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Mechanosignalling pathways that regulate endothelial barrier function

Anna McQueen and Christina M. Warboys

Abstract

Blood vessels are lined by a single layer of endothelial cells that provide a barrier between circulating plasma and the underlying tissue. Permeability of endothelial cells is tightly regulated, and increased permeability is associated with a number of diseases including atherosclerosis. Endothelial cells are continuously exposed to mechanical forces exerted by flowing blood and are particularly sensitive to shear stress, which is a key determinant of endothelial function. Undisturbed flow promotes endothelial resilience and reduces permeability to macromolecules whereas disturbed flow promotes endothelial dysfunction and barrier disruption. This review will outline recent advances in our understanding of how disturbed and undisturbed flow regulate paracellular and transcellular permeability and will highlight potential cellular targets that could form the basis of therapies to limit the development of cardiovascular disease.

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Endothelial cells and atherosclerosis

The endothelium forms a continuous monolayer that lines the interior walls of all blood vessels and regulates many processes that control vascular function including inflammation, angiogenesis, haemostasis, and vasodilation. Endothelial cells also provide a semipermeable barrier between circulating plasma and the underlying tissue, allowing the transport of gases and nutrients while restricting the leakage of macromolecules from the circulation [1]. Endothelial permeability is tightly regulated to meet the demands of the tissue and varies depending on the vessel type. Venous endothelial cells exhibit increased permeability when compared to arterial endothelium with enhanced responsiveness to permeability factors [2]. Additionally, there are tissue specific differences, with vessels of the blood-brain and blood-retinal barrier exhibiting the tightest barriers which severely restricts transport [1,3].

Endothelial dysfunction and increased permeability play an important role in many diseases [1] and is a key factor in the development of atherosclerosis [4], a chronic inflammatory condition characterised by the development of lipid-rich plaques within the vessel wall. The development of atherosclerosis is highly focal with plaques forming in the regions of arteries exposed to non-uniform (disturbed) flow revealing the importance of haemodynamic stresses in atherosclerosis [4].

Mechanical forces regulate vascular function

Endothelial cells are continuously exposed to mechanical forces exerted by flowing blood. These include tensile stresses arising from circumferential stretch of the vessel wall and the stress exerted perpendicularly on the wall by hydrostatic pressure. Endothelial cells are particularly sensitive to shear stress, which is the mechanical drag exerted by blood flow along the vessel wall and is defined as the tangential frictional force of blood flow per unit area. Shear stress is a key determinant of endothelial function, and both the magnitude and directionality of shear stress are important.

Whilst is widely accepted that unidirectional, high magnitude shear stress (10–40 dyn/cm^2) is associated with the activation of cytoprotective mechanisms and protection from endothelial dysfunction and atherosclerosis, there continues to be debate regarding the contribution of low magnitude (<5 dyn/cm^2), oscillatory (bidirectional), and multidirectional flow to atherogenesis. Recent work has highlighted the importance of multidirectional flow in promoting endothelial dysfunction [5] and atherosclerosis [6]; however, the role of low time-averaged wall shear stress is less clear with conflicting evidence regarding its role in plaque formation [6,7].

It is also important to consider the different *in vitro* methods that are used to study the effects of shear stress on endothelial cells since the haemodynamic profiles

generated can vary significantly and will depend on the configuration of the system being used, as reviewed elsewhere [8]. Parallel-plate flow chambers, cone and plate viscometers, and microfluidic devices are commonly used to expose cells to unidirectional or oscillatory flow, whereas multidirectional flow can be achieved using the swirling well (orbital shaker) method or through the modification of parallel-plate flow chambers [8]. Most commonly used *in vitro* systems are not yet able to induce multidirectional flow without also resulting in low time-averaged wall shear stresses, and so for the purposes of this review, disturbed flow denotes flow that is low in magnitude and oscillatory/multidirectional.

Shear stress-dependent regulation of endothelial function

Undisturbed flow activates homeostatic pathways and promotes endothelial resilience. Conversely, disturbed flow is associated with endothelial dysfunction and atherosclerosis [9]. The differential effects of disturbed flow and undisturbed flow on endothelial cells may be attributed to the activation of different mechanosensitive transcription factors, reviewed elsewhere [10]. The transcription factors Kruppel-like factor-2 and -4 (KLF-2, KLF-4) and nuclear factor erythroid 2-related factor 2 (Nrf-2) are preferentially activated in endothelial cells exposed to flow, whereas endothelial cells exposed to disturbed flow exhibit enrichment of nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB), activator protein-1 (AP-1), YAP/TAZ/TEAD, and hypoxia-inducible factor 1α (HIF-1α), which contribute to endothelial dysfunction [10]. Disturbed flow promotes inflammatory signalling, apoptosis, oxidative stress, endothelial-to-mesenchymal transition, and senescence [11]. Shear stress also regulates endothelial barrier function, with *in vivo* and *in vitro* studies both demonstrating that endothelial cells exposed to disturbed flow exhibit increased permeability to albumin and low-density lipoprotein (LDL) [4,12,13].

Shear stress-dependent regulation of endothelial permeability

Substances can be transported across endothelial cells via paracellular or transcellular routes [14]. Endothelial paracellular permeability is predominantly regulated by the two types of intercellular junctions: tight junctions and adherens junctions [1,3] (see Figure 1). Endothelial permeability is increased following a loss of junctional integrity and formation of focal gaps between endothelial cells.

Adherens junctions

Adherens junctions are formed by Ca^{2+}-dependent homophilic interactions between the transmembrane protein vascular endothelial (VE)-cadherin on adjacent endothelial cells and between VE-cadherin and associated cytoplasmic proteins (β-, γ-, and p120-catenin). These proteins associate with the actin cytoskeleton via interaction with α-catenin [1]. The regulation of VE-cadherin plays a key role in determining junctional integrity and barrier function [1,15]. Increased permeability of venous endothelial cells arises due to internalisation and degradation of VE-cadherin following low shear-mediated phosphorylation of VE-cadherin [2]. Adherens junctions can also be stabilised by binding of Rac to VE-cadherin in response to undisturbed flow [16]. Aside from flow-dependent regulation, adherens junction proteins also play a direct role in endothelial mechanosensing [17].

Tight junctions

Tight junctions determine the tightness of the endothelial barrier and are formed by homophilic interactions between the transmembrane proteins claudins, occludin, and junctional adhesion molecules on adjacent cells. Membrane-spanning proteins associate with cytoplasmic scaffold proteins such as zonula occludens proteins (ZO-1, ZO-2, ZO-3), cingulin, and paracingulin that interact with the cytoskeleton [1]. Crosstalk between adherens junctions and tight junctions enables barrier function to be regulated in a co-ordinated manner. VE-cadherin regulates the expression of claudin-5 whilst ZO-1 interacts with VE-cadherin to increase stability [18]. Little is known about the mechanoregulation of tight junctions by shear stress in endothelial cells [19].

Transcellular permeability

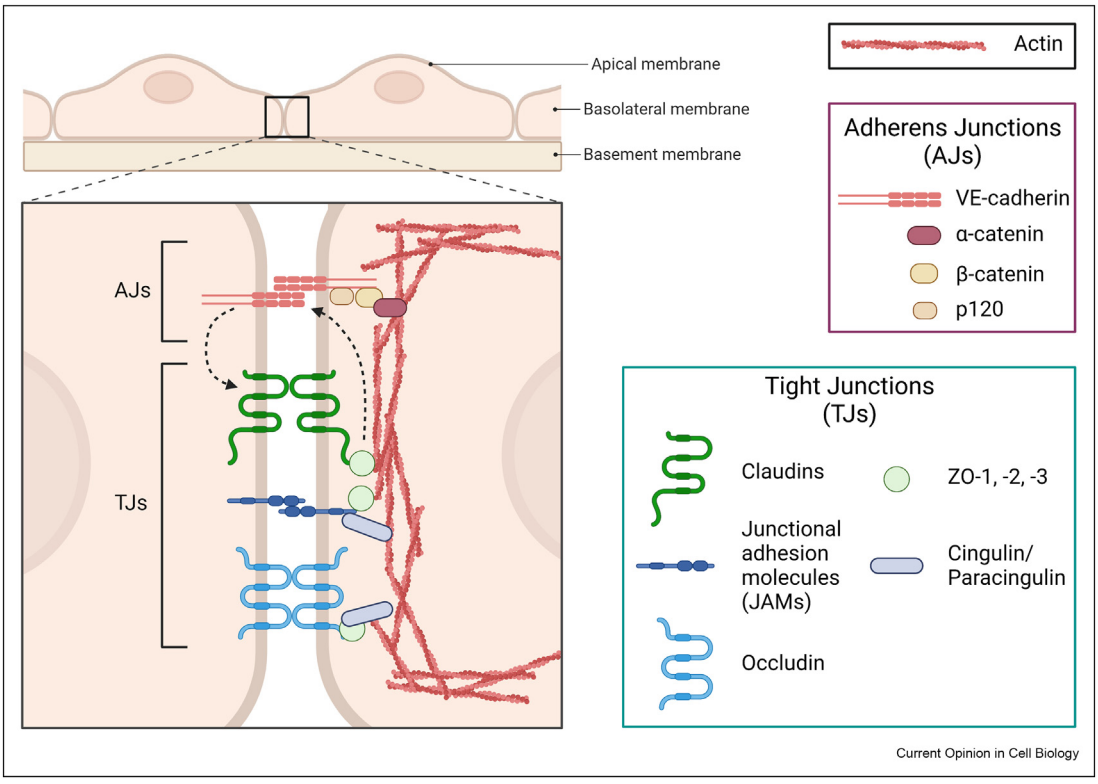
LDL is transported across endothelial cells via a transcellular route [20]. Given the importance of LDL transport to the development of atherosclerosis, it is surprising that the mechanisms underlying flow-dependent alterations in endothelial transcellular transport are poorly defined.

This review will summarise recent advances in our understanding of how mechanosignalling pathways activated in response to either undisturbed flow or disturbed flow regulate endothelial permeability. Although it is increasingly recognised that shear stress plays a critical role in the formation and maintenance of the blood-brain barrier [21–23], the focus of this review will be on the peripheral vasculature.

New insights into endothelial transport under physiological flow conditions

There has long been a debate about the predominant transport routes for macromolecules across endothelial cells and how these may be influenced by shear stress [14]. A long-favoured hypothesis was that macromolecules cross the endothelial barrier at 'leaky junctions' arising from cells undergoing mitosis or apoptosis [12]. The development of a method to directly visualise transport routes across cultured endothelial cells has

Figure 1



Paracellular permeability is regulated by adherens junctions and tight junctions. Adherens junctions are formed by homophilic interactions between membrane-spanning VE−cadherin on adjacent endothelial cells. VE−cadherin is associated with the actin cytoskeleton via interactions with β- and α-catenin. Tight junctions are formed by homophilic interactions between membrane-spanning claudins, occludin, and junctional adhesion molecules on adjacent endothelial cells. Membrane-spanning proteins associate with cytoplasmic scaffold proteins such as zonula occludens proteins (ZO-1, ZO-2, ZO-3), cingulin, and paracingulin that interact with the cytoskeleton. Crosstalk between adherens junctions and tight junctions enables barrier function to be regulated in a co-ordinated manner. Image created with BioRender.com and published with a BioRender content licence for use in academic journals.

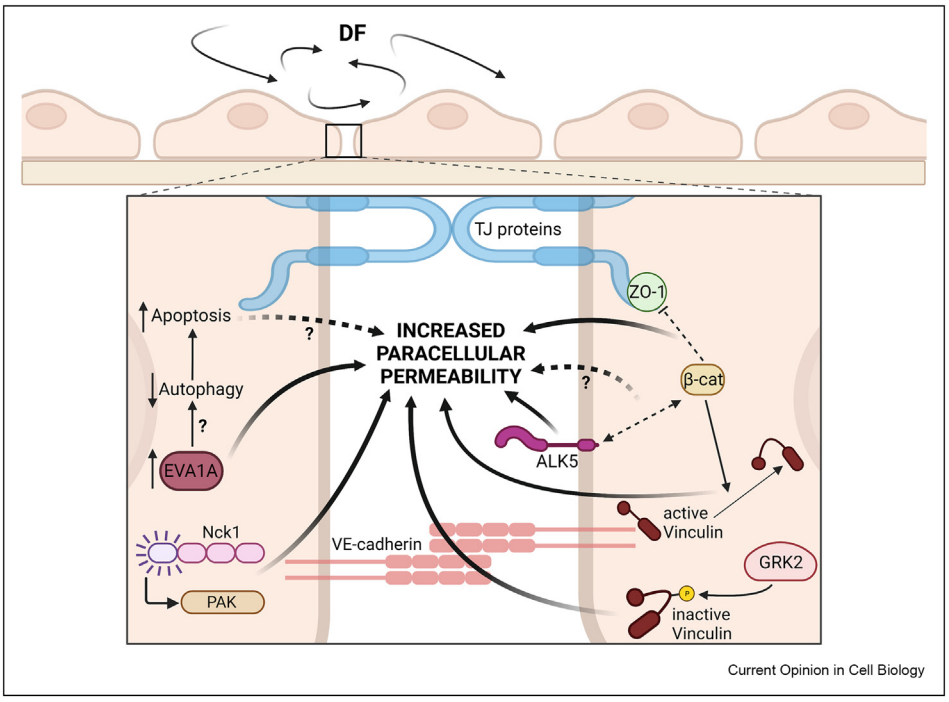
clarified many of these questions [24]. Using this method in combination with substrate-binding tracers of different sizes, the presence of three transport pathways was observed: albumin-sized tracers cross endothelial cells via a paracellular route through bicellular and tricellular junctions, transport of high-density lipoprotein-sized tracers occurs only at tricellular junctions, and LDL-sized tracers are transported across endothelial cells via transcytosis [13]. A subsequent study on the transport of albumin-sized tracers revealed that most (>80%) transport occured at tricellular junctions [25]. This study also revealed that in endothelial cells exposed to disturbed flow, the number of leaky tricellular junctions was increased compared to undisturbed flow and that the permeability of tricellular junctions was increased [25]. The role of tricellular junctions in endothelial dysfunction and atherogenesis has received little attention to date and is, therefore, an important area for further study.

To clarify the role of 'leaky junctions' in endothelial transport, a cell-by-cell analysis was conducted to probe for evidence of transport around endothelial cells undergoing apoptosis or proliferation [26]. Whilst a positive correlation was observed between apoptosis or proliferation and increased permeability, less than 5% of paracellular transport was associated with these events. Thus, whilst apoptosis and proliferation of endothelial cells can alter junctional integrity, the increase in permeability associated with disturbed flow cannot be explained by these events alone [26].

Mechanosignalling pathways that promote barrier disruption

Disturbed flow is known to promote endothelial dysfunction and to increase endothelial permeability; however, relatively little is known about the mechanosignalling pathways that lead to barrier disruption [27,28]. Several recent papers have identified new permeability-regulating pathways or provided additional insight into previously described pathways (see Figure 2). For example, p21-activated kinase (PAK) has long been known to increase paracellular permeability in endothelial cells exposed to disturbed flow [29];

Figure 2



Mechanosignalling pathways that destabilise endothelial barrier function. Disturbed flow induces endothelial barrier disruption by destabilising adherens junctions and tight junctions. Nck1 is activated by disturbed flow which promotes the association of PAK with VE-cadherin. PAK destabilises adherens junctions and increases permeability. Disturbed flow also increases the expression of EVA1A which promotes barrier disruption. Disturbed flow also increases permeability via increased β-catenin signalling which reduces the expression of ZO-1 and reduces localisation of vinculin to cell junctions. Re-distribution of vinculin away from cell junctions is also facilitated by GRK2 which is activated in response to disturbed flow. Permeability is also increased following disturbed flow-mediated activation of ALK5. DF; disturbed flow, Nck; noncatalytic region of tyrosine kinase-1, PAK; p21-activated kinase, EVA1A; Eva-1 Homolog A, ZO-1; zonula occludens-1, GRK2; G-protein-coupled receptor kinase 2, ALK5; activin receptor-like kinase-5. Image created with BioRender.com and published with a BioRender content licence for use in academic journals.

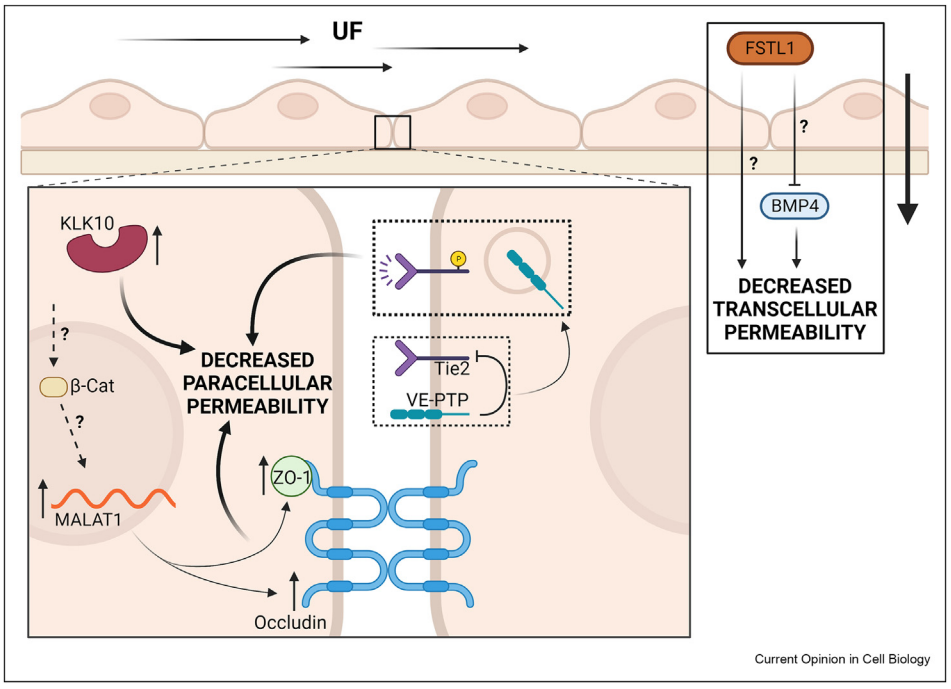
however, the mechanism was unclear. It was recently shown that the SH2/SH3 domain containing adapter protein, noncatalytic region of tyrosine kinase-1 (Nck1), is activated in response to disturbed flow and that this mediates disturbed flow-dependent PAK activation [30]. The deletion of Nck1 prevents the recruitment of PAK to cell junctions and significantly inhibits disturbed flow-induced paracellular permeability [30].

Similarly, TWIST1 was previously shown to increase the permeability of endothelial cells exposed to disturbed flow [31]. A new study by the same group demonstrates that under disturbed flow, TWIST1 increases the expression of EVA1A (Eva-1 Homologue A) which promotes barrier disruption [32]; however, the mechanisms by which EVA1A regulates permeability are not yet clear. Increased expression of EVA1A was associated with a reduction of autophagic flux and consequent increase in apoptosis although, as discussed above, this is not likely to account for the changes in permeability observed [26].

We have recently shown that disturbed flow increases paracellular permeability via a Frizzled-4-β-catenin-dependent mechanism [33]. Inhibition of β-catenin-dependent transcriptional activity reduced permeability in endothelial cells exposed to disturbed flow and was associated with increased expression of ZO-1 and altered junctional and cytoskeletal organisation [33]. Inhibition of β-catenin signalling also resulted in redistribution of vinculin away from focal adhesions with increased localisation around endothelial junctions [33] which is associated with increased stability [34]. Consistent with our study, it has been shown that in endothelial cells exposed to disturbed flow, G-protein-coupled receptor kinase 2 (GRK2) increased the phosphorylation of vinculin on Ser721. This results in the inactivation of vinculin, disruption of adherens junctions, and increased paracellular permeability [35].

We have also shown that transforming growth factor-β (TGF-β) Type I receptor, also known as activin receptor-like kinase-5 (ALK5), contributes to the disturbed flow-dependent barrier disruption [36]. Crosstalk and synergy between β-catenin and TGF-β signalling has been documented in endothelial cells [37], and therefore, it will be important to establish if the effects of β-catenin on barrier disruption are mediated by ALK5 or vice versa.

Figure 3



Mechanosignalling pathways that stabilise endothelial barrier function. Undisturbed flow promotes stabilisation of endothelial cell junctions by increasing the expression of the lnc-RNA *MALAT-1*, which increases the expression of ZO-1 and occludin. Permeability is also reduced following up-regulation of KLK10 in response to undisturbed flow and via increased internalisation of VE-PTP. This results in enhanced activation of Tie2 which stabilises VE-cadherin. Undisturbed flow also reduces transcellular permeability via increased expression of FSTL-1. FSTL-1 reduces permeability in part via inhibition of BMP4 signalling. BMP-4; bone morphogenic protein-4, FSTL-1; Follistatin-like 1, KLK10; Kallikrein-related peptidase-10, UF; undisturbed flow, VE-PTP; vascular endothelial protein tyrosine phosphatase, ZO-1; zonula occludens-1. Image created with BioRender.com and published with a BioRender content licence for use in academic journals.

Mechanosignalling pathways that stabilise endothelial barrier function

Whilst it is widely accepted that atheroprotective shear stress reduces permeability to macromolecules and stabilises endothelial barrier function [38], there have been few studies into the precise mechanical signalling pathways involved. Previous research has shown that the long non-coding RNA (lnc-RNA), *SENCR* stabilises proteins within adherens junctions and reduces permeability [39]. A new study has highlighted the role of a second lnc-RNA, *MALAT1*, in maintaining barrier integrity [40]. Acute exposure to undisturbed flow caused upregulation of *MALAT1* which increased the expression of ZO-1 and occludin. This is inferred to result in improved barrier function although this was only studied under static conditions. The authors demonstrated that acute exposure to undisturbed flow also increased the nuclear localisation of β-catenin, in a Nesprin-dependent manner, and that this was responsible for the increase in *MALAT1* expression. These observations contrast our recent findings on the role of β-catenin in regulating permeability in endothelial cells exposed to disturbed flow [33] and may reflect a difference between acute and chronic exposure to flow [41]. Alternatively, the use of a different extracellular matrix may account for divergent findings [28]. The *in vivo* permeability assays using a tankyrase inhibitor (XAV-939) also contrast with our finding that β-catenin mediates barrier disruption; however, it is not clear that the inhibitor reduces the β-catenin activity or whether the effects demonstrated *in vivo* could occur via modulation of other tankyrase targets [42].

Other studies have highlighted important roles for Tie2 [43] and Kallikrein-related peptidase-10 (KLK10) [44] in enhancing barrier function in endothelial cells exposed to undisturbed flow. Previous studies have shown that the activation of Tie2 can increase junctional stability and reduce permeability and that Tie2 can be inhibited by vascular endothelial protein tyrosine phosphatase (VE-PTP). A new study demonstrated that endocytosis of VE-PTP was increased in endothelial cells exposed to undisturbed flow, sequestering it from Tie2, resulting in enhanced Tie2 activity [43]. KLK10, a secreted serine protease, was also shown to be upregulated by undisturbed flow [44]. Studies using recombinant KLK10 demonstrated that KLK10 reduced the permeability of endothelial cells and could reverse

barrier disruption induced by disturbed flow, although the mechanism of action is not yet known [44]. These pathways are summarised in Figure 3.

Mechanosignalling pathways that regulate transcellular transport

Using a novel technique to restrict endothelial growth to either the centre or edge of swirling wells, where flow is disturbed or undisturbed, respectively [45], it has been discovered that endothelial cells exposed to undisturbed flow secrete a soluble mediator, Follistatin-like 1 (FSTL1) that reduces transcellular transport of LDL-sized particles across endothelial cells [46]. FSTL1 mediates these effects partly via inhibition of BMP4 signalling, although data obtained using a BMP4 inhibitor suggest additional mechanisms are present [46]. These findings have not been confirmed *in vivo* although it was shown that the expression of FSTL1 was greater in endothelial cells exposed to undisturbed flow [46].

Conclusions and future perspectives

Recent research has revealed important new insights into the regulation of permeability in endothelial cells by shear stress (see Figures 2 and 3) and highlighted a number of signalling molecules that could be targeted to stabilise junctions and thereby reduce paracellular permeability and endothelial dysfunction [26,30,35,36,43,44]. These could be utilised as part of a preventative strategy, alongside statins and anti-inflammatory therapies, to further limit the development and progression of atherosclerosis. The modification of LDL transport may also provide another important target to limit the deposition of cholesterol within the vessel wall [46] and protect against cardiovascular disease. To date, this has been an understudied area, especially given its importance in atherogenesis; the recent identification of its dominant transport route in endothelial cells under physiological flow conditions will hopefully provide a platform for further studies into its regulation. Before translation can be considered, it is necessary to replicate many of these findings in animal models since cultured endothelial cells exhibit permeability values that are orders of magnitude higher than those observed *in vivo* [47]. Whilst numerous *in vivo* permeability assays are available [48], to explore specific transport routes in different haemodynamic environments, the use of substrates that become immobilised in the subendothelial space (in a manner similar to *in vitro* methods [24]) would be the most informative.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data were used for the research described in the article.

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