nature portfolio

Peer Review File

Increased CSF drainage by noninvasive manipulation of cervical lymphatics

Corresponding Author: Professor Gou Young Koh

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)
The critical importance of cerebrospinal fluid (CSF) drainage to cervical lymph nodes has been intensely studied and discussed in recent years, but the precise drainage routes are still enigmatic. This has also precluded to some extent a better understanding of reduced CSF flow with increasing age, and a rational attempt at ameliorating this age-related phenomenon.
In the current study the authors make significant strides to overcome the lack in our understanding of CSF flow, and establish the drainage routes of CSF from different intracranial origins. In particular, the importance of superficial cervical lymphatics is firmly established.
The results are novel and have the potential to serve as a hallmark reference in the field. Other than the carefully documented anatomical and functional results, the authors report additional results of significance: (1) the equivalent studies in Macaca fascicularis support the findings in mice. (2) aged mice show reduced CSF outflow through cervical lymph nodes and present with a shift to fibrosis-associated fibroblasts adjacent to scLVs, possibly explaining the functional impairment. (3) CSF impairment in aged individuals can be improved significantly by external, non-invasive mechanical stimulation of scLVs.
The approaches chosen are robust and valid, the quality of the data is excellent. I find little to criticize other than a couple of minor points for clarification (see below).

The conclusions are robust and follow from the data, the text is well presented and clear. I find the study extremely well done, and using a rather straight-forward set of experiments, the authors have succeeded in compiling an important set of data that will be extremely useful to colleagues in the immediate field, but also insightful to a wider audience. The finding of enabling improved CSF flow through mechanical forces on the skin is remarkable.

Minor comments:
(1) Regarding the findings in Fig.9a/b, the increase of Nos3high LECs in aged mice is remarkable. The authors compare the ratio of these cells in comparison to all other cells (incl. capillaries and valve cells), but I would think that the ratio of Nos3high LECs in comparison to only the collecting LECs is more informative.
(2) The discussion in lines 489-497 is relevant, but limited to one example in its attempt to address the translational relevance of understanding the connections to the meningeal lymphatic network. If space permits, other examples would help to support the relevance of the authors' findings.
(3) There have been many discussions in the field about the technicalities of introducing tracer dyes to follow lymphatic routes in mice. The respective M&M section (lines 596 onwards) shows no references. The authors should make an attempt to relate their methodology to the existing literature.

Referee #2

(Remarks to the Author)
The report from Jin et al presents experimental data from mice and primates regarding the routes of CSF lymphatic drainage to the superficial cervical lymph nodes. This research follows upon another report from Yoon et al that was published in Nature in January of this year that elucidated the pathways through the nasopharyngeal lymphatic plexus to the deep cervical lymph nodes. The authors describe the morphological and functional differences in the superficial lymphatic network that exist between young and aged mice and present a method using mechanical stimulation that can acutely

increase the transport of CSF tracers to the cervical lymph nodes. As with the authors' previous papers, the presentation of the data, including high resolution images of fluorescently labeled decalcified tissue sections or cleared samples, is spectacular. However, this reviewer is not convinced that the method introduced and suggested to improve the CSF drainage deficit seen in aging mice would allow for prolonged increases in clearance beyond the acute effect that is demonstrated. There are also open questions regarding the relevance of the superficial lymphatic pathways in humans or during neuropathology.

Major comments:

1. It is unclear why the authors decided to attempt to stimulate the afferent pathways to the superficial cervical lymph nodes as an approach to enhance CSF clearance in aged mice. There were no apparent differences found in the contractile apparatus of these vessels between aged and young mice at either the functional or transcriptomic level. The only overt difference found when comparing aged vs young mice appeared to be less VEGFR3+ lymphatic vessels in the nasal mucosa in aged mice. What do the authors envision that the mechanical device is stimulating? Is this targeting a direct enhancement of the pumping function of these vessels or is it "squeezing" lymph from the initial or precollecting lymph vessels and/or promoting drainage of interstitial fluid from subcutaneous tissue into the collectors? The latter appears more likely as the collecting vessels inherently have the ability of spontaneous contractility. In this scenario, how can the authors be sure that they are truly enhancing CSF clearance from the subarachnoid space?
2. The mechanical stimulation led to an acute effect of drainage that could be immediately measured by CSF tracer accumulation in the downstream lymph node. It is still unclear how such an approach could alleviate a long-term decline in CSF clearance, such as that seen in aging or aging-related neurodegenerative disorders. The authors claim that "Importantly, the results also show that the reduction in CSF drainage to cervical lymph nodes in aged mice can be reversed by mechanical stimulation of the skin." However, the approach would appear to be more well-suited to alleviate an acute functional blockage of CSF drainage that might develop after a traumatic injury.
3. The authors have demonstrated that microbeads delivered into the CSF of primates can also drain into the cervical lymph nodes in this species. This is a notable finding, as data is lacking on lymphatic routes for CSF clearance in high-order species. From this finding, the authors claim "CSF drainage routes through lymphatics similar to mice" for monkeys. However, from the data presented it is not entirely clear whether this is true. The authors show fluorescence signal emanating from the foramina at the hard and soft palate, but they have not demonstrated the downstream lymphatic pathways. Do the lymphatic vessels also track closely to the skin in monkeys as they do in mice? This is important information if the authors want to make the claim that a mechanical stimulation of the skin might alleviate impaired CSF clearance through superficial lymphatics in the clinic.

Minor comments:

1. The authors make the claim that 50% of the drainage to the cervical lymph nodes occurs through the superficial lymphatic network. I'm not sure that one can make this claim from the data presented. The estimate of 50% is made from quantification of intensity of TMR-dextran between the two submandibular lymph nodes and deep cervical lymph nodes at a single time point. CSF clearance is a dynamic process with multiple routes active simultaneously, each carrying a portion of the volumetric outflow.
2. The authors repeatedly make the statement that the drainage to the pathways documented in the paper originates at upstream meningeal lymphatic vessels. However, despite the many images presented with tracer clearly present within the extracranial lymphatics, this reviewer does not see any equivalent imaging of the tracer within the intracranial meningeal lymphatics. The figures referenced for these statements are Figs. 1a, b (which are schemes) and Fig. 4a-g. Furthermore, Extended Data Fig 10e shows extensive microbead coverage on the intracranial side but this signal does not appear to be particularly associated with lymphatic vessels.
3. Regarding the decreased coverage of VEGFR3 lymphatic vessels in the nasal mucosa in aged mice, can the authors indicate where on Figure 7, Panel c that these quantifications were performed (as was done for ROI 1 and ROI 2 in Panel a)? Can the authors comment on what appears to be an increase in coverage in Prox1-GFP vessels in the aged maxilloturbinates?
4. Beyond the image of the device shown in Extended Data Fig. 24, the authors should provide a video showing the mechanical stimulation therapy in mice.

Referee #3

(Remarks to the Author)
Jin et al investigate cerebrospinal fluid (CSF) drainage pathways, which have been a topic of intense investigations and debate recently, focusing on the role of superficial cervical lymphatics that they identify as key routes for CSF outflow. Using fluorescent tracers in Prox1-GFP reporter mice and in monkeys, they provide a comprehensive anatomical mapping of this CSF outflow pathway and its age-related alterations. In addition, they develop a force-regulated mechanical device to manipulate superficial cervical lymphatics through the intact skin, which significantly enhances CSF outflow.

This work offers important insights into the anatomy of lymphatic pathways involved in CSF drainage and introduces a promising non-invasive therapeutic strategy for enhancing CSF flow, which decreases with age. The detailed anatomical descriptions are supported by beuatiful, convincing imaging and clear illustrations that provide an anatomy textbook-like reference for further research. Apart from these strengths, the study is largely descriptive, and the molecular findings, such as those from single-cell RNA sequencing, lack validation, which reduces the overall value and impact of this data as presented.

Specific comments:

1. Limited experimental manipulation was performed to assess the relationship between the previously identified CSF drainage mechanisms and the superficial drainage routes. It would be valuable to assess if the superficial drainage pathways are affected upon manipulation of meningeal lymphatic vessels, for example through VEGF-C overexpression or trapping, or photoablation. The reverse experiment to that shown in Extended Data Fig. 2a-d (lines 146-150), with ligation of scLV1 and scLv2 and analysis of drainage to dcLN would strengthen the data.

2. scRNAseq data lacks experimental validation, limiting its value and impact. For example, three clusters of collecting vessels are annotated in Fig 9a, but their identity at the tissue level is unclear. Do they represent different collecting vessels, calibres of vessels, or specific anatomic positions within individual vessels? Heatmaps of top cluster markers are shown in extended data, but all data including statistics should also be provided as supplementary data.

3. Similarly, several clusters of smooth muscle cells and fibroblasts are annotated but not validated at the tissue level. Is increased number or fibrosis-associated fibroblasts and associated fibrosis observed in aged mice?

4. Age-related transcriptional changes are primarily characterized using GO term analysis, without experimental validation, limiting the strength of the conclusions. The full results of the GO term analysis should be included in supplementary data.

5. It is not clear from the method description if the scRNAseq data is based on a single pooled sample including six mice per condition, or individual samples from six mice per condition (adult, aged). It would be important to confirm that all samples contribute to all clusters, particularly those that are increased in aged mice (Nos3-high LEC and Postn-high fibroblasts)?

6. Certain speculative statements should be backed up by data or toned down to reflect limited experimental validation, including line 45: '…surrounded by more abundant fibrosis-associated fibroblasts, which could contribute to the impaired CSF outflow, line 347: 'Upregulation of Nos3 and Gch1 expression during ageing could be compensation for the reduced responsiveness to the nitric oxide'.

7. Detailed information on how the mechanical stimulator was applied is lacking, including 1) if the device was applied to awake or sedated mice, 2) how the device was positioned, and 3) how the device strokes the areas indicated in Extended Data Fig. 24. A figure or movie showing the mechanical stimulation would be informative.

8. Line 61: the following articles should be discussed and cited to provide a balanced view on the role of CSF drainage through meningeal lymphatics in CNS pathologies: Antila et al, Nat Cardiovasc Res 3, 474–491 (2024), Zhilin et al, Sci Immunol 8,eabq0375 (2023).

9. Lines 136-138 and Fig. 2b: As discussed in lines 142-144 and Extended Data Fig. 1 there are anatomical variations in the location of smLN, asmLN and ptLN. It should be clarified what type of LN antomy the mice undergoing these drainage experiments had. As ptLN and asmLN are fused and connected in 7% of the case, it is surpising that the uptake of tracer into ptLN is never observed.

10. Line 174, 179-181: The authors state that drainage to smLN occurs first through scLV1 (30 min) and later to scLV2 (60 min) and that asmLN receives CSF from the nasopharyngeal plexus at 60 min. Since in 60% of mice smLN and asmLN are fused, it should be again clarified what type of LN antomy the mice undergoing these drainage experiments had, and if and how the fusion affects the drainage patters.

11. Line 386-388: It is unclear why electrical stimulation of muscle contractions would involve surgery or anaesthesia. Transcutaneous electrical nerve stimulation (TENS) is widely used and does not involve invasive procedures. Would it have a similar impact? The mechanical device developed here presumably also involves anaesthesia.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
The authors have responded to my queries, and also have addressed most of the queries raised by the other reviewers. The manuscript has improved, and I have no further comments.

Referee #2

(Remarks to the Author)
The authors have performed a substantial number of additional experiments in response to the reviewer comments. The manuscript is much improved, and my major concerns have been addressed. In particular, the additional experiment with ICG injections into the macaques is very exciting and shows the close similarity in the pathways of CSF lymphatic clearance between the rodent and primate. This is very important data to help to bridge the gap towards the clinic by making the critical

point that the superficial lymphatic networks may indeed be a relevant therapeutic target. I have only a couple more minor comments that could be addressed by changes to the text.

Minor comments:

1. Regarding previous Minor comment #1: The authors have performed an additional study covering additional timepoints that show the dynamics of tracer outflow to the different cervical lymph nodes (Fig 2g). They then use AUC calculations to continue to make the claim that "Tracer studies in adult mice revealed that ~50% of total CSF outflow to the neck drained to superficial cervical lymph nodes." The authors should still be careful how they interpret this data. The deep cervical lymph node showed much higher peak signal intensity and a more rapid wash-through of the tracer bolus compared to the superficial cervical lymph nodes. Thus, the AUC measurements are skewed toward inflating the values of the slower drainage routes, which retain tracer for a longer period. A lymphatic cannulation approach using a sustained low-rate infusion of tracer would be one way to accurately estimate the proportion of CSF outflow that the specific drainage routes carry. However, this experiment would be extremely challenging in mice from a technical standpoint. The AUC data in the Fig 2g are still valuable. However, the authors should adjust the language in the Abstract, Results and Discussion to indicate that the superficial cervical lymph node network carries a "significant portion" of CSF flow rather than making any quantitative claim.
2. In the sentence, on Page 17, lines 493-495: "To determine whether mechanical stimulation of facial and neck skin increased CSF clearance from the SAS, instead of just pushing CSF in scLV-1 and scLV-2 into cervical lymph nodes" – shouldn't this be written "lymph" or "lymphatic fluid" in scLV-1 and scLV-2 rather than CSF?

Referee #3

(Remarks to the Author)
The authors have carefully addressed the reviewers' comments and improved the study. I have no further concerns and would like to congratulate the authors on an excellent piece of work.

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Referees' comments:

Referee #1 (Remarks to the Author)

The critical importance of cerebrospinal fluid (CSF) drainage to cervical lymph nodes has been intensely studied and discussed in recent years, but the precise drainage routes are still enigmatic. This has also precluded to some extent a better understanding of reduced CSF flow with increasing age, and a rational attempt at ameliorating this age-related phenomenon.

In the current study the authors make significant strides to overcome the lack in our understanding of CSF flow, and establish the drainage routes of CSF from different intracranial origins. In particular, the importance of superficial cervical lymphatics is firmly established.

The results are novel and have the potential to serve as a hallmark reference in the field. Other than the carefully documented anatomical and functional results, the authors report additional results of significance: (1) the equivalent studies in *Macaca fascicularis* support the findings in mice. (2) aged mice show reduced CSF outflow through cervical lymph nodes and present with a shift to fibrosis-associated fibroblasts adjacent to scLVs, possibly explaining the functional impairment. (3) CSF impairment in aged individuals can be improved significantly by external, non-invasive mechanical stimulation of scLVs.

The approaches chosen are robust and valid, the quality of the data is excellent. I find little to criticize other than a couple of minor points for clarification (see below).

The conclusions are robust and follow from the data, the text is well presented and clear. I find the study extremely well done, and using a rather straight-forward set of experiments, the authors have succeeded in compiling an important set of data that will be extremely useful to colleagues in the immediate field, but also insightful to a wider audience. The finding of enabling improved CSF flow through mechanical forces on the skin is remarkable.

Response: We appreciate the reviewer's enthusiastic, favorable, and encouraging comments and thorough, insightful, and careful review. We are delighted the reviewer found the findings novel and have the potential to serve as a hallmark reference in the field.

Minor comments:

(1) Regarding the findings in Fig.9a/b, the increase of Nos3high LECs in aged mice is remarkable. The authors compare the ratio of these cells in comparison to all other cells (incl. capillaries and valve cells), but I would think that the ratio of Nos3high LECs in comparison to only the collecting LECs is more informative.

Response: We agree that comparing the relative size of the *Nos3*^{high} subcluster to other subclusters in collecting LEC in adult and aged mice is more informative. The revised analysis is described in the main text (lines 376-377), pie charts (**Fig. 9b**), and revised figure legend.

(2) The discussion in lines 489-497 is relevant, but limited to one example in its attempt to address the translational relevance of understanding the connections to the meningeal lymphatic network. If space permits, other examples would help to support the relevance of the authors' findings.

Response: We agree and have revised this section of the Discussion (lines 589-591).

(3) There have been many discussions in the field about the technicalities of introducing tracer dyes to follow lymphatic routes in mice. The respective M&M section (lines 596 onwards) shows no references. The authors should make an attempt to relate their methodology to the existing literature.

Response: To address this suggestion, we added the relevant references to lines 701-705 (Methods reference 3), lines 730-733 (Methods references 3-5), and lines 752-754 (Methods reference 6) in Materials and Methods section.

Additional information for Reviewers #1, #2, and #3 at the end of the point-by-point responses: Please see at the end of our point-by-point responses the list of new experiments performed to address issues raised by the reviewers. Because of the overlapping nature of comments, this list was prepared for all reviewers to complement the point-by-point responses to comments made by individual reviewers.

Referee #2 (Remarks to the Author)

The report from Jin et al presents experimental data from mice and primates regarding the routes of CSF lymphatic drainage to the superficial cervical lymph nodes. This research follows upon another report from Yoon et al that was published in Nature in January of this year that elucidated the pathways through the nasopharyngeal lymphatic plexus to the deep cervical lymph nodes. The authors describe the morphological and functional differences in the superficial lymphatic network that exist between young and aged mice and present a method using mechanical stimulation that can acutely increase the transport of CSF tracers to the cervical lymph nodes. As with the authors' previous papers, the presentation of the data, including high resolution images of fluorescently labeled decalcified tissue sections or cleared samples, is spectacular. However, this reviewer is not convinced that the method introduced and suggested to improve the CSF drainage deficit seen in aging mice would allow for prolonged increases in clearance beyond the acute effect that is demonstrated. There are also open questions regarding the relevance of the superficial lymphatic pathways in humans or during neuropathology.

Response: We appreciate the reviewer's favorable and insightful comments. We are pleased that the reviewer appreciated the images of immunohistochemically stained tissue whole mounts, as the preparation of these whole mounts proved to require considerable technical innovation.

Major comments:

1. It is unclear why the authors decided to attempt to stimulate the afferent pathways to the superficial cervical lymph nodes as an approach to enhance CSF clearance in aged mice. There were no apparent differences found in the contractile apparatus of these vessels between aged and young mice at either the functional or transcriptomic level. The only overt difference found when comparing aged vs young mice appeared to be less VEGFR3+ lymphatic vessels in the nasal mucosa in aged mice.

Response: The reviewer raises an important issue regarding the rationale for using mechanical stimulation of superficial cervical lymphatics to increase CSF clearance in aged mice. This rationale is now clarified in the Introduction (lines 107-131). In brief, as the reviewer points out, no morphological, functional, or transcriptomic differences between young and aged mice were found in the contractile apparatus of superficial cervical lymphatics (**Extended Data Figs. 20a-e, 21a,b** and **Figs. 8a,b, 9f-j**). Because the contractile apparatus remained relatively normal in aged mice, superficial collecting lymphatics were considered a reasonable target for increasing CSF clearance by mechanical stimulation in the presence of atrophic upstream lymphatics and impaired CSF clearance.

What do the authors envision that the mechanical device is stimulating? Is this targeting a direct enhancement of the pumping function of these vessels or is it "squeezing" lymph from the initial or precollecting lymph vessels and/or promoting drainage of interstitial fluid from subcutaneous tissue into the collectors? The latter appears more likely as the collecting vessels inherently have the ability of spontaneous contractility.

Response: These are important questions. New experiments were designed to test the hypothesis that mechanical stimulation increases CSF outflow by inducing repeated compression and relaxation of superficial upstream head and neck lymphatics and downstream superficial cervical collecting lymphatics, with little effect on their spontaneous contractility.

Results obtained from intravital imaging analyses were consistent with this hypothesis. These findings are described in the Summary (lines 46-48), Results (lines 493-515), and Discussion (560-565) and are illustrated in **Extended Data Figs. 30-32**.

In brief, low-magnitude mechanical stimulation increased CSF outflow from the SAS into superficial cervical lymphatics (lines 493-499 and **Extended Data Fig. 30**) with little effect on multiple parameters of spontaneous contractility (lines 501-509 and **Extended Data Fig. 31**). Importantly, CSF outflow was increased not only after a single session of massage but also after repeated sessions over four days, indicative of the resilience of the effect (lines 511-515 and **Extended Data Fig. 32**).

In this scenario, how can the authors be sure that they are truly enhancing CSF clearance from the subarachnoid space?

Response: This is a critical point. To determine whether mechanical stimulation simply squeezes CSF out of superficial cervical lymphatics or truly increases CSF clearance from the subarachnoid space, we injected TMR-dextran into a lateral ventricle and applied 10 sessions of low-magnitude mechanical stimulation. We then measured the residual TMR-dextran concentration in CSF sampled at the cisterna magna. As described in the Results (lines 493-499) and illustrated in **Fig. 10g,h** and **Extended Data Fig. 30**, the concentration of TMR-dextran in CSF was significantly lower after stimulation, consistent with increased CSF clearance from the subarachnoid space. The approach is described in the Methods (lines 847-869).

2.The mechanical stimulation led to an acute effect of drainage that could be immediately measured by CSF tracer accumulation in the downstream lymph node. It is still unclear how such an approach could alleviate a long-term decline in CSF clearance, such as that seen in aging or aging-related neurodegenerative disorders. The authors claim that “Importantly, the results also show that the reduction in CSF drainage to cervical lymph nodes in aged mice can be reversed by mechanical stimulation of the skin.” However, the approach would appear to be more well-suited to alleviate an acute functional blockage of CSF drainage that might develop after a traumatic injury.

This excellent comment led us to perform new experiments that compared CSF outflow after one session of mechanical stimulation and after daily sessions over 4 days. CSF outflow was increased not only after a single session but also after daily sessions over four days (lines 511-515 and **Extended Data Fig. 32**). These findings demonstrate the reproducibility and resilience of the effect over four days and justify longer studies in aged mice and models of neurodegenerative conditions (lines 654-656).

3.The authors have demonstrated that microbeads delivered into the CSF of primates can also drain into the cervical lymph nodes in this species. This is a notable finding, as data is lacking on lymphatic routes for CSF clearance in high-order species. From this finding, the authors claim “CSF drainage routes through lymphatics similar to mice” for monkeys. However, from the data presented it is not entirely clear whether this is true. The authors show fluorescence signal emanating from the foramina at the hard and soft palate, but they have not demonstrated the downstream lymphatic pathways. Do the lymphatic vessels also track closely to the skin in monkeys as they do in mice? This is important information if the authors want to make the claim that a mechanical stimulation of the skin might alleviate impaired CSF clearance through superficial lymphatics in the clinic.

Response: This important question was addressed by performing new experiments where lymphatics in the head and neck of *Macaca fascicularis* monkeys were imaged intravitally after an intracisternal infusion of indocyanine green. Indeed, the tracer was clearly visible in periorbital lymphatics, nasal sidewall lymphatics, superficial cervical lymphatics (scLV), and in submandibular lymph nodes (smLN) in the monkeys at 30 min after infusion (lines 257-262 and **Extended Data Fig. 16**). The new approaches are described in the Methods (lines 750-767).

Minor comments:

1.The authors make the claim that 50% of the drainage to the cervical lymph nodes occurs through the superficial lymphatic network. I'm not sure that one can make this claim from the data presented. The estimate of 50% is made from quantification of intensity of TMR-dextran between the two submandibular lymph nodes and deep cervical lymph nodes at a single time point. CSF clearance is a dynamic process with multiple routes active simultaneously, each carrying a portion of the volumetric outflow.

Response: We agree that a single time point cannot fully reflect the dynamics of CSF clearance. Therefore, to address this issue in new experiments, TMR-dextran was injected intracisternally and measured in cervical lymph nodes at 15, 30, 60 and 120 min later, the longest time point chosen to reflect when little tracer remained in the lymph nodes. The amount of CSF outflow was represented as the area under the time-course curve in a pie chart that compares superficial and deep cervical lymph nodes (**Fig. 2g**). This section of the Results (lines 138-155) was rewritten to show that the new findings are consistent with the initial claim of superficial and deep cervical lymph nodes each receiving about half of the CSF drainage to lymph nodes in the neck.

2.The authors repeatedly make the statement that the drainage to the pathways documented in the paper originates at upstream meningeal lymphatic vessels. However, despite the many images presented with tracer clearly present within the extracranial lymphatics, this reviewer does not see any equivalent imaging of the tracer within the intracranial meningeal lymphatics. The figures referenced for these statements are Figs. 1a, b (which are schemes) and Fig. 4a-g. Furthermore, Extended Data Fig 10e shows extensive microbead coverage on the intracranial side but this signal does not appear to be particularly associated with lymphatic vessels.

Response: The reviewer makes an excellent point about the importance of providing unambiguous evidence of tracer movement from the SAS into dural lymphatics in these regions. To address this comment, we performed new experiments and added the findings (lines 232-247) and images of CSF tracers in dural lymphatics near the olfactory bulb and cribriform plate (**Extended Data Fig. 12d-j**) and pterygopalatine artery (**Extended Data Fig. 12b,c**), which are intracranial components of the CSF outflow route through superficial cervical lymphatics. To our knowledge, **Extended Data Fig.12d** is the first direct demonstration of a particulate CSF tracer in dural lymphatics around the olfactory bulb after injection into the SAS. Also shown is the widespread trapping of the red tracer in the arachnoid (**Extended Data Fig. 12b,d,e,g**), and the tracer in downstream extracranial lymphatics in the nasal mucosa (**Extended Data Fig. 12e**).

3. Regarding the decreased coverage of VEGFR3 lymphatic vessels in the nasal mucosa in aged mice, can the authors indicate where on Figure 7, Panel c that these quantifications were performed (as was done for ROI 1 and ROI 2 in Panel a)? Can the authors comment on what appears to be an increase in coverage in Prox1-GFP vessels in the aged maxilloturbinates?

Response: We thank the reviewer for finding that ROI-3 was not marked and now mark ROI-3, which shows the region in **Fig. 7c** where measurements of nasal Prox1^{+}/VEGFR3^{+} lymphatics were made for data in **Fig. 7d**. The apparent increase in *Prox1*-GFP vessels in the aged maxilloturbinate region reflects changes in venous sinusoids, which are Prox1^{+}, as described in our recent report (Hong SP et al. *Nat Cardiovasc Res*. 2023 2(5):449-466. PMID: 39196043). This feature is now described in the legend for **Fig. 7c** (lines 1562-1567). The new approaches used to measure the abundance and diameter of lymphatics are described in the Methods (lines 968-976).

4. Beyond the image of the device shown in Extended Data Fig. 24, the authors should provide a video showing the mechanical stimulation therapy in mice.

Response: We have added a video (**Supplementary Video 2**) showing the use of the mechanical stimulator to increase CSF drainage by messaging superficial cervical lymphatics in mice.

Additional information for Reviewers #1, #2, and #3 at the end of the point-by-point responses: Please see at the end of our point-by-point responses the list of new experiments performed to address issues raised by the reviewers. Because of the overlapping nature of comments, this list was prepared for all reviewers to complement the point-by-point responses to comments made by individual reviewers.

Referee #3 (Remarks to the Author)

Jin et al investigate cerebrospinal fluid (CSF) drainage pathways, which have been a topic of intense investigations and debate recently, focusing on the role of superficial cervical lymphatics that they identify as key routes for CSF outflow. Using fluorescent tracers in Prox1-GFP reporter mice and in monkeys, they provide a comprehensive anatomical mapping of this CSF outflow pathway and its age-related alterations. In addition, they develop a force-regulated mechanical device to manipulate superficial cervical lymphatics through the intact skin, which significantly enhances CSF outflow.

This work offers important insights into the anatomy of lymphatic pathways involved in CSF drainage and introduces a promising non-invasive therapeutic strategy for enhancing CSF flow, which decreases with age. The detailed anatomical descriptions are supported by beautiful, convincing imaging and clear illustrations that provide an anatomy textbook-like reference for further research. Apart from these strengths, the study is largely descriptive, and the molecular findings, such as those from single cell RNA sequencing, lack validation, which reduces the overall value and impact of this data as presented.

Response: We appreciate the reviewer's favorable assessment of the manuscript and images. This is especially meaningful to us as it acknowledges our effort to prepare images of CSF drainage in regions of lymphatics that have proven difficult to image by previous methods.

Specific comments:

1. Limited experimental manipulation was performed to assess the relationship between the previously identified CSF drainage mechanisms and the superficial drainage routes. It would be valuable to assess if the superficial drainage pathways are affected upon manipulation of meningeal lymphatic vessels, for example through VEGF-C overexpression or trapping, or photoablation. The reverse experiment to that shown in Extended Data Fig. 2a-d (lines 146-150), with ligation of scLV1 and scLv2 and analysis of drainage to dcLN would strengthen the data.

Response: To address these insightful comments, we first performed new experiments to determine the effects of VEGF-C overexpression on intracranial and superficial cervical lymphatics by infusing AAV9-mCherry-VEGF-C (experimental) or AAV9-mCherry (control) into the subarachnoid space at the cisterna magna. Regions where VEGF-C did or did not expand the lymphatic network are described in the Result (lines 277-291), illustrated in **Extended Data Fig. 17a-c,** and commented on in the Discussion (lines 606-609). The approach is described in the Methods (lines 769-778).

Next, we performed the reverse experiment by ligating scLV-1 and scLV-2 and measuring CSF drainage to superficial and deep cervical lymph nodes. This intervention markedly reduced CSF drainage to the submandibular lymph node but not to the accessory submandibular or deep cervical lymph node. The findings are described in the Results (lines164-175) and illustrated in **Extended Data Fig. 3a-d**. The new approaches are described in the Methods (lines 741-748).

2. scRNAseq data lacks experimental validation, limiting its value and impact. For example, three clusters of collecting vessels are annotated in Fig 9a, but their identity at the tissue level is unclear. Do they represent different collecting vessels, calibres of vessels, or specific anatomic

positions within individual vessels? Heatmaps of top cluster markers are shown in extended data, but all data including statistics should also be provided as supplementary data.

Response: To address these important issues and related issues raised in comments 3 and 5, we performed new experiments, reanalyzed earlier data to add to the validation of the scRNA-seq data, and rewrote this section of the Results (lines 365-409). New analyses of the six LEC subgroups are described in the text (lines 396-409) and illustrated in a new figure (**Extended Data Fig. 24**). The new experiments included *in-situ* hybridization for *Nos3* mRNA and immunofluorescence labeling of eNOS protein and FOXP2 protein in superficial cervical collecting LECs and also elucidated differences between superficial cervical collecting LECs and initial and pre-collecting LECs in the same region. References for *Foxp2*^{high} LEC (Hernández et al, 2021, EMBO) and valve LEC (Abe et al, 2022, Nature Cell Biology) are now cited as references 47 and 48 (lines 370 and 372). The new approaches are described in the Methods (lines 926-931, 937-944, and 979-981).

In addition, all data and statistics for the top 15 cluster markers used in the heatmaps for the LEC, mural cells, and fibroblasts are included in **Supplementary Tables 4, 5, and 6**, as described in the legends for **Extended Data Figs. 23, 25, and 27** on lines 2220-2221, 2273-2274, and 2328-2329. Furthermore, the code availability for measuring algorithms is described in lines 1127-1135).

Briefly, as is now described in the Results (lines 396-409) and shown in **Extended Data Fig. 24**, *Nos3* mRNA *in-situ* hybridization was greater in the LEC of superficial cervical lymphatics of aged mice, but eNOS immunoreactivity was lower (lines 396-401). This discrepancy is considered in the Summary (lines 42-44), Results (lines 444-450), and Discussion (lines 651-654). FOXP2 immunoreactivity was present in luminal LEC and stronger in valve LEC of superficial cervical lymphatics (**Extended Data Fig. 24f**). Regional lymphatics, consisting of initial lymphatics (with blunt ends) and pre-collecting lymphatics (with valves), were identified by the combination of *Prox1*-GFP and LYVE1 staining (**Extended Data Fig. 24h**), whereas collecting lymphatics lacked LYVE1 but had smooth muscle cell coverage marked by αSMA$^+$ (**Extended Data Fig. 24i**). Arteries and large veins were also marked by αSMA$^+$ but not by *Prox1*-GFP (**Extended Data Fig. 24i**). The mural cell annotation included pericytes and vascular smooth muscle cells (lines 411-415 and 423-431, **Fig. 9f,g**) because the facial vein was closely adherent to the superficial cervical lymphatics**.**

3. Similarly, several clusters of smooth muscle cells and fibroblasts are annotated but not validated at the tissue level. Is increased number or fibrosis-associated fibroblasts and associated fibrosis observed in aged mice?

Response: New experiments confirmed that ageing-related fibrosis was not restricted to lymphatics. As described in the revised Results (lines 433-442), cells with RNA *in-situ* hybridization for *Postn* (periostin) mRNA were more abundant around cervical lymphatics of aged mice than younger adults (**Extended Data Fig 26e,f).** However, IHC staining for type 1 collagen was stronger in the adventitia of veins than lymphatics (**Extended Data Fig 26h,i)**. We also added that lymphatic smooth muscle cell (LSMC) clusters and vascular smooth muscle cell (VSMC) clusters were annotated according to a previous report (Lei et al., Developmental Cell 2024, reference 51, lines 411-413). Immunohistochemical confirmation that Nr4a2 and Scn3a levels can be used to distinguish between LSMC and VSMC was not possible because of lack of availability of the corresponding antibodies.

4. Age-related transcriptional changes are primarily characterized using GO term analysis, without experimental validation, limiting the strength of the conclusions. The full results of the GO term analysis should be included in supplementary data.

Response: The complete dataset for the GO term analysis is presented in **Supplementary Tables 2** and **3**, as described in the legends of **Fig. 9d** (lines 1617-1618) and **Extended Data Fig. 26c** (lines 2294-2295). Multiple experimental validations are also presented in these tables to support the conclusions.

5. It is not clear from the method description if the scRNAseq data is based on a single pooled sample including six mice per condition, or individual samples from six mice per condition (adult, aged). It would be important to confirm that all samples contribute to all clusters, particularly those that are increased in aged mice (Nos3-high LEC and Postn-high fibroblasts)?

Response: This is an important question. We now specify (lines 350-355) that the original scRNAseq data were obtained from pooled samples of tissues from six adult mice (3 males and 3 females) and six aged mice (3 males and 3 females). This approach was used because the number of LECs per mouse was too small for meaningful analysis of individual mice. Analyzed samples were confirmed as representative by examining the distribution of *Xist*, a female-specific non-coding RNA, in the LEC UMAP plot **(Extended Data Fig. 24a**). RNA *in-situ* hybridization for *Nos3* mRNA and *Postn* mRNA was done on tissues from 4-6 mice per group **(Extended Data Figs. 24b,c, 26e-g**).

6. Certain speculative statements should be backed up by data or toned down to reflect limited experimental validation, including line 45: '…surrounded by more abundant fibrosis-associated fibroblasts, which could contribute to the impaired CSF outflow, line 347: 'Upregulation of Nos3 and Gch1 expression during ageing could be compensation for the reduced responsiveness to the nitric oxide'.

Response: We agree and deleted this speculation in the revised manuscript.

7. Detailed information on how the mechanical stimulator was applied is lacking, including 1) if the device was applied to awake or sedated mice, 2) how the device was positioned, and 3) how the device strokes the areas indicated in Extended Data Fig. 24. A figure or movie showing the mechanical stimulation would be informative.

Response: The mechanical stimulator was used on mice anesthetized with urethane (lines 826-829). **Supplementary Video 2** was added to show how the mechanical stimulator was used to massage the facial and neck skin to increase CSF drainage through superficial cervical lymphatics.

8. Line 61: the following articles should be discussed and cited to provide a balanced view on the role of CSF drainage through meningeal lymphatics in CNS pathologies: Antila et al, Nat Cardiovasc Res 3, 474–491 (2024), Zhilin et al, Sci Immunol 8,eabq0375 (2023).

Response: We agree and provide a more balanced consideration of the roles of CSF drainage through lymphatics in CNS pathologies and potential therapeutic interventions in the Introduction (lines 59-64) and Discussion (lines 583-587 and 644-658). The recommended articles are now cited as references 9 and 10 (line 584).

9. Lines 136-138 and Fig. 2b: As discussed in lines 142-144 and Extended Data Fig. 1 there are anatomical variations in the location of smLN, asmLN and ptLN. It should be clarified what type of LN anatomy the mice undergoing these drainage experiments had. As ptLN and asmLN are fused and connected in 7% of the cases, it is surprising that the uptake of tracer into ptLN is never observed.

Response: To address these important issues, we determined the location and amount of tracer in the three types of superficial lymph nodes from 15 to 120 min after tracer infusion and described the new findings in the Results (lines 145-155) and figures (**Fig.2f,g**, **Extended Data Fig. 1d-f**). In Type 3 scLN, where the asmLN was fused to the ptLN, the tracer was detected in the asmLN and adjacent half – but not the distal half - of the ptLN at 30 min after intracisternal tracer infusion (**Extended Data Fig. 1e**).

10. Line 174, 179-181: The authors state that drainage to smLN occurs first through scLV1 (30 min) and later to scLV2 (60 min) and that asmLN receives CSF from the nasopharyngeal plexus at 60 min. Since in 60% of mice, smLN and asmLN are fused, it should be again clarified what type of LN anatomy the mice undergoing these drainage experiments had and if and how the fusion affects the drainage patterns.

Response: We appreciate this comment and address the issue by performing the new analyses described in the response to comment 9, where the amount of tracer was measured in the three types of superficial lymph nodes (scLN) from 15 to 120 min. The effect of smLN-asmLN fusion on CSF drainage was determined by comparing the three types is described in the Results (lines 145-155) and illustrated in **Extended Data Fig. 1d-f**.

11. Line 386-388: It is unclear why electrical stimulation of muscle contractions would involve surgery or anaesthesia. Transcutaneous electrical nerve stimulation (TENS) is widely used and does not involve invasive procedures. Would it have a similar impact? The mechanical device developed here presumably also involves anaesthesia.

Response: We appreciate the reviewer's critique of the requirement of anesthesia for electrical stimulation and have deleted this section of the text. The revised text describes the approach we used (lines 462-472). Parenthetically, transcutaneous electrical nerve stimulation could reduce CSF outflow - opposite to the effect of mechanical stimulation – by stimulating adrenergic nerves located along superficial cervical lymphatics. As described in the response to comment 7, mice were sedated with urethane before mechanical stimulation of neck lymphatics, but such sedation would not be necessary in humans during gentle massage, which is not fundamentally different from deep skin massage regularly done in non-medical settings.

Additional information for Reviewers #1, #2, and #3 at the **end of the point-by-point responses:** Please see at the end of these point-by-point responses the list of new experiments performed to address issues raised by the reviewers. Because of the overlapping nature of comments, this list was prepared for all reviewers to complement the point-by-point responses to comments made by individual reviewers.

Additional information for Reviewers #1, #2, and #3

The following is a list of new experiments performed to address issues raised by the reviewers. Because of the overlapping nature of comments, this list was prepared for all reviewers to complement the point-by-point responses to comments made by individual reviewers.

1. The relative amounts of CSF drainage to Type 1 and Type 2 superficial cervical lymph nodes and deep cervical lymph nodes were determined by measuring the temporal changes of TMR-dextran fluorescence in each lymph node on both sides over 120 min after intracisternal infusion of the tracer (**Fig. 2g**).

2. CSF was found to reach the parotid lymph nodes indirectly from afferent lymphatics to the ipsilateral accessory submandibular lymph node by measuring temporal changes in TMR-dextran fluorescence in Type 3 superficial cervical lymph nodes over 120 min after intracisternal infusion of the tracer (**Extended Data Fig. 1d-f**).

3. Additional evidence that mechanical stimulation increases CSF clearance from the subarachnoid space, rather than simply squeezing CSF from superficial cervical lymphatics, was obtained by (a) infusing 1.0 µl TMR-dextran over 1 min into a lateral ventricle of adult *Prox1*-GFP mice, (b) followed 10 min later by low-magnitude mechanical stimulation for 10 min, and (c) at 30 min measuring TMR-dextran fluorescence in the CSF removed at the cisterna magna (**Fig. 10g,h** and **Extended Data Fig. 30**).

4. Ligation of the superficial cervical lymphatic vessels, to assess the compensatory redistribution of CSF drainage from superficial cervical lymph nodes to deep cervical lymph nodes, revealed that the ligation significantly reduced drainage to the submandibular lymph node and overall drainage to superficial and deep cervical lymph nodes but did not change drainage to nasopharyngeal lymphatics (**Extended Data Fig. 3**). These experiments underscored the greater importance of superficial cervical lymphatics to overall CSF drainage than deep cervical lymphatics.

5. FluoSpheres or Qdot 705 were found in intracranial lymphatics in the meninges around the olfactory bulb and pterygopalatine artery at 20, 30 or 60 min after intracisternal infusion into *Prox1*-GFP mice (**Extended Data Fig. 12**).

6. Drainage of CSF through lymphatics in the periorbital, nasal sidewall, hard palatal, and superficial cervical areas of the face and neck of *Macaca fascicularis* monkeys was confirmed by measurement of indocyanine green in the face and neck at 30 min after intracisternal infusion of indocyanine green (ICG) at 2.5 ml over 10 min (**Extended Data Fig. 16**).

7. VEGF-C induced plasticity of lymphatics in the nasal mucosa, hard palate, meninges, and neck was revealed by intracisternal infusion of 1 x 10^{13} genome copies of AAV9-mCherry or AAV9-mVEGF-C-mCherry in 1 µl of PBS over 1 min into adult *Prox1*-GFP mice (**Extended Data Fig. 17**)

8. Data from scRNA-seq analyses were validated in cervical lymphatic vessels by RNA *in-situ* hybridization for *Nos3, Postn* mRNA and immunofluorescence staining for PROX1, FOXP2, LYVE1, αSMA, and Type 1 collagen (**Extended Data Fig. 24a-i** and **26e-i**).

9. Measurements of superficial cervical lymphatics made during intravital imaging in *Prox1*-GFP mice revealed that low-magnitude mechanical stimulation increased CSF outflow to superficial cervical lymphatics and lymph nodes by promoting a small increase in the diameter of superficial cervical lymphatics and a transient increase in the amplitude of spontaneous contractions but no consistent change in spontaneous contraction ejection fraction, frequency, or fractional pump flow. (**Extended Data Fig. 31a-d**). Experiments comparing CSF tracer outflow after one or four daily sessions of stimulation revealed that repeated stimulation did not change multiple parameters of spontaneous contraction or impair the stimulation-mediated increase in CSF outflow through superficial cervical lymphatics, indicating the resilience of this effect over time (**Extended Data Fig. 32a-d**).

Responses to Referees' comments
Reference: Nature manuscript 2024-09-20226A; Increased CSF drainage by noninvasive manipulation of cervical lymphatics

Referee #1 (Remarks to the Author):

The authors have responded to my queries, and also have addressed most of the queries raised by the other reviewers. The manuscript has improved, and I have no further comments.

We appreciate the reviewer's encouraging comments.

Referee #2 (Remarks to the Author):

The authors have performed a substantial number of additional experiments in response to the reviewer comments. The manuscript is much improved, and my major concerns have been addressed. In particular, the additional experiment with ICG injections into the macaques is very exciting and shows the close similarity in the pathways of CSF lymphatic clearance between the rodent and primate. This is very important data to help to bridge the gap towards the clinic by making the critical point that the superficial lymphatic networks may indeed be a relevant therapeutic target. I have only a couple more minor comments that could be addressed by changes to the text.

We appreciate the reviewer's encouraging comments.

Minor comments:

1. Regarding previous Minor comment #1: The authors have performed an additional study covering additional timepoints that show the dynamics of tracer outflow to the different cervical lymph nodes (Fig 2g). They then use AUC calculations to continue to make the claim that "Tracer studies in adult mice revealed that ~50% of total CSF outflow to the neck drained to superficial cervical lymph nodes." The authors should still be careful how they interpret this data. The deep cervical lymph node showed much higher peak signal intensity and a more rapid wash-through of the tracer bolus compared to the superficial cervical lymph nodes. Thus, the AUC measurements are skewed toward inflating the values of the slower drainage routes, which retain tracer for a longer period. A lymphatic cannulation approach using a sustained low-rate infusion of tracer would be one way to accurately estimate the proportion of CSF outflow that the specific drainage routes carry. However, this experiment would be extremely challenging in mice from a technical standpoint. The AUC data in the Fig 2g are still valuable. However, the authors should adjust the language in the Abstract, Results and Discussion to indicate that the superficial cervical lymph node network carries a "significant portion" of CSF flow rather than making any quantitative claim.

We agreed with this point and changed the quantitative percentage to "a significant portion" in the revised Abstract and Discussion.

2. In the sentence, on Page 17, lines 493-495: “To determine whether mechanical stimulation of facial and neck skin increased CSF clearance from the SAS, instead of just pushing CSF in scLV-1 and scLV-2 into cervical lymph nodes” – shouldn’t this be written “lymph” or “lymphatic fluid” in scLV-1 and scLV-2 rather than CSF?

We agreed with this point and changed CSF to lymph in lines 493-495.

Referee #3 (Remarks to the Author):

The authors have carefully addressed the reviewers’ comments and improved the study. I have no further concerns and would like to congratulate the authors on an excellent piece of work.

We appreciate the reviewer's encouraging comments.