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Fluids and Barriers of the CNS

LETTER TO THE EDITOR RESPONSE

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Response to Commentary on "Structural characterization of SLYM – a 4th meningeal membrane" by Julie Siegenthaler and Christer Betsholtz

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Abstract

Histological studies have for decades documented that each of the classical meningeal membranes contains multiple fibroblast layers with distinct cellular morphology. Particularly, the sublayers of the arachnoid membranes have received attention due to their anatomical complexity. Early studies found that tracers injected into the cerebrospinal fluid (CSF) do not distribute freely but are restricted by the innermost sublayer of the arachnoid membrane. The existence of restrictions on CSF movement and the subdivision of the subarachnoid space into several distinct compartments have recently been confirmed by in vivo 2-photon studies of rodents, as well as macroscopic imaging of pigs and magnetic resonance imaging of human brain. Based on in vivo imaging and immunophenotyping characterization, we identified the structural basis for this compartmentalization of the subarachnoid space, which we term 'Subarachnoid lymphatic-like membrane', SLYM. The SLYM layer engages the subarachnoid vasculature as it approaches the brain parenchyma, demarcating a roof over pial perivascular spaces. Functionally, the separation of pial periarterial and perivenous spaces in the larger subarachnoid space is critical for the maintenance of unidirectional glymphatic clearance. In light of its close apposition to the pial surface and to the brain perivascular fluid exit points, the SLYM also provides a primary locus for immune surveillance of the brain. Yet, the introduction of SLYM, in terms of its anatomic distinction and hence functional specialization, has met resistance. Its critics assert that SLYM has been described in the literature by other terms, including the inner arachnoid membrane, the interlaminate membrane, the outer pial layer, the intermediate lamella, the pial membrane, the reticular layer of the arachnoid membrane or, more recently, BFB2-3. We argue that our conception of SLYM as an anatomically and functionally distinct construct is both necessary and warranted since its functional roles are wholly distinct from those of the overlying arachnoid barrier layer. Our terminology also lends clarity to a complex anatomy that has hitherto been ill-described. In that regard, we also note the lack of specificity of DPP4, which has recently been introduced as a 'selected defining marker' of the arachnoid barrier layer. We note

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that DPP4 labels fibroblasts in all meningeal membranes as well as in the trabecula arachnoides and the vascular adventitial layers, thus obviating its utility in meningeal characterization. Instead, we report a set of glymphatic-associated proteins that serve to accurately specify SLYM and distinguish it from its adjacent yet functionally distinct membranes.

The human meningeal membranes have been classically divided into dura, arachnoid and pia mater during macroscopic dissections. Yet, several histological analyses have documented an overlap in the fibroblast characteristics in each layer (see Coles et al., 2017 [1] for a thoughtful review, consider the "*pia-arachnoidal cells*" described in McLone and Bondareff, 1975 [2] as an example of cells subdividing the subarachnoid compartment). Already in 1983, Krisch et al., concluded: "*Due to the development of the pia mater and the arachnoidea from a common matrix primitiva and due to the cytologic characteristics common to the pia mater and the arachnoidea, one should avoid the terms "pia" and "arachnoidea". Both terms, having their origin in gross anatomy, should be replaced by the terms of "inner, intermediate", and "outer leptomeninges" encompassing the inner and outer leptomeningeal spaces*" [3]. In other words, it was already acknowledged, more than 40 years ago, that the subarachnoid space consists of 2 compartments.

Our interest in the meningeal layers sprang from studies on cerebrospinal fluid transport. Multiple lines of work have documented a high degree of organization of fluid transport in mouse, rat, pig and human brains using a variety of techniques, including optical imaging, magnetic resonance and SPECT studies (reviewed in Rasmussen et al., 2022 [4]). In all species, periarterial influx of cerebrospinal fluid (CSF) from the subarachnoid space (SAS) fuels glymphatic flow whereas efflux follows the perivenous spaces. This highly organized unidirectional brain fluid transport system prompted the question: *Why does CSF in the SAS act as a sink for both CSF influx and efflux?* The concept that the SAS plays a double role by

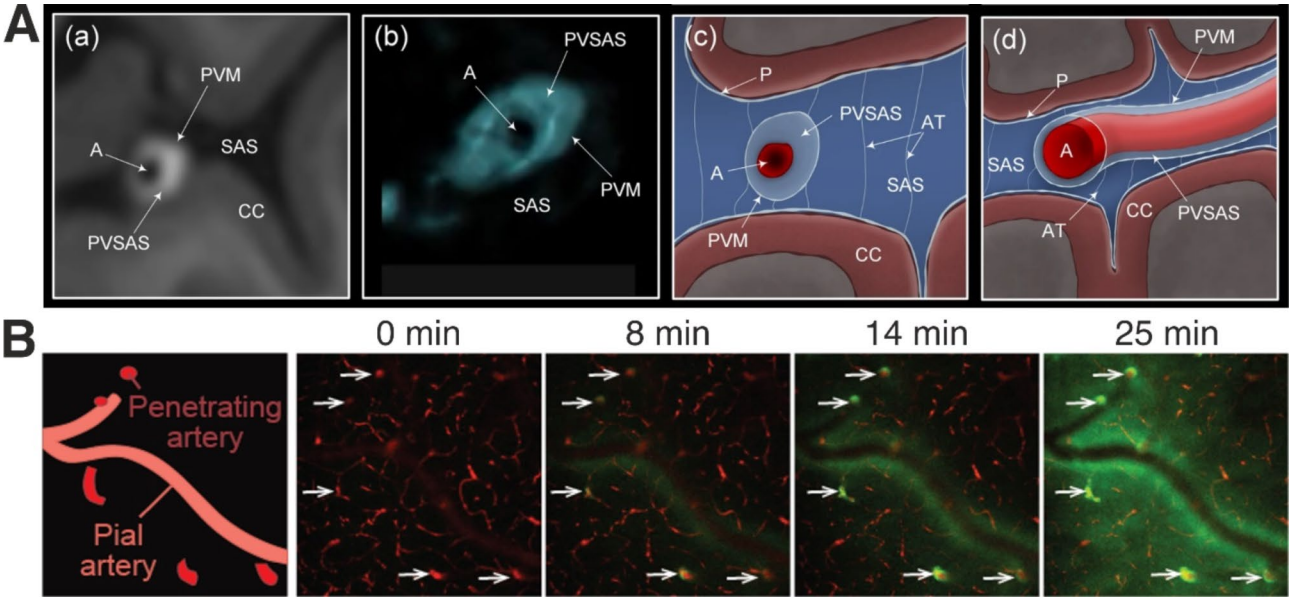

Fig. 1 Tracers enter the subarachnoid space preferentially around pial arterioles. (**A**) From Per Eide et al., 2024 [6]: "The human subarachnoid space is compartmentalized by a perivascular subarachnoid space. The MRI contrast agent (gadobutrol) was used as CSF tracer to study compartmentalization of the subarachnoid space in the human brain. a, b In MR image planes orthogonal to the vessels, the CSF tracer that was administered intrathecal formed a donut-shaped form around the arteries (A). This perivascular subarachnoid space (PVSAS) is thus represented by the contrast-enriched perivascular compartment, delineated by a perivascular membrane (PVM) semipermeable to the CSF tracer. Tracer enrichment in PVSAS preceded tracer enrichment in surrounding subarachnoid space (SAS) and thereafter in cerebral cortex (CC). In b is shown a 3D representation of the PVSAS residing within the SAS. c, d Schematic illustrations show the artery (A), perivascular subarachnoid space (PVSAS), delineated by the perivascular membrane (PVM), and surrounding SAS. Provided the PVM is part of the leptomeninges (arachnoid and pia), we may anticipate that the perivascular membrane (PVM) is attached to the arachnoid trabecula (AT) and further towards the pia mater (P) and the arachnoid barrier cell layer towards the dura mater (not shown here)". **(B)** Images extracted from Iliff et al., 2012 [27]: 2-photon imaging of adult mouse cortex (sensory-motor) after injection of a green tracer (FITC-dextran, 40 kDa) in cisterna magna and TexasRed-dextran (70kD) in blood. The left panel is a sketch of the field of view depicting the position of the pial and penetrating arterioles. The cerebrospinal fluid tracer (green) enters the field of view along the pial arteriole and flows into the Virchow-Robin spaces surrounding the penetrating arterioles before mixing with CSF in the larger subarachnoid space. *Arrows indicate penetrating arterioles*. (**A**): Adapted from Eide et al., 2024 [6]; (**B**): Adapted from Iliff et al., 2012 [27]

serving as a reservoir for CSF inflow and outflow is surprising since the intraparenchymal transport of tracers from the arterial to venous perivascular spaces is directional and highly organized [4]. Mixing 'clean' and 'dirty' CSF within one large compartment will lead to recirculation of metabolic products and less efficient waste clearing. In fact, functional data shows that CSF tracers delivered either into cisterna magna or intracortically (mice) [5] or intrathecally (human) [6] appear to follow the periarterial spaces surrounding penetrating arterioles before being, at least initially, excluded from the larger CSF filled compartment (Fig. 1A). Furthermore, several murine studies have shown that tracers injected into cisterna magna are clearly hindered from accessing the outer CSF-filled SAS (Fig. 1B).

Supporting the hypothesis that waste containing CSF ('*dirty*') outflows to the outer SAS, a recent study showed that bridging veins traverse the SAS and pierce through the arachnoid barrier layer to directly connect the brain and dura, enabling protein export and directed immune trafficking [7]. Similar observations were made many decades ago by Krisch, Leonhardt and Oksche (1983, 1984) [3, 8], in which the meninges surrounding mouse cortex were studied by tracer injection. Using their original nomenclature and moving down from the skull to the brain, the meningeal layers were defined here as: (1) dura, (2) the inner dura layer, (3) the neurothelium and (4) the outer arachnoid layer. Below the outer arachnoid layer is the (5) CSF-filled arachnoid space, followed by (6) the inner arachnoid layer and (7) the outer pial layer. The inner arachnoid layer and the outer pial layer are often fused, and the resultant dual layer is called the *intermediate lamella*. Below the intermediate lamella is (8) the pial space, the floor of which is created (9) by the inner pial layer. (10) A subpial space is also described (Fig. 2A).

Injection of a tracer, horseradish peroxidase (HRP, 40 kDa) in either the arachnoid or pial spaces demonstrated that the intermediate lamella is indeed a barrier that separates the CSF-filled arachnoid and pial compartments [3, 8]. Figure 2B-C displays the original data by which Ichimura et al., 1991 [9] demonstrated the existence of two separate CSF-filled compartments: the arachnoid space (A) and the pial space (P) separated by the intermediate lamella (the inner arachnoid layer and the outer pial layer) [3, 8]. Studies from the Cserr group described: "*The pia mater of the brain reflects onto blood vessels within the subarachnoid space, forming a perivascular compartment bounded externally by the pia and internally by the smooth muscle of the vessel wall. This subarachnoid perivascular space is in continuity with the perivascular space of the brain and the subpial space*" [9]. The authors injected colloidal gold-tagged albumin into the perivascular space of surface arteries and veins and observed that the "pial membrane" covering the vasculature prevented albumin from entering the larger subarachnoid space (Fig. 2C, D).

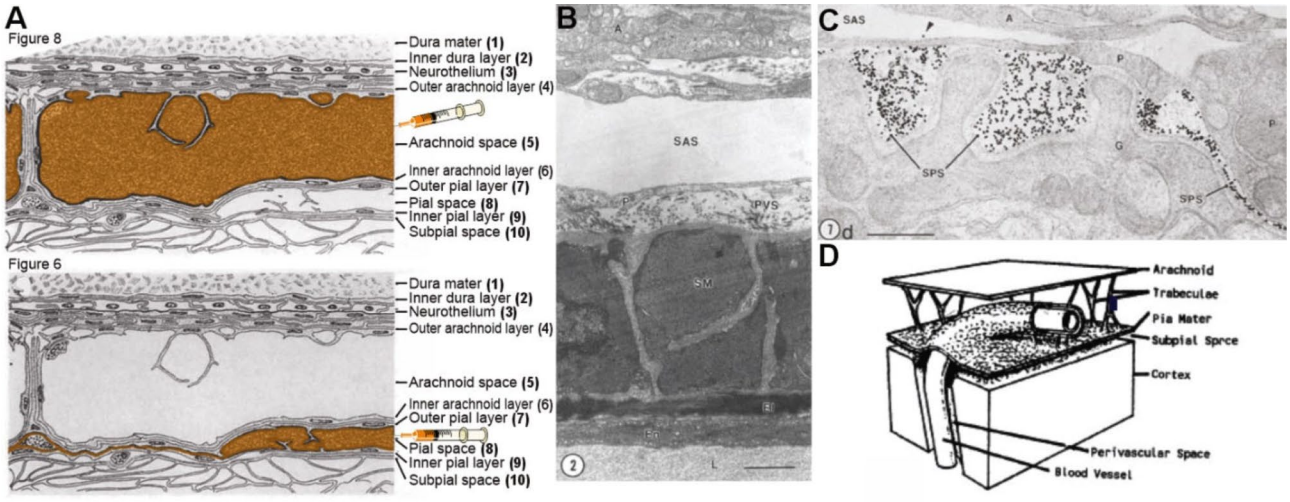

Fig. 2 The subarachnoid compartment is subdivided into two functional compartments in vivo. (**A**) The top diagram (Fig. 8) shows HRP injection in the arachnoid space. Note that the dual-layered intermediate lamella consisting of the inner arachnoid layer (top) and the outer pial layer (lower) creates a barrier that prevents HRP from entering the pial space. The lower diagram (Fig. 6) shows HRP injection in the pial space. The authors conclude that intermediate lamella again acts as a barrier that prevents HRP from entering the arachnoid space. (**B**) Electron micrograph showing the perivascular space (PVS) lies between the pial membrane and the outer wall of the vessel. The smooth muscle cells (SM), the elastic lamina (El) and the endothelial cell (En) adjacent to the lumen (L) of the vessel can be seen below. Bar = 1 µm. (**C**) Electron micrograph showing a cross section of the wall of the middle cerebral artery and the overlying meninges near the tracer injection site. The arachnoid (annotated as A) forms the dorsal boundary and the pial membrane (P) the ventral boundary of the subarachnoid space (SAS). (**D**) Schematic drawing illustrating the perivascular space surrounding a vessel on the surface of the cortex and showing that this subarachnoid portion of the perivascular space is continuous with the perivascular space of the brain and the subpial space but separated from the subarachnoid space by the pia mater. (**A**): Adapted from Krisch et al.,. 1983 [3]. (**B-D**): Adapted from Ichimura et al., 1991 [9]

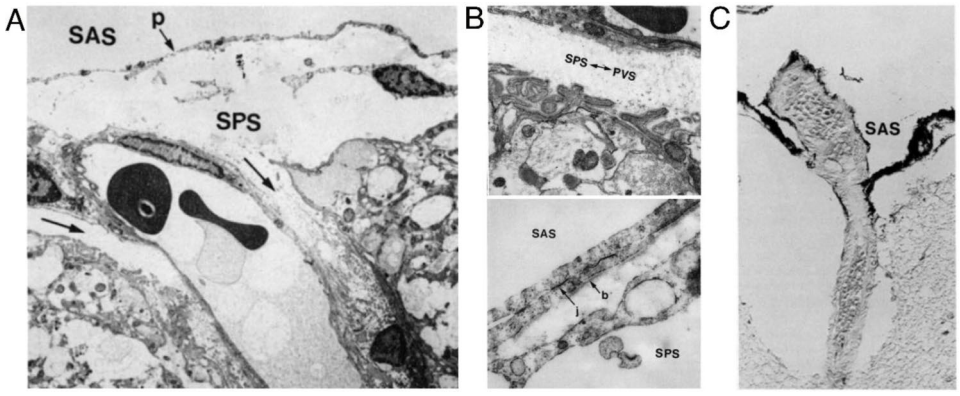

Fig. 3 (Outer) SAS is not continuous with the PVS. From Hutchings and Weller, 1986 [11].: (**A**) "Transmission electron micrograph (TEM) showing the entry zone of a small blood vessel at the surface of normal cerebral cortex. A continuous layer of pia mater (p) separates the subarachnoid space (SAS) from the subpial space (SPS). As the vessel enters the cortex, the subpial space extends along the outside of the vessel as the perivascular space (long arrows). x2200". (**B**) *Top*: "Higher-power TEM of the region marked by the lower of the two long arrows in upper left. The irregular surface of the cortical glia limitans can be seen at the bottom of the figure, and its basement membrane is separated from the vessel wall by the subpial-perivascular spaces (SPS-PVS) containing sparse collagen fibers. x10,700. *Bottom*: Higher-power TEM of the pia mater. Thin cell processes partly coated by a structure similar to basement membrane (b) separate the subarachnoid space (SAS) from the subpial space (SPS). The intercellular junctions (j) are also shown. x33,150". (**C**) "Photomicrograph following India ink injection [*NOTE: subdural injection in freshly dissected human brains*] into the cerebrospinal fluid of normal brain. There is a funnellike invagination of the subarachnoid space (SAS) by the ink but no penetration of the perivascular space as the vessel enters the cortex. The brain is artifactually retracted from the vessel. H & E, x 300". (**A**-**C**): Adapted from Hutchings and Weller, 1986 [11]

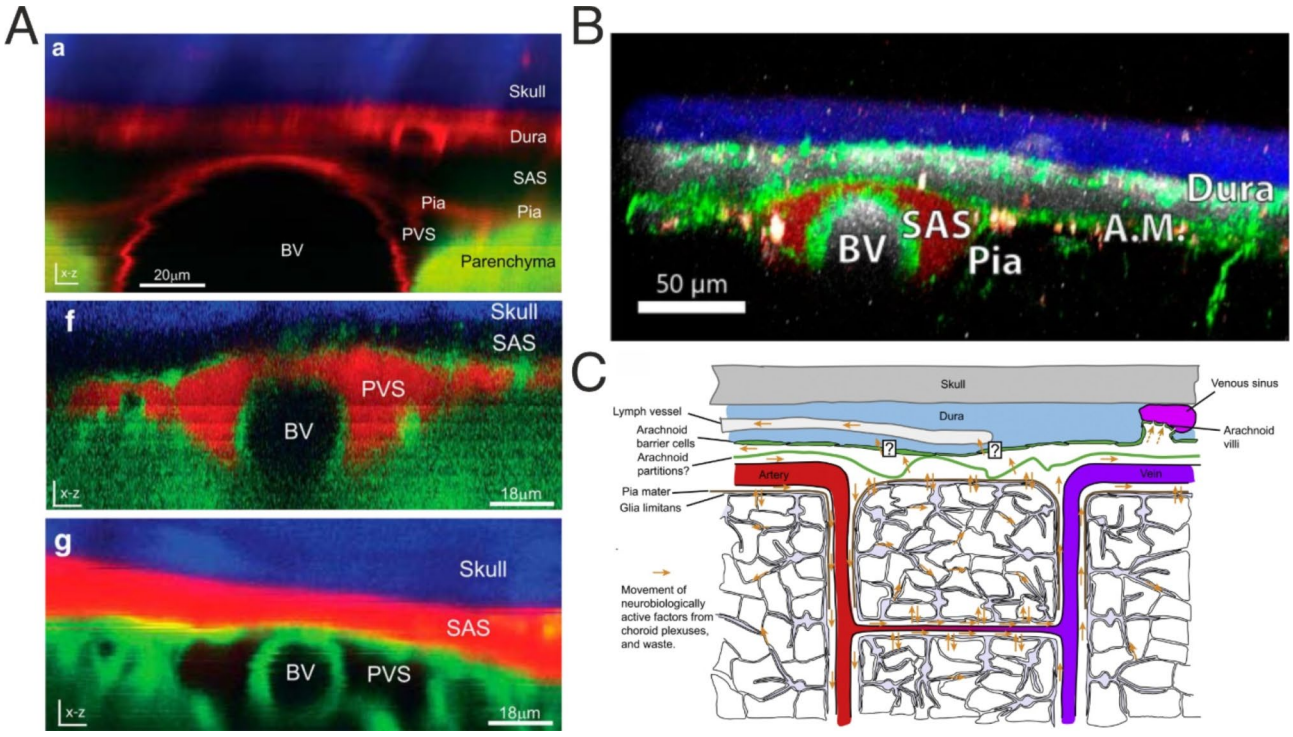

Fig. 4 Previous publications already showing SAS compartmentalization. (**A**) Text extracted from Schain et al., 2017 [28]: "When labeling the PVS with TRD via intracortical injection, we observed that the pia was a functional barrier to 3 kDa TRD as dye did not readily enter the adjacent SAS. To further test this barrier, we also injected TRD directly into the SAS (n = 3), and found that it did not freely enter the underlying PVS (Fig. 2f)". (**B**) While Mapunda et al., 2023 [29] refer to the membrane defining the PVS as "pial membrane", the authors describe a separation between that compartment and SAS, limiting tracer free movement: "The arachnoid mater (green, AM) forms an impermeable barrier between blood (gray) in the dura mater (blue), and CSF (red) in the subarachnoid space (SAS). The pial membrane (green) defines the floor of the SAS, and surrounds blood vessels (BV) that cross this space". (**C**) Pathways of CSF flow in the cortex and meninges as sketched by Coles et al., 2017 [1] after revisiting the current knowledge of the anatomy of the meninges, particularly as it appears in intravital imaging. The authors depicted the possible existence of an arachnoid partition layer that might explain the compartmentalization of the SAS into a perivascular space for CSF flow along arteries, capillaries and veins, and a subarachnoid space for CSF exit. (**A**): Adapted from Schain et al., 2017 [28] (**B**): Adapted from Mapunda et al., 2023 [29] (**C**): Adapted from Coles et al., 2017 [1]

Thus, a wealth of tracer injection studies in mice, rats and human brain carefully documents the compartmentalization of the larger SAS [10]. Notably, Hutchings and Weller, 1986 [11] concluded that "*the subarachnoid space does not seem to connect with the perivascular spaces of the cortex*" after investigating the impermeability of a "*pial membrane*" by injecting India ink into the subarachnoid space of normal humans brains at autopsy (before formalin fixation) (Fig. 3). A few more recent examples are included in Fig. 4. It is also clear that the past nomenclature is highly variable, with the membrane covering the pial vasculature named either the inner arachnoid membrane, the interlaminate membrane, the outer pial layer, the intermediate lamella, the pial membrane, the reticular layer of the arachnoid membrane. These observations inspired us to study the structural basis for the membrane that covers the pial vasculature and prevents mixing of CSF in pial periarterial spaces [now translated into "inner subarachnoidal spaces"] with fluid exiting the brain along the pial perivenous spaces. In other words, our aim was to describe this poorly recognized membrane both functionally and immunophenotypically. Based on 2-photon in vivo imaging combined with tracer and microsphere injections, as well as histological analysis of decalcified whole mouse heads, we introduced the concept of the subarachnoid lymphatic-like membrane (SLYM) – a 4th meningeal layer surrounding the brain [12, 13] that supports the existence of an unidirectional organization of brain fluid transport.

In essence, SLYM separates the subarachnoid space into two principal compartments: (a) an inner compartment that consists of perivascular spaces and the cisterns where SLYM fuses with the pial membrane and separates CSF in the periarterial and perivenous spaces, thereby supporting unidirectional glymphatic flow; and (b) an outer subarachnoid space. SLYM is most well-developed as a membrane at the ventral side of the brain where it consists of a 3 layered lamina containing short unorganized collagen bundles [13]. The ventral side of the brain receives the large diameter internal carotid and vertebral arteries which form the Circle of Willis. Here, SLYM supports those arteries in the basal cistern and separates the inflowing 'fresh' CSF, that is transported from cisterna magna to the pial periarterial space [now translated into "inner subarachnoidal space"], from the 'dirty' fluid leaving the brain along perivenous spaces. Yet, in some areas, such as in regions of the dorsal cortex or below the foramen magnum, SLYM merges with the ABCL, forming a double membrane adjoining dura, that we already described in Plá et al., 2024 [13].

SLYM is phenotypically characterized by labeling for Prox1-eGFP^{+} and podoplanin (PDPN^{+}), in contrast to the arachnoid barrier which is negative for these markers and instead positive for E-cadherin (E-Cad) and Claudin-11 (Cld-11) ([13] and Fig. 5). The premise for our follow-up study [13] was to respond to online unreviewed critiques [14–16] by further characterizing the whole brain structural arrangement and phenotypical markers of SLYM and meningeal layers. We took advantage of Betsholtz group's report stating that dipeptidyl peptidase 4 (DPP4) is a selective marker of the arachnoid barrier layer. DPP4 was described as the defining marker of the arachnoid barrier layer using single cell transcriptomic analysis additionally validated by immunohistochemistry [17]. We used two DPP4 antibodies when preparing the data presented in Fig. 2 in Plá et al., 2024 [12] and showed our results with the marker using brain and skull whole mounts for immunofluorescence. Yet, an extended analysis using a more sensitive peroxidase-based method has shown that DPP4 immunoreactivity is not limited to the arachnoid barrier layer, but otherwise has a widespread distribution. Figures 5 and 6 show that positive signals can be found in pia, trabecula arachnoides, adventitia and SLYM, yet in some preparations signal intensity can be more intense in the ABC layer (Fig. 6D, inset). In consequence, we advise AGAINST the use of DPP4 as a selective marker of the arachnoid barrier layer, as reported by the Betsholtz group, and, therefore, the use of DPP4 should be limited to pan-fibroblast studies.

Response to critique

This manuscript is written in reply to Drs. Siegenthaler and Betsholtz's letter to FBCNS. Below is our detailed response to the author's concerns:

Additional immunolabeling controls and methodology optimization

We agree that the best characterized marker for the ABCL is E-Cadherin (E-Cad), in fact that has motivated its inclusion in both publications describing SLYM [12, 13]. Yet, in response to their request, we have included additional images documenting the absence of E-Cad labeling in the SLYM layer, while positive signal is observable in the arachnoid layer, in agreement with previous publications (Figs. 5, 6 and 7). In case that the ABCL immunolabeling is not acceptable as positive control for the antibody, additional areas corresponding to the epithelium of the rhino- and nasopharynx are included, where a very distinctive signal is present (Fig. 6C). Again, in response to the authors' request, we have included new double immunofluorescence images (complementing those already published in Møllgård et al., 2023 [12]), where E-Cad and Prox1-eGFP signal are shown simultaneously (Fig. 7). However, we do not agree with their critique stating that the use of serial sections and HRP-based immunohistology is not the best method to identify marker expression: well-established chromogenic IHC methods have been widely used in

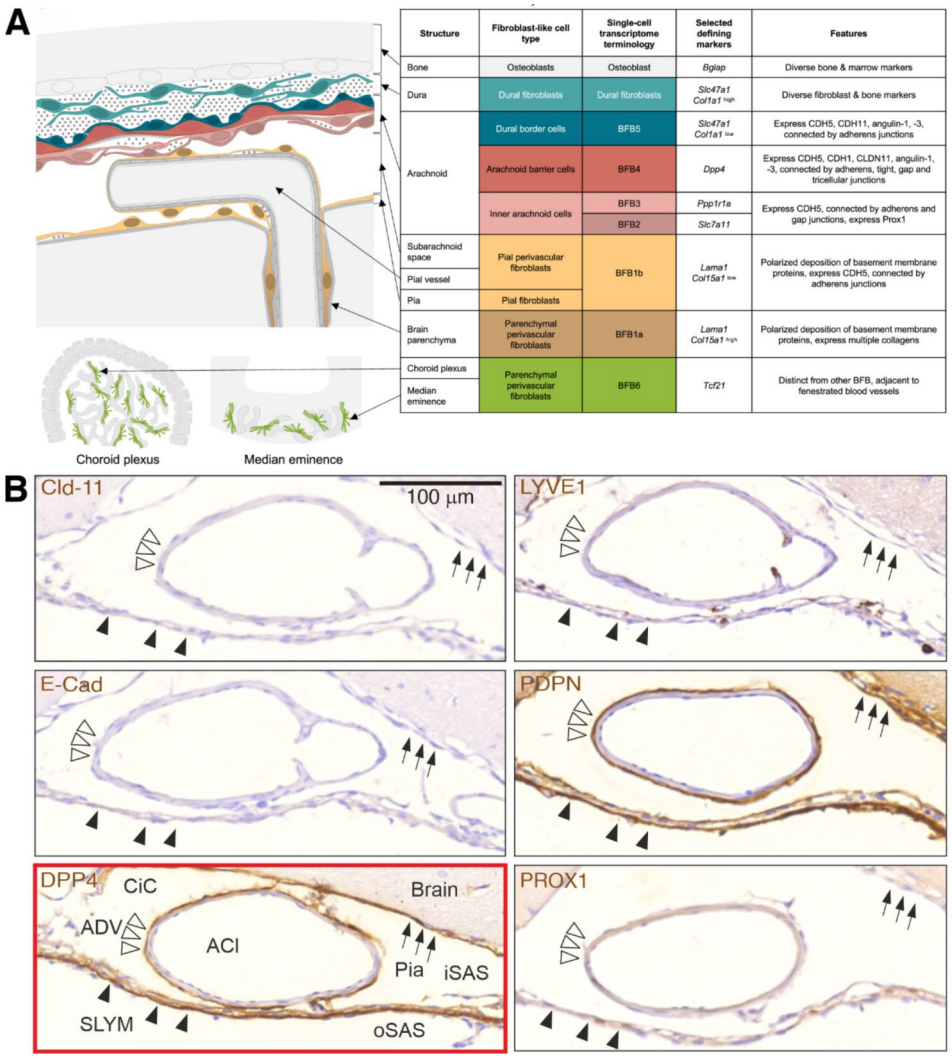

Fig. 5 DPP4 is NOT a specific marker for arachnoid barrier cells. (**A**) Schema from Pietilä et al., 2023 [17] illustrating the numerous and vastly heterogeneous fibroblast populations present at the brain meningeal layers. Note that DDP4 is indicated as 'selected defining marker' for arachnoid barrier cells. (**B**) Coronal sections through cisterna caroticus (CiC) from adult mice comparing immunophenotypical marker expression across the meningeal layers. Note that we include previously published images from [13] together with the new immunohistochemistry results for DPP4 (red colored border) with the objective of drawing a global set of SLYM markers. As expected, no positive signal of the arachnoid specific marker *E-Cad* was detected in either SLYM (arrowheads) or pial layers (arrows). *DPP4* staining demonstrated the extensive positive immunoreactivity of this marker – promoted as 'selected staining' for arachnoid barrier cells. There is specific staining of pia (arrows), trabecula arachnoidales, Adventitia (ADV, open arrowheads) of the internal carotid artery (ACI) and SLYM. (**A**): Adapted from Pietilä et al., 2023 [17] (**B**): Adapted from Plá et al., 2023 [13]

pathology laboratories for decades [18] and are the gold standard for clinical studies. Additionally, the possibility of visualizing the tissue morphology helps to put the signal into context, identifying expression patterns in anatomical structures with better sensitivity and without interference of autofluorescence background from fluorescent multiplexing. In fact, DPP4 peroxidase IHC clearly demonstrates a lack of specificity for ABCL that is not evident with fluorescent antibodies, as shown in Figs. 5 and 6. Serial sections, just a few microns apart from each other, allow clear identification of the layers without any antibody cross-reactivity and keeping the best possible signal-to-noise ratio. We have always shown macroscopic to microscopic images, indicating the high magnification inset original location and showing both positive and negative results for each antibody (see [12, 13] and Fig. 6). As an example, in supplementary Fig. 9 of [12] lymph node tissue is included as positive control for all the used antibodies, even highly cited and validated antibodies.

Meningeal membranes exhibit developmental, inter-species and inter-regional specificity

The authors repeatedly refer to other publications describing observations from studies from developmental stages, different species and alternative central

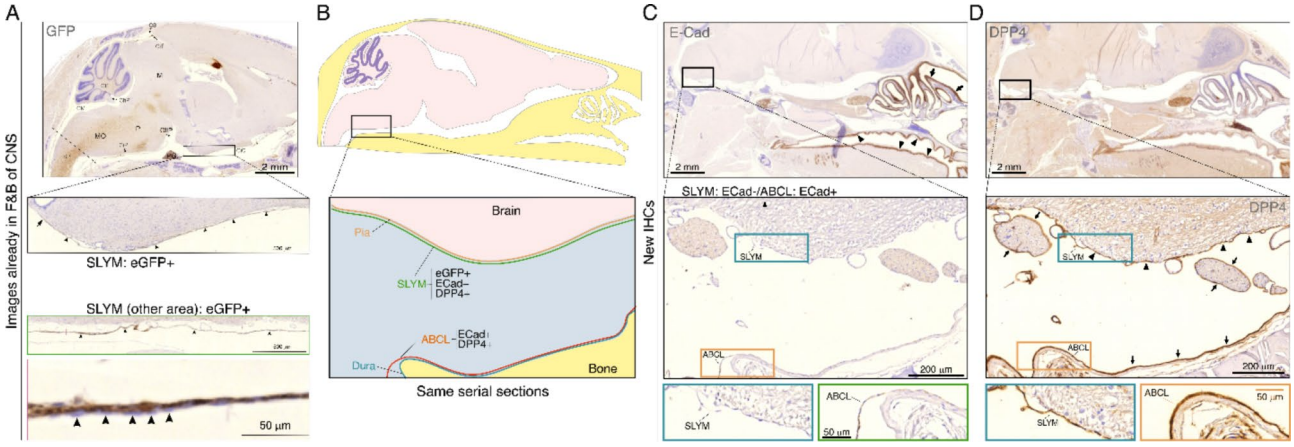

Fig. 6 SLYM is negative for the specific ABCL marker E-Cadherin. (**A**) Already published [13] immunohistochemistry images for GFP, with the objective to illustrate univocally SLYM location in the serial sections. (**B**) Schema of the anatomical location together with the layer identification markers to aid in the tissue orientation. (**C**) Sagittal sections from the series shown in Fig. 3 of Plá et al., 2023 [13] in question demonstrating the positive staining for E-cadherin (Abcam, ab231303, 1:100) in the epithelium of the nasal (arrows) and oral (arrowheads) cavities. The insert (black box) which is shown in higher magnification below, depicts a lack of E-cadherin reactivity of membranes in the lower brain stem. Blue and orange box high magnification insets focus on SLYM and ABCL respectively. (**D**) DPP4 (R&D Systems, AF954, 1:200) staining shows a distinct immunoreactivity in pia (arrowheads), in the perineurium surrounding nerve rootlets (small arrows) around blood vessels and of SLYM (large arrows). Blue and orange box high magnification insets are focusing on SLYM and ABCL respectively. (**A**): Adapted from Plá et al., 2023 [13]

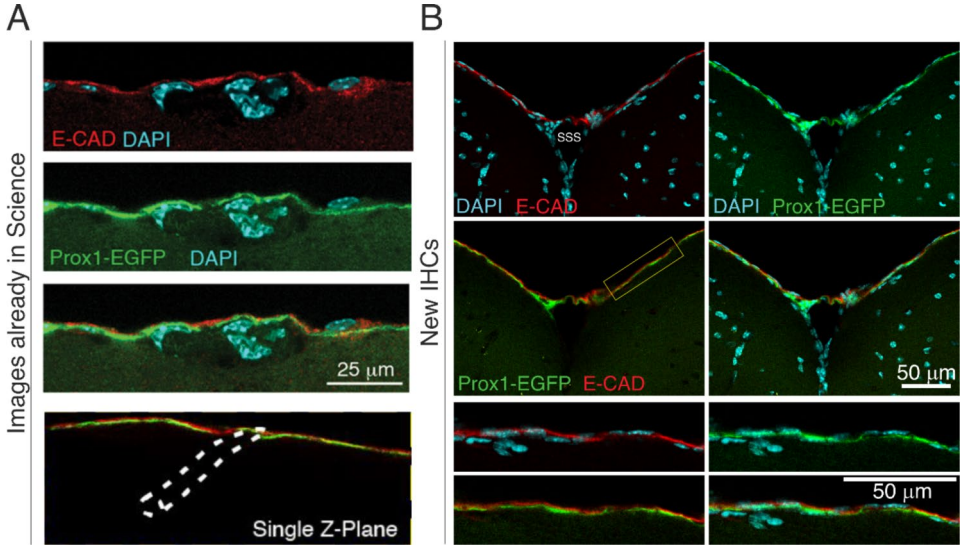

Fig. 7 E-Cadherin and Prox1-eGFP markers do not overlap. (**A**) Already published confocal images in [12] showing the distinctive localization of E-Cadherin and Prox1-eGFP markers at the murine cortex brain tissue. Both arachnoid barrier cell layer and SLYM appear fused as it is described in some areas of the brain and as expected in fixated tissue. (**B**) New confocal images further depicting the lack of overlapping between E-Cadherin and Prox1-eGFP markers at the tissue surrounding the superior sagittal sinus (SSS). (**A**): Adapted from Møllgård et al., 2023 [12]

nervous system (CNS) regions. As widely demonstrated, the organization of meningeal membranes exhibit considerable developmental, inter-species and inter-regional specificity [19]. Our studies are restricted to the adult mouse and fetal human brains, and we have never stated otherwise. We have not studied SLYM in other species or during development. Our histological analysis provided a limited view of meningeal layers surrounding the rostral aspect of the spinal cord in mice, but it is nor comprehensive. Furthermore, we have methodically gone over the letter mentioned citations – none of the cited publications have dissected the brain out to provide evidence for that the arachnoid barrier layer remains attached to pia or the brain surface. Additionally, Siegenthaler and Betsholtz refer to the Allen Brain Atlas to indicate that Cdh1 (mRNA encoding CADH1, also known as E-Cadherin) presents a continuous pattern, which they consider that proves the location of the ABCL on the brain surface. The authors neglected to mention that they were citing a developmental age brain section (p28) in which,

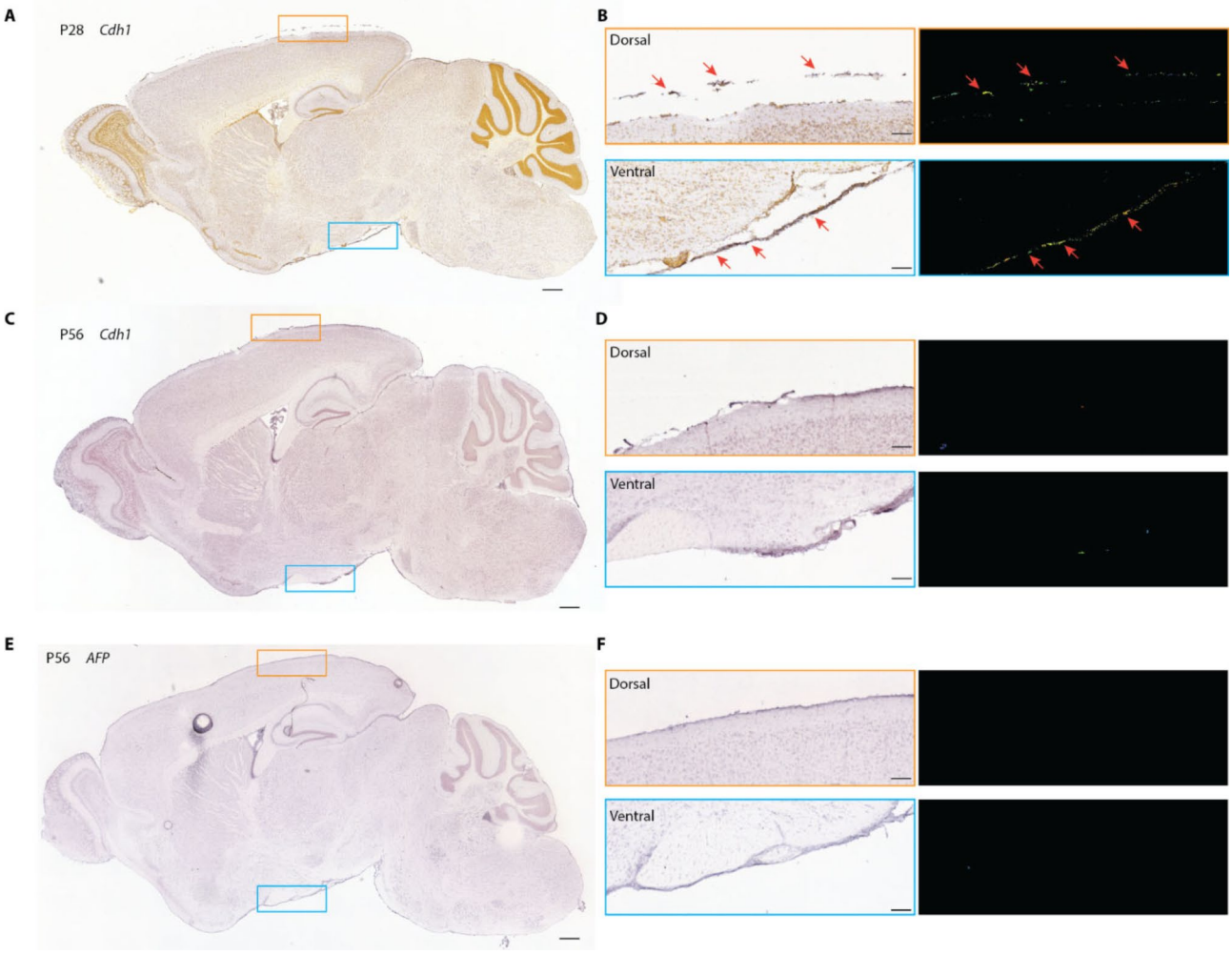

Fig. 8 In situ hybridization of *Cdh1* demonstrates inconsistent localization of ABCL to mouse brain surface. (**A**) Sagittal section of p28 mouse brain ISH for *Cdh1* transcripts. Orange (dorsal) and blue (ventral) boxes represent insets in panel B. (**B**, top) *Cdh1* expression in patches (red arrows) distant from the dorsal surface of the brain and ventral surface of the brain (bottom). Left panels represent brightfield and right panels are heatmap for expression. (**C**) Sagittal section of p56 mouse brain ISH for *Cdh1* transcripts. Orange (dorsal) and blue (ventral) boxes represent insets in panel D. (**D**) High magnification views of dorsal (top) and ventral (bottom) brain surface showing absence of *Cdh1* transcripts. Left panels are brightfield and right panels are heatmaps. (**E**) Sagittal section of p56 mouse ISH for liver specific *AFP* transcripts, that is not actually present in the brain. Orange (dorsal) and blue (ventral) boxes represent insets in panel D. (**F**) High magnification views of dorsal (top) and ventral (bottom) brain surface. Left panels show ISH brightfield images with some apparent darkening on edges of brain surface, right panels show heatmap with absence significant expression. Scale bar 500 mm (panels ACE) and 100 mm (panels BDF). All images were adapted from Allen Brain Atlas: (**A**-**B**): https://developingmouse.brain-map.org/experiment/show/100042112. (**C**-**D**): https://mouse.brain-map.org/experiment/show/70918862. (**E**-**F**): https://mouse.brain-map.org/experiment/show/69524431

upon close examination, the Cdh1 staining is not continuous as described (Fig. 8A, B). Moreover, the Cdh1 transcript might be staining the meninges distant from the brain surface (Fig. 8A, B). In contrast, when a comparable tissue is examined (adult (p56) mouse), there is no significant Cdh1 staining on the mouse brain (Fig. 8C, D). Still, this ISH data should be interpreted with caution, as a liver specific transcript AFP also appears to have darker staining on the edges of the brain section (Fig. 8E, F), and the freezing and sectioning process used in the study could displace the delicate membranes.

The brain fluid-filled compartments cannot be studied in dehydrated ex vivo slices due to significant histological distortion

The premise of our work is to study functional brain fluid transport in live mice. We have documented that the osmolarity of 4% paraformaldehyde is ~5-fold higher (~1,500 mOsm) than the osmolarity in biological fluids and tissues (~300 mOsm) [20]. Consequently, histological sections are prone to significant shrinkage and tissue distortion. This is particularly evident for the fragile membranes of meninges facing the large CSF-filled SAS compartment. As a consequence, the positioning or distance between the meningeal layers cannot be studied

using either light or electron microscopy (EM). Our functional studies of SLYM are therefore based on in vivo imaging. Histology is only utilized to characterize SLYM immunophenotypically.

The presence of adherent junctions and gap junctions between arachnoid barrier and inner arachnoid cells

Our histological analysis indeed demonstrates that the SLYM and ABCL exhibit fusion in several locations, which naturally implies the presence of such junctions. We documented that in the spinal cord and parts of the dorsal cortex, the SLYM fuses with the arachnoid barrier layer. This fusion is particularly evident below the foramen magnum, where the two layers merge to form a double membrane adjoining the dura. This is contrasted by the anatomical arrangement rostral to the foramen magnum, where the SLYM and ABCL separate (see Fig. 4 in Plá et al., 2024 [13]). The functional reason for the varying fusion of these layers is not settled, but one purpose could be to segregate CSF spaces between different brain regions. The CSF spaces around the brain [21], the eye [22], the spinal cord [23, 24] are organized to optimize fluid circulation and clearance of metabolic waste. It is not surprising that CSF flow is organized differently given the widely different function of these structures. Thus, given the variability in anatomical relationships across different regions, it is crucial to specify the exact area being studied when describing meningeal membranes.

Unfortunately, our colleagues are misciting the references that are claimed to " [4, 7, 9, 11, 12] *provide evidence that these layers [arachnoid barrier and inner arachnoid cells] are naturally linked, without an intervening space, in a live animal*". Below are a few extracts of those 5 references to clarify what the publications actually show:

a. Reference 4 in the letter, Pietilä et al., 2023 [17]: "*A caveat of our observations is that TEM tracer studies or freeze fracture replicas would be needed for confirmation of tight junction identification.*"

b. Reference 7 in the letter, Vandenabeele et al., 1996 [21]: This study provides evidence for clear junctions between ABCL and inner arachnoid, but focuses on the spinal meninges. Also, the Schachenmayr et al., 1978 [22] study of cranial meninges in human cited in the publication shows a compartmentalization of the SAS.

c. Reference 9 in the letter, Oda et al., 1984 [23]: "*The subarachnoid space, which is believed to be a specialized channel exclusively containing the cerebrospinal fluid, was not recognized*". The study only demonstrates junctions between cells of the same layer.

d. Reference 10 in the letter, McLone and Bondareff, 1975 [2]: Fig. 5 from adult mouse shows a "*pial-arachnoid*" layer that contributes to both the pial and arachnoid layers.

e. Reference 11 in the letter, Nabeshima et al., 1975 [24]: Most of plates shows junctions in spinal meninges. Plate 4 is from macaque monkey cranial meninges.

Placement and alignment of 'SLYM' in context of prior studies of the meninges

Regarding the authors' critique about a possible misalignment of images from different species, we would like to clarify that the alignment of the images in Fig. 8 in Plá et al., 2024 [13] was originally present in Orlin et al., 1991 [25]. Our contribution was exclusively to color-code the existing sketch to highlight what we believe corresponds to the SLYM, as described in the classical literature, which subdivides the subarachnoid space (SAS) into two compartments. If the authors had reviewed the Orlin study, they would have seen that the alignment was not an initiative taken by us. Our intention was to clarify the anatomical relationships within the meninges not to misalign or misrepresent these features as suggested by the authors (Fig. 4C, extracted from Coles et al., 2017 [1]).

Conclusion

We value the opinions of our colleagues and recognize that robust academic discussion and the exchange of differing viewpoints are essential for the advancement of our field. We appreciate the critiques and have welcomed the opportunity to respond to them in various forums, including the Alzheimer's Forum, Science eLetters, our preprint on bioRxiv, X (formerly Twitter) and now in the recent commentary on our study in Fluids and Barriers of the CNS. The same critiques and arguments have been posted on 4 different online forums, none of them following academia rules of scientific debate. None of these platforms are peer-reviewed, published in an indexed journal that ensures wide-public availability and future inclusion in the scientific literature, evidence-based and constructive. We have refrained from commenting on the detailed critique in the 3 tables provided by Julie Siegenthaler and Christer Betsholtz. All the

Table 1 Summary of critiques raised against SLYM studies on different not per-reviewed platforms

	AlzForum [15]	Science eLetters [14]	bioRXiv [26]	X (Twitter) [16]
SLYM as a 4th membrane independent of ABCL	✓	✓	✓	✓
Barrier properties of SLYM	✓	✓	✓	✓
SAS compartmentalization	✓	✓	✓	✓
Name SLYM	✓	✓	✓	✓

points have been addressed before in the online forums listed (Table 1). The repetitive presentation of the same critiques, without acknowledging our detailed responses, not acknowledging the importance for in vivo observation and the absence of newly generated data to support alternative conclusions are becoming counterproductive. This is exemplified by the authors' description of their critique as '*expert opinion*', while our published data and conclusions are described as '*the authors claim*' or the '*authors insist*'. We urge our colleagues to engage in forward-looking scientific dialogue and publication of data, rather than repeating points that have already been thoroughly discussed.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Competing interests
The authors declare no competing interests.

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