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REVIEW

Endothelial permeability, LDL deposition, and cardiovascular risk factors—a review

Santa Mundi[1], Marika Massaro[2], Egeria Scoditti[2], Maria Annunziata Carluccio[2], Victor W.M. van Hinsbergh[3], Marial Luisa Iruela-Arispe[4], and Raffaele De Caterina[5]*

[1]Department of Biological and Environmental Science and Technology (DISTEBA), University of Salento, via Monteroni, 73100, Lecce, Italy; [2]National Research Council (CNR), Department of Biomedical sciences, Institute of Clinical Physiology, Via Monteroni, 73100, Lecce, Italy; [3]Department of Physiology, Institute for Cardiovascular Research, VU University Medical Center, van der Boechorststraat, NL-1081 BT, Amsterdam, The Netherlands; [4]Department of Molecular, Cell and Developmental Biology and Molecular Biology Institute, University of California, 610 Charles E Young Dr S, 90095, Los Angeles, USA; and [5]Department of Neuroscience, Imaging and Clinical Science and Institute of Advanced Biomedical Technologies, University G. D'Annunzio, via dei Vestini, 66100 Chieti, Italy

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Early atherosclerosis features functional and structural changes in the endothelial barrier function that affect the traffic of molecules and solutes between the vessel lumen and the vascular wall. Such changes are mechanistically related to the development of atherosclerosis. Proatherogenic stimuli and cardiovascular risk factors, such as dyslipidaemias, diabetes, obesity, and smoking, all increase endothelial permeability sharing a common signalling denominator: an imbalance in the production/disposal of reactive oxygen species (ROS), broadly termed oxidative stress. Mostly as a consequence of the activation of enzymatic systems leading to ROS overproduction, proatherogenic factors lead to a pro-inflammatory status that translates in changes in gene expression and functional rearrangements, including changes in the transendothelial transport of molecules, leading to the deposition of low-density lipoproteins (LDL) and the subsequent infiltration of circulating leucocytes in the intima. In this review, we focus on such early changes in atherogenesis and on the concept that proatherogenic stimuli and risk factors for cardiovascular disease, by altering the endothelial barrier properties, co-ordinately trigger the accumulation of LDL in the intima and ultimately plaque formation.

Keywords Atherosclerosis • Endothelium • Vascular permeability • Cardiovascular risk factors

1. Introduction

The vascular endothelium is the main regulator of the selective exchanges of solutes and cells between the flowing blood and the surrounding tissues. Small molecules may cross this inner vascular layer gaining access to the tunica intima according to concentration gradients, whereas the passage of larger molecules and cells can only occur via vesicles and receptors, or when the endothelial junctions are impaired.[1] Although the term 'increased vascular permeability' broadly refers to a compilation of structural and functional changes in the entire vessel wall, the passage of macromolecules, fluids, and cells into the intima, occurring at the inception of atherosclerosis, is primarily due to changes in endothelial barrier function. Endothelial dysfunction(s)[2] initiate a dysregulated transendothelial flux, which lead(s) to abnormal deposition of molecules and cells in the intima. This results in intimal enlargement and local inflammation, both featuring in early atherosclerosis.

Low-density lipoproteins (LDL) are the main vehicle of blood cholesterol transport, and their accumulation in the intima characterizes the formation of the fatty streak,[3] the earliest morphological change occurring in atherosclerosis.[4] Understanding the control of endothelial permeability to LDL in the broader context of changes in endothelial function is therefore crucial to an understanding of atherosclerosis.

We have here compiled the most relevant information on mechanisms by which the vascular endothelium allows molecules or cells to permeate the intima. This process involves changes in three compartments: (i) the 'glycocalyx', which is the surface layer of glycoproteins, proteoglycans, and glycosaminoglycans that together create a scaffold on the endothelial surface[5]; (ii) the energy-dependent vesicular trafficking, referred to as the 'transcellular pathway'[6]; and (iii) the opening or rearrangements of cell-to-cell junctions, also known as the 'paracellular pathway'.[7,8] An enhanced vesicular trafficking and/or widened intercellular spaces in the presence of a disarrayed or overtly disintegrated glycocalyx may all facilitate the transendothelial flux of LDL, paving the way to their subendothelial retention.[9,10]

We also herein review the molecular changes involved in endothelial permeability to LDL caused by cardiovascular risk factors; specifically dyslipidaemia, diabetes, hypertension, obesity, and smoking. All these factors have been reported to promote transendothelial uptake of LDL through mechanisms likely related to increased oxidative stress, highlighting the concept that all such risk factors promote atherosclerosis—at least in part—by inducing changes in endothelial permeability to LDL.

* Corresponding author. Tel: +39 0871 41512; fax: +39 0871 402817, E-mail: rdecater@unich.it

2. Literature search methods

For the literature search, we have used the following terms: 'Dyslipidaemia AND atherosclerosis AND endothelial permeability', with 56 output articles; 'Diabetes AND atherosclerosis AND endothelial permeability', with 97 output articles; 'Hypertension AND atherosclerosis AND endothelial permeability', with 99 output articles; 'Obesity AND atherosclerosis AND endothelial permeability', with 8 output articles; and 'Oxidative stress AND atherosclerosis AND endothelial permeability', with 51 output articles; 'smoking AND atherosclerosis AND endothelial permeability' with 21 output articles. Based on the title or abstract, articles were chosen for text review, and cited when appropriate. Additional records were identified and cited from the analysis of references cited in each article and from the authors' own expertise.

3. Increased endothelial permeability and intimal LDL accumulation

In the late 1970s, Minick *et al.*[11,12] tested the hypothesis that persistent absence of the endothelium favours intimal thickening, lipid accumulation, and atherosclerosis. Their results showed that, in rabbit aortas, the greatest accumulation of lipids and the largest intimal thickening was present in areas covered by a regenerated endothelium, and not in adjacent denuded areas, i.e. intimal areas lacking an endothelial lining. These experiments apparently supported the hypothesis that in areas of regenerated endothelium severe endothelial hyperpermeability is a major factor favouring intimal lipid uptake, more so than in areas with the complete absence of endothelium. Subsequent studies in moderately hypercholesterolaemic rabbits[13] showed that the influx of labelled LDL into the aortic tunica intima mainly proceeds through focal sites, where inter-endothelial junctions are impaired. In Sprague-Dawley rats, impaired junctional function may occur as the consequence of cell damage and/or cell division[14,15] and is found for months in the regenerated endothelium.[14,15]

An important reason why an endothelium with impaired barrier function may cause lipid accumulation more than in its complete absence is potentially linked to the mechanisms by which LDL accumulate in the intima. The relatively small fenestrae in an intact intimal elastic lamina (IEL) limit LDL flux into the media as compared to that of fluids.[16] A sieving of the IEL with intimal accumulation of LDL was hypothesized to occur in the presence of what we would now call 'dysfunctional' endothelium,[2] particularly in areas of recent endothelial regeneration. Such sieving of the IEL was actually found in specimens of human aortas, where concentration of unbound LDL in the intima markedly exceeded that of plasma.[17,18] In an elegant physical model, Fry[19] explained the transport and accumulation of atherogenic agents in elastic and muscular arteries ignoring at that time chemical reactions occurring in the intima. He demonstrated that both 'mild' increases in endothelial permeability and subjacent interstitial sieving were required for the accumulation of LDL in conditions known to promote atherosclerosis, such as hypercholesterolaemia and hypertension. *Figure 1* summarizes the transport of LDL to and from the arterial intima, including binding of LDL to glycosaminoglycans, by which LDL can become more vulnerable to oxidation, favouring their uptake by phagocytes.[20] These monocyte/macrophages see bound and modified LDL as foreign particles, initiating an immune response. In addition, oxidised LDL (ox-LDL) also promote endothelial activation with the release of soluble and membrane-bound mediators that further fuel the inflammatory response.[21]

Subsequent studies further underpinned the role of endothelial permeability in atherosclerosis. In reviewing these data, Nielsen[22] summarized a great number of findings as follows: 'In laboratory animals, the regional variation in the arterial wall permeability predicts the pattern of subsequent dietary induced atherosclerosis. Moreover, mechanical or immunological injury of the arterial wall increases the LDL permeability and is accompanied by accelerated development of experimental atherosclerosis'. Based on these statements, it is still unclear whether the increase in permeability is a cause or a consequence of the inflammatory activation that overtakes the tunica intima.

4. The intimal uptake of LDL—methods of investigation

In vitro studies on LDL transport using endothelial monolayers consist in growing such cell monolayers on filters and placing them in chemotactic chambers. Under these conditions, the fluid above and below the monolayer can be readily sampled and evaluated for lipoprotein transport.[23,24] The extent of LDL transcytosis can be measured using a non-radioactive '*in vitro*' method, by which confluent endothelial cells (ECs) grown in culture in the upper compartment of a chemotaxis chamber are exposed to a solution of labelled LDL.[25,26] The passage of labelled LDL from the upper to the lower compartment can be evaluated by measuring the concentration of LDL in the lower compartment by fluorimetry. To distinguish whether the passage of LDL is mediated by transcytosis or by impaired cell junctions allowing for paracellular transport phenomena, the permeation rate can also be evaluated after treatment of cells with inhibitors of transcytosis. It is possible to carry-out the analysis of LDL uptake in EC incubating EC with labelled LDL under appropriate culture conditions before the uptake assessment by fluorescence microscopy.[27] These methods ignore the contribution of the glycocalyx, as this is poorly developed in culture.

Vascular wall permeability to LDL can now also be measured using *in vivo*/*ex vivo* methods. *In vivo*/*ex vivo* measurements of LDL uptake in carotid and aorta can be assessed by administering radiolabelled LDL, followed by autoradiography.[28,29] This method consists in obtaining LDL from animal or human plasma *in vivo*, labelling the lipoprotein fraction, and then injecting both LDL and serum albumin into patients then undergoing surgery. Subsequently, surgical or endarterectomy specimens are fixed and sliced transversely, and processed by autoradiography.[29] It is also possible to calculate LDL transport from the blood into the arterial wall after obtaining *in vivo* anatomy data from computed tomography images.[30] Alternatively, local changes in arterial wall permeability to LDL,[13,31,32] as well as the contribution of the glycocalyx,[5] can be assessed by electron microscopy.

5. Structures involved in endothelial permeability to LDL

The first regulator of LDL transendothelial passage is the glycocalyx,[33] a thick and negatively charged matrix layer, that lines the inner wall of healthy blood vessels[34] (*Figure 1*). Once through the glycocalyx, LDL can cross the endothelium via transcytosis, a process that occurs through vesicles, which transport lipoproteins from the apical to the basolateral aspect of ECs.[31] In Sprague-Dawley rats under pathological conditions,

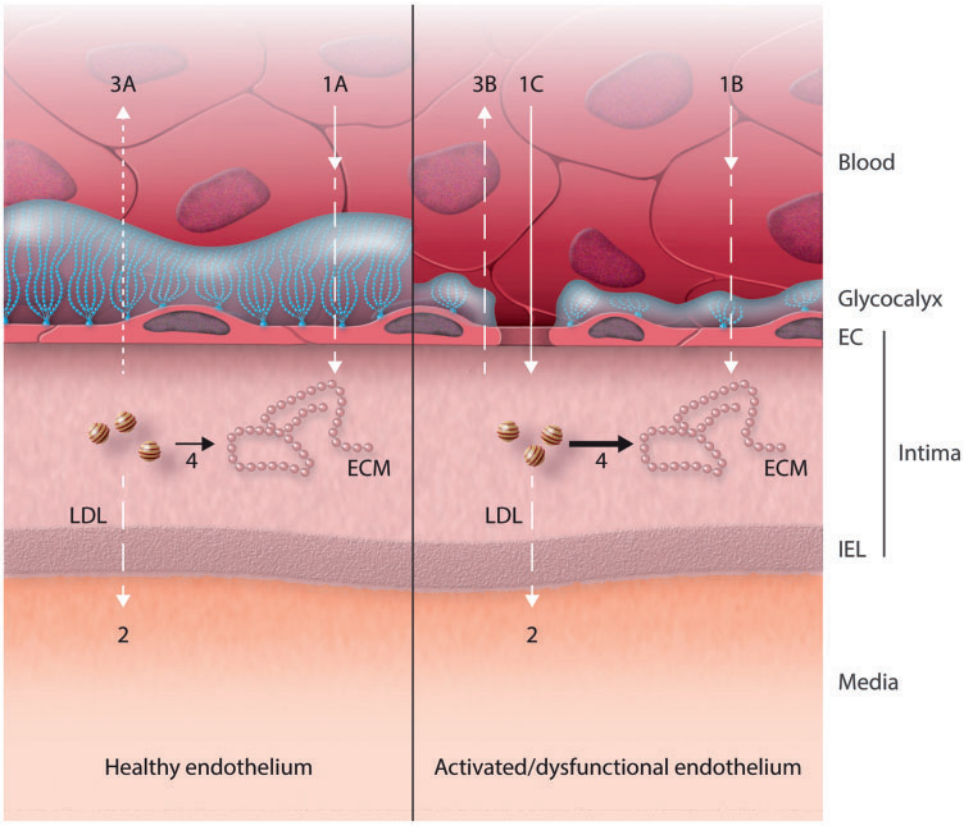

Figure 1 The entrance of LDL in the arterial intima of healthy vessels is determined by the glycocalyx and endothelial vesicles (1A). In vessels where the glycocalyx is compromised, the entrance of LDL in the arterial intima is determined by endothelial vesicles (1B) and open endothelial junctions (1C). The efflux occurs through the IEL (2) and the backward diffusion (3A and 3B), which may be of minor relevance as it goes against hydraulic conductivity. LDL can be trapped by binding to the extracellular matrix (ECM) (4). Adapted from Fry.[19]

LDL may also, however, cross the endothelium through junctions with a wider inter-junctional space, the so-called 'leaky junctions', associated with dying or dividing cells.[35,36]

5.1 The glycocalyx

The glycocalyx is a thick layer of glycoproteins, proteoglycans, and hyaluronan (HA) that lines the endothelial surface. It is firmly attached to the luminal surface, as many of these molecules are membrane-bound and extend ~0.05 to 0.4 μm from the endothelium towards the lumen.[37] In addition, a second layer, partly intertwined within the former, extends by an additional ~0.3 to 0.5 μm, and is composed mostly by HA. This second layer of the glycocalyx traps plasma- and endothelium-derived proteins and is in direct contact with the bloodstream.[34] The glycocalyx plays a role in the regulation of vascular permeability, in the transmission of shear stress to the endothelial surface, provides a barrier to pathogens, and prevents blood cell margination towards the vessel wall.

In murine models, there is evidence that composition and thickness of the glycocalyx depend on local haemodynamic shear forces from the flowing blood.[37–39] Moreover, in a pig model, laminar flow, likely through mechanical distortions of the glycocalyx, enhances nitric oxide (NO) production by the underlying ECs, a process that may become insufficient when shear forces are altered.[40] As shown in male Syrian golden hamsters, the glycocalyx, as a selective barrier to solutes, allows anionic and protein molecules to gain access to the membrane bilayer, depending on size, charge, and structure,[41] while water and electrolytes can pass freely. The binding and intercalation of plasma constituents within the structural elements of the glycocalyx create the so-called endothelial surface layer.[42] The glycocalyx also plays a critical role in regulating LDL transport into the arterial wall, although the exact nature of the interactions between LDL and the glycocalyx is still under investigation.

Initially in coronary arteries of cholesterol-fed White Carneau pigeons, and subsequently in carotid arteries of hypercholesterolaemic mice, it was shown that the thickness of the glycocalyx was reduced in plaque regions of the arteries (45–64% and 71%, respectively).[37,43,44] In addition, the glycocalyx coverage was reduced by 29% in atherosclerotic areas.[44] These changes are dependent both on the plaque-inducing diet and location in the vessel. Reducing the thickness of the glycocalyx, or decreasing its barrier function would both favour the access of LDL to the endothelial surface, expose endothelial receptors and enhance binding of monocytes,[45–47] which lead to enhanced accumulation of LDL into the intima[5,10] (*Figure 1*). In specific conditions such as hypertension this process benefits from convective transmural fluxes, causing concentration and polarization of LDL at the luminal surface of arteries.[48]

The reduction in glycocalyx in atherosclerotic regions is probably due to both decreased synthesis and increase breakdown. Exposure to

laminar flow is required for the continued production of proteoglycans, and when laminar flow ceases, rapid shedding of proteoglycans occurs.[38] Furthermore, enhanced breakdown of the glycocalyx can also occur by enhanced generation of reactive oxygen species (ROS) and inflammatory activation of the endothelium, which both can lead to increased activity of proteoglycan-shedding metalloproteinases. It is known that the glycocalyx is severely impaired in diabetes, a disease accompanied by enhanced ROS production. Furthermore, ox-LDL reduce the effective thickness of the glycocalyx.[49] Finally, Son *et al.*[50] showed that disturbed flow targets and downregulates the inhibitor of metalloproteinases TIMP3 via induction of mouse-specific miR-712 (and human miR-205). Under laminar flow, TIMP3 limits the proteolytic activity of and the shedding by several metalloproteinases.[51] Thus, laminar flow protects the glycocalyx by several mechanisms: it enhances NO production, which in turn counteracts ROS and inflammatory activation; and limits the activity of metalloproteinases in shedding the glycocalyx by maintaining TIMP3 synthesis.

5.2 LDL transcytosis and the role of caveolae

To reach the subendothelial space, after crossing the glycocalyx, LDL also have to pass the EC barrier (*Figure 1*). The passage of LDL through normal endothelial junctions is not possible for a variety of reasons, including the mere steric hindrance, being LDL diameter (20–30 nm) larger than the inter-junctional space (3–6 nm). Most of LDL transport is mediated by clathrin-coated and caveolin vesicles, both originated from invaginations of the plasma membrane and playing a major role in the continuous exchange of molecules, including LDL.[52] Under physiological conditions, open caveolae and clathrin-coated vesicles at the luminal aspect of the endothelium bind LDL through LDL receptors, specifically scavenger receptor class B member 1 (SBR1), and more recently also activin receptor-like kinase 1 receptors.[53,54] Once bound, LDL become internalized, shuttled through the endothelium, and delivered at the abluminal membrane.[31] This process is called *transcytosis*.[55] Pathological stimuli can upregulate endothelial transcytosis, and caveolin vesicles are key regulators of the endothelial barrier function.[55] Particularly, silencing caveolin-1 (Cav-1) in ECs prevents the endocytosis of LDL, suggesting that Cav-1 function is critical for the endocytosis of LDL and potentially their transcytosis across EC. Compared with mice with the Cav-1$^{+/+}$ background, mice silenced for Cav-1 displayed increased endothelial vascular cell adhesion molecule (VCAM)-1 expression, suggesting an important role for Cav-1 in vascular inflammation, and explaining its involvement in the inception of atherosclerosis.[56]

Transcytosis is a two-step process: first, receptor-mediated endocytosis into specific vesicles must take place, followed by exocytosis, an event that requires their recognition, docking and binding into the correct plasma membrane. This second step is facilitated by the soluble *N*-ethylmaleimide-sensitive factor (NSF) Attachment Protein (SNAP) receptors.[55] In cultured EC, the transport of LDL cholesterol is largely controlled by protein kinase C (PKC) and the tyrosine kinase Src (Src)[26] (*Figure 2*). Exocytosis requires integral membrane proteins members of the SNAp REceptor complex (SNARE), which comprises soluble SNAP receptors,[61] and mediates the docking and fusion of vesicles with the cell membrane in order for vesicles to release their contents (*Figure 2*). The capacity of vesicle-mediated exchange of LDL over the endothelium is, however, quite limited.

Once released from the basolateral aspect of the cell, LDL are trapped in the subendothelium, where they may undergo oxidative modifications, becoming strongly pro-atherogenic and able to induce the expression of adhesion molecules on the endothelial surface.[63] The enhanced vesicular trafficking induced by atherogenic risk factors facilitates the transendothelial flux of LDL, enhancing their sub-endothelial retention, an early step in atherogenesis[9] (*Figure 2*).

Importantly, the transport of LDL by low density lipoprotein receptor (LDLR) and SBR1 has been shown also to mediate increased transcytosis of high molecular weight compounds.[64] This has been shown by exposing endothelial monolayers to LDL and visualizing the incorporation and transcytosis of high molecular weight dextrans.[64] These findings clarify the effect of high levels of LDL and cholesterol on the endothelial barrier function, and highlight the possible implications of high level cholesterol to disease.

5.3 Endothelial cell–cell junctions

In animal model, LDL may also cross the endothelium through the *paracellular pathway*, when the barrier 'leaks'.[35,36] The paracellular pathway relies on structural changes in cell-cell junctions. Early electron microscopy studies[65] revealed numerous and highly ordered junctional complexes between EC. These structures vary between distinct vessels, being more developed in arteries and capillaries and less so in post-capillary venules, where cell extravasation and exchange of plasma constituents are particularly prominent.[65] The main groups of junctional complexes that control permeability are *adherens junctions* (AJ) and *tight junctions* (TJ),[66] composed of membrane proteins connected to the cytoskeleton through transmembrane and cytosolic proteins.[67] Their role is to impede the diffusion of molecules between the apical and the basolateral membrane of EC through tight contact of juxtaposed membranes[68] (*Figure 3*).

TJ bring plasma membrane from adjacent cells into very close proximity, and were first incorrectly thought to be a fusion between the outer leaflets of plasma membranes.[74] Ultrastructurally, TJ appear as networks of linear fibrils circumscribing the cell, intersected by short transversal fibrils,[74,75] with plasma membranes in tight contact with each other. TJ are formed by occludins, claudins, and junctional adhesion molecules (JAMs)[73] and contain many intracellular components, such as the *zonula occludens-1* (ZO-1), which assembles molecular complexes and connects junctional structures with the cytoskeleton.[71] *Table 1* lists the most important molecules and the resulting structures involved in TJ regulation of permeability.

Serial-section electron microscopy studies revealed that—with the exception of the blood–brain barrier—TJ in the capillary endothelium of the rat heart are mosaic structures, organized as irregular networks between neighbouring cells, and provided with discontinuities about 4 nm wide.[97] This supports the notion that, in most healthy continuous endothelia, the paracellular pathway is only viable for small solutes, and is responsible for the relatively low electrical resistances when compared to epithelia and the brain endothelium, which are sealed by a TJ belt.[98,99] Although macromolecules may also pass these TJ mosaic discontinuities, a subsequent belt of closed AJs only allows the passage of small solutes, but not of macromolecules.

AJ are primarily composed of clusters of cadherin–catenin complexes typical of all epithelial and endothelial layers. Within the many types of cadherins, vascular-endothelial (VE)-cadherin or cadherin-5[100] is specific to ECs. Cadherins in general are transmembrane proteins that extend into the extracellular domain and, in the case of VE-cadherin, form homodimeric interactions via their C-terminal domains. After such engagement, the cytosolic regions of these proteins become connected with the actin cytoskeleton.[69] Such interactions are reinforced by a group of molecules that include, on the cadherin side, β-catenin,

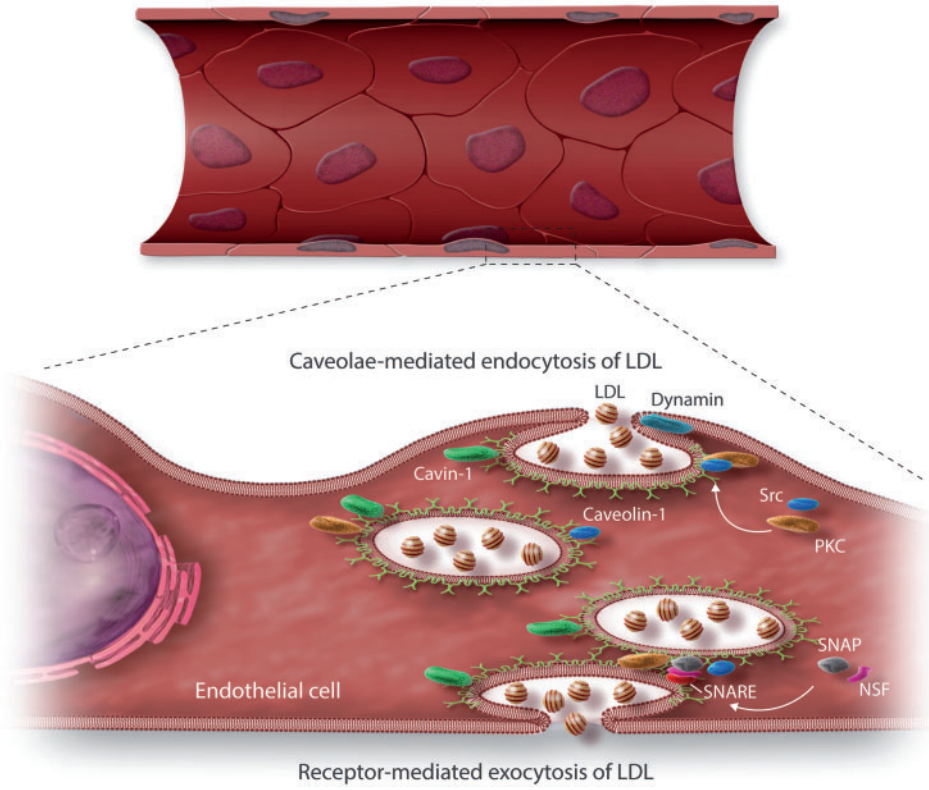

Figure 2 Caveolae-/Cav-1-/cavin-1-mediated endocytosis and exocytosis of LDL particles in endothelial cells. The vesicular transport of LDL mediated by the caveolae/Cav-1/cavin-1 complex is triggered by PKC or Src,[26] with the contribution of dynamin, a target protein for Src[57] and PKC.[58] Dynamin acts as a guanosine triphosphatase in the fixing of caveolae,[59] and enables signals for the vesicle internalization.[60] LDL endocytosis is further influenced by signalling pathways that further regulate LDL uptake. In turn, exocytosis is mediated by an integral membrane protein family, called SNARE, which comprises SNAP receptors[61] and promotes the docking and fusion of vesicles with the cell membrane in order to release their contents through its interaction with *N*-Ethyl Maleimide (NEM)-Sensitive Factor,[55] which is activated after the recruitment by its SNAP receptor.[62]

plakoglobin, and p120.[69] β-Catenin and plakoglobin, in turn, link α-catenin, which further connects with actin via α-actinin and perhaps another still uncertain protein—eplin—mediating the anchorage of actin microfilaments to AJ (for reviews see[101,102]).

When α-catenin is stretched, a new domain is exposed, binding vinculin and enabling the formation of a second bridge to the actin cytoskeleton. An additional group of proteins have been recently acknowledged to contribute to AJ, including the nectin–afadin protein complexes, which itself includes nectin, afadin, and ponsin.[71] Nectin binds to afadin, which in turn binds ponsin, connecting nectin to the actin cytoskeleton[103] (*Figure 3*). For a more comprehensive list of the molecules and structures involved in the regulation of permeability by AJ, see *Table 2*. The expression of VE-cadherin, which is essential for maintenance of the endothelial barrier function,[115] is altered in early atherosclerosis and specifically in ECs of the human carotid artery and aorta that are involved in intra-plaque neovascularization.[116] It is worth noting that an increased density of 'leaky' intercellular clefts have a much more limited effect on transendothelial LDL transport when the glycocalyx is present.[10] Thus, a joint perturbation of several structural components of the endothelial barrier is necessary for a substantial subendothelial accumulation of LDL.

Other junctional contacts include *gap junctions* (GJ), formed by connexins (Cx), which are members of a large family of transmembrane proteins. In contrast to AJ and TJ, GJ allow ECs to communicate through the exchange of small molecular weight solutes between neighbouring cells[117] (*Figure 3*). Endothelial GJ are formed by three Cx: Cx43, Cx40, and Cx37.[100] GJ have also been proven to play an important role in atherogenesis, but have no direct effect on endothelial permeability. In fact, significant changes in the expression of vascular Cx have been described during the formation of atherosclerotic plaques in murine and human atherosclerotic plaques,[118] and this expression is influenced by atherosclerotic risk factors, modifying the GJ channel or the hemichannel-mediated communication between cells, and influencing the progression of atherosclerosis [119] (*Figure 3*).

6. Atherosclerotic risk factors and endothelial permeability

The impairment of endothelial function(s) is widely recognized as the first clinical correlate of atherosclerosis and the first step in its inception.[2,120] In fact, atherosclerotic risk factors are known to promote endothelial dysfunction(s) in the earliest phase of the process,[2] affecting structure and function of the glycocalyx and NO production, as well as promoting the underlying critical changes that lead to the atheroma.[33] Conditions of enhanced cardiovascular risk, such as dyslipidaemias,

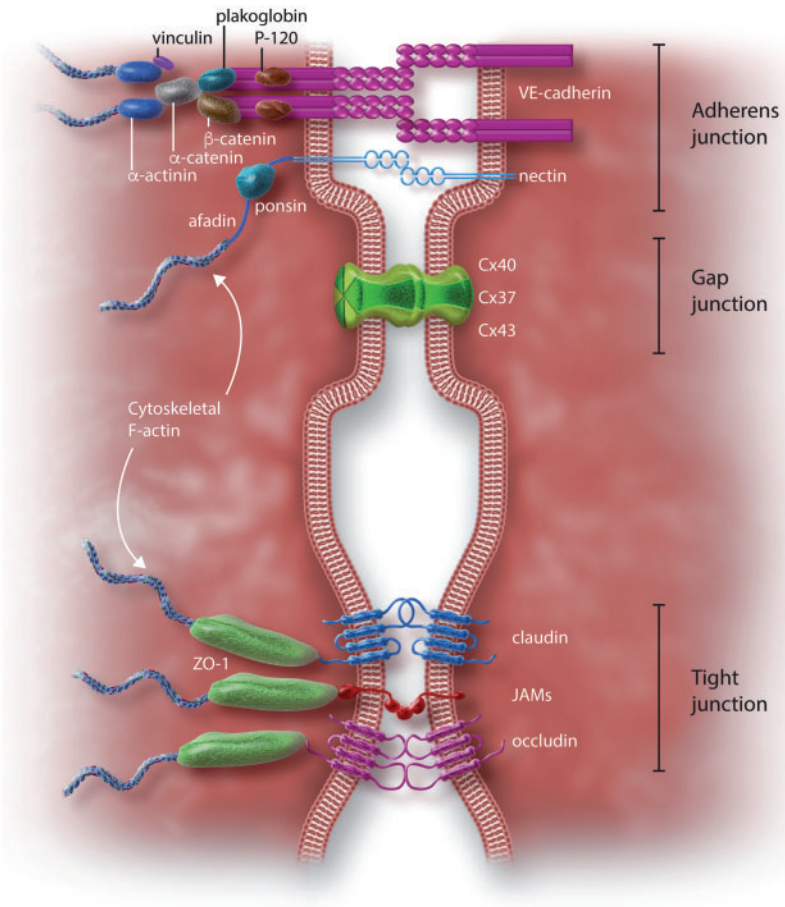

Figure 3 Proteins involved in endothelial junctions. AJ are represented by the cadherin–catenin complex and the nectin–afadin complex. VE-cadherin binds to β-catenin and plakoglobin (probably these two different molecules both connect to the actin cytoskeleton by a still uncertain mechanism that probably involves alpha-actinin and eplin, as it does for E-cadherin), and p120 through its cytoplasmic tail.[69] β-Catenin and plakoglobin in turn link α-catenin, a filamentous (F) actin-binding protein, which is the primary link between the AJ and the actin cytoskeleton.[70] α-Catenin can bind vinculin and α-actinin, stabilizing the anchorage to F-actin microfilaments (for a review see[71]). When α-catenin is stretched (upon tension on the junctions), a latent vinculin binding site becomes available and initiates a second interaction of the cadherin–catenin complex with the F-actin cytoskeleton.[72] Nectin binds afadin, which in turn binds ponsin, connecting nectin to the F-actin cytoskeleton.[73] TJ are formed by occludin, claudin, JAMs,[66] and by many intracellular components, such as ZO-1, which assemble molecular complexes and connect junctional structures to the cytoskeleton.[67] GJ are formed by three Cx: Cx43, Cx40, and Cx37[71] (see also *Table 1*).

diabetes, hypertension, obesity, and smoking, all increase vascular permeability to LDL. Furthermore, micro-mechanical forces, such as fluid shear stress or cyclic strain, also influence endothelial permeability.[121] We here therefore highlight the effect of cardiovascular risk factors on endothelial permeability. Importantly, all of these conditions affect cholesterol transfer across the endothelium, having increased oxidative stress as a common denominator.[2]

6.1 Dyslipidaemias

Dyslipidaemias, in their various forms, lead to EC dysfunction, thus increasing cardiovascular risk.[120,122,123] The most common types of dyslipidaemia associated with increased cardiovascular risk are those characterized by high levels of LDL cholesterol.[124] High levels of LDL generate a prominent increase in lipoprotein transcytosis[125] that, together with changes in aortic endothelial permeability, as seen in cholesterol-fed rabbits,[22] are the two major determinants of the LDL flux into the arterial wall and their deposition in the intima.[126] Sprague-Dawley rats fed a high-cholesterol diet or normal diet for 12 months featured significant differences in the intercellular cleft morphology and the associated junctional complexes compared with rats fed a normal diet for 1 month.[127] In such conditions, the density of GJ decreases, while the density of TJ and the other junctional complexes increase after 12 months compared with 1 month of normal diet.[127] Although it is unlikely that LDL molecules can pass through GJ, it is recognized that the GJ assembly is modulated by exposure to LDL and apolipoprotein B.[128] Hypercholesterolaemia may weaken the endothelial barrier function by activating Ras Homolog Family Member (Rho)A, while statins decrease permeability by suppressing Rho function in cultured human ECs.[129] Hypercholesterolaemic serum may increase endothelial permeability of cultured ECs through the activation of phosphatidylinositol (PI)3-kinase.[130] This latter is a major intracellular step regulating cell functions, such as cell growth, survival, and intracellular trafficking. Moreover, the thickness and function of the endothelial

Table 1 Structural and functional factors involved in the control of endothelial permeability: *tight junctions*

Name	Acronim/short name	Role and molecular relationship	References
Integral membrane proteins			
Occludin	OCLN	Occludin is a 65 kDa protein containing two extracellular loops and four membrane-spanning regions, localizes at the TJ of epithelial and endothelial cells and might contribute to intercellular adhesion. Its endothelial expression correlates with the permeability of vascular tree	71
Claudin	CLDN	Claudin is a 22 kDa protein containing four membrane-spanning regions, two extracellular loops, and two cytoplasmic termini, and has central role in TJ formation in various tissues. Claudin-5 is typical of endothelium	76,77
Junctional adhesion molecule-A	JAM-A	JAMA-A is a 32-kDa glycoprotein composed of an extracellular region, a transmembrane segment, and a short cytoplasmic tail. JAM-A interacts with ZO-1, AF-6/Afadin, PAR-3/ASIP, CASK/Lin-2, and MUPP-1. Togeter with occludin and claudin, JAM-A binds cytoplasmic and cytoskeletal proteins involved in cell signalling, cell polarity, transcriptional regulation, and membrane trafficking	78–80
Endothelial cell selective adhesion molecule	ESAM	ESAM is a protein of 55 kDa localized at the TJ trough its colocalization with ZO-1 in brain and muscle capillaries. It participates in TJ assembly and in the regulation of paracellular permeability	81
Coxsackie and adenovirus receptor	CAR	CAR also participates in TJ assembly and in the regulation of paracellular permeability	82
Intracellular proteins			
Zonula occludens-1	ZO-1	A 220–225 kDa protein belonging to the family of membrane-associated guanylate	83
Calcium/calmodulin-dependent serine protein kinase	CASK	CASK is a MAGUK protein, associating indirectly JAM-A to the cytoskeleton, via the actin/spectrin binding protein	84,85
Membrane-associated guanylate kinase inverted-1	MAGI-1	MAGI-1 is a MAGUK protein and has been recently shown to bind α-actinin-4 and associated to TJ	86
Afadin	AF6	Afadin is a multi-domain protein involved in signalling and organization of cell junctions during embryogenesis. It contains PDZ domains and has been reported either at the TJ or at the AJ, also found in association with TJ proteins, such as JAM-A	79
Proteinase-activated receptor 3/Agouti-signalling protein	PAR-3/ASIP	PAR-3/ASIP has been localized at the TJ of enterocytes and MDCK cells	87
Cingulin	CGN	Cingulin is a 140- to 160-kDa component of endothelial and epithelial TJ and does not contain PDZ domain associated to TJ	88
Tight junction-associated antigen	7H6	7H6 is a 155-kDa antigen localized at the TJ which likely plays a role in the barrier function of TJ in epithelial and endothelial cells	89
Tight junction associated protein 1 provided	PILT	PILT is a 61-kDa protein only recruited to TJ following the formation of claudin-based strands, not directly interacting with claudin	90
Serine–threonine kinase	WNK-4	WNK-4 is the localization at the TJ and its mutations cause a form of secondary hypertension in humans	91
Junction-enriched and -associated protein	Jeap	JEAP is a 98-kDa protein that contains a carboxy-terminal consensus motif for binding PDZ domains. It is specifically expressed in epithelia of exocrine glands	92
Ras-related protein Rab3b	Rab3b	Monomeric G protein localized to the TJ with a role in membrane and vesicle trafficking	93
Ras-related protein Rab13	Rab13	Monomeric G protein localized to the TJ with a role in membrane and vesicle trafficking	94
Vesicle-associated membrane protein	VAP-33	Monomeric G protein localized to the TJ with a role in membrane and vesicle trafficking	95
Exocyst complex component 3/4	Sec6/Sec8	Monomeric G proteins localized to the TJ with a role in membrane and vesicle trafficking	96

Many additional factors forming complexes with TJs and implicated in their regulation could not be here summarized.
MUPP-1, multi-PDZ domain protein 1; MDCK, Madin–Darby canine kidney epithelial cells.

Table 2 Structural and functional factors involved in the control of endothelial permeability: *adherens junctions*

Name	Acronym/short name	Role and molecular relationship	References
The cadherin–catenin complex			
Vascular endothelial-cadherin	VE-cadherin	VE-cadherin is a calcium-dependent protein involved in membrane permeability which binds with its cytoplasmic domain the β-catenin, plakoglobin, and catenin-p120. This protein complex interacts with receptors of VEGFR2 growth factors, FGF-R1, and TGFb-R	100,101,104
Catenin (cadherin-associated protein), beta 1	β-Catenin	β-Catenin binds α-catenin, homologous to vinculin, which binds to α-actinin. This protein is involved in the canonical Wnt signalling pathway	105
Plakoglobin	JUP	β-Catenin binds α-catenin, homologous to vinculin, which can binds to α-actinin	105
Catenin-P120	P120	Catenin-P120 is an additional VE-cadherin partner which is a Src substrate and is homologous to β-catenin and plakoglobin	106
Alfa-catenin	α-Catenin	α-Catenin can be bounded by plakoglobin and β-catenin, which is homologous to vinculin and anchors the complex to actin	71,105
Vinculin	VCL	vinculin is bounded by α-catenin (340), and may further stabilize AJ anchorage to actin	107
Alfa-actinin	α-Actinin	α-Actinin can be bounded by α-catenin and further stabilize AJ anchorage to actin	108
Rac guanosine exchange factor	Tiam	Tiam codistributes with VE-cadherin at AJ	109
Density-Enhanced Phosphatase 1	DEP-1	VE-cadherin binds VEGFR2 inducing its dephosphorylation through the action of DEP-1	110
Vascular endothelial-protein tyrosine phosphatase	VE-PTP	VE-PTP interacts with VE-cadherin modulating the cadherin/catenin complex and its phosphorylation and/or its intracellular partners. VE-PTP also associates with Tie-2, dampening the tyrosine kinase activity of this receptor that can support stabilization of endothelial junctions	111,112
Protein Tyrosine Phosphatase	PTPN11 or SHP2	SHP2 could be associated with VE-cadherin and decrease its phosphorylation, enhancing endothelial barrier function	113
Beta-arrestin 2	β-arrestin 2	The β-arrestin-dependent endocytosis of VE-cadherin is promoted by VEGF and it controls endothelial-cell permeability	114
Neural cadherin	N-cadherin	N-cadherin is a member of the classical cadherin family of transmembrane glycoproteins that mediate cell-to-cell adhesion via a homophilic binding mechanism. It's interaction with actin-based cytoskeleton, which is important for adhesion, is mediated by the catenins	114
Heart cadherin	T-cadherin	T-cadherin is a glycosyl phosphatidylinositol anchored cell-surface glycoprotein and it promotes intercellular adhesion. It's expression in the human vasculature may be relevant to control of the normal vascular architecture	87
Nectin–afadin complex			
Nectin cell adhesion molecule 1, adherens junction formation factor, SH3-DOMAIN Protein 5	Nectin, afadin, ponsin	The nectin–afadin complex is a family of cell calcium-independent adhesion molecules. The nectin binds afadin that connects the nectin to the cytoskeleton. The ponsin binds afadin and vinculin and α-catenin. Nectin and afadin are localized at the cadherins and can interact with each other through the catenin	100

Many additional factors forming complexes with TJs and implicated in their regulation, could not be summarized.
VEGFR2, vascular endothelial growth factors receptor 2; FGF-R1, fibroblast growth factor receptor 1; TGFβ-R, transforming growth factor beta receptor.

glycocalyx are profoundly reduced in patients with heterozygous familial hypercholesterolaemia, and such changes may contribute to increased vulnerability to atherosclerosis. Such perturbations are partially reversed by an even short-term statin therapy.[131]

6.2 Diabetes

Diabetic subjects have a 2- to 4-fold higher risk of cardiovascular events,[132] and cardiovascular disease causes nearly 80% of diabetes-associated deaths.[133] Mechanisms by which diabetes mellitus contributes to atherosclerosis are multiple and are still only partly understood.[134,135]

A damage to the vascular glycocalyx and the hyperglycaemia-related loss of glycocalyx function, followed by glycocalyx thinning and subsequent disease of the underlying vascular tissue, might be of particular importance in diabetes or in the metabolic syndrome and in situations of insulin resistance.[33] Systemic hyperglycaemia has been shown to cause a generalized glycocalyx thinning in human subjects,[136] probably because of the interaction of glucose with the glycoproteinaceous constituents of

the glycocalyx.[33] In type 1 diabetic patients, plasma HA, a main glycocalyx constituent, and hyaluronidase are both increased, resulting in increased synthesis and shedding of HA.[136,137] This has led to the conclusion that type 1 diabetes is characterized by a damage to the endothelial glycocalyx.[137] Moreover, hyperglycaemia attenuates the shear stress-dependent dilatation in distal pig arteries, consistent with the loss of mechanotransducing properties of the endothelial glycocalyx by hyperglycaemia.[138] All these changes might represent one of the first steps in atherogenesis in the presence of diabetes.[33]

Several *in vitro* and *in vivo* studies have demonstrated that hyperglycaemia alters endothelial functions,[139] activates PKC chronically,[140] and markedly increases vascular permeability, monocyte adhesion, the expression of cell adhesion molecules, the generation of ROS, and the activation of nuclear factor (NF)-κB.[141] High glucose *in vitro*, simulating hyperglycaemia, increases endothelial permeability of human umbilical vein ECs (HUVECs) by activating the RhoA-Rho Associated Coiled-Coil Containing Protein Kinase (ROCK) signalling pathway,[142] a crucial set of mediators of endothelial barrier function.[143] Treatment of ECs with high glucose also leads to tyrosine phosphorylation of VE-cadherin, with a consequent dissociation from β-catenin, barrier disruption, and increased trans-endothelial migration of monocytes.[139]

Vascular endothelial growth factor (VEGF) is regarded as the 'master regulator of angiogenesis'.[144] It typically induces migration and proliferation of ECs, modulates thrombogenicity and, most of all, enhances vascular permeability[145] through the activation of VEGF receptor (VEGFR)-2 and the following calcium influx, activation of phospholipase C (PLC) and of NO synthase, all converging in the guanylyl cyclase-mediated activation of the Rho–Ras-Related C3 Botulinum Toxin Substrate (Rac) pathway and in the subsequent functional alteration of the junctional proteins cadherins, ZOs and occludins linked to the actin cytoskeleton.[145] In the arteries of diabetic patients with atherosclerosis, VEGF is overexpressed, and treatment of endothelial monolayers with VEGF downregulates ZO-1 expression, thus favouring the formation of intercellular gaps and the increase in permeability to LDL.[146] Exposure to high glucose concentrations has also been associated with increased expression of toll-like receptor (TLR)2 and 4 in ECs. It is likely that such activation contributes to increase endothelial permeability because of the downregulation of junctional proteins through an extracellular signal-regulated kinase (ERK) 1/2 dependent mechanism.[147]

6.3 Hypertension

Arterial hypertension is the most prevalent risk factor associated with increased cardiovascular morbidity and mortality.[148,149] Hypertension accelerates the development, progression and complication of atherosclerosis, through mechanisms involving endothelial dysfunction, vascular oxidative stress, inflammation and remodelling.[150,151] Hypertension is primarily characterized by morphological changes in the arterial endothelium and by hypertrophy of the smooth muscle layer in the tunica media.[152] Many studies on hypertensive animals have reported increased transendothelial LDL permeability in arteries, and indicated that hypertension may lead to enhanced LDL entry into the intima by altering the permeability of the endothelium rather than by increasing the filtration rate.[153,154] Mechanisms for the enhancement of endothelial permeability under hypertension in the rat aorta might consist of increased EC mitosis and apoptosis, and the associated transient formation of leaky junctions, which increase endothelial permeability to macromolecules.[154] A quantitative model revealed important details about the influence of hypertension on LDL transport and its accumulation in the subendothelial space: LDL fluxes across the leaky junction, the intima, the IEL, and the media are all highly affected by the transmural pressure, which, in turns, affects EC turnover and compaction of the intima.[155] Another mathematical model has shown that a seriously damaged glycocalyx augments the flux of plasma solvent and solutes, and both fluxes are further increased in the presence of hypertension.[156] Furthermore, angiotensin II, the principal effector molecule of the renin angiotensin system (RAS), the activation of which is a key regulator of blood pressure and cardiovascular function, has been recently shown to increase LDL transcytosis across ECs and accelerate LDL retention in the subendothelial space of human umbilical venous walls. Mechanistically, proteins involved in caveolae-mediated transcytosis, including LDLR, Cav-1, and cavin-1, were found to be tightly associated with angiotensin II-induced LDL transcytosis across ECs.[157]

The local chemically-active interstitial concentration of a particular atherogenic molecule is a fundamental driving force in a system of reactions that produces local atherogenic changes. Such concentration of specific molecules was used as a measure of the potential for lesion development—an expression of local 'risk'—in another model whereby transport processes interact with a tissue barrier. Such model explained that sites along the arterial tree that were characterized by 'mild' increases in endothelial permeability to macromolecules make the arterial intima particularly prone to increased accumulation of larger macromolecules, including LDL, thus developing high chemical activities. These sites were at high risk even in the absence of other risk factors, and this risk was dramatically increased with hypertension and/or elevated serum concentration of atherogenic soluble factors and/or pre-existing intimal thickening.[19]

A further possible mechanism explaining changes in endothelial permeability caused by hypertension might involve the role of calcium. Indeed, a mathematical model has been developed, predicting a sigmoidal dependence of calcium influx from shear stress.[158] In cultured ECs, it has been shown that these changes in cytosolic calcium concentration might lead to calcium-dependent activation of myosin light chain (MLC) kinase, as well as RhoA/Rho kinase-dependent inhibition of the myosin phosphatase facilitating MLC phosphorylation, resulting in contraction of the cells and, finally, in endothelial barrier disruption.[143]

6.4 Obesity

The imbalance in energy homeostasis resulting in increased adipose tissue mass favours the initiation of cardiovascular and other diseases.[159] This occurs because the adipose tissue acts as an endocrine organ, secreting molecules with profound local and systemic effects,[160] including increased endothelial permeability.[161] Abdominal obesity results in the enhanced expression of systemic circulating proinflammatory cytokines and growth factors, including, but not limited to, tumour necrosis factor (TNF)-α, interleukin (IL)-6, resistin, leptin, VEGF, and free fatty acids (FFA), which predispose to the onset of endothelial dysfunction(s).[162] Obesity also results in the reduced expression of an anti-inflammatory cytokine, adiponectin,[163] which normally prevents endothelial dysfunction(s).[164]

TNFα was among the first cytokines recognized to be expressed by the adipose tissue.[165] Its levels correlate with the degree of adiposity and the associated insulin resistance,[166] as well as with the risk of coronary artery disease.[167] Many experimental data have accumulated regarding how TNFα contributes to boosting atherogenesis (for an exhaustive review see[168]). However, its regulatory effect on endothelial permeability is a quite novel acquisition. In addition to stimulating proinflammatory canonical pathways leading to deep changes in vascular cell gene expressions,[168] TNFα activates the Rho A/ROCK axis. ROCK phosphorylates and inhibits MLC phosphatase, which promotes MLC phosphorylation, which in turn triggers the acto-myosin contraction,[169] thus changing the

endothelium from a pavement-like monolayer, with ECs containing a belt of peripheral actin, into a monolayer formed by elongated cells enriched in actin stress fibres.[170]

More recently TNFα was also demonstrated to increase the transcytosis of LDL across the undamaged endothelial barrier, and promote LDL retention in the subendothelial space through a mechanism involving the activation of NF-κB and of peroxisome proliferator activated receptor (PPAR)γ, which, upon a co-ordinated activation by TNFα, in concert increase the gene expression of Cav-1 and -2 and of the LDLR.[26]

Although the binding of TNFα to its membrane receptor activates many intracellular signalling pathways,[168] the activation of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex and the following signalling sequelae leading to the oxidative modification of junctional proteins are considered the pathway most impacting the endothelial barrier properties.[171]

In more recent times, also the adipocyte-secreted cytokine resistin has been linked to obesity, insulin resistance, and atherosclerosis[172] and has been specifically shown to increase endothelial permeability in cardiovascular patients.[161] Mechanistic *in vitro* data suggest for this cytokine the ability to downregulate the endothelial expression of the junction proteins ZO-1 and occluding, through the stimulation of a signalling pathway enhancing the production of ROS and the activation of the mitogen activated protein kinases (MAPK) p38.[173] Interestingly adiponectin suppresses multiple cellular effects known to be associated with TNFα-mediated endothelial hyperpermeability including actin stress fibre development, intercellular gap formation, and tubulin disassembly, through the activation of PKA and the increase of cAMP,[174] known downregulators of RhoA GTPases activity.[175]

Other direct or indirect links between adipokines and cell junctional proteins that could alter endothelial permeability are plausible, and should be further investigated as well. Adiponectin, for example, is an important mediator of vascular disease, as it activates AMP-activated protein kinase and the subsequent increases in NO production in ECs. Because of its markedly reduced production in obesity, adiponectin can be responsible of the loss of the NO-mediated protection *in vitro* and *in vivo*.[176]

The body mass index and visceral fat accumulation in human subjects associates, among others, with elevated serum levels of VEGF.[177] Although all human adipose depots may express VEGF[178] the omentum was shown to express the highest levels,[178] to the point that it has been hypothesized that the omental production of VEGF mostly determines serum levels of VEGF in obese patients.[179] In addition and possible synergism with other adipokines, including resistin and leptin,[180] VEGF contributes to increase endothelial permeability again by upregulating the intracellular production of ROS.[180] However, it is also interesting to report that VEGF may mediate LDL transport across the endothelial barrier through an 'uncanonical signalling pathway', as recently demonstrated.[181] It was indeed observed that LDL, by binding to LDLR, induce the autophosphorylation of VEGFR1 and its internalization together with both LDL and LDLR,[181] thus providing an additional mechanistic route for LDL transport.[182]

An alternative pathway for the destabilization of endothelial permeability is also effected by saturated FFA such as palmitate.[183] Plasma levels of saturated FFA are often elevated in obese patients[184] and are considered critical contributors for obesity-induced pathological conditions, including diabetes and accelerated atherosclerosis. It has been recently shown that palmitate affects endothelial barrier functionality by activating the Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat And Pyrin Domain Containing (Nlrp)3 inflammasome complex[183] through a mechanism involving the increased release of mitochondrial ROS, leading to reduced expression of the inter-endothelial junction proteins ZO-1 and -2.[183]

Overall, these data provide important mechanistic links between the adipose tissue secretome and impairment of endothelial barrier permeability.

6.5 Smoking

Cigarette smoking has been strongly associated with subclinical atherosclerosis in multiple vascular beds.[185] Smoking injures the blood vessel wall by damaging ECs, thus potentially increasing permeability to lipids and other blood components,[186] and affects all phases of atherosclerosis, from endothelial dysfunction(s) to acute occlusive clinical events.[187]

It has been reported that tobacco smoke cooperates with IL-1β to alter β-catenin trafficking in the vascular endothelium, eventually increasing permeability and inducing the expression of cyclooxygenase-2 *in vitro* and *in vivo* through a mechanism involving the overproduction of intracellular ROS.[187] It has indeed been shown that the unsaturated aldehydes acrolein and crotonaldehyde contained in the gas phase of cigarette smoke are able to activate the endothelial activity of NADPH oxidase and the subsequent production of superoxide anion (O_2^-).[188] More recently, it has been shown that tobacco smoke interacts with IL-1β determining suppression in the activity of *protein deleted on chromosome 10 phosphatase and tensin homolog* (PTEN), leading to increased VE-cadherin and β-catenin phosphotyrosine and to a disassembly of VE-cadherin/β-catenin membrane complexes. These events lead to the accumulation of β-catenin within the nucleus.[189] Moreover, cigarette smoke contains metals that catalyse the oxidation of cellular proteins, causing a loss of microtubule function in turn culminating in microtubule depolymerization, the proteasome-dependent degradation of cytoskeletal α-tubulin, and eventually a contraction of vascular ECs and endothelial leakiness.[190]

The 'cell turnover-leaky junction' theory states that dying or dead ECs enhance endothelial permeability and provide a site for the localization of atherosclerosis.[35] In fact, the long-term exposure to nicotine increases aortic EC death and promotes the transendothelial transport of macromolecules in rats.[191] These findings are consistent with the observation that, although open junctions occupy less than 10^{-5}% of the *en face* area of the endothelium, endothelial permeability in larger arteries can increase by 50–100% due to the experimentally observed regional variations in cell turnover.[36]

6.6 Haemodynamic forces

Although atherosclerosis is associated with systemic risk factors, it is a focal disease preferentially developing in predisposed, athero-prone regions,[192] such as in the vicinity of branch points, the outer wall of bifurcations, and the inner wall of curvatures, generally characterized by low, disturbed, or oscillating blood flow. Local factors, such as haemodynamic forces, indeed play a major role in the regional localization of atherosclerosis,[193] being able of modulating, through complex mechanoreception and mechanotransduction processes, endothelial functions and phenotype.[194] These haemodynamic forces include flow-generated endothelial shear stress—a tangential frictional force, which plays the most fundamental role in atherosclerosis—and blood pressure-derived circumferential tensile/cyclic stress, acting perpendicularly to the vessel wall. The current consensus is that physiologic laminar flow and high shear stress (>15 dyn/cm^2) suppress several endothelial proatherogenic genes and favour the expression of atheroprotective genes and bioactive products, including vasodilators (NO and prostacyclin) and antithrombotic agents

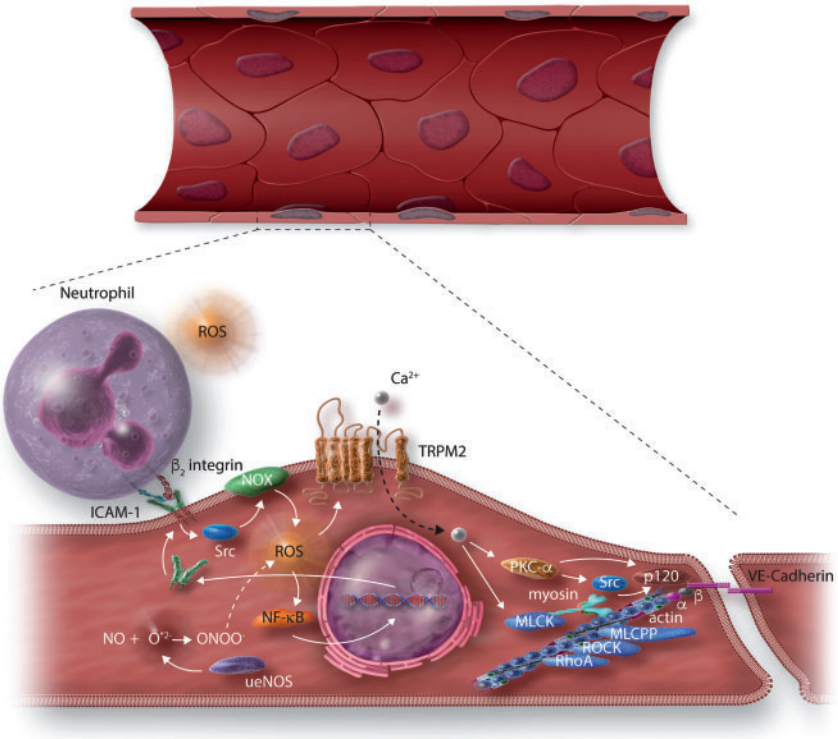

Figure 4 Increased oxidative stress as the final common pathway of the effects of cardiovascular risk factors, and its effects on the endothelial permeability in endothelial cells. ROS stimulate a NF-κB-dependent upregulation of intercellular adhesion molecule (ICAM)-1 expression. ICAM-1 binds the β_2 integrins of neutrophils, inducing activation of endothelial NADPH oxidase (NOX) via Src, and enhancing ROS production.[218] ROS release from neutrophils and NOX activates the redox sensitive Ca^{2+} permeability *transient receptor potential cation channel subfamily M member 2* (TRPM2), leading to Ca^{2+} entry into endothelial cells (ECs) and promoting PKC-α activation, which, in turn, may regulate EC permeability via the phosphorylation of p120 and its dissociation from VE-cadherin,[219] and the activation of Src,[220] leading to tyrosine phosphorylation of VE-cadherin and β-catenin, eventually resulting in AJ destabilization.[218] Ca^{2+} entry (bound to calmodulin) activates MLC kinase, which phosphorylates MLC, mechanism through which myosin starts to move along the actin fibres[221] and eliciting a co-ordinated spatial activation of RhoA and a global reorganization of the actin cytoskeleton, resulting in endothelial barrier dysfunction.[102] RhoA, via activation of Rho kinase (ROCK), inhibits MLC phosphatase, making the effect of MLC kinase activity longer lasting.[222] A loss of endothelial nitric oxide (NO) bioavailability is caused by its reaction with $O_2^{\bullet-}$, produced by uncoupled endothelial nitric oxide synthase (eNOS) (ueNOS), yielding $ONOO^-$production, which contributes to vary the cellular redox state.[223]

(thrombomodulin). Conversely, disturbed flow and low shear stress (<4 dyn/cm^2), occurring at arterial branching points and curvatures, stimulate EC turnover and apoptosis, and the expression of genes and gene products that promote atherogenesis, including cytokines and growth factors, adhesion molecules, ROS, and prothrombotic factors.[195–197]

An important underlying mechanism for the effects of local haemodynamic forces on atherogenesis is through the flow pattern-mediated regulation of endothelial permeability. Atheroprone areas, where blood flow is disturbed, feature increased permeability to macromolecules, including fluorescent, radiolabelled and chromogenic tracer-labelled particles, such as albumin and LDL. In porcine iliac arteries *in vivo*, sites exposed to low wall shear stress were found more likely to exhibit elevated permeability to albumin.[198] Besides inducing SREBPs-dependent LDL uptake and synthesis, disturbed flow and low shear stress increase endothelial permeability to LDL, as demonstrated in different areas of the vasculature for both rabbits and pigs, as well as in a computational model of LDL transport in human coronary arteries.[199,200] Conversely, laminar flow and high shear stress appear to limit endothelial permeability to LDL.[201]

In addition to the influence on glycocalix turnover discussed above, altered expression and discontinuous distribution of intercellular junction proteins, such as VE-cadherin,[202] and induction of VEGF[203] in ECs by disturbed flow and low shear stress provide possible molecular mechanisms for the increased endothelial permeability in regions of disturbed flow or low shear stress. The pronounced EC mitosis and apoptosis demonstrated both in the rabbit thoracic aorta *in vivo* and in cultured ECs,[204,205] as well as the morphological changes of ECs from elongated to more rounded shape associated with low shear stress compared with high shear stress, might also be responsible for the greater permeability of ECs in segments exposed to disturbed flow.[204,206,207] In these regions, the increased residence time of LDL due to flow stagnation also contributes to the infiltration LDL across the arterial wall.[208]

Another emerging mechanism related to local differences in shear stress profiles with impact on endothelial permeability is the generation of mesenchymal cells from the endothelium, known as endothelial-mesenchymal transition (EndoMT). This transition process, during which ECs can achieve a fibro-proliferative phenotype, is characterized on the one hand by the loss of EC markers, including VE-cadherin, PECAM-1;

and by the increased expression of mesenchymal cell markers, including α-smooth muscle actin (α-SMA) and vimentin, on the other. This is accompanied by a loss of cell–cell adhesions and cell polarity, with consequent damage of endothelial junction stability and increased vascular permeability. Several lines of evidence indicate that EndoMT contributes to atherosclerotic pathobiology, from its inception to plaque destabilization,[209] through mechanisms including endothelial barrier dysfunction. Pulmonary artery EC monolayers undergoing endoMT indeed failed to form integral biological barriers and featured enhanced leakage.[210] The presence of ECs with mesenchymal characteristics has also been identified as overlying human and animal atherosclerotic plaques[211,212] in relation to atherosclerosis severity. EndoMT in atherosclerosis may be driven by inflammatory stimuli, including an imbalance in transforming growth factor (TGF)-β and fibroblast growth factor receptor 1 (FGFR1) signalling.[211] Furthermore, compared with LDL, ox-LDL increased radiation-induced EndoMT in human aortic ECs and in atherosclerotic tissues of irradiated ApoE$^{-/-}$ mice.[213] Accordingly, EndoMT was induced by ox-LDL-induced foam cells via the CCL-4/CCR5/TGF-β axis.[214] EndoMT is also sensitive to haemodynamic forces, as demonstrated both in cultured ECs exposed to flow and in animal models with varying levels of flow. Disturbed flow promotes EndoMT via the GATA4-TWIST1 signalling and the transcription factor Snail,[215] while laminar uniform shear stress prevents EndoMT via activation of MEK5/ERK5 signalling.[212] This shear stress-modulating effect on EndoMT might therefore contribute to explain the focal nature of atherosclerosis.

7. Oxidative stress: a common feature of atherogenic stimuli affecting endothelial permeability

Increased ROS production and dysregulated redox balance, also known as 'oxidative stress', contribute to many of the molecular events underlying atherogenesis and, correspondently, to the loss of endothelial barrier function.

Low levels of ROS, derived from various enzymatic sources, are the product of the normal cellular metabolism. In the vascular endothelium, sources of ROS include the xanthine oxido-reductase system, the mitochondrial respiratory chain, and the NADPH oxidase complex.[216]

Originally NADPH oxidase was considered an exclusive expression of phagocytic cells. It is now evident that a homologous of phagocytic NADPH oxidases is also functionally active in the vascular endothelium. The prototypical gp91phox-containing phagocytic NADPH oxidase is in the endothelium termed Nox2, and comprises 5 subunits: p47phox p67phox, p40phox, p22phox, and the catalytic subunit gp91phox.[217] In basal conditions, p47phox, p67phox, and p40phox are in the cytosol, while p22phox and gp91phox are in the membrane in a heterodimeric flavoprotein conformation known as cytochrome b558. Upon proinflammatory and proatherogenic conditions, p47phox and p67phox, supported by the activation of the small Rho GTPase Rac-1, forms a complex that translocate to the plasma membrane, where it associates with cytochrome b558 constituting the active enzymatic complex able to transfers electrons to O_2, thus producing both superoxide anion (O_2^-) and hydrogen peroxide[217] (*Figure 4*).

As revised above, despite differences existing in upstream signalling among the various proatherogenic risk factors, most of them share the ability to activate NADPH oxidase and increase the production of ROS as a common denominator, directly linking such factors to endothelial injury in terms of altered endothelial permeability.

Mechanistically, ROS regulate vascular permeability by directly attacking cellular cytosolic and membrane components, such as increasing the tyrosine phosphorylation of VE-cadherin,[224] which prevents the association of β-catenin and p120-catenin and results in the inhibition of endothelial barrier function.[225] On the other hand, ROS may also contribute to increased endothelial permeability by inducing the activation of NF-κB. It has been shown that the activation of NF-κB alters the distribution of vascular junctional proteins ZO-1 and -2, and of occludins, thus increasing endothelial permeability independently from the induction of proinflammatory gene expression.[226]

Moreover, enhanced NO degradation by ROS has been found in animals[227–229] and humans[230–232] in the presence of hypertension, diabetes, cigarette smoking, and heart failure. Among ROS, superoxide ($O_2^{\bullet-}$) can be produced by endothelial NO synthase (eNOS), under conditions of substrate or cofactor deficiency (referred to as an 'uncoupled' state).[233] Because $O_2^{\bullet-}$ and NO* are both radicals and contain unpaired electrons in their outer orbitals, they undergo a radical–radical reaction, leading to the formation of peroxynitrite ($ONOO^-$), which can be protonated to peroxynitrous acid—the cleavage products of which are among the most abundant ROS in biological systems[223] and contribute to alter the cellular redox state. As tyrosine phosphatases contain a reactive cysteine group in their active centre, oxidant species can shift the balance between tyrosine kinases and phosphatases,[234] and hence influence VE-cadherin phosphorylation and function (*Figure 4*).

Oxidative stress also affects the abundance and function of junctional molecules: in cultured ECs, hydrogen peroxide promotes a reduced expression of cadherins and occludin in intercellular junctions, suggesting a destabilizing role of ROS-mediated signalling and oxidative stress on vascular integrity[235,236] (*Figure 4*).

Oxygen-derived free radicals also mediate the disruption of the glycocalyx surface layer and increase vascular wall adhesiveness by ox-LDL, as shown in the hamster cremaster muscle preparation.[49] In the same model, ox-LDL decrease the effective glycocalyx dimensions by removal of proteoglycans or adsorbed proteins from the endothelial surface; and increase capillary volume accessible to red blood cells in the absence of changes in anatomic capillary diameter.[237] These effects may be mediated by the action of ox-LDL on NO bioavailability, which further disturbs the balance between oxygen radical production and NO at the endothelial surface, as demonstrated by the protective effect of the administration of superoxide dismutase and catalase[237].

The endothelial redox state is also orchestrated by different flow patterns and shear stresses. While several papers in the literature report increased and sustained endothelial NADPH oxidase activation and ROS production under conditions of disturbed flow and low shear stress, steady laminar shear stress is commonly associated with an induction of antioxidant defences (superoxide dismutase, haeme oxygenase-1, NF erythroid 2-like 2, etc.) and a suppression of ROS generation.[238–240] The modulation of ROS production by mechanical forces may mediate their downstream effects on the vasculature. Accordingly, the 'physiologic' shear stress-mediated suppression of the cellular redox stress may contribute in part to the attenuation of endothelial barrier dysfunction, as demonstrated in human microvascular ECs challenged by proinflammatory cytokines.[241]

Recently, it has been shown that ROS are induced by low shear stress via the angiotensin II type 1 receptor/eNOS/NO pathway,[242,243] and their effects are suppressed by the bradycardic agent ivabradine in cultured HUVECs.[244] Cyclic strain[245] or endothelin-1-induced ROS in animal cultured ECs,[246] modulated gene expression in HUVECs. These

findings highlight that the relationship between the micromechanical environment and the development of atherosclerosis[242,247] are likely due to a direct influence of haemodynamic forces on EC morphology, metabolism, and inflammatory phenotype through various signal transduction mechanisms altering specific expressions, with ROS as a likely common downstream effector,[248] In micro- and macrovascular ECs, ROS have been implicated in the EndoMT induced by inflammatory stimuli, paving the ground for more detailed studies aimed at identifying the relationship between mechanical endothelial ROS production, EndoMT and atherosclerosis.[209–213]

8. Conclusions and clinical perspectives

On the basis of the evidence summarized above, atherosclerosis may indeed be promoted, at its early stages, by changes in the transendothelial permeability to LDL. The 'response to retention hypothesis' model states that a subendothelial retention of lipoproteins is in fact an early step in atherogenesis. The practical consequence of such a reconstruction of pathogenetic events is therefore that a primary therapeutic focus might be to prevent the entry and subsequent subendothelial retention of LDL.[249] This strategy would be complementary to the much more widespread current approaches aimed at reducing LDL since, as discussed above, all the main recognized modifiable atherosclerosis risk factors have the ability to alter endothelial permeability to these molecules.

Great breakthroughs have been made in our understanding of signalling mechanisms and mediators regulating endothelial permeability in normal and diseased states, as well as in elucidating the dynamic interactions between EC barrier dysfunction and atherosclerotic risk factors. These results on the one hand can help the development of new methods to distinguish dysfunctional or activated ECs from healthy or quiescent ECs; and, on the other hand, they can foster the development of novel therapeutic targets and drugs against atherosclerosis.

The *in vivo* assessment of endothelial barrier function/dysfunction through molecular imaging or monitoring circulating components of the pertinent regulating systems may hold promise to discover biomarkers early detecting and/or monitoring the progression of atherosclerotic vascular disease. Appropriate criteria for the appraisal of these potential biomarkers need to be fulfilled, including reproducible measurement, as well as early—and specific—detection of otherwise subclinical vascular disease. Monitoring *in vivo* endothelial permeability through molecular imaging of nanoparticle deposition within plaque has been recently demonstrated to specifically identify atheromas susceptible to thrombosis in an experimental atherothrombosis model in rabbits, as well as in human coronary artery atheroma *in vivo*.[250]

Since glycocalyx degradation is strongly correlated with vascular disease progression, a clinical monitoring of glycocalyx integrity/thickness, as well as the assessment of the glycocalyx fragments, such as syndecan-1 and/or HA, are being examined as reliable diagnostic or prognostic indicators of vascular endothelial damage in various pathological conditions,[251] including atherosclerosis. Circulating components of the endothelial glycocalyx have been demonstrated in humans undergoing vascular surgery associated with ischaemia/reperfusion injury, and are proposed as sensitive markers of early EC distress.[252]

The direct targeting of endothelial hyperpermeability can be considered as a promising approach to prevent vascular damage during inflammation and atherogenesis. Pharmacological interventions to prevent glycocalyx degradation have been considered, including the use of HA to repair the glycocalyx, although further studies are here certainly needed. Hydroxyethyl starch has been reported to prevent capillary leakage in the early stages of the acute respiratory distress syndrome,[253] either by acting on endothelial surface layer pores caused by glycocalyx degradation,[254] or by specific interaction with the glycocalyx.[255] RhoA, through its downstream kinase ROCK, plays a central role in the loss of endothelial barrier integrity. Therefore fasudil, a derivative of isoquinoline, has been proposed as a safe and clinically approved inhibitor of ROCK,[256] and—due to its acceptable safety profile[257,258]—may be a candidate for reversing endothelial barrier dysfunction, although in some animal studies it also appears to increase blood flow-mediated vasodilation, with demonstrated therapeutic benefits in the treatment of hypertension.[259,260]

Previous studies have shown that platelet-endothelial cell adhesion molecule-1 (PECAM-1) plays a key role in maintaining EC junctional integrity (for a review see[261]). Thus, antibody-driven affinity modulation of PECAM-1 has been proposed as a strategy to regulate EC barrier function, suggesting a novel approach for controlling EC migration and changes in barrier function in a variety of vascular permeability disorders.[262]

The extravasation of 125I-LDL through rabbit aortic ECs was significantly increased by VEGF and decreased by salvianolic acid B (SalB): VEGF here reduced TJ-associated proteins occludin and claudin-5, and increased expression of caveolar structural proteins CAV-1 and -2, an effect abolished by SalB.

Incubation of bovine coronary ECs with VEGF significantly increased their permeability to ^{164}I-ox-LDL, an effect significantly inhibited by extract of Ginkgo biloba leaves, suggesting that such extracts may have clinical applications in the treatment of vascular disease.[263,264] Cryptotanshinone, a major compound derived from the Chinese herb *Salvia miltiorrhiza*, also attenuates the increased endothelial permeability, likely due to the restoration of NO bioavailability in ECs.[265]

Despite all these many attempts at exploiting current knowledge to design and develop new pharmacological compounds active on the pathways described above, important gaps of knowledge still remain, and need to be filled to best develop endothelial barrier-targeted treatments for atherosclerosis. First, the improvements in endothelial barrier function demonstrated in experimental models need to translate into clinically meaningful anti-atherogenic effects in humans. In this context, a major challenge is to conduct interdisciplinary research that combines bioengineering, physiology, and clinical medicine; and possibly using a systems biology approach, integrating information at the molecular, cellular, and organ levels. Second, as in the case of the inhibition of key players of endothelial permeability such as ROCK or VEGF, the ability of a therapeutic to prevent EC barrier dysfunction without interfering with basic barrier formation and functions in response to physiologic stimuli should be specifically shown. Third, although ROS overproduction is recognized as a key common switch in EC hyperpermeability and cardiovascular disease, it seems extremely difficult to finely and specifically modulate pathological ROS overproduction while leaving the physiological role of ROS as second messengers unaffected.[266] Fourth, although novel potential therapeutic targets are increasingly recognized, including EndoMT reversal, the hypothesis to treat atherosclerosis via these targets should be addressed in future and eventually human studies.

Therefore, information related to mechanisms that promote changes in endothelial permeability holds the promise to lead to new diagnostic markers, and the development of new therapeutic strategies—including drugs—targeting such processes. More research on such strategies is therefore certainly warranted.

Conflict of interest: none declared.

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