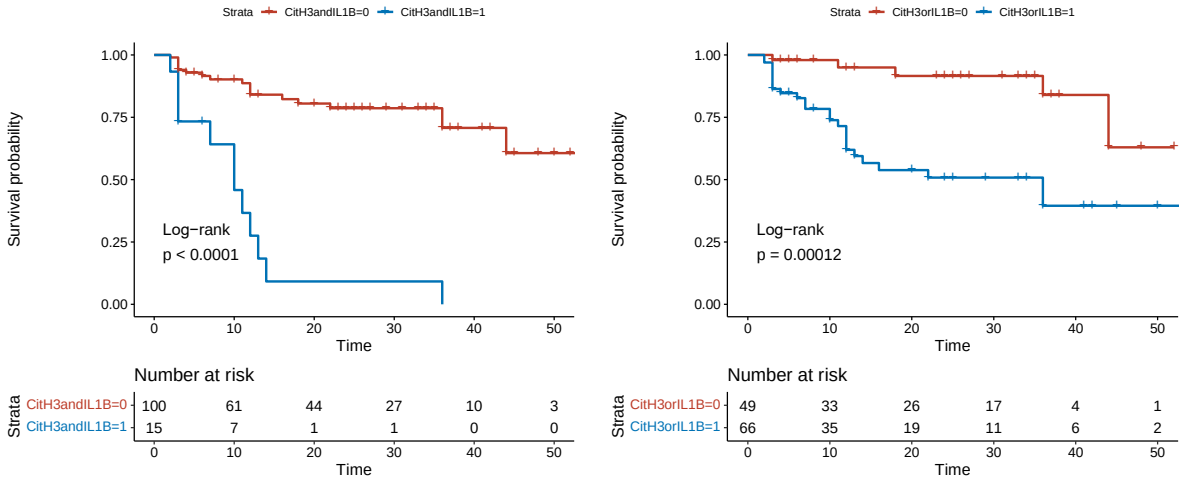
Previous study pinpointed the macrophage subset expressed several cytokine genes as the predominant origin of detrimental molecules for AD, including IL1RN/TREM1 M1-like macrophage subcluster, which also expressed high levels of proinflammatory chemokines such as CXCL2, CCL3, and CCL4. While our study showed an inflammatory chemokines and cytokines highly-expressed macrophage subcluster, but limited expression of TREM1 and a moderate expression of IL1RN in macrophages, which may not qualify as a proinflammatory macrophage population and further indicate the intrinsic difference in the underlying pathologic mechanism between aneurysm and dissection. (Lines 499-507 in revised manuscript)

2. In figure 5E, could authors provide Kaplan-Meier curves of patients with combining high CitH3 and high IL-1β, compared to patients with other conditions?

Response: Thank you for the comment. We conducted the analyses according to the comment combining high CitH3 and high IL-1β patients into one group. As you can see in the following two figures, the left one represents both high CitH3 **and** high IL-1β patients were divided into one group, while the right one depicts patients with high CitH3 **or** high IL-1β patients were grouped together. Both two classifying methods yields statistically significant differences and diverged survival

curves. Besides, we added these results into the supplementary material. We propose that in some certain clinical scenario, combining CitH3 and IL-1β could further improve the accuracy of prognostic evaluation than CitH3 alone does.

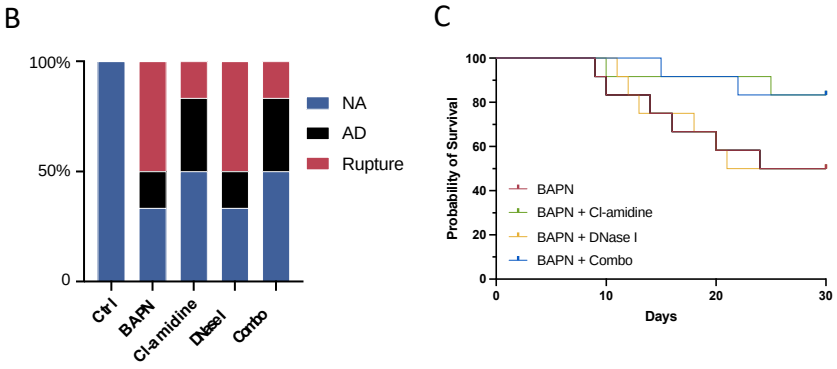
Revision: Supplementary Figure 7. The presence of NETs formation and CitH3 as an independent risk factor for AAEs following endovascular treatment of AD.
(I) Kaplan-Meier curves of patients with both high CitH3 (≥ 6.91 ng/ml) and high IL-1β (≥ 90.86 pg/ml) compared to remaining patients. Below the survival curves showing the number of exposed subjects at each time point.
(J) Kaplan-Meier curves of patients with either high CitH3 (≥ 6.91 ng/ml) or high IL-1β (≥ 90.86 pg/ml). Below the survival curves showing the number of exposed subjects at each time point. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 by Mann-Whitney U test.



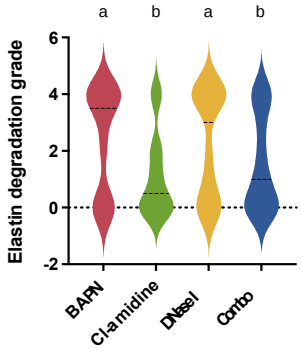
3. In figure 6D, for the BAPN-induced aortic dissection model, mice should have a statistical graph of the incidence of aortic dissection and elastic fiber fracture grades to reflect the severity of the dissection. Elastic fiber fracture grades: I: 0~25%; II: 26%~50%; III: 51%~75%; IV: 76%~100%

Response: Thank you for the suggestion. We have quantified the incidence of AD, shown in Figure 4B in revised manuscript. We also evaluated the elastic degradation grade according to the criteria provided, and the result is added as Figure 6I.

Revision: Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD.
(B) The incidence of AD and aortic rupture in each group (n = 12).
(C) Mice survival due to aortic rupture of each indicated group.



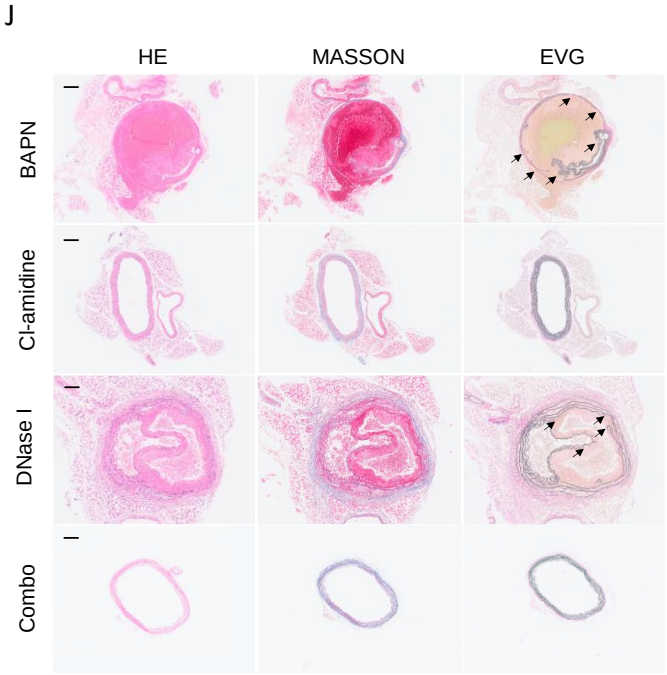
(I) Elastin degradation grading evaluation of aorta in each group.



4. In figure 6D, the aorta of AD and DNase I groups seems to be relatively normal from EVG. In theory, the false lumen should be surrounded by elastic fibers, yet the EVG staining displayed a relatively intact aorta and the elastic fibers of outer side of "false lumen" called by authors was not observed. Is there a more suitable representative diagram? (Refer to Circulation. 2022. DOI: 10.1161/CIRCULATIONAHA.121.056640, Figure 3)

Response: Thank you for this valuable suggestion, which literally help us improve the quality and accuracy of Figures. We have looked through all mice aorta staining images and replaced original images with more suitable ones, in which aortic false lumens are surrounded by several layers of elastic fibers, just like the given Circulation paper. We present them as follows as well.

Revision: Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD.
(J) Representative immunohistochemistry images showing aortic dilation, false lumen formation, and elastin degradation within aortas in each group. Arrow indicating broken elastin.



5. In this study, elevated NETosis was observed in patients with AD, and proved to be predictive of the post-TEVAR outcomes. The BAPN animal model reflects the incidence and development of AD. Therefore, in the presentation of results, mouse model should correspond to the findings in patients with AD. This section (mouse model) can be placed following the result "Elevated NETosis occurs in aortic lesions and peripheral blood from patients with AD." Additionally, the prediction of TEVAR outcomes should be discussed in the last paragraph of the results section.)

Response: Thank you for the suggestion. We have rearranged the order of the paragraphs regarding the mouse model and biomarkers validation. The section “Inhibition of NETosis ameliorates dissection progression in AD mice” is now ahead of the part “NETs-associated markers serve as prognostic risk factors”.

Revision: Manuscript headlines

1. AD is marked by systemic inflammation and neutrophil activation in peripheral blood
2. A phenotype-associated macrophage subset orchestrates neutrophil within aortic microenvironment
3. Neutrophil heterogeneity and differentiation trajectories
4. Elevated NETosis occurs in aortic lesions and peripheral blood from patients with AD
5. Inhibition of NETosis ameliorates dissection progression in AD mice
6. Macrophage fosters NETs formation via CXCL3/CXCR2 axis
7. NETs-associated markers serve as prognostic risk factors

6. The patient tissue used in the study should provide basic information about the patient and healthy controls (such as age, base height, weight, underlying disease, etc.)

Response: Thank you for the suggestion. We have collected and analyzed the baseline information of patients, whose aortic tissue and venous blood were used in this study. The tables of baseline characteristics are presented as follows.

Revision: We conducted a proteomic analysis comparing the plasma samples of 30 patients with acute AD and 30 matched healthy individuals, which were screened from a total of 128 subjects, with the data-independent acquisition quantitative proteomic approach using mass spectrometry. The age at onset and gender did not differ between patients with AD and matched controls after an exact matching, indicating a proper matching quality. (Supplementary Table 1). Other baseline characteristics including AD etiologically related history of hypertension, inflammatory indicators, and D-dimer were statistically significant between two groups (Supplementary Table 1). (Lines 123-130 in revised manuscript)

Supplementary Table 1. Baseline characteristics of patients with acute AD and healthy individuals involved in mass spectrometry.

	AD (N=30)	CL (N=30)	p-value
Age (y)	57.1 ± 12.0	52.8 ± 8.1	0.113
Gender	0.9 ± 0.3	0.9 ± 0.3	1
Hypertension	0.7 ± 0.5	0.0 ± 0.0	<.001
Smoking	0.5 ± 0.5	0.2 ± 0.4	0.012
Neutrophil ($\times 10^9$ /L)	7.0 ± 2.1	3.8 ± 0.8	<.001
Monocyte ($\times 10^9$ /L)	0.8 ± 0.4	0.6 ± 0.6	0.189
Lymphocyte ($\times 10^9$ /L)	1.3 ± 0.5	2.0 ± 0.9	0.006
Platelet ($\times 10^9$ /L)	234.9 ± 93.5	259.9 ± 54.4	0.26
D-dimer (mg/L)	5.1 ± 5.2	0.2 ± 0.1	<.001
CRP (mg/L)	45.5 ± 54.9	71.7 ± 53.3	0.23
ALT (U/L)	29.7 ± 38.4	34.8 ± 16.9	0.542
AST (U/L)	23.1 ± 19.8	27.7 ± 7.3	0.272
Cholesterol (mmol/L)	4.7 ± 1.0	5.2 ± 0.6	0.115
Triglyceride (mmol/L)	1.2 ± 0.4	1.9 ± 0.6	0.001

CRP: C-reactive protein; ALT: Alanine transaminase; AST: Aspartate aminotransferase.

Minor concerns:

1. For page 28 line 776, the content in "()" is missing.

Response: Thank you for the notification. We have revised the missing and deleted the "()" behind the "KEGG", which is illustrated in the ending of the legends.

2. For page 5 line 143, 149 and page 8 line 226, supplementary figures need to be marked accurately.

Response: Thank you for the comment. We have corrected the supplementary figures numbers Revised:

Revision:
Despite their prevalence as the largest cell population within the aorta, the comprehensive clustering analysis revealed overlapping gene expression patterns among different macrophage populations (Supplementary Figure 3B-F), making it difficult to definitively identify distinct subsets of macrophages within the aortas. (Lines 166-170 in revised manuscript)

We found that FCGR3A$^+$ macrophages and OLR1$^+$ macrophages, which comprised most of the phenotype-specific macrophages, exhibited strong interactions with other macrophage populations, fibroblasts, macrophage-like fibroblasts and neutrophils (Supplementary Figure 4A and 4B). (Lines 370-373 in revised manuscript)

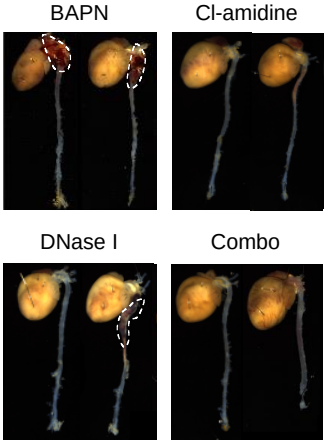
3. For page 5 line 152, cluster 13 does not appear to be macrophage_TCR mentioned behind. Is the cluster 4?

Response: Thank you for the comment. We have checked that cluster 13 does represent macrophage_TCR and cluster 4 is neutrophil (marked in green). It may not be distinctly obvious to the naked eye on the UMAP plot because cluster 13, marked in dark green, is sparse and slightly dispersed into other subpopulations.

4. In figure 6D, if possible, could you provide a suitable representative diagram of the aorta? Additionally, all aorta diagram for every group should be presented in the supplement figure.

Response: Thank you for the comment. We have added representative images of the aorta from mice challenged with different agents, in revised Figure 4E as follows.

Revision: Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD.
(E) Macrographs of aortas in each group.



5. The role of NETosis also contributes to autoimmune diseases, the initiation and facilitation of metastasis of malignant tumors. Therefore, under these conditions, does the predictive capacity of biomarker NETosis decrease?

Response: Thank you very much for this valuable suggestion. NETs formation plays a role in a variety of diseases and its level may be influenced by the comorbidity of patients, which may skew our results. To further validate our conclusion, we additionally analyzed the baseline characteristics of patients with AD and healthy controls, as well as patients with or without postoperative aorta-related adverse events (AAEs). The results are provided in the supplementary data. Although our data in the cohort establish the value of NETs biomarkers in predicting the prognosis of patients with AD, chances are that NETs biomarkers may not precisely function in some certain clinical scenario. If a patient with malignant tumor history, which might possess a higher baseline NETs level, receive endovascular therapy and are followed-up to monitor AAEs. When NETs levels are found increased, patients may develop AAEs or suffer from cancer relapse or metastasis. If AAEs could be excluded via CTA, the elevated NETs levels might indicate a cancer relapse or metastasis. Therefore, though the predictive capacity of NETs biomarker for cardiovascular complications decreases, they could also indicate other disease progression like cancer or autoimmune diseases.

6. Please specify the statistical testing approach for each figure in the corresponding legend, avoiding general descriptions.

Response: Thank you very much for the suggestion. We have added the statistical testing approach for each figure in the corresponding legend.

Revision:

Figure 3. Elevated NETosis occurs in aortic lesions and peripheral blood from patients with AD. (C) NETs associated markers as detected by ELISA in the plasma from patients with AD (n = 187) and healthy individuals (n = 58). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by Mann-Whitney U test.

Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD. Statistical significance was set at $p < 0.05$ by Mann-Whitney U test or Fisher's exact test.

Figure 5. $CXCL3^+$ macrophages were positively correlated with $CXCR2^+$ neutrophils within AD lesion. Statistical significance was set at $p < 0.05$ by Mann-Whitney U test or Fisher's exact test.

Figure 6. Macrophage fosters NETs formation via CXCL3/CXCR2 axis. Statistical significance was set at $p < 0.05$ by Mann-Whitney U test or Fisher's exact test.

Figure 7. NETs-associated markers serve as prognostic risk factors. (A) The levels of NETs-associated markers in plasma of patients with (n = 31) or without AAEs (n = 84) after endovascular treatment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ by Mann-Whitney U test.

Reviewer #2

Yufei Zhao et al. have applied a multi-omics approach to investigate the cell type composition and gene expression profile in blood and tissue samples of patients with aortic dissection (AD) and have deduced an interplay of macrophages and neutrophils, including the formation of neutrophil extracellular traps (NETs). Furthermore, the biomarker potential of neutrophil- and NET-associated parameters was evaluated in a diagnostic and prognostic setting for adverse events after thoracic endovascular aortic repair (TEVAR). In more detail:

Proteomic analysis from plasma samples of 30 AD patients and 30 matched healthy individuals was based on mass spectrometry and revealed an accumulation of proteins related to protein-DNA complex formation and NET formation in blood of AD patients. Overall, inflammatory mediators of leukocyte chemotaxis and activation were enriched.

Single-cell RNA sequencing (scRNAseq) was performed for cells isolated from 3 resected healthy aortas versus 3 AD samples. Data were compared to a publicly available bulk sequencing set from 7 AD patients and 5 healthy controls to delineate cell populations which were predominantly associated with the AD phenotype. While smooth muscle cells (SMCs) and fibroblasts dominated in aorta samples of healthy individuals, about 50-80% of cells isolated from AD samples were identified as macrophages which were further divided into 5 subsets. Two of these subsets (macrophage_FCGR3A and macrophage_OLR1) constituted the majority of cells associated with the AD phenotype. Among various proinflammatory and immunoregulatory pathways, these macrophage subtypes showed gene expression profiles of neutrophil chemotaxis and activation.

The authors then investigated putative cell-cell communication pathways based on matching ligand-receptor pairs in scRNAseq analysis. While the two macrophage subtypes were found to have the highest potential for interactions with other macrophages, fibroblasts and endothelial cells, they also presented putative cross-talk with neutrophils (based on the expression of CXCL2, -3, and -8 and the pertaining receptors CXCR1 and -2 on neutrophils).

The study then focused on the detection of NETs in blood and tissue samples of AD patients and found gene expression modules (in scRNAseq) associated with extracellular trap formation to be elevated in the 3 AD patients versus 3 healthy controls – not only in neutrophils but also in all 5 macrophage subsets. By immunofluorescence staining of FFPE sections, neutrophil/NET markers were further detected in 4 AD tissues but not in 4 healthy aortas of transplant donors. Additionally, neutrophil and NET parameters (citrullinated histone H3 = citH3, cell-free DNA and neutrophil elastase) were measured by ELISA in the plasma of 187 AD patients and were significantly elevated compared to 58 healthy controls.

Based on this diagnostic biomarker assessment, the authors subsequently explored the prognostic potential of these plasma molecules in 115 AD patients. Among the 31 patients who developed adverse events after endovascular repair, 70% had citH3 levels exceeding the defined cut-off level, and plasma citH3 was found to significantly predict adverse events in ROC analysis with an area

under the curve of 0.751 (sensitivity of 60% and specificity of 90% at the defined cut-off). In addition to citH3 also IL-1β was identified as independent predictor for post-TEVAR complications.

To investigate the relevance of NETs in vivo, aorta dissection and concomitant aneurysm formation were induced in male wildtype Black6 mice over a time course of 6 weeks by administration of an inhibitor of lysyl-oxidase (BAPN). Anti-NET therapy by either DNase I or Cl-amidine was given for 2 weeks (in week 2 and 3 of BAPN treatment). While BAPN led to high rates of aortic ruptures (63%), anti-NET therapy reduced animal death to 13-33%. Furthermore, aortic dilatation (aneurysm formation) was significantly decreased by the combined anti-NET treatment. Histological stainings and immunofluorescence imaging were conducted to document aorta degeneration and NET accumulation in the experimental groups.

While this is a well-written, comprehensive study based on multi-omics of AD specimen which has resulted in a variety of interesting observations, there is no clear evidence for the importance of the proposed ***macrophage-NET link*** *in AD patients. The omics data offer cues to potential cellular functions and interactions. The biomarker analyses are clearly of interest, yet need to be put into the context of* ***multivariable analysis with potential confounders****. Finally, the data of the conducted mouse model do not resolve the impact of* ***anti-NET therapy on aneurysm formation versus aortic dissection****, thus making it difficult to support the authors´ conclusion that NETs promote AD.*

Originality and significance to the field/related fields:
What is already known:
• scRNAseq has previously been conducted to characterize the cellular composition and gene expression profile of thoracic AD [1] and has resulted in the identification of macrophage subsets distinct from the results in this study.
• Various blood parameters for diagnosis of acute AD have previously been proposed, among them D-dimer with a sensitivity and specificity of 96% and 70%, respectively (AUC 0.940) as calculated in a recent meta-analysis [2]. The authors themselves have recently developed a systemic immune inflammation index to predict adverse events after TEVAR of AD [3].
• Anti-NET therapy has been shown to prevent the combined AD+aneurysm development in mouse models similar to the model conducted by the authors. Thus, angiotensin II administration to ApoE k.o. mice results in aortic dissection and thoracic as well as abdominal aortic aneurysms. The formation and progression of aortic disease is reportedly reduced by anti-NET therapy [4, 5].

What this study adds:
• The identification of novel AD-specific macrophage subsets by scRNAseq.
• The detection of NETs (NET associated molecules) in tissue and blood of AD patients.
• The biomarker potential (diagnostic and prognostic after TEVAR) of the plasma NET parameter citH3 for AD patients – although multivariable analysis with potential confounders is missing.

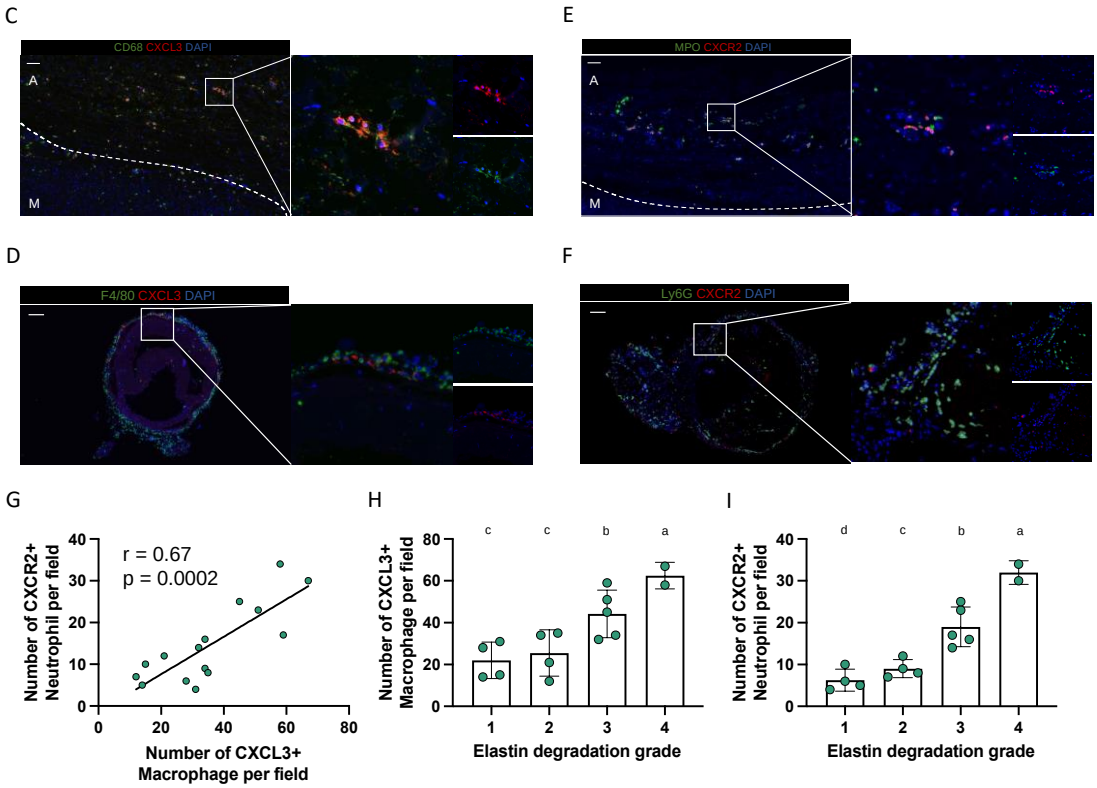
What is not resolved/addressed by this study:
• The impact (and therapeutic potential) of the proposed macrophage-NET link on disease development in AD patients. Of note, the mouse model does not differentiate between aneurysm formation and AD – main readouts are aorta rupture and aortic dilatation.

Response: We really appreciate the reviewers' constructive suggestions on our manuscript. Several points are not resolved by our previous study, including mouse model on aneurysm formation rather than AD, the putative CXCL3/CXCR2 macrophage-NET link in AD patients, and the potential confounders in multivariable regression model. Therefore, we have conducted additional experiments to further support our conclusion.

We totally agree with reviewers that the mouse model induced by BAPN only was not proposed to differentiate between aneurysm formation and AD. We also acknowledge the considerable difference between aneurysm and AD, clinically or mechanistically, and importance of excluding aneurysm from AD study. We then first re-performed the AD mouse experiment with a slight refinement to the study design according to reviewers' comments. We raised the number of mice per group from n = 8 to n = 12, a larger sample size allowed us to observe the incidence of AD or aneurysm, and extended the NETs inhibitor administration till experiment endpoint. We abandoned the previous BAPN administration to induce AD, rather adopted BAPN and Ang II with mini-osmotic pumps co-administration instead. We conducted H&E staining for each mouse aorta to confirm AD or aneurysm occurrence, the former one had more or less elastin degradation and rupture in aortic media layer. It was observed that the incidence among each group did not differ statistically. However, we noted a significant reduction in the incidence of aortic rupture due to AD, which meant death before the experiment's endpoint, in the NETs-inhibited group. (**Figure 4 in revised manuscript**)

The interaction between macrophages and neutrophils was questioned in our previous results, thereby we supplemented with fluorescence staining and corresponding quantification to observe the correlation between these two. (**Figure 5 in revised manuscript**)

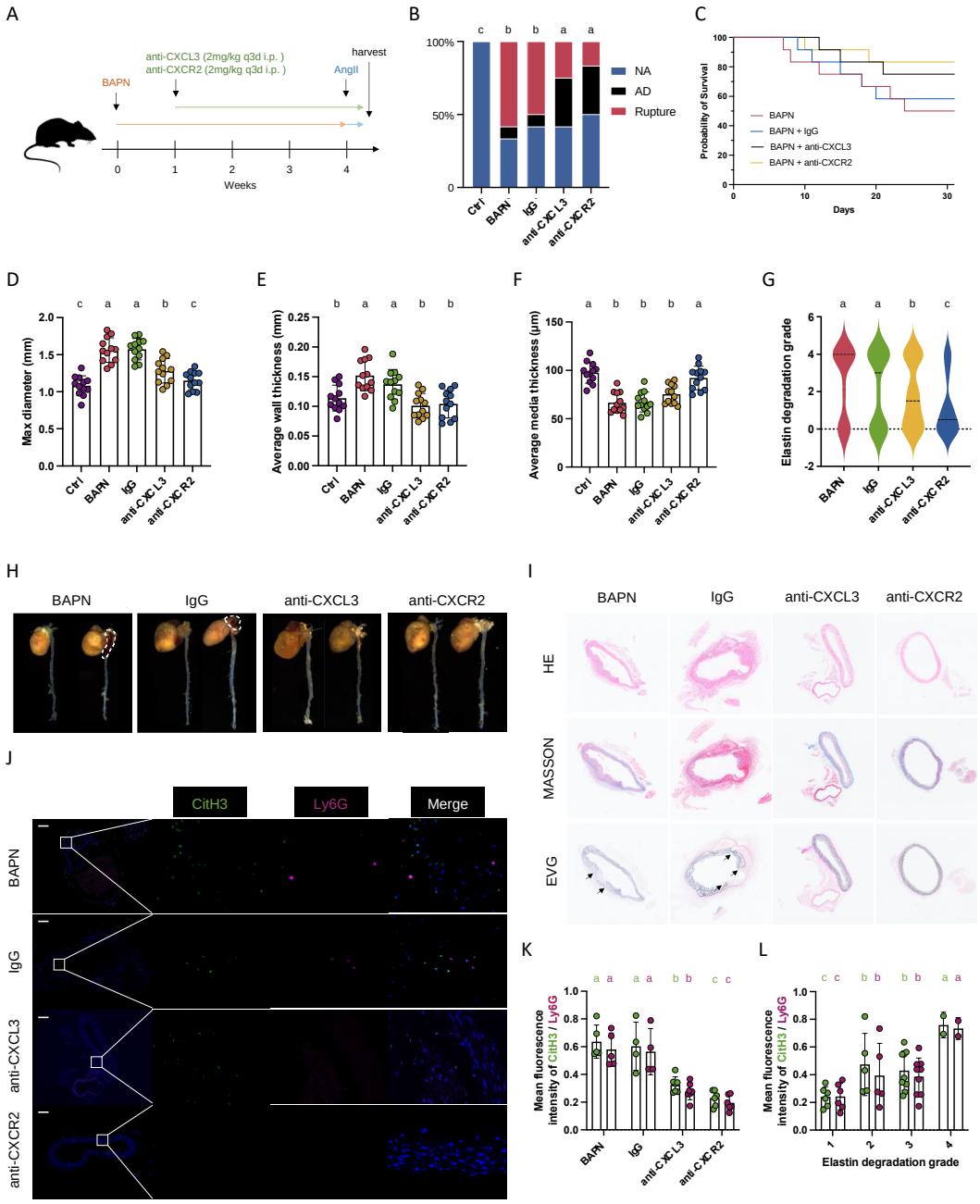
Figure 5. $CXCL3^+$ macrophages were positively correlated with $CXCR2^+$ neutrophils within AD lesion. (A) Circle plot showing counts of ligand-receptor interactions between macrophages and neutrophils by Cellphone DB analysis. (B) The ligand-receptor interactions between macrophages and neutrophils in AD compared with NA. (C-D) Representative images of the presence of $CXCL3^+$ macrophages within human (C) and mice (D) dissected aorta. Scale bars = 100 μm. (E-F) Representative images of the presence of $CXCR2^+$ neutrophils within human (E) and mice (F) dissected aorta. Scale bars = 100 μm. (G) Correlation analysis showing positive linear correlation between the number of $CXCL3^+$ macrophages and the number of $CXCR2^+$ neutrophils. (H-I) Bar plot showing the positive correlation between elastin degradation grading and $CXCL3^+$ macrophages (H) and $CXCR2^+$ neutrophils (I).



We further confirmed the indispensable role of CXCL3/CXCR2 axis in AD progression in mouse models, by separately blocking CXCL3 and CXCR2. There was 50.0% (6/12) of the mice in the BAPN group succumbing due to aortic rupture. In contrast, the mortality rate due to aortic rupture in the anti-CXCL3 group was 25.0% (3/12), while in the anti-CXCR2 group it was 16.7% (2/12). The survival analysis showed improved mortality due to aortic rupture in anti-CXCL3 and anti-CXCR2 groups. (**Figures 6 in revised manuscript**)

Figure 6. Macrophage fosters NETs formation via CXCL3/CXCR2 axis. (A) Schematic overview of experimental design. Mice received intraperitoneal injection of IgG anti-body, anti-CXCL3 anti-body (2mg/kg), and anti-CXCR2 anti-body (2mg/kg) at a fixed time once a day until death or the end point of the experiment. (B) The incidence of AD and aortic rupture in each group (n = 12). (C) Mice survival due to aortic rupture of each indicated group. (D-F) The average of max diameter (D), media thickness (E), and aortic wall thickness of aortas (F) in each group. (G) Elastin degradation grading evaluation of each aorta. (H) Representative macrographs of aortas in each group. (I) Representative immunohistochemistry images showing aortic dilation, false lumen formation, and elastin degradation within aortas in each group. Scale bar = 100 μm. (J) Representative immunofluorescence images showing NETs formation within aortas of mice. Scale bar = 100 μm. (K) Immunofluorescence quantification of NETs markers among each group. (H-I) Immunofluorescence quantification of NETs markers within aortas with four elastin degradation grades.

Figure 6



We compared baseline characteristics of patients with acute AD and healthy individuals involved in mass spectrometry. Moreover, we performed analyses of baseline characteristics of patients with AD and healthy individuals in diagnostic cohort, and conducted LASSO and multivariable logistic regression analyses. Last, baseline characteristics of patients with AD and healthy individuals of patients receiving TEVAR in prognostic cohort were analyzed, followed by univariable and multivariable Cox proportional hazard regression analyses of patients. The additional statistical analyses yielded solid conclusion that CitH3 is an independent risk factor for AAEs following endovascular treatment of AD. (**Supplementary Tables 1-5 in revised manuscript**)

Finally, we have additionally investigated the neutrophil heterogeneity and differentiation trajectories in single-cell data. We found two subsets of neutrophils highly expressed CXCR1/2, $CXCR4^+$ neutrophil and $S100A12^+$ neutrophil, and are also positively correlated to inflammation activation, innate immune response, smooth muscle cell apoptosis, and angiogenesis. The differentiation trajectory analysis revealed a highly overlapping at the starting point while bifurcating at the endpoint differentiation path from $S100A12^+$ neutrophil to $CXCR4^+$ neutrophil and ultimately two subsets of MGP^+ and $ANXA1^+$ neutrophil, respectively. The results illustrated divergent neutrophil phenotypes and bifurcating differentiation trajectories in this acute inflammatory condition, wherein neutrophils demonstrated a dual nature and bipotent function, encompassing both naïve pro-inflammatory and anti-inflammatory profiles. (**Supplementary Figure 4 in revised manuscript**)

Below is our point-by-point response.

Conclusions and claims:

1. It is not entirely clear to me how the data shown in Suppl. Figure 1 support the conclusion that neutrophil activation is increased (rather than other components of inflammation) in the blood of AD patients, as indicated by the title of the Figure. Please specify the processes or components recognized in these analyses to conclusively identify neutrophils and their activation.

Response: Thank you very much for raising this concern. We looked at the data and figures again. As you mentioned, Suppl. Figure 1 indeed does not directly indicate increased neutrophil activation, though they reveal elevated cytokine production and leukocyte adhesion. And it may not be appropriate to conclude neutrophil activation based solely on Suppl. Figure 1. Therefore, we removed this statement in the revised manuscript. Thank you very much for pointing out this inadequacy.

Revision: Supplementary Figure 1. Systemic inflammation ~~and neutrophil activation are~~ is elevated in peripheral blood from patients with AD.

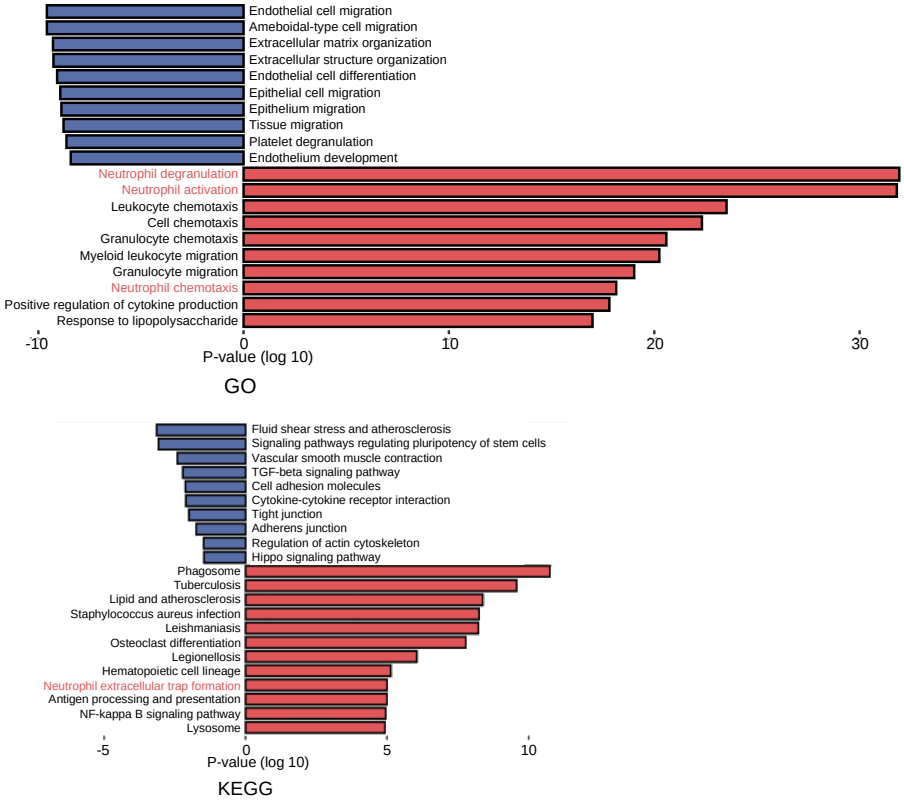
2. In Figure 3C/D the authors have conducted a direct comparison of gene expression in cell clusters predominantly found in AD aorta samples (i.e. mainly macrophages) with the prevailing cell cluster associated with healthy aortas (i.e. mostly SMCs and ECs). I don´t see the benefit of this analysis, since it is to be expected – as found by the authors – that macrophages have more hallmarks of inflammation, including neutrophil chemotaxis and activation among many other immune pathways, yet a lack of SMC/EC differentiation markers (when compared to SMCs/ECs). So the authors have essentially based the main conclusion of their scRNAseq analyses ("An AD phenotype associated macrophage subpopulation promotes NETosis within the aortic microenvironment.") on the comparison of macrophages with SMCs/ECs which does, in fact, tell us that macrophages are more potent regulators of neutrophil recruitment and activation than the stromal cells.

Response: Thank you very much for raising this concern. We understand you may regard our phenotype-associated cell cluster finding step by Scissor as an inappropriate way. Indeed, we found macrophages comprised of the majority of positive phenotype-associated cells and the negative one was largely consisted of SMCs/ECs, and thereby we directly compared their gene expression, showed in heatmap (Figure 2E, revised manuscript). While the next GO/KEGG analyses (Figures 2F and 2G, revised manuscript) was not done by comparing the macrophages within positive phenotype-associated cells to SMCs/ECs negative phenotype-associated cells, they were done by extracting all positive phenotype-associated cells only, not SMCs/ECs at all, and then performed the downstream analyses to see what kind of role they played and what the mechanism may be. The red bar indicated the increased pathway within phenotype-associated macrophages, and blue bar depicted the reduced pathway within phenotype-associated macrophages. Thereby, it turned out that phenotype-associated macrophages interacted with neutrophil and promote their activation. In addition, we revised these figure legends to avoid misunderstandings and enhance clarity. We apologize for our any confusion caused by our previous ambiguous description, and hope we explain the methods and results clearly. Thank you very much for pointing out this question.

Revision: Figures 2. A phenotype-associated macrophage subset orchestrates neutrophil within aortic microenvironment

(F) A bar plot of the GO enrichment of biological processes showing the significantly enriched pathways in Scissor^{+} cells.

(G) A bar plot of the KEGG enrichment showing the significantly enriched pathways in Scissor^{+} cells.



3. The authors have attempted to illustrate the presence of CXCL3 expressing macrophages

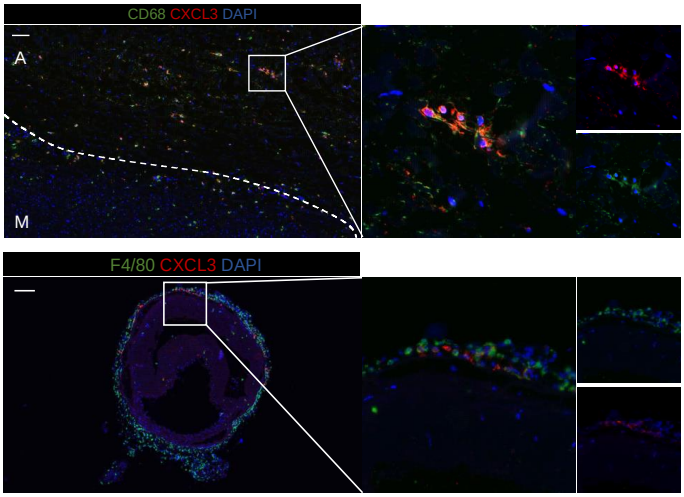
in human AD tissue by immunofluorescence staining, but only show a single cell with co-staining of CD68 and CXCL3 (in – what seems to be the adventitia – of the aortic wall). This illustration is neither suited to document the accumulation of macrophages in the AD sample nor their abundant/frequent expression of CXCL3. A whole-tissue scan (as for H&E stainings and as performed for mouse aorta sections in Figure 6) is required in comparison with the healthy control aorta staining to provide convincing evidence.

Response: Thank you very much for the comments. We have added the whole-tissue scan of H&E staining and IF staining of CD68 and CXCL3 markers in dissected aorta and healthy aorta from both patient- and mouse-derived aortic tissue for comparison. In the Figures 5C and 5D, and Supplementary Figures 5F and 5G, there was significantly more the co-staining of CD68 and CXCL3 in AD than in normal aorta.

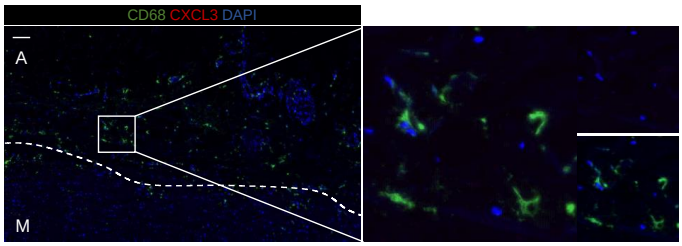
Revision:

Figure 5. CXCL3$^+$ macrophages were positively correlated with CXCR2$^+$ neutrophils within AD lesion.

(C-D) Representative images of the presence of CXCL3$^+$ macrophages within human (C) and mice (D) dissected aorta. Scale bars = 100 μm.



Responding figure. Representative images of the presence of CXCL3$^+$ macrophages within **normal aorta**, determined by CD68 (green) and CXCL3 (red) staining in human tissue. Scale bars = 100 μm.

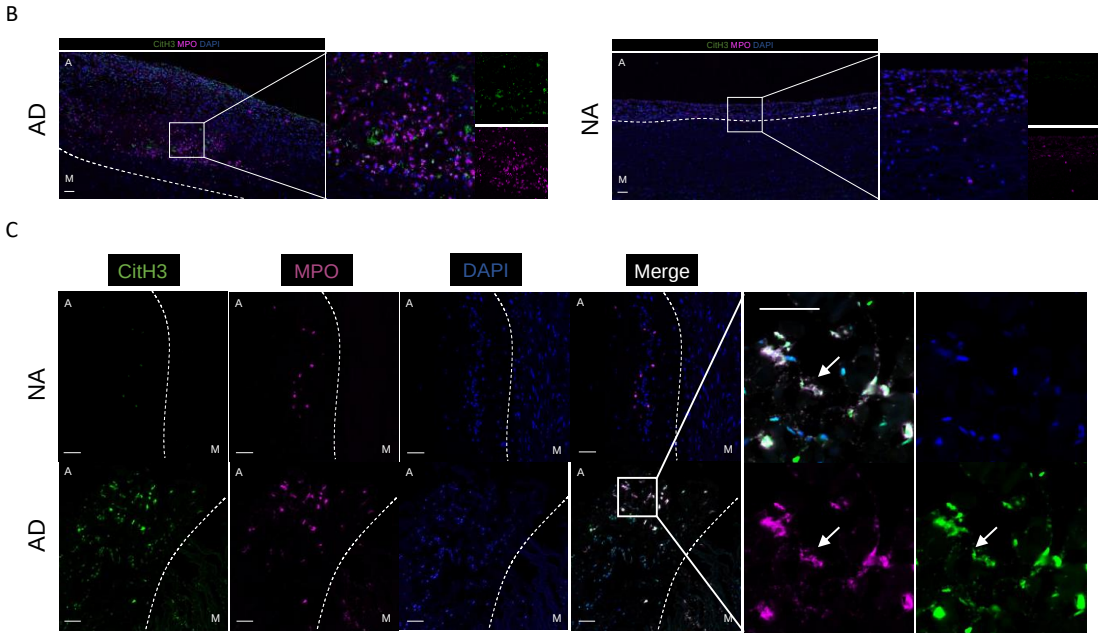


4. Similarly, Figure 4B documents the presence of citH3 and MPO staining in an AD tissue sample (while these markers are not detected in the healthy control). To be able to follow the conclusion that NETs are more abundant in AD than in healthy aortas, whole-tissue scans

of both, histology slides (H&E or MTC) and the immunofluorescence staining of citH3 and MPO should be provided – indicating the zoom-in regions seen in Figure 4B.

Response: Thank you very much for the comments. We have supplemented the whole-tissue scan of H&E staining and IF staining of CitH3 and MPO markers in dissected aorta and healthy aorta from human. As shown in Figures 3B and 3C, the co-staining of CitH3 and MPO, while without DAPI was significantly more prevalent in AD compared to the normal aorta.

Revision: Figure 3. Elevated NETosis occurs in aortic lesions and peripheral blood from patients with AD.
(B) Representative immunofluorescence images of NETs identified by co-localization of MPO (red) and CitH3 (green). Scale bars = 1 mm.
(C) Zoom-in immunofluorescence images of NETs determined by co-staining of MPO (red) and CitH3 (green). Scale bars = 100 μm.
M indicated media, A indicated adventitia, and the white dotted line indicated the boundary between media and adventitia.



5. In line 275, the authors mention that IL-1β is significantly higher in plasma of AD patients than healthy controls. Yet, the data presented in Figure 4C do not support this conclusion.

Response: Thank you very much for this inconsistency. We reviewed the data on plasma NETs marker levels and found that our previous statement was incorrect. We intended to delete "IL-1β" from the sentence but mistakenly deleted the marker "MPO" instead. We have made the correction in the revised manuscript.

Previous: Our findings revealed that the levels of plasma NETs markers, including CitH3, cf-DNA, NE, IL-6, and ~~**IL-1β**~~ were significantly higher in patients with AD than those observed in healthy subjects (Figure 4C)

Revision: Our findings revealed that the levels of plasma NETs markers, including CitH3, cf-DNA, **MPO**, NE, and IL-6, were significantly higher in patients with AD than those observed in healthy subjects (Figure 4D).

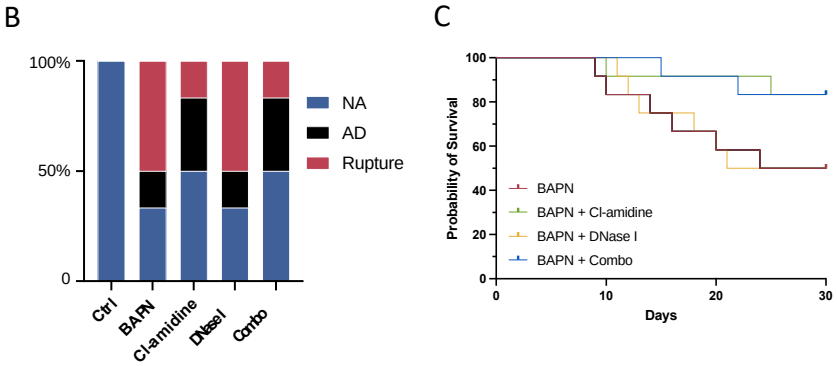
> ***6. The mouse study is based on a model of combined AD and aneurysm formation. The authors document the efficacy of anti-NET treatment to reduce aortic rupture/animal death and aorta dilatation as well as wall thickness, but it does not provide sufficient evidence for the impact on aorta dissection. Only 1 histological sample/section is given per treatment group showing AD and thrombus formation in the untreated animal as opposed to no dissection in the Cl-amidine treated mice. The frequency of AD occurrence should be calculated and appropriately illustrated for each experimental group (investigating the entire aorta) and statistically evaluated – which might necessitate higher n numbers per group.***

Response: Thank you very much for this constructive comment. We fully agree with you that our experiment requires a higher number of samples per group. We used a group of mice (n = 12) after removing aneurysm aortas for our study. Consequently, we meticulously documented and calculated the incidence and mortality of AD by thoroughly examining the entire aorta in each group. Our findings indicated that in this study, the incidence of AD was not reduced by the NETs inhibitor. However, the rate of aortic rupture was significantly decreased in the NETs inhibitor group compared to the BAPN group (Figures 4B and 4C in revised manuscript).

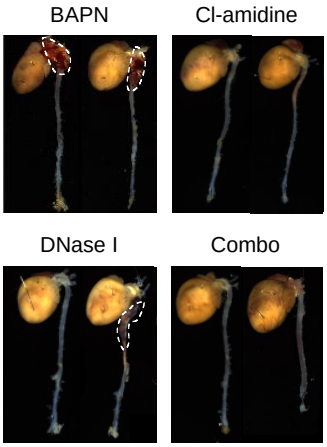
Additionally, we have included representative images of three whole histological mouse aortas for each group (Figure 4E in revised manuscript). We initially planned to dissect the adventitial tissues of each aorta for gross representation, as shown in Figure 4E. However, if we had done so, the loss of adventitial tissues would have prevented us from investigating the immune cells, which are predominantly located in the adventitial layer.

Furthermore, we assessed the grade of elastic degradation in each group (Figure 4I in revised manuscript).

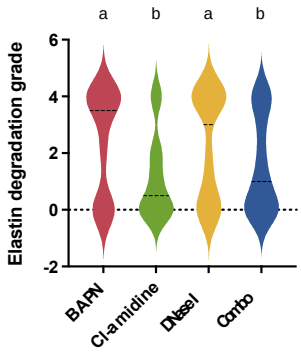
Revision: Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD.
(B) The incidence of AD and aortic rupture in each group (n = 12).
(C) Mice survival due to aortic rupture of each indicated group.



(E) Macrographs of aortas in each group.



(I) Elastin degradation grading evaluation of aorta in each group.

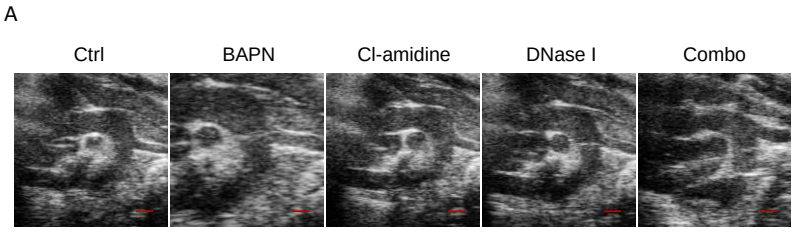


7. Since the authors interpret the effects of anti-NET therapy to "ameliorate dissection progression", it would be essential to additionally document the rate of dissection and max. aortic diameter dilatation at treatment start (i.e. 1 week after AD induction by BAPN, immediately before therapy initiation). Without this comparison, it is difficult to deduce the extent of reduction/blockade of disease progression.

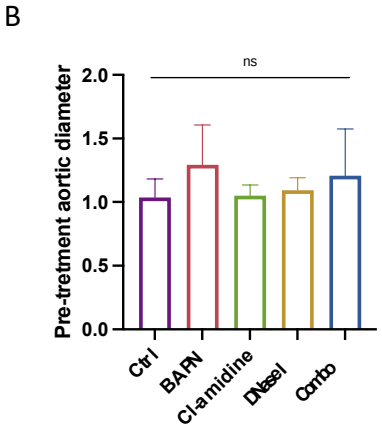
Response: Thank you very much for this constructive suggestion. In the second animal experiment, we carefully measured the pre-treatment largest aortic diameter using vascular ultrasound. Representative images of each group and corresponding quantification are provided in Supplementary Figures 6A and 6B. We confirmed that the aortic diameter dilations at the start of treatment did not differ between groups.

Revision: Baseline aortic diameters of each mouse were measured by vascular ultrasound at day 7 before the administration of NETs inhibitors, and no significant difference was observed among groups (Supplementary Figures 6A and 6B). Of note, we happened to notice two mice with enlarged section located in proximal descending aorta in BAPN and combination treatment groups (Supplementary Figure 6C). (Lines 345-350 in revised manuscript)

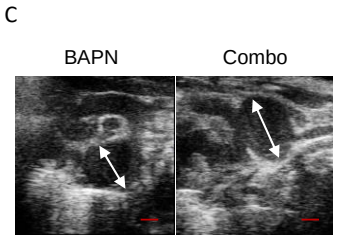
Supplementary Figures 6. Inhibition of NETs attenuates dissection progression in mice with AD. (A) Representative pre-treatment vascular ultrasound images of aorta in each group. Scale bar = 1 mm.



(B) Baseline aortic diameters of each mouse measured by vascular ultrasound at day 7 before the administration of NETs inhibitors.



(C) The representative images of two mice with enlarged section located in proximal descending aorta (indicated by white arrow) in BAPN and combination treatment groups. Scale bar = 1 mm.



Data and methodology:

8. Proteomic analysis was conducted for plasma samples of AD patients and healthy controls. Please give more information as to when the blood samples were retrieved from AD patients with respect to the occurrence of acute AD and treatment interventions. Please also provide the basic demographics of the two collectives (sex and age distribution, co-morbidities, medication, etc.) to document how well the two groups were matched. Observed differences might be due to confounders, if groups were not well matched. Furthermore, if routine blood parameters (such as D-dimer, CRP, blood lipids etc.) were measured (as indicated in Fig. 1A), a supplementary table with the additional information – including p-values - would be helpful in assessing group differences.

Response: Thank you very much for this valuable suggestion. We have collected and analyzed the baseline characteristics of patients with acute AD and matched healthy individuals involved in mass

spectrometry. After performing propensity score matching with a 1:1 ratio and exact matching for age at onset and gender (p-values: Age = 0.113 and Gender = 1), we found appropriate matching quality. Additionally, we identified several statistically significant baseline characteristics, including AD-related history of hypertension, inflammatory indicators, and D-dimer levels, all of which are closely associated with the onset of AD. We have incorporated this information into the revised manuscript and supplementary table.

Revision: We conducted a proteomic analysis comparing the plasma samples of 30 patients with acute AD and 30 matched healthy individuals, which were screened from a total of 128 subjects, with the data-independent acquisition quantitative proteomic approach using mass spectrometry. The age at onset and gender did not differ between patients with AD and matched controls after an exact matching, indicating a proper matching quality (Supplementary Table 1). Other baseline characteristics including AD etiologically related history of hypertension, inflammatory indicators, and D-dimer were statistically significant between two groups (Supplementary Table 1). (Lines 123-130 in revised manuscript)

Proteomic analysis. Proteomic data was obtained from plasma samples of 30 patients with AD and 30 matched healthy individuals. To reduce the confounding bias of unintended variables, propensity score matching was conducted with the following parameters: nearest-neighbor matching (1:1) with a caliper of 0.2; exact matching for age at onset and gender. Matching quality was evaluated by comparing baseline characteristics between patients with AD and matched healthy subjects. (Lines 645-650 in revised manuscript)

Supplementary Table 1. Baseline characteristics of patients with acute AD and healthy individuals involved in mass spectrometry.

	AD (N=30)	CL (N=30)	P-value
Age (y)	57.1 ± 12.0	52.8 ± 8.1	0.113
Gender	0.9 ± 0.3	0.9 ± 0.3	1
Hypertension	0.7 ± 0.5	0.0 ± 0.0	<.001
Smoking	0.5 ± 0.5	0.2 ± 0.4	0.012
Neutrophil ($\times 10^9$ /L)	7.0 ± 2.1	3.8 ± 0.8	<.001
Monocyte ($\times 10^9$ /L)	0.8 ± 0.4	0.6 ± 0.6	0.189
Lymphocyte ($\times 10^9$ /L)	1.3 ± 0.5	2.0 ± 0.9	0.006
Platelet ($\times 10^9$ /L)	234.9 ± 93.5	259.9 ± 54.4	0.26
D-dimer (mg/L)	5.1 ± 5.2	0.2 ± 0.1	<.001
CRP (mg/L)	45.5 ± 54.9	71.7 ± 53.3	0.23
ALT (U/L)	29.7 ± 38.4	34.8 ± 16.9	0.542
AST (U/L)	23.1 ± 19.8	27.7 ± 7.3	0.272
Cholesterol (mmol/L)	4.7 ± 1.0	5.2 ± 0.6	0.115
Triglyceride (mmol/L)	1.2 ± 0.4	1.9 ± 0.6	0.001

CRP: C-reactive protein; ALT: Alanine transaminase; AST: Aspartate aminotransferase.

9. The same holds true for the 187 plasma samples of AD patients and 58 samples of healthy individuals which were used for evaluation of inflammation, neutrophil and NET parameters by ELISA: Please provide a comparison of demographic/routine variables of the

study collectives and specify the time point of blood withdrawal for the AD group. A multivariable analysis would strengthen the authors´ conclusion that the elevated neutrophil/NET parameters are indeed associated with AD rather than potential confounders.

Response: Thank you very much for this constructive comment. All venous blood was drawn within three days before TEVAR. We conducted comparisons of demographic variables among patients and performed univariable/multivariable logistic regression analyses, which are presented as follows.

Supplementary Table 2. Baseline characteristics of patients with AD and healthy individuals in diagnostic cohort.

	AD (N = 187)	CL (N = 59)	P-value
Gender	29 (15.5%)	27 (45.8%)	<.001
	158 (84.5%)	32 (54.2%)	
Age	56.6 ± 13.6	46.7 ± 14.6	<.001
D-dimer	4.5 ± 5.2	3.6 ± 5.1	0.227
WBC ($\times 10^9$ /L)	7.5 ± 4.4	7.3 ± 1.8	0.459
Neutrophil ($\times 10^9$ /L)	6.4 ± 2.6	4.4 ± 2.2	<.001
Monocyte ($\times 10^9$ /L)	0.8 ± 0.8	0.4 ± 0.2	<.001
Lymphocyte ($\times 10^9$ /L)	1.3 ± 0.5	2.4 ± 0.6	<.001
Platelet ($\times 10^9$ /L)	202.3 ± 71.1	255.7 ± 65.2	<.001
Albumin (g/L)	40.7 ± 4.8	46.2 ± 2.7	<.001
Creatinine (μmol/L)	91.9 ± 92.2	74.0 ± 17.2	0.012
GFR (ml/min)	71.3 ± 32.6	81.3 ± 30.0	0.038
Cholesterol (mmol/L)	4.1 ± 1.1	4.8 ± 1.0	<.001
Triglyceride (mmol/L)	1.4 ± 0.9	1.5 ± 1.4	0.646
LDL (mmol/L)	2.3 ± 0.8	3.2 ± 0.9	<.001
HDL (mmol/L)	1.4 ± 0.9	1.2 ± 0.3	0.01
CRP (mg/L)	56.5 ± 52.7	45.7 ± 49.2	0.165
CitH3 (ng/mL)	4.4 ± 3.0	1.8 ± 1.3	<.001
cf-DNA (ng/mL)	2.0 ± 0.7	1.7 ± 0.6	0.006
MPO (ng/mL)	4.4 ± 2.5	2.8 ± 1.7	<.001
NE (ng/mL)	4.1 ± 1.9	2.2 ± 1.3	<.001
IL-1β (pg/mL)	94.2 ± 29.0	86.6 ± 26.7	0.074
IL-6 (pg/mL)	45.2 ± 19.7	37.7 ± 17.4	0.01
Hypertension	0.8 ± 0.4	0.1 ± 0.2	<.001
Smoking	0.6 ± 0.5	0.1 ± 0.2	<.001
Alcohol abuse	0.4 ± 0.5	0.1 ± 0.2	<.001
Diabetes mellitus	0.4 ± 0.5	0.1 ± 0.2	<.001
Coronary heart disease	0.0 ± 0.2	0.0 ± 0.0	0.004
Stroke	0.0 ± 0.1	0.0 ± 0.0	0.319
Chronic kidney disease	0.0 ± 0.2	0.0 ± 0.0	0.004

WBC: White blood cell; GFR: Glomeruar filtration rate; LDL: Low density lipoprotein; HDL: High

density lipoprotein; CRP: C-reactive protein; CitH3: citrullinated histone H3; cf-DNA: cell free-DNA; MPO: myeloperoxidase; NE: Neutrophil elastase.

Supplementary Table 3. LASSO and multivariable logistic regression analyses of patients with AD and healthy individuals in diagnostic cohort.

	OR (95% CI)	P-value
Gender	1.98 (0.37-10.75)	.416
Age	1.01 (0.96-1.07)	.638
Neutrophil	0.75 (0.53-1.03)	.079
Monocyte	7.36 (1.71-9.67)	< .001
Lymphocyte	0.79 (0.10-0.89)	< .001
Platelet	0.99 (0.98-1.01)	.325
LDL	0.41 (0.18-0.87)	.025
HDL	1.03 (0.42-4.10)	.952
CRP	1.00 (0.99-1.01)	.976
CitH3	2.02 (1.39-3.38)	.002
cf-DNA	0.84 (0.22-3.20)	.791

LDL: Low density lipoprotein; HDL: High density lipoprotein; CRP: C-reactive protein; CitH3: citrullinated histone H3; cf-DNA: cell free-DNA.

10. The multivariable Cox regression model calculated in the prognostic study (for prediction of adverse events after AD repair by TEVAR) included all investigated plasma proteins of inflammation (IL-1β, IL-6) and neutrophil activation (elastase, MPO) or NET formation (citH3, cell-free DNA) – even though the majority of these factors did not differ significantly between patients with and without adverse events. Analyzing the two significant parameters (citH3 and MPO) with previously reported risk factors (such as identified in [6]) and prognostic variables for complications post-TEVAR would provide more information as to the usefulness of the investigated neutrophil/NET parameters. The authors have recently published on a systemic immune inflammation index to predict adverse events after AD-TEVAR [3]. How do NET parameters perform in comparison to this predictive index?

Response: Thank you very much for this constructive comment. We have compared the baseline characteristics between patients and reanalyzed the prognostic value of NETs associated markers with previously reported risk factors including D-dimer, NLR, SIRI, etc, based on LASSO regression to cherry-pick the important variable and to reduce the potential bias. We found that though recently established predictors (D-dimer, NLR, SIRI) showed close association with poor prognosis, CitH3 still exhibited excellent predictive accuracy with HR of 1.18 (95% CI 1.05-1.33) and p-value of .004 after adjusting the confounding factors in the multivariable analysis. While MPO seemed to lose its superiority, particularly when competing with other predictive indexes (p = .847). In addition, our previously established systemic inflammatory response index showed seemingly good but not very stable predictive accuracy with HR of 15.33 (95% CI 3.82-61.53) with p value of .001 in univariable regression, and even a wider range of HR of 14.23 (95% CI 1.82-111.31) and p-value of .011 after adjusting confounding factors. Therefore, CitH3 exhibited the strongest performance among the previously reported risk factors.

Revision:

Supplementary Table 4. Baseline characteristics of patients with AD and healthy individuals of patients receiving TEVAR in prognostic cohort.

		non-AAEs (N=85)	AAEs (N=31)	P-value
AAEs		1 (1.2%)	0 (0%)	<.001
	AAEs	0 (0%)	31 (100%)	
	non-AAEs	84 (98.8%)	0 (0%)	
Gender		1 (1.2%)	0 (0%)	0.077
	Female	12 (14.1%)	10 (32.3%)	
	Male	72 (84.7%)	21 (67.7%)	
Age		56.2 ± 13.7	54.0 ± 14.5	0.468
Phase		2 (2.4%)	3 (9.7%)	0.328
	Acute	70 (82.4%)	22 (71%)	
	Subacute	9 (10.6%)	4 (12.9%)	
	Chronic	4 (4.7%)	2 (6.5%)	
Hypertension		0.9 ± 0.3	1.0 ± 0.2	0.259
D-dimer		4.5 ± 5.1	7.2 ± 7.0	0.056
Neutrophil		6.3 ± 2.6	7.2 ± 2.4	0.088
Monocyte		0.7 ± 0.3	0.7 ± 0.3	0.413
Lymphocyte		1.3 ± 0.4	1.2 ± 0.4	0.366
Platelet		211.4 ± 79.5	192.1 ± 70.8	0.238
ALB		40.5 ± 4.4	40.5 ± 3.5	0.97
Cholesterol		4.1 ± 1.2	4.1 ± 0.7	0.734
Triglyceride		1.4 ± 0.9	1.1 ± 0.5	0.036
LDL		2.2 ± 0.8	2.4 ± 0.6	0.101
HDL		1.5 ± 1.1	1.3 ± 0.9	0.267
NLR		5.0 ± 3.2	6.2 ± 3.4	0.086
PLR		166.8 ± 66.3	179.7 ± 69.8	0.362
MLR		4.3 ± 3.0	5.0 ± 2.4	0.234
SII		1014.1 ± 702.5	1163.4 ± 572.1	0.291
SIRI		0.6 ± 0.2	0.7 ± 0.3	0.018
CitH3		4.3 ± 2.4	6.9 ± 3.2	<.001
cf-DNA		2.0 ± 0.6	2.0 ± 0.7	0.65
MPO		4.8 ± 2.3	6.0 ± 2.5	0.02
NE		4.1 ± 1.9	4.2 ± 1.7	0.834
IL-1β		94.9 ± 29.1	93.0 ± 26.8	0.755
IL-6		43.8 ± 20.0	43.5 ± 17.6	0.946

LDL: Low density lipoprotein; HDL: High density lipoprotein; NLR: Neutrophil-to-lymphocyte ratio; MLR: Monocyte-to-lymphocyte ratio; PLR: Platelet-to-lymphocyte ratio; SII: Systemic immune inflammation index; SIRI: Systemic inflammatory response index; CitH3: citrullinated histone H3; cf-DNA: cell free-DNA; MPO: myeloperoxidase; NE: Neutrophil elastase.

Supplementary Table 5. Univariable and multivariable Cox proportional hazard regression

analyses of patients receiving TEVAR.

	Univariable		Multivariable	
	HR (95% CI)	P-value	HR (95% CI)	P-value
D-dimer	1.04 (1.00-1.09)	.049	1.02 (0.97-1.07)	.510
Neutrophil	1.17 (1.03-1.33)	.017	1.13 (0.95-1.35,)	.174
NLR	1.13 (1.04-1.22)	.004	1.07 (0.87-1.30,)	.524
MLR	1.14 (1.04-1.25)	.007	0.89 (0.68-1.16)	.379
SIRI	15.33 (3.82-61.53)	.001	14.23 (1.82-111.31)	.011
CitH3	1.24 (1.11-1.37)	< .001	1.18 (1.05-1.33)	.004
MPO	1.18 (1.01-1.39)	.036	1.02 (0.84-1.23)	.847

NLR: Neutrophil-to-lymphocyte ratio; MLR: Monocyte-to-lymphocyte ratio; SIRI: Systemic inflammatory response index; CitH3: citrullinated histone H3; MPO: myeloperoxidase.

11. In accordance with the journal guidelines, please include the sex/gender distribution of your human samples and possibly explain the focus on male mice in your experimental model.

Response: Thank you very much for this comment. The baseline characteristics of patients included in this study are additionally present in the supplementary materials. There were three females in both the AD and the healthy group included in the proteomics cohort of 60 subjects. In the prospective AAEs cohort, consisting of a total of 115 patients, there were 12 females among the 84 non-AAEs patients and 10 females among the AAEs patients.

We focused on male mice since sexual dimorphism has been reported in AD formation in humans and mice. In humans, AD has been more prevalent in men than in women.[1-4] Likewise, in several mouse models, the incidence and severity of aortic pathologies are sexually dimorphic, with greater severity in male mice.[5-7] While no studies have reported sexual dimorphism when BAPN is administered alone, a few studies have indicated a lower incidence of AD in female mice co-administered BAPN and Angiotensin-II.[8,9] Taking these factors into consideration, similar to other studies published in top-tier cardiovascular journals[10-12], we chose to use male mice for our experiments.

12. Please specify whether tissues were processed immediately after retrieval from surgical intervention or were stored (for how long? under which conditions?) prior to cell isolation. Was the time between resection and processing consistent or did it vary (and to which extent)?

Response: Thank you very much for this comment. All of our fresh tissues were processed within 3 hours after retrieval from surgical intervention, before which they were kept in RPMI 1640 containing 10% FBS on ice. We have included this description in the Methods section.

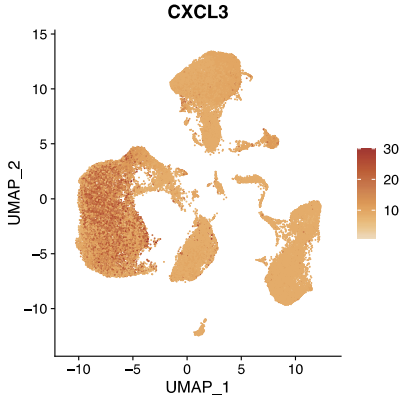
Revision:

Tissue dissociation and cell purification. Fresh tissues were stored in RPMI 1640 containing 10% FBS on ice immediately after retrieval from surgical intervention. All tissues were further processed within 3 hours. (Lines 580-584 in revised manuscript)

13. Please add CXCL3 as a gene expression – projected UMAP plot to Suppl. Fig. 2B, since it would be of interest to see whether the two identified AD-associated macrophage clusters are the main producers.

Response: Thank you very much for this comment. We have included the UMAP plot that the expression level of CXCL3 projected on into Supplementary Figure 4F. As we can see as follows, the phenotype-associated macrophages highly express CXCL3 compared with other cell populations.

Revision: Supplementary Figure 4. Cell-cell interactions in aortic lesions in AD.
(E) The expression level of CXCL3 projected on UMAP plot.



14. In our experience, most CTAD plasma samples of healthy individuals range close to the detection limit of the applied commercial citH3 ELISA. Please provide the information, how many samples of the AD and healthy control groups were within the detection range of the ELISA (and at which sample dilution).

Response: Thank you very much for this comment. The majority of our plasma samples were obtained from patients with acute AD, with a few from those with subacute and chronic AD. In the prospective AAEs cohort, there were 92 patients with acute AD, 13 patients with subacute AD, and 6 patients with chronic AD (shown in supplementary tables). In our experience, all of the plasma sample were within the detection range. The ELISA used in this paper adopts the sandwich method at 1:2 sample dilution.

Revision:
Measurement of human plasma. Peripheral, human venous blood was drawn into prechilled citrate, theophylline, adenosine, dipyridamole containing tubes for processing within 60 minutes of collection. The plasma was obtained by centrifugation of whole blood at 8,000 x g at 4℃ for 20 minutes and stored in aliquots at -80℃. The NETs markers MPO, NE, IL-1β, IL-6 were measured by ELISA kit (Thermo Fisher). Circulating cell-free DNA was quantified in NET samples by using Quant-iT™ PicoGreen® dsDNA (P7589, Invitrogen). Citrullinated histone H3 (CitH3) was measured as previously described.[46]

REFERENCE

46. Thålin, C., et al. Quantification of citrullinated histones: Development of an improved assay to reliably quantify nucleosomal H3Cit in human plasma. J Thromb Haemost 18, 2732-2743 (2020).

15. BAPN is frequently administered in drinking water. Please specify the mode and frequency of BAPN "infusion" mentioned in the methods section – and the applied drug concentration.

Response: Thank you very much for this comment. In our study, BAPN was administered in drinking water at a dose of 5 mg/mL (0.5% wt/vol). This is a more direct description compared to recent studies that report a dose of 1 g/kg/day BAPN in drinking water, where the calculation method for this dose is unclear.[13]

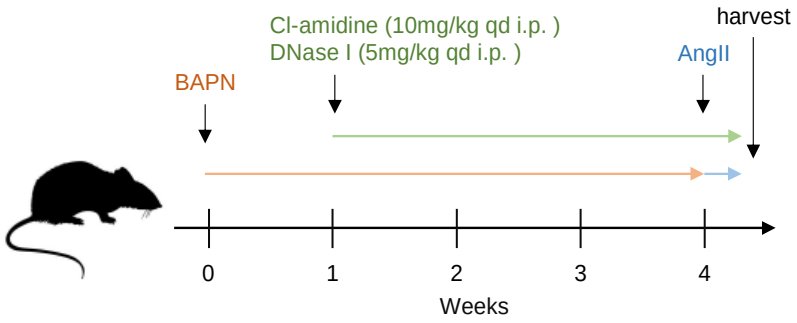
Revision: **Mice and In Vivo Experimental Strategies.** All animal experiments were approved by the Institutional Animal Care and Use Committee at Zhongshan Hospital. Wild-type C57BL/6J male mice were used in this study and were purchased from the Beijing Vital River Laboratory Animal Technology. Mice were housed in the animal facility of Zhongshan Hospital under a 12/12-h light/dark cycle, in a temperature-controlled manner with free access to food and water. Unchallenged 3-week-old male control mice were fed a chow diet and infused with saline. Three-week-old male WT mice were challenged with β-aminopropionitrile (BAPN), an inhibitor of lysyl oxidase (LOX) activity, for 4 weeks. BAPN was administered in drinking water with the dose at 5 mg/mL (0.5% wt/vol). (Lines 689-697 in revised manuscript)

16. The information on the time frame of BAPN treatment is inconclusive. It is mentioned that BAPN was administered for 4 weeks (line 586) but a 6-week period is indicated in Figure 6A and in the Methods (line 591) and Results section (line 319). It is also unclear to me why anti-NET therapy was only given for 2 weeks (according to Fig. 6A in week 2 and 3 of BAPN administration) and was apparently discontinued in experimental weeks 4-6 prior to animal sacrifice!?

Response: Thank you for your insightful observations. We apologize for any confusion regarding the time frame of BAPN treatment. BAPN was administered for 4 weeks from the 3 weeks of age. Initially, we extended another two-week period in order to observe possible subsequent aortic rupture of mice. However, we found that the 6-week strategy is not widely adopted in the AD model. Consequently, we revised our approach in our second attempt to a 4-week BAPN administration period, aligning it with more commonly used protocols.

We at first applied a 2-week-period of anti-NET therapy for the reason that, as an acute disease, AD is characterized by neutrophil activation and NETs formation in the early-phase of disease onset. And mice are prone to develop aortic rupture, especially when they are under stress that intraperitoneal injections may induce. To minimize potential stress on the mice, we tried to reduce the frequency of injections. Therefore, we applied a 2-week-period of anti-NET therapy in the original design. After receiving your suggestion, we read more high-quality studies concerning this topic, and found most of them have employed the strategy of blocking NETs as you mentioned.[14,15] We then refined our experiment design. Thank you again for this valuable suggestion.

Revision: Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD. (A) Schematic overview of experimental design. Each mouse received intraperitoneal injection of saline, Cl-amidine (10mg/kg), DNase I (5mg/kg) or both drugs at a fixed time once a day during 4-7 weeks of age (1-4 week of experiment) until death or the end point of the experiment.



Mice and In Vivo Experimental Strategies. All animal experiments were approved by the Institutional Animal Care and Use Committee at Zhongshan Hospital. Wild-type C57BL/6J male mice were used in this study and were purchased from the Beijing Vital River Laboratory Animal Technology. Mice were housed in the animal facility of Zhongshan Hospital under a 12/12-h light/dark cycle, in a temperature-controlled manner with free access to food and water. Unchallenged 3-week-old male control mice were fed a chow diet and infused with saline. The challenged three-week-old male WT mice were challenged with β-aminopropionitrile (BAPN) for 4 weeks. BAPN was administered in drinking water with the dose at 5 mg/mL (0.5% wt/vol). Four weeks later, the mice were infused with Ang II (1,000 ng/kg/min) with mini-osmotic pumps (Model 1003D, Alzet) for 3 days to induce AAD.

During the four-week of BAPN infusion period, the challenged mice were also given daily intraperitoneal injections of either PBS (n = 12), or Cl-amidine (10 mg/kg, Sigma-Aldrich) (n = 12), or DNase I (5 mg/kg, Roche Diagnostics) (n = 12) or the combination of Cl-amidine and DNase I (n = 12) for **3 weeks from the 4 weeks of age**. The dead mice were immediately dissected and the surviving mice were euthanized at the end of the **4-week study period**. The aortas were exposed and rinsed with cold PBS, and the periaortic tissues were removed. The aorta was then excised, further cleaned, and rinsed with cold PBS to remove any residual blood in the lumen. Next, aortas were imaged for diameter measurement and disease evaluation. Each mouse aorta was stained for H&E to observe AD occurrence, characterized by more or less elastin degradation and rupture in aortic media layer. (Lines 698-713 in revised manuscript)

17. Considering the high death rate in murine AD induction, is the group size of n=8 per experimental group referring to surviving animals to the experimental endpoint after 6 weeks?

Response: Thank you very much for this comment. We conducted a second mouse experiment and increased the number of mice in each group (n = 12). The group size of n = 12 per experimental group is referring to mice at the beginning of the experiment.

The murine AD model usually has a high mortality rate, which poses a tricky problem: if we do not promptly dissect the mice after death, the mouse carcasses may be eaten by their cage mates,

rendering the aortas unavailable for study. We tried our best to minimize these potential losses, we checked the survival condition of the mice five to six times a day, including during both daytime and nighttime. Therefore, we successfully collected the aortas from all the mice.

> ***18. Regarding "Immunofluorescence analysis of NETs in tissue sections" the authors describe the application of a mouse antihuman Ly6G antibody (ab238132, Abcam). To the best of my knowledge, anti-Ly6G antibodies can only be used to detect neutrophils in murine but not in human tissues as there is no human orthologue for Ly6G [7]. According to Abcam, ab238132 is a rabbit anti-mouse Ly6G antibody. Hence, I assume that the description relates to the separate IHC stainings for citH3 or Ly6G in mouse AD sections (Suppl. Figure 5B). However, since these two antibodies are rabbit-derived, they should not be combined for IF co-stainings as shown in Figure 6E. Therefore, the data shown in Figure 6E are difficult to interpret. Especially the citH3 staining of all nuclei in the Cl-amidine treated mouse is highly unexpected and looks more like a staining/autofluorescence artefact. Please give more details for the mouse IF co-stainings of Figure 6E – and also provide information on the IF staining method of human sections for citH3 and MPO (Figure 4B). Please also indicate in Figure 6 and Suppl. Fig. 5B from which part of the murine aorta the sections were taken, and what you consider the true versus false lumen. Were measurements of maximum diameter focused on the thoracic part of the aorta (ascending/arch/descending)?***

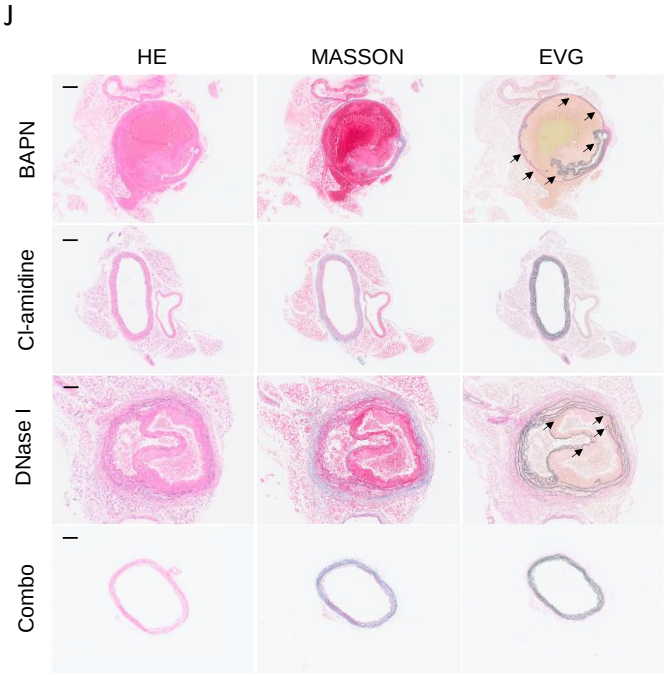
Response: Thank you very much for this valuable comment. We used two rabbit antibodies to indicate NETs formation, determined by staining CitH3 and Ly6G. **Tyramide signal amplification** was applied to enhance the fluorescence amplification signal and to avoid limit to the species origin of primary antibody.[16] Previous studies have also employed this method, and we have listed them for your reference.[17-19]

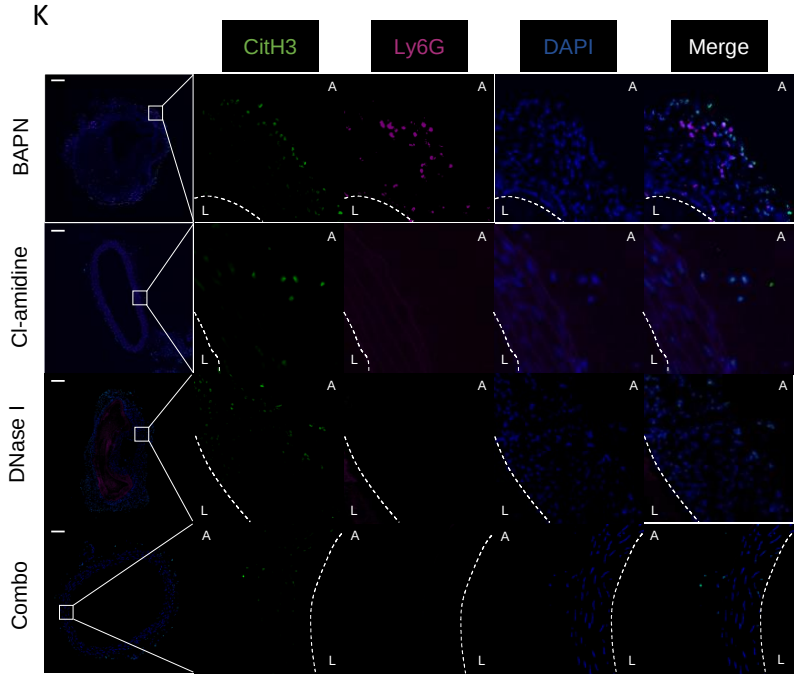
Regarding the point you mentioned that "the citH3 staining of all nuclei in the Cl-amidine treated mouse is highly unexpected and looks more like a staining/autofluorescence artefact", we adjusted the intensity of the fluorescence, and found staining in and out of a subset of cells, particularly in the adventitial aorta, confirming the authenticity of NETs formation.

The measurements of maximum diameter focused on both the ascending and descending thoracic section of the aorta, since AD occurred at all thoracic part in our study. In revised Figures 4-6 and Supplementary Figures concerning mouse aorta, the representative four case of H&E and IF staining imagines were taken from descending part of the murine aortas. Thank you again for this valuable comments.

As for true versus false lumen, the true lumen is covered with endothelial cells that could prevent thrombosis, while we usually observe large thrombosis and broken elastin in the false lumen

Revision: Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD.
(J) Representative immunohistochemistry images showing aortic dilation, false lumen formation, and elastin degradation within aortas in each group. Arrow indicating broken elastin.
(K) Representative immunofluorescence images showing NETs formation within aortas of mice, determined by CitH3 (citrullinated histone H3, green) and neutrophil (Ly6G, red) staining. Scale bar = 100 μm.





Minor issues:

19. Methods/Proteomic Analysis: The word "entially" is used twice. Please explain. Or is it erroneously shortened and derived from "differentially"?

Response: Thank you very much for pointing out the error. We apologize for not completely deleting the word "differentially" It has been corrected in the revised manuscript.

Revision:

Proteomic analysis. Proteomic data was obtained from plasma samples of 30 patients with AD and 30 matched healthy individuals. To reduce the confounding bias of unintended variables, propensity score matching was conducted with the following parameters: nearest-neighbor matching (1:1) with a caliper of 0.2; exact matching for age at onset and gender. Matching quality was evaluated by comparing baseline characteristics between patients with AD and matched healthy subjects. The difference of protein expression patterns was quantitatively analyzed based on 4D data-independent acquisition (DIA) mass spectrometry. edegR was used for differential gene analysis among samples, p values were calculated and corrected by multiple hypothesis testing. The threshold of p values was determined by controlling FDR (False discovery rate). The corrected p value (p.adjust value) was q value. A Fold change (FC), calculated based on the FPKM value, was defined as a **differentially** expressed gene (DEG) with a value of q (or p.adjust) < 0.05 after the comparison of 2 data, The absolute value of Log2 expression ratio is greater than 1. Genomes The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis results are displayed as bubble graphs. (Line 645-659 in revised manuscript)

20. Results: Paragraphs in the Results section contain background information (e.g. page 4, line 113-119) which is normally given in the Introduction. Furthermore, conclusions (e.g. page 4, line 120-123) should be moved from the Results to the Discussion section.

Response: Thank you very much for your comment. We completely agree with you and have moved this paragraph to the Discussion section.

Revision: Previous studies have demonstrated the ability of neutrophils to undergo NETosis, a process by which they produce and release NETs, which are lattices composed of DNA filaments interspersed with enzymes that effectively immobilize and eradicate bacteria. However, the role of NETosis extends beyond just protection from infectious conditions, as it also contributes to autoimmune diseases, the initiation and facilitation of metastasis of malignant tumors, the progression of cardiovascular diseases, the promotion of abnormal coagulation and thrombosis, aiding in wound healing and the response to traumatic events. (Lines 524-530 in revised manuscript)

21. Supplementary Figure 2: Mast cells seem to be included in the heatmap but are not mentioned in the color code for cell clusters (as in Figure 2).

Response: Thank you very much for the question. We missed the mat cell legend in the heatmap plot. We correct it in the revised manuscript.

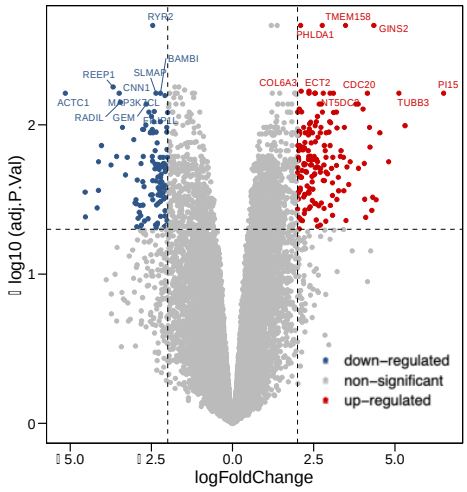
Revision: Supplementary Figure 2. Single-cell transcriptomic analyses show cell populations with expression patterns in aortic lesions in AD.
(A) Heatmap showing the most upregulated genes (ordered by decreasing p-value) in each cluster and selected enriched genes used for biological identification of each cluster (Scale: $\log_2$ Fold Change).

22. Legend to Supplementary Figure 3G: Please indicate more clearly that these data refer to the publically available bulk RNAseq data set, while all other parts of Suppl. Fig. 3 seem to relate to the self-conducted scRNAseq analysis of aortic tissue from 3 AD patients vs 3 healthy controls.

Response: Thank you for the comment. We have denoted the data in volcano plot refer to publicly available bulk RNA-seq dataset, which could be assessed from GEO database.

Revision: Supplementary Figure 3. Single-cell transcriptomic analyses show cell populations with putative functions in aortic lesions in AD.
(G) Volcano plot shows the differentially expressed genes between patients with AD (n = 7) and healthy individuals (n = 5) from a publicly available bulk RNA-seq dataset (GSE52093).

G



23. Supplementary Figure 1A-D: Please indicate more clearly, whether a positive or negative enrichment score indicates association with AD. It seems that the negative score indicates upregulation in AD patients.

Response: Thank you for the comment. A positive enrichment score indicates association with AD in Supplementary Figures 1A-1D. We have denoted it in the revised manuscript.

Revision: Supplementary Figure 1. Systemic inflammation is elevated in peripheral blood from patients with AD. (A) GSEA of biological processes of differently expressed proteins. (B) GSEA of cellular component in differently expressed proteins. (C) GSEA of molecular function in differently expressed proteins. (D) KEGG analysis in differently expressed proteins. **A positive enrichment score indicates association with AD.** KEGG: Kyoto Encyclopedia of Genes and Genomes; GSEA: Gene set enrichment analysis. (Lines 1050-1057 in revised manuscript)

24. Under Methods (line 566) it is mentioned that the clinical data of AD patients with endovascular repair were retrospectively analyzed in the prognostic study, while it is defined as a prospective cohort study (line 288) in the Results section. Please specify.

Response: Thank you for your comment. Our cohort was prospectively established, and we inadvertently wrote otherwise in the Methods section. We have already corrected this error. Thank you for bringing it to our attention.

Revision:

Prognostic cohort design. We **prospectively** collected and analyzed the clinical data of patients with type B aortic dissection who underwent endovascular therapy in the Zhongshan Hospital of Fudan University between November 2016 and November 2020. (Lines 675-677 in revised manuscript)

Reference to previous literature:

25. The entire introduction is lacking references, i.e. please support your statements by the relevant literature citations on AD and TEVAR.

Response: Thank you for your comment. We have reorganized our Introduction part and cited relevant literature accordingly.

Revision:

INTRODUCTION

Over the past few decades, significant progress has been made in the treatment of aortic dissection (AD), a life-threatening condition caused by a tear in the intimal layer of the aorta that allows blood to surge into the wall of the tissue, leading to the formation of true and false lumens. Minimally invasive thoracic endovascular aortic repair (TEVAR) has proven effective in stabilizing the dissected aorta with benefit for patients in the early stages of AD.[1,2] However, TEVAR adeptly seals the proximal tear and leaves distal entries open, resulting in an imbalanced blood flow distribution to the distal aorta. In certain patients, persistent blood flow from the true lumen into the false lumen via distal entries contributes to suboptimal or adverse aortic remodeling, thereby increases the risk of aorta-related adverse events (AAEs), and subsequently diminishes overall prognosis of AD.[3-6] Thus, predicting the risk factors for future AAEs in patients with AD is crucial to determine a therapeutic strategy. A comprehensive understanding of the onset and progression of AD is necessary to facilitate the exploration of risk factors that can predict the prognosis of patients with AD and identify those at high risk of postoperative AAEs, ultimately optimizing the efficacy of TEVAR.

To monitor the aortic remodeling, recent studies mainly focus on the clinical manifestation and anatomical and morphological changes of aorta based on imaging methods. Several clinical and imaging-related risk factors have been shown to be associated with early disease progression.[7-12] Established risk factors such as the thrombus in the false lumen[13] and the angle of aortic arch[14] are closely associated with postoperative aortic remodeling. The evaluation of morphological change of aorta often relies on computed tomography angiography or magnetic resonance angiography, while can be applied only under certain circumstances due to large radiation dose, high cost of examination, contrast agent-induced nephropathy, and failing to provide functional or dynamic assessment. Serum biomarkers could accurately reflect the pathophysiological changes in the process of disease occurrence and development. In recent years, some progress has been made concerning the diagnosis and prognosis of AD, including D-dimer, inflammation and lipid metabolism associated markers, smooth muscle cell and extracellular matrix related markers, microRNA, and cell-free DNA, which may act synergistically with existing clinically known risk factors to identify individuals at high risk for poor aortic remodeling.[15,16] Due to the limited sensitivity and specificity, inconsistent results between different studies, and the lack of case of subacute and chronic acute dissection, quite a part of the existing candidate biomarkers are still in the preclinical stage.

Single-cell sequencing enables the comprehensive characterization of cells in a complex and heterogeneous tissue ecosystem, which is of indispensable importance to facilitate prognostic biomarker discovery and cell type targeted therapy.[17-19] However, most expression-based assays

that are clinically used rely on bulk transcriptional analysis. Bulk RNA sequencing measures the averaged properties of whole tissue from large samples and provides comprehensive clinical and pathologic phenotype information.[20,21] Therefore, leveraging valuable and widely available phenotype information in bulk data to guide cell subpopulation identification from single-cell data is a promising approach to exploring and interpreting single-cell data from a new perspective.

Here, we performed the multi-omics profiling to unveil highly associated cell types and disease-relevant signaling pathways implicated in the onset progression of AD. This approach allowed us to identify prognostic biomarkers and potential therapeutic targets, providing a more objective and detailed theoretical basis for the development of effective personalized and precision medicine for patients with AD.

REFERENCE

1. Erbel, R., et al. 2014 ESC Guidelines on the diagnosis and treatment of aortic diseases: Document covering acute and chronic aortic diseases of the thoracic and abdominal aorta of the adult. The Task Force for the Diagnosis and Treatment of Aortic Diseases of the European Society of Cardiology (ESC). Eur Heart J 35, 2873-2926 (2014).
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3. Afifi, R.O., et al. Outcomes of Patients With Acute Type B (DeBakey III) Aortic Dissection: A 13-Year, Single-Center Experience. Circulation 132, 748-754 (2015).
4. Garbade, J., et al. Outcome of patients suffering from acute type B aortic dissection: a retrospective single-centre analysis of 135 consecutive patients. Eur J Cardiothorac Surg 38, 285-292 (2010).
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17. Yofe, I., Dahan, R. & Amit, I. Single-cell genomic approaches for developing the next generation of immunotherapies. Nat Med 26, 171-177 (2020).
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21. Weinstein, J.N., et al. The Cancer Genome Atlas Pan-Cancer analysis project. Nat Genet 45, 1113-1120 (2013).

26. Conversely, I did not find the in-text citations for references 1-3 on single cell omics.

Response: Thank you for your comment. We reorganized our Introduction part and cited relevant literature accordingly. We revised the citations about single-cell omics. Please see the revised Introduction placed after Q25.

27. The discussion is very well written but predominantly focusses on the diversity and role of macrophage subsets and neutrophils/NETs in atherosclerosis, while comparably little is discussed on what is known for aneurysms. Since aneurysm formation is closely linked to AD, the pertaining literature may be more relevant to compare.

Response: Thank you for your comment. We additionally discussed several studies concerning aneurysms.

Revision:

DISSCUSSION

Previous study pinpointed the macrophage subset expressed several cytokine genes as the predominant origin of detrimental molecules for aneurysm and AD, including IL1RN/TREM1 M1-like macrophage subcluster, which also expressed high levels of proinflammatory chemokines such as CXCL2, CCL3, and CCL4.[32,33] While our study showed an inflammatory chemokines and cytokines highly-expressed macrophage subcluster, but limited expression of TREM1 and a moderate expression of IL1RN in macrophages, which may not qualify as a proinflammatory macrophage population and further indicate the intrinsic difference in the underlying pathologic mechanism between aneurysm and dissection. (Lines 499-507 in revised manuscript)

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32. Liu, X. et al. Single-cell RNA sequencing identifies an Il1rn(+)/Trem1(+) macrophage subpopulation as a cellular target for mitigating the progression of thoracic aortic aneurysm and dissection. Cell Discovery 8, doi:10.1038/s41421-021-00362-2 (2022).

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28. Reference 16 is incomplete, i.e. is lacking the article title and further publication details.

Response: Thank you for your comment. We have revised the previous reference 16, which is currently listed as reference 33.

Revision:

REFERENCE

33. Daniel, C., Leppkes, M. & Muñoz, L.E. Extracellular DNA traps in inflammation, injury and healing. Nat Rev Nephrol Sep15, (9):559-575 (2019).

Reference of Responding Letter

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4 Hagan, P. G. *et al.* The International Registry of Acute Aortic Dissection (IRAD): new insights into an old disease. *Jama* **283**, 897-903, doi:10.1001/jama.283.7.897 (2000).

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Reviewer #3

The paper presents potentially valuable and clinically relevant research data in the field of aorta related pathologies and risk assessment. However, below suggested revisions may significantly increase visibility and overall quality of the publication.

> ***1. The title should be reconsidered to attract attention of multi-professional groups to this very important topic and proposed technological solutions such as "The multi-omics profiling is instrumental to facilitate risk assessment, patient stratification and targeted treatments in patients undergoing aortic dissection".***

Response: Thank you for your suggestion to consider the title to attract attention from multi-professional groups. Your proposed title, "The multi-omics profiling is instrumental to facilitate risk assessment, patient stratification, and targeted treatments in patients undergoing aortic dissection," indeed highlights the significance of the topic and the technological solutions involved. We appreciate your insight and agree that a title emphasizing the importance and utility of multi-omics profiling in aortic dissection (AD) could be more engaging for a diverse audience. After careful consideration, we propose the following revised title to maintain a balance between specificity and broad appeal: "Integrated Multi-Omics Profiling Reveals Neutrophil Extracellular Traps Potentiate Aortic Dissection Progression". Thank you very much for your kind suggestion!

> ***2. As presented, the paper is rather technology-driven, although "the identification of individuals at elevated risk for postoperative AAEs, and therapeutic targets to optimize the efficacy of TEVAR for patients with AD" is envisaged as stated in the Abstract. This evident "gap" has to be closed by the thoroughly performed revision - the authors are kindly requested to provide clear explanatory paragraphs on how the presented achievements may improve healthcare quality in the area benefiting affected individuals and improving individual outcomes. These statements have to be presented in Abstract together with clear rationale why an application of proteomics and transcriptomics has been chosen to reach the goals. Clinical relevance of the proposed approach and achievements has to be detailed in "Conclusions with Outlook".***

Response: Thank you for your detailed and constructive feedback. To address these concerns, we have revised the manuscript to include clear explanatory paragraphs on how the presented technological achievements may improve healthcare quality, benefiting affected individuals and improving individual outcomes.

Revision:

ABSTRACT

Background and Aims: Adverse aortic remodeling increases the risk of aorta-related adverse events (AAEs) after thoracic endovascular aortic repair (TEVAR) and affects the overall prognosis of aortic dissection (AD). It is imperative to delve into the exploration of prognostic indicators to streamline the identification of individuals at elevated risk for postoperative AAEs, and therapeutic targets to optimize the efficacy of TEVAR for patients with AD.

Methods: We performed proteomic and single-cell transcriptomic analyses of peripheral blood samples and aortic lesions, respectively, from patients with AD and healthy subjects. We performed in vivo experiments to further confirm the effect of inhibiting NETs. Kaplan-Meier and Cox regression analysis were used to identify independent risk factors for AAEs.

Results: Integrated multi-omics profiling identified highly phenotype-associated macrophages, which frequently interacted with neutrophils via CXCL3/CXCR2 axis, and promoted neutrophil extracellular traps (NETs) in driving and fueling the development of AD. Increased NETs formation is a defining feature of systemic immunity and aortic microenvironment of AD. Furthermore, we demonstrated that the level of citrullinated histone H3 (CitH3), a NETs associated marker, could serve as a risk factor for AAEs following endovascular therapy. Inhibiting NETs formation through the blockade of CitH3 alleviated the progression and rupture of AD in mice.

Conclusions: The multi-omics profiling reveals NETs formation features in the development of AD. NETs associated markers could facilitate the risk stratification and prognostic evaluation, and might serve as potential therapeutic targets of patients with AD.

3. "Phenotyping" is correctly mentioned in the paper as being crucial for risk assessment and targeted treatments". However, too little information is provided on the phenotyping criteria with clinically relevant examples. To this end, below listed references might be supportive for the authors to discuss "phenotyping", "innovative technologies in disease prediction" "risk assessment", "individualised patient profile", "predictive and preventive approach", "targeted treatments" which are highly relevant to the presented study and the field in general:

- ***https://pubmed.ncbi.nlm.nih.gov/36505894/***
- ***https://pubmed.ncbi.nlm.nih.gov/36061830/***
- ***https://pubmed.ncbi.nlm.nih.gov/36415625/***
- ***https://pubmed.ncbi.nlm.nih.gov/34876936/***

Response: Thank you for your insightful feedback regarding the discussion of phenotyping in our manuscript. To address this, we have revised the manuscript to provide a comprehensive overview of the phenotyping criteria used in our study. We have included clinically relevant examples to illustrate these criteria more clearly. We have also incorporated the references you suggested, which help support our discussion and provide a broader context for the importance of phenotyping in the field.

Revision:

In recent years, some progress has been made concerning the diagnosis and prognosis of AD, including D-dimer, inflammation and lipid metabolism associated markers, smooth muscle cell and extracellular matrix related markers, microRNA, and cell-free DNA, which may act synergistically with existing clinically known risk factors to identify individuals at high risk for poor aortic remodeling.[15-17] (Line 97-100 in revised manuscript)

During the paradigm changing from reactive to predictive, preventive, and personalized medicine[51-53], such dichotomous functions of neutrophils should be taken into account when designing therapeutic drugs that target neutrophils. (Lines 520-523 in revised manuscript)

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52. Zhan, X., Li, J., Guo, Y. & Golubnitschaja, O. Mass spectrometry analysis of human tear fluid biomarkers specific for ocular and systemic diseases in the context of 3P medicine. Epma j 12, 449-475 (2021).

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4. Introduction is too short and does not provide sufficient information to support the study rationale and working hypothesis. This section has to be extended providing sub-titles as messages from corresponding paragraphs such as

- Unmet patient needs in AD

- Risk assessment tools to be developed

- Technological solutions

- Working hypothesis and expectations

Sufficient references have to be provided which are currently missing in the "Introduction".

Response: Thank you for your detailed feedback regarding the Introduction section. We have revised the Introduction to include the given sub-sections. Additionally, we have ensured that sufficient references are included to support the information presented and to enhance the robustness of the Introduction. Please see the revised Introduction after the sixth comment.

5. Discussion has to be presented in a reader-friendly manner providing sub-titles as messages from corresponding paragraphs (see explanation under the above point 4).

Response: Thank you for your valuable feedback regarding the Discussion section. We understand the importance of presenting the Discussion in a reader-friendly manner. To improve clarity and readability, we have re-organized this section according to the sub-titles below that encapsulate the key messages of the corresponding paragraphs.

-Summary of Key Findings

-Clinical Implications

-Comparison with Previous Studies

-Strengths and Limitations

-Future Directions

This structured approach will help readers easily navigate and comprehend the main points and implications of our findings. Thank you again for your constructive feedback.

6. References' numbering has to be started with 1 in Introduction instead of 4 in Results. Further, references have to be updated: no any references originated from 2023 are presented.

Response: Thank you for your valuable feedback regarding the numbering and updating of references. We apologize for the oversight in starting the reference numbering at 4 in the Results section instead of 1 in the Introduction. We will correct this to ensure that the numbering begins appropriately in the Introduction and follows sequentially throughout the manuscript.

Regarding the inclusion of more recent references, we understand the importance of incorporating the latest research to support our findings and ensure the relevance of our work. We have reviewed and updated the references to include recent studies from 2023, ensuring that our manuscript reflects the most current advancements and discussions in the field.

Revision:

INTRODUCTION

Over the past few decades, significant progress has been made in the treatment of aortic dissection (AD), a life-threatening condition caused by a tear in the intimal layer of the aorta that allows blood to surge into the wall of the tissue, leading to the formation of true and false lumens. Minimally invasive thoracic endovascular aortic repair (TEVAR) has proven effective in stabilizing the dissected aorta with benefit for patients in the early stages of AD.[1,2] However, TEVAR adeptly seals the proximal tear and leaves distal entries open, resulting in an imbalanced blood flow distribution to the distal aorta. In certain patients, persistent blood flow from the true lumen into the false lumen via distal entries contributes to suboptimal or adverse aortic remodeling, thereby increases the risk of aorta-related adverse events (AAEs), and subsequently diminishes overall prognosis of AD.[3-6] Thus, predicting the risk factors for future AAEs in patients with AD is crucial to determine a therapeutic strategy. A comprehensive understanding of the onset and progression of AD is necessary to facilitate the exploration of risk factors that can predict the prognosis of patients with AD and identify those at high risk of postoperative AAEs, ultimately optimizing the efficacy of TEVAR.

To monitor the aortic remodeling, recent studies mainly focus on the clinical manifestation and anatomical and morphological changes of aorta based on imaging methods. Several clinical and imaging-related risk factors have been shown to be associated with early disease progression.[7-12] Established risk factors such as the thrombus in the false lumen[13] and the angle of aortic arch[14] are closely associated with postoperative aortic remodeling. The evaluation of morphological change of aorta often relies on computed tomography angiography or magnetic resonance angiography, while can be applied only under certain circumstances due to large radiation dose, high cost of examination, contrast agent-induced nephropathy, and failing to provide functional or dynamic assessment. Serum biomarkers could accurately reflect the pathophysiological changes in the process of disease occurrence and development. In recent years, some progress has been made concerning the diagnosis and prognosis of AD, including D-dimer, inflammation and lipid metabolism associated markers, smooth muscle cell and extracellular matrix related markers, microRNA, and cell-free DNA, which may act synergistically with existing clinically known risk factors to identify individuals at high risk for poor aortic remodeling.[15,16] Due to the limited sensitivity and specificity, inconsistent results between different studies, and the lack of case of

subacute and chronic acute dissection, quite a part of the existing candidate biomarkers are still in the preclinical stage.

Single-cell sequencing enables the comprehensive characterization of cells in a complex and heterogeneous tissue ecosystem, which is of indispensable importance to facilitate prognostic biomarker discovery and cell type targeted therapy.[17-19] However, most expression-based assays that are clinically used rely on bulk transcriptional analysis. Bulk RNA sequencing measures the averaged properties of whole tissue from large samples and provides comprehensive clinical and pathologic phenotype information.[20,21] Therefore, leveraging valuable and widely available phenotype information in bulk data to guide cell subpopulation identification from single-cell data is a promising approach to exploring and interpreting single-cell data from a new perspective.

Here, we performed the multi-omics profiling to unveil highly associated cell types and disease-relevant signaling pathways implicated in the onset progression of AD. This approach allowed us to identify prognostic biomarkers and potential therapeutic targets, providing a more objective and detailed theoretical basis for the development of effective personalized and precision medicine for patients with AD.

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19. Sattler, S. Single-Cell Immunology in Cardiovascular Medicine: Do We Know Yet What We Do Not Know? Circulation 144, 843-844 (2021).
20. Sun, D., et al. Identifying phenotype-associated subpopulations by integrating bulk and single-cell sequencing data. Nat Biotechnol 40, 527-538 (2022).
21. Weinstein, J.N., et al. The Cancer Genome Atlas Pan-Cancer analysis project. Nat Genet 45, 1113-1120 (2013).

7. Overall terminology has to be rechecked utilising an appropriate wording such as "fracture" instead of "tear".

Response: Thank you for your suggestion to recheck the terminology and consider using "fracture" instead of "tear." We appreciate your attention to detail and the importance of precise language in our work. Upon reviewing the terminology, we recognize that accurate and consistent wording is crucial for clarity and understanding, especially in multi-professional contexts. In this case, the term "tear" was chosen because it more accurately describes the specific pathology of the condition being discussed. "Fracture" typically refers to the breaking of hard tissues like bone, while "tear" is commonly used to describe the ripping or splitting of softer tissues, such as those involved in aortic dissection.[20]

However, we understand the need for clear and precise language. If there are specific instances where the term "tear" might lead to confusion or if there are other terms you believe would be more appropriate, we are open to further discussion and refinement.

Manuscript # NCOMMS-23-59187

Title: Integrated Multi-Omics Profiling Reveals Neutrophil Extracellular Traps Potentiate Aortic Dissection Progression

Response to Reviewers

We sincerely appreciate the reviewers' thorough and constructive feedback on our manuscript. In response, we have undertaken additional statistical and computational analyses and revised the manuscript to further strengthen our study. Our point-by-point response is as follows.

Reviewer #1

The authors have effectively addressed the issues we raised in their revised manuscript. Throughout the study, the authors utilized multi-omics approaches to examine the cellular composition and gene expression profiles in blood and tissue samples from aortic dissection (AD) patients. They inferred the interactions between macrophages and neutrophils, including the formation of neutrophil extracellular traps (NETs). Additionally, the biomarker potential of neutrophil and NET-related parameters was assessed in the context of diagnosing and prognosticating adverse events post-thoracic endovascular aortic repair (TEVAR). Furthermore, the authors validated in animal models that inhibiting NETs provides protective effects against AD in mice, and supplemented these findings by demonstrating the protective effects of CXCL3/CXCR2 monoclonal antibody inhibition on mice AD, further illustrating the involvement of the CXCL3/CXCR2 axis in NETs formation and the promotion of AD progression. The study combines clinical, animal, and bioinformatics screenings to identify biomarkers that potentially exacerbate AD progression. The article is fairly complete in its logical structure, I have no further questions to raise.

Response: We sincerely thank you for your thoughtful and encouraging feedback on our revised manuscript. Your comments have been invaluable in strengthening our study.

Reviewer #2

The authors have comprehensively addressed my major points of critique by additional experiments and by manuscript changes – thank you and congratulations on the impressive amount of work! Of note, the newly introduced parts have raised some additional (minor) issues in the following manuscript parts:

Response: We are deeply grateful to the reviewer for their thoughtful and constructive feedback on our revised manuscript. Your insights have been invaluable in enhancing the quality of our study. Our point-by-point response is as follows.

1. AD is marked by systemic inflammation and neutrophil activation in peripheral blood (Fig. 1, Suppl. Fig. 1, Suppl. Table 1).

Suppl. Table 1: Categorical variables such as gender or occurrence of co-morbidities seem to be listed as mean±SD values, e.g. gender of AD patients: 0.9±0.3. It seems rather strange how gender could have a standard deviation? Please convert the presentation of categorical variables to the usual number of patients and percent frequency – as e.g. given in Suppl. Table 2. Also, how were the p-values calculated for these categorical variables – by Chi square/Fisher´s exact test?
While plasma proteomics analysis identified CRP as significantly upregulated in AD patients vs. controls (Fig. 1B), Suppl. Table 1 lists a lower CRP value for AD patients than controls. Please address this discrepancy.

Response: Thank you for the comment. We have revised the statistical results concerning gender in Supplementary Table 1. Fisher's exact test was used to analyze categorical variables. According to the general rule that if the number of cases is less than 40, or the theoretical number is less than 2, the Fisher's exact test should be chosen.

Thank you for pointing out this discrepancy. Upon thorough re-examination of the raw data, I discovered that CRP levels in the AD and control groups were measured using different analytical platforms with different reference ranges, since patients with AD were hospitalized in vascular surgery inpatient department and healthy subjects underwent blood tests at the hospital's health check-up center. This discrepancy likely accounts for the observed differences and should prevent a direct comparison between the groups. To address this issue, I have decided to remove the CRP results from the analysis. This ensures that all reported data are comparable. The revised manuscript reflects this change, and I have updated the figures and tables accordingly. Thank you for your understanding, and I appreciate your attention to this detail.

Revision: Supplementary Table 1. Baseline characteristics of patients with AD and healthy individuals involved in mass spectrometry.

	AD (N = 30)	CL (N = 30)	P-value
Age (y)	57.1 ± 12.0	52.8 ± 8.1	0.113†
Gender			1‡
M	27 (90.0%)	27 (90.0%)	

F	3 (10.0%)	3 (10.0%)	
Hypertension	21 (70.0%)	0	< .001‡
Smoking	11 (36.7%)	0	< .001‡
Diabetes mellitus	5 (16.7%)	0	0.052‡
Alcohol abuse	13 (53.3%)	0	< .001‡
Stroke	2 (6.7%)	0	0.491‡
Coronary heart disease	6 (20%)	0	0.023‡
Chronic kidney disease	0	0	1‡
Neutrophil ($\times 10^9$ /L)	7.0 ± 2.1	3.8 ± 0.8	< .001†
Monocyte ($\times 10^9$ /L)	0.8 ± 0.4	0.6 ± 0.6	0.189†
Lymphocyte ($\times 10^9$ /L)	1.3 ± 0.5	2.0 ± 0.9	0.006†
Platelet ($\times 10^9$ /L)	234.9 ± 93.5	259.9 ± 54.4	0.260†
D-dimer (mg/L)	5.1 ± 5.2	0.2 ± 0.1	< .001†
ALT (U/L)	29.7 ± 38.4	34.8 ± 16.9	0.542†
AST (U/L)	23.1 ± 19.8	27.7 ± 7.3	0.272†
Cholesterol (mmol/L)	4.7 ± 1.0	5.2 ± 0.6	0.115†
Triglyceride (mmol/L)	1.2 ± 0.4	1.9 ± 0.6	0.001†

† Mann-Whitney test

‡ Fisher's exact test

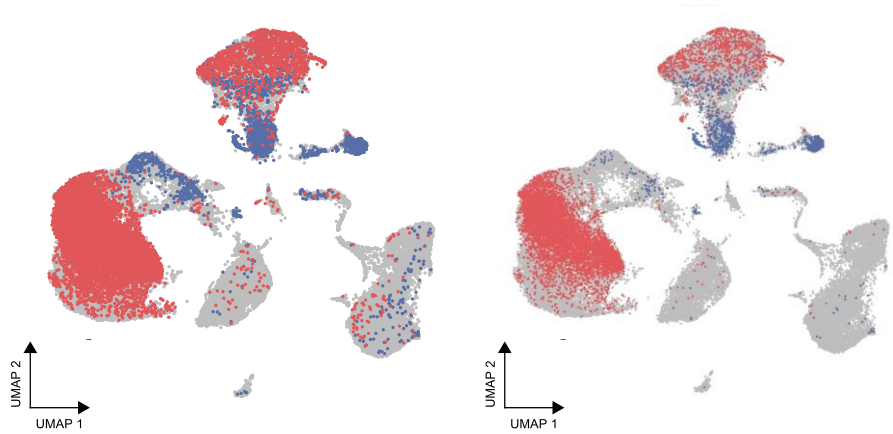
Values are expressed as means ± SD.

ALT: Alanine transaminase; AST: Aspartate aminotransferase.

2. A phenotype-associated macrophage subset orchestrates neutrophils within the aortic microenvironment (Fig. 2, Suppl. Fig. 2 and 3).

Please discuss the finding that "Scissor-positive cells" did not include neutrophils, yet the highlighted enriched pathways were neutrophil chemotaxis, activation, degranulation and NET formation. Why did the scRNA-seq analyses not reflect the accumulation of activated neutrophil cell clusters in AD?

Response: Thank you for the constructive comment. We applied Scissor algorithm to perform integrated analysis. The Scissor algorithm establish the similarity network between cells based on scRNA-seq and Pearson correlation coefficient between the scRNA-seq and bulk-seq expression data. We set a stringent alpha value of 0.05 in the regression model established by the correlation matrix. Therefore, the Scissor-selected cells include only macrophage and SMCs. Theoretically, when lowering the alpha value, we should observe more Scissor-selected cells, which may include neutrophils. Thereby, we further performed the Scissor process with alpha value of 0.01, and Scissor turned out to select around 25% of cells among all cells, which is higher than that when alpha value equalled to 0.05. In these selected-cells, we found more neutrophils were included in Scissor-positive cells.



Responding figures. Scissor selected-cells at alpha value of 0.01 (left) and 0.05 (right).

In addition, we applied 10x Genomics' scRNA-seq technology to analyze transcriptomes of aortas. While human neutrophil has a short half-life *in vivo*, and is extremely delicate and vulnerable *ex vitro*. Compared to BD Rhapsody, the 10x technique possess a well-accepted disadvantage that neutrophil is less susceptible to 10x Chromium technical issues and clearly underrepresented, which can complicate experimental results and interpretations.[1,2] We acknowledge that this is a limitation of our study, which we have addressed in the Limitation section. A more in-depth investigation of neutrophils during the development of AD is planned for future research.

Revision: As with all sequencing studies, ours also has some limitations. Firstly, phenotype information from a single bulk dataset of 12 human subjects may skew the results, which warrants further evaluation of results in larger datasets with abundant phenotype information. **Secondly, Scissor-positive cells included only a subset of neutrophils, which may be attributed to the inherent properties of neutrophils, such as their short half-life in vivo, extreme delicacy and vulnerability ex vivo, and the limitations of the 10x Chromium platform technical issues that clearly underrepresents neutrophil.** Thirdly, determining exactly how NETs formation promotes inflammation and aggravates AD progression is beyond the scope of this study and require further investigation. (Lines 570-573)

3. Neutrophil heterogeneity and differentiation trajectories (Suppl. Fig. 4).

This new chapter is solely based on scRNA-seq data, it documents heterogeneity among neutrophils in AD and shows that only 2 of 5 neutrophil subsets express the CXCR1/2 receptors. Since these subsets are proposed to be the target of macrophage regulation by CXCL3 leading to NET formation, it would be of interest whether these subsets are characterized by processes of NET formation (elevated "NET score") according to the expression data – see point 4.

Response: Thank you for the comment. Please see our response below.

4. Elevated NETosis occurs in aortic lesions and peripheral blood from patients with AD (Fig. 3, Suppl. Table 2 and 3).

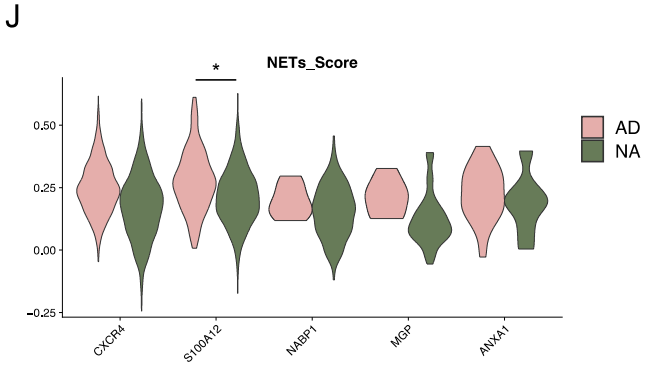
Please specify the determinants of the NET score and whether the NET score was indeed significantly higher in the CXCR1/2-positive neutrophil subsets of AD patients versus controls.

Response: Thank you for the valuable comment. We adopted determinant gene set of the NETs as previously mentioned. We cited this paper as reference in the revised manuscript (no.32). Upon this gene set, we found significantly higher NETs score in the $S100A12^{+}$ $CXCR1/2^{+}$ neutrophil subsets of AD patients versus controls. Although there was no significant difference of NETs score in the $CXCR4^{+}$ $CXCR1/2^{+}$ neutrophil (p-value = 0.0603), we can still observe a higher level of that in AD versus normal aortas. We reckon this result can be explained by the fact that NETs formation is a relatively newly investigated biological process. Many details and genes involved still warrant further study to fully characterize the comprehensive picture of NETs formation.

Revision: To investigate the association between NETs and different cell subgroups, we calculated the NETs-associated gene module scores[32] for both dissected and normal aortas using single-cell data. (Lines 289-290)

32. Zhang Y, Guo L, Dai Q, Shang B, Xiao T, Di X, Zhang K, Feng L, Shou J, Wang Y. A signature for pan-cancer prognosis based on neutrophil extracellular traps. J Immunother Cancer. 2022 Jun;10(6):e004210. doi: 10.1136/jitc-2021-004210. PMID: 35688556; PMCID: PMC9189842.

Supplementary Figure 4. Neutrophil heterogeneity and differentiation trajectories.
(J) NETs-associated gene module score of neutrophil subsets in AD and normal aortas.



5. Plasma measurements of CitH3 are briefly mentioned in the Methods section - with reference to the publication by Thalin et al. 2020. In this report Thalin et al. introduced the "nucleosomal CitH3" ELISA detecting complexes of CitH3 and DNA in human plasma (with healthy reference values around 30 ng/ml). Since the authors consistently mention measurements of CitH3 plasma levels (which ranged at 2 ng/ml in healthy individuals and 4 ng/ml in AD patients), please specify whether indeed CitH3-DNA complexes were detected or whether the previously published CitH3 protein ELISA by Thalin et al. (2017) was applied – and at which detection limits?

Response: Thank you for the comment. We originally intended to cite the article Thalin et al. (2017), but accidentally cited a different one, that is Thalin et al. (2017). We sincerely apologize for citing the incorrect paper and for any confusion caused by the lack of clarification. We diluted the plasma samples at 1:2 ratio, and CitH3 levels were measured by ELISA, which is confirmed effective and reliable by Thalin et al. (2017). We utilized the Citrullinated Histone H3 ELISA Kit (501620, Cayman Chemical) and carefully followed the manufacturer's instructions to ensure accuracy in our experimental procedures. The technical information indicates a detection limit of 0.15-10 ng/ml. In our research, the working concentrations of the diluted samples ranged from 0.237797 to 6.94058 ng/ml, while the stock concentrations ranged from 0.475594 to 13.88116 ng/ml.

Revision:
The plasma samples were diluted 1:2, and CitH3 levels were measured using the Citrullinated Histone H3 ELISA Kit, following the manufacturer's instructions (501620, Cayman Chemical). (Lines 686-686)

> ***6. Please note that the entire chapter cites Figure 4 while the respective data is displayed in Figure 3. The legend to Fig. 3D, E, F seems wrong (E/F do not exist and D is duplicated with a second text). Also, H&E stainings of longitudinal aorta sections are mentioned to be displayed in Suppl. Fig. 5A. Yet, it seems they are actually shown in Suppl. Fig. 6D? The statistics section mentions that continuous variables were either presented as mean and SD or as median and IQR. Please specify what holds true in the legends to the various Tables. In Suppl. Table 2, please attribute male and female gender to the respective lines. In the text, 58 controls are mentioned while Suppl. Table 2 lists 59 healthy controls – what is correct? As mentioned for Suppl. Table 1, please give the number of affected patients (or controls) and calculated percent frequency for the occurrence of co-morbidities listed in Suppl. Table 2 and verify that the correct statistical test for categorical variables has been applied.***

Response: Thank you for the comment. We apologize for the error in citing Figure 4 throughout the entire chapter. We have corrected this mistake.

Revision:
Notably, we observed a significant increase in NETs scores within the dissected aorta compared to the normal aorta. Furthermore, among the 20 cell subgroups analyzed, the highest NETs scores in neutrophils were observed (**Figure 3A**). (Lines 290-293)

High-magnification images obtained through hematoxylin and eosin (H&E) staining displayed longitudinal sections of both dissected and normal aortas (**Supplementary Figure 6D**). (Lines 298-300)

In contrast, limited areas positive for CitH3 were observed in tissues obtained from the healthy aorta (**Figure 3B**). (Lines 304-305)

We detected NETs-associated markers in the plasma from 187 patients with AD and 59 healthy individuals with balanced baseline characteristics (Supplementary Table 2). Our findings revealed

that the levels of plasma NETs markers, including CitH3, cell-free DNA (cf-DNA), MPO, NE, and IL-6, were significantly higher in patients with AD than those observed in healthy subjects (**Figure 3C** and Supplementary Table 3). (Lines 307-311)

Supplementary Table 1. Baseline characteristics of patients with AD and healthy individuals involved in mass spectrometry.

	AD (N = 30)	CL (N = 30)	P-value
Age (y)	57.1 ± 12.0	52.8 ± 8.1	0.113†
Gender			1‡
M	27 (90.0%)	27 (90.0%)	
F	3 (10.0%)	3 (10.0%)	
Hypertension	21 (70.0%)	0	< .001‡
Smoking	11 (36.7%)	0	< .001‡
Diabetes mellitus	5 (16.7%)	0	0.052‡
Alcohol abuse	13 (53.3%)	0	< .001‡
Stroke	2 (6.7%)	0	0.491‡
Coronary heart disease	6 (20%)	0	0.023‡
Chronic kidney disease	0	0	1‡
Neutrophil ($\times 10^9$ /L)	7.0 ± 2.1	3.8 ± 0.8	< .001†
Monocyte ($\times 10^9$ /L)	0.8 ± 0.4	0.6 ± 0.6	0.189†
Lymphocyte ($\times 10^9$ /L)	1.3 ± 0.5	2.0 ± 0.9	0.006†
Platelet ($\times 10^9$ /L)	234.9 ± 93.5	259.9 ± 54.4	0.260†
D-dimer (mg/L)	5.1 ± 5.2	0.2 ± 0.1	< .001†
ALT (U/L)	29.7 ± 38.4	34.8 ± 16.9	0.542†
AST (U/L)	23.1 ± 19.8	27.7 ± 7.3	0.272†
Cholesterol (mmol/L)	4.7 ± 1.0	5.2 ± 0.6	0.115†
Triglyceride (mmol/L)	1.2 ± 0.4	1.9 ± 0.6	0.001†

† Mann-Whitney test

‡ Fisher's exact test

Values are expressed as means ± SD.

ALT: Alanine transaminase; **AST**: Aspartate aminotransferase.

Supplementary Table 2. Baseline characteristics of patients with AD and healthy individuals in diagnostic cohort.

	AD (N = 187)	CL (N = 59)	P-value
Gender			<.001‡
M	29 (15.5%)	27 (45.8%)	
F	158 (84.5%)	32 (54.2%)	
Age	56.6 ± 13.6	46.7 ± 14.6	<.001†
D-dimer	4.5 ± 5.2	3.6 ± 5.1	0.227†
WBC ($\times 10^9$ /L)	7.5 ± 4.4	7.3 ± 1.8	0.459†
Neutrophil ($\times 10^9$ /L)	6.4 ± 2.6	4.4 ± 2.2	<.001†
Monocyte ($\times 10^9$ /L)	0.8 ± 0.8	0.4 ± 0.2	<.001†
Lymphocyte ($\times 10^9$ /L)	1.3 ± 0.5	2.4 ± 0.6	<.001†

Platelet ($\times 10^9$ /L)	202.3 ± 71.1	255.7 ± 65.2	<.001†
Albumin (g/L)	40.7 ± 4.8	46.2 ± 2.7	<.001†
Creatinine (μmol/L)	91.9 ± 92.2	74.0 ± 17.2	0.012†
GFR (ml/min)	71.3 ± 32.6	81.3 ± 30.0	0.038†
Cholesterol (mmol/L)	4.1 ± 1.1	4.8 ± 1.0	<.001†
Triglyceride (mmol/L)	1.4 ± 0.9	1.5 ± 1.4	0.646†
LDL (mmol/L)	2.3 ± 0.8	3.2 ± 0.9	<.001†
HDL (mmol/L)	1.4 ± 0.9	1.2 ± 0.3	0.010†
CRP (mg/L)	56.5 ± 52.7	45.7 ± 49.2	0.165†
CitH3 (ng/mL)	4.4 ± 3.0	1.8 ± 1.3	<.001†
cf-DNA (ng/mL)	2.0 ± 0.7	1.7 ± 0.6	0.006†
MPO (ng/mL)	4.4 ± 2.5	2.8 ± 1.7	<.001†
NE (ng/mL)	4.1 ± 1.9	2.2 ± 1.3	<.001†
IL-1β (pg/mL)	94.2 ± 29.0	86.6 ± 26.7	0.074†
IL-6 (pg/mL)	45.2 ± 19.7	37.7 ± 17.4	0.010†
Hypertension	146 (78.1%)	3 (1.6%)	<.001‡
Smoking	106 (56.7%)	3 (1.6%)	<.001‡
Alcohol abuse	80 (42.8%)	3 (1.6%)	<.001‡
Diabetes mellitus	75 (40.1%)	3 (1.6%)	<.001‡
Coronary heart disease	8 (4.3%)	0	0.204‡
Stroke	1 (0.5%)	0	1‡
Chronic kidney disease	8 (4.3%)	0	0.204‡

† **Mann-Whitney test**

‡ **Fisher's exact test**

Values are expressed as means ± SD.

WBC: White blood cell; **GFR**: Glomeruar filtration rate; **LDL**: Low density lipoprotein; **HDL**: High density lipoprotein; **CRP**: C-reactive protein; **CitH3**: citrullinated histone H3; **cf-DNA**: cell free-DNA; **MPO**: myeloperoxidase; **NE**: Neutrophil elastase.

Supplementary Table 4. Baseline characteristics of patients with AD and healthy individuals of patients receiving TEVAR in prognostic cohort.

		non-AAEs (N=85)	AAEs (N=31)	P-value
Age		56.2 ± 13.7	54.0 ± 14.5	0.468†
Gender				0.077‡
	Female	12 (14.1%)	10 (32.3%)	
	Male	72 (84.7%)	21 (67.7%)	
Phase				0.328‡
	Acute	70 (82.4%)	22 (71%)	
	Subacute	9 (10.6%)	4 (12.9%)	
	Chronic	4 (4.7%)	2 (6.5%)	
D-dimer		4.5 ± 5.1	7.2 ± 7.0	0.056†
Neutrophil		6.3 ± 2.6	7.2 ± 2.4	0.088†
Monocyte		0.7 ± 0.3	0.7 ± 0.3	0.413†
Lymphocyte		1.3 ± 0.4	1.2 ± 0.4	0.366†

Platelet	211.4 ± 79.5	192.1 ± 70.8	0.238†
Albumin	40.5 ± 4.4	40.5 ± 3.5	0.970†
Cholesterol	4.1 ± 1.2	4.1 ± 0.7	0.734†
Triglyceride	1.4 ± 0.9	1.1 ± 0.5	0.036†
LDL	2.2 ± 0.8	2.4 ± 0.6	0.101†
HDL	1.5 ± 1.1	1.3 ± 0.9	0.267†
NLR	5.0 ± 3.2	6.2 ± 3.4	0.086†
PLR	166.8 ± 66.3	179.7 ± 69.8	0.362†
MLR	4.3 ± 3.0	5.0 ± 2.4	0.234†
SII	1014.1 ± 702.5	1163.4 ± 572.1	0.291†
SIRI	0.6 ± 0.2	0.7 ± 0.3	0.018†
CitH3	4.3 ± 2.4	6.9 ± 3.2	<.001†
cf-DNA	2.0 ± 0.6	2.0 ± 0.7	0.650†
MPO	4.8 ± 2.3	6.0 ± 2.5	0.020†
NE	4.1 ± 1.9	4.2 ± 1.7	0.834†
IL-1β	94.9 ± 29.1	93.0 ± 26.8	0.755†
IL-6	43.8 ± 20.0	43.5 ± 17.6	0.946†

† **Mann-Whitney test**

‡ **Fisher's exact test**

Values are expressed as means ± SD.

LDL: Low density lipoprotein; **HDL**: High density lipoprotein; **NLR**: Neutrophil-to-lymphocyte ratio; **MLR**: Monocyte-to-lymphocyte ratio; **PLR**: Platelet-to-lymphocyte ratio; **SII**: Systemic immune inflammation index; **SIRI**: Systemic inflammatory response index; **CitH3**: citrullinated histone H3; **cf-DNA**: cell free-DNA; **MPO**: myeloperoxidase; **NE**: Neutrophil elastase.

5. Inhibition of NETosis ameliorates dissection progression in AD mice (Fig. 4, Suppl. Fig. 6).

Please acknowledge that a substantial fraction of CitH3 signal is detected without co-localization of neutrophil markers (in both the human – Fig. 3B – and mouse AD sections – Fig. 4K, also seen in Fig. 6J) which would point to additional essential sources of extracellular trap formation in AD.

Response: Thank you for the valuable comment. To date, NETs is the most comprehensively investigated type of ETosis. Although neutrophils are the main source of extracellular DNA traps, other types of granulocytes, as well as mononuclear phagocytes, reportedly share the ability to form extracellular traps (ETosis) and/or to externalize chromatin in response to various stimuli.[3-5] We have also included the relevant insights in the discussion section. We will continue to monitor molecular and translational research concerning ETosis and actively consider and explore whether relevant advances can further assist in the diagnosis and treatment of AD.

Revision:

Interestingly, a considerable portion of CitH3 signal is detected without co-localization with neutrophil markers, hinting at other critical sources of extracellular trap formation in AD. While

neutrophils are the primary contributors of extracellular DNA traps, other granulocytes, as well as mononuclear phagocytes and eosinophils, have also demonstrated the ability to form extracellular traps and externalize chromatin in response to various stimuli. Thus, the mechanism and the potential diagnostic or prognostic role of extracellular DNA traps originating from other immune cells in AD are highly promising and warrant further exploration. (Lines 543-550)

Several of the results are mentioned twice within this chapter. Please try to streamline and shorten the chapter.

Response: Thank you for the comment. We have revised our description in the manuscript. Please see our revision below.

Revision:
~~We further investigated the potential of blocking NETs to ameliorate AD and improve survival in the AD mouse model, with t~~Two drugs Cl-amidine (PAD4 inhibitor) and DNase I were adopted to inhibit NETs formation and digest NETs after their formation, respectively. (Lines 339-341)

Moreover, the average max aortic diameter, overall thickness and media thickness of aortas, and elastic fiber degradation from Cl-amidine-treated mice were significantly **reversed** compared to AD mice, suggesting reduced aortic expansion and maintained elastin integrity (Figures 4F-4I). The average max diameter of aortas from DNase I-treated mice showed a slight reduction, but without statistical significance when compared to AD mice (Figures 4F-4I). ~~In the Cl-amidine-treated mice, there was compelling evidence of reduced aortic expansion and maintained elastin integrity (Figures 4F-4I), while the DNase I-treated mice exhibited moderate elastin rupture and partial thrombosis in the false lumen, resulting in partial compression of the true lumen (Figures 4F-4J).~~ Furthermore, The Cl-amidine- and DNase I-treated group (Combo) demonstrated a significant reduction in CitH3 and Ly6G or MPO positive areas when compared to BAPN-induced AD mice (Figure 4K). (Lines 348-356)

With respect to the Methods applied, please specify whether aortic diameter assessment was based on US analysis or ex vivo measurements, and explain how wall thickness and media thickness were defined. While in the AD mouse model, wall thickness and media thickness are inversely regulated, the results text suggests that both are reduced by the applied inhibitors (lines 350 and 401).

Response: Thank you for the comment. Measurements of maximum aortic diameter were performed both *in vivo* by vascular ultrasound or *ex vivo*. Baseline aortic diameter of each mouse was measured by ultrasound before the administration of drugs. Then the dead mice were immediately dissected and the surviving mice were euthanized at the end of the 4-week study period. The aortas were exposed and rinsed with cold PBS, and the periaortic tissues were removed. The aorta was then excised, further cleaned, and rinsed with cold PBS to remove any residual blood in the lumen. Next, aortas were imaged for diameter measurement and disease evaluation.

Aortic wall thickness and media thickness were measured through the images determined by H&E, elastic and collagen fiber staining. We measured the maximum and minimum values of the

wall thickness and media thickness, respectively, in each image and calculated the averages of them as values provided in the Figures.

The dissected aorta usually had a thinner media due to elastin rupture and thicker wall partly due to inflammatory edema, which can illustrate that wall thickness and media thickness are inversely regulated. We found Cl-amidine could ameliorate AD condition and reverse parameters of aortas. Therefore, we should use "reversed" instead, not "reduced", to articulately describe the changes between groups. Thank you again for help point out this error!

Revision:

Baseline aortic diameter of each mouse was measured by ultrasound. The dead mice were immediately dissected and the surviving mice were euthanized at the end of the 4-week study period. The aortas were exposed and rinsed with cold PBS, and the periaortic tissues were removed. The aorta was then excised, further cleaned, and rinsed with cold PBS to remove any residual blood in the lumen. Next, aortas were imaged for diameter measurement and disease evaluation. Each mouse aorta was stained for H&E to observe AD occurrence, characterized by elastin degradation and rupture in aortic media layer. Aortic wall thickness and media thickness were measured through the images determined by H&E, elastic, and collagen fiber staining. Maximum and minimum values of the wall thickness and media thickness were measured in each image and calculated the averages. (Lines 710-719)

Moreover, the average max aortic diameter, overall thickness and media thickness of aortas, and elastic fiber degradation from Cl-amidine-treated mice were significantly **reversed** compared to AD mice, suggesting reduced aortic expansion and maintained elastin integrity (Figures 4F-4I). (Lines 348-352)

> ***Importantly, Figures 4, 5 and 6 do not include significance levels. It seems that letters (a-d) were used instead but are not explained in any of the figure legends. In Figure 4, the labels of G and H are reversed in the legend, and in 4K the merged image for the DNase I treated aorta is missing (i.e. is replaced by another DAPI image). Suppl. Fig. 6F is labeled by "Ly6G" but shows MPO staining according to the legend. Please show single mice values in Suppl. Fig. 6B and H (as in Figure 4F), since it seems that at treatment start, only 2/48 animals had a detectable aorta dissection.***

Response: Thank you for the comment. We initially used different letters to mark statistical significance in bar chart plots. Upon further review, we discovered that the asterisk (*) method to denote significance is more commonly used and widely acceptable. Consequently, we have updated our style to align with this standard practice. We have revised the Suppl fig 6B, 6F and 6H accordingly.

Revision:

Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD.
(F-H) The average of max diameter (F), **aortic wall thickness of aortas (G),** and **media thickness (H)** in each group. Statistical significance was set at $p < 0.05$ by Mann-Whitney U test or Fisher's exact test. Different letters between groups indicate statistical significance.

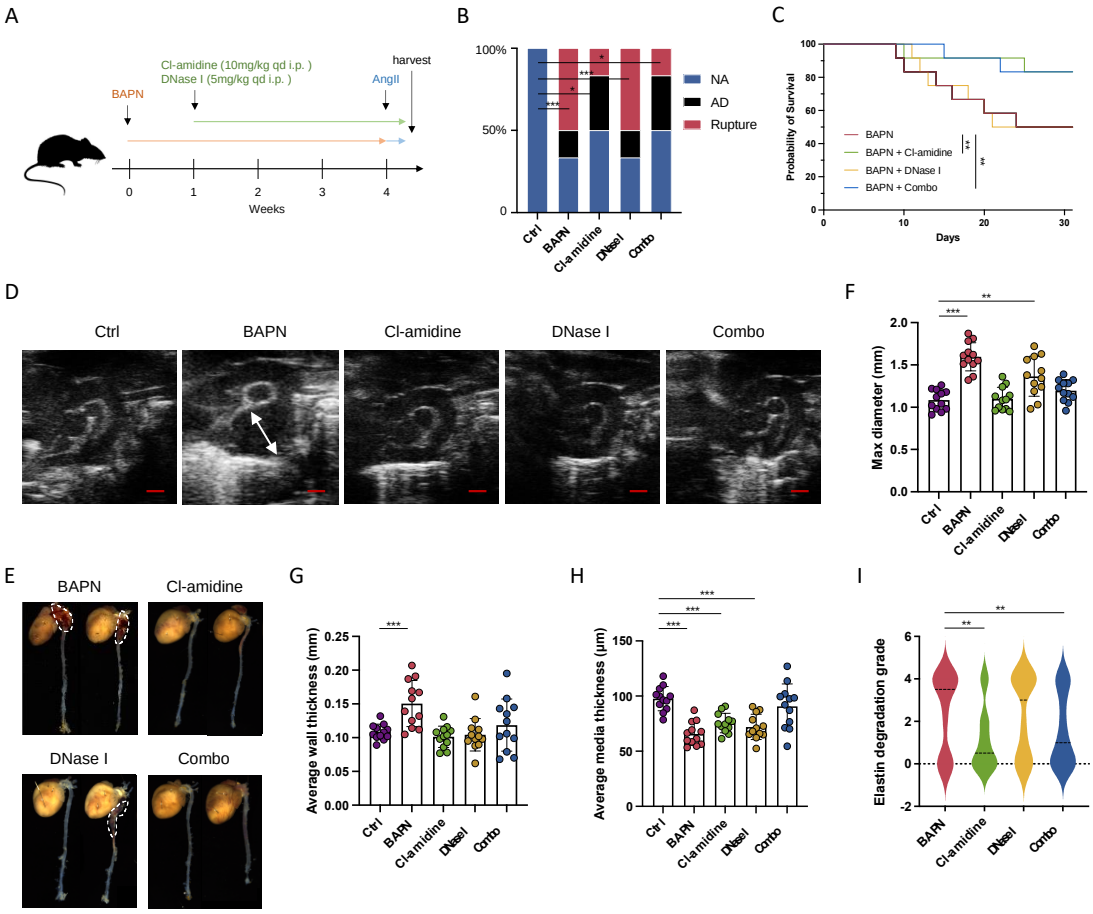


Figure 5. CXCL3^{+} macrophages were positively correlated with CXCR2^{+} neutrophils within AD lesion.

Statistical significance was set at $p < 0.05$ by Mann-Whitney U test or Fisher's exact test.

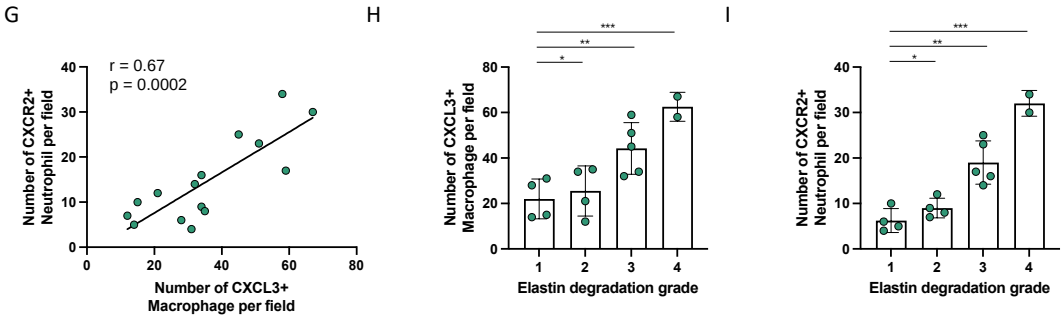


Figure 6. Macrophage fosters NETs formation via CXCL3/CXCR2 axis.

Statistical significance was set at $p < 0.05$ by Mann-Whitney U test or Fisher's exact test.

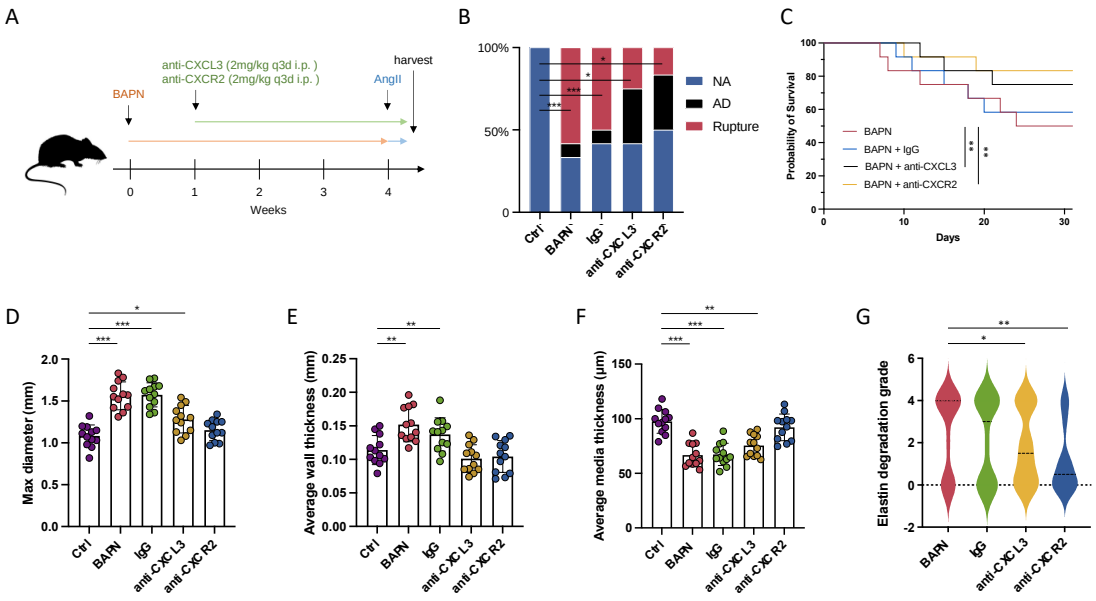
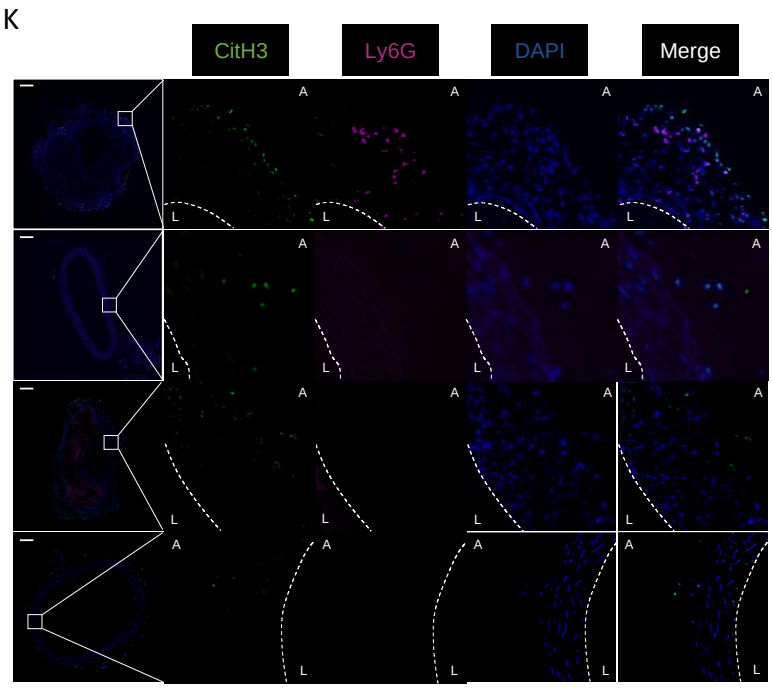


Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD.

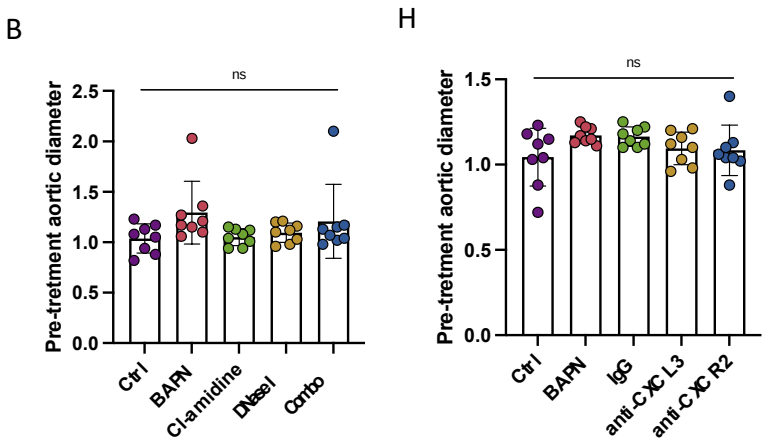
(K) Representative immunofluorescence images showing NETs formation within aortas of mice, determined by CitH3 (citrullinated histone H3, green) and neutrophil (Ly6G, red) staining. Scale bar = 100 μm.



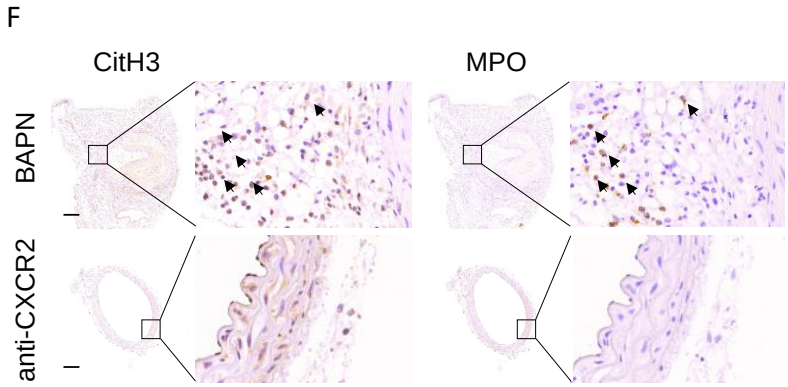
Supplementary Figure 6. Inhibition of NETs attenuates dissection progression in mice with AD.

(B) Baseline aortic diameters of each mouse measured by vascular ultrasound at day 7 before the administration of NETs inhibitors.

(H) Baseline aortic diameters of each mouse measured by vascular ultrasound at day 7 before the administration of anti-CXCL3 and anti-CXCR2 antibodies.



(E-F) The immunohistochemistry images showing NETs formation within aortas in mice with BAPN and NETs inhibition, determined by staining of CitH3 and Ly6G, and CitH3 and MPO, respectively. Scale bar = 100 μm.



6. Macrophages foster NET formation via the CXCL3/CXCR2 axis (Fig. 5 and 6, Suppl. Fig. 5).

The communication matrix in Suppl. Fig. 5B would indicate that the proposed interactions between macrophages and neutrophils were rather comparable in AD patients and controls, while communication between macrophages and myofibroblasts were highly increased in AD. Please discuss whether CXCR1/2 positive (myo)fibroblasts could be an additional target of macrophage-derived CXCL3 in AD pathogenesis.

Response: Thank you for the comment. CXCR1 and CXCR2 chemokine receptors are expressed in a variety of cells including, leukocytes, fibroblasts, endothelial cells, and smooth muscle cells.[6,7] Macrophages are proposed to interact with fibroblast and SMCs during AD development. We acknowledge that cell-cell communications showed a significantly increased number of interactions between macrophages and (myo)fibroblasts in AD compared with that in NA. Though recent studies revealed CXCL3/ CXCR2 axis in pancreatic ductal adenocarcinoma and its receptor CXCR2 was almost exclusively expressed in cancer-associated fibroblasts[8], we found exclusive expression of CXCR1/2 on neutrophils rather than other cells in both AD and normal aorta (NA) (Supplementary

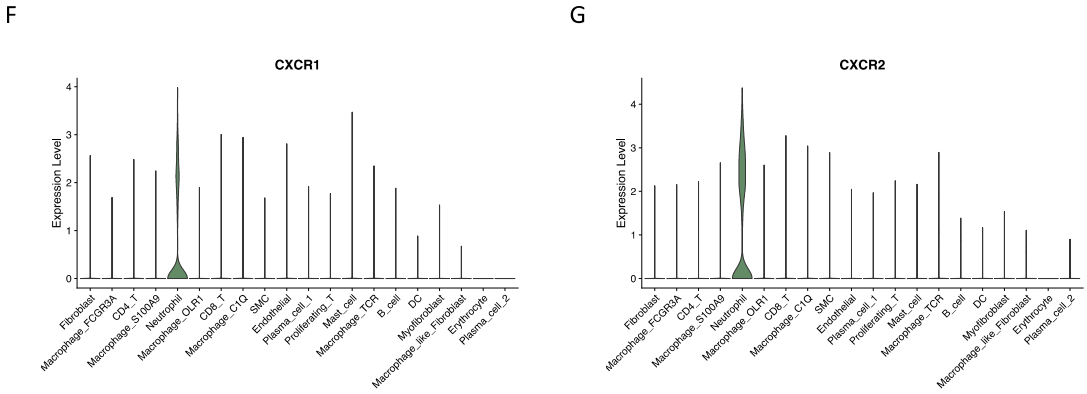
Figures 5F-5G). Thank you for your valuable suggestions. We also believe that the connection between macrophages and fibroblasts is critically important, which may act thorough ligand/receptor pairs. In fact, our team is already conducting in-depth research on the roles and mechanisms of smooth muscle cells and fibroblasts in the progression of AD.

Revision:

Supplementary Figure 5. Cell-cell interactions in aortic lesions in AD.

(F) Violin plot showing the expression level of CXCR1 among all cell populations.

(G) Violin plot showing the expression level of CXCR2 among all cell populations.



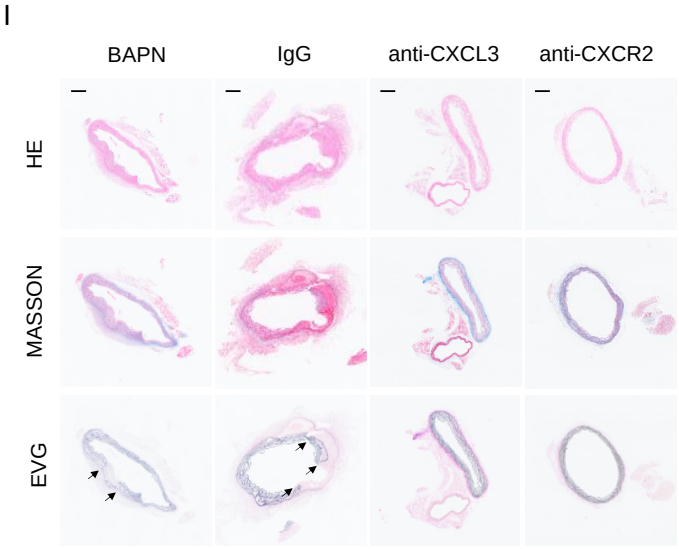
In Figure 6, the labels of E and F are reversed in the legend, and there is no scale bar of 100 μm in 6I.

Response: Thank you for the comment. We have made the revision accordingly.

Revision: Figure 6. Macrophage fosters NETs formation via CXCL3/CXCR2 axis.

(D-F) The average of max diameter (D), aortic wall thickness (E), and aortic media thickness (F) in each group.

(I) Representative immunohistochemistry images showing aortic dilation, false lumen formation, and elastin degradation within aortas in each group. Scale bar = 100 μm.



7. NET-associated markers serve as prognostic risk factors (Fig. 7, Suppl. Fig. 6, Suppl. Table 4 and 5).

Several parameters in Suppl. Table 4 (AAEs, gender, phase) have 3 categories. Please specify the third category – presumably "unknown"? Also, the title of Suppl. Table 4 is incorrect and the title for Suppl. Table 5 should mention AAE prediction. The "numbers at risk" given for Kaplan-Meier curves in Figure 7E specify time in days which is likely months and which should also be labeled in Suppl. Fig. J and K.

Response: Thank you for the comment. Due to our incorrect organization, Suppl. Table 4 erroneously showed several three-category variables, including AAEs, gender, and phase. We sincerely apologize for the inconvenience and misunderstanding caused by the previous table! We have now revised our Suppl. Table 4. In the current table, gender is composed of two variables (male/female), and phase is composed of three variables (acute/subacute/chronic). Two patients with AAEs and three patients with non-AAEs are excluded from the comparison of phase due to inability to obtain their intervention phase in the medical records. Thank you very much for help us improve the tables!

Revision:

Supplementary Table 4. Baseline characteristics of patients with AD ~~and healthy individuals of patients~~ receiving TEVAR ~~in prognostic cohort~~.

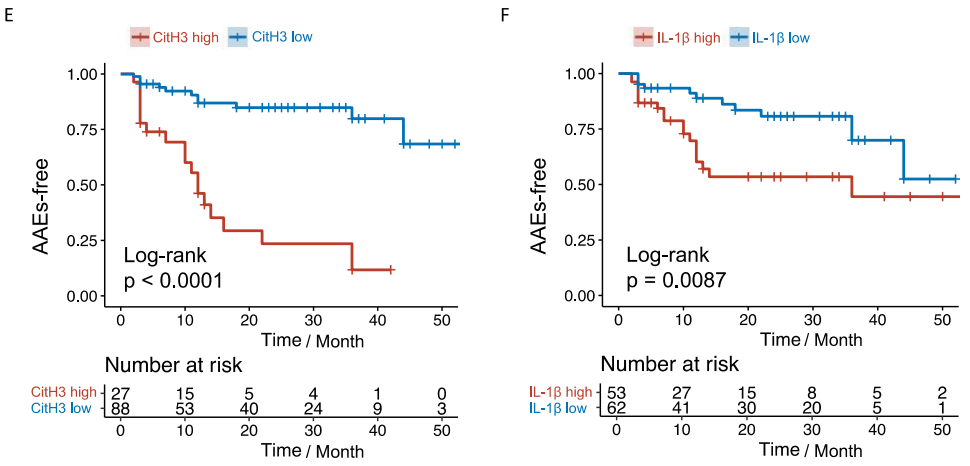
		non-AAEs (N=85)	AAEs (N=31)	P-value
Age		56.2 ± 13.7	54.0 ± 14.5	0.468†
Gender				0.077‡
	Female	12 (14.1%)	10 (32.3%)	
	Male	72 (84.7%)	21 (67.7%)	
Phase				0.328‡
	Acute	70 (82.4%)	22 (71%)	
	Subacute	9 (10.6%)	4 (12.9%)	
	Chronic	4 (4.7%)	2 (6.5%)	

Supplementary Table 5. Univariable and multivariable Cox proportional hazard regression analyses of patients receiving TEVAR **in predicting AAEs**.

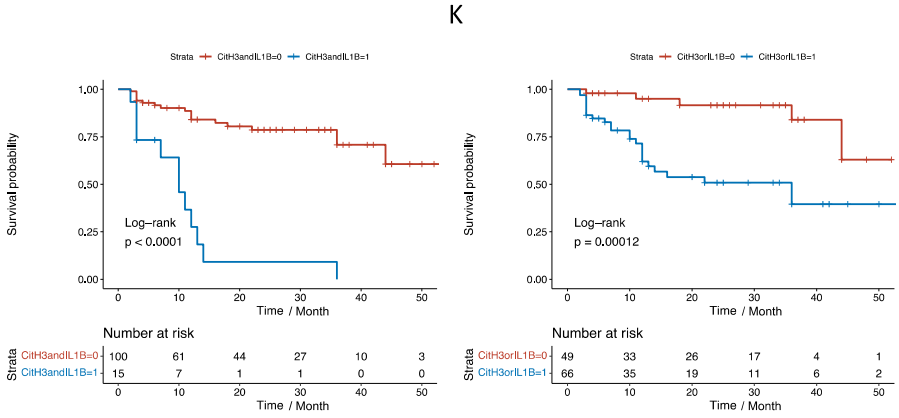
Figure 7. NETs-associated markers serve as prognostic risk factors.

(E) Kaplan-Meier curves of patients with high levels of CitH3 (≥ 6.91 ng/ml) and the incidence of AAEs compared to patients with low levels of CitH3 (< 6.91ng/ml). Below the survival curves showing the number of exposed subjects at each time point.

(F) Kaplan-Meier curves of patients with high IL-1β (≥ 90.86 pg/ml) and the incidence of AAEs compared to patients with low IL-1β (< 90.86 pg/ml). Below the survival curves showing the number of exposed subjects at each time point.



Supplementary Figure 6. Inhibition of NETs attenuates dissection progression in mice with AD.
(J) Kaplan-Meier curves of patients with both high CitH3 (≥ 6.91 ng/ml) and high IL-1β (≥ 90.86 pg/ml) compared to remaining patients. Below the survival curves showing the number of exposed subjects at each time point.
(K) Kaplan-Meier curves of patients with either high CitH3 (≥ 6.91 ng/ml) or high IL-1β (≥ 90.86 pg/ml). Below the survival curves showing the number of exposed subjects at each time point.



Additional minor issues:

• The Abstract could be improved by more precise wording, e.g. "Inhibiting NET formation through the blockade of histone citrullination or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The plasma level of citrullinated histone H3 (CitH3) could serve as a risk factor for AAEs following endovascular therapy for patients with AD."

Response: Thank you for the comment. We have already revised the Abstract according to your suggestions.

Revision: Conclusions: The multi-omics profiling reveals NETs formation features in the development of AD. Inhibiting NET formation through the blockade of histone citrullination or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The plasma level

of citrullinated histone H3 (CitH3) could serve as a risk factor for AAEs following endovascular therapy for patients with AD.

- ***While mostly well-written, the manuscript could profit from another scientific language check, since several sentences are difficult to understand or appear incomplete (e.g. lines 101, 249, 262, 499-507).***

Response: Thank you for your feedback. We appreciate your suggestion regarding the scientific language check. We have carefully reviewed the manuscript to improve clarity and ensure that all sentences are complete and easy to understand. Below are our revisions.

Revision:
In recent years, some progress has been made concerning the diagnosis and prognosis of AD, including D-dimer, inflammation and lipid metabolism associated markers, smooth muscle cell and extracellular matrix related markers, microRNA, and cell-free DNA, which may act synergistically with **established clinical risk factors** to identify individuals at high risk for poor aortic remodeling. (Lines 98-103)

We proceeded to survey the expression of CXCR1/2 in neutrophils ~~due to their potential roles as chemotactic receptors when interacting with phenotype-associated macrophages~~. (Lines 248-249)

While $ANXA1^+$ neutrophil exhibited quiescent phenotype that promote T cell proliferation, and inhibit mast cell chemotaxis and activation, **playing a potential role in** inducing chronic inflammation (Supplementary Figure 4B). (Lines 258-261)

Previous study pinpointed the **macrophage subset as the predominant origin** of detrimental molecules for aneurysm and AD, including IL1RN/TREM1 M1-like macrophage subcluster, which also expressed high levels of proinflammatory chemokines such as CXCL2, CCL3, and CCL4. **While our study uncovered the macrophage subcluster that highly expressing inflammatory chemokines and cytokines, but moderately expressing of TREM1 and IL1RN**, which further indicates the intrinsic difference in the underlying pathologic mechanism between aneurysm and dissection. Recently, $CXCL3^+$ inflammatory macrophages have been detected primarily in acute coronary culprit plaques rather than in chronic plaques, exhibiting potential therapeutic targets for preventing acute coronary syndrome. Likewise, to further contextualize our findings, it would be interesting to test the effects of specific depletion of $CXCL3^+$ macrophage in an AD mouse model. (Lines 494-504)

- ***Reference #33 has no title or publication details.***

Response: Thank you for the comment. We have made the revision.

Revision:
33. Daniel, C., et al. Extracellular DNA traps in inflammation, injury and healing. Nat Rev Nephrol 15, 559-575 (2019).

• ***Methods: The IF chapter accidentally mentions 5 mm rather than 5 µm sections.***

Response: Thank you for pointing that out. We have corrected the error.

Revision:

All aortic tissue samples were formalin fixed, paraffin embedded, and **5 µm** sections were subjected to antigen retrieval in citrate buffer, then permeabilized and stained with mouse anti-human. (Lines 729-731)

References of responding letter

1. Salcher, S.*, et al.* High-resolution single-cell atlas reveals diversity and plasticity of tissue-resident neutrophils in non-small cell lung cancer. *Cancer Cell* **40**, 1503-1520.e1508 (2022).
2. Wang, L.*, et al.* Single-cell RNA-seq analysis reveals BHLHE40-driven pro-tumour neutrophils with hyperactivated glycolysis in pancreatic tumour microenvironment. *Gut* **72**, 958-971 (2023).
3. Daniel, C.*, et al.* Extracellular DNA traps in inflammation, injury and healing. *Nat Rev Nephrol* **15**, 559-575 (2019).
4. Okubo, K.*, et al.* Macrophage extracellular trap formation promoted by platelet activation is a key mediator of rhabdomyolysis-induced acute kidney injury. *Nat Med* **24**, 232-238 (2018).
5. Pertiwi, K.R.*, et al.* Extracellular traps derived from macrophages, mast cells, eosinophils and neutrophils are generated in a time-dependent manner during atherothrombosis. *J Pathol* **247**, 505-512 (2019).
6. Dhayni, K., Zibara, K., Issa, H., Kamel, S. & Bennis, Y. Targeting CXCR1 and CXCR2 receptors in cardiovascular diseases. *Pharmacol Ther* **237**, 108257 (2022).
7. Sitaru, S., Budke, A., Bertini, R. & Sperandio, M. Therapeutic inhibition of CXCR1/2: where do we stand? *Intern Emerg Med* **18**, 1647-1664 (2023).
8. Sun, X.*, et al.* Inflammatory cell-derived CXCL3 promotes pancreatic cancer metastasis through a novel myofibroblast-hijacked cancer escape mechanism. *Gut* **71**, 129-147 (2022).

Reviewer #3

The proposed round of revisions has been accurately performed. The revised manuscript is acceptable for publishing as is.

Response: We sincerely thank you for your positive evaluation and for recognizing the efforts we made in revising the manuscript. We are delighted that you find the revised manuscript acceptable for publication. Your constructive feedback has been greatly appreciated throughout the review process.

Manuscript # NCOMMS-23-59187

Title: Integrated Multi-Omics Profiling Reveals Neutrophil Extracellular Traps Potentiate Aortic Dissection Progression

Response to Reviewers

We sincerely appreciate the detailed feedback provided by the reviewers and the editorial team. We have carefully addressed all the concerns and suggestions raised by Reviewer #2 to ensure that the revised version meets the journal's guidelines. Our point-by-point response is as follows.

Reviewer #2

The authors have conducted a comprehensive second round of revisions to address all raised concerns. In view of their explanation on the underrepresentation of neutrophils in the applied omics technique, I was surprised that an entire chapter is devoted to the characterization of neutrophil subsets and their differentiation trajectories. Yet, based on my limited experience with omics technology, I do not feel sufficiently qualified to evaluate the aptness of these analyses and interpretations.

After carefully reading the manuscript once more, I do not suggest any further major revisions. Yet, there were several minor errors and issues of scientific phrasing which have remained and which are listed below. Pending journal policy and Editor´s assessment, I would recommend to possibly include these corrections at the type setting (page proof) stage rather than going through another lengthy round of revisions and review.
Please note: The manuscript text was inserted twice in the merged article file (with slightly distinct line numbering). The line numbers listed below refer to the first inserted manuscript version.

Response: We are deeply grateful to the reviewer for their thoughtful and constructive feedback on our revised manuscript. Your insights have been invaluable in enhancing the quality of our study. Our point-by-point response is as follows.

1. Abstract:
An error was accidentally introduced by misinterpreting my statement of the last review, i.e. an entire sentence was duplicated and is now shown twice in results as well as conclusions of the Abstract (rather than improving the phrasing of the results text).
Text now reads:
Results: Integrated multi-omics profiling identified highly phenotype-associated macrophages orchestrate neutrophil extracellular traps (NETs) through CXCL3/CXCR2 axis in fueling the development of AD. Increased NETs formation is a defining feature of systemic immunity and aortic microenvironment of AD. Inhibiting NETs formation through the blockade of CitH3 or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The level of citrullinated histone H3 (CitH3) could serve as a risk factor for AAEs following endovascular therapy for patients with AD.
Conclusions: The multi-omics profiling reveals NETs formation features in the development of AD. Inhibiting NET formation through the blockade of histone citrullination or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The plasma level of citrullinated histone H3 (CitH3) could serve as a risk factor for AAEs following endovascular therapy for patients with AD.
Text suggested by me:
Results: Integrated multi-omics profiling identified highly phenotype-associated macrophages to orchestrate neutrophil extracellular traps (NETs) through the CXCL3/CXCR2 axis in fueling the development of AD. Increased NET formation is a defining feature of systemic immunity and aortic microenvironment of AD. Inhibiting NET formation through the blockade of histone citrullination or the CXCL3/CXCR2 axis could

ameliorate the progression and rupture of AD in mice. The plasma level of citrullinated histone H3 (CitH3) predicted AAEs following endovascular therapy for patients with AD. Conclusions: The multi-omics profiling revealed NET formation features in the development of AD. NET-associated markers could facilitate the risk stratification and prognostic evaluation, and might serve as potential therapeutic targets of patients with AD.

Response: Thank you for the comment. We have revised the Abstract section according to your suggestion. Abstract now reads as follows.

Revision:

Abstract

Background and Aims: Adverse aortic remodeling increases the risk of aorta-related adverse events (AAEs) after thoracic endovascular aortic repair (TEVAR) and affects the overall prognosis of aortic dissection (AD). It is imperative to delve into the exploration of prognostic indicators to streamline the identification of individuals at elevated risk for postoperative AAEs, and therapeutic targets to optimize the efficacy of TEVAR for patients with AD.

Methods: We performed proteomic and single-cell transcriptomic analyses of peripheral blood and aortic lesions, respectively, from patients with AD and healthy subjects. We performed in vivo experiments and multiplex immunofluorescence to further confirm the effect of inhibiting NETs. Kaplan-Meier and Cox regression analysis were used to identify independent risk factors for AAEs.

Results: Integrated multi-omics profiling identified highly phenotype-associated macrophages orchestrate neutrophil extracellular traps (NETs) through CXCL3/CXCR2 axis in fueling the development of AD. Increased NETs formation is a defining feature of systemic immunity and aortic microenvironment of AD. Inhibiting NETs formation through the blockade of CitH3 or CXCL3/CXCR2 axis could ameliorate the progression and rupture of AD in mice. The plasma level of citrullinated histone H3 (CitH3) predicted AAEs following endovascular therapy for patients with AD.

Conclusions: The multi-omics profiling revealed NETs formation features in the development of AD. NET-associated markers could facilitate the risk stratification and prognostic evaluation, and might serve as potential therapeutic targets of patients with AD.

2. Apparent errors:

Line 301: Figure 4A not 5A

Line 354: Supplementary Figures 5D-E not 4D-E

Line 372: The BAPN + IgG group (not the BAPN group) shows 50% aortic rupture according to Figure 6B

Line 375: Figure 6C not 6F

Line 936: n=59 not n=58 (based on Suppl. Table 2)

Line 1046: "sequenced mesenchymal lineage cells" should be neutrophil subsets?

Line 1112: "in prognostic cohort" was not replaced by "in predicting AAEs"

Methods – IF analysis of NETs in tissue sections: The authors state that "All aortic tissue samples were formalin fixed, paraffin embedded, and 5 µm sections were subjected to antigen retrieval in citrate buffer, then permeabilized and stained with mouse anti-human." Yet, all listed IF antibodies were apparently of rabbit origin!?

Errors in English spelling/grammar: in particular
Lines 229-247 (chapter on neutrophil heterogeneity)
Lines 474-484 (Discussion)

Response: Thank you for the comment. We have revised these errors and spellings according to your advice.

Revision:
The mice in the experimental group were harvested after 4-week induction of BAPN to observe the subsequent AD occurrence or immediately after death (Figure 4A). (Lines 319-321)

Next, we found that expression of CXCL3 was higher in AD than in normal aorta (Supplementary Figure 5C), and phenotype-associated macrophages, especially FCGR3A$^+$ macrophages and OLR1$^+$ macrophages, displayed elevated expression compared to other clusters (Supplementary Figures 5D-E). (Lines 368-371)

At the end of the 4-week period, there was 50.0% (6/12) of the mice in the BAPN plus IgG group succumbing due to aortic rupture. (Lines 388-389)

The survival analysis showed improved mortality due to aortic rupture in anti-CXCL3 and anti-CXCR2 groups (Figure 6C). (Lines 391-393)

(D) NETs associated markers as detected by ELISA in the plasma from patients with AD (n = 187) and healthy individuals (n = 59). (Lines 979-981)

(G-H) trajectory and heatmap of neutrophils with markers by Monocle. (Lines 1090-1091)

Supplementary Table 4. Baseline characteristics of patients with AD receiving TEVAR in predicting AAEs. (Lines 1145-1146)
Supplementary Table 5. Univariable and multivariable Cox proportional hazard regression analyses of patients receiving TEVAR in predicting AAEs. (Lines 1156-1157)

All aortic tissue samples were formalin fixed, paraffin embedded, and 5 μm sections were subjected to antigen retrieval in citrate buffer, then permeabilized and stained ~~with mouse anti-human~~. (Lines 728-730)

3. Cases to improve the accuracy of scientific phrasing:
Legend to Figures 4 and 6: (C) Mice survival due to aortic rupture of each indicated group. (It is mouse death rather than survival that is due to aorta rupture.)

Response: Thank you for the comment. We have revised the phrasing according to your advice.

Revision:

Figure 4. Inhibition of NETosis attenuates dissection progression in mice with AD. (C) Mice death due to aortic rupture of each indicated group. (Lines 990-991)
Figure 6. Macrophage fosters NETs formation via CXCL3/CXCR2 axis. (C) Mice death due to aortic rupture of each indicated group. (Lines 1121-1122)

> ***4. Lines 286-288: We detected NETs-associated markers in the plasma from 187 patients with AD and 59 healthy individuals with balanced baseline characteristics (Supplementary Table 2).***
> ***(Suppl. Table 2 shows that the two groups were not balanced, i.e. differed significantly in almost all baseline characteristics – including sex and age distribution. Yet, these potentially confounding factors were partly included in the multivariable analysis in Suppl. Table 3.)***

Response: Thank you for the comment. We have revised the phrasing according to your advice.

Revision:
We detected NETs-associated markers in the plasma from 187 patients with AD and 59 healthy individuals ~~with balanced baseline characteristics~~ (Supplementary Table 2). (Lines 304-306)

> ***4. Lines 333-335: Furthermore, both Cl-amidine-treated and DNase I-treated group (Combo) demonstrated a significant reduction in CitH3 and Ly6G or MPO positive areas when compared to BAPN-induced AD mice (Figure 4K).***
> ***(There is no quantitation but representative confocal images of these immunofluorescence tissue stainings, i.e. the term "significant reduction" is somewhat misleading and should rather read "substantial reduction".)***

Response: Thank you for the comment. We have made the revision according to your advice.

Revision:
Both Cl-amidine-treated and DNase I-treated group (Combo) demonstrated a substantial reduction in CitH3 and Ly6G or MPO positive areas when compared to BAPN-induced AD mice (Figure 4K). (Lines 350-352)

> ***5. Lines 336-338: Taken together, our findings suggested that alleviating the progression and rupture of AD in mice can be achieved by inhibiting NETs formation through the blockade of CitH3, while inhibiting NETs function via DNase I treatment is not as effective.***
> ***(Cl-amidine blocks citrullination of histones and other proteins; CitH3 is the chosen read-out parameter i.e. is not the sole target and not the only citrullinated histone in NET formation. Hence, "blockade of histone citrullination" would seem more appropriate.)***

Response: Thank you for the comment. We have made the revision according to your advice.

Revision:

Taken together, our findings suggested that alleviating the progression and rupture of AD in mice can be achieved by inhibiting NETs formation through the blockade of histone citrullination, while inhibiting NETs function via DNase I treatment is not as effective. (Lines 353-355)

6. Lines 382-384: These findings suggested that the CXCL3/CXCR2 axis plays an indispensable role in the macrophage-neutrophil interaction, thereby contributing to the progression and rupture of AD.
(The incidence of AD and aorta rupture are reduced but not abrogated by CXCL3/CXCR2 blockade, i.e. the word "indispensable" seems too strong.)

Response: Thank you for the comment. We have made the revision according to your advice.

Revision:
These findings suggested that the CXCL3/CXCR2 axis plays a crucial role in the macrophage-neutrophil interaction, thereby contributing to the progression and rupture of AD. (Lines 399-401)

7. Line 376: The authors forgot to substitute the word "reduction" by "reversal" to indicate that the various investigated disease parameters were either decreased or increased by therapy.

Response: Thank you for the comment. We have made the revision according to your advice.

Revision:
Moreover, significant reversal of the average max aortic diameter, overall and media thickness, elastic fiber degradation, and NETs marker presence were observed in anti-CXCL3 and anti-CXCR2 groups (Figures 6D-6K and Supplementary Figure 6F). (Lines 393-398)

8. Line 399 and legend to Figure 7B specify a cut-off level at 6.91 ng/ml but do not mention the parameter, i.e. 6.91 ng/ml CitH3.

Response: Thank you for the comment. We have made the revision according to your advice.

Revision:
(B) ROC curve analysis showing that at the cut-off level defined as 6.91 ng/ml CitH3, the sensitivity and specificity for predicting AAEs are 0.6 and 0.9, respectively, with the area under the curve of 0.75.

9. Results chapter on "Inhibition of NETosis ameliorates dissection progression in AD mice": data are presented twice (lines 301-309 and lines 318-323)

Response: Thank you for the comment. We have made the revision according to your advice.

Revision:

Two drugs Cl-amidine (PAD4 inhibitor[1]) and DNase I were adopted to inhibit NETs formation and digest NETs after their formation, respectively. The mice in the experimental group were harvested after 4-week induction of BAPN to observe the subsequent AD occurrence or immediately after death (Figure 4A). At the end of the 4-week period, it was observed that 66.7% (8/12) of the mice developed AD in the BAPN group and 50% (6/12) of them died of aortic rupture, with the first mouse succumbing on the 9th day. In Cl-amidine group, 50.0% (6/12) of the mice developed AD and 16.7% (2/12) of them died of aortic rupture before the end of the experiment, showing Cl-amidine-treated mice had significantly less AD rupture rate and improved survival rate (Figures 4B and 4C). While in DNase I group, 66.7% (8/12) of the mice developed AD and 50.0% (6/12) of them died of aortic rupture, which showed a moderate improvement in survival rate (Figures 4B and 4C). Whereas the combined group of Cl-amidine and DNase I (Combo) group showed an incidence of 50.0% (6/12) and mortality of 16.7% (2/12) due to aortic rupture (Figures 4B). (Lines 318-329)

There was strong evidence of aortic expansion, elastin degradation and thrombosis in the false lumen, resulting in the compression of the true lumen, in BAPN-induced AD mice (Figure 4G and 4J). Abundant staining of CitH3 and moderate staining of Ly6G were observed in AD mice (Supplementary Figure 6E). ~~Since not only neutrophils but also macrophages, eosinophils and mast cells are sources of extracellular traps[2], NETs formation needed to be further verified.~~ Moreover, the average max aortic diameter, overall thickness and media thickness of aortas, and elastic fiber degradation of mice from Cl-amidine-treated and Combo group were significantly reversed compared to AD mice, suggesting reduced aortic expansion and maintained elastin integrity (Figures 4F-4I). The average max diameter of aortas from DNase I-treated mice showed a slight reduction, but without statistical significance when compared to AD mice (Figures 4F-4I). (Lines 337-347)