Trends in Neurosciences

CellPress OPEN ACCESS

Review

A Vessel for Change: Endothelial Dysfunction in Cerebral Small Vessel Disease

Sophie Quick,[1,3] Jonathan Moss,[1,3] Rikesh M. Rajani,[2] and Anna Williams[1,*]

The blood vessels of the brain are lined with endothelial cells and it has been long known that these help to regulate blood flow to the brain. However, there is increasing evidence that these cells also interact with the surrounding brain tissue. These interactions change when the endothelial cells become dysfunctional and have an impact in diseases such as cerebral small vessel disease, the leading cause of vascular dementia. In this review, we focus on what endothelial dysfunction is, what causes it, how it leads to surrounding brain pathology, how researchers can investigate it with current models, and where this might lead in the future for dementia therapies.

Highlights

Endothelial cells lining the brain's blood vessels have been under-appreciated as players in brain diseases: they not only control blood flow and blood–brain barrier function, but also, their cellular interactions influence surrounding brain tissue.

Cerebral small vessel disease (SVD) is a common form of vascular dementia, where dysfunctional endothelial cells lead to surrounding white matter changes, an early feature of the disease, which associates with clinical severity.

We describe preclinical models reflecting genetic and/or environmental influences of SVD and summarise the links found between endothelial dysfunction and white matter changes, with suggestions to improve choice and use of these models.

How Do Endothelial Cells (ECs) Affect the Brain?

ECs form the luminal surface of every blood vessel in the body, but are not simply the inner lining of these tubes. They have different characteristics depending on the organ the blood vessels serve and they play an active role in maintaining the health and function of the vasculature and tissue. Brain ECs maintain the integrity of the blood–brain barrier (BBB) and regulate transport across it, but are also functionally active in controlling processes such as blood clotting and matching blood flow to neural activity (neurovascular coupling). Importantly, they also interact with brain cells on their abluminal side. When these cells are compromised, it can lead to widespread consequences for the brain, causing and exacerbating diseases such as cerebral small vessel disease (SVD).

SVD is a heterogeneous disease, which has been proposed to be divided into different subtypes on the basis of aetiology and pathology [1] (Box 1). In this review, we have chosen to confine ourselves to the arteriolosclerotic type and genetic forms of the same, but for brevity, will simply refer to this as SVD. It is within this grouping that EC dysfunction has recently been implicated in the mechanism of parenchymal change through cellular interactions and secretions, in addition to the critical role of ECs in neurovascular coupling and BBB function (Box 2). Here, we shift the focus to the direct impact of EC dysfunction on white matter (WM) change via cellular interactions. We describe the signature of endothelial dysfunction at the molecular level, animal models of SVD that exhibit these hallmarks, and the methods best suited to probe these features, to demonstrate how researchers might find ways to limit or prevent its effects in SVD.

What Is Cerebral SVD?

Understanding the pathogenesis of SVD is important as SVD is the leading cause of vascular dementia, triples the risk of stroke, and frequently compounds Alzheimer's disease [2]. SVD can lead to cognitive decline, particularly affecting executive function early (rather than memory such as in Alzheimer's disease), but can also manifest as abnormal gait, depression, or be subclinical and incidentally detected on brain magnetic resonance imaging (MRI). Furthermore, symptoms are often poorly recognised and can be mistaken as features of the poorly defined 'normal ageing process'. Diagnosis of SVD in this context is made with MRI, showing **WM hyperintensities** (see Glossary), **microbleeds**, and **small subcortical infarcts**, which are

[1]Centre for Regenerative Medicine, Institute for Regeneration and Repair, The University of Edinburgh, Edinburgh EH16 4UU, UK
[2]UK Dementia Research Institute at UCL, University College London, London, UK
[3]These authors are equal first authors

*Correspondence: anna.williams@ed.ac.uk (A. Williams).

Box 1. SVD Heterogeneity

SVD is a highly heterogeneous disease that can be subcategorised by different pathology and aetiology. Pantoni, in 2010 [1], proposed grouping SVD into six subtypes:

(i) Arteriolosclerosis, also known as age-related or vascular risk factor-related small vessel disease. This form is common and systemic, associated with ageing, diabetes, and hypertension. In this form, the vessels lose smooth muscle cells, but have thicker, stiffer walls, with increased hyaline deposits and narrowed lumens.
(ii) Cerebral amyloid angiopathy (CAA), which is characterised by accumulation of the immunoreactive protein β-amyloid in the walls of arteries and arterioles. In severe cases, this leads to vessel disruption and leakage from the circulation into the parenchyma. This specific vessel pathology can be sporadic or hereditary, is also seen in Alzheimer's disease and Down's syndrome, and increases in frequency with age in the general population. These first two forms are the most common overall.
(iii) Inherited or genetic forms, distinct from CAA, of which cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and Fabry's disease are the most common.
(iv) Vasculitides, all characterised by the presence of inflammatory cells in the vessel walls. These rare forms are typically systemic and their pathology is inflammatory and immunologically mediated.
(v) Venous collagenosis, where veins and venules close to lateral ventricles have increased wall thickness, mainly due to excess collagen, resulting in a narrowed lumen and sometimes occlusion.
(vi) Other SVDs, including postradiation angiopathy, where hyaline deposition leads to thickening of vessel walls after irradiation therapy, with resultant degeneration of myelin sheaths.

thoroughly described elsewhere [3]. Risk factors associated with SVD include ageing, hypertension, smoking, diabetes, and hypercholesterolaemia, similar to the cardiovascular risk factors associated with large vessel strokes and heart disease [4], however, the pathology is different. Pathologically the small perforating arterioles of the brain (around 40–200 μm in diameter [5]) are thickened and stiff (arteriolosclerosis), with a reduced lumen size but not containing clots or occlusions. Compromised BBB integrity is speculated to lead to both microbleeds and reduced blood flow distally, leading to ischemia and small infarcts. These microinfarcts can be subcortical or cortical and are clearly different from atherosclerotic/embolic strokes characteristic of large vessel disease [6]. These ischaemic changes may contribute to the loss of **oligodendrocytes** and thinner **myelin** sheaths found in SVD [7,8], which are thought to reflect the WM hyperintensities seen by MRI. However, recently, EC dysfunction has been shown to be key in the pathogenesis of SVD, occurring prior to BBB breakdown [8], and features of EC dysfunction appear as a common theme underlying pathological changes in genetic and sporadic SVD. However, what defines this state and how does this affect the brain parenchyma?

What Is EC Dysfunction?

Endothelial Function, Dysfunction, and Activation

Normally functioning ECs lining vessels provide a separation between tissue and blood, allowing small molecules through, regulating blood flow, and responding to signals involved in inflammatory processes. ECs of the brain vasculature have additional properties with more stringent barrier functions: the BBB. These specialised ECs form tight junction (TJ) complexes between each other as part of a protective barrier for the brain parenchyma. They also have specific transporter proteins to allow for selective movement of certain molecules across this barrier and into the brain tissue or into adjacent cells.

Dysfunctional ECs lose these properties and gain others, leading to consequences for the vessels themselves and for the organ that they serve. Endothelial dysfunction is usually defined in relation to endothelium-derived nitric oxide (NO), which promotes blood vessel dilation, with dysfunctional ECs synthesising and releasing less NO [9], but other additional markers have also been described. The terms endothelial dysfunction and vascular dysfunction are often used interchangeably, as NO regulates local vascular responses as well as ECs, but the two are distinct and do not always coexist (e.g., vascular dysfunction can be caused by EC-independent

Glossary

Laser speckle contrast imaging: technique used to visualise perfusion in live tissue through the dynamic changes in the interference patterns of light as a result of interaction between red blood cells and the illumination, which is backscattered onto a detector.
Matrix metalloproteinases (MMPs): a large subfamily of proteins that have many substrates and can break down elements of the extracellular matrix. They have roles in normal biological functions, including wound healing and angiogenesis.
Microbleeds: small intracerebral haemorrhages, associated with small vessel disease, identifiable on MRI T2* scans as a round, 2–10-mm sized area of no signal.
Myelin: concentric layers of membrane from an oligodendrocyte (in the central nervous system) around axons to form myelin sheaths separated by nodes of Ranvier, allowing saltatory conduction of nerve impulses and metabolic support to the axon. Most myelin is in the white matter of the brain and is damaged in SVD.
Oligodendrocytes: glial cells of the central nervous system that can extend processes and wrap multiple membranes around axons to form a myelin sheath, important for saltatory conduction of nerve impulses and metabolic support to the axon.
Small subcortical infarcts: loss of blood supply in the territory of one perforating arteriole, identifiable on MRI scan as a lesion less than 20 mm at its largest diameter.
Transendothelial electrical resistance (TEER): ohmic resistance measured between two electrodes on either side of a monolayer of cells seeded on a semipermeable membrane.
WM hyperintensities: lesion in white matter, associated with small vessel disease, identifiable as an area of high intensity on MRI T2 scans, usually nonsymmetrical.

mechanisms rooted in pericytes or smooth muscle cells). Endothelial dysfunction is thought to be distinct from, but linked to, endothelial activation [10], which is defined by the increased expression of adhesion molecules such as VCAM-1, ICAM-I, and E-selectin, typically occurring in response to inflammatory stimuli [11]. In this review, we will focus on and use the term endothelial dysfunction to describe the chronic, less inflammatory changes seen in SVD. It is also worth noting the recently used term 'endothelial-BBB-dysfunction', which describes a similar group/module of transcriptional changes in ECs from areas of BBB disruption in mouse models of neurological diseases, including large vessel stroke [12].

In the context of SVD, identifying the signature of EC dysfunction will help understand the role of ECs in its mechanism and may also lead to identification of biomarkers in patients. The exact order of events that leads an EC from normal function to dysfunction is unclear and may vary

Box 2. The Neurovascular Unit (NVU)

The NVU (Figure I) describes the structure connecting central nervous system neurons and the vasculature and includes the cells of the blood vessel, surrounding extracellular matrix, glia, and neurons. The NVU is sometimes called the neurogliovascular unit, to emphasise the role of glia. This structure forms the basis of the blood–brain barrier (BBB) at the blood vessel end and allows neurovascular coupling, which is the response of the blood vessel to neural activity, providing functional hyperaemia, which may be measured on functional MRI (fMRI) (reviewed in [91]). The small vessels (arterioles) of the brain comprise a highly specialised layer of endothelial cells (ECs), surrounded by pericytes, basement membrane, and the end-feet of astrocytes, which encircle the vessel and provide a direct link with neurons through the astrocytes. Surrounding this are neighbouring oligodendroglia and microglia, which are in direct and indirect communication with the other cells of the unit. This structure is involved in SVD pathology, with all cells implicated.

ECs are not simply the cells lining the blood vessel. Instead, they are active players in controlling intravascular blood coagulation, blood flow, the BBB and neurovascular coupling, as well as communicating with surrounding glia, controlling the health of the brain parenchyma. The TJs between ECs contribute to the BBB and ion channels (e.g., Kir2.1, TRPV4) present on their membrane allow propagation of information about neuronal activity along the blood vessel, from the capillaries in close proximity to each neuron, to bigger vessels with smooth muscle around for vasodilation/constriction [114,115]. SVD leads to EC dysfunction (the main topic of the present review), interfering with all of these functions.

On the brain parenchymal side of ECs lie pericytes, which regulate BBB function during development and adulthood [116,117] and are involved in SVD. Pericyte-deficient mice develop WM atrophy, show a loss of oligodendrocytes and myelin, and have impairments in cognitive function, which all worsen with age [118].

Astrocytes have fine specialised processes that extend from the cell body to form end-feet in contact with the basement membrane surrounding ECs and with pericytes [119]. As other astrocyte processes contact neurons, synapses, and other glia, including oligodendroglia, they provide a link between these cells and structures. The end-feet encircle the vessels but are not coupled together by TJs and so do not act as a significant physical barrier. However, Aquaporin 4 (AQP4) is located at the end-feet [120], helping to regulate cerebral water balance, normally and after traumatic brain injury at least, as AQP4KO mice survive with a better neurological outcome after this insult [121,122]. Astrocytes interact with other local cells of the unit, including oligodendroglia, with evidence that, depending on their functional state, they can promote maturation (more 'A2-like') or reduce maturation (more 'A1-like') of oligodendroglia [123] in models of chronic hypoperfusions and SVD and promote myelination and even remyelination after demyelination by their interactions [124].

Oligodendrocyte precursor cells (OPCs) 'walk along' vasculature to migrate through brain tissue in development [125] and in pathology, where they can acquire aberrant Wnt tone, fail to detach, and interfere with astrocyte end-feet and EC TJ integrity, disrupting the BBB [126]. OPCs can develop into mature oligodendrocytes, which extend plasma membrane protrusions to ensheath axons with myelin sheaths. In SVD, there is a lack of myelin seen on postmortem histology correlating with white matter hyperintensities seen by MRI scans of patients [127], and myelin loss both reduces axon conduction velocities and leaves axons vulnerable to degradation and damage (reviewed in [128]). Axonal damage can lead to neuronal damage, which is then irreversible and characterises neurodegenerative diseases such as dementias, including SVD.

The development of the concept of the NVU has integrated the research of the various cell types and provided evidence that their interplay is highly important. Although the endpoint of neurovascular degeneration is neuronal loss, the other cells of the NVU may offer opportunities and insights into earlier targets for therapy for diseases such as SVD.

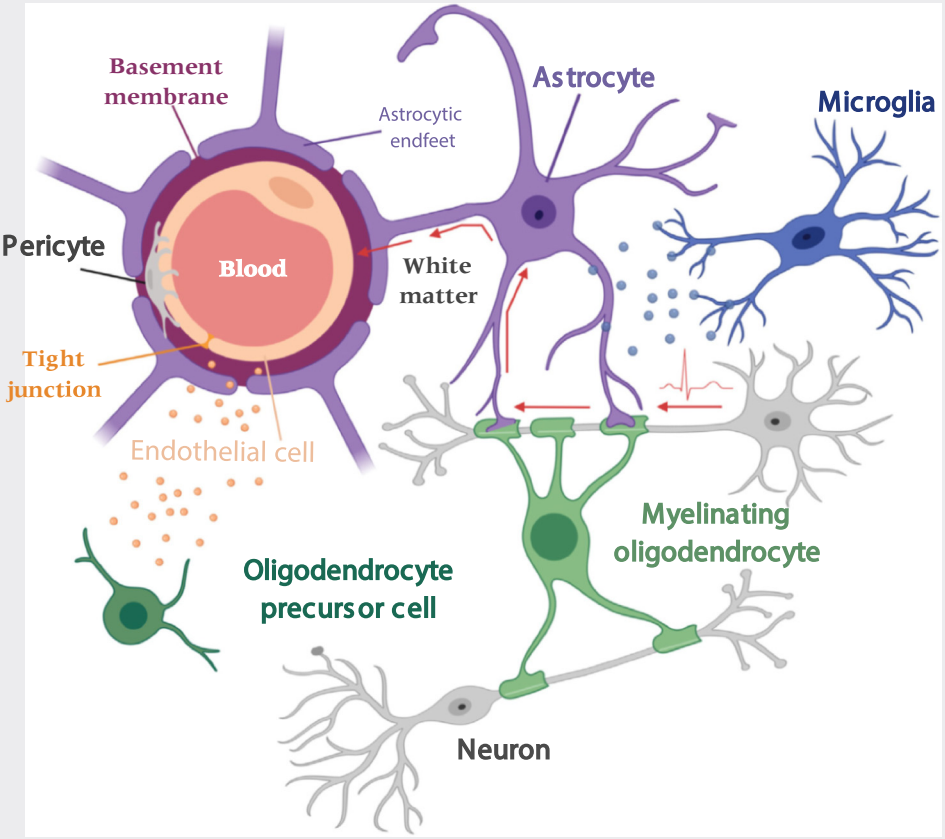

Figure I. The Neurogliovascular Unit: A Dynamic Connection between the Blood, the White Matter, and Neurons Responsive to Energy Demands. At the blood vessel end of the unit is the blood–brain barrier, consisting of endothelial cells, pericytes, astrocytic end-feet, and the basement membrane. Endothelial cells have tight junctions between them, separating the blood from the brain parenchyma. Pericytes maintain the integrity at this interface by regulating gene expression of endothelial cells and polarising the surrounding astrocytic end-feet and, through these, link the blood vessel to other glia and neurons. Through this link, changes in neural activation can affect cerebral blood flow, sending signals to intermediary cells to contract and dilate vessels in response to energy demands, in a process called neurovascular coupling. The identity of these intermediary cells is a matter of ongoing controversy, with accounts of involvement of pericytes on the smaller vessels, smooth muscle cells on larger arterioles, and endothelial cells themselves mediating the response directly from endothelial cell to endothelial cell along the length of the vasculature. Microglia survey the brain parenchyma, interacting with other cells of the neurogliovascular unit mainly through bidirectional paracrine signalling. Oligodendrocyte precursor cells migrate along and interact with endothelial cells altering their function. These can mature into myelinating oligodendrocytes, forming myelin around axons, impacted by the actions of astrocytes and microglia. The architecture of the neurogliovascular unit and the complex interactions between its cell types underscores the idea that disorder of any of its components may lead to broader pathology. Figure created using BioRender.

depending on the cause of the dysfunction. Nevertheless, using a panel of protein markers, one can distinguish between the states. Here we will describe some commonly used markers and describe why they are relevant in the context of SVD research (Figure 1).

Markers of EC Dysfunction

Responsive vasodilation of vessels is necessary for proper blood supply to the tissue and is controlled in part by the release of endothelial-derived NO from ECs into smooth muscle cells, where NO activates guanylate cyclase to cause cGMP-mediated relaxation of the vessel.

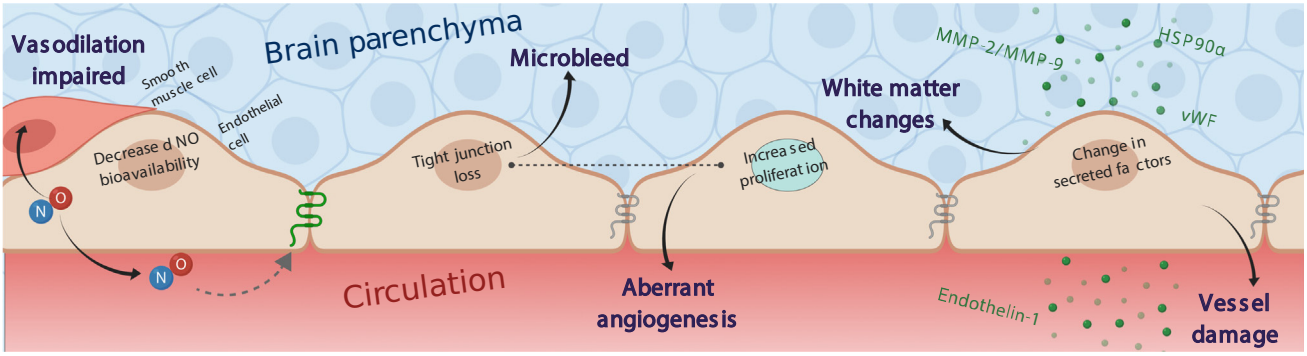

Figure 1. Markers of Dysfunction in Endothelial Cells (ECs) of the Blood–Brain Barrier (Shown within Cells), with the Links to Specific Features of Small Vessel Disease (SVD) (Shown Surrounding the Cells). From left to right: decreased nitric oxide (NO) bioavailability is a commonly used marker of EC dysfunction. Reduced levels of NO produced by ECs means less is received by neighbouring smooth muscle cells, leading to less vasodilation. Experimentally, decreased NO bioavailability can be assessed by measuring levels of nitrate/nitrite in the Griess reaction on plasma or cell supernatant but also through levels of endothelial nitric oxide synthase (eNOS) in tissue or cells. As NO can modify tight junction proteins via nitrosylation or nitrosation, this may cause another marker of dysfunction, tight junction loss. This impacts the permeability of the vascular system, providing a link between dysfunction and the microbleeds that are seen in SVD by magnetic resonance imaging. Experimentally, this can be shown by reduced levels of proteins, such as Claudin-5. Intercellular changes, like tight junction loss, that decrease overall barrier integrity can be linked to intracellular changes, such as increased proliferation. This may contribute to the aberrant angiogenesis seen in SVD and is measured by increased levels of proliferation markers such as Ki67 and PCNA. Finally, dysfunctional ECs secrete a different profile of factors compared with healthy functional ECs, which can be measured by the levels of secreted proteins in serum or supernatant. These secreted factors are secreted both into the circulation and the brain parenchyma, so can have direct effects on the surrounding cells in the white matter or in the vessel itself, both of which are features of SVD. Figure created using BioRender. Abbreviation: vWF, von Willebrand factor.

Reduced release of NO occurs in dysfunctional ECs and may lead to pathological vasoconstriction, impaired blood flow, and, ultimately, tissue ischemia. Bioavailability of NO is possible to measure experimentally, typically utilising the Griess reaction to quantify the nitrite and nitrate in biological samples. However, a reduction in levels of the enzyme endothelial NO synthase (eNOS), which converts L-arginine to NO, is also used as a proxy for decreased production of NO. Reduced eNOS has been identified in animal models of SVD and in cerebrospinal fluid of living SVD patients [13,14]. Vascular risk factors associated with SVD, such as aging and hypertension, increase reactive oxygen species and have a direct effect on eNOS, causing the enzyme to shift from producing NO to producing the destructive superoxide O_2^-, which degrades any persisting bioavailable NO. Changes in NO affect the permeability of the vascular system, through modification of TJ proteins via nitrosylation or nitrosation [15], providing a link between dysfunction and microbleeds that are seen in SVD by MRI.

The integrity of the BBB depends in part on the formation and maintenance of these TJ protein complexes between ECs that limit and control the passage of cells and molecules. These proteins include transmembrane proteins such as Claudin-5 and Occludin, as well as cytoplasmic plaque proteins of adherens junctions such as Zona Occludens-1. Lower expression of the TJ protein Claudin-5 is seen in dysfunctional ECs *in vitro*, in animal models with consequent BBB breach [16,17], and in humans with SVD [8]. Decreased expression levels of these TJ proteins leads to decreased barrier integrity and microbleeds, but are also related to the higher proliferation seen in dysfunctional ECs.

ECs in adulthood are normally quiescent, but EC dysfunction leads to increased proliferation, seen in SVD models [18,19] and in human SVD [20], which may contribute to aberrant angiogenesis. Aberrant retinal angiogenesis, seen as increased branching on fundoscopy, has been recently described as associated with SVD in patients [21] and it may be speculated that this increased

branching is a compensatory mechanism for hypoxia related to reduced blood flow and/or microbleeds and necessitates disruption of TJs between ECs.

Another potential way for dysfunctional ECs to cause pathology in SVD is to increase the secretion of **matrix metalloproteinases (MMP)**. There are many different types of these, but MMP-9 and MMP-2 are particularly interesting in the context of SVD as they are constitutively expressed at low levels in the brain, including by ECs [22] and are elevated in serum in other diseases characterised by endothelial dysfunction, such as renal disease and the lysosomal storage disorder Fabry's disease [23,24]. MMP-2 and MMP-9 also show increased expression in rat models of SVD, leading to a decrease in the TJ proteins Claudin-5 and Occludin, which can be prevented with MMP inhibitors [25]. As well as secreting MMPs into the lumen of the blood vessel, ECs secrete MMPs abluminally into surrounding brain tissue with implications for WM myelin in SVD: increased MMP-9 was associated with decreased levels of the major myelin protein, myelin basic protein (MBP) [26]. Dysfunctional ECs from a rat model of SVD (SHRSP; Table 1) secrete more of the MMP chaperone protein Heat Shock Protein 90α (HSP90α; cleaved form) than control ECs, which leads to arrest of oligodendrocyte maturation, contributing to WM changes [8]. Furthermore, in humans, a single nucleotide polymorphism (SNP) in MMP-2 was identified as a risk factor for WM change on MRI scans [27].

Other factors secreted by ECs into the serum and brain include the endothelins (primarily endothelin-1; ET1) and von Willebrand factor (vWF). ET1 binds to endothelin receptor-A on smooth muscle cells abluminally to cause vasoconstriction, but is also able to bind to endothelin receptor-B on adjacent ECs, where it stimulates NO production to cause vasodilation. The balance between these counteracting effects normally maintains homeostasis in the vessel, but elevated serum ET1 due to endothelial dysfunction [28] upsets the balance and contributes to pathological vasoconstriction. vWF is expressed constitutively by ECs, but is stored in granules and released from normal ECs in response to injury, aiding coagulation and platelet adhesion [29]. However, in dysfunctional ECs, vWF is released in a more complex multimeric form, which prevents its normal function and instead promotes vessel damage [30].

These markers allow identification of dysfunctional ECs but also give clues to the different pathways involved in SVD, potential biomarkers, and therapeutic targets. Serum biomarkers investigated for diagnosis of SVD in humans have been comprehensively reviewed [31] but there is no one marker found yet that defines SVD. There could be various reasons for this, including that the human population is heterogeneous, that endothelial dysfunction from different causes gives a different signature, that the brain endothelial signal is diluted out by the systemic signal, or that techniques to measure each biomarker vary between laboratories. It is conceivable that a panel of biomarkers may be more robust than a single biomarker, particularly when aided by better understanding of the dysfunction of ECs and their supporting cells. To best determine the contributions of each factor/pathway, it is important to understand the different animal models available and their relevance to human disease, as discussed next.

Animal Models of SVD and EC Dysfunction

There are various animal models for investigating SVD and they offer different perspectives on a strikingly multifaceted disease [32]. To examine the applications of these models, with particular reference to endothelial dysfunction, we will focus on those most commonly used, which fall into three main methodological categories: those developed by selective breeding, genetic mutation, and surgical induction, and which are illustrated in detail in Table 1.

Table 1. Murine Models of Small Vessel Disease Displaying Features of Endothelial Dysfunction

Model: description	Development (species)	Features of endothelial cell (EC) dysfunction (and timing of emergence, if applicable)		Refs
Selective breeding models				
SHR: Spontaneously hypertensive rats. Bred from Wistar Kyoto (WKY) rats to develop high blood pressure from 12 weeks onwards that further increases at week 18.	Selective breeding (Rat)	Early EC dysfunction (<20 weeks): ▪ Proliferation: >WKY control rats ▪ Tight junction disruption: present ▪ Membrane perturbations: >controls ▪ Nitric oxide bioavailability: <controls	Late EC dysfunction (>20 weeks): ▪ Proliferation: progressively ↑ versus controls ▪ Membrane perturbations: more extensive ▪ Activated platelet adhesion: present ▪ Degeneration: present	[33–35]
SHRSP: Spontaneously hypertensive stroke-prone rats. Established through selective breeding of SHRs for those most stroke-prone.	Selective breeding (Rat)	Early EC dysfunction (<20 weeks): ▪ Proliferation: >WKY control rats ▪ Tight junction disruption: present ▪ Nitric oxide bioavailability: <controls	Late EC dysfunction (>20 weeks): ▪ Membrane perturbations: present ▪ Activated platelet adhesion: present ▪ Tight junction disruption: present ▪ Degeneration: present	[8,17,101–104]
Surgical models				
Rose bengal: Systemic injection, via tail vein, of photosensitizing rose bengal, and 2 minute photoactivation with 560 nm light to generate singlet oxygen that leads to multiple thromboemboli.	Acute injection (Rat)	Shorter latency EC dysfunction (2 minutes after photoactivation): ▪ Membrane perturbations: >controls[a] ▪ Activated platelet adhesion: present	Longer latency EC dysfunction (15 minutes after photoactivation): ▪ Membrane perturbations: more extensive ▪ Activated platelet adhesion: present	[66]
Detergent: Systemic injections of sodium laurate via carotid artery to lyse endothelial membranes, causing microthrombi and multiple infarcts.	Acute injection (Rat)	EC dysfunction (1 hour postinjection): ▪ Membrane perturbations: present[b] ▪ Activated platelet adhesion: present		[68]
Vasoconstrictor: Direct injection into the brain of vasoconstrictive endothelin-1 with L-NAME (nitro-L-arginine methyl ester); an inhibitor of endothelial nitric oxide synthase (eNOS) that leads to EC dysfunction.	Acute injection (Rat)	▪ L-NAME-induced EC dysfunction		[69]
BCAS: Bilateral common carotid artery stenosis. Surgery to wrap metal microcoils around arteries to narrow them by 50%, producing a chronic global hypoperfusion less severe than total occlusion.	Chronic hypoperfusion (Mouse)	Shorter latency EC dysfunction (1–28 days post-BCAS surgery): ▪ Cell adhesion molecules[c] > controls[d]	Longer latency EC dysfunction (6 months post-BCAS surgery): ▪ Claudin-5+ tight junctions: < controls[d] EC-related changes: ▪ Blood–brain barrier (BBB) disruption	[70–74]
Genetic models				
CADASIL Tg*Notch3*: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy caused by germline *Notch3* mutations.	Single mutation (Mouse)	EC-related changes: ▪ Recruitment of extracellular matrix proteins around blood vessels, including tissue inhibitor of metalloproteinases 3 (TIMP3), possibly leading to increased eNOS expression and activation.		[42–47]
COL4A1/2 mutations: Mutations in alpha 1 or alpha 2 chains of collagen IV, the most abundant component of the basement membrane, that prevents the normal formation of the heterotrimeric protein, causing disruption to normal interactions.	Single mutation (Mouse)	EC-related changes: ▪ Reduced NOS activity (at least in aorta) ▪ BBB leakage		[55–58]

(continued on next page)

Table 1. (continued)

Model: description	Development (species)	Features of endothelial cell (EC) dysfunction (and timing of emergence, if applicable)	Refs
CARASIL: Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy caused by loss of function mutation in the high-temperature requirement A serine peptidase 1 (*HTRA1*) gene.	Single mutation (Mouse)	EC-related changes: ▪ Reduced retinal capillary density ▪ Reduced transforming growth factor beta (TGF-β) activity	[60,61]
R+/A+: Overexpresses human renin and angiotensin, developing haemorrhages but only when mice are treated with high-salt diet and L-NAME, leading to EC dysfunction.	Single mutation (Mouse)	▪ L-NAME-induced EC dysfunction	[63]
eNOS +/–: Heterozygous mutation in eNOS, resulting in reduced activity that leads to decreased overall nitric oxide bioavailability and EC dysfunction.	Single mutation (Mouse)	▪ Reduced eNOS-induced EC dysfunction Other EC-related changes: ▪ Increased BBB leakage and BBB transport	[64,65]

[a]Solely rose bengal or photoactivation.
[b]Absent from control brain hemisphere.
[c]Intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 (ICAM-1 and VCAM-1).
[d]Sham-injected animals.

Selective Breeding Models of SVD: SHR and SHRSP

The widely used model of SVD, the stroke-prone spontaneously hypertensive rat (SHRSP), was developed by selective breeding from the spontaneously hypertensive rat (SHR) [33–35] and has a striking similarity of brain pathology to human SVD [36]. Recent papers have shown that EC dysfunction precedes other SVD pathology [8,17] and is related to a deletion mutation in the *Atp11b* gene, which causes EC dysfunction when knocked-down in ECs cultured *in vitro*. Furthermore, a SNP in *ATP11B* associates with the presence of WM hyperintensities on MRI scans in humans [8], suggesting it may be involved in sporadic SVD. ATP11B is a phospholipid flippase that maintains membrane asymmetry by keeping phosphatidylserine more on the inner cytoplasmic side [37,38] and which has been implicated in synaptic plasticity [39], but it is not yet clear how changes in ATP11B cause EC dysfunction and contribute to SVD.

Genetic Models of SVD

In addition to in-bred models, there are two types of genetic models of SVD: those which model rare single gene mutations known to cause inherited SVD in humans and those which genetically induce risk factors or pathways thought to be associated with SVD.

The most common genetic human SVD is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [40], caused by mutations in the *NOTCH3* gene, most commonly introducing an extra cysteine within the extracellular domain (ECD) of the protein (NOTCH3^{ECD}) [41]. The best characterised model of CADASIL is the Tg*Notch3*^{R169C} mouse, which overexpresses a form of the *Notch3* gene containing a common human mutation and shows cerebrovascular dysfunction and WM lesions [42] but no lacunar infarcts [43]. NOTCH3^{ECD} accumulates around blood vessels and recruits extracellular matrix proteins [44], such as tissue inhibitor of metalloproteinases-3 (TIMP3). TIMP3 may induce EC dysfunction through its inhibition of A disintegrin and metalloproteinase 17 (ADAM17) [45], leading to shedding of Heparin binding epidermal-like growth factor, which in turn increases eNOS expression and activation [46,47]. However, direct evidence of a generalised EC dysfunction in these mice is not clear. While BBB leakage has previously been shown in this model [48], this has recently been disputed [49]. Similarly, while this model has a reduced global cerebral blood flow and an impaired vascular response to pressure changes [42], the vascular responses to L-NAME were unaffected [50]. As this is an important genetic model of SVD, further study of EC dysfunction

is merited to resolve this discrepancy. If there is indeed no EC dysfunction in this model, then this may explain why it fails to reflect some aspects of the human disease such as lacunes and microinfarcts with BBB leakage [49].

COL4A1/A2 related angiopathies are caused by mutations in the alpha-1 or alpha-2 chains of collagen IV, a key component of the basement membrane supporting blood vessels [51]. These affect multiple organs, but in the brain cause SVD-like changes to the vasculature and WM [52–54]. Multiple mouse models of COL4-related angiopathies have been generated with different mutations [55–57], leading to cerebral micro- and macro-haemorrhages [57,58] and signs of endothelial dysfunction, with both reduced NOS activity and BBB leakage [55,58]. SNPs in COL4A2 are associated with sporadic SVD [59], suggesting that subtle COL4A2 changes may also contribute to sporadic disease.

Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) is caused by a loss of function mutation in the High-Temperature Requirement A Serine Peptidase 1 (*HTRA1*) gene. *Htra1* knockout mice have been generated, showing reduced retinal capillary density [60] and reduced TGF-β activity [61], suggesting possible EC dysfunction, but these are still not fully characterised. An adult-onset disease, Cathepsin A-related arteriopathy with strokes and leukoencephalopathy (CARASAL), is caused by mutations in Cathepsin-A, which normally degrades ET1. ET1 was found to be upregulated in these patients' brains and associated with an increased number of immature oligodendroglia [62], again suggesting a link between ECs and WM oligodendrocytes.

Other genetic models of SVD are generated by inducing hypertension, such as in the R^+/A^+ mouse, which overexpresses human renin (R) and angiotensin (A) and develops cerebral haemorrhages in a similar pattern to in human SVD [63]. However, these changes were only induced when the mice were treated with a high-salt diet and nitro-L-arginine methyl ester (L-NAME), an inhibitor of eNOS function, suggesting pathology may be a consequence of L-NAME-induced EC dysfunction, rather than hypertension. This is supported by the fact that heterozygous $eNOS^{+/-}$ mice also display many features of SVD [64,65], suggesting that direct modification of endothelial function alone may be a promising avenue for modelling SVD.

Surgically Induced Models of SVD

Models of SVD can be induced in wild type animals by either acute injections of toxic substances or induction of stenosis in the brain blood vessels, causing chronic hypoperfusion.

Systemic injections of toxic substances can affect the EC luminal membrane, leading to SVD-like pathology with a clinical phenotype, and include the photo-sensitizing agent, rose bengal [66,67], and the detergent, sodium laurate [68]. Alternatively, direct injections of ET-1 in combination with the NOS inhibitor L-NAME into the brain induce vasoconstriction of small vessels, SVD-like pathology, and a clinical deficit [69]. The identification of vasoconstriction in SVD led to the development of chronic hypoperfusion rodent models, which focus on reducing cerebral blood flow with pathological changes of SVD such as gliosis and demyelination, though some have also been demonstrated to show EC dysfunction, but should be distinguished from acute blood flow interruption causing large vessel ischaemic stroke [70]. Of these, the most commonly used model is that of bilateral carotid artery stenosis (BCAS), using microcoils applied around both common carotid arteries, which restricts blood flow to the forebrain [70]. Within days of surgery, the cell adhesion molecules ICAM-1 and VCAM-1 are upregulated in ECs [71] but treatment with cilostazol, a phosphodiesterase inhibitor, which helps normalise EC function, can prevent this [72]. There is subsequent Claudin-5+ TJ loss, BBB disruption, WM change, and clinical

deficits akin to later-stage SVD [73,74]. Variations on this theme, where carotid blood flow is reduced gradually using ameroid constrictor devices, progressively restrict vessel diameter with intimal thickening over a period of weeks [75]. Here, there are signs of reduced cerebral blood flow, with subsequent increases in parenchymal inflammation and demyelination, but endothelial dysfunction has not yet been studied. Other murine models, designed to exhibit risk factors for human SVD, such as those with hyperhomocysteinaemia, are not included here as EC dysfunction has not yet been confirmed.

All of these models play an important role in understanding the mechanisms of SVD, but all also have drawbacks, making it important to consider the choice carefully to address the appropriate research question. Models of familial SVD provide vital insights into the mechanisms of genetic forms of the disease, but may not always be applicable to sporadic SVD. Hypertensive models do not contain all of the key features of SVD and may suffer from presuming an origin which may not be the primary driver of disease. Moreover, no model fully recapitulates the human disease. One reason for this may be that many are in mice, which have a much lower ratio of WM to grey matter (WM:GM) than humans and so may not fully show human WM changes [76]. The angioarchitecture of mouse WM is also different, with no arterioles being present in the subcortical WM, only capillaries. Rats have a slightly higher WM:GM ratio than mice and also contain some larger vessels in their subcortical WM and may therefore best reflect the sporadic forms of SVD. With technologies allowing easier genetic modification of rats, these may provide better genetic models of SVD than the current mouse models in the future.

Having chosen the most appropriate from this multitude of models to address the research question at hand, how can researchers then test whether these translate into salient clinical outputs relevant to human SVD?

Behavioural Testing

The relevance of any disease model is increased when the animal shows phenotypic changes that reflect those seen in patients. However, behavioural tests present challenges in SVD models, as even in humans, many patients are apparently asymptomatic, or have subtle symptoms, such as mood disturbances [77], or symptoms may be misinterpreted as ‘normal ageing’. In hypoperfusion models and older selective breeding animals (e.g., SHRSP), behavioural changes seen are usually seen late, poststroke. Specific links between behaviour and endothelial dysfunction seen in early stages of SVD has so far received relatively little attention. Here, we briefly discuss the different methods used to describe the clinical phenotypes generally observed in the models outlined earlier and highlight some studies that may have relevance for future characterisation of the existing SVD models. For those unfamiliar with the logistics of these tests, we refer to a comprehensive summary [78].

Learning and Memory Tests

As SVD classically causes cognitive impairment, useful clinical tests for a rodent model of SVD include cognitive tests, aiming to demonstrate deficits in spatial or working memory, or learning. Early characterisation of the SHRSP model showed delayed learning and persistent memory impairment poststroke, with worse performance on the three-panel runway [79], but also learning and memory deficits in passive avoidance tests even at 15–20 weeks old [80]. In hypoperfusion models, many studies show impaired memory through a range of different tests, including the Morris water maze (MWM) and Barnes maze [81].

While useful in showing behavioural deficits relevant to stroke, these examples do not link endothelial dysfunction to cognitive changes. However, EC dysfunction induced in mice fed a high-salt

diet for 12 weeks led to changes in nonspatial memory, as shown by the novel object recognition test, and in spatial memory, as shown by the Barnes maze, which was reversible by treatment with the NO precursor L-arginine to correct EC dysfunction, highlighting an association between EC dysfunction and changes in cognition [82].

Basic Motor and Sensory Function

SVD patients exhibit altered gait [77], making it relevant to assess motor function in rodent models using techniques such as rotarod and CatWalk. One study using a hypoperfusion model showed a lack of significant improvement in performance from baseline on the rotarod, even 8 weeks after surgery, compared with sham animals, suggesting motor deficits or perhaps learning deficits [83]. However, other similar studies report no significant difference in motor deficits between groups [84] and are only observed poststroke in both CADASIL [85] and SHRSP [86]. This paucity of reports of motor deficits in SVD models is indicative perhaps of negative results rather than an untested paradigm.

Anxiety and Depression-like Behaviours

Patients with SVD often experience anxiety and depression [87] but such symptoms have rarely been investigated in animal models and could be an additional useful measure [88]. Interestingly, the SHRSP model has been presented as a potential animal model of attention-deficit/hyperactivity disorder [89], as juvenile males exhibited higher levels of anxiety in the elevated plus maze. Early work with the SHRSP model indicated that at 15 and 30 weeks old, there was increased daily ambulation (prestroke) thought to represent delirium [90] but which may actually reflect anxiety-like behaviour.

It may seem surprising that rodents remain relatively or even completely apparently asymptomatic despite substantial pathology. This may speak to their simpler nervous system, resilience to injury, or perhaps to the lack of sensitivity in tests used to measure these outcomes. As the nature of SVD means that many human cases go undiagnosed due to the lack of clear symptoms, an animal model with no obvious changes in cognition or motor function does not necessarily preclude these animals' efficacy in modelling SVD. In humans, the phenotype is subtle, therefore, the most relevant behaviour in animal models may also be subtle.

By contrast, MRI changes in SVD are more overt. Next, we will consider which imaging modalities can identify endothelial dysfunction and other SVD-relevant changes in animal models, again focussing on investigating the link between EC dysfunction and parenchymal changes, rather than neurovascular coupling/BBB function, which has been reviewed elsewhere [91].

Imaging of Endothelial Dysfunction

There are multiple *in vivo* and *ex vivo* imaging modalities available to visualise brain pathology in animal models of SVD; MRI and intravital **laser speckle contrast imaging** reveal dynamic changes in the live brain, light microscopy allows interrogation of these changes marking proteins of interest, and electron microscopy (EM) shows the ultrastructure.

MRI

As rodent brains are smaller than human ones, inevitably the structural details visible by MRI are fewer, even with higher field strength animal scanners. MRI was previously not able to detect the subtle changes in the early stages of SVD in the SHRSP model [92], but careful study has now shown smaller corpus callosum volumes and enlarged ventricles in this model as early as 7 weeks old [93]. Later features of SVD are detectable in the BCAS mouse model, namely WM

disruption and subcortical alterations after 6 months of hypoperfusion [73]. Correlation of human and animal model MRI changes allows use of immunohistochemistry in the animal model to determine the cellular and molecular bases of these changes and extrapolate to human [94].

Intravital Microscopy

Despite the obvious advantages of imaging the whole brain with MRI, individual small vessels, affected in SVD, are at the limit of MRI resolution. Instead, researchers have been increasingly using intravital microscopy. Early evidence using a video image shearing device to measure pial arteriolar diameter, demonstrated that endothelial-dependent dilation (by acetylcholine or serotonin) is impaired in 6–8-month-old SHRSP animals [95]. Laser speckle contrast imaging can now be used to more accurately visualise changes in cerebral blood flow. Using this method, reductions in functional capillary density and microvascular cerebral blood flow were detected in the SHR model, indicating endothelial dysfunction, and these were reversed by treatment with the cholesterol-lowering drug, simvastatin [96]. Simvastatin can also reverse signs of EC dysfunction and WM damage in the SHRSP model [8] and restoring endothelial function, vessel integrity, and reactivity in a transgenic mouse overexpressing TGF-1β [97]. Observing vessels *in situ* adds the key dimension of dynamic vessel behaviour to the static image of complementary imaging techniques. Despite cranial windows increasing in size and microscopy gaining better resolution at greater depths in the brain [98], deep WM, where SVD pathology classically lies, is still difficult to image *in vivo*. Here, *ex vivo* immunohistochemical light microscopy is still required to describe finer features of the changes seen, using markers as described earlier for endothelial dysfunction either on brain sections, or the whole brain using clearing techniques [99,100]. The ultrastructure of these features can then be examined using EM.

EM

EM has been used extensively to describe ultrastructural features of dysfunctional ECs in the SHRSP model: increased trans-endothelial transport of tracers to adjacent phagocytes or perivascular spaces [101], leakage of plasma components [102,103], numerous endothelial intracellular vesicles and protrusions, disrupted basement membranes [103], disrupted TJs [104], and activated platelets adhering to dysfunctional ECs [102,103]. These features reflect changes in the EC plasma membrane, linking back to the deficit of the phospholipid flippase ATP11B in these animals [8] (described earlier).

These imaging modalities reveal the interactions between dysfunctional ECs, other cell types, and their environment. However, sometimes a reductionist approach of modelling these interactions between individual cells *in vitro* helps to understand and manipulate the key actors in EC dysfunction and its consequences.

In Vitro Modelling of SVD

ECs can be isolated/derived and cultured *in vitro* from different sources, comprehensively reviewed in [105] and summarised in Box 3, often with the intention of recreating the BBB for drug screening studies. Here, we focus on the use of these techniques in modelling the effect of endothelial dysfunction on surrounding brain cells.

Culture of primary murine ECs extends the usefulness of transgenic SVD models by studying ECs in isolation, but also to understand the two-way interactions between dysfunctional ECs and other specific brain cells such as astrocytes and pericytes (wild type or transgenic) in cocultures. Indeed, it is now understood that when a monolayer of ECs is cultured with astrocytes, or with astrocytes and pericytes, the ECs show enhanced levels and functionality of TJ proteins [106,107], suggesting that these cells are crucial to the development and maintenance of the

Box 3. Endothelial Cells *in Vitro* to Study Dysfunction

Primary Cells

A technique to isolate and grow ECs from rat brains was developed over 40 years ago, allowing the study of these cells in monocultures. Pure cultures can be obtained by seeding with puromycin-containing media, which selects for ECs as they express high levels of P-glycoprotein, and so are able to export the toxic substrate at a higher rate than contaminating fibroblasts and pericytes [129].

Primary human ECs can be obtained from neurosurgical biopsies, first used over 25 years ago. However, the drive for readily available robust cells led to the development of cell lines.

Immortalised Cell Lines

Murine EC lines bEND.3 and bEND.5 [130], cENd and cerebEND [131] were developed from mouse brain ECs with the aim of improving BBB studies that used primary cell sources. bEND.3 cells were recently used in two studies of dysfunctional ECs induced by hyperglycaemia or nicotine (both associated with SVD), highlighting changes of mitochondrial function [132,133].

HBMECs are human brain microvascular ECs transfected with SV40 antigen to immortalise them. These were used to study the observed clinical association between high circulatory phosphate levels and increased WM hyperintensities on MRI, by exposing HBMECs to high phosphate concentrations and resulting in reduced TJ proteins such as Claudin5 [134], though previous studies have stated that HBMECs already express rather low levels of this protein [135]. The hCMEC/d3 cell line are from human temporal lobe ECs transduced with both human telomerase and SV40 T antigen. Monolayers of hCMEC/d3 exposed to environmental factors such as altered glycaemia or cigarette smoke extract have shown loss of TJ proteins and an upregulation of proinflammatory markers [136,137]. Recent work with hCMEC/D3 cells has also identified the role of cAMP-degrading enzyme phosphodiesterase 3 in endothelial dysfunction in SVD [138]. However, as these are cell lines, researchers have turned to deriving ECs from stem cells.

ECs Induced from Pluripotent Stem Cells

There are now various protocols to derive ECs from human stem cells. One protocol uses a series of growth factor changes to drive Wnt signalling in human induced pluripotent stem cells and embryonic stem cells to differentiate into ECs [139]. Another uses cord blood-derived haematopoietic stem cells as the source, activating Notch signalling [140], suggesting that both pathways may be involved. However, these induced ECs can be immature and it is important to determine whether they are truly brain-like.

BBB (Box 2). Primary human ECs are arguably the most relevant source, but these samples are pathological, either being postmortem or from neurosurgical biopsies from patients with disease (usually epilepsy), which may of course alter cellular functioning. Additionally, this tissue is intermittently available and has limited longevity in culture.

This has led to the development of immortalised cell lines (mouse and human) offering a readily available, constant, robust supply for SVD research. Yet, however useful, the inevitable caveat is that these are transformed cell lines, with concomitant increased proliferation and biological differences compared with normal ECs *in situ*. Transcriptional profiling has indeed emphasised this, by showing many differences between the hCMEC/d3 cell line and isolated human brain ECs [108]. This prompted the development of protocols to differentiate ECs from pluripotent stem cells. However, here the challenge is to obtain suitably mature and brain-like ECs to be comparable with *in vivo* cells. One key indicator of mature brain ECs is the expression of TJ proteins such as Claudin-5, but Claudin-5+ TJs are also present in ECs in other tissues, such as the kidney. Another common indicator of maturity, at least in terms of BBB function, is the formation of a functional barrier in a cultured monolayer, using **transendothelial electrical resistance (TEER)**. However, *in vitro* TEER values do not measure up to TEER measurements seen *in vivo* [109], suggesting it is not necessarily an ideal way to demonstrate the relevance of these cells.

Stem cell-differentiated ECs also differ from primary ECs transcriptionally [110], but if this approach can be improved, it will provide a virtually unlimited source, with the possibility of

disease-specific lines, from iPS cells from patients with SVD caused by single gene mutations. Better understanding of what distinguishes a brain EC from other ECs should facilitate the development of improved differentiation protocols. Comparison of the transcriptomes of ECs from 11 different mouse tissues using scRNAseq identified the transcription factors Foxq1 and Foxf2 and the marker Pglyrp1 as brain-selective [111], with the latter surprising, as this is typically found in bone marrow and lymphoid tissues. These data also give signatures of artery, capillary, and venous ECs, which will help the generation of more appropriate ECs for modelling SVD and to determine whether any subtype has a greater propensity to become dysfunctional.

Concluding Remarks and Future Perspectives

EC dysfunction is increasingly recognised as important in brain diseases and in SVD it may be the initial driver of the disease. Here, we have sought to describe how to identify EC dysfunction at a molecular level, how to choose from the range of SVD rodent models and cell culture sources available that show these molecular changes, and how to measure its impact on the clinical phenotype in rodents. Much remains to be clarified, both in terms of better understanding of the processes of EC dysfunction and its impact on surrounding brain tissue, and in determining the effects on the blood vessel itself and surrounding tissues (see Outstanding Questions). Advances in transcriptomics, particularly at the single cell level, may help resolve the question of heterogeneity in ECs and provide an EC dysfunction signature, which may not be identical to the signature obtained from ECs at a BBB breach. A resurgence in the use of electron microscopy to study the brain is providing new hypotheses about the dysfunction of ECs, for example, regarding changes in vesicle release, which need to be tested further. There is also need to validate behavioural tests in SVD models to find measurable clinical outcomes, if possible, for therapeutic testing. There are drugs already available that normalise EC dysfunction and early pathology [8,96,97], which may help clinical outcomes [112]. The features of SVD that are seen using imaging may be reversible [113] and there are ongoing clinical trials testing this hypothesis (e.g., LACI2-NCT03451591). Our hope for the future is that clinicians may be able to treat SVD by delivering therapeutics into the systemic blood stream, directed at normalising EC function in the blood vessels supplying the brain, to improve the abnormalities of the surrounding brain parenchyma and improve clinical symptoms of SVD.

Acknowledgements

A.W., J.M., and R.M.R. are supported by the UK Dementia Research Institute, which receives its funding from DRI Ltd., funded by the Medical Research Council, Alzheimer's Society, and Alzheimer Research UK. S.Q. is supported by a Wellcome Trust Tissue Repair PhD fellowship. A.W. is also supported by the Medical Research Council and the MS Society UK. Figure 1 and Figure I in Box 2 were created using BioRender.

References

1. Pantoni, L. (2010) Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. *Lancet Neurol.* 9, 689–701
2. Kivipelto, M. *et al.* (2001) Midlife vascular risk factors and Alzheimer's disease in later life: longitudinal, population based study. *Br. Med. J.* 322, 1447–1451
3. Wardlaw, J.M. *et al.* (2013) Neuroimaging standards for research into small vessel disease and its contribution to ageing and neurodegeneration. *Lancet Neurol.* 12, 822–838
4. Wardlaw, J.M. *et al.* (2014) Vascular risk factors, large-artery atheroma, and brain white matter hyperintensities. *Neurology* 82, 1331–1338
5. Fisher, C.M. (1991) Lacunar infarcts — a review. *Cerebrovasc. Dis.* 1, 311–320
6. Wardlaw, J.M. *et al.* (2003) Is breakdown of the blood-brain barrier responsible for lacunar stroke, leukoaraiosis, and dementia? *Stroke* 34, 806–811
7. Peters, A. (2007) The effects of normal aging on nerve fibers and neuroglia in the central nervous system. In *Brain Aging: Models, Methods, and Mechanisms* (Riddle, D.R., ed.), pp. 97–125, CRC Press/Taylor & Francis
8. Rajani, R.M. *et al.* (2018) Reversal of endothelial dysfunction reduces white matter vulnerability in cerebral small vessel disease in rats. *Sci. Transl. Med.* 10, eaam9507
9. Harrison, D.G. (1997) Cellular and molecular mechanisms of endothelial cell dysfunction perspective series: nitric oxide and nitric oxide synthases cellular and molecular mechanisms of endothelial cell dysfunction. *J. Clin. Invest.* 100, 2153–2157
10. Liao, J.K. (2013) Linking endothelial dysfunction with endothelial cell activation. *J. Clin. Invest.* 123, 540–541
11. Zhang, J. *et al.* (2010) Biomarkers of endothelial cell activation serve as potential surrogate markers for drug-induced vascular injury. *Toxicol. Pathol.* 38, 856–871
12. Munji, R.N. *et al.* (2019) Profiling the mouse brain endothelial transcriptome in health and disease models reveals a core

Outstanding Questions

How can endothelial dysfunction be better defined across all functional roles? The definition of endothelial dysfunction currently varies depending on whether the focus is blood flow (vascular dysfunction), blood–brain barrier (barrier dysfunction), or changes in vessel cell–brain cell interactions (cell biology dysfunction). It will vary across tissue and maybe even within different blood vessels in the brain (e.g., arteriole versus venule). Better definition crossing-over between these research themes is needed.

Is all endothelial dysfunction the same or does it depend on the cause? Direct comparisons in a variety of models and diseases will determine the similarities and differences in dysfunctional features within and between those caused by different genetic or environmental factors and in different tissues.

How does endothelial dysfunction impact the surrounding brain tissue? There is lack of understanding of the mechanistic link between endothelial dysfunction and presence of white matter changes, which needs to be addressed by combining the expertise of different disciplines and using a variety of preclinical models and disease tissue.

How much of SVD pathology is reversible? Ultimately, the goal of preclinical SVD models is to better reflect the human disease and to provide a platform that can identify therapeutic targets to slow, stop, or reverse SVD and translate these into effective human therapies. MRI suggests that some features, such as WM hyperintensities, may be reversible, which is promising compared with other dementias and should focus research in this area for greater impact. However, do neuroradiological improvements translate into clinical improvements? Clinical trials are beginning to answer this question, but better measures of clinical changes in preclinical models and human patients are needed to provide meaningful outcomes from therapeutic trials in both.

blood–brain barrier dysfunction module. *Nat. Neurosci.* 22, 1892–1902
13. Lavallée, P.C. *et al.* (2013) Circulating markers of endothelial dysfunction and platelet activation in patients with severe symptomatic cerebral small vessel disease. *Cerebrovasc. Dis.* 36, 131–138
14. Deplanque, D. *et al.* (2013) Cerebral and extracerebral vasoreactivity in symptomatic lacunar stroke patients: a case-control study. *Int. J. Stroke* 8, 413–421
15. Daneman, R. and Prat, A. (2015) The blood–brain barrier. *Cold Spring Harb. Perspect. Biol.* 7, a020412
16. Nitta, T. *et al.* (2003) Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *J. Cell Biol.* 161, 653–660
17. Bailey, E.L. *et al.* (2011) Cerebral small vessel endothelial structural changes predate hypertension in stroke-prone spontaneously hypertensive rats: a blinded, controlled immunohistochemical study of 5- to 21-week-old rats. *Neuropathol. Appl. Neurobiol.* 37, 711–726
18. Drab, M. *et al.* (2001) Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science* 293, 2449–2452
19. Rajashekhar, G. *et al.* (2006) Continuous endothelial cell activation increases angiogenesis: evidence for the direct role of endothelium linking angiogenesis and inflammation. *J. Vasc. Res.* 43, 193–204
20. Grinberg, L.T. and Thal, D.R. (2010) Vascular pathology in the aged human brain. *Acta Neuropathol.* 119, 277–290
21. McGrory, S. *et al.* (2019) Retinal microvasculature and cerebral small vessel disease in the Lothian Birth Cohort 1936 and Mild Stroke Study. *Sci. Rep.* 9, 6320
22. Yong, V.W. *et al.* (2001) Metalloproteinases in biology and pathology of the nervous system. *Nat. Rev. Neurosci.* 2, 502–511
23. Gheissari, A. *et al.* (2018) Association of matrix metalloproteinase-2 and matrix metalloproteinase-9 with endothelial dysfunction, cardiovascular disease risk factors and thrombotic events in children with end-stage renal disease. *Iran. J. Kidney Dis.* 12, 169–177
24. Loso, J. *et al.* (2018) Serum biomarkers of endothelial dysfunction in Fabry associated cardiomyopathy. *Front. Cardiovasc. Med.* 5, 108
25. Yang, Y. *et al.* (2007) Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J. Cereb. Blood Flow Metab.* 27, 697–709
26. Jalal, F.Y. *et al.* (2012) Myelin loss associated with neuroinflammation in hypertensive rats. *Stroke* 43, 1115–1122
27. Smith, J.A. *et al.* (2009) Complexity in the genetic architecture of leukoaraiosis in hypertensive sibships from the GENOA study. *BMC Med. Genet.* 2, 16
28. Galatius, S. *et al.* (1999) Endothelin and von Willebrand factor as parameters of endothelial function in idiopathic dilated cardiomyopathy: different stimuli for release before and after heart transplantation? *Am. Heart J.* 137, 549–554
29. Blann, A. (1993) von Willebrand factor and the endothelium in vascular disease. *Br. J. Biomed. Sci.* 50, 125–134
30. Lip, G.Y. and Blann, A. (1997) von Willebrand factor: a marker of endothelial dysfunction in vascular disorders? *Cardiovasc. Res.* 34, 255–265
31. Poggesi, A. *et al.* (2015) Circulating biologic markers of endothelial dysfunction in cerebral small vessel disease: a review. *J. Cereb. Blood Flow Metab.* 36, 72–94
32. Hainsworth, A.H. and Markus, H.S. (2008) Do in vivo experimental models reflect human cerebral small vessel disease? A systematic review. *J. Cereb. Blood Flow Metab.* 28, 1877–1891
33. Hazama, F. *et al.* (1977) Autoradiographic investigation of cell proliferation in the brain of spontaneously hypertensive rats. *Acta Neuropathol.* 37, 231–236
34. Ozaki, T. *et al.* (1977) Scanning electron microscopic study on the endothelial cells of the cerebral arteries in SHR. *Jpn. Heart J.* 18, 556–557
35. Qadri, F. *et al.* (2003) Brain nitric oxide synthase activity in spontaneously hypertensive rats during the development of hypertension. *J. Hypertens.* 21, 1687–1694
36. Yamori, Y. *et al.* (1976) Pathogenetic similarity of strokes in stroke-prone spontaneously hypertensive rats and humans. *Stroke* 7, 46–53
37. Pomorski, T. *et al.* (2001) Lipid distribution and transport across cellular membranes. *Semin. Cell Dev. Biol.* 12, 139–148
38. Wang, J. *et al.* (2018) Proteomic analysis and functional characterization of P4-ATPase phospholipid flippases from murine tissues. *Sci. Rep.* 8, 10795
39. Wang, J. *et al.* (2019) ATP11B deficiency leads to impairment of hippocampal synaptic plasticity. *J. Mol. Cell Biol.* 11, 688–702
40. Chabriat, H. *et al.* (2009) CADASIL. *Lancet Neurol.* 8, 643–653
41. Joutel, A. *et al.* (1996) Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. *VHNature* 383, 707–710
42. Joutel, A. *et al.* (2010) Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease. *J. Clin. Invest.* 120, 433–445
43. Joutel, A. and Faraci, F.M. (2014) Cerebral small vessel disease. *Stroke* 45, 1215–1221
44. Monet-Leprêtre, M. *et al.* (2013) Abnormal recruitment of extracellular matrix proteins by excess Notch3 ECD: a new pathomechanism in CADASIL. *Brain* 136, 1830–1845
45. Amour, A. *et al.* (1998) TNF-α converting enzyme (TACE) is inhibited by TIMP-3. *FEBS Lett.* 435, 39–44
46. Lee, D.C. *et al.* (2003) TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase. *Ann. N. Y. Acad. Sci.* 995, 22–38
47. Mehta, V.B. *et al.* (2008) HB-EGF stimulates eNOS expression and nitric oxide production and promotes eNOS dependent angiogenesis. *Growth Factors* 26, 301–315
48. Ghosh, M. *et al.* (2015) Pericytes are involved in the pathogenesis of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Ann. Neurol.* 78, 887–900
49. Rajani, R.M. *et al.* (2019) Blood brain barrier leakage is not a consistent feature of white matter lesions in CADASIL. *Acta Neuropathol. Commun.* 7, 187–201
50. Dabertrand, F. *et al.* (2015) Potassium channelopathy-like defect underlies early-stage cerebrovascular dysfunction in a genetic model of small vessel disease. *Proc. Natl. Acad. Sci. U. S. A.* 112, E796–E805
51. Pöschl, E. *et al.* (2004) Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development* 131, 1619–1628
52. Kuo, D.S. *et al.* (2012) COL4A1 and COL4A2 mutations and disease: insights into pathogenic mechanisms and potential therapeutic targets. *Hum. Mol. Genet.* 21, R97–110
53. Verbeek, E. *et al.* (2012) COL4A2 mutation associated with familial porencephaly and small-vessel disease. *Eur. J. Hum. Genet.* 20, 844–851
54. Gould, D.B. *et al.* (2006) Role of COL4A1 in small-vessel disease and hemorrhagic stroke. *N. Engl. J. Med.* 354, 1489–1496
55. Van Agtmael, T. *et al.* (2010) Col4a1 mutation in mice causes defects in vascular function and low blood pressure associated with reduced red blood cell volume. *Hum. Mol. Genet.* 19, 1119–1128
56. Chen, Z. *et al.* (2016) HANAC syndrome Col4a1 mutation causes neonate glomerular hyperpermeability and adult glomerulocystic kidney disease. *J. Am. Soc. Nephrol.* 27, 1042–1054
57. Gould, D.B. *et al.* (2005) Mutations in Col4a1 cause perinatal cerebral hemorrhage and porencephaly. *Science* 308, 1167–1171
58. Ratelade, J. *et al.* (2018) Severity of arterial defects in the retina correlates with the burden of intracerebral haemorrhage in COL4A1-related stroke. *J. Pathol.* 244, 408–420
59. Rannikmäe, K. *et al.* (2017) COL4A2 is associated with lacunar ischemic stroke and deep ICH: meta-analyses among 21,500 cases and 40,600 controls. *Neurology* 89, 1829–1839
60. Zhang, L. *et al.* (2012) High temperature requirement factor A1 (HTRA1) gene regulates angiogenesis through transforming

growth factor-β family member growth differentiation factor 6. *J. Biol. Chem.* 287, 1520–1526
61. Beaufort, N. *et al.* (2014) Cerebral small vessel disease-related protease HtrA1 processes latent TGF-β binding protein 1 and facilitates TGF-β signaling. *Proc. Natl. Acad. Sci. U. S. A.* 111, 16496–16501
62. Bugiani, M. *et al.* (2016) Cathepsin A-related arteriopathy with strokes and leukoencephalopathy (CARASAL). *Neurology* 87, 1777–1786
63. Iida, S. *et al.* (2005) Spontaneous stroke in a genetic model of hypertension in mice. *Stroke* 36, 1253–1258
64. Didion, S.P. (2017) *Effects of eNOS haploinsufficiency on the cerebral circulation*. 2 pp. 6–9
65. Tan, X.-L. *et al.* (2015) Partial eNOS deficiency causes spontaneous thrombotic cerebral infarction, amyloid angiopathy and cognitive impairment. *Mol. Neurodegener.* 10, 24
66. Dietrich, W.D. *et al.* (1987) Photochemically induced cerebral infarction. *Acta Neuropathol.* 72, 326–334
67. Alexis, N.E. *et al.* (1995) Nonocclusive common carotid artery thrombosis in the rat results in reversible sensorimotor and cognitive behavioral deficits. *Stroke* 26, 2338–2346
68. Toshima, Y. *et al.* (2000) A new model of cerebral microthrombosis in rats and the neuroprotective effect of a rho-kinase inhibitor. *Stroke* 31, 2245–2250
69. Uchida, H. *et al.* (2015) Experimental model of small subcortical infarcts in mice with long-lasting functional disabilities. *Brain Res.* 1629, 318–328
70. Shibata, M. *et al.* (2004) White matter lesions and glial activation in a novel mouse model of chronic cerebral hypoperfusion. *Stroke* 35, 2598–2603
71. Huang, Y. *et al.* (2010) Is endothelial dysfunction of cerebral small vessel responsible for white matter lesions after chronic cerebral hypoperfusion in rats? *J. Neurol. Sci.* 299, 72–80
72. Kitamura, A. *et al.* (2017) Long-term cilostazol treatment reduces gliovascular damage and memory impairment in a mouse model of chronic cerebral hypoperfusion. *Sci. Rep.* 7, 4299
73. Holland, P.R. *et al.* (2015) Gliovascular disruption and cognitive deficits in a mouse model with features of small vessel disease. *J. Cereb. Blood Flow Metab.* 35, 1005–1014
74. Reimer, M.M. *et al.* (2011) Rapid disruption of axon-glial integrity in response to mild cerebral hypoperfusion. *J. Neurosci.* 31, 18185–18194
75. Hattori, Y. *et al.* (2016) Gradual carotid artery stenosis in mice closely replicates hypoperfusive vascular dementia in humans. *J. Am. Heart Assoc.* 5, e002757
76. Bailey, E.L. *et al.* (2009) Potential animal models of lacunar stroke: a systematic review. *Stroke* 40, e451–e458
77. Ter Telgte, A. *et al.* (2018) Cerebral small vessel disease: From a focal to a global perspective. *Nat. Rev. Neurol.* 14, 387–398
78. Quillfeldt, J.A. (2015) Behavioral methods to study learning and memory in rats. In *Rodent Model as Tools in Ethical Biomedical Research* (Aridersen, M. and Tufik, S., eds), pp. 271–311, Springer International Publishing
79. Yamaguchi, M. *et al.* (1994) Memory deficit accompanying cerebral neurodegeneration after stroke in stroke-prone spontaneously hypertensive rats (SHRSP). *Acta Neurochir. Suppl. (Wien)* 60, 200–202
80. Togashi, H. *et al.* (1996) Cholinergic changes in the hippocampus of stroke-prone spontaneously hypertensive rats. *Stroke* 27, 520–526
81. Sarti, C. *et al.* (2002) Persistent impairment of gait performances and working memory after bilateral common carotid artery occlusion in the adult Wistar rat. *Behav. Brain Res.* 136, 13–20
82. Faraco, G. *et al.* (2018) Dietary salt promotes neurovascular and cognitive dysfunction through a gut-initiated TH17 response. *Nat. Neurosci.* 21, 240–249
83. Truong, D.T. *et al.* (2012) Deficits in auditory, cognitive, and motor processing following reversible middle cerebral artery occlusion in mice. *Exp. Neurol.* 238, 114–121
84. Linden, J. *et al.* (2014) Assessment of behavioral flexibility after middle cerebral artery occlusion in mice. *Behav. Brain Res.* 258, 127–137
85. Wallays, G. *et al.* (2011) Notch3 Arg170Cys knock-in mice display pathologic and clinical features of the neurovascular disorder cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Arterioscler. Thromb. Vasc. Biol.* 31, 2881–2888
86. Kimura, S. *et al.* (2000) Pathogenesis of vascular dementia in stroke-prone spontaneously hypertensive rats. *Toxicology* 153, 167–178
87. Herrmann, L.L. *et al.* (2007) White matter hyperintensities in late life depression: a systematic review. *J. Neurol. Neurosurg. Psychiatry* 79, 619–624
88. Ueno, M. *et al.* (2002) Blood–brain barrier disruption in white matter lesions in a rat model of chronic cerebral hypoperfusion. *J. Cereb. Blood Flow Metab.* 22, 97–104
89. Ueno, K. *et al.* (2003) Behavioral and pharmacological studies of juvenile stroke-prone spontaneously hypertensive rats as an animal model of attention-deficit/hyperactivity disorder. *Nihon Shinkei Seishin Yakurigaku Zasshi* 23, 47–55
90. Minami, M. *et al.* (1985) Changes in ambulation and drinking behavior related to stroke in stroke-prone spontaneously hypertensive rats. *Stroke* 16, 44–48
91. Kaplan, L. *et al.* (2020) Neuronal regulation of the blood–brain barrier and neurovascular coupling. *Nat. Rev. Neurosci.* 21, 416–432
92. Mencl, S. *et al.* (2013) Early microvascular dysfunction in cerebral small vessel disease is not detectable on 3.0 Tesla magnetic resonance imaging: a longitudinal study in spontaneously hypertensive stroke-prone rats. *Exp. Transl. Stroke Med.* 5, 8
93. Koundal, S. *et al.* (2019) Brain morphometry and longitudinal relaxation time of spontaneously hypertensive rats (SHRs) in early and intermediate stages of hypertension investigated by 3D VFA-SPGR MRI. *Neuroscience* 404, 14–26
94. Humphreys, C.A. *et al.* (2019) A protocol for precise comparisons of small vessel disease lesions between ex vivo magnetic resonance imaging and histopathology. *Int. J. Stroke* 14, 310–320
95. Mayhan, W.G. *et al.* (1987) Impairment of endothelium-dependent responses of cerebral arterioles in chronic hypertension. *Am. J. Physiol. Circ. Physiol.* 253, H1435–H1440
96. Freitas, F. *et al.* (2017) Acute simvastatin treatment restores cerebral functional capillary density and attenuates angiotensin II-induced microcirculatory changes in a model of primary hypertension. *Microcirculation* 24, e12416
97. Tong, X.-K. and Hamel, E. (2015) Simvastatin restored vascular reactivity, endothelial function and reduced string vessel pathology in a mouse model of cerebrovascular disease. *J. Cereb. Blood Flow Metab.* 35, 512–520
98. Koletar, M.M. *et al.* (2019) Refinement of a chronic cranial window implant in the rat for longitudinal in vivo two-photon fluorescence microscopy of neurovascular function. *Sci. Rep.* 9, 5499
99. Chung, K. *et al.* (2013) Structural and molecular interrogation of intact biological systems. *Nature* 497, 332–337
100. Poinsatte, K. *et al.* (2019) Visualization and quantification of post-stroke neural connectivity and neuroinflammation using serial two-photon tomography in the whole mouse brain. *Front. Neurosci.* 13, 1055
101. Tagami, M. *et al.* (1983) Increased transendothelial channel transport of cerebral capillary endothelium in stroke-prone SHR. *Stroke* 14, 591–596
102. Tagami, M. and Yamori, Y. (1988) Morphological analysis of the pathogenesis of hypertensive cerebrovascular lesions: role of monocytes and platelets in intracerebral vessel occlusions. *Jpn. Circ. J.* 52, 1351–1356
103. Fredriksson, K. *et al.* (1988) Cerebral microangiopathy in stroke-prone spontaneously hypertensive rats. *Acta Neuropathol.* 75, 241–252
104. Held, F. *et al.* (2017) Vascular basement membrane alterations and β-amyloid accumulations in an animal model of cerebral small vessel disease. *Clin. Sci.* 131, 1001–1013
105. Helms, H.C. *et al.* (2016) In vitro models of the blood-brain barrier: an overview of commonly used brain endothelial cell culture models and guidelines for their use. *J. Cereb. Blood Flow Metab.* 36, 862–890
106. Nakagawa, S. *et al.* (2009) A new blood–brain barrier model using primary rat brain endothelial cells, pericytes and astrocytes. *Neurochem. Int.* 54, 253–263

107. Li, G. *et al.* (2010) Permeability of endothelial and astrocyte cocultures: in vitro blood-brain barrier models for drug delivery studies. *Ann. Biomed. Eng.* 38, 2499–2511
108. Urich, E. *et al.* (2012) Transcriptional profiling of human brain endothelial cells reveals key properties crucial for predictive in vitro blood-brain barrier models. *PLoS One* 7, e38149
109. Appelt-Menzel, A. *et al.* (2017) Establishment of a human blood-brain barrier co-culture model mimicking the neurovascular unit using induced pluri- and multipotent stem cells. *Stem Cell Reports* 8, 894–906
110. McCracken, I.R. *et al.* (2020) Transcriptional dynamics of pluripotent stemcell-derived endothelial cell differentiation revealed by single-cell RNA sequencing. *Eur. Heart J.* 41, 1024–1036
111. Kalucka, J. *et al.* (2020) Single-cell transcriptome atlas of murine endothelial cells. *Cell* 180, 764–779
112. Becker, C.E. *et al.* (2019) Association between endothelial cell stabilizing medication and small vessel disease stroke: a case-control study. *Front. Neurol.* 10, 1029
113. Wardlaw, J.M. *et al.* (2015) What are white matter hyperintensities made of? Relevance to vascular cognitive impairment. *J. Am. Heart Assoc.* 4, 1140
114. Harraz, O.F. *et al.* (2018) Endothelial GqPCR activity controls capillary electrical signaling and brain blood flow through PIP2 depletion. *Proc. Natl. Acad. Sci. U. S. A.* 115, E3569–E3577
115. Harraz, O.F. *et al.* (2018) PIP2 depletion promotes TRPV4 channel activity in mouse brain capillary endothelial cells. *eLife* 7, e38689
116. Armulik, A. *et al.* (2010) Pericytes regulate the blood-brain barrier. *Nature* 468, 557–561
117. Daneman, R. *et al.* (2010) Pericytes are required for blood-brain barrier integrity during embryogenesis. *Nature* 468, 562–566
118. Montagne, A. *et al.* (2018) Pericyte degeneration causes white matter dysfunction in the mouse central nervous system. *Nat. Med.* 24, 326–337
119. Mathiisen, T.M. *et al.* (2010) The perivascular astroglial sheath provides a complete covering of the brain microvessels: an electron microscopic 3D reconstruction. *Glia* 58, 1094–1103
120. Nielsen, S. *et al.* (1997) Specialized membrane domains for water transport in glial cells: high- resolution immunogold cytochemistry of aquaporin-4 in rat brain. *J. Neurosci.* 17, 171–180
121. Manley, G.T. *et al.* (2000) Aquaporin-4 deletion in mice reduces brain edema after acute water intoxication and ischemic stroke. *Nat. Med.* 6, 159–163
122. Ikeshima-Kataoka, H. *et al.* (2015) Aquaporin 4-dependent expression of glial fibrillary acidic protein and tenascin-C in activated astrocytes in stab wound mouse brain and in primary culture. *J. Neurosci. Res.* 93, 121–129
123. Miyamoto, N. *et al.* (2020) The effects of A1/A2 astrocytes on oligodendrocyte linage cells against white matter injury under prolonged cerebral hypoperfusion. *Glia* 68, 1910–1924
124. Domingues, H.S. *et al.* (2016) Oligodendrocyte, astrocyte, and microglia crosstalk in myelin development, damage, and repair. *Front. Cell Dev. Biol.* 4, 71
125. Tsai, H.H. *et al.* (2016) Oligodendrocyte precursors migrate along vasculature in the developing nervous system. *Science* 351, 379–384
126. Campana, D. *et al.* (1987) The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. *J. Immunol.* 138, 648–655
127. Maniega, S.M. *et al.* (2015) White matter hyperintensities and normal-appearing white matter integrity in the aging brain. *Neurobiol. Aging* 36, 909–918
128. Franklin, R.J.M. and Ffrench-Constant, C. (2017) Regenerating CNS myelin - from mechanisms to experimental medicines. *Nat. Rev. Neurosci.* 18, 753–769
129. Perrière, N. *et al.* (2005) Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood-brain barrier-specific properties. *J. Neurochem.* 93, 279–289
130. Wagner, E.F. and Risau, W. (1994) Oncogenes in the study of endothelial cell growth and differentiation. *Semin. Cancer Biol.* 5, 137–145
131. Burek, M. *et al.* (2012) Generation of an immortalized murine brain microvascular endothelial cell line as an in vitro blood brain barrier model. *J. Vis. Exp.* 66, e4022
132. Suzuki, K. *et al.* (2011) Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function. *Proc. Natl. Acad. Sci. U. S. A.* 108, 13829–13834
133. Kaisar, M.A. *et al.* (2018) Conventional and electronic cigarettes dysregulate the expression of iron transporters and detoxifying enzymes at the brain vascular endothelium: in vivo evidence of a gender-specific cellular response to chronic cigarette smoke exposure. *Neurosci. Lett.* 682, 1–9
134. Chung, C.-P. *et al.* (2019) High circulatory phosphate level is associated with cerebral small-vessel diseases. *Transl. Stroke Res.* 10, 265–272
135. Eigenmann, D.E. *et al.* (2013) Comparative study of four immortalized human brain capillary endothelial cell lines, hCMEC/D3, hBMEC, TY10, and BB19, and optimization of culture conditions, for an in vitro blood–brain barrier model for drug permeability studies. *Fluids Barriers CNS* 10, 33
136. Sajja, R.K. *et al.* (2014) Impact of altered glycaemia on blood-brain barrier endothelium: an in vitro study using the hCMEC/D3 cell line. *Fluids Barriers CNS* 11, 8
137. Prasad, S. *et al.* (2015) Impact of cigarette smoke extract and hyperglycemic conditions on blood-brain barrier endothelial cells. *Fluids Barriers CNS* 12, 18
138. Yasmeen, S. *et al.* (2019) miRNA-27a-3p and miRNA-222-3p as novel modulators of phosphodiesterase 3a (PDE3A) in cerebral microvascular endothelial cells. *Mol. Neurobiol.* 56, 5304–5314
139. Cecchelli, R. *et al.* (2014) A stable and reproducible human blood-brain barrier model derived from hematopoietic stem cells. *PLoS One* 34, 382–393
140. Ponio, J.B.-D. *et al.* (2014) Instruction of circulating endothelial progenitors in vitro towards specialized blood-brain barrier and arterial phenotypes. *PLoS One* 9, e84179