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**‘Small vessels, dementia and chronic diseases –
molecular mechanisms and pathophysiology’
Workshop proceedings, Jan 25-26th 2017**

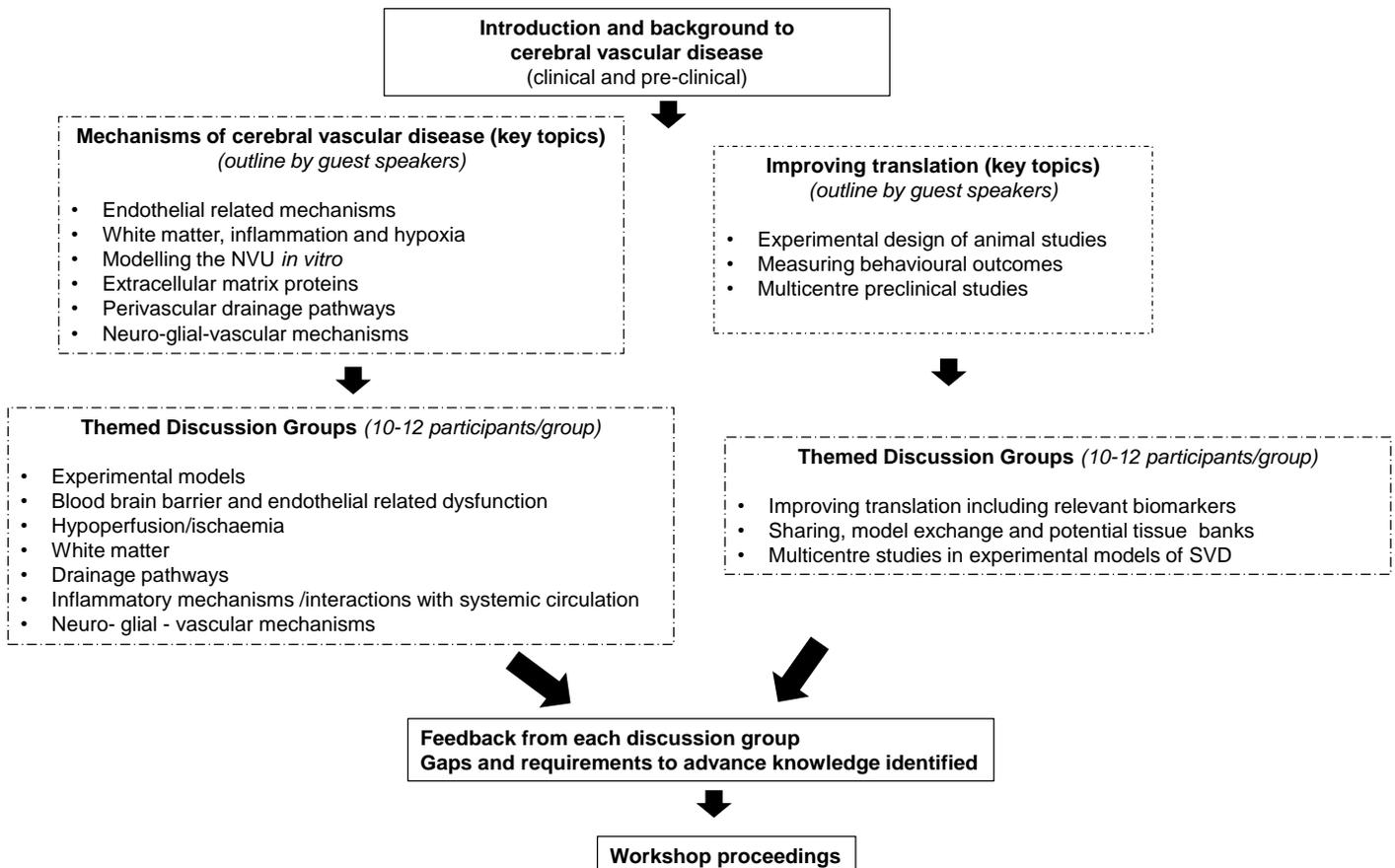
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INTRODUCTION

Cerebral small vessel disease (SVD) is a major health challenge. Therapeutic approaches remain limited, hampered by the lack of mechanistic understanding and identification of therapeutic targets. Relevant animal models could provide a cornerstone to basic scientific studies of disease mechanisms and pre-clinical studies of potential therapies, but there is a critical need to improve the current translational gap that exists between pre-clinical research and treatments in patients. The Medical Research Council Dementias Platform UK (MRC DPUK) Vascular Experimental Medicine Theme identified that a comprehensive assessment of the latest developments in animal models and of their contribution to understanding of cerebral microvascular disease would reduce the translational gap. In response to this, a two day workshop took place in late January 2017 at the British Heart Foundation Centre of Research Excellence in Glasgow, Scotland in conjunction with MRC DPUK and brought together experts from several disciplines in cerebrovascular disease, dementia and cardiovascular biology, to highlight current advances in these fields, explore synergies and scope for development. There were presentations from UK and international researchers and a specific focus on animal models of cerebral microvascular disease and dementia, considering vascular biology, neuroglial coupling, blood-brain barrier function, neuroinflammation, cerebral drainage pathways, and methodological and translational challenges (see Figure 1 for the general organisation of the meeting including the key topics and themes discussed). This overview provides a summary of the key talks, with a particular focus on mechanisms of cerebral vascular disease (see Figure 2) and improving translation. These talks were followed by related themed discussion groups on the gaps in knowledge and requirements to advance knowledge, the outcomes of which are highlighted in Table 1. Additional related articles are published in the Special Edition of Clinical Science (<http://www.portlandpresspublishing.com/cc/small-vessels>).

Figure 1 General organisation of the meeting



CEREBRAL VASCULAR DISEASE: CLINICAL CONTEXT

Of the approximate 17 million strokes per year worldwide, 20-25% are due to small vessel disease (SVD). Additionally, a large burden of silent SVD contributes to up to 45% of the 35-42 million new cases of dementia globally each year. SVD is difficult to research, small vessel stroke being neglected in stroke research, and vascular causes are neglected in dementia research. Human pathology often reflects late disease stages with few relevant experimental models identified [1,2] Consequently, treatment is empirical and probably suboptimal. The individual SVD features on neuroimaging are now well-described with standardised terminology [3]. These include acute (recent) small subcortical infarcts and clinically covert features (lacunes, white matter hyperintensities (WMH), microbleeds, perivascular spaces, microinfarcts, and cerebral atrophy), and many haemorrhagic strokes. From early neuropathology studies onwards, SVD has been the vascular lesion most strongly associated with vascular cognitive impairment (VCI) [4], an observation confirmed consistently through more recent large cohort studies of donated brain tissue, showing that neuropathological SVD is associated with cognitive impairment [5]

SVD features are associated with age and several vascular risk factors (hypertension, smoking, diabetes, hypercholesterolemia), but vascular risk factors explain only a small proportion of the variance [6] leaving a large proportion unexplained. Potential pathophysiological mechanisms include vessel stiffening, blood brain barrier failure,

poorer premorbid white matter integrity [7,8] a cross-sectional association with reduced cerebral blood flow [9,10], and several underlying genetic contributors [11].

Progress in reducing SVD requires questioning of conventional wisdom. Firstly, the effects of SVD on the brain are both diffuse and more dynamic than appreciated previously [12,13]. Subcortical lesions have remote effects; lesions can appear silently or cause symptoms, or can resolve completely, cavitate, or form a WMH [14]. WMH, lacunes and microbleeds may increase, but also decrease [15,16] which suggests that these lesions do not indicate permanent brain damage only. Perivascular spaces are crucial for cerebral interstitial fluid drainage and waste clearance [17]. They are a site of inflammation and amyloid protein build-up, linking to Alzheimer's disease. Other cell types and structures are involved in SVD, not just the vascular endothelium and smooth muscle cells, but also microglia, astrocytes, oligodendrocytes, pericytes, matrix proteins all play a role. Finally, data from humans and experimental models (see below) indicate a vulnerability to SVD in part [18] explaining why vascular risk factor exposure in some people is far more devastating than in others.

CEREBRAL VASCULAR DISEASE: RODENT MODELS

Whilst there is no one model that captures the complexities of SVD and VCI, different animal models have been studied that encompass many hypothesised causes (e.g. SVD); hypoxic hypoperfusion injury secondary to large artery disease; or genetic influences (see recent review [2] and previous systematic review [19]).

A widely studied model is the spontaneously hypertensive stroke prone (SHRSP) rat which develops malignant hypertension. This model has been reported to develop SVD-like features including white matter changes, lipohyalinosis and fibrinoid necrosis [20,21]. Endothelial and the earliest measured myelin changes have also been shown to predate hypertension [22]. Analysis of gene and protein expression before the onset of hypertension highlighted several genes and proteins associated with SVD and pathways that may underlie these changes (e.g. matrix and vascular integrity, inflammation), and suggests a potential predisposition of SHRSPs to development of SVD-like pathology which is later exacerbated by hypertension [23]. However these findings vary and others have failed to identify white matter damage in older SHRSP in the absence of stroke [24] and in other models of hypertension such as an inducible transgenic model [25]. Additional factors, such as added dietary salt, hypoperfusion and high fat diet have been shown to exacerbate white matter pathology in SHRSP [26] similar to such factors accelerating human SVD.

Alternative models of hypertension have been developed including the effects of angiotensin (ANG) administration. Low doses of angiotensin-II (ANGII) in rodents results in a slow-developing rise in blood pressure which impairs neuroglial vascular (NGVU) coupling and endothelial responses before the onset of hypertension and in the absence of SVD like changes [27]. More recently, simultaneous administration of ANGI and L-NAME (NG-nitro-L-arginine methyl ester) led to chronically elevated blood pressure and produced early SVD-like features [28]. Key features relevant to human SVD were reported in this model including hypertrophy of the cerebral vessel wall, structural impairment of the NGVU, hippocampal atrophy, blood brain barrier (BBB) impairment and occasional microinfarcts and microbleeds.

To investigate vascular insufficiency as a central mechanism in the pathophysiology of VCI, models of cerebral hypoperfusion were developed [29]. These models have been refined over the years to induce hypoperfusion whilst avoiding severe reductions in blood flow that would cause immediate ischaemia. The recent models involve stenosis of the common carotid arteries which immediately restricts blood flow to the forebrain on application of microcoils [30],

or gradually restricts blood flow by the use of constrictor devices [31]. These models have been widely studied with behavioural measures of cognition which demonstrate particular impairments in spatial working memory reminiscent of the disrupted frontal cortical circuitry found in VCI [29, 32]. In these models, reduced perfusion is thought to cause a slowly evolving diffuse white matter pathology that can be detected using a variety of approaches such as immunohistochemistry and in vivo magnetic resonance imaging (MRI). The oxygen levels are reduced to hypoxic levels in white matter, which is thought to be the main driver of the ensuing pathological changes [29]. These models have been widely studied to understand the pathophysiology of hypoperfusion-induced white matter changes that are often accompanied by indices of inflammation. Carotid stenosis induced using microcoils in the mouse has also been shown to culminate in marked changes to microvascular structure, BBB breakdown, glio-vascular damage and microinfarcts/microbleeds relevant to human SVD [33]. These studies are predominantly conducted in C57Bl/6J mice which have poor collateral flow through the Circle of Willis and the resultant effects of microcoil/constrictor application assigned to reduced cerebral perfusion. However other, albeit ill-defined, effects on vascular stiffness/pulsatility and cerebrospinal fluid (CSF) drainage may also contribute to the pathology. In a longitudinal study in humans, there is an absence of a direct association between carotid stenosis and WMH or cognitive decline and instead a link with vascular stiffness [34].

Notably, these models of carotid stenosis and hypertension often lack the overt white matter lesions (WML) characteristic of SVD. However, WML are difficult to detect pathologically and a combination of different risk factors may be required to produce WML. As indicated above, the effects of hypoperfusion and diet have been studied in the SHRSP model [26] which exacerbate white matter changes, as compared to individual factors, and precipitate overt WML. Models with co-morbidities may more accurately reflect clinical SVD and be considered as a basis for testing therapeutic interventions.

A number of different mouse models that express highly penetrant gene mutations linked to familial SVD (*NOTCH3*, *COL4A1*, *COL4A2*, *HTRA1*) have provided important insights into causal pathways related to these gene mutations. Interestingly, investigation of these models has now indicated that these different gene mutations may lead to common convergent pathways involving the impairment of extracellular matrix (ECM) function [35]. Further genome wide association studies (GWAS) have shown common variants associated with familial SVD, such as *COL4A1/4A2*, are also risk factors for common sporadic SVD [36]. Thus, an emerging view is that these models may be useful for probing mechanisms important not only in rare monogenic forms of SVD, but also in the more common sporadic forms of SVD, opening up avenues of potential therapeutic intervention.

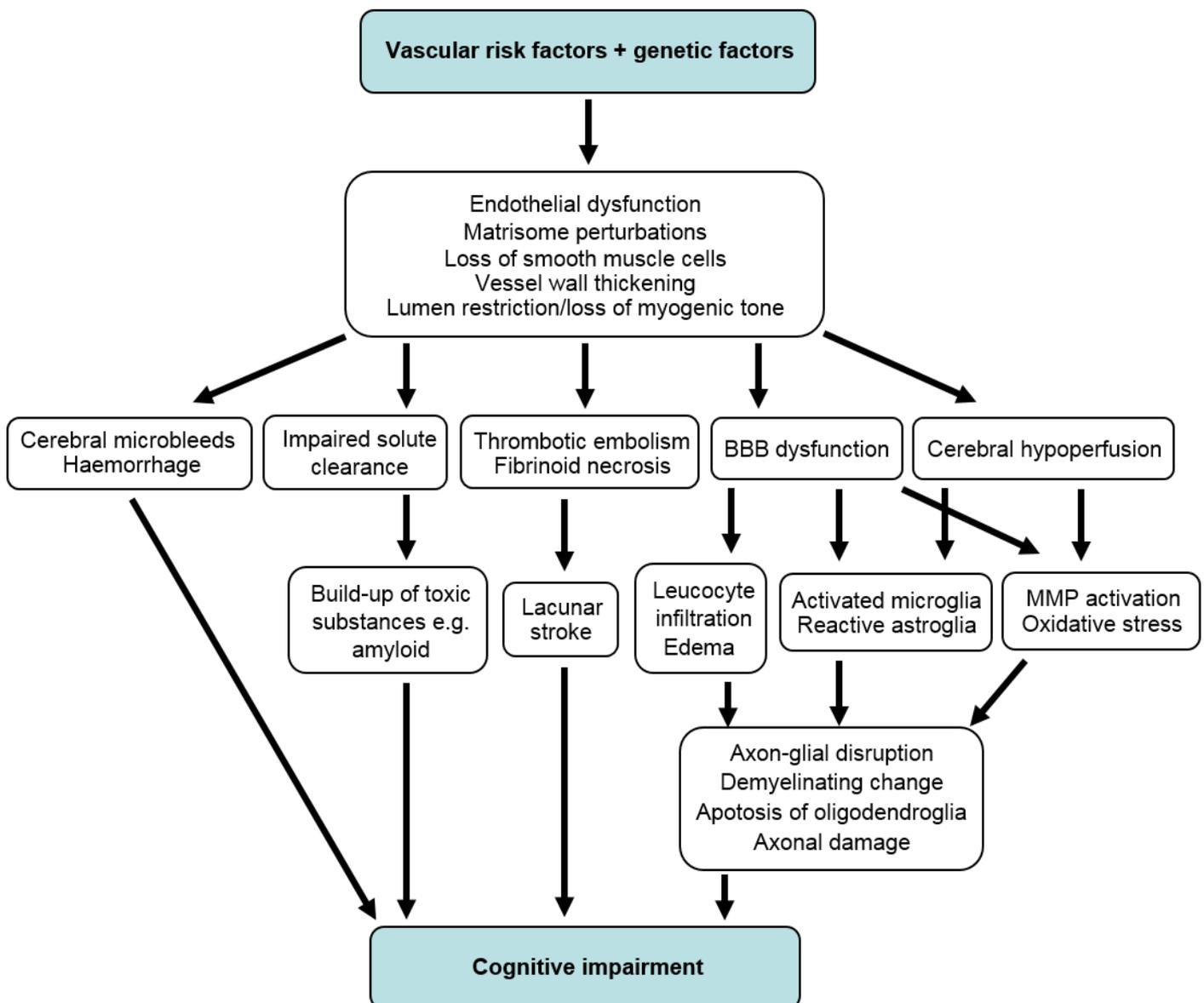
Both vascular and Alzheimer's disease pathologies appear to work synergistically to promote neurodegeneration and cognitive impairments [37]. Further, there is considerable overlap between risk factors for cerebral microvascular disease, cardiovascular disease and Alzheimer's disease, and yet the synergies between these areas are so far largely unexplored in models. Animal models could provide a powerful basis to tease out molecular interactions. Amyloid is believed to be a key driver of the pathophysiology of AD so most work has focused on models that accumulate amyloid in the brain. Rodents do not naturally develop and deposit amyloid, but genetically modified mice were developed that harbour mutations in human amyloid precursor protein associated with familial forms of AD (TgAPP) and that lead to age-dependent accumulation of amyloid. The study of these TgAPP models revealed that a prominent cerebrovascular dysfunction occurs prior to the onset of amyloid deposition, which would be consistent with impaired vasoreactivity and reduced CBF seen in AD patients [37]. TgAPP models are now being probed to study interactions with risk factors such as hypertension and high fat diet which in general have been shown to exacerbate the cerebrovascular dysfunction and progression of amyloid pathology [37,38].

Across the various models, vascular haemodynamics and NGVU coupling are impaired [see 28] and related to breakdown in cell-cell communication and signalling within the NGVU and supporting ECM. Whilst animal models are limited in their ability to fully recapitulate human SVD, they remain pertinent to tease out specific questions that are impossible to address in human studies and to identify molecular targets.

MECHANISMS RELEVANT TO CEREBRAL VASCULAR DISEASE

Figure 2: Mechanisms relevant to cerebral vascular disease

An outline of mechanisms relevant to cerebral vascular disease and VCI. These mechanisms were highlighted during the workshop and described in more detail in the text. In general, downstream of risk factors, a cascade of key events are indicated, of which some may be considered as primary events (eg endothelial changes, inflammation, oxidative stress) and may also be bidirectional, leading to cognitive impairment. (Abbreviations: BBB, blood brain barrier, MMP, matrix metalloproteinase)



a) Endothelial-related mechanisms

The exact molecular and cellular mechanisms underlying cerebral microvascular disease remain elusive, but endothelial dysfunction seems to be especially important. Cerebral endothelial cells regulate vascular tone and blood flow, protect against thrombosis, inflammation and fibrosis, control exchange across the blood-brain barrier, modulate innate immunity and influence clearance of amyloid peptides [39,40]. In addition, and unique to the cerebrovascular system, the microvascular endothelium is critically involved in blood brain function and neuroprotection, through autoregulatory mechanisms that protect against barotrauma. Consequences of cerebrovascular autoregulatory dysfunction include structural injury to capillaries, microvascular rarefaction, exaggerated disruption of the blood brain barrier, neuroinflammation, neurodegeneration and increased susceptibility to intracerebral haemorrhage. This link between impaired autoregulation and downstream microvascular injury has been demonstrated experimentally and clinically.

Endothelial-cell (EC) signalling is mediated through production of numerous endothelial cell-derived vasoactive agents, including nitric oxide (NO), endothelium-derived hyperpolarizing factors (EDHF), endothelium-derived relaxing factors (EDRF), eicosanoid mediators (EETs, HETEs), prostaglandins, and endothelin-1 (ET-1) [41,42]. Endothelial-derived NO is not only a potent vasodilator, but also inhibits platelet aggregation inflammation, apoptosis, fibrosis and vascular smooth muscle cell proliferation as well as modulates mitochondrial function, neuronal metabolism and synaptic transmission [43]. Cerebrovascular endothelial cells possess reactive oxygen species (ROS)-generating enzymes, such as NADPH oxidases (Nox) and mitochondrial oxidases that produce superoxide and hydrogen peroxide, important in redox signalling and endothelial function [44,45]. Because the endothelium is in direct contact with blood flow, it 'senses' haemodynamic changes and adapts accordingly through production of EC-derived factors, such as NO, EDRF and prostacyclins to induce vasodilation and prevent vascular damage or ET-1 and 20-hydroxyeicosatetraenoic acid to promote vasoconstriction. At the capillary level, endothelial cells are in direct contact with neurons via astrocytes and as such there is 'cross-talk' between vascular and neural cells through NGVU coupling, important in regulating neural activity and cerebral function [46].

Ageing, cardiovascular disease and ischaemia/hypoxia are associated with endothelial damage that impacts microvascular function, moment-to-moment adjustment of regional blood flow and neuronal function [47]. Molecular mechanisms involved include reduced microvascular eNOS-derived NO production with consequent decline in cerebrovascular, neuronal, astrocyte, microglial and angiogenic function. Mitochondrial damage, production of arachidonic metabolites and increased oxidative stress are also important. Decreased NO bioavailability in microvascular endothelial cells increases expression of amyloid precursor protein (A β PP) and β -site A β PP cleaving enzyme 1 (BACE-1) as well as A β formation, suggesting a role for endothelial dysfunction in neurodegenerative pathologies, such as Alzheimer's disease [48,49]. Moreover chronic reduction of NO generation and increased production of pro-inflammatory cytokines (TNF- α , IL-1 β , interferon γ) increases endothelial permeability, transmigration of pro-inflammatory cells and blood brain barrier dysfunction, promoting neuroinflammatory and neurodegenerative processes [50,51].

Increased ROS production in the cerebral microvasculature is due primarily to hyperactivation of Noxs (particularly Nox2 and Nox4) [52-54], but also to disruption of mitochondrial respiratory chain oxidases, decreased anti-oxidant capacity, reduced nuclear factor erythroid 2-related factor 2 (Nrf-2) activity, uncoupled eNOS, and increased levels of asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor [55,56]. Vascular oxidative stress, activation of endothelial cell cation channels, such as transient receptor potential (TRP) melastatin 2 [57] and TRP vanilloid 4 cation channels [58], and induction of calcium and redox-sensitive signalling pathways promote oxidative

modification of lipids, proteins and DNA causing endothelial, pericyte and neuronal cell injury [59]. Overexpression of the anti-oxidant superoxide dismutase -1 rescues cerebral endothelial dysfunction associated with neurodegeneration [60], while resveratrol treatment improves cerebrovascular endothelial function, reduces Nox activity and rescues NVGU coupling in aged mice [61].

Thus, endothelium-mediated mechanisms of cerebro-microvascular dysfunction involve a complex network of factors and systems. There is important interplay between molecular and cellular elements that regulate cerebral blood flow, blood brain function and the microvascular endothelium. Perturbations of these interconnections promote cerebral hypoperfusion, blood brain barrier damage and endothelial dysfunction, with consequent neurodegeneration and clinical manifestations of cognitive decline and vascular dementia. To date there are still no therapies to target the microvascular endothelium and hence management of these patients remains sub-optimal.

b) White matter, inflammation and hypoxia

Demyelinating lesions in the CNS white matter are a feature common to both small vessel disease (SVD) and multiple sclerosis (MS). MS has been studied in much greater detail than SVD, raising the possibility that knowledge gained in MS may help in understanding the demyelination in SVD. Demyelinating MS lesions have been classified into four types depending on their morphological features during lesion development [62]. Two types, Patterns I and II, have morphological features consistent with an autoimmune aetiology, but a third type, Pattern III, has features suggesting an important role for tissue hypoxia, based on the similarity with demyelination in the penumbra around ischaemic foci. Since demyelination in SVD is likely to involve tissue hypoxia, it may be instructive to consider the factors determining the formation of Pattern III lesions in MS.

Our understanding of Pattern III demyelination has been greatly advanced by the introduction of a model lesion formed in the rodent spinal white matter by the direct intraspinal injection of the pro-inflammatory agent lipopolysaccharide (LPS) [63]. The lesion has been validated as an accurate model for Pattern III lesions in MS [62], and it results from innate, rather than autoimmune, mechanisms [65]. A surprising feature of the experimental Pattern III lesion is its location, because it does not form precisely at the site of injection (in this case in the middle of the dorsal columns), as is usual for demyelinating agents, [66,67] but rather it forms near the base of the dorsal columns, and after a delay of 7-10 days from the time of injection [68]. It is as if the LPS does not directly cause the demyelination, but rather sets in motion a train of events that culminates in the lesion forming at a nearby site. This peculiarity focusses attention on the features at the base of the dorsal columns that make it vulnerable, and these include a paucity of blood vessels, and location at a watershed between three arterial trees formed by long, fine, end arteries. All these features render the site particularly vulnerable to hypoxia, especially if the blood supply is impaired. The hypoxia kills the most vulnerable cells first, and these include the oligodendrocytes, resulting in the demyelination observed. The suspected key role of tissue hypoxia in causing Pattern III demyelination has been confirmed by the observation that breathing raised oxygen avoids tissue hypoxia, and prevents the demyelination [68].

In summary, impaired vascular perfusion, as occurs in cerebral SVD and MS, appears to cause tissue hypoxia that is most severe at sites rendered vulnerable by the vascular architecture, notably at poorly vascularised sites located at vascular watersheds, such as those in the periventricular white matter. The most vulnerable cells, oligodendrocytes, die first, resulting in demyelination, but the tissue can be protected from demyelination, in rodents, if hypoxia is avoided by breathing air enriched with oxygen.

c) Microvascular extracellular matrix and small vessels diseases of the brain.

The extracellular matrix (ECM) of cerebral blood vessels, and especially the basement membrane, is a key component of the NGVU that occupies a very strategic location, at the interface between the cerebral microcirculation and astrocytes [35]. Proteins constituting or associated with the ECM, referred to as “the matrisome”, have not only structural but also biochemical and signaling roles [69]. Hence, any change in the microvascular matrisome could have profound impact on the brain parenchyma. A common feature in both monogenic and sporadic forms of SVDs of the brain is the presence of major remodeling of the brain microvascular ECM, with prominent fibrosis, associated with severe degeneration of vascular smooth muscle cells [35]. Over the past ten years, genetic studies have revealed that most monogenic forms of SVDs are caused by mutations in genes encoding matrisome proteins, namely dominant mutations in the coding region of $\alpha 1$ (COL4A1) or $\alpha 2$ (COL4A2) chains of collagen type IV, a major component of vascular basement membranes, in COL4A1/COL4A2-related hemorrhagic stroke [70,71], dominant gain-of-function mutations in the 3'UTR of COL4A1 in Pontine Autosomal Dominant Microangiopathy with Leukoencephalopathy (PADMAL) [72], recessive or dominant loss-of-function mutations in HTRA1, a serine protease regulating the TGF β pathway, in Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CARASIL) and HTRA1-related SVDs respectively [73-75], and dominant mutation in Cathepsin A in Cathepsin A-related arteriopathy with strokes and leukoencephalopathy (CARASAL) [76]. Moreover, recent work established that cerebrovascular manifestations of CADASIL, the most frequent hereditary SVD and the most aggressive vasculopathy, are driven by perturbations of the microvascular matrisome, as a consequence of an abnormal accumulation of the extracellular domain of Notch3 receptor [77,78]. Specifically, elevated levels of tissue inhibitor of metalloproteinases 3 (TIMP3), an ECM regulator, blunts the activity of the ADAM17/HB-EGF/(ErbB1/ErbB4) pathway leading to the upregulation of voltage-gated potassium channels subfamily 1 in cerebral arterial myocytes, thereby attenuating myogenic responses in brain arteries and compromising cerebral blood flow regulation in CADASIL [79].

Yet, much work remains to be done to understand in detail the mechanisms linking perturbations of the microvascular ECM and cerebrovascular manifestations in these hereditary SVDs. Remarkably, these recent findings suggest that similar mechanisms may be at work in sporadic SVDs. Therefore, work is needed to characterize the 'prefibrotic/transitional changes' of the brain microvascular matrisome in response to age or hypertension, two major risk factors of sporadic SVDs, and investigate their contribution to disease initiation. Also, with respect to fibrosis of small vessels in the brain, major gaps remain in our understanding of the mechanisms initiating the fibrosis response, in identifying the molecular and cellular mediators as well as the effector cells, and in understanding the relationship with SMC degeneration. Improvement of animal models of SVDs are needed to address these gaps.

d) Intramural periarterial drainage pathways

Arteries that supply the brain are vital for cerebral blood flow but they also play a major role in the drainage of interstitial fluid (ISF) from the brain. As arteries penetrate the brain from the subarachnoid space, they acquire a layer of pia mater that separates the arterial wall from the glia limitans. After injections of particulate and soluble tracers into the subarachnoid space, tracers are entering by glymphatic/convective influx within the arterial pial-glial basement membranes of cortical arteries [80]. When soluble fluorescent tracers are injected into the brain parenchyma, they rapidly drain out of the brain with ISF along basement membranes in the walls of cerebral capillaries and basement membranes surrounding smooth muscle cells of arteries, a process named intramural periarterial drainage (IPAD)

[81]. Changes in arterial basement membranes related to genotype (apolipoprotein E4), age, or blockage due to immune complexes lead to a failure of intramural perivascular drainage pathways [82,83]. A recent study on a canine brain using electron microscopy demonstrated that all arterioles are enveloped in one complete layer of leptomeningeal cells, often with a second incomplete layer in the white matter, whereas venules in the white matter only occasionally possessed one layer only of leptomeningeal cells [84]. It is possible that dilated perivascular spaces observed in the white matter are created between the two adventitial layers of pia mater as a result of the blocked drainage pathways in the overlying grey matter [85]. The extensive fibrosis and hyaline change seen in small vessel disease disrupts not only the basement membrane pathways carrying ISF out of the brain parenchyma but is also likely to contribute to the stiffening of the artery walls that would also impair drainage of ISF from cerebral white matter. Clarifying the key factors involved in the impairment of perivascular drainage of ISF such as the role of vascular smooth muscle cells and modulators of extracellular matrix are essential therapeutic strategies. It is currently unknown whether there is a pattern of anatomical or topographical changes in the cerebrovascular basement membrane and perivascular compartment architecture in brains susceptible to different degrees or subtypes of vascular dementia.

e) The glymphatic fluid transport system (and interactions with amyloid)

Lymphatic circulation is essential in peripheral organs for the removal of excess fluid and metabolic waste products; but the brain, in spite of having the highest metabolic activity of all tissues, has no conventional lymphatic system. A fundamentally novel pathway for fluid transport has recently been identified: the glymphatic system consisting of a periarterial cerebrospinal fluid (CSF) influx path and a perivenous interstitial fluid (ISF) clearance route, which are coupled through convective interstitial bulk flow supported by astrocytic AQP4 water channels [86]. It is designated the “glymphatic system” because it operates analogously to the peripheral lymphatic system and the CSF/ISF fluxes depend on astroglial AQP4 channels [87]. This brain-wide pathway serves as a waste disposal system of fluid, as well as larger solutes and proteins, such as amyloid and tau, from the CNS. Recent work has extended the glymphatic concept to show that outflow occurs along both cervical and meningeal/pial lymphatic vessels [88,89]. An interesting observation is that the sleep-wake cycle regulates glymphatic activity, which is primarily active during sleep, or the use of some anesthetic agents [90]. Glymphatic drainage is similar to lymphatic depending upon position, and the lateral and supine positions, which are the most popular sleep positions among human and most animal species, appear to be superior for amyloid-beta clearance compared with the prone position [91]. Ageing is linked to a sharp decline in glymphatic activity in wildtype mice [92]. Moreover, APP/PS1 mice – a murine model of AD exhibited reduced glymphatic activity in young mice without amyloid plaques as well as in older mice with plaques. Mice with multiple mini-strokes induced by intracarotid injection of cholesterol crystals similarly showed a global decrease in glymphatic function [93]. Also, traumatic brain injury triggered a sustained suppression of glymphatic activity [94], whereas middle-aged rats with diabetes exhibit increased influx, but reduced clearance, of contrast agents in an MRI study [95]. The latter observation can best be explained by trapping of the contrast agent within the neuropil due to pathological changes in the extracellular matrix and reactive gliosis, since the volume of fluid influx and efflux must match each other. Very recent small studies, one with Gadolinium injected into the CSF, the other using a novel MR technique (MREG), provided interesting evidence for the existence of glymphatic fluid transport in the human brain [96,97]. Altogether, glymphatic function has been shown to be altered in all disease states studied so far.

GAPS IN KNOWLEDGE AND REQUIREMENTS TO ADVANCE KNOWLEDGE

Following the focussed lectures the participants assembled in small groups and discussed the gaps in the different research areas and opportunities for development which are summarised in Table 1. There were several key themes to emerge from the discussion groups which are highlighted.

INSERT TABLE 1

METHODOLOGICAL APPROACHES

a) Modelling the neurogliovascular unit in vitro

The neurogliovascular unit (NGVU) is arguably the most elementary representative functional unit of the brain and specific alterations in the NGVU underpin much of the pathophysiology of cerebrovascular disease. The ideal in-vitro model will depend upon the particular use and research question to be addressed. However, to probe disease mechanisms, attempts should be made to recapitulate the complex micro-environment of the NGVU, including the key cell types and the extracellular matrix. The constituent cells should also display mature properties of the NGVU such as the specialisation of endothelial cells to impart effective BBB function, specific transport mechanisms and the requisite cell-cell interactions including those that underpin NGVU coupling. NGVU models should be robust, yield reproducible results and be scalable if they are to be incorporated into drug screening programs. Current models can be broadly divided into easily adopted static compartment models such as Transwell cultures and more sophisticated flow models based upon a microfluidic design [98]. The latter have emerged in recognition of the importance of flow and shear stress of maintaining NGVU properties.

More than 80% of drug candidates tested in animal models for stroke, and a large proportion so far of drugs tested for AD, have failed in clinical trials. Better in-vitro mimics of human systems might help increase the likelihood of successful drug discovery. Notably there have been few trials to date of any drugs for SVD, therefore it would be premature to level the same criticism at human microvascular disease. Nonetheless, human stem cell technologies offer the opportunity to generate patient specific NGVU unit models for improved drug discovery and personalized medicine [99]. Differentiation protocols have been described that would produce all the constituent cells of the NGVU [100-102]. However further work is required to demonstrate the reproducibility of these methods and the ideal co-culture conditions.

NGVU in vitro models should enable assessment of physiological and pathophysiological mechanisms that would be difficult to dissect in vivo. Molecular 'omics' technologies in particular provide a means to evaluate disease processes in an unbiased approach and identify new drug targets. However, molecular studies require careful planning as they can be subject to significant technical confounds and non-relevant biological processes which are only beginning to be fully understood. Conducting studies with sufficient samples to allow data normalisation methods and remove nuisance variance is important. Single cell 'omics' are also a powerful technique to deal with heterogeneity in cell culture models as well as reveal cell-specific disease processes. These recent advances in tissue engineering, stem cell methods and novel 'omics' platforms are anticipated to enable unprecedented mechanistic insights into NGVU brain disorders such as dementia [103].

b) Measuring behavioural outcomes in animal models

In human studies, different cognitive domains can be assessed with a battery of visual touchscreen-based tasks using similar stimuli and responses, thus facilitating between-task comparison and potentially reducing confounds [104-106]. Conversely, in the rodent, comprehensive cognitive batteries are seldom employed, and different cognitive domains are assessed by tasks that typically vary widely in the nature of stimuli, responses, reinforcers, and testing environment. Moreover, these tasks often bear no resemblance to the tests used with humans. Finally, the measurement of rodent behaviour is notoriously variable across labs and experimenters [107]. These factors reduce the likelihood of rodent and human studies assessing consistent and comparable cognitive functions, thus compromising the efficacy of translation. There is a recognised need to share standardised operating procedures for common outcomes and to improve translation.

One approach to addressing these issues is by using a touchscreen-based cognitive testing method for rodents, which has the potential to achieve more accurate, efficient, and reproducible phenotyping of rodents, and help bridge the translational divide between animal and human studies of cognition. This method uses an automated operant chamber with a computer monitor for the presentation of visual stimuli and an infrared touchscreen assembly to record the animal's responses. The apparatus allows for the flexible presentation of visual stimuli at any location on the screen. Rats or mice respond directly to the stimuli by breaking the infrared beams overlaying the touchscreen with their nose. Appetitive reinforcers such as strawberry milkshake are delivered in a reward magazine to the rear of the chamber.

An extensive battery of tests using this touchscreen cognitive testing apparatus has been developed and validated. The tasks closely parallel human tests and have high translational face validity. Although face validity does not guarantee translational neurocognitive validity (i.e., the same cognitive constructs and circuits mediating the tasks across species), minimizing methodological differences enables back- and forward-translational opportunities and improves the likelihood of neurocognitive validity of touchscreen tasks [108-111]. Touchscreen-based tests now form a core component of recommended cognitive test batteries for models of schizophrenia (e.g., NEWMEDS and CNTRICS) and AD (e.g., PHARMACOG). The system is presented as a key method in the standard guide to behavioural testing for mouse researchers [112], and protocols for an extensive battery of tests have been published [113-115].

c) Experimental Design

Findings from in vivo research may be less reliable if those studies do not adopt and report measures to reduce the risk of bias in the experiments reported. The experimental stroke community have been at the forefront of implementing changes to improve the conduct and reporting of studies, but the recent impact of these efforts is not clear. A systematic identification of recent literature was conducted describing animal experiments inducing middle cerebral artery occlusion or lacunar stroke, and text mining approaches developed automatically to ascertain risk of bias reporting from full text articles [116]. There were substantial improvements in the reporting of middle cerebral artery occlusion studies since the first systematic report in 2007 [117]. However, in reports of experiments in lacunar

stroke there was no substantial improvement since the first systematic report of the field to 2012 [118]. This may in part reflect that there has been less work on SVD models. The accuracy (true positive plus true negative as a percentage of the total) of automated risk of bias annotation ranged from 67% (randomisation, lacunar stroke) to 100% (sample size calculation, middle cerebral artery occlusion). There therefore remains substantial opportunity for improvement in the reporting, and probably the conduct, of animal research modelling stroke, particularly lacunar stroke. Automated tools are sufficiently accurate to identify whether studies report the blinded assessment of outcome, but improvements are required in the tools to ascertain whether randomisation and a sample size calculation were reported.

d) Multi-centre pre-clinical studies

There are many steps in the translational pathway from original mechanistic discoveries through to successful translation of a therapy to the clinic. Progress in this pathway has been poor across a range of CNS diseases despite increased knowledge of the mechanistic basis of diseases, identification of drug targets and positive drug studies using in vitro and in vivo models. This failure to translate led to criticism of the quality of in vivo drug study design and reporting, and the publication and uptake of guidelines (e.g. STAIR, ARRIVE, RIGOR, IMPROVE) [119-123] to improve the translational potential of pre-clinical studies. However, single centre studies, although useful for proof of concept of a particular therapy, are unlikely to be rigorous enough to predict the therapies which will also be effective in man. What is needed is an additional step in the pathway where promising therapies which demonstrate efficacy in a number of single centre studies are then tested more rigorously on a multi-site platform. Multi-PART (Multicentre Preclinical Animal Research Team, <http://www.dcn.ed.ac.uk/multipart/>) is such a platform, set up with funding from EU FP7 for ischaemic stroke. However, the structure of the platform could easily be adapted for multi-site studies for any CNS disease.

The key objective of Multi-PART was “to implement and establish a platform for international multicentre preclinical stroke trials using the repertoire of randomised clinical trial design and the complexities of a multicentre, multimodel paradigm”. This was made possible by the experience within the Multi-PART team of scientists and clinicians with expertise in a range of stroke models and outcome measures, regulation and ethics of in vivo research, precision, reproducibility & external validity in animal research, design and running of clinical trials, statistics, database and web site management, good laboratory practice (GLP), and standard operating procedures (SOPs), development & costing structures etc. The design of the Multi-PART platform also benefitted from improvements in clinical trial design by incorporating strategies to minimize bias, biostatistical advances, data monitoring and auditing, etc.

Multicentre pre-clinical randomised control trials (pRCTs) in models relevant to SVD and VCI could be performed after single centre laboratory studies and a systematic review of all existing data, prior to clinical testing. The need for pRCTs reflects the repeated failure of translation from preclinical to clinical development and lack of reproducibility between laboratories. Since new healthcare interventions require one or more positive large multicentre clinical RCTs prior to introduction, pRCTs should largely follow the design of phase III clinical RCTs involving randomisation, treatment and outcome blinding [124-126]. Coordination would need a Trial Steering Committee and central database for management of randomisation and data storage. Outcomes would need central adjudication of imaging (e.g. MRI, histology for lesion volume, microbleeds) and videos (behavioural & functional testing). Studies can utilise advanced trial techniques such as adaptive design, randomisation, and statistical analysis, and the concept extended to designing observational and characterisation studies across multiple laboratories.

Key differences exist between preclinical and clinical RCTs, many of which could be ameliorated by adopting specific design elements in preclinical RCTs. Since patients are very heterogeneous, a variety of SVD models would be needed. Preclinical RCTs may need to incorporate both positive and neutral control comparator groups. Classically, trials have a single primary outcome but co-primary outcomes, composite outcomes, or statistical integration of several outcomes are alternatives that may be preferred. The Data Monitoring Committee will assess futility during the course of the study with the aim of stopping further development if data are not positive, thus preventing the need for further preclinical and clinical testing, and their considerable expense. Conversely, a positive multicentre pRCT will considerably enhance the justification for proceeding to clinical development.

Changing the views of industry, funding agencies and grant reviewers may be necessary to overcome perceptions that pRCTs are incremental and not novel or ground-breaking. For large pRCTs, authorship should be discussed prior to commencing studies and presents a potential issue for many basic science academics where decisions on hiring, promotion and returns on the research excellence framework (REF) are taken on publications and author position.

e) **Biomarkers**

Biomarkers can give insights into pathogenesis and suggest potential drug-able targets. They can inform prognosis and be used to 'enrich' trial populations with participants who are most likely to have outcomes of interest such as progression to dementia. Biomarkers can be used as surrogate outcome measures for phase II intervention trials, to screen treatment prior to large and expensive definitive phase 3 trials. Finally, biomarkers can be used for diagnostics. In this regard researchers were mindful of the continued debate around the value of biomarkers in diagnosis of Alzheimer's disease (AD) [127,128]. The necessary properties and performance characteristics of a biomarker will vary with the proposed purpose.

MRI modalities were regarded as offering the greatest utility as SVD biomarkers. A number of different MRI markers of SVD may represent useful markers of disease including recent small subcortical infarcts, lacunes, white matter hyperintensities, brain volume, and white matter ultrastructure estimated on diffusion tensor imaging (DTI) or other quantitative methods [129,130]. Increasingly sophisticated approaches to MRI are moving beyond gross anatomy to assess function and connectivity using approaches such as network analysis and these show promise in the mouse pre-clinical hypoperfusion model to predict outcome [131] but need further evaluation in prospective patient cohorts [132]. Computerised Tomography (CT) has value, particularly as it is routinely available but lacks sensitivity to early SVD change. For imaging, there is no suitable SVD target for radiolabelling that is analogous to amyloid imaging although PET approaches may be useful to assess parts of the pathophysiological process such as neuroinflammation. For tissue based markers, use of cerebrospinal fluid (CSF) is under-researched in SVD in direct contrast to its importance in AD. However, the dynamics of the exchange between the peptides in the interstitial fluid of the brain and the CSF and their routes in the perivascular compartment still require investigation. To date no blood based biomarker has shown utility, although with 'omics' based technologies new markers may be discovered. Electroencephalogram (EEG) has a role in certain neurodegenerative diseases but has limited application in SVD.

External validation, replication and standardisation of putative biomarkers is critical particularly for multicentre work. At present there is no SVD biomarker suitable for clinical use. Future trials and observational cohorts should create biobanks of tissue (blood, CSF) and imaging data that can be interrogated to validate existing biomarkers or discover potential new markers. If biomarkers are to be used as reliable surrogate markers in clinical trials, they must fulfil the following criteria: (1) they must be able to predict clinical outcome, as changes induced by a therapy on a

surrogate marker are expected to reflect changes in a clinically meaningful end point; (2) change in a surrogate marker must be detectable prospectively; and (3) the sample size required to show therapeutic efficacy should be feasible in the setting of a clinical trial [133].

f) **Sharing, model exchange and potential tissue banks**

There is a growing need to address standardisation across models, as far as this is possible. This could include standardised protocols for surgery, behavioural testing and clearly defined genetic details in relation to models. Central facilities for provision of genetically modified mice would reduce the genetic drift inherent in many of the frequently used models. However, their utility is dependent on phenotypes. Human tissue banks are well established and provide a useful source of translational material. The development of a tissue bank for preclinical models may be a useful resource which could range from cut sections of tissues through to whole organs with standardisation of pathological approaches. Potential issues in relation to animal licensing (eg UK Home office) is acknowledged. Tissue banks may facilitate validation of published work in a low cost way, by providing tissues prepared by one published group for laboratory studies to be undertaken by a second group in a remote laboratory. An open access resource where data could be deposited would be particularly useful for negative studies that may not otherwise be published.

INDUSTRY PERSPECTIVE ON THE WORKSHOP

Independent of the shape of future research efforts there are a number of critical areas that need to be kept in mind that will help facilitate drug discovery and reduce the overall level of risk. Firstly, the generation of high quality targets/ pathways with strong supporting neuropathological, genetic, and/ or epidemiological data. This will require the generation and interrogation of well characterised cohorts, using best practices for data generation and sharing that can be found in other pre-competitive settings; these cohorts will be additionally important for longitudinal tracking of disease and the identification of potential biomarkers that can be used to make early Go/NoGo decisions within drug development programs.

Secondly, there should be a strong focus on generating a robust translational pharmacology package to bridge between preclinical models and clinical development. A number of basic principles underpin this area: understand the temporal expression of target in both the model and the human disease; match the preclinical intervention paradigm to the intended therapeutic intent in the clinic (i.e. prevention versus treatment); demonstrate in preclinical studies drug exposure at the site of action/ target engagement, expression of downstream target pharmacology and importantly generate exposure- response relationships (e.g. EC50 values).

Finally, as far as possible standardise procedures/ protocols to reduce variability and improve reproducibility of preclinical studies. With this in mind the emergence of the EU-funded MultiPART initiative provides a network capability for the conduct of randomised preclinical studies in experimental models relevant to SVD. Together these approaches will all serve to “de-risk” clinical development and encourage further industry and academic investment in SVD research in dementia and stroke.

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CONFLICTS OF INTEREST

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Table 1: **Gaps in knowledge and requirements to advance knowledge**

A summary of outcomes of discussion groups at the workshop are outlined. Several key mechanisms, the gaps in knowledge and requirements for early advances in knowledge are highlighted. (Abbreviations: AD, Alzheimer's disease; BBB, blood brain barrier; CBF, cerebral blood flow; CSF, cerebrospinal fluid; DTI, Diffusion Tensor Imaging; EEG, electroencephalogram; ISF, interstitial fluid; MRI, Magnetic Resonance Imaging; MMP, matrix metalloproteinase; NVU, neurovascular unit; SVD, small vessel disease; TIMPs, tissue inhibitor of metalloproteinase; VCI, Vascular Cognitive Impairment; VaD, Vascular Dementia)

Mechanism	Gaps in knowledge	Requirements for early advances in knowledge
Common to all mechanisms	<ul style="list-style-type: none"> • The most relevant model for human SVD is unclear • Regional variation in CBF, in vulnerability to low flow, hypoxia or inflammation, and in flow-metabolism coupling, in grey and white matter (periventricular and deep), in normal tissue and in SVD, is not well established. • Influence of regional differences (e.g. in arteriolar morphology between lenticulostriate and cortical perforating arterioles) in microvessel and perivascular structures on lesion development is poorly understood. 	<ul style="list-style-type: none"> • Use different models to recapitulate components of SVD appropriate to the specific research question • Better <i>in vitro</i> and <i>in vivo</i> models of BBB function and dysfunction, including under conditions of shear stress, in cell co-cultures (3D >2D) and including the glycocalyx • A reliable 'neuro-glio-vascular unit on a chip' would help accelerate understanding of pathophysiological mechanisms, identify targets and allow high throughput testing of potential interventions; • Better molecular imaging probes to study different NVU cell types, determine molecular tissue changes and probe inflammatory processes; • Use larger mammalian models, eg well characterised porcine models, since rodents have low white matter:grey matter ratio.
BBB	<ul style="list-style-type: none"> • A dynamic complex structure whose dysfunction is a common feature of neurological diseases including cerebral SVD and related dementia syndromes (e.g. AD and VCI/VaD). • Lack of understanding of: <ol style="list-style-type: none"> a) how cellular morphology relates to function b) the temporal changes and sequence of damage c) how BBB dysfunction leads to brain parenchymal lesions. 	<ul style="list-style-type: none"> • Core set of reference standard techniques for preclinical, neuropathological and clinical studies; • <i>In vivo</i> and <i>in vitro</i> methods to model small and transient BBB changes; • More sensitive neuropathological methods to identify WMH as seen on MRI; • New molecular imaging probes for pericytes and endothelial cell function status; • More use of the retina routinely in rodent models (as in human SVDs) could help visualise arteriolar/venular, retinopathic and nerve fibre layer changes that could advance understanding of brain changes in rodent models (as suggested in human SVDs); • Alternatives to gadolinium for human <i>in vivo</i> BBB imaging.
Hypoperfusion and ischaemia	<ul style="list-style-type: none"> • Often poor characterisation in models • Poor understanding of the influence of structural differences in perforating arterioles in different brain regions and arterial territories. • The primary trigger, whether inflammation, 	<ul style="list-style-type: none"> • Longitudinal investigations in models • Better characterisation of tissue metabolic changes (eg pO₂, glucose, metabolites) (particularly white matter); • More standard use of terminology for SVD in <i>in vivo</i> models and neuropathology, and for studying components of NVU <i>in vitro</i> would help, as

	<p>altered flow, other, and their interactions.</p> <ul style="list-style-type: none"> • Is flow-metabolism coupling altered in SVD? 	has been implemented in clinical studies [3]
Inflammation	<ul style="list-style-type: none"> • How do microglia, pericytes, perivascular macrophages, fibroblasts, oligodendrocytes all interact to maintain homeostasis? • Are soluble inflammatory mediators (complement, chemokines, cytokines) involved in perivascular inflammation in SVD? • What is the role of fibrosis in SVD? • How is the matrix environment perturbed to influence cell function and signalling? 	<ul style="list-style-type: none"> • Determine the contribution of inflammation in SVD and define whether there is a classic innate response and/or contribution of systemic inflammation or both • Undertake longitudinal studies across models to profile microglial proliferation and migration and the broader molecular/cellular neuroinflammatory environment including measures of circulating immune cells. • In relation to above a wider investigation of the 'matrisome'
White matter: axons, myelin sheaths	<ul style="list-style-type: none"> • The role of oligodendrocytes and myelinated axons in the pathogenesis of SVD is unclear as is their interaction with other cells, such as endothelial cells. • The extent of damage to myelinated axons in cortical grey matter in SVD is poorly defined • The relationship of demyelination and axonal loss to CBF changes in SVD is unclear 	<ul style="list-style-type: none"> • Comparative studies of potential SVD mechanisms across models, species, and diseases of white matter that have potential similarities to SVD (eg multiple sclerosis) using the most innovative molecular, cellular and imaging approaches • Use MRI/DTI of white matter status as routinely as possible to link in vivo to tissue pathology more clearly; • Link white matter pathology stages and burden to vascular pathology load and CBF alterations more clearly • Investigate integrity of architecture of myelinated axons in cortical grey matter and white matter
Perivascular and interstitial fluid drainage	<ul style="list-style-type: none"> • To what extent are rodents relevant models of human drainage pathways and mechanisms? • What is the driving force for ISF clearance: <ul style="list-style-type: none"> a) vasomotion of cortical arteries?; b) perivascular innervation? c) does the smooth muscle action in veins have a functional role? • How does ISF clearance relate to SVD development or progression? • Does extracellular remodelling vary during the sleep-wake cycle? 	<ul style="list-style-type: none"> • Determine how CSF and ISF and clearance pathways all relate to each other; • Determine if ISF and perivascular drainage vary with sleep-wake cycle; • Better methods to detect perivascular and ISF drainage in humans • Can CSF or ISF drainage pathways be targeted to inform treatment for SVD? • Does targeting of matrix proteins (TIMP3-TIMPs or MMPs) or vasomotion increase drainage?
Neuro-glia-Vascular mechanisms	<ul style="list-style-type: none"> • How do vascular changes drive glial and neuronal dysfunction and damage? 	<ul style="list-style-type: none"> • Target white matter more in rodents; • Standardise anaesthetics and stimulation procedures; • Identify better ways to measure neuronal responses than blood oxygen dependent (BOLD) and related imaging methods (arterial spin labelling, blood volume measures, O2 metabolism, EEG and magnetoencephalography);

		<ul style="list-style-type: none">• Expand cohort imaging similar to that achieved in the AD Neuroimaging Initiative;• Make more use of optogenetics and genetically encoded calcium indicators.
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