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The role of pericytes and endothelial cells in small vessel disease: From animal models to human disease

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Doctor of Philosophy

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Declaration

I declare that I have written and composed this thesis and that it has not been submitted, in whole or in part, for any other degree or professional qualification. I confirm that the work presented is entirely my own, except when stated otherwise by reference or acknowledgement, or where work forms part of a published paper authored by several individuals. In instances of joint authorship, I have clearly delineated my own contributions and those of other authors below.

The work presented in **Chapter 2** was published in the International Journal of Molecular Sciences in August 2023 (available at: **https://doi.org/10.3390/ijms241713114**), titled 'A scoping review on biomarkers of endothelial dysfunction in small vessel disease: molecular insights from human studies'. Co-authors were Audrey Chagnot, Joanna Wardlaw, and Axel Montagne. DJG designed the protocol and search strategy under the supervision of AM and JMW. DJG conducted the literature search and reviewed the resulting title, abstracts and full-text articles for inclusion/exclusion. AM and JMW were consulted on articles that were uncertain or on matters of quality control and aided in the interpretation of data. DJG drafted the first version of the manuscript. AC, DJG, and AM helped design the main Figure 2 in the publication and AC constructed the figure. All authors contributed to the review and editing of later versions of the manuscript and all authors accepted the final manuscript.

The work in **Chapter 5** is awaiting final decision from the journal Alzheimer's & Dementia after minor suggested revisions during peer-review as an original research article titled 'Blood biomarkers of endothelial and pericyte function in small vessel disease: A longitudinal study'. Co-athors were Una Clancy, Carmen Arteaga, Maria C. Valdés-Hernandez, Francesca M. Chappell, Angela C.C. Jochems, Yajun Cheng, Junfang Zhang, Michael J. Thrippleton, Michael S. Stringer, Emilie Sleight, Ellen V. Backhouse, Stewart Wiseman, Rosalind Brown, Fergus N. Doubal, Axel Montagne, and Joanna M. Wardlaw. We also

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The work in Chapter 6 is prepared for publication. DJG drafted the original manuscript and thesis chapter. The co-authors are Stephen D.J. Makin, Caroline A. McHutchson, Vera Cvoro, Francesca M. Chappel, Fergus N. Doubal, and Joanna M. Wardlaw. The work was conceptualised by DJG under the supervision of JMW. Mild Stroke Study 2 (MSS2) study methodology was devised by FC, SDJM, CL, CAM, VC, FND, and JMW. MSS2 funding was acquired by FND and JMW, who also managed and supervised the project. Data acquisition was done by CL, SDJM., CAM, VC, DJG, FND, and JMW. Manuscript methodology was developed by DJG, CL, FND and JMW. Images, graphs, and figures were created by DJG. DJG drafted the first version of the manuscript, and all authors contributed to later versions of the manuscript.

The work in Chapters 3 & 4 is being prepared for joint submission to publication. DJG drafted the original manuscript and thesis chapter. The co-authors are Audrey Chagnot, Conor McQuaid, Karina McDade, Justyna Cholewa-Waclaw, Dorota Stefancova, Colin Smith, Joanna M. Wardlaw and Axel Montagne. The work was conceptualised by DJG under the supervision of JMW and AM. Study methodology was devised by DJG, AC, DS, KC, CS, JCW, JMW, and AM. Data acquisition was done by DJG, AC, CM, DS, KM, and JCW. Manuscript methodology was developed by DJG, AM, and JMW. Images, graphs, and figures were created by DJG. DJG drafted the first version of the manuscript, and all authors are contributing to later versions of the manuscript.

Daniela Jaime Garcia

18 December, 2024

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Finally, this thesis is dedicated to the best person I know, my grandfather. I was only starting to study neuroscience when we both first recognised the early signs of what would later progress into dementia. In those early days, I made him a promise; this thesis is the first step in the journey to honour that vow.

Lay Summary

Cerebral small vessel disease (SVD) is a disease affecting the smallest blood vessels in the brain. It is very common, particularly as we age. SVD is one of the main causes of stroke, and it is also the leading cause of vascular dementia, the second most common type of dementia worldwide. It can cause a type of stroke called lacunar stroke, as well as haemorrhagic stroke, and it is also associated with problems relating to mobility, thinking and memory, and changes in behaviour, personality, or mood. Currently, there are no treatments that exist for SVD.

SVD is caused by problems in the blood supply to the brain because of damaged blood vessels, depriving the brain of the oxygen and nutrients it needs in order to function properly. Although we can see signs of SVD on brain scans, we do not know what causes the brain blood vessels to begin to accumulate damage. Identifying how and why the blood vessels become dysfunctional could help uncover potential treatments. In this thesis, I focus on two cell types that we believe play an important role in the development of SVD, the endothelial cells, which make up the lining of the small brain vessels themselves, and pericytes, which are cells that wrap around these vessels and help protect them (and brain cells) and regulate blood flow.

Some studies suggest that as we age or develop SVD, we begin to lose pericytes, which play an important role in protecting endothelial cells. At the same time, endothelial cells begin to express certain proteins which affect their ability to function, leading to a loss of their specialised properties that serve to protect the brain. To determine whether we could observe evidence of this in ageing, we measured brain pericyte blood vessel coverage in both young and ageing mice. We also assessed endothelial dysfunction by measuring levels of vascular cell adhesion molecule-1 (VCAM-1), a protein expressed by dysfunctional endothelial cells. Our results showed a 7.5% reduction in pericyte coverage and nearly double the amount of VCAM-1 in ageing mice compared to younger mice. We then analysed human brain tissue samples

from deceased healthy controls, as well as individuals with mild, moderate, and severe SVD, and found an 85% reduction in pericyte coverage and nearly a three-fold increase in VCAM-1 in moderate-to-severe SVD cases compared to controls. We also observed a potential proportional relationship between pericyte loss and increase in VCAM-1 expression, suggesting a connection between these two processes.

I then aimed to determine whether these pericyte and endothelial cell dysfunctions could be detected in living patients using blood samples and whether they could be related to SVD severity and progression over one year. We found several associations between markers of endothelial and pericyte function in the blood and symptoms or other markers of SVD. For instance, patients with higher levels of Vascular Endothelial Growth Factor (VEGF) in the blood were more likely to have new brain lesions visible on a brain scan over 1 year. In addition, patients with higher levels of P-Selectin were at a higher risk of having thinking and memory problems at 1 year.

Finally, I report the results from a study where we followed-up patients with a mild stroke who frequently have signs of SVD, for 9 years. One in three patients had at least one other stroke since joining the study, and a quarter of all patients had died. About half of patients were struggling with everyday activities and reported experiencing anxiety and/or depression and problems with thinking and memory. The results from this study show us how significant and life-altering the impact of SVD and mild stroke can be on patient quality of life.

This research supports the idea that the dysfunction of two key cell types, endothelial cells and pericytes, plays a major role in the development of SVD and highlights how these cells may interact to influence disease. A major challenge in developing treatments and understanding SVD better, is the lack of specific blood tests to detect and track disease. If these findings are confirmed in larger studies, they could offer an exciting opportunity to connect the physical signs of SVD to hidden biological processes, helping to bridge the gap between lab research and clinical care.

Publications and Conferences

Publications presented in this thesis:

Jaime Garcia, D., Chagnot, A., Wardlaw, J.M. and Montagne, A., 2023. A scoping review on biomarkers of endothelial dysfunction in small vessel disease: molecular insights from human studies. *International Journal of Molecular Sciences*, *24*(17), p.13114.

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Publications I contributed to that were published throughout the course of my PhD:

Cheng, Y., Clancy, U., Arteaga, C, **Jaime Garcia, D.,** Stringer, M., Thrippleton, M.J., Chappell, F.M., Doubal, F., Wardlaw, J.M., et al., 2024, Clinical relevance of 'cap' and 'track' development after recent small subcortical infarct. *Accepted for publication in Annals of Neurology.*

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Conferences: oral presentations

Cerebrovascular Research Group Forum,
Edinburgh, UK, March 2024

UK Dementia Research Institute: Vascular Theme Early Career Researcher Meeting,
York, UK, March 2024
Prize: Best talk

Alzheimer's Research UK Oxford Drug Discovery Institute,
Oxford, UK, February 2024
Lecture

Small Vessel Disease Network Meeting,
Edinburgh, UK, November 2023

Leducq Research Network Meeting,
Kent, UK, September 2023

Leducq Research Network Meeting,
Kent, UK, September 2023

Prize: Best talk

UK Dementia Research Institute: Vascular Theme Early Career Researcher workshop,
Edinburgh, UK, February 2023
chair and moderator for translational fluid biomarker working group

Leducq Research Network Meeting,
New York, USA, February 2023

Leducq Research Network Meeting,
New York, USA, February 2023

European Stroke Organisation Conference,
Munich, Germany, May 2023

UK Dementia Research Institute: Connectome,
Brighton, UK, November 2022

Mechanisms of Small Vessel Disease: Challenge Workshop: Neuroscience School of Advanced Studies,
Crans-Montana, Switzerland, July 2022

UK Dementia Research Institute Board Meeting,
Edinburgh, UK, May 2022

UK Research Institute Board Meeting,
Edinburgh, UK, May 2022

European Molecular Imaging Meeting,
Thessaloniki, Greece, March 2022

11th UK and Ireland Blood-Brain Barrier Symposium,
Online, November 2021

UK Dementia Research Institute Early Career Researcher: dissecting the role of the neurogliovascular unit in disease: pericytes & perivascular spaces,
Edinburgh, UK, November 2021

New horizons in Alzheimer's Disease,
Leuven, Belgium, October 2021

UK Dementia Research Institute: Connectome,
Brighton, UK, October 2021

Conferences: poster presentation

UK Dementia Research Institute: Connectome,
Harrogate, UK, November 2024

European Stroke Organisation Conference,
Basil, Switzerland, May 2024

UK Dementia Research Institute: Connectome,
Brighton, UK, October 2023

European Stroke Organisation Conference,
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Abstract

Cerebral small vessel disease (SVD) is a prevalent disorder of the brain's perforating microvessels and the underlying cause of a quarter of all ischaemic strokes and most haemorrhagic strokes and the commonest cause of vascular dementia. Despite its increasing incidence and importance in the public health domain, the underlying pathophysiology of SVD remains poorly understood and there are currently no available treatments beyond secondary stroke-prevention. An abundance of recently published evidence suggests the involvement of capillary-enwrapping pericyte and brain microvessel endothelial cell dysfunction in SVD pathogenesis, although significant controversy remains, and translational research is required to reconcile what we know about disease mechanisms on the basis of preclinical studies and human studies.

This thesis begins with an introduction to brain pericytes, endothelial cells, and an in-depth exploration into how they potentially contribute to SVD mechanisms. Subsequently, through a scoping review featuring 49 eligible publications, I provide a holistic overview of the existing literature on the molecular mechanisms underlying endothelial dysfunction in SVD, including discussions around gaps in literature and potential sources of inconsistencies amongst study results.

There are discrepancies in the literature regarding pericyte loss and endothelial activation in ageing and in the context of SVD, and the nature of the relationship between endothelial and pericyte function remains to be elucidated. Seeking to address some of these inconsistencies, I quantified pericyte coverage (%) and marker of endothelial activation, vascular cell adhesion molecule-1 (VCAM-1) expression across 9 brain regions in young (n=8, 3 months-old) and ageing (n=8, 12 months-old) mice. We found a 7.5% reduction in global pericyte coverage and more than a two-fold increase in VCAM-1 coverage in aged animals compared to their younger counterparts, after adjusting for sex and brain region using linear mixed effects models. We

also demonstrated a potential inverse relationship between VCAM-1 capillary coverage (%) and pericyte signal intensity, wherein a 10% decrease in pericyte signal was associated with a 0.20% increase in VCAM-1 capillary coverage.

To corroborate the murine findings within a human SVD context, I used *postmortem* human brain tissue from 5 healthy control cases (mean age: 33 years (SD: 9.3)), as well as 3 mild (mean age: 66 (SD: 20)), 3 moderate (mean age: 80 (SD:6.2)), and 5 severe (mean age: 83 (SD:8.8)) SVD cases. We report an estimated 83-86% reduction in pericyte coverage and a 2-to-3-fold increase in VCAM-1 amongst those with moderate-to-severe SVD compared to healthy controls in linear mixed effects models after adjusting for potential covariates, including vascular risk factors. We also demonstrate a significant inverse relationship between VCAM-1 and pericyte signal intensity, wherein every 10% increase in pericyte signal was associated with a 0.80% decrease in VCAM-1 signal.

I then aimed to determine whether these dysfunctions could be detected *in vivo* and if they were associated with distinct manifestations of SVD burden and progression. In these exploratory analyses, I examined the relationship between a panel of biomarkers of vascular, endothelial, and pericyte function and SVD structural and vascular function neuroimaging measures and cognitive outcomes in 181 patients with mild, non-disabling ischaemic stroke, enriched for SVD features (mean age: 65 years (SD: 11), 31% female). Outcomes were measured at baseline and throughout 1 year and included several MRI markers of SVD, blood-brain barrier (BBB) permeability, cerebrovascular reactivity (CVR), and cognition. After adjusting for relevant covariates, we identified a novel association between higher circulating levels of Vascular Endothelial Growth Factor (VEGF) and incident lesions visible on diffusion-weighted imaging appearing during the first year post-stroke. We also detected associations between higher Intercellular Adhesion Molecule-1 (ICAM-1) and lower CVR, as well as elevated concentrations of P-Selectin and a higher risk of cognitive impairment at 1-year follow-up.

Finally, I report results from a longitudinal observational cohort study, the Mild Stroke Study 2, where we followed-up a cohort of patients with mild ischaemic stroke, enriched for SVD features, 8.5-years post-stroke (n=243, mean age: 67 years (SD:12), 42% female). During the 7-9 years of follow-up, despite the lacunar strokes being slightly younger at baseline, a similar proportion of cortical and lacunar stroke participants were diagnosed with dementia (11%), at least one recurrent stroke (32%), or died (26%). Cortical stroke participants exhibited higher mortality rates, particularly from cardiovascular causes, compared to those with lacunar stroke. Cardiac conditions, such as ischaemic heart disease, arrhythmias, and heart failure, were also more prevalent in cortical stroke participants. Conversely, hypertension, hypercholesterolaemia, and diabetes were more frequent amongst lacunar stroke participants. These profiles coincide with previous research results and likely reflect the differing vascular pathologies underlying the two stroke subtypes: large artery atherothrombotic disease associated with cortical strokes and small vessel disease characteristic of lacunar strokes. Importantly, 45% of patients reported struggling with everyday activities. Half of lacunar stroke patients and a third of cortical stroke patients reported experiencing anxiety and/or depression, and over half of lacunar stroke patients reported experiencing subjective cognitive difficulties or concerns. Results from this study highlight the far-reaching and life-altering impacts on patient quality of life that persist for long after a mild stroke.

This translational work reinforces the view that pericyte and endothelial cell dysfunction play key roles in ageing and SVD mechanisms and provides further evidence supporting the modulating influence of endothelium-pericyte crosstalk on disease pathophysiology. A major challenge impending the advancement of therapeutics and the mechanistic understanding of SVD arises from the absence of elucidative and specific blood biomarkers. If validated in larger cohorts, these findings may offer a particularly exciting prospect to link manifestations of disease to otherwise unseen biological mechanisms and could help bridge the gap between what we know of SVD from preclinical and clinical investigations.

Abbreviations and Definitions

Abbreviation	Definition
ACE-R	Addenbrooke Cognitive Examination-Revised
AD	Alzheimer's disease
Aβ	amyloid beta
Aβ-CAA	amyloid-beta cerebral angiopathy
AT1	angiotensin II type 1 receptor
A CC	anterior corpus callosum
AQP4	Aquaporin 4
AE	astrocyte end-feet
ADMA	asymmetric dimethylarginine
AF	atrial fibrillation
β-amyloid1–40	beta-amyloid1–40
BBB	blood-brain barrier
CRP	C-reactive protein
Ca2+	calcium
CP	caudate putamen
Cav-1	caveolin-1
CD40	CD40 ligand
CNS	central nervous system
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CBF	cerebral blood flow
CSF	cerebrospinal fluid
CVR	cerebrovascular reactivity
CDR	Clinical Dementia Rating Scale
CV	coefficients of variability
CT	computerised tomography
CI	confidence interval
CA1	Cornu Ammonis 1
CA2/3	Cornu Ammonis 2/3

DG	dentate gyrus
DAB	Diaminobenzidine
DTI	diffusion tensor imaging
DWI	diffusion-weighted imaging
DCE	dynamic contrast-enhanced
EtCO2	end-tidal carbon dioxide
EC	endothelial cell
eNOS	endothelial nitric oxide synthase
NOSTRIN	endothelial nitric oxide synthase traffic inducer
E-selectin	endothelial selectin
EDE	endothelial-derived exosome
ET-1	endothelin-1
Endo-1	endothelin-1
EF	executive function
FLAIR	fluid-attenuated inversion recovery
GLUT-1	glucose transporter-1
GP	general practitioner
HbA1c	haemoglobin A1c
Hcy	homocysteine
IL-6	interleukin-6
IC	internal capsule
ICAM-1	intracellular cell adhesion molecule-1
ICV	intracranial volume
ICH	intracranial haermorrhage
IHD	ischeamic heart disease
IQR	interquartile range
LAT-1	large neutral amino acid transporter-1
Lp-PLA2	lipoprotein-associated phospholipase A2-mass
LBC	Lothian birth cohort
LINCHPIN	Lothian study of intracerebral haemorrhage, pathology, imaging, and neurological outcome

MFSD2A	major facilitator superfamily domain-containing protein 2A
MRI	magnetic resonance imaging
MI	myocardial infarction
MT	masson trichrome
MMP-9	metalloproteinase-9
MCI	mild cognitive impairment
MSS2	mild stroke study 2
MSS3	mild stroke study 3
mRS	modified Rankin Score
MoCA	Montreal Cognitive Assessment
NART	National Adult Reading Test
NHS	National Health Services
NIHSS	National Institute of Health Stroke Scale
NO	nitric oxide
NOS	nitric oxide synthase
NAWM	normal-appearing white matter
OR	odds ratio
OPCs	oligodendrocyte precursor cells
PC	pericyte
PVS	perivascular spaces
PS	permeability surface area product
P-GP	permeability-glycoprotein
PBS	phosphate buffer saline
PlGF	placental growth factor
Vp	plasma volume fraction
PAI-1	plasminogen activator inhibitor-1
PDGFRβ	platelet derived growth factor receptor beta
PDGF-BB	platelet derived growth factor subunit B
PF-4	platelet factor-4
P-selectin	platelet selectin
P CC	posterior corpus callosum

PMI	postmortem interval
K+	potassium
ROS	reactive oxygen species
RSSI	recent small subcortical infarcts
SVD	small vessel disease
sPDGFRβ	Soluble platelet derived growth factor receptor beta
sVCAM-1	soluble vascular cell adhesion molecule-1
S1P	sphingosine-1-phosphate
SD	standard deviation
SE	standard error
SIS	Stroke Impact Scale
SWI	susceptibility-weighted imaging
Thal	thalamus
NIHSS	The National Institutes of Health Stroke Scale
TIA	transient ischaemic attack
TM	thrombomodulin
TJ	tight junctions
TF	tissue factor
TFPI	tissue factor pathway inhibitor
t-PA	tissue-type plasminogen activator
TMT	Trail Making Test
TGF-β1	transforming growth factor- β 1 TM
TNF-α	tumour necrosis factor alpha
TNFR1	tumour necrosis factor receptor 1
TNFR2	tumour necrosis factor receptor 2
UKBBN	UK Brain Banks Network
VCAM-1	vascular cell adhesion molecule-1
VCING	Vascular cognitive impairment neuropathology guidelines
VEGF	vascular endothelial growth factor
vWF	von Willebrand Factor

WM	white matter
WMH	white matter hyperintensities

Table of Contents

Declaration ..... *i*

Acknowledgments ..... *v*

Lay Summary ..... *vii*

Publications and Conferences ..... *ix*

Abstract ..... *xvii*

Abbreviations and Definitions ..... *xxi*

List of Tables ..... *xxxiii*

List of Figures ..... *xxxv*

Chapter 1 Introduction *1*

1.1 Sporadic cerebral small vessel disease **1**

1.1.1 Clinical manifestations 2

1.1.2 Radiological markers 3

1.2 Small vessel disease pathophysiology **6**

1.2.1 Historical context and pathology 6

1.3 The blood-brain barrier **9**

1.4 The neurogliovascular unit **11**

1.5 Pericytes **13**

1.5.1 What are pericytes? 13

1.5.2 Why are capillaries and pericytes important? 15

1.5.3 Pericytes, blood flow regulation, and the blood-brain barrier 15

1.5.4 Signalling and the endothelium 17

1.5.5 Pericyte dysfunction in SVD 19

1.6 Endothelial cells **21**

1.6.1 What are endothelial cells? 21

1.6.2 Endothelial cell dysfunction in SVD 24

1.7 My thesis **27**

Chapter 2 A scoping review on biomarkers of endothelial dysfunction in small vessel disease: Molecular insights from human studies 29

2.1 Context 29

2.2 Introduction 29

2.3 Methods 31

2.3.1 Literature review 31

2.4 Results 32

2.4.1 Biomarkers of endothelial dysfunction and SVD: molecular insights from human studies 33

2.4.1.1 Immunoglobulin Superfamily—Cell adhesion molecules and Selectins 33

2.4.1.2 Blood-brain barrier transporters 40

2.4.1.3 Cytokines and chemokines 42

2.4.1.4 Other pro-inflammatory markers 47

2.4.1.5 Coagulation factors 52

2.4.1.6 Growth factors 58

2.4.1.7 Nitric oxide pathways 61

2.4.1.8 Other relevant biomarkers 64

2.5 Conclusions and future perspectives 69

2.5.1 Implications for clinical practice 77

2.5.2 Implications for future research 78

2.6 Concluding statements 80

Chapter 3 Ageing mice study: pericyte coverage and endothelial activation . 81

3.1 Introduction 81

3.1.1 Ageing 81

3.1.2 Pericyte loss with ageing and in the context of dementia 81

3.1.3 Endothelial activation in ageing 83

3.1.4 Aims of the ageing mice study 85

3.2 Methods 85

3.2.1 Animals 85

3.2.2 Termination and tissue processing 86

3.2.3 Immunohistochemistry 86

3.2.4 Microscopy 87

3.2.5 Image processing and quantification 88
3.2.6 Statistical analysis 91

3.3 Results 91

3.3.1 Vessel density 91
3.3.2 Vessel diameter 94
3.3.3 Pericyte coverage 96
3.3.4 VCAM-1 coverage 99
3.3.5 Vessel segments 102

3.4 Conclusions 109

Chapter 4 Examining pericyte and endothelial cell dysfunction in SVD: Insights from postmortem human brain tissue analyses 113

4.1 Introduction 113

4.1.1 Pericyte loss and endothelial activation in SVD 113

4.2 Methods 115

4.2.1 Design and subjects 115
4.2.2 Tissue 116
4.2.3 Clinical Information and post-mortem neuropathological examination 116
4.2.4 Immunohistochemistry 117
4.2.5 Multispectral Imaging and Microscopy 118
4.2.6 Image Quantification 120
4.2.7 Statistical Analyses 122

4.3 Results 123

4.3.1 Vessel Density 125
4.3.2 Vessel Diameter 126
4.3.3 Pericyte Coverage 128
4.3.4 VCAM-1 Coverage 130
4.3.5 Vessel segments 134

4.4 Conclusions 136

Chapter 5 Blood biomarkers of vascular dysfunction in SVD progression: Insights from a longitudinal neuroimaging study 141

5.1 Context 141

5.2 Introduction 141

5.3 Methods 144
5.3.1 Participants and Design 144
5.3.2 MRI Acquisition 145
5.3.3 MRI Processing and Analysis 145
5.3.4 Blood Biomarkers 146
5.3.5 Clinical and Cognitive Assessments 147
5.3.6 Statistical Analyses 147
5.3.6.1 Cross-sectional Analyses 148
5.3.6.2 Longitudinal Analyses 149
5.4 Results 149
5.4.1 Imaging Outcomes 153
5.4.1.1 WMH: Cross-sectional and longitudinal WMH volumes and baseline Fazekas score 153
5.4.1.2 Prevalent lacunes and microbleeds at baseline 155
5.4.1.3 Incident DWI-positive lesions longitudinally 155
5.4.1.4 Summary SVD scores at baseline 156
5.4.1.5 Blood-brain barrier permeability at baseline 157
5.4.1.6 Cerebrovascular reactivity at baseline 159
5.4.2 Cognitive Outcomes 161
5.4.2.1 Montreal Cognitive Assessment scores and Mild Cognitive Impairment 161
5.4.2.2 Executive function: Trail Making Test ratio (B/A) 163
5.4.3 Discussion 163
5.5 Concluding Remarks 172
Chapter 6 7-9-year longitudinal outcomes of mild lacunar and cortical ischaemic stroke: dementia incidence, mortality, recurrent stroke, and functional well-being 173
6.1 Context 173
6.2 Introduction 173
6.3 Methods 174
6.3.1 Participants and Study Design 174
6.3.2 Baseline assessments and, 1-, 3-, and 7-9-year follow-up 175
6.3.3 Imaging and Stroke Diagnosis 176
6.3.4 MRI Processing 176

6.3.5 Death 177
6.3.6 Statistics 177
6.4 Results 178
6.4.1 Clinical outcomes: dementia, recurrent stroke, death, cardiac disease, and vascular risk factors 181
6.4.1.1 Dementia 181
6.4.1.2 Recurrent stroke 184
6.4.1.3 Death 185
6.4.1.4 Cardiac diseases 187
6.4.1.5 Vascular risk factors 187
6.4.2 Functional and subjective well-being outcomes 188
6.4.2.1 Disability and dependence 188
6.4.2.2 Activities of daily living and subjective well-being 189
6.4.3 Discussion 193
Chapter 7 Conclusion, Discussion, and Future Perspectives 197
7.1 In focus: Molecular insights into endothelial dysfunction in SVD from human biomarker studies 197
7.2 Evidence of endothelial activation and pericyte coverage loss in ageing mice 198
7.3 Evidence of endothelial activation and pericyte coverage loss in SVD: Findings from postmortem human brain tissue 199
7.4 Blood biomarkers of vascular dysfunction in SVD progression: Insights from a longitudinal neuroimaging study 201
7.5 Widening the lens: Unveiling the burden of mild stroke and SVD by investigating 8.5-year longitudinal outcomes and patient impact 202
7.6 Summary 203
7.7 The role of pericytes and endothelial cells in SVD: Possible mechanisms 203
7.7.1 Strengths and limitations 206
7.7.2 Research implications, clinical implications and future perspectives 210
7.7.3 Final remarks 212
References 215
Appendix A. Supplemental material to Chapter 2 241

Appendix B. Supplemental material to Chapter 3 277

Appendix C. Supplemental material to Chapter 4..... 279

Appendix D. Supplemental material to Chapter 5 294

Supplemental Appendix A. 324

Appendix E. Supplemental material to Chapter 6..... 329

List of Tables

Table 2-1. Associations between individual biomarkers and distinct radiological manifestations of SVD present in the literature reviewed. 73
Table 3-1. Linear mixed effects model results examining vessel density (mm/mm^3) and age whilst controlling for sex, brain region and random effects stemming from individual animals. 93
Table 3-2. Linear mixed effects model results examining vessel diameter (μm) and age whilst controlling for sex and brain region as fixed effects as well as any random effects stemming from individual animals. 95
Table 3-3. Linear mixed effects model results examining pericyte coverage (%) and age whilst controlling for sex and brain region as fixed effects as well as any random effects stemming from individual animals. 98
Table 3-4. Linear mixed effects model results examining VCAM-1 coverage (%) and age whilst controlling for sex and brain region as fixed effects as well as any random effects stemming from individual animals 100
Table 3-5. Linear mixed effects model results examining log-transformed VCAM-1 coverage and log-transformed pericyte signal adjusting for brain region and sex. 108
Table 4-1. Sample Characteristics: healthy control, mild SVD, moderate SVD and severe SVD cases. 124
Table 4-2. Linear mixed model results examining vessel density (mm/mm^3) and SVD status 126
Table 4-3. Linear mixed model results examining vessel diameter (um) and SVD status adjusting for sex, risk factor score, brain matter type (grey versus white) and random effects stemming from repeated measures/images from each individual case. 128
Table 4-4. Linear mixed model results examining pericyte coverage (%) and SVD status adjusting for sex, risk factor score, brain matter type (grey versus white) and random effects stemming from repeated measures/images from each individual case. 130

Table 4-5. Linear mixed model results examining VCAM-1 coverage (%) and SVD status adjusting for sex, risk factor score, brain matter type (grey versus white) and random effects stemming from repeated measures/images from each individual case. 134
Table 4-6. Linear mixed model results examining log VCAM-1 immunofluorescent signal and log pericyte signal adjusting for age, sex and vascular risk factors as well as random effects stemming from repeated measures/images from each individual case. 136
Table 5-1. Cohort characteristics (n=181). 151
Table 6-1. Baseline cohort characteristics. 180
Table 6-2. Stroke subtype and clinical outcomes 7-9 years after lacunar or mild cortical ischaemic stroke. Logistic regression results for the association between dementia, recurrent stroke, death, and cardiac disease and possible predictors, including stroke subtype, age, sex, vascular risk factors, baseline WMH volume, and NIHSS where appropriate. 182
Table 6-3. Self-reported functional, neuropsychiatric, and subjective well-being outcomes 7-9 years post- lacunar or mild cortical ischaemic stroke (n=96). 190

List of Figures

Figure 1-1. Panel depicting key SVD neuroimaging features. 4
Figure 1-2. Recent small subcortical infarcts on diffusion-weighted imaging (DWI) sequences. 5
Figure 1-3. Pathological features of SVD 7
Figure 1-4. Visual depicting Ehrlich's and Goldmann's first (A) and second (B) experiments. (..... 10
Figure 1-5. Illustration of the constituents of the NGVU 12
Figure 1-6. Photon image of a pericyte (red) enwrapping a Atp13a5 tdT mouse brain capillary (green) 14
Figure 1-7. Visual representation of PDGF-BB and PDGFRβ signalling by pericytes enveloping the endothelial cells making up a capillary wall in the brain. 19
Figure 1-8. The attributes of a healthy BBB in a cognitively unimpaired brain versus what is hypothesised to occur in the context of SVD and AD, including the loss of pericytes. 20
Figure 1-9. Endothelial cells and the BBB. 22
Figure 1-10. Cell adhesion molecules, Selectins, and the BBB. 24
Figure 2-1. Study screening and selection process. 33
Figure 2-2. Biomarker roles in the gliovascular unit. 70
Figure 3-1.Immunohistochemistry and Opera Phenix High-Content Screening System microscopy. 88
Figure 3-2. VasOMatic FIJI Macro demonstration 90
Figure 3-3. Average global vessel density (mm/mm^3) in both young and aged animals. 92
Figure 3-4. Average vessel density (mm/mm^3) by brain region in both young and aged animals. 93
Figure 3-5. Average global vessel diameter (μm) in both young and aged animals. 94
Figure 3-6. Average vessel diameter (μm) by brain region in both young and aged animals. 95

Figure 3-7. Average pericyte coverage (%) in 3-month-old mice and 12-month-old mice. 97
Figure 3-8. Average pericyte coverage (%) in all brain regions observed in both young and aged animals. 97
Figure 3-9. Average VCAM-1 coverage (%) in 3- and 12-month-old animals. 99
Figure 3-10. Average VCAM-1 coverage (%) in all brain regions observed in both young and aged animals. 100
Figure 3-11. All images were stained using β-Dystroglycan (vasculature), CD13 (pericytes, green), and VCAM-1 (endothelial activation, magenta). (101
Figure 3-12. Density Hexbin plots from A) young mice (3-months) and B) Aged mice (12-months) showing the relationship between pericyte coverage (%), capillary diameter (μm), and age, in vessel segments extracted from all 8 brain regions of interest. 103
Figure 3-13. Pericyte coverage (%) and capillary vessel diameter (μm) from vessel segments from both young (3-months) and aged (12-months) animals in each of the 8 brain regions observed. 104
Figure 3-14. Density Hexbin plots from A) young mice (3-months) and B) Aged mice (12-months) showing the relationship between VCAM-1 coverage (%), capillary diameter (μm), and age, in vessel segments extracted from all 8 brain regions of interest. 105
Figure 3-15. VCAM-1 coverage (%) and capillary vessel diameter (μm) 106
Figure 3-16. (A) Section of the thalamus from a 3-month-old female mouse (B) Section of the thalamus from a 12-month-old female mouse. 107
Figure 3-17. Density Hexbin plot showing the relationship between local VCAM-1 coverage (%) and local pericyte signal intensity in vessel segments extracted from all 8 brain regions of interest in young (3-month-old) and aged (12-month-old) mice. 109
Figure 4-1. Visual representation of the segmentation of eight 929.74 x 697.31 μm images per tissue slice in borderzone grey and white matter areas 119
Figure 4-2. Visual representation of the unmixing of brightfield images into fluorescent-like composite images using multispectral imaging and the Vectra

Polaris Automated Quantitative Pathology Imaging System for accurate data quantification. 120
Figure 4-3. VasOMatic[328] FIJI Macro demonstration.....122
Figure 4-4. Average vessel density (mm /mm3) in healthy control, mild SVD, moderate SVD, and severe SVD cases. 125
Figure 4-5. Average vessel diameter (μm) in healthy control, mild SVD, moderate SVD, and severe SVD cases. 127
Figure 4-6. Average pericyte coverage (%) in healthy control, mild SVD, moderate SVD, and severe SVD cases. 129
Figure 4-7. Average VCAM-1 coverage (%) in healthy control, mild SVD, moderate SVD, and severe SVD cases. 131
Figure 4-8. All images were stained using CD-31 (vasculature, DAB), PDGFRβ (pericytes, magenta), and VCAM-1 (endothelial activation, green). A 4μm section from the precentral gyrus of a healthy control (top row), a mild SVD case (second row from top), a moderate SVD case (third row from top), and a severe SVD case (bottom row) denoting pericyte loss (magenta) and enhanced endothelial activation (green, white arrowheads) in SVD. 132
Figure 4-9. Images stained using CD-31 (vasculature, DAB), PDGFRβ (pericytes, magenta), and VCAM-1 (endothelial activation, green). Sections from the precentral gyrus of a healthy control (top row), a mild SVD case (second row from top), a moderate SVD case (third row from top), and a severe SVD case (bottom row) denoting pericyte loss and enhanced endothelial activation in SVD in individual microvessels.....133
Figure 4-10. Hexbin density plots showing average VCAM-1 signal and pericyte (PDGFRβ) signal per disease group.....135
Figure 5-1. Panel of serum biomarkers extracted and their putative involvement in pericyte and endothelial cell (dys)function in the cerebral small vessels.....143
Figure 5-2. Flow diagram of study recruitment and data collection. 150
Figure 5-3. Blood biomarkers and WMH volume.....154
Figure 5-4. Blood biomarkers and newly appearing incident lesions (DWI-positive) over one year. 156

Figure 5-5. Blood biomarkers and BBB permeability (PS) from DCE-MRI. 158
Figure 5-6, Blood biomarkers and CVR. 160
Figure 5-7. Blood biomarkers and MoCA score. 161
Figure 5-8. Relationship between 13 biomarkers and 24 markers of disease grouped by effect type. 165
Figure 6-1. Participant flow chart for clinical outcomes (left) based on a combination of general practitioner records, hospital record data, and participant questionnaire responses and for functional and subjective well-being outcomes (right) based on a postal questionnaire sent to participants 7-9 years after initial study recruitment. 179
Figure 6-2. Stroke subtype and clinical outcomes at 7-9-years. Logistic regression results for the association of possible predictors (including stroke subtype) and clinical outcomes: dementia, recurrent stroke, death and cardiac diseases. 184
Figure 6-3. Kaplan-Meier survival curves comparing death rates between cortical and lacunar stroke 7-9 years post-stroke (n=143). The log-rank test indicated a significant difference in survival probabilities between the groups, where cortical stroke may have worse survival outcomes compared to lacunar stroke (X2=8.2; p=0.004). 186
Figure 6-4. Lacunar and cortical stroke participants with diagnosed hypertension, diabetes, hypercholesterolemia, and/or atrial fibrillation at baseline, along with new diagnoses acquired between baseline and the 7-9 year follow-up. 188
Figure 6-5. Longitudinal disability and functional independence at baseline and 7-9 years after minor ischaemic lacunar (dark pink) or cortical (light pink) stroke (n=93). 189

Chapter 1 Introduction

The purpose of this chapter is to 1) introduce cerebral small vessel disease (SVD) and describe the clinical manifestations and radiological markers associated with it and 2) discuss our current understanding of SVD pathophysiology, particularly as it relates to pericyte and endothelial cell dysfunction. I describe brain pericytes and endothelial cells and elaborate on how they relate to BBB integrity and SVD development based on what is known and highlight knowledge gaps in the literature that require further investigation. This chapter concludes with the aims and outline of this thesis.

1.1 Sporadic cerebral small vessel disease

SVD is a highly prevalent disorder of the small perforating arterioles, capillaries and venules in the brain, mainly affecting the cerebral white and grey deep matter[1]. It is associated with cerebrovascular dysfunction and characterised by structural and functional alterations in the brain's perforating vessels, in blood flow responsiveness to changing metabolic demands, and a compromised BBB, which precedes visible brain lesions[2]. SVD causes a quarter of all ischaemic strokes, most haemorrhagic strokes, and is the commonest cause of vascular dementia, contributing to approximately half of all dementia cases worldwide[3–5].

SVD is associated with a series of signature lesions visible on neuroimaging and on pathological examination. Clinically, SVD can manifest as lacunar or haemorrhagic stroke, and is associated with neuropsychiatric symptoms, mobility problems, cognitive impairment, and dementia[3,6–8]. Although SVD is associated with ageing, it is representative of a pathological process which may accumulate covertly over time culminating in a diffuse disruption in brain connectivity and neuronal function. Although associated with traditional vascular risk factors, namely hypertension, hypercholesterolaemia, smoking, and diabetes, these account for little variability in disease severity amongst

patients and epidemiological study results are inconsistent[9–14]. The precise causes of SVD remain largely unclear.

SVD can be characterised as sporadic or familial and can be further differentiated from haemorrhagic (i.e. cerebral amyloid angiopathy) and non-haemorrhagic or ischaemic subtypes, which may involve distinct pathological mechanisms. The most common type of SVD caused by a genetic variant is Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL), which is caused by mutations in the NOTCH3 gene.

With a growing ageing population with increasing morbidity and disability, it is important to consider the substantial public health and financial burdens posed by SVD, stroke, and dementia. In the UK, the cost of dementia is forecast to more than double in the next 20 years, increasing to £90 billion, and stroke incidence is projected to increase by 59%, with the annual NHS costs projected to treble, surpassing £10 billion[15,16]. SVD more than doubles future risk of dementia and stroke, including recurrent stroke, and is associated with worse outcomes after stroke[3,17]. Additionally, in a prospective cohort of adults aged 60-90 years, the authors report identifying SVD features on neuroimaging in 92% of participants, the vast majority of which were undiagnosed[18]. With better detection and management of SVD, it is estimated that the incidence rate of stroke can be lowered by 5% starting next year, reducing the number of people living with stroke by over 70,000[15]. This burden also extends beyond its direct impact on health services and reaches widely across society, affecting the quality of life of patients, loved ones, and carers, and impacting societal participation and productivity.

1.1.1 Clinical manifestations

The clinical manifestations of SVD are diverse, and a comprehensive understanding of its overall clinical picture remains elusive. In part, this can be attributable to the clinically subtle and oftentimes largely ignored mild neurological symptoms associated with the gradual accumulation of SVD

pathology and damage to the brain, particularly in what is already a clinically complex population prone to comorbidities.

Clinical features of SVD include lacunar ischaemic stroke, intracerebral haemorrhage, cognitive impairment and progressive decline, dementia, mobility problems (including gait and balance dysfunction), and neuropsychiatric symptoms (including depression and apathy)[3,6,8,19–22]. Emerging research increasingly paints SVD as a global brain disease involving many systems and domains and resulting in impaired function, prolonged hospitalisation, institutionalisation, and increased mortality[23].

Currently, there are no very effective available treatment options for SVD[24,25]. Management therefore relies on SVD detection and risk reduction strategies including prescribing secondary stroke prevention medication when appropriate (i.e. antiplatelets, antihypertensives, and statins), the management of relevant comorbid conditions (e.g. diabetes), and adherence to healthy lifestyle recommendations (e.g. smoking cessation, exercise).

1.1.2 Radiological markers

SVD is associated with a range of lesions visible on neuroimaging with CT or MRI; these are widely recognised and were standardised in the Standards for Reporting Vascular Changes on Neuroimaging (STRIVE) guidelines, recently updated in STRIVE-2[26,27]. The most commonly observed SVD features on neuroimaging include white matter hyperintensities (WMH), lacunes, enlarged perivascular spaces, microbleeds, superficial siderosis , and cortical atrophy **(Figure 1-1).**

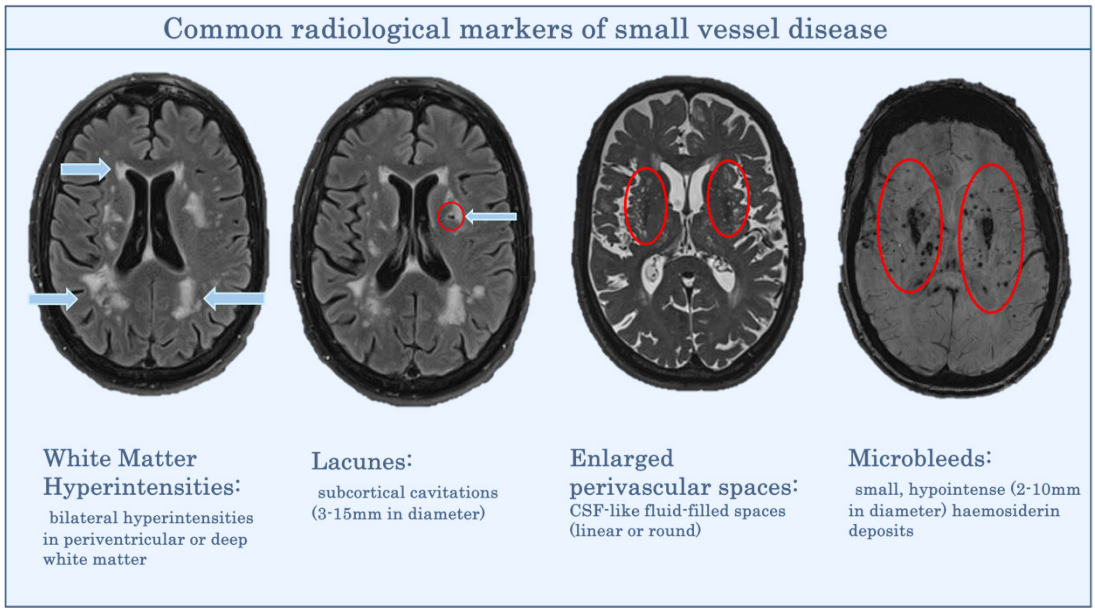


Figure 1-1. Panel depicting key SVD neuroimaging features. These include (from left to right): bilateral WMH visible as hyperintense lesions in the periventricular and deep white matter on a FLAIR sequence; lacune on a FLAIR sequence; enlarged PVS in the basal ganglia visible on a T2-weighted image; microbleeds on susceptibility-weighted imaging (SWI).

The most commonly observed and reported SVD feature on neuroimaging is WMH of presumed vascular origin, which are hyperintense lesions on T2-weighted imaging sequences and are normally visible surrounding the ventricles or in the deep white matter. They are variable in size and shape and are mostly bilaterally symmetrical. WMH are believed to represent areas of permanent brain damage including demyelination and increased interstitial fluid[28,29]. Emerging evidence strongly points to the dynamic nature of radiological features of SVD, suggesting lesions can change, progress and even regress over time, including WMH[30].

Lacunes are small (3-15 mm diameter), round or ovoid fluid-filled subcortical cavitations. They are believed to result from acute, small subcortical infarcts or haemorrhages within a single perforating artery perfusion territory. They appear as hypointense on fluid-attenuated inversion recovery (FLAIR), T1- and T2-weighted images.

PVS are small (<3 mm diameter) tubular CSF-like fluid-filled spaces which appear as linear (if parallel to the imaging plane) or round (if perpendicular) hyperintense lesions on T2-weighted images or hypointense on T1-weighted images when enlarged[27]. They are visible in the basal ganglia, midbrain and centrum semiovale and are thought to represent extensions of extracerebral fluid space enveloping the perforating arterioles.

Microbleeds are small (2-10 mm diameter) haemosiderin deposits consistent with blood leakage into the brain tissue. They can be detected as hypointense lesions in imaging sequences sensitive to blood, such as susceptibility-weighted imaging (SWI)[27]. Other radiological findings associated with SVD include cortical superficial siderosis, and deep and superficial brain atrophy[27].

Recent small subcortical infarcts (RSSI) are small (<20 mm) lesions visible as focal subcortical hyperintensities on diffusion-weighted imaging (DWI) on MRI within the territory of a single perforating arteriole[26]. They are commonly an incidental MRI-related finding and are not necessarily associated with overt neurological symptoms or stroke syndromes **(Figure 1-2).**

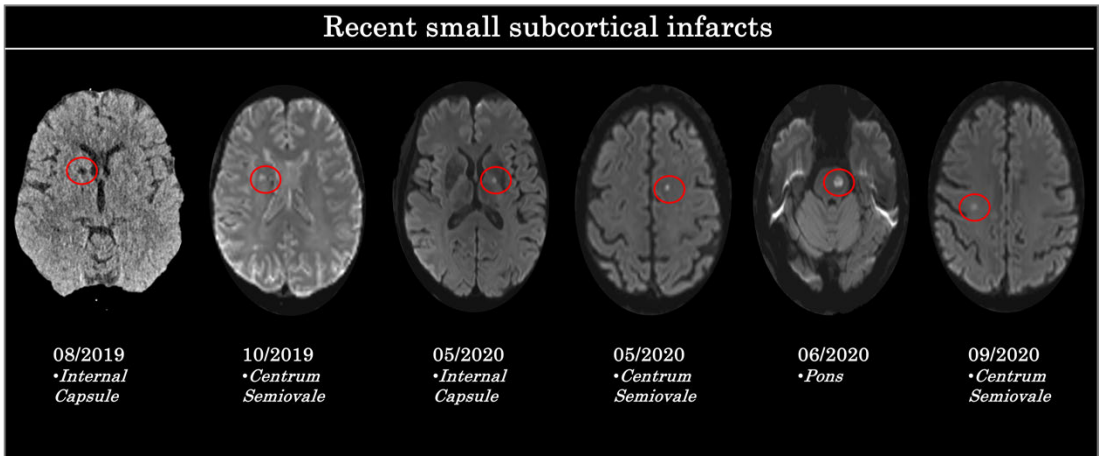


Figure 1-2. Recent small subcortical infarcts on diffusion-weighted imaging (DWI) sequences. These are scans from one participant who presented to hospital with stroke symptoms on 08/2019 (CT scan on left); at least one RSSI was evident in each of their study visit scans from 10/2019 up until their final study visit on 09/2020. Only one of these instances (06/2020) was associated with clinically overt focal neurological symptoms reported by the patient.

1.2 Small vessel disease pathophysiology

1.2.1 Historical context and pathology

Charles Miller Fisher, a prominent neurologist, is known for describing what we now recognise as the pathological hallmarks of SVD in the 1950s and 60s and is credited to be the first to describe lacunar stroke[31–35]. However, clinicopathological reports of SVD pathology date back to as early as the mid-1800s, when Otto Binswanger described a progressive, chronic subcortical disease state characterised by white matter and cortical atrophy as well as cognitive and mood-related changes and acute sudden-onset hemiparesis in 8 postmortem cases; less than two decades later, Pierre Marie portrayed a lacunar disease state, which they described as a progressive neurological disorder characterised by hemiparesis episodes, dysarthria, gait and balance disturbances and dementia associated with the presence of lacunes on pathological examination[36,37]. Neuropathology studies as early as 1902 describe signs of inflammation in the arteriolar wall and the perivascular tissue with segmental non-atheromatous thickening of arteriolar walls with little to no evidence of infarction or vessel occlusion[38,39].

Prior to the invention of the MRI, and indeed even CT, the clinical and pathological assessment of SVD and SVD pathophysiology was a challenging endeavour; perhaps it comes to no surprise that pathological studies of SVD are scarce. Charles Miller Fisher himself stated in 1991 that the introduction of the MRI will likely settle the debate around the 'criticism of the concept of lacunar disease', commenting on the practical challenges of vascular clinical and pathological studies up until that point:

> ''Developing a better clinicopathological experience with lacunar infarcts was a long-drawn-out process because of the irregularity with which patients examined in life came to have a satisfactory pathological examination of their brains. Between 1955 and 1975 only 20 clinicopathological vascular investigations were undertaken. Since the introduction of CT in 1973, no further vascular studies have been made''[31].

Most vascular pathological research at the time was aimed at large-artery atherosclerotic disease, and to date, there are few studies focussing on human lacunar stroke pathology. This is likely due to several factors: the small size of lacunar infarcts, the low early-disease mortality (meaning pathology is often examined long after an acute event), the challenge of identifying a specific infarct and the affected arteriole, and the 'clinically silent' nature of early disease stages.

Pathologic depictions of SVD include the description of abnormalities in arterioles, namely arteriosclerosis, lipohyalinosis, and fibrinoid necrosis **(Figure 1-3)**[31]. Arteriosclerosis involves the thickening of the vessel walls and concurrent lumen narrowing. Lipohyalinosis, characterised as 'segmental arterial wall disorganisation', appears as hyaline and fat-filled macrophages within the wall of penetrating arteries, particularly in the basal ganglia and thalamus, often co-occurring with the appearance of proximal microatheroma[40–43]. Fibrinoid necrosis is an aggressive form of arteriolosclerosis and involves the deposition of granular fibrinoid material, which consists of fibrin, fibrinogen, and necrotic smooth muscle cells within the vessel [40].

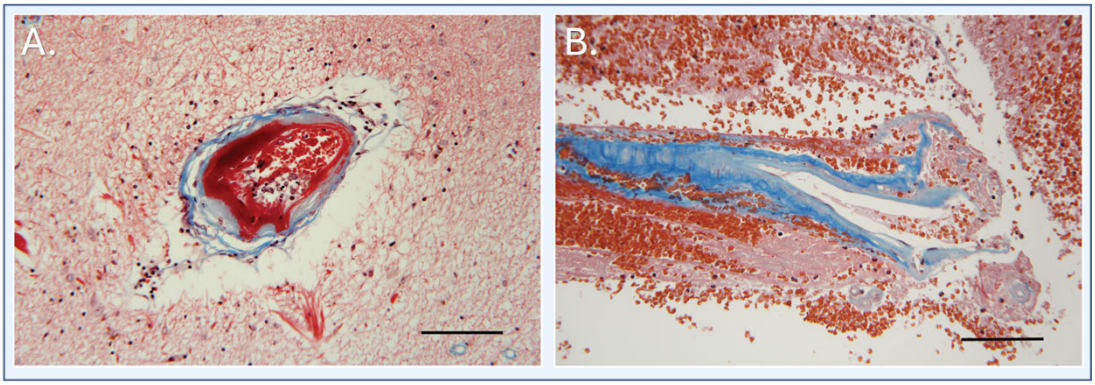


Figure 1-3. Pathological features of SVD, adapted from Ogata et al. 2014[40] (A) An arteriole depicting fibrinoid necrosis and thrombus in red and hyalinosis in blue (Masson trichrome (MT), Bar = 100 μm) (B) A ruptured arteriole showing arteriosclerosis in a cerebellar haemorrhage (Masson trichrome (MT), Bar = 100 μm).

Another commonly described feature includes the ‘segmental’ appearance of vessels, with some sections of narrowing due to arteriolar wall thickening, and other sections showing luminal dilation, perhaps due to a loss of scaffolding structure in the smooth muscle layer and perivascular inflammation[31,39,44].

Vessel damage can result in a more permeable BBB, which facilitates the extravasation of blood components into vessel walls and perivascular tissue. Plasma leakage across the BBB could potentially account for perivascular oedema, neuronal damage/death, and possibly even arterial wall damage[42]. This process of inciting and exacerbating perivascular oedema and the increase in BBB permeability can also be toxic to the collection of cells which serve to orchestrate all aspects of brain function, the neurogliovascular unit (NGVU). This pathology sequence can result in vessel wall thickening and stiffening, occlusion, and a loss of autoregulative perfusion properties. This vascular, perivascular, BBB, and NGVU dysfunction may eventually culminate in more severe manifestations of chronic cerebrovascular dysfunction, including ischaemia/focal brain infarction, perfusion irregularities, a diminished response to fluctuating metabolic demands, intracerebral haemorrhage and later stage global brain and diffuse connectivity dysfunction.

What instigates this cerebrovascular dysfunction, and the sequential downstream cascade of events and their interaction, remains elusive. There is evidence that BBB and endothelial dysfunction precede visible brain lesions[2]. The precise cause of endothelial dysfunction remains unclear, and it is likely that multiple pathways can lead to this dysfunction, all converging on the same outcome. It is important to acknowledge the overlap between SVD and AD, not only in terms of clinical manifestations, but also in terms of their pathological mechanisms. In a recent postmortem pathological examination, nearly 80% of clinically diagnosed AD cases had, in fact, evidence of vascular pathology, either independently or in tandem with AD pathology[45].

1.3 The blood-brain barrier

Rather than being one single structure, the blood-brain barrier (BBB) refers to the highly specialised and dynamic set of characteristics in the vessels that vascularise the brain. It consists of a tightly connected monolayer of endothelial cells lining the blood vessel lumen, which employs selective permeability to closely regulate the exchange of substances between the blood and the brain. Amongst its many roles, the BBB is integral to maintaining brain homeostasis, it actively transports nutrients in and out of the brain, even across concentration gradients through extrusion pumps. It has low rates of transcytosis and protects the central nervous system (CNS) by limiting toxic molecules, pathogens, and immune cells into the brain. Interestingly, there appears to be something unique within the brain cellular milieu which instructs vessels to form this barrier; embryonic gut endothelial cells transplanted to the brain will act like the BBB and even begin to form tight junctions[46].

The BBB was initially discovered (albeit reluctantly and entirely by happenstance) by German physician and Nobel Laurate, Paul Ehrlich, in 1885[47]. Fuelled by curiosity about oxygen consumption rates in different organs in the body, he injected blue dye (trypan blue) into mice intravenously and observed how the animal's organs turned blue- with the exception of the brain and the spinal cord. This essentially suggested that although the vessels in other places in the body are leaky, and allow things in and out, the brain vessels do not (Dr Ehrlich, however, was not an enthusiastic advocate of this hypothesis). Some years later, in 1913, one of his graduate students, Edwin Goldmann, repeated these experiments, this time injecting the blue due intrathecally directly into the CSF **(Figure 1-4)**. The brain parenchyma and spinal cord turned blue, and the rest of the organs were spared. With the invention of electron microscopy, Reese and Karnovsky were able to observe more concrete evidence of the BBB, including morphological constituents such as sealing tight junctions[48].

Figure 1-4. Visual depicting Ehrlich's and Goldmann's first (A) and second (B) experiments. (A) shows an adult rat after intravenous injected of trypan blue dye, and (B) shows the blue brain and spinal cord following lumbar injection of trypan blue. Adapted from Saunders et al.[49].

The function of the BBB is closely linked to and influenced by the components of the NGVU, including the basement membrane, pericytes, and astrocyte end-feet. Optimal BBB functioning is essential for healthy brain function, as it allows for the adequate delivery of nutrients and oxygen to neurons, promotes drainage of metabolic waste, and protects neurons from pathogens and neurotoxic compounds[50,51].

In the pathological context of SVD, and indeed to an extent in normal ageing, there is a loss of some of the properties that maintain BBB integrity which leads to a cascade of effects that impact the function of surrounding cells and tissue, contributing to disease pathophysiology and burden[2,7,52–55]. Reports suggest the BBB can be twice as permeable in 70–80-year-old adults compared to young controls[55].

BBB dysfunction is a central mechanism and characteristic of SVD; it has been linked to cognitive impairment, lacunar stroke, worse functional outcome, and cognitive decline longitudinally, and various SVD features on neuroimaging, including increasing WMH burden, and is also supported by neuropathological evidence[2,53,54,56–58]. In dysfunction, the BBB will allow neurotoxic molecules into the perivascular tissue; there is infiltration and activation of immune cells, loss of tight junctions, an increase in cell adhesion molecules with a concurrent increase in immune cells that are crossing the barrier, inflammation, thickening of arteriolar walls, ion dysregulation, and an interruption in the efficacious delivery of oxygen and energy to nearby tissue and neurons[50,59–61]. Endothelial cells and the pericytes that envelop them play key roles in maintaining the integrity and functioning of the BBB, and it is therefore likely that they play a role in BBB breakdown mechanisms; perhaps endothelial cells begin to express pro-inflammatory profiles, and their enveloping pericytes concurrently become dysfunctional.

1.4 The neurogliovascular unit

To understand the mechanisms of SVD, it is important to consider how the lumen-facing cells of the brain vasculature (i.e. endothelial cells) interact with the rest of the NGVU, comprised of pericytes, astrocytes, microglia, oligodendrocytes, basement membranes, and neurons **(Figure 1-5)**.

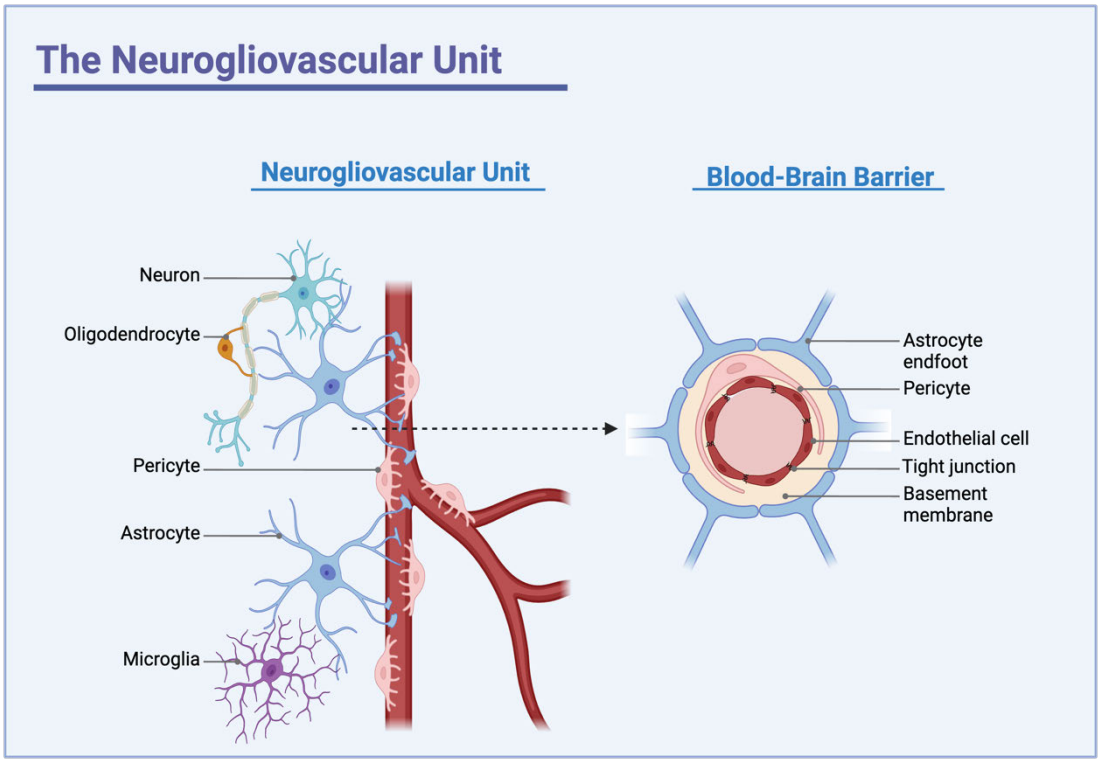


Figure 1-5. Illustration of the constituents of the NGVU, including endothelial cells, pericytes, oligodendrocytes, microglia, and astrocyte end-feet. Cross-section of the components of the blood-brain barrier on the left. Figure was edited with BioRender.

Astrocytes and their end-feet form a connecting bridge between the outer endothelial cell layer of a capillary and proximal neurons, modulating local blood and energy supply depending on fluctuating neuronal activity. There is evidence of their involvement in myelination and interaction with other cells, including glia and neurons. Due to water channel proteins, Aquaporin 4 (AQP4), in their end-feet, astrocytes are also implicated in regulating brain water dynamics, which might be relevant in SVD mechanisms[62,63].

Microglia, the resident immune cells of the brain, are the primary orchestrators of the brain's inflammatory response. They respond to peripheral inflammation by altering the BBB to facilitate the transendothelial migration of leukocytes, and inducing the expression of cytokines, chemokines, and cell adhesion molecules by endothelial cells[64–66]. Microglial activation and neuroinflammation have been associated with SVD and hypertension[67]. Animal models of SVD and studies with postmortem human tissue suggest

distinct morphological microglial features specific to SVD, including an increase in number of vascular-associated microglia, microglia with larger cell bodies, and an increase in the areas occupied by thickened microglial processes[68].

In the brain, white matter is distinguishable from grey matter by the myelin sheath which envelops neuronal axons in the deep brain matter. Oligodendrocytes are responsible for producing the myelin that ensheathes axons. Evidence from postmortem and neuroimaging studies supports an association between demyelination and SVD[69]. One possible mechanism is through endothelial dysfunction, which may hinder the maturation of oligodendrocyte precursor cells (OPCs) into mature myelin-producing oligodendrocytes, thereby blocking myelination and axonal myelin repair[70]. This process could affect the efficacy of action potential mechanisms and conduction velocities as well as exacerbate axonal and eventual neuronal damage.

I consider pericytes and endothelial cells and their potential roles in SVD pathophysiology in detail in the next sections, **1.5** and **1.6**.

1.5 Pericytes

1.5.1 What are pericytes?

Pericytes are contractile cells with small cell bodies and long extending processes that envelop the walls of pre-capillary arterioles, capillaries, and post-capillary venules and play essential roles in vascular function and BBB maintenance **(Figure 1-6).** Pericytes cover approximately 80% of the brain capillaries in the human cortex, with some variation depending on subregions[71].

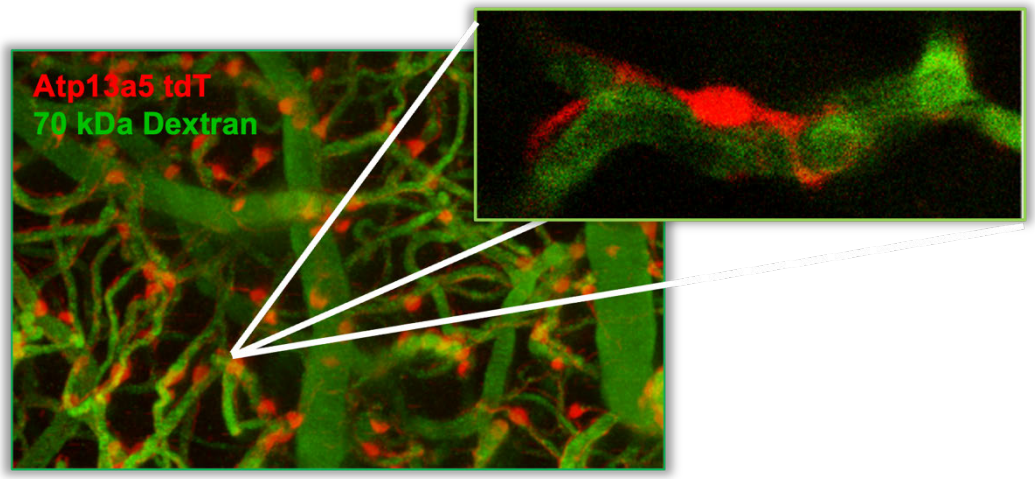


Figure 1-6. Photon image of a pericyte (red) enwrapping a Atp13a5 tdT mouse brain capillary (green); image taken in collaboration between the Montagne Lab and Dr Juraj Koudelka (Research Fellow, Microscopy Platform Manager & Imaging Lead in the UK Dementia Research Institute at the University of Edinburgh).

In the brain, they are present at intervals (i.e. bump-on-a-log morphology) along the walls of capillaries, sandwiched between the endothelial cells and astrocytes. Amongst their many roles, they help maintain the integrity of the BBB by regulating the entry of immune cells, help regulate cerebral blood blow by modulating vessel diameter in response to metabolic demands, and aid with blood vessel formation, maintenance, and repair[72,73]. They can be identified by their expression of growth factor receptor beta (PDGFRβ). There are diverse subtypes of pericytes found along the microvasculature, including ensheathing pericytes in the pre-capillary arterioles, thin-strand pericytes in the capillaries, and mesh pericytes in the post-capillary venules. Although pericytes were first identified by Carl Eberth and Charles Rouget in the 1870s in the frog and later characterised and termed by Karl Zimmermann in 1923, much about their morphology and role remains unknown[74,75]. This is in large part due to the challenges in visualising and distinguishing brain pericytes, which in recent years has been mitigated by the invention of *in vivo* microscopy and single-cell transcriptomics. Currently, there are many known pericyte markers, though none that are entirely cell-specific[76].

1.5.2 Why are capillaries and pericytes important?

The brain is a remarkably energy-expensive organ, and despite it taking up only 2% of body mass, it consumes a great deal of resources, accounting for approximately 20% of the base metabolic rate, requiring 15% of total cardiac output and 20% of daily oxygen and glucose consumption[77,78]. The effective vascularisation and perfusion of the brain is a formidable endeavour, and penetrating capillaries play a crucial role. Capillaries make up the vast majority of the brain's vasculature, approximately 85%; the total length of capillaries in the human brain spans a staggering 400 miles (644 km)[79,80]. An injected contrast will take less than 5 seconds to reach the entirety of the capillary network, highlighting the efficiency of the microcirculation[81]. It is estimated that no neuron is ever more than 20 μm away from a capillary, presumably to aid in the rapid brain perfusion in response to increases in zonal neuronal activity[82].

It was previously believed that smooth muscle cells were responsible for the rapid dilating of *arterioles* in response to increased oxygen demand in areas of heightened neuronal activity. However, recent research indicates that it is the relaxation of contractile pericytes around *capillaries* that is not only quicker, but possibly the leading driving force in this process[83,84]. Interestingly, pericytes can exert their influence over brain perfusion in subtle ways too, and a change in vessel diameter is not always evident. Capillaries can be narrow enough that red blood cells passing through must expand the vessel walls. The relaxation of a pericyte cell body, or indeed its processes, may affect the rigidity of the vessel walls.

1.5.3 Pericytes, blood flow regulation, and the blood-brain barrier

Along with endothelial cells and astrocyte end-feet, pericytes are one of the main building blocks of the BBB and play a major role in its formation. Genetic deletion of pericytes results in defective or no BBB development and, in viable cases, a leaky and dysfunctional BBB proportional to the degree of pericyte deficiency[85]. Within their roles in maintaining the BBB, pericytes help preserve

the tight junctions that keep endothelial cells together and form the scaffolding architecture of the BBB; the loss of pericytes can cause aberrant and disorganised tight junction morphology and alignment, and pericyte detachment with ageing has been associated with a decrease in tight junction expression[86,87]. In models of pericyte deficiency, the increase in the space between endothelial cells in the BBB can be reversed *in vitro* with the introduction of pericytes[86].

Pericytes can also regulate transcytosis within the BBB. Evidence indicates that a leaky BBB resulting from pericyte deficiency is not only due to a loss of tight junctions that maintain the barrier's sealed integrity, but also due to an increase in transcytosis (vesicle trafficking) in endothelial cells[88–90]. A possible, but controversial, mechanism is through the regulation of Major facilitator superfamily domain-containing protein 2A (MFSD2A), which facilitates the suppression of transcytosis expressed by endothelial cells proportional to pericyte coverage[76,89–91]. Another way through which pericytes influence transcytosis and BBB regulation, is through their inflammatory reaction. Pericytes are sensitive to inflammatory cues, which cause them to increase cell adhesion molecule expression in the endothelium, facilitating the activation and migration of leukocytes through vascular walls[92]. Pericyte ablation in experimental models leads to acute BBB breakdown, blood flow changes, and neuronal loss[93].

A compelling testament to the importance of pericytes in maintaining BBB homeostasis and blood flow is evident in instances where their dysfunction leads to undesirable effects, such as in the case of ischaemic stroke. In this context, pericytes are known to cause the 'no-flow phenomenon' by tightly constricting capillaries post-stroke, even after reperfusion[84]. Despite pericyte cell bodies covering less than 10% of total capillary surface area, over 80% of BBB leakage occurring post-stroke centres around pericyte cells' soma[76,84]. Even so, increased pericyte coverage leads to improved outcome after stroke, and pericyte knockout experimental models tend to suffer greater neurological

deficits and inflammation post-stroke, possibly due to pericytes' role in vascular remodelling and repair[94,95].

In addition to helping with BBB maintenance, pericytes also play an important role in modulating blood flow within the microcirculation in the brain. In hypoxic conditions, for instance, pericytes are able to dilate capillaries, possibly through nitric oxide (NO) or astrocytic-modulated prostaglandin E2 expression, before arteriolar dilation occurs, accounting for over 80% of the increased blood flow within the brain[84]. However, much about the roles of pericytes in influencing cerebral blood flow (CBF), and how this may differ depending on pericyte subtype remains unknown and some controversy exists in the literature, with some maintaining that it is vascular smooth muscle cells and arteries, and not pericytes and capillaries, that exert the greatest influence in CBF[76]. Some studies indicate that capillary pericytes provide a substantial contribution to the modulation of blood flow and basal blood flow resistance, albeit through slower kinetics than arterioles[96]. Animal models of both, acute and chronic pericyte loss show a reduction in blood flow as well as BBB breakdown, and in patients with cognitive impairment, the observed reduction in CBF correlates positively with pericyte loss[53,71,93,97,98].

It is also worth noting that pericytes are highly plastic and dynamic cells within the CNS. The removal of one pericyte can engender bilateral neighbouring pericytes to extend their processes and reclaim the vascular territory[83,99,100]. It is believed their plastic remodelling abilities are compromised in the aged brain and contribute to impaired capillary blood flow and structure[99].

1.5.4 Signalling and the endothelium

Pericytes are an essential component of the NGVU and they are constantly engaged in interactions with other cells, including the endothelium, to maintain homeostasis in their cellular environments. For instance, pericytes can help regulate the polarisation of astrocyte end-feet, facilitate the maturation of OPCs into myelinating oligodendrocytes, promote neurogenesis, support neuronal survival, and there is even research suggesting that under certain

conditions such as stroke, inflammation, or in the presence of a compromised BBB, pericytes may be able to transition or spontaneously differentiate into CD11B-positive microglial-like cells[86,101–103]. Through their interactions with astrocyte end-feet and AQP4, it is also possible that pericytes play a role in the glymphatic system, the process implicated in waste and neurotoxin removal throughout the CNS through the drainage of cerebrospinal fluid (CSF) and extracellular interstitial fluid. In this process, AQP4 helps mediate CSF clearance, and pericytes help regulate AQP4 expression and polarisation in astrocyte end-feet; it is therefore not unlikely that pericytes may participate in glymphatic function[104,105].

Pericytes are embedded within the basement membrane of capillaries and rely on gap and adherens junctions to communicate with endothelial cells at sites where the basement membrane thins[106]. These gap junctions between endothelial cells and pericytes allow ionic currents to pass through cell cytoplasm and through adhesion plaques which help anchor pericytes to their corresponding endothelial cells[107]. In addition, pericytes and endothelial cells are also able to communicate through basement membrane-free peg-and-socket junctions[107,108].

Brain pericytes rely on PDGF-BB and PDGFRβ signalling to effectively maintain BBB function[86,109,110]. PDGF-BB is secreted by the endothelium and serves to recruit pericytes to endothelial cells, whilst its receptor, PDGFRβ, is enriched in pericytes **(Figure 1-7)**. An elevation in soluble PDGFRβ secretion is considered an indicator of pericyte injury and has been proposed as a potential biomarker for increased BBB breakdown[53,111–113]. In the context of SVD and Alzheimer's disease (AD), increased CSF concentrations of PDGFRβ have been implicated in mild cognitive impairment (MCI) and BBB dysfunction[53,114,115]. PDGFRβ is the most widely used pericyte marker, and although it is a non-specific surface protein also expressed in glia, neuronal progenitor cells, and fibroblasts, RNA-sequencing data in mice and humans has demonstrated a significantly higher expression of PDGFRβ in pericytes compared to other cell types[106,116].

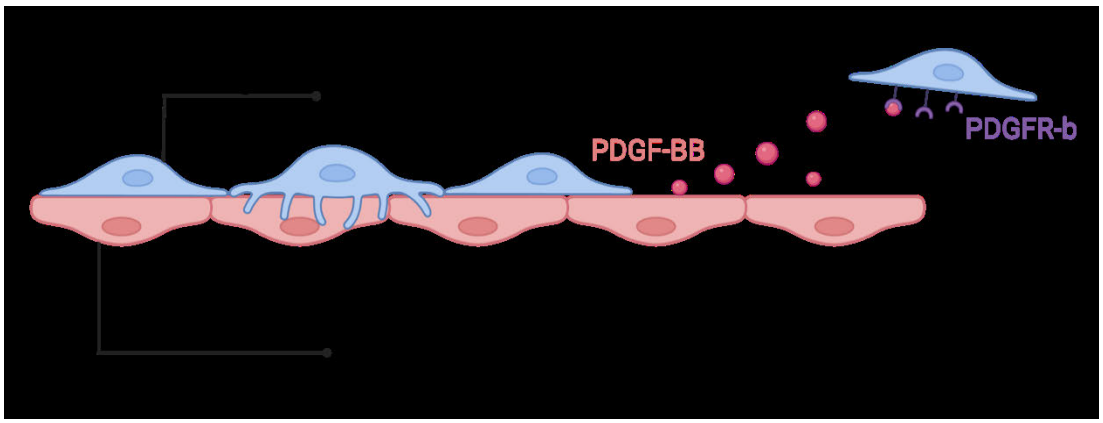


Figure 1-7. Visual representation of PDGF-BB and PDGFRβ signalling by pericytes enveloping the endothelial cells making up a capillary wall in the brain. Figure was edited with BioRender.

1.5.5 Pericyte dysfunction in SVD

Pericyte coverage in the young, healthy brain is estimated to hover between 80-90%, however, recent research indicates that pericytes are sensitive to cerebrovascular pathology and mechanisms of ageing, even in the absence of pathology[99]. Pericyte degeneration with ageing has been reported in mice, rats, monkeys, and humans[53,117–122]. Pericyte loss evident in SVD is associated with neurovascular uncoupling, impaired blood flow, white matter damage, neuronal dysfunction, hypoxia, and correlates with BBB breakdown, and cognitive decline **(Figure 1-8)**[53,54,84,93,114,123,124]. Several studies have reported a reduction in pericyte cell count and capillary pericyte coverage in the context of neurodegenerative diseases, including vascular dementia and AD as well as in acute stroke[114,124–128]. The exact mechanisms responsible for pericyte loss remain poorly understood, but may involve the endothelial sphingosine-1-phosphate (S1P) receptor, which modulates N-cadherin transport to the vessel membrane, where it facilitates pericyte-to-endothelium adhesion[129–131]. Evidence suggests an activated endothelium can impair pericyte function and pericyte loss can modify endothelial activation; however, the mechanisms underlying this dysfunctional interplay and their effect on BBB function remain unknown[132]. A more in-depth discussion of the available evidence of pericyte loss in ageing and SVD can be found in the introduction section of **Chapter 3**.

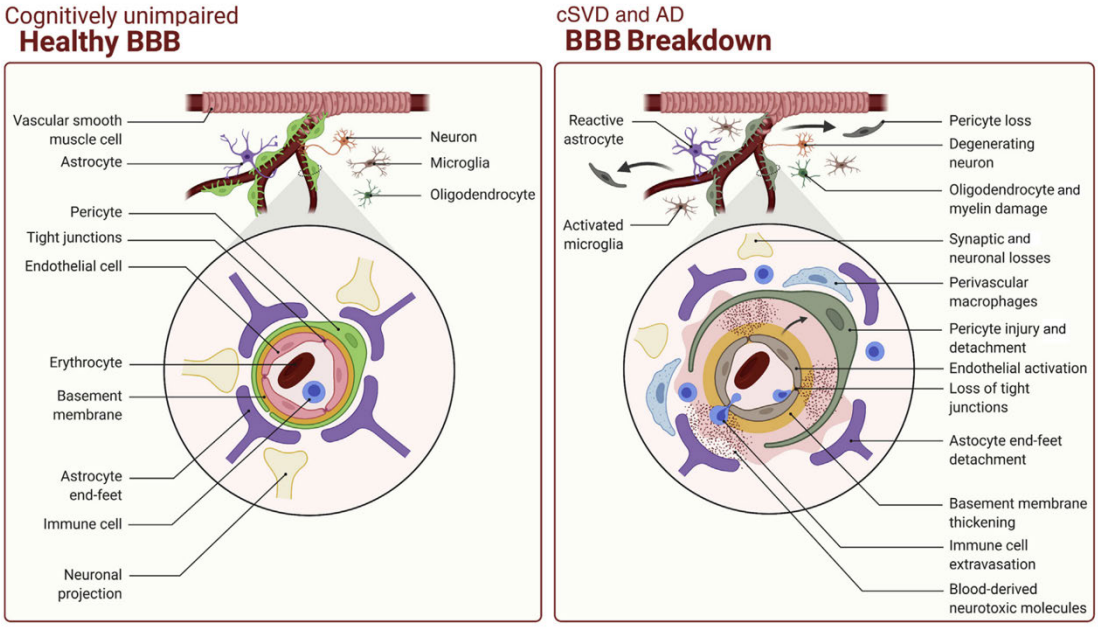


Figure 1-8. The attributes of a healthy BBB in a cognitively unimpaired brain versus what is hypothesised to occur in the context of SVD and AD, including the loss of pericytes. Taken from Procter, Williams & Montagne, 2021[129].

In summary, pericytes control key neurovascular functions that are crucial for BBB maintenance and neuronal function. Their loss in ageing and in the context of SVD may represent a major event in disease pathophysiology, likely preceding visible brain lesions and clinical manifestations, through the following plausible mechanisms:

1) Disrupting brain perfusion by causing reduced capillary circulation, CBF, and dysfunctional blood flow responses to activation (functional hyperaemia), and a loss of autoregulation, causing chronic hypoperfusion and hypoxia (there is a possible cyclic component to this, wherein, as in the case of ischemic stroke, hypoxia may lead to further pericyte contraction and hence more compromised perfusion);
2) Rendering capillaries more vulnerable to damage and ischemia because of stiffness, inflammation, and loss of tight junctions;
3) Inciting and exacerbating heightened BBB permeability, allowing for leaky vessels, immune cell crossing, microvessel ruptures, perivascular oedema, and the unregulated entry of neurotoxic and vasculotoxic

macromolecules into brain tissue, leading to secondary neuronal degeneration;

4) Inciting and exacerbating endothelial activation, including the release of cell adhesion molecules, which facilitate the transendothelial migration of leukocytes across vascular walls and further disrupt the BBB;
5) Disrupting the glymphatic system through astrocyte end-feet and AQP4 interaction;
6) Hindering the maturation of OPCs into myelinating oligodendrocytes, disturbing myelination and myelin repair.

In tandem and exerted over a period of time in what represents a chronic disease process, these pathological microvascular changes may culminate in CBF and BBB changes, infarction, stroke, white matter damage, and global brain connectivity problems, which may account for eventual cognitive and/or functional impairment, and the heterogenous and multi-system clinical picture of SVD.

1.6 Endothelial cells

1.6.1 What are endothelial cells?

The brain endothelium forms the blood luminal side of the BBB and is an important structure for conserving brain and neuronal functioning **(Figure 1-9).** Endothelial cells line the walls of all vascular segments, including arteries and arterioles, which deliver blood to the tissue, capillaries and the capillary bed, which form part of the microvasculature and aid in perfusion and nutrient/molecule exchange, and veins and venules, which drain blood from tissue. Most vessels within the CNS are continuous non-fenestrated vessels, which are tightly joined together by cell junctions, have an intact and complete basement membrane lacking pores; they also contain an extra set of specialised characteristics that allow them to regulate the exchange of ions and molecules between the blood and the brain[50,60]. The smallest capillaries in the brain are formed by one single endothelial cell monolayer[133]. Brain

endothelial cells serve to maintain the integrity of the BBB as well as play a role in modulating vascular tone, blood flow, inflammation, thrombosis, and immune cell adhesion and transmigration[50,51,134,135]. Endothelial cells in the brain are sensitive and responsive to subtle environmental changes and can sense and adapt to fluctuations in blood flow or pressure through mechanoreceptors, as well as communicate with their cellular environments through ion channels and signalling molecules and proteins, influencing brain vessel function[129,136].

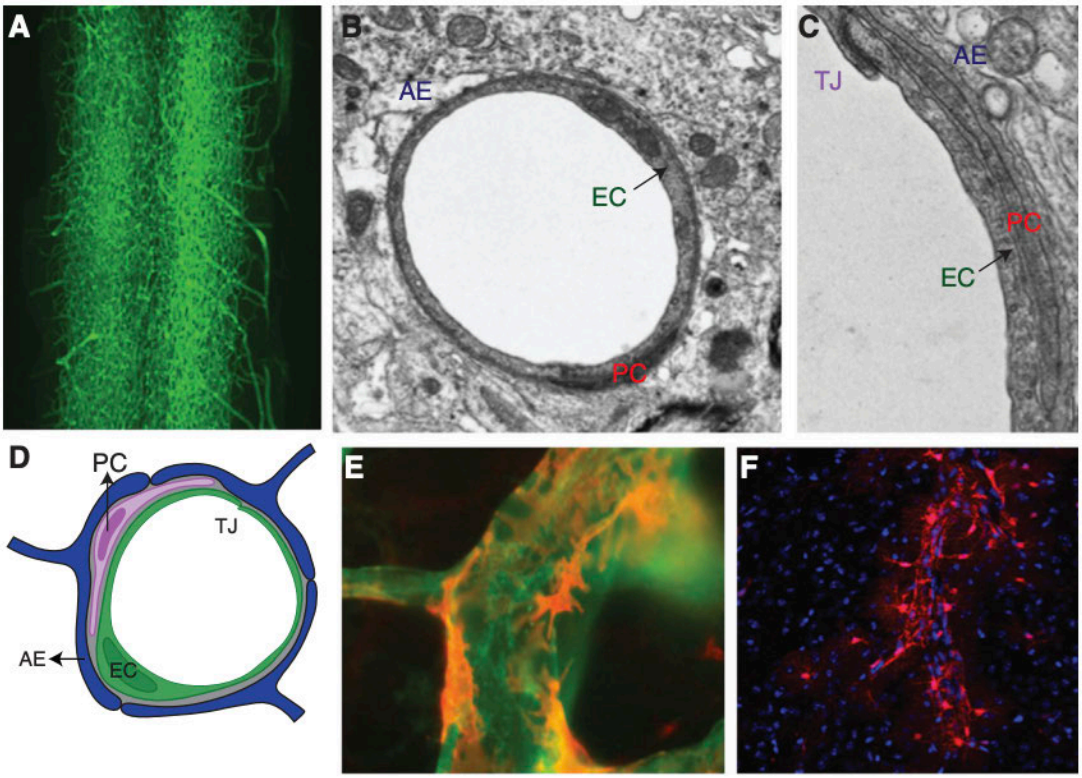
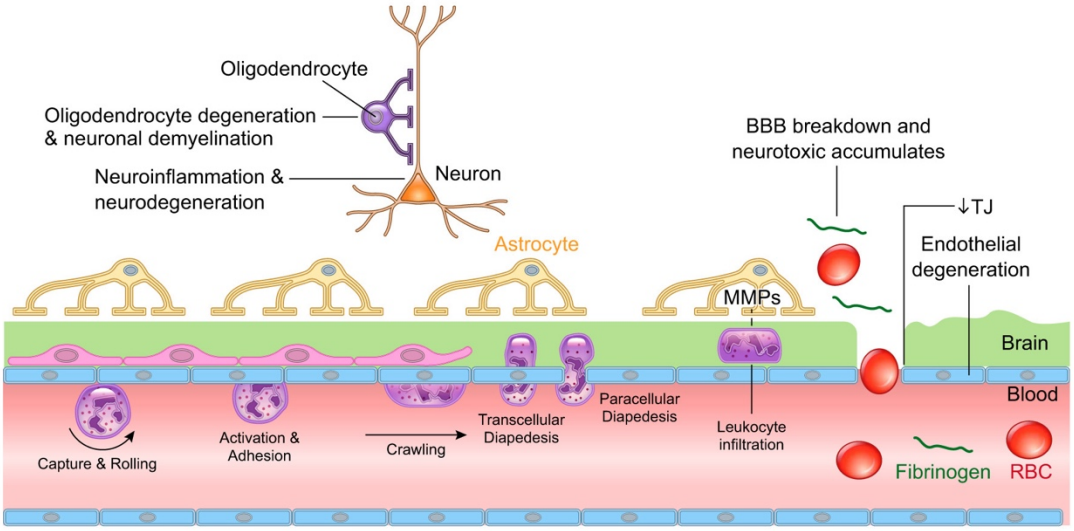


Figure 1-9. Endothelial cells and the BBB. Taken from Daneman and Prat, 2015[50], courtesy of Matthew Boisvert and Nicola Allen. Components of the BBB. A) Vascular cast of a spinal cord showing vessel density network B) Electron micrograph of a brain vessel showing an endothelial cell (EC), pericyte (PC), and astrocytes (AE) C) Electron micrograph close-up of endothelial cells, showing EC, PC, AE, and tight junctions (TJ) and the basement membrane D) Schematic representation of the BBB with the cellular components E) immunofluorescence micrograph depicting a capillary (green) and pericytes (red) F) Micrograph showing astrocytes (red) with blood vessels (unstained).

One of the unique qualities present in brain endothelial cells that allow them to make up the BBB is the presence of tight junctions, including claudins, occludin, and junctional adhesion molecules. Tight junctions hold endothelial cells together and tightly regulate the efflux of ions, molecules, and cells between the blood and the brain by providing a physical barrier to molecules and polarising the luminal and abluminal sections of the vessel[50]. Tight junctions also play a role in regulating calcium efflux in the BBB and leukocyte extravasation[137,138].

In a homeostatic state, the endothelial cells in the BBB have a low rate of transcytosis, predominantly mediated through receptor-specific, vesicle-mediated transcellular transport of molecules. In pathology, including within the context of SVD, transcytosis rates increase and there is a shift towards non-specific, caveolin-based vesicle trafficking through Caveolin-1 expression[60]. Endothelial cells in the CNS express efflux transporters and specific nutrient transporters, which aid in the transport of lipophilic molecules towards the blood and nutrients from the blood to the brain, respectively.

Cell adhesion molecules are constitutively expressed at low levels by brain endothelial cells in a homeostatic state, and endothelial cells will usually have a limited adhesiveness for leukocytes[50,133]. However, an activated endothelium in pathological conditions can express cell-surface adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), and Selectins, both of which play important roles in diapedesis. Selectins, for instance, are important for the recruitment and rolling of leukocytes whilst cell adhesion molecules facilitate the adhesion and transmigration of leukocytes through vascular walls **(Figure 1-10)**[139].



Leukocyte Infiltration Step	Capture & Rolling	Activation & Adhesion	Crawling	Transcellular Diapedesis	Paracellular Diapedesis
Endothelial cell adhesion molecules	E-selectin P-selectin	ICAM-1 VCAM-1	ICAM-1 ICAM-2	ICAM-1 JAM-A Caveolin-1 PECAM CD99	VE-cadherin JAM-A ESAM PECAM1 CD99
Leukocyte adhesion molecules	PSGL1 L-selectin	LFA-1 VLA-4	LFA-1 VLA-4 Mac-1	LFA-1 CD49 PECAM CD99	LFA-1 PECAM1 CD99

Figure 1-10. Cell adhesion molecules, Selectins, and the BBB. Figure from Sweeney et al. 2018[139] outlining the specific roles several cell adhesion molecules and Selectins play in diapedesis and, ultimately, the breakdown of the BBB through neurotoxic fibrinogen accumulation, tight junction loss, and endothelial damage.

1.6.2 Endothelial cell dysfunction in SVD

Although there is increasing evidence advocating for the role of endothelial dysfunction in SVD and as a major driver of the vascular contributions to dementia, the specific molecular mechanisms involved, and better methods of detecting these dysfunctions *in vivo,* remain unclear.

Endothelial *dysfunction* traditionally refers to the decreased activity of endothelium-derived NO activity, whilst endothelial *activation* refers to the increased expression of endothelial cell-surface adhesion molecules, such as

VCAM-1, and leukocyte adhesion molecules induced by pro-inflammatory cytokines. These molecules serve to facilitate the diapedesis, adhesion and migration of leukocytes across the BBB. Clinically, endothelial dysfunction can also manifest as BBB leakage, pulsatility, and blood flow changes, or impaired cerebrovascular reactivity (CVR), which refers to the ability of cerebral blood vessels to change their diameter in response to fluctuations in carbon dioxide levels, blood pressure, or neuronal activity. Endothelial dysfunction and damage can be detected *in vivo* in several ways, including by measuring soluble blood and CSF markers released into circulation and offer a window into the diverse facets of endothelial function that contribute to homeostasis, including cell adhesion molecules, BBB transporter proteins, cytokines, chemokines, pro-inflammatory markers, coagulation factors, growth factors, and nitric-oxide pathway participants. A comprehensive description and review of the molecular mechanisms associated with endothelial dysfunction within the context of SVD is included in **Chapter 2** of this thesis.

Dysfunctional endothelial cells also have reduced NO bioavailability, which is required for pericyte-regulated vasodilation via cGMP-dependent pathways[129,140]. Evidence also points to abnormal angiogenesis and increased endothelial cell proliferation in both rodent models and human cases of SVD, which may represent a compensatory mechanism to protect against changes in blood flow, including hypoperfusion or hypoxia[141–143].

In addition, endothelial cells play an important role in the clearance of substances, such as amyloid beta (Aβ), from the brain out to the circulation through receptor expression; a process which may malfunction in the context of SVD and AD, leading to Aβ buildup in the perivascular space and cerebral amyloid angiopathy pathology[50].

A substantial body of evidence supports the influence of endothelial dysfunction in SVD mechanisms. In plasma proteomics, VCAM-1 is one of the proteins that shows the most significant increase in expression in ageing[144]. Human brain endothelial cells cultured with exposure to VCAM-1 show

increased permeability of a soluble tracer with a concurrent reduction in tight junctions[145]. In addition, increased circulating levels of endothelial dysfunction markers, including cell adhesion molecules, have been associated with SVD and linked to lacunar stroke, WMH lesion volume and grade, BBB breakdown and permeability, and cognitive decline[145–149].

Interestingly, sequencing capillary endothelial cells of pericyte-deficient mice demonstrated a gene expression shift towards a venous pattern, with low levels of MFSD2A which inhibits transcytosis[150]. A more comprehensive review of existing evidence on the molecular mechanisms underlying endothelial dysfunction in SVD can be found in **Chapter 2** and the introduction to **Chapter 3** of this thesis.

In brief, endothelial cells and endothelial cell dysfunction may contribute to SVD via:[151,152]

1) Increased caveolar, non-specific transcytosis and BBB permeability, including through the loss of tight junctions, causing perivascular tissue and vessel wall damage and the extravasation of immune cells as well as an increase in neurotoxic blood-derived molecules in the brain tissue;
2) Blood flow dysregulation and the loss of autoregulative properties, including the ability to contract/dilate in response to metabolic demands in the brain;
3) Increased stiffness, which may increase vascular pulsatility and pulse wave velocity, thereby also decreasing the flow of perivascular space fluid for waste clearance;
4) Contribute to or exacerbate pericyte loss;
5) Endothelial cell activation, inflammation and perivascular oedema, and extracellular matrix breakdown.

The precise role of pericytes in regulating endothelial activation and vice-versa remains unclear, and the possibility of an impaired endothelial-pericyte

crosstalk in the early stages of BBB breakdown in SVD remains a feasible hypothesis and a particularly promising potential therapeutic target to explore.

1.7 My thesis

In this chapter, I have introduced SVD and elaborated on what is known about its clinical, radiological, and pathological manifestations. I have also discussed available evidence on what we know about disease pathophysiology, particularly as it pertains to the dysfunction of brain pericytes and endothelial cells, and highlighted what remains to be explored. The overarching aim of this thesis is to examine the role of brain pericyte and endothelial cell dysfunction in the development and progression of SVD mechanisms. This is further broken down into the following aims, each of which is represented by a thesis chapter:

Aim 1: To thoroughly review existing literature and identify the molecular mechanisms underlying endothelial dysfunction in SVD.

Aim 2: To investigate whether ageing mice exhibit signs of pericyte loss and/or endothelial cell activation and explore the potential relationship between these two processes.

Aim 3: To validate the murine findings from Aim 2 within the context of human SVD using postmortem brain tissue.

Aim 4: To determine how endothelial and pericyte function blood biomarkers measured *in vivo* associate with SVD burden and progression over time.

Aim 5: To acknowledge the gravity and importance of SVD and SVD research by taking a step back from the cellular level and exploring the longitudinal outcomes and far-reaching consequences of SVD.

To achieve these aims, I begin by presenting a thorough scoping literature review in **Chapter 2.** Here, I discuss existing evidence of endothelial dysfunction in SVD, emphasising established findings, identifying areas that

require further investigation, and addressing potential sources of reproducibility challenges and inconsistencies in study results. In **Chapter 3**, I investigate whether ageing mice (12 months old) exhibit signs of pericyte loss and endothelial cell activation (as indicated by cell adhesion molecule expression) compared to young mice (3 months old) across 8 distinct brain regions. I also explore the potential relationship between these two processes by assessing their co-localisation within brain capillary segments.

In **Chapter 4**, I endeavour to validate the murine findings from the previous **Chapter 3** by investigating whether the same endothelial pro-inflammatory (activated) phenotype and concurrent pericyte loss can be observed in human postmortem brain tissue across varying degrees of SVD severity compared to healthy controls. I then aim to determine whether pericyte and endothelial cell dysfunction can be detected *in vivo* and explore how they might associate with disease burden and longitudinal progression in **Chapter 5**. In this study, I examine the relationships between a panel of vascular, endothelial, and pericyte function blood biomarkers and SVD-related structural and vascular function neuroimaging measures and cognitive outcomes in highly-phenotyped participants from the Mild Stroke Study 3 (MSS3).

In **Chapter 6**, I shift my perspective from the cellular level to a broader context by acknowledging the gravity of SVD and the importance of SVD research by exploring its longitudinal effects in a patient population. Here, I present findings from a longitudinal observational study where we follow-up participants from the Mild Stroke Study 2 (MSS2) 9 years after mild ischaemic stroke. I report health and functional outcomes and describe some of the significant and far-reaching effects SVD and mild stroke can have on patient quality of life.

Finally, in **Chapter 7,** I summarise and interpret the findings outlined in previous chapters and discuss potential strengths and limitations of this research, concluding with the clinical and research implications of the findings presented in this thesis.

Chapter 2 A scoping review on biomarkers of endothelial dysfunction in small vessel disease: Molecular insights from human studies

2.1 Context

The previous chapter served to contextualise the research presented by providing an overview of SVD and its underlying mechanisms, particularly those pertaining to pericyte and endothelial cell dysfunction. In this chapter, I consolidate the substantial body of research focussing on the molecular mechanisms that drive cerebrovascular dysfunction in SVD. More specifically, I focus principally on clinical studies that link biochemical markers of endothelial dysfunction detectable in blood or CSF to clinical or neuroimaging manifestations of SVD in various populations. The aim of this chapter is to comprehensively review existing evidence of endothelial dysfunction in SVD, elucidating known aspects and highlighting areas which require further investigation or potential sources which might be hindering result extrapolation or reproducibility.

This chapter was published as a scoping literature review in the International Journal of Molecular Sciences in August of 2023[152]; a PDF copy of the publication is supplied in Appendix A.

2.2 Introduction

Small vessel disease (SVD) is a highly prevalent disorder of the brain's perforating microvessels. It is the underlying cause of a quarter of all ischaemic strokes and most haemorrhagic strokes, and is the commonest cause of vascular dementia, contributing to approximately 45% of all dementias worldwide[153]. Radiological signs of SVD include lacunes, small subcortical infarcts, white matter hyperintensities (WMH), microbleeds, brain atrophy, and

enlarged perivascular spaces (PVS), and are clinically associated with lacunar stroke, cognitive decline, neuropsychiatric symptoms and mobility problems[7,154–156]. Despite its increasing incidence and importance in the public health domain, the underlying pathophysiology of SVD remains poorly understood and there is currently no effective therapeutic intervention beyond secondary stroke-prevention strategies.

SVD is associated with cerebral vascular dysfunction, characterised by structural and functional alterations in cerebral blood vessels, in blood flow responsiveness to changing metabolic demands, and a compromised and permeable blood-brain barrier (BBB), which precede visible brain lesions[157]. The BBB is a highly specialised and dynamic structure referring to the set of characteristics in the vessels that vascularise the brain. It is formed by a tight monolayer of endothelial cells that line the blood vessel lumen, which uses selective permeability to closely regulate the exchange of substances between the circulating blood and the brain. Its function is also influenced by the constituents of the neurogliovascular unit, including pericytes, astrocyte endfeet, microglia, oligodendrocytes, the basal membrane, and neurons. Optimal BBB functioning is required to maintain healthy brain function by allowing for the adequate delivery of nutrients and oxygen to neurons, promoting drainage of metabolic waste, and protecting neurons from pathogens and neurotoxic compounds[50,52].

Brain endothelial cells are joined by tight junctions and serve to maintain the integrity of the BBB as well as play a role in modulating vascular tone, blood flow, inflammation, thrombosis, and immune cell adhesion and transmigration[50–52,134]. Endothelial cells are responsive to changes in their environment and can sense and adapt to fluctuations in blood flow or pressure through surface mechanoreceptors as well as communicate with their cellular environments through ion channels and signalling molecules and proteins that can alter their signature and thus influence brain vessel function[135,136]. Endothelial malfunction can be detected *in vivo* by quantifying soluble blood and cerebrospinal fluid (CSF) markers that are released into the circulation

and offer a window into the diverse facets of endothelial function that contribute to homeostasis; these include cell adhesion molecules, BBB transporters, cytokines and chemokines, pro-inflammatory markers, coagulation factors, growth factors, and markers pertaining to the nitric-oxide pathway.

In the pathological context of SVD, and indeed to an extent in normal ageing, there is a loss of some of the properties that maintain BBB integrity and a propensity towards endothelial dysfunction and activation, both of which carry a downstream set of consequences influencing the function of surrounding cells and tissue and are associated with disease burden and progression[7,129,156–167]. Within this review, the term 'endothelial dysfunction' encompasses the established definitions of both, endothelial dysfunction, marked by decreased endothelium-derived nitric oxide (NO) activity, and endothelial activation, which refers to the increased expression of endothelial cell-surface adhesion molecules and leukocyte adhesion molecules induced by pro-inflammatory cytokines[134,151]. Though there is mounting evidence advocating for the role of endothelial dysfunction in SVD, the underpinning molecular mechanisms, and better methods to detect dysfunction *in vivo*, remain unclear. In this scoping review, we aim to summarise the findings from clinical studies examining the role of the molecular mechanisms underlying endothelial dysfunction and activation in SVD, focussing on biochemical markers of endothelial dysfunction detectable in blood or CSF.

2.3 Methods

2.3.1 Literature review

We reviewed the literature examining biomarkers pertaining to endothelial dysfunction and radiological features of SVD in clinical studies. The literature search was conducted using MEDLINE and EMBASE from database inception to June 1st, 2023 using key terms relating to small vessel disease (lacunes, small subcortical infarcts, white matter hyperintensities, microbleeds, and enlarged perivascular spaces) combined with terms relating to the endothelium

(endotheli*). All articles were screened for eligibility criteria. We included publications relating to humans and examining biochemical markers of endothelial dysfunction detectable in blood or CSF and known radiological markers of SVD. We excluded review articles/meta-analyses, publications relating to study rationale and design, publications based exclusively on case studies or monogenic forms of SVD (including Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy (CADASIL)), as well as articles solely pertaining to mechanisms of cardiovascular or large-artery disease (e.g. coronary artery disease, atherosclerosis). Duplicate articles were removed. Additional studies examining the relationship between radiological signs of SVD and biomarkers of endothelial dysfunction from reference lists were also manually added to reinforce the search. We extracted summary data for each paper, including main aims, study population and sample size, methods employed, adjustment for covariates, and primary findings.

2.4 Results

The search yielded 380 results, 135 of which were classified as reviews and were thus removed, as were 2 duplicate publications and 4 papers pertaining to excluded publication types **(Figure 2-1).** Of the 239 remaining results, 194 publications described clinical studies, of which 38 were eligible for inclusion based on the criteria outlined above. In addition, 11 papers were manually added from reference lists.

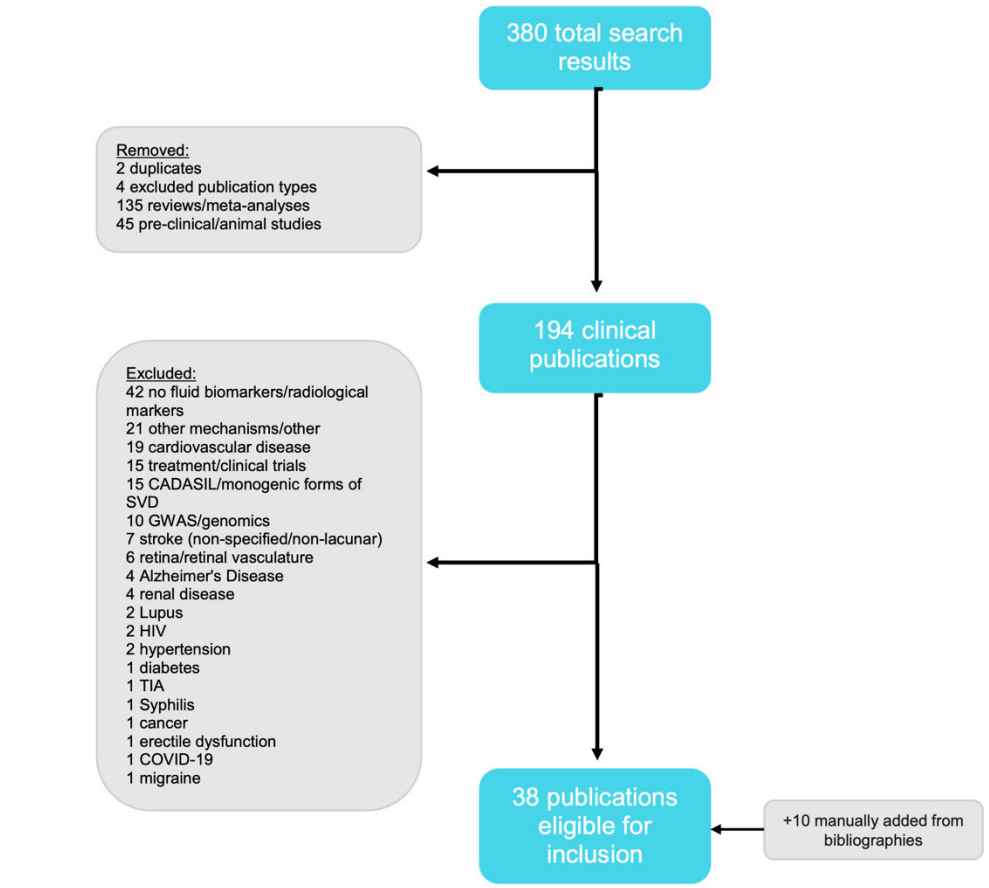


Figure 2-1. Study screening and selection process. A total of 49 publications were included in this review, including 11 manually selected from bibliographies.

2.4.1 Biomarkers of endothelial dysfunction and SVD: molecular insights from human studies

2.4.1.1 Immunoglobulin Superfamily—Cell adhesion molecules and Selectins

In a homeostatic state, most cell adhesion molecules are constitutively expressed at low levels in the brain vasculature, and endothelial cells have a limited adhesiveness for leukocytes. However, a cytokine-activated endothelium can express cell-surface adhesion molecules that facilitate the recruitment, rolling, adhesion and transendothelial migration of leukocytes through the endothelium of vascular walls; the soluble forms of these molecules are released from the cell surface into the circulation and can serve

as biomarkers of endothelial activation[168–170]. Endothelium-leukocyte interactions engender a cyclic activation of both components, leading to the production of neopterin and cytokines, which can also instigate hepatic production of C-reactive protein (CRP), thus exacerbating an inflammatory response[171,172]. There is also evidence suggesting the involvement of other constituents of the neurogliovascular unit, particularly pericytes, in a dysfunctional crosstalk which can further aggravate the endothelium[129].

Vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are transmembrane glycoproteins from the immunoglobulin gene superfamily which bind to integrins on the cell surface, whilst endothelial-leukocyte adhesion molecules, Endothelial-selectin (E-selectin) and Platelet-selectin (P-selectin), interact with leukocyte surfaces via carbohydrate structures[170]. Circulating cell adhesion molecules have been linked to ageing and a series of age-related neurodegenerative disorders, including Alzheimer's Disease (AD) and SVD, and there is substantial evidence suggesting their expression can lead to downstream signalling events that are associated with increased BBB permeability[166,173–179]. For instance, cultured human brain microvascular endothelial cells exposed to soluble VCAM-1 (sVCAM-1) exhibit increased permeability of a soluble tracer accompanied by evidence of actin stress fibre formation and a decrease in tight junctions[174].

We found 19 published studies investigating the relationship between cell adhesion molecules and radiological features of SVD featuring a total of 7,364 study participants (average sample size: 368.2).

In a study with 110 lacunar stroke patients and 50 community controls with similar vascular risk-factor profiles, those with lacunar stroke had higher levels of circulating ICAM-1[162]. These associations remained significant after controlling for conventional vascular risk factors and were not found to be associated with age, sex, blood pressure, diabetes, myocardial infarction, or time from vascular event to blood draw (median: 210 days). They also found

comparable ICAM-1 concentrations amongst patients with isolated lacunar infarcts and those with WMH lesions.

Similarly, circulating levels of ICAM-1, VCAM-1 and P-selectin (though not E-selectin) were significantly higher in a group of 163 first-ever lacunar stroke patients compared to essential hypertensive patients and healthy controls[177]. Those with extensive WMH lesions had higher concentrations of sVCAM-1 than those with mild or absent WMH and those with radiological evidence of both, a lacunar infarct and WMH lesions had the highest levels of sVCAM-1 and sICAM-1 on average. Though a marginal portion of the essential hypertensive group were found to have asymptomatic lacunes (n=13/183) and an extensive WMH burden (n=22/183) on magnetic resonance imaging (MRI), the distinct biomarker signatures between these groups provide further evidence in support of SVD-specific pathology beyond hypertension-related vascular injury. In the same study, multivariate analyses controlling for age, sex, pre-existing large-artery disease, and patient group revealed a positive association between higher levels of sE-selectin and number of microbleeds, perhaps suggesting a haemorrhagic phenotype-specific role. This is further supported by another study with 126 first-ever ischaemic stroke patients, where higher levels of sE-selectin within 24-hours of hospital admission were associated with the prevalence of microbleeds after adjusting for demographic and clinical variables[180]. No significant differences in P-selectin-positive platelets were found amongst 74 lacunar stroke patients and 74 controls matched for age, sex, and diabetes and hypertension status with no history of stroke, though the possible presence of radiological signs of SVD in controls was not ruled out nor was the extent of SVD radiological burden taken into account in these analyses[181].

We found few studies examining the relationship between fluid biomarkers and enlarged PVS, though in 100 mild ischaemic stroke patients, PVS burden in the basal ganglia was not associated with ICAM-1 levels. While this might indicate different pathological processes involved in the appearance of PVS, further investigation is needed[182].

Including mild ischaemic stroke patients as controls can help parse out potential effects independent of those stemming from an acute ischaemic event or large-artery pathology. Regression analyses in a group of 263 thrombolysed acute ischaemic stroke patients showed that a combined score comprised of number of lacunes and WMH lesion severity as detectable on computerised tomography (CT) was associated with increased levels of sICAM-1 and sVCAM-1 at 90 days post-stroke after adjusting for age, sex, and vascular risk factors[183]. Interestingly, levels of these biomarkers were comparable between patients with WMH, cerebral atrophy and/or lacunes compared to those without at the time of stroke-symptom onset, suggesting possible fluctuations in circulating cell adhesion markers during an acute stroke phase. Compared to a group of 67 age and sex-matched controls, 64 SVD patients and 109 patients with large-artery atherosclerosis had higher concentrations of sE-selectin and sICAM-1 (though not sVCAM-1), with the highest concentrations found in patients with intracranial macroangiopathy[184]. However, these analyses did not account for clinical covariates despite substantial group differences- for instance, nearly half of patients with large-artery atherosclerosis were smokers, compared to 13% of SVD patients. In addition, some participants in every group had a history of stroke, including 21% of controls, which may have affected results.

In a small study of 29 patients with SVD and 18 patients with recent ischaemic stroke on the basis of internal carotid artery stenosis, levels of sVCAM-1 and sP-selectin were significantly higher in patients in the highest quartile of periventricular WMH lesion burden compared to those in the lower quartiles[166]. This relationship did not extend to subcortical white matter lesions. To account for potential differences relating to the stroke itself, the authors performed a sub-analysis comparing cell adhesion molecule concentrations with degree of white matter lesions separately in patients with acute stroke and those with carotid atherosclerosis. Their results indicated that the increased levels of sVCAM-1 and sP-selectin in those with severe periventricular WMH lesions in the group was driven by the subgroup of patients with carotid atherosclerosis. It is plausible that this is a result of compounded chronic vascular damage,

which might be attenuating the relationship between WMH and cell adhesion molecules. It is important to note that cell adhesion molecule plasma concentrations in this small study sample were highly variable and the dichotomisation of white matter lesions to 'mild' (including the lowest three quartiles of WMH lesion burden) and 'severe' (including the highest quartile), could potentially overlook a nuanced relationship between WMH volume and endothelial activation. In addition, though the study was comparing symptomatic SVD with large-artery atherosclerosis, both groups had comparable levels of WMH lesion burden, and the analyses did not account for potential confounding variables, including vascular risk factors.

Similar results are mirrored in studies with healthy ageing cohorts. In 175 healthy adults ≥ 60 years old with no history of stroke or cognitive impairment, those in the highest quartile of sICAM-1 levels had a four-fold increased risk of having WMH lesions on MRI after adjusting for age, sex and traditional vascular risk factors[185]. In a large Chinese community-based cohort study (n=960; average age: 56.02), grouped endothelial dysfunction biomarkers including sE-selectin, sP-selectin, sICAM-1 and sVCAM-1 were associated with WMH volume and the presence of lacunes, though not microbleeds or PVS, independent of age, sex and vascular risk factors; backward elimination regression retained sVCAM-1 as an independent predictor of WMH volume[186]. In the Framingham Offspring cohort, analyses with 1,763 stroke-free individuals (average age: 60.2) showed higher levels of sICAM-1 (though not sP-selectin) in those with radiological signs of SVD, defined as the presence of lacunes and/or extensive WMH lesions on MRI. Though these results survived additional adjustment for clinical covariates, they did not withstand Bonferroni correction for multiple comparisons. It is worth noting that the mean time from blood draw to MRI was substantial, averaging at 5.4 years, and thus it is possible that biomarker profiles were not representative of temporal disease progression. In addition, the study excluded those with a history of stroke, limiting the participation of individuals with symptomatic or more severe SVD; indeed, only 12-21% of participants had lacunes or extensive WMH lesions[187].

Longitudinal studies also support the involvement of cell adhesion molecules in SVD progression and adverse clinical outcomes. In a 10-month follow-up study, 35 SVD patients receiving a monthly MRI were stratified into those with SVD progression and those without, based on the appearance of incident lacunes, microbleeds or an increase in WMH volume. After adjusting for age, sex, and hypertension, circulating levels of sVCAM-1 and sE-selectin were higher in those with SVD progression compared to those without, though only the latter was statistically significant[188]. Changes in sICAM-1 and sVCAM-1 levels were positively associated with an increased risk of SVD progression in a 69-month longitudinal study with 1,396 participants and were independently associated with WMH grade and number of new appearing lacunes and microbleeds[189]. Likewise, amongst a cohort of 123 SVD patients followed-up 2 years, those with radiological disease progression had a higher compound z-score comprised of markers of vascular inflammation, including sICAM-1 and sP-selectin, after adjusting for age, sex, blood pressure and total SVD radiological load[190]. Circulating levels of sICAM-1 were significantly higher in 113 acute lacunar stroke patients compared to 43 healthy controls[191]. Although potential effects stemming from an acute ischaemic event cannot be discounted, they also found that within those with a lacunar stroke, sICAM-1 levels were higher in those who had neurological deterioration within 48-72 hours post-stroke (defined as a ≥ 1 point fall in the motor items of the Canadian Stroke Scale) than those with non-progressive strokes. After adjusting for potential confounding variables, sICAM-1 levels were also independently associated with poor outcome 3 months post-stroke, including lower functional independence and death, though infarct volume and radiological disease burden could have mediated this association. Intriguingly, in an 8-year longitudinal cohort study of 495 cognitively unimpaired elderly individuals (average age: 70.02) and 247 participants (average age: 70.89) with mild cognitive impairment (MCI), CSF levels of ICAM-1 and VCAM-1 had independent effects on declining cognition, even after adjusting for WMH volume, potentially suggesting a distinct pathway involved in cognitive decline[192].

In the Austrian Stroke Prevention Study cohort, 296 community-dwelling participants received an MRI at baseline and again at 3 and 6 years[193]. After adjusting for age, sex, CRP levels, baseline WMH volume and traditional vascular risk factors, WMH lesion progression at both follow-up time points was associated with higher levels of circulating sICAM-1. In a longitudinal study of 190 type-2 diabetes patients, baseline levels of sICAM-1 were higher in patients with radiological signs of SVD than those without and were also associated with the increased presence of incident lacunes and WMH lesion progression at 3 and 6 years after adjusting for age, sex, hypertension, duration of diabetes, baseline MRI findings and medication use[194]. They also demonstrated a significant trend association between higher levels of sICAM-1 and lower scores in a letter-digit psychomotor task, though this could be mediated by radiological disease burden.

Examining brain endothelial-specific molecular mechanisms of SVD in patients represents a significant challenge, and while quantifying biomarkers from circulating blood can reflect possible dysregulated pathways, it provides an indirect path of examination. Studies zoning in on brain endothelial cell signatures from SVD patients provide some conflicting evidence. A post-mortem study examining vessels from 17 SVD cases and 12 aged controls found little evidence of positive endothelial ICAM-1 vessels in either group[195]. The authors hypothesise that elevated circulating levels of sICAM-1 in SVD patients are therefore unlikely brain-derived and may indicate inflammatory activity in distal peripheral vascular territories. However, this study limited analyses to one cell adhesion molecule and small arteries within the caudate putamen gray matter areas, excluding other vascular territories directly impacted by SVD, namely the capillaries feeding into the white matter. While it remains feasible that high sICAM-1 levels in SVD patients could stem from beyond the brain endothelium, ICAM-1 is a heterogeneous molecule and can influence endothelial function through dysfunctional crosstalk involving other cells in the brain that can also derive it, including monocytes, macrophages, smooth muscle cells and pericytes[196]. Elahi *et al.* used plasma membrane-derived vesicles released by cells (plasma endothelial-derived exosomes

(EDE)) in 26 functionally intact older adults (average age: 75.4) to compare protein cargo between those with and without WMH lesions on MRI[197]. Though SVD cases showed higher levels of brain-expressed EDE cargo proteins, and these were associated with lower cognitive function, there were no significant group differences detected in VCAM-1 levels specifically after adjusting for age.

Although there is evidence in support of the involvement of cell adhesion molecules in diverse manifestations of SVD, the lack of clarity in the underlying mechanisms through which they might influence disease and where the molecules derive from and how they engender and interact with pathological mechanisms warrants further examination.

2.4.1.2 Blood-brain barrier transporters

Membrane transport proteins at the BBB mediate the influx and efflux of solutes from the circulation through endothelial cells and into the brain and vice-versa. Glucose transporter-1 (GLUT-1) is expressed in brain vascular endothelial cells and transports glucose to the brain via facilitative diffusion. GLUT-1 levels are implicated in metabolic dysregulations and are known to decrease with ageing, possibly disturbing neurovascular coupling processes based on glucose demand[198]. Permeability-glycoprotein (P-GP) is an adenosine triphosphate binding cassette transporter expressed by the luminal surface of endothelial cells. It is known for its role in pharmacokinetics and drug-delivery to the BBB by strictly limiting the exposure of pharmaceuticals to the brain by actively transporting undesired substrates from the BBB back to the bloodstream against the concentration gradient; it has also been implicated in the accumulation of amyloid-β (Aβ) in AD pathology[182,199]. Large neutral amino acid transporter-1 (LAT-1) is expressed in both the luminal and abluminal membranes of endothelial cells and carries large essential amino acids across the BBB[200]. Caveolin-1 (Cav-1) is the principal scaffold protein comprising caveolae, which are invaginations abundant on the cell membrane of endothelial cells, facilitating vesicle trafficking and signal transduction. Cav-1 activity is related to oxidative stress, the attenuation of inflammatory

pathways, and BBB permeability, perhaps through the modulation of tight junction-associated proteins[201,202]. Pro-inflammatory cytokines can also upregulate BBB transporter protein expression in endothelial cells, affecting vascular function. For instance, increases in GLUT-1 in murine smooth muscle cells can impair vascular contractility and instigate an inflammatory response to vascular injury[203]. GLUT-1 deficiency in mice overexpressing Aβ-peptide precursor protein leads to significant reductions in blood flow, BBB and microvascular breakdown, accelerated Aβ accumulation, cognitive deficits and progressive neurodegeneration[204].

In an attempt to circumvent the low specificity of fluid biomarkers for the assessment of endothelial dysfunction, a case-control study with 26 functionally intact older adults (average age: 75.4) used endothelial-derived biomarkers to examine the relationship between WMH lesions and endothelial-expressed BBB transporter proteins GLUT-1, P-GP and LAT-1[197]. EDE cargo protein quantification offers a non-invasive way of observing cellular molecular changes *in vivo* using exosomes, which are cell-released vesicles containing cargo molecules such as proteins, mRNAs and lipids from their cell of origin. Levels of GLUT-1, LAT-1 and P-GP were significantly higher in participants with WMH than in those without. Age-adjusted logistic regression analyses demonstrated a robust predictive power of GLUT-1, LAT-1 and P-GP to differentiate between participants with and without WMH, with the area under the curve for the strongest predictor, GLUT-1, being 0.89. LAT-1 levels were significantly inversely associated with total grey matter volumes after controlling for intracranial volume (ICV) and age and correcting for multiple comparisons. In addition, all brain-expressed EDE cargo proteins were associated with lower cognitive performance in a set-shifting speed task after adjusting for age. Cytokines and chronic states of inflammation could up-regulate some BBB transporter proteins in endothelial cells and interestingly, levels of GLUT-1, P-GP and LAT-1 were associated with levels of systemic inflammatory cytokine Interleukin-6 (IL-6) in this cohort[197].

In 156 first-ever ischaemic stroke patients, circulating Cav-1 levels were lower in patients with microbleeds compared to those without, and lowest in patients with multiple microbleeds or microbleeds in deep or infratentorial areas compared to those with a single microbleed or strictly lobar microbleeds[205]. Patients below their established Cav-1 concentration cut-off (≤5.25 ng/mL) had a near four-fold increased risk of having microbleeds after adjusting for age, sex, vascular risk factors, anti-thrombotics use, glucose levels, lipids and fibrinogen. There was no relationship detected between Cav-1 levels and WMH lesions or lacunes.

Clinical research studies investigating the role of transport proteins in SVD pathogenesis are scarce. However, transporter proteins play a crucial role in BBB homeostasis and can potentially serve as promising targets to ameliorate the BBB dysfunction observed in the early stages of disease. The studies mentioned here suggest a relationship between transporter protein dysregulation and some radiological manifestations of SVD, though more studies are required to validate and further uncover the nature of this relationship.

2.4.1.3 Cytokines and chemokines

2.4.1.3.1 Interleukin-6

IL-6 is a pro-inflammatory cytokine with pleiotropic effects produced by monocytes and endothelial cells. Once synthesised in a lesion site, it migrates to the liver where it incites the release of several acute-phase proteins, including CRP and fibrinogen[206]. It also instigates platelet production and is thus involved in the development of atherosclerosis. Pre-clinical studies suggest IL-6 can engender endothelial dysfunction through an increase in Angiotensin II type 1 (AT1) receptor activity, an interaction with tumour necrosis factor alpha (TNF-α), and the induced production of reactive oxygen species (ROS), which can cause enhanced vascular superoxide production, exacerbate oxidative stress, and impair endothelium-dependent vasodilation[207,208]. Numerous clinical studies have examined the association between IL-6 and cardiovascular health, particularly in stroke, with many lines

of evidence suggesting it serves as a strong predictor for stroke, atherosclerosis and adverse cardiovascular events[209,210].

In a Japanese study with 194 neurologically asymptomatic participants at a high risk of stroke, mean serum levels of IL-6 were higher in patients with lacunes visible on MRI than in those without, with one standard deviation increase in IL-6 leading to a near two-fold increased risk of having at least one visible lacune after adjusting for age, sex, vascular risk factors, medication use and carotid atherosclerosis[211]. In the Cardiovascular Health Study, both white (n=3,073) and black (n=571) participants with higher IL-6 levels had a mildly increased risk of having WMH lesions and brain infarcts independent of age, sex and cardiovascular risk factors[212]. Likewise, in 247 elderly participants with MCI, though not in 495 cognitively unimpaired participants, baseline CSF IL-6 levels were associated with greater WMH volume after adjusting for age, sex, ICV and vascular risk factors[192]. Adjusting for CSF Aβ-40 attenuated the relationship. Intriguingly, lower IL-6 levels were associated with increasing WMH burden over 7 years in the same study. It is possible that the greater incidence and severity of WMH lesions in the MCI group increased the likelihood of finding significant relationships within this group due to enhanced statistical power.

In univariate analyses, plasma IL-6 concentrations were significantly higher in 113 lacunar stroke patients within 24-hours of stroke onset compared to 43 healthy controls, though the possible confounding effects of an acute ischaemic event, demographic characteristics or comorbidities were not taken into account in these analyses[191]. In this same study, after adjusting for potential confounding variables, there was no association found between circulating levels of IL-6 and worsening clinical progression or poor outcome at 3 months post-stroke. A study comparing 65 lacunar stroke patients with 60 cortical stroke patients reported no significant differences in circulating IL-6 levels between the groups, possibly suggesting the association between IL-6 and recent stroke might not be specific to SVD pathology[213]. It is important to consider that medication use (including aspirin treatment), glutamate and

GABA levels, the presence or timing of stroke, and the degree of large-artery atherosclerosis are all elements that could influence circulating pro-inflammatory biomarker levels.

In line with previous research examining the relationship between IL-6 levels and cardiovascular event risk, IL-6 concentrations were associated with an increased risk (OR: 1.4, 95% CI: 1.1-2.2) of adverse vascular events, including stroke, cardiovascular events and/or vascular death in a 24-month longitudinal study with 130 functionally independent SVD patients[214]. This association was further confirmed by findings from the LIMITS study, which observed an association between elevated levels of circulating IL-6 and the risk of recurrent ischaemic stroke, myocardial infarction or vascular death in 1,244 radiologically confirmed lacunar stroke patients[215]. However, a small 10-month longitudinal study with 35 SVD patients reported no significant association between baseline IL-6 levels and the appearance of new lesions on diffusion-weighted MR imaging, incident lacunes and/or microbleeds or WMH progression after adjusting for age, sex and hypertension[188].

Although studies examining the relationship between IL-6 and other radiological markers of SVD were lacking, one study reported finding no association between IL-6 and the presence of basal ganglia PVS on MRI after adjusting for potential confounding variables in 100 mild ischaemic stroke patients, both lacunar and cortical[216].

2.4.1.3.2 Tumour necrosis factor alpha and its receptors

TNF-α is a pro-inflammatory cytokine primarily secreted by macrophages and microglia, that functions via cell membrane receptors, including tumour necrosis factor receptors 1 and 2 (TNFR1/TNFR2). TNF-α promotes the secretion of endothelial cell adhesion molecules, facilitating leukocyte adhesion and reduced blood flow[217]. High levels of TNF-α have been observed in patients with acute ischaemic stroke[218]. In both clinical and pre-clinical investigations, TNF-α has been associated with increased BBB permeability and dysfunction, inflammation, oligodendrocyte death and myelin damage[219–

[221]. In addition, increased levels of TNF-α can inhibit NO production in the endothelium, induce ROS production, and impair vasodilation[222,223].

Results from the LIMITS study provide some evidence of the relationship between TNFR1 and worse clinical outcomes in SVD patients. The risk of major vascular events, though not recurrent stroke, increased with elevated concentrations of TNFR1 (HR: 1.68, 95% CI: 0.93-3.04) after adjusting for demographics, risk factors and statin use in 1,244 radiologically-confirmed lacunar stroke patients (average age: 63.3)[215]. There was no evidence that TNFR1 was associated with the risk of having a recurrent lacunar stroke.

Average plasma concentrations of TNF-α were significantly higher in 113 acute lacunar stroke patients compared to 43 healthy controls in univariate analyses[191]. In addition, within lacunar stroke patients, levels of TNF-α were higher in those who had neurological deterioration (defined as a fall of ≥ 1 point in the motor items of the Canadian Stroke Scale within inclusion and 48 hours) and served as an independent predictor for worse clinical outcome (Barthel index < 85 or death). However, it is worth noting that the control group was considerably younger (average age: 55) compared to the lacunar stroke group (average age: 69.7). Moreover, comparing an acute-stroke group to a non-stroke group provides limitations in the interpretation of results, particularly as it relates to the variation of pro-inflammatory molecules that may fluctuate as a direct consequence to an acute ischaemic event. Indeed, results from another study reported no significant differences in circulating TNF-α levels between 60 cortical stroke patients and 65 lacunar stroke patients 1-3 months post-stroke[213].

Studies examining the relationship between TNF-α and radiological markers of SVD beyond WMH lesions are sparse. In a prospective study with 100 mild ischaemic stroke patients, there were no significant associations between TNF-α and PVS after adjusting for potential covariates[216]. In the Framingham Heart Study cohort (sample size: 1,763, average age: 60.2), higher levels of circulating TNFR2 were associated with the presence of deep cerebral microbleeds after adjusting for age, sex, time from blood draw to MRI (average:

5 years), hypertension, WMH volume, number of lacunes and medication use[187]. In this study, increases in TNFR2 related to a 3.3-fold increase in odds of having more than 1 microbleed and a 5.7-fold increase in odds of having more than 2 microbleeds, possibly suggesting distinct mechanistic pathways involved in ischaemic and haemorrhagic manifestations of SVD.

Dobrynina and colleagues performed a cluster comparison in 40 SVD patients with a Fazekas score of 3 to categorise patients into 2 radiological subtypes of SVD[224]. Patients in the MRI subgroup characterised by more severe deep WMH, lacunes in the white matter as opposed to dispersed, a lack of microbleeds or atrophy and less severe clinical manifestations had significantly higher levels of TNF-α compared to those in the other MRI group and healthy controls.

2.4.1.3.3 Osteoprotegerin

Osteoprotegerin is a cytokine expressed by endothelial cells and smooth muscle cells involved in inflammation and vascular injury. High levels have been associated with large-artery atherosclerosis and stroke, though there is limited research regarding its relation to SVD[225]. Results from the Framingham Offspring cohort study (sample size: 1,763; average age: 60.2) showed that participants with lacunes and/or extensive WMH lesions had higher levels of osteoprotegerin than those without after adjusting for age, sex, vascular risk factors and the presence of microbleeds; the relationship also withstood Bonferroni correction for multiple comparisons[187].

2.4.1.3.4 Platelet factor-4

Platelet factor-4 (PF-4) is a chemokine stored in the alpha granules of platelets. It is released on activation, whereupon is exerts angiostatic effects by inhibiting the binding of specific growth factors, such as vascular endothelial growth factor (VEGF) to cells[226]. PF-4 has previously been associated with stroke risk, infarct size and worse clinical outcomes post-stroke[227]. A prospective study featuring 123 SVD patients aged over 60 years demonstrated that patients with radiological disease progression, as measured by an increase in WMH severity, had higher levels of PF-4 after adjusting for age, sex, blood pressure

and total SVD radiological load[190]. The precise mechanisms underpinning the interaction between SVD and PF-4 remain unclear, and larger studies are required to reach more robust and definitive conclusions.

2.4.1.4 Other pro-inflammatory markers

2.4.1.4.1 C-reactive protein

CRP is a hepatic acute-phase reactant protein known to increase in concentration as a response to IL-6 secretion and systemic inflammation. Though little is known about its direct role in SVD, there is an established connection between CRP and cardiovascular-event risk, including stroke[228,229]. CRP can influence endothelial dysfunction in conjunction with its involvement in systemic inflammation through a variety of diverse mechanisms. For instance, CRP activity can inhibit angiogenesis, promote atherosclerosis, mediate circulating cytokine levels, inhibit NO, decrease endothelial nitric oxide synthase (eNOS) activity in endothelial cells, increase Endothelin-1 production, attenuate endothelial-dependent vasodilation and increase endothelial cell adhesion molecule expression, including VCAM-1 and E-selectin[230–232].

We found 12 studies investigating the relationship between CRP levels and radiological signs of SVD featuring a total of 9,794 study participants (average sample size: 816.16).

Most studies featuring acute and sub-acute stroke patients do not report strong associations between CRP levels and SVD disease burden, though it is important to note that CRP levels are influenced by ischaemic events. For instance, baseline CRP levels were higher in 74 lacunar stroke patients within a month of symptom onset compared to 74 controls matched for age, sex, hypertension and diabetes; however, this difference was attenuated by 3 months post-stroke, possibly suggesting that the observed elevated CRP levels in lacunar stroke patients were attributable to an acute ischaemic event rather than chronic SVD disease mechanisms[181]. In a study with 65 lacunar stroke patients and 60 cortical stroke patients serving as controls, CRP levels did not vary significantly between the groups after adjusting for age, sex and

the presence of traditional vascular risk factors. However, incorporating a cluster of inflammatory markers including CRP, TNF-α and IL-6 into their base model for predicting WMH volume mildly improved model fit (from R2=0.289 to R2=0.291)[213].

Though WMH lesion burden and the presence of lacunes constituted the main outcomes in most of the publications we found, a study examining the association between PVS in the basal ganglia and circulating CRP levels at 1-3 months post mild cortical or lacunar stroke in 100 patients reported no significant associations after controlling for age, sex and vascular risk factors[216]. In addition, a recent study reported significantly higher levels of CRP in SVD patients with cognitive impairment (n=40) compared to SVD patients without cognitive impairment (n=38) and healthy controls (n=35), respectively[233]. However, these analyses did not account for potential covariates and included a relatively small sample size.

In line with existing evidence, longitudinal studies with SVD patients support the relationship between baseline CRP levels and recurrent stroke. In 1,422 lacunar stroke patients with no prior history of cortical stroke or carotid stenosis, patients in the highest quartile of circulating CRP levels measured in the subacute post-stroke phase (median: 60 days) had more than twice the risk of recurrent ischaemic stroke at 3 year follow-up (OR: 2.32, 95% CI: 1.15-4.68) after adjusting for age, sex, race-ethnicity, history of cardiac disease, vascular risk factors and statin use[234]. Although approximately 70% of recurrent ischaemic strokes were of lacunar aetiology, there was no evidence of a statistically significant predictive effect of CRP on recurrent lacunar stroke specifically. In addition, a small study with 130 SVD patients (including 28 vascular Parkinsonism patients) reported a marginal association between baseline CRP levels and recurrent stroke, death or cardiovascular events at 24-month follow-up after adjusting for age, sex, vascular risk factors, statin or antiplatelet use and radiological SVD burden (HR 1.02, 95% CI: 0.98-1.7)[214].

Larger population-based studies with stroke-free participants have yielded conflicting results. Findings from the Rotterdam cohort (n=1,033, average age: 72) showed that those in the highest quartile of CRP levels at baseline had more extensive WMH lesions compared to those in the lowest quartile after adjusting for age, sex, cardiovascular risk factors and carotid atherosclerosis[235]. In the 636 participants who had a repeat MRI at 3 years, CRP levels at baseline were also associated with WMH lesion progression and number of new lacunes, though the latter association was not statistically significant. A small study with 194 neurologically asymptomatic patients with vascular risk factors showed similar results, with circulating levels of CRP being higher in those with lacunar infarcts compared to those without and CRP levels functioning as an independent predictor for lacunar infarcts after adjusting for risk factors and carotid atherosclerotic vascular disease (OR 1.85 per SD increase in CRP)[211]. Similarly, in 3,644 participants (average age: 74.65) in the Cardiovascular Health Study, both white (n=3,073) and black (n=571) participants with higher CRP levels had a mildly increased risk of having WMH lesions (OR: 1.13, CI: 1.06-1.22) independent of age, sex and cardiovascular risk factors- though statistical significance was reached only in white participants. Plasma CRP levels were only marginally associated with the presence of brain infarcts in the two groups, though infarct subtype was not specified. Moreover, there was no association found between CRP haplotypes or genotypes and the appearance of brain infarcts or WMH lesions[212].

Contrastingly, in 1,763 stroke-free individuals in the Framingham Offspring cohort (average age: 60.2), there were no significant associations found between circulating CRP levels and the presence of lacunes, extensive WMH lesions or microbleeds[187]. In a longitudinal study of 190 type-2 diabetic patients, baseline levels of CRP were comparable between patients with radiological signs of SVD and those without[194]. In addition, there was no relationship between CRP levels at baseline and SVD progression at 3 or 6 years. However, previous research has shown an association between glycaemic control and systemic inflammation in people with diabetes, with increasing

levels of haemoglobin A1c (HbA1c) being significantly associated with elevated CRP levels after adjusting for covariates[236]. Therefore, it is possible that CRP levels were elevated within the control group comprised of diabetic patients, and HbA1c levels were not incorporated into these analyses.

2.4.1.4.2 Neopterin

Neopterin is a pteridine immune modulator produced by activated monocytes and macrophages and can serve as a marker for inflammation. Higher neopterin levels have been detected in patients with cardiovascular disease and are associated with increased risk of stroke[237]. Due to endothelium-leukocyte interactions, neopterin may play a direct role in inciting endothelial activation, possibly by promoting cell adhesion molecule expression mediated by oxygen free radicals[172,238]. Research relating neopterin to SVD is lacking, though one study with 163 lacunar stroke patients and 183 essential hypertensive patients reported higher levels of circulating neopterin in patients with a lacunar infarct and/or WMH lesions compared to those without[177]. In addition, neopterin levels were independently associated with a higher number of PVS in the basal ganglia after controlling for age, sex, pre-existing large-artery disease and patient group.

2.4.1.4.3 CD40 ligand

CD40 ligand (CD40) is a transmembrane glycoprotein expressed by endothelial cells and activated T-cells in an inflammatory state. It has been implicated in the activation of endothelial cells and leukocyte adhesion; its anchoring to the endothelium not only encourages the production of pro-inflammatory cytokines, but also enhances the expression of VCAM-1, ICAM-1 and E-selectin[239]. CD40 has previously been associated with stroke risk, infarct size and worse clinical outcomes post-stroke[227].

Findings from the Framingham Heart Study suggested no association between plasma CD40 levels and WMH, lacunes or microbleeds in 1,763 stroke-free participants[187]. In a prospective cohort study with 123 SVD patients aged over 60, those with radiological disease progression at 2-year follow-up had a higher z-score comprised of markers of vascular inflammation, which included

CD40 ligand, after adjusting for age, sex, blood pressure, and total SVD radiological load score[190]. However, there were no reported independent associations between CD40 and radiological features of SVD or disease progression. Moreover, analyses with 1,244 lacunar stroke patients in the LIMITS study demonstrated no association between circulating levels of CD40 and recurrent ischaemic stroke (including lacunar stroke), myocardial infarction, or vascular death, though analyses exploring CD40 and radiological SVD burden were not performed as part of this study[215].

Though a wealth of evidence suggests the involvement of systemic inflammation in stroke and cerebrovascular dysfunction, it is important to consider certain elements when dissecting the discrepant results from these studies. Firstly, a variety of factors can influence circulatory inflammatory markers, including comorbidities, concurrent infection, malignancy, medication use and rheumatologic disease. Though some of these can be plausibly controlled for in certain study conditions, it remains unclear whether circulating biomarkers of systemic inflammation can serve as an adequate proxy for brain endothelial dysfunction. MRI markers of SVD reflect evidence of an accumulation of chronic disease, whereas blood biomarkers can fluctuate rapidly in response to acute events or homeostatic changes, something that bears particular relevance in the acute and subacute stages of stroke. For this reason, it is important to note the variability in the time window between stroke onset, serology and MRI acquisition in stroke studies and whether the control group was comprised of non-lacunar stroke or non-stroke participants. In addition, CRP levels have a known association with atherosclerosis and other large-artery-related pathology, which not only shares a vascular risk factor profile overlap with SVD, but might also chronically exert effects on the microvasculature. Whether SVD could be partly caused by the release of pro-inflammatory molecules into the brain microvessels and cellular milieu or whether it induces the synthesis and release of these molecules also remains unclear.

2.4.1.5 Coagulation factors

Physiological haemostasis is preserved through blood constituents, blood flow regulation and the integrity of the vessel wall. Endothelial cells play a crucial role in the maintenance of physiological haemostasis and the modulation of thrombosis through the tight control of both pro-coagulant and anti-coagulant mechanisms. Not only can the endothelium provide a surface for thrombosis formation, but it also regulates blood flow and mediates the expression of vasoactive factors relating to platelet reactivity, coagulation and fibrinolysis. At sites of vascular injury, platelets migrate to the endothelium and adhere to the injury site where they form clots to effectively seal the barrier and prevent excessive blood loss. Several studies have investigated the relationship between circulating coagulation factors and radiological markers of SVD.

2.4.1.5.1 Von Willebrand factor

Von Willebrand Factor (vWF) is a large polymeric blood glycoprotein synthesised by endothelial cells. Although it plays a role in haemostasis and thrombus formation by sensing and responding to shear blood flow changes, one of its main functions is to facilitate platelet adhesion to the endothelium in sites of vascular injury. States of chronic inflammation or endothelial activation can encourage excessive secretion of vWF from secretory granules within dysfunctional endothelial cells and increase platelet binding to vWF. Furthermore, high levels of circulating vWF are associated with prothrombotic complications, diabetes, stroke, and inflammatory cardiovascular disease[240]. Though some evidence suggests plasma vWF can function as a biomarker of endothelial dysfunction, considerable inconsistencies remain amongst clinical studies[241].

Average vWF levels were higher in a group of 74 recent lacunar stroke patients compared to 74 controls matched for age, sex, hypertension and diabetes; however, vWF levels had significantly decreased by 3 months post-stroke and were at comparable levels between groups, perhaps suggesting that chronic vWF elevation was not playing a central role in SVD pathophysiology[181]. A study of 263 thrombolysed acute ischaemic stroke patients further supports

these findings, with vWF levels found comparable between patients with WMH lesions, cerebral atrophy or lacunes visible on CT compared to those without at the time of stroke onset[183]. Adjusted analyses at 90-day post-stroke showed similar results. Likewise, a previous study reported that vWF levels were increased to a similar extent in patients of acute-phase lacunar stroke as in patients of large-artery atherosclerotic and cardioembolic strokes[242]. There were also no significant differences in vWF levels between 65 lacunar stroke patients and 60 cortical stroke patients 1-3 months post-stroke after adjusting for age, sex and vascular risk factor profiles[213]. Collectively, these studies provide evidence for the hypothesis that although vWF might serve as a marker of endothelial dysfunction consequent to an acute endothelial response to an ischaemic insult, it might not reflect any SVD-specific pathogenic effect.

Notably, some studies suggest that an active form of vWF can be distinguished from a latent form, and though the use of total circulating vWF has yielded conflicting results, it is possible that differentiating between these subtypes could prove insightful in the context of SVD[243]. The ability to control vWF secretion from endothelial cells might serve as a future therapeutic target, though more research, particularly in the form of large longitudinal studies, is required to adequately differentiate the role of vWF from an acute response to an ischaemic insult versus a marker of chronic endothelial dysfunction pertinent to SVD disease mechanisms.

2.4.1.5.2 Tissue factor and tissue factor pathway inhibitor

Tissue factor (TF) binds to coagulation factors and thrombin to form fibrin. TF is normally concealed within the intact vessel wall until it becomes exposed after vascular injury, wherein it initiates the clotting response with the recruitment of platelets and coagulation factors. Though it is most commonly expressed by perivascular cells and platelets, it can be secreted by the endothelium, particularly under pathophysiological conditions in which endothelial cell-secreted miR-126 inhibits thrombosis by attenuating TF expression[244]. Decreased levels of TF have been found in patients of ischaemic stroke, including lacunar stroke[245]. Tissue factor pathway inhibitor (TFPI) is an

anticoagulation protein primarily secreted by endothelial cells which can inhibit TF activity and thereby modulate thrombosis[246].

In the 6-year longitudinal neuroimaging Austrian Stroke Prevention Study, no significant associations were found between SVD features or disease progression and TFPI in 296 community-dwelling participants after adjusting for age, sex, CRP levels, baseline WMH volume and traditional cardiovascular risk factors[193]. Importantly, as this cohort was not an SVD cohort, WMH severity grading was 'none' or 'punctate' in the vast majority (86.1%) of the sample, with only 24 participants out of 259 being classified as having SVD progression by 3 years. Another study found that TFPI levels were higher on average after adjusting for traditional vascular risk factors in a group of 110 symptomatic SVD patients compared to 50 healthy controls[162]. Patients with widespread WMH lesions had lower TFPI levels and a higher TF/TFPI ratio compared to those with isolated lacunar infarction. These results suggest that endothelial prothrombotic changes may be important in mediating an ischaemic SVD phenotype.

2.4.1.5.3 Tissue-type plasminogen activator and plasminogen activator inhibitor-1

Tissue-type plasminogen activator (t-PA) is a serine protease synthesised by endothelial cells which plays a critical role in fibrinolysis. It is associated with endothelial activation and increased BBB permeability[247]. Overexpression of t-PA is a known risk factor for future ischaemic stroke[248]. Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor primarily produced by endothelial cells involved in the suppression of fibrinolysis through the inhibition of t-PA. Physiological levels of PAI-1 are required for haemostasis and increased concentrations of PAI-1 are associated with atherosclerosis, thrombosis, cellular senescence and endothelial dysfunction[249,250]. Studies using *in vitro* BBB models provide further support regarding the involvement of t-PA in endothelial dysfunction, showing a dose-dependent effect on BBB permeability and endothelial cell morphological changes through the activation of the Rho-kinase pathway[247].

Studies investigating the relationship between t-PA, PAI-1 and SVD are limited and inconsistent. Lacunar stroke patients (n=65) had higher levels of t-PA 1-3 months post-stroke compared to cortical stroke patients (n=60) after adjusting for age, sex and vascular risk factors, perhaps indicating a role in the development or progression of mechanisms specific to SVD[213]. Dobrynina *et al.* also reported a significant positive correlation between t-PA levels and WMH severity and PVS burden in 71 SVD patients and 21 healthy controls; however, they did not adjust for potential clinical covariates in this study[251]. Conversely, a study with 100 patients of recent cortical or lacunar ischaemic stroke failed to find a significant relationship between plasma t-PA concentrations and PVS count or volume after adjusting for age, sex and vascular risk factors[216]. No association was found between PAI-1 levels and WMH volume or the presence of lacunes in 651 community-based elderly participants or in 71 SVD patients and 21 healthy controls[251,252].

2.4.1.5.4 Prothrombin factors 1 and 2

Prothrombin factors 1 and 2 are coagulation factors that can cleave to form thrombin, which is the key enzyme that transforms fibrinogen into fibrin and facilitates platelet activation. There was no reported association between circulating levels of prothrombin factor 1 and 2 and PVS in the basal ganglia in 100 ischaemic stroke patients, including lacunar stroke patients[216]. In the aforementioned Austrian Stroke Prevention Study, no significant associations were detected between prothrombin factors 1 and 2 and SVD features or WMH lesion progression at 3- or 6-year follow-up in 296 community-dwelling participants[193]. However, as participants in this study were asymptomatic, it remains a possibility that relationships between SVD and coagulation factors could go undetected in this cohort; subsequent power analyses also revealed larger sample sizes would be necessary to detect significant differences if a subtle effect size were to be assumed.

2.4.1.5.5 D-Dimer

D-dimer is a by-product of blood clotting and fibrin degradation produced when fibrin is lysed with plasmin. High levels are indicative of plasmin activation and

fibrin production and have been associated with cardiovascular disease and future atherothrombotic events[253]. There was no reported association between levels of D-dimer and PVS in the basal ganglia in 100 recent ischaemic stroke patients, including lacunar stroke patients[216]. There was also no relationship found between circulating levels of D-dimer and SVD features or progression in 296 community-dwelling participants in a 6-year longitudinal study[193]. Albeit perhaps unrelated to pathophysiological mechanisms specific to SVD, one study with 102 SVD patients did find a relationship between baseline serum concentrations of D-dimer and an increased risk of a cardiovascular event (including recurrent stroke) or death after adjusting for age, sex and radiological markers of SVD[214].

2.4.1.5.6 Thrombomodulin

Thrombomodulin (TM) is a vasoactive factor expressed on the surface of endothelial cells. It engenders a powerful anticoagulation effect by decreasing circulating levels of thrombin and inhibiting factors Va and VIIIa through the production of activated protein C[250,254]. TM can reflect systemic endothelial damage, although some studies suggest it can protect the endothelium by attenuating inflammation-inducing injury[162,250]. Increased circulating TM concentrations were significantly associated with a higher number of lacunes and more severe WMH burden in 110 lacunar stroke patients, with higher TM levels reported in SVD patients compared to 50 healthy controls after adjusting for vascular risk factors[162]. Similarly, a cross-sectional analysis with 651 community-based ageing participants (average age: 67.4) demonstrated higher average serum concentration levels of TM present in those with lacunar infarcts or moderate WMH compared to those without[252]. In contrast, no association was found between levels of TM and WMH lesion progression at 6-year follow-up after adjusting for potential confounding variables in 296 community-dwelling participants in the Austrian Stroke Prevention Cohort[193].

Intriguingly, an *ex vivo* pathology study examining small penetrating cerebral arteries in 17 SVD cases, 12 aged controls and 4 young controls found significantly higher levels of TM immunoreactivity in SVD cases compared to

aged controls, and in vessels with a higher sclerotic index; TM was entirely absent or sparse in young controls [195].

2.4.1.5.7 Endothelin-1

Endothelin-1 (ET-1) is a potent exogenous vasoconstrictor peptide produced by endothelial cells in response to hypoxia, shear stress, and exposure to cytokines, growth factors and thrombin. ET-1 can induce vasoconstriction, hypertrophy, fibrosis and increased BBB permeability and can also mediate the release of NO, implicating it in hypertension and endothelial dysfunction[250]. ET-1 has been associated with synaptic dysfunction and consequent cognitive deficits in pre-clinical investigations featuring a mouse model of transient vasoconstriction via bilateral injections of ET-1[255]. Research examining the possible role of ET-1 in SVD is limited. A large (n=1,396) 6-year longitudinal study found baseline serum ET-1 levels to be positively associated with worsening WMH burden (Fazekas scale score ≥ 2) and the presence of lacunes and microbleeds after adjusting for age, sex, vascular risk factors, medication use, carotid stenosis and baseline WMH burden[189].

2.4.1.5.8 Fibrinogen

Fibrinogen is a blood plasma glycoprotein produced in the liver. It is the main structural component of a blood clot and functions as a coagulation factor. Fibrinogen can also indicate capillary leakage and can disrupt BBB integrity by converting to toxic blood-derived fibrin deposits, which disrupt blood flow and result in downstream consequences affecting other constituents of the neurogliovascular unit, eventually compromising white matter integrity[256]. Participants with elevated fibrinogen levels tended to have a more severe WMH burden and a greater number of lacunes in a Japanese community-based neuroimaging study (n=651)[257]. Logistic regression analyses adjusted for age, sex, vascular risk factors, CRP levels and carotid artery atherosclerosis revealed an independent association between fibrinogen levels and WMH burden. In the same cohort, levels of fibrinogen were inversely correlated with cognitive performance, albeit only in patients without WMH. Findings from the Framingham Heart Study (n=1,763) did not support an

association between circulating levels of fibrinogen and WMH volumes or the presence of lacunes or microbleeds[187]. Furthermore, there was no association between circulating levels of fibrinogen and basal ganglia PVS in 100 patients with recent ischaemic stroke[216].

2.4.1.6 Growth factors

Vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and transforming growth factor-β1 (TGF-β1) are potent angiogenic factors crucial for endothelial cell proliferation, migration, maturation and survival; they play a key role in angiogenesis, microvascular permeability and overall vessel function[258,259]. A fundamental feature of ageing involves angiogenic signalling failure and possible subsequent alterations in blood flow[260]. Mice with a genetic deletion of VEGF display endothelial cell degeneration and death[261]. There is evidence to suggest that abnormally raised or decreased levels of VEGF and TGF-β1 are associated with cardiovascular disease, perhaps via their induction through hypoxia-pertinent pathways or through stimulation of NO production[262]. There is also evidence to suggest VEGF and PlGF encourage vascular permeability and inflammation by uncoupling endothelial cell-to-cell junctions[263,264]. Injections of VEGF can result in fibrinogen and plasma clotting, increased BBB permeability and vascular inflammation[265].

Few clinical studies have investigated the link between growth factors and SVD. We identified 8 studies with a total of 5,176 participants (average sample size: 739.43).

In a cross-sectional study with 293 thrombolysed acute ischaemic stroke patients, higher serum VEGF levels at 90 days post-stroke, though not baseline levels at stroke onset, were associated with the presence of lacunes and greater global SVD disease burden on CT after adjusting for age, sex, and vascular risk factors; there was no association with WMH burden specifically[183]. One study reported higher VEGF levels in a group of 91 large vessel atherosclerotic disease patients compared to 89 SVD patients within 24-hours of acute ischaemic stroke onset[266]. However, serum VEGF levels were

proportional to infarct volume after adjusting for potential confounding variables, which might explain the increased levels in the group with strokes of large-artery aetiology, which have larger volumes. Group differences in VEGF levels were attenuated by 3 months post-stroke, though higher serum levels of VEGF in the acute stroke phase were associated with a better functional outcome (National Institute of Health Stroke Scale (NIHSS) score decrease > 4 points) after adjusting for potential covariates including initial NIHSS and infarct volume, possibly due to their neuroprotective and angiogenic effects. However, it is important to note that the well-established roles of VEGF in acute-ischaemic stroke render the extrapolation of findings from these populations arguably unreliable in the context of SVD, as significant fluctuations in growth factor levels after an acute ischaemic event cannot be discounted.

Some large studies in stroke-free ageing populations report no significant associations between VEGF and radiological markers of SVD. For instance, no connection was reported between circulating levels of VEGF and brain atrophy, WMH volume or white matter tract integrity (fractional anisotropy via Diffusion Tensor Imaging (DTI) sequence on MRI) in a young (average age: 46) subset of 1,853 participants in the Framingham Heart Study cohort after adjusting for demographic and clinical variables[267]. Likewise, Shoamanesh *et al.* did not observe an association between circulating VEGF levels and WMH burden, microbleeds or lacunes in 1,763 stroke-free adults (average age: 60.2) in the Framingham offspring cohort[187].

In contrast, higher baseline levels of the VEGF family and PlGF in CSF were associated with greater WMH volume after adjusting for age, sex, ICV and vascular risk factors in 247 elderly participants with MCI (average age: 70.89), though not in 495 cognitively unimpaired participants; adjusting for CSF Aβ-40 attenuated the relationship for VEGF but not PlGF[192]. PlGF was amongst the strongest independent predictors of WMH at baseline in cognitively impaired and unimpaired individuals, independent of Aβ status, providing evidence of the overlapping implications between inflammation and cerebrovascular

disease. Curiously, lower levels of VEGF and PlGF were associated with increasing WMH lesions over 7 years, leading the authors to hypothesise that vascular injury might inflict enough damage to endothelial cells leading to a depletion in growth factor production and a subsequent decline in angiogenesis. Results from the ongoing MarkVCID consortium also indicate that in individuals at risk for cognitive impairment and dementia, PlGF may function as a candidate biomarker measuring the severity of vascular injury and WMH lesion burden and may serve as an accurate cross-sectional diagnostic biomarker and contribute as a prognostic susceptibility biomarker longitudinally[268]. In this study, Hinman et al. observed a significant relationship between circulating plasma PlGF levels and WMH lesion burden (measured volumetrically and using the Fazekas scale) as well as cognitive impairment as measured by the Clinical Dementia Rating Scale (CDR) after adjusting for age and sex. Encouragingly, PlGF levels showed increasing diagnostic accuracy for all progressive disease states: WMH only (AUC: 0.66), mild cognitive impairment with WMH (AUC: 0.74) and dementia with WMH (AUC: 0.89). The authors posit that SVD mechanisms might be driving an increase in angiogenic factor circulation paired with a failure in downstream receptor signalling in cerebral microvessels, possible involving Flt-1 receptor activity.

To determine whether the heterogeneity observed in the clinical and radiological manifestations of SVD could be attributed to different pathogeneses, Dobrynina *et al.* categorised 40 SVD patients with a Fazekas score of 3 into two subgroups of radiological manifestations based on a cluster comparison and examined whether they were related to different blood biomarkers[224]. Univariate analyses revealed lower levels of VEGF in patients in an MRI subgroup with widespread WMH and lacunes, microbleeds, atrophy and severe cognitive and gait impairments compared to 21 healthy controls or those in the other MRI subgroup, categorised by more severe deep WMH, lacunes in the white matter, no microbleeds or atrophy and less severe clinical manifestations, though the latter was not statistically significant. Whilst it is possible that VEGF could contribute to a specific SVD radiological profile amongst patients with severe WMH lesions, it is worth noting that these

analyses did not include important clinical covariates that may influence blood biomarker concentrations, despite significant differences between SVD patients and controls. Notably, SVD patients were substantially more likely to be hypertensive (85.4% versus 43.5%) and diabetic (17.7% versus 0%).

In a 5-year longitudinal imaging study with 35 SVD patients, 43 age-matched participants with a history of stroke (further subdivided into lacunar and atherothrombotic) and 46 age-matched controls, TGF-β1 levels were highest among those with deep WMH lesions which had progressed at 5-year follow-up[269]. Logistic regression analyses indicated that the odds ratio for the presence of elevated TGF-β1 was significant after adjusting for age, sex and major vascular risk factors. It is not entirely clear why this increase was not observed in the subset of participants with a history of lacunar stroke or why it was unrelated to periventricular WMH lesion presence and progression.

2.4.1.7 Nitric oxide pathways

2.4.1.7.1 Nitric oxide

NO, an endothelium-derived vasodilator, inhibits platelet activation and thrombosis and limits adhesion of inflammatory cells. Dysfunctional endothelial cells decrease NO bioavailability by reducing NO synthesis and secretion, which can lead to defective vasodilatory processes, impaired blood flow, platelet aggregation, proliferation of smooth muscle cells and increased blood pressure[270,271]. In the endothelium, NO is produced by eNOS through calcium-dependent binding of calmodulin, hence, intracellular calcium can influence eNOS and NO production. NO can alter the calcium (Ca2+) in smooth muscle cells through second messenger and potassium (K+) channel activation pathways that facilitate contraction and relaxation. Levels of endothelial-derived factors, including NO, must be tightly regulated to allow for healthy BBB and brain function[272,273]. A population-based longitudinal study (average follow-up: 69.7 months) with 1,396 stroke-free adults reported that changes in NO levels were significantly negatively associated with an increased risk of new incident WMH lesions, lacunes, and microbleeds on MRI after adjusting

for age, sex, vascular risk factors, medication, carotid stenosis and baseline WMH volume[189].

2.4.1.7.2 NOSTRIN

Endothelial nitric oxide synthase traffic inducer (NOSTRIN) is involved in the regulation of perfusion and inflammation and is thought to inhibit the production of NO by sequestering and thus attenuating the activation of eNOS[274]. In addition, NOSTRIN may influence the expression of secreted cytokines and can restrict endothelial cell proliferation and adhesion[275]. Using EDE biomarkers, Elahi *et al.* examined the cargo proteins within plasma membrane-derived vesicles of brain endothelial cells from 26 neurologically-normal older adults[197]. They found higher levels of NOSTRIN in participants with WMH lesions compared to those without, though they did not find significant associations between NOSTRIN levels and global grey matter volumes or cognitive performance; it is important to note that only 11 patients within this small sample size had signs of SVD.

2.4.1.7.3 ADMA

Asymmetric dimethylarginine (ADMA) is a nitric oxide synthase (NOS) inhibitor associated with traditional vascular risk factors, stroke and endothelial dysfunction, presumably via a reduction in cerebral blood flow or the promotion of oxidative stress and inflammation[276]. NOS is an endogenous enzyme involved in the conversion of substrate L-arginine to NO, the former of which is involved in various processes crucial to the maintenance of cerebrovascular health, including vasodilation, platelet aggregation inhibition, smooth muscle cell generation and vascular cell adhesion. Due to their structural affinity, ADMA and L-arginine compete for NOS binding, hence reducing the bioavailability of NO[277].

Serum ADMA levels were significantly higher in a group of 210 SVD patients compared to 207 healthy controls; however, this difference was no longer significant when adjusting for potential confounders including age, sex, vascular risk factors and coronary heart disease[278]. ADMA levels differed significantly between SVD-associated lesion types, with higher levels reported

in patients with an isolated lacune compared to those with multiple visible lacunes and significantly lower levels in those with WMH lesions compared to those with WMH lesions and accompanying cerebral atrophy. Though the study sample size is relatively small, one possible explanation for these findings relates to the potential role of ADMA in the development of atherosclerosis, which might play a more direct role in isolated lacunar infarction. Though no significant correlation was found between levels of ADMA and cognitive impairment, subgroup analyses revealed that in patients with WMH lesions specifically, levels of ADMA were significantly correlated with Montreal Cognitive Assessment (MoCA) scores. These results suggest that though ADMA may not play a key role in instigating SVD pathophysiology, it could exacerbate disease progression, conceivably through the inhibition of NO production and consequent cerebral blood flow changes.

Khan *et al.* observed higher levels of circulating ADMA levels in 47 SVD patients compared to 38 controls with similar vascular risk factor profiles[279]. ADMA levels were not correlated with number of lacunes, though WMH severity grade was positively correlated with ADMA (OR 10.04, 95% CI: 1.70-59.27) after controlling for age, sex, vascular risk factors and creatinine clearance. Though the authors hypothesised that ADMA might mediate the relationship between homocysteine (Hcy) and endothelial activation, they found no significant associations between ADMA and Hcy levels and the relationship between ADMA and SVD was only slightly attenuated after controlling for Hcy. Along the same vein, a study with young, asymptomatic patients (average age: 51.1) also reported significantly higher levels of ADMA amongst those with SVD compared to controls[280]. ADMA levels were positively correlated with WMH presence and severity after adjusting for potential confounding variables, further supporting a role of ADMA and endothelial dysfunction in early disease pathology.

2.4.1.7.4 Myeloperoxidase

Myeloperoxidase is an oxidizing enzyme secreted by neutrophils, leukocytes and microglia following neuronal injury. It restricts NO bioavailability by

consuming it and also contains hypochlorous acid, and can thus aggravate inflammation, incite tissue damage and has been associated with BBB and endothelial dysfunction[281]. In the Framingham Heart Study, circulating levels of myeloperoxidase were measured as a proxy biomarker for oxidative stress in 1,763 stroke-free participants (average age: 60.2)[187]. After controlling for age, sex, vascular risk factors and time from blood draw to MRI (average: 5 years), they observed significantly higher levels of myeloperoxidase in the presence of microbleeds (OR 1.5, 95% CI: 1.1-2.0), with each additional unit increase resulting in a 1.9-fold increased risk of having more than 1 microbleed. The authors also reported a non-significant association between lower myeloperoxidase levels and the presence of WMH lesions and/or lacunes. It remains unclear why lower levels of myeloperoxidase were associated with ischaemic SVD-related lesions and higher levels with haemorrhagic disease manifestations, and it remains feasible that these might have distinct pathophysiological causes. In addition, it is possible that chronic SVD could lead to an overburdened inflammatory pathway, which might eventually deplete circulating myeloperoxidase availability.

2.4.1.8 Other relevant biomarkers

2.4.1.8.1 Homocysteine

Hcy is a sulphur-containing protein usually excreted from the body via the kidneys. It is associated with cardiovascular event and stroke risk as well as cognitive impairment and is indicative of endothelial dysfunction[282,283]. Increased levels of Hcy are thought to exert deleterious effects on the endothelium and can facilitate oxidative stress, impair NO-mediated blood flow processes, up-regulate redox-sensitive inflammatory pathways, as well as promote atherogenesis, thrombogenicity and the aggregation of ADMA[284–286].

Higher levels of Hcy were reported in 74 lacunar stroke patients 1 month post-stroke compared to 74 healthy controls matched for age, sex, hypertension status and diabetes[181]. Though many inflammatory markers had normalised by 3 months post-stroke, Hcy levels remained unchanged, suggesting it was not a by-product of the acute ischaemic event itself. In a UK-based study of 457

black stroke patients and 179 black community controls, elevated serum Hcy concentrations were observed in SVD patients compared to controls after adjusting for age, sex, vascular risk factors and renal function[287]. In addition, Hcy levels within SVD patients were positively correlated with WMH severity and were associated with the presence of lacunes with confluent WMH lesions. Hcy levels were also found to be independently associated with a greater WMH volume burden after adjusting for demographic and vascular risk factors in 809 acute ischaemic stroke patients (average age: 65.6), with each quartile increase in plasma Hcy being associated with a WMH volume increase of 11%[288]. Also, Hcy levels were positively correlated with the number of lacunes (OR 2.14, 95% CI: 1.4-3.27) in a large population-based cohort study of 1,023 participants (average age: 56.7) after adjusting for age, sex and vascular risk factors[289]. Another study also reported higher circulating Hcy in 47 SVD patients compared to 38 healthy controls with similar risk factor profiles and were positively correlated with WMH severity grade and number of lacunes after controlling for demographic and clinical covariates as well as creatinine clearance[279].

In a large Chinese community-based cohort study (n=960, average age: 56.02), grouped endothelial dysfunction biomarkers including Hcy were independently associated with WMH volume and number of lacunes, though not microbleeds or PVS; backward elimination regression retained Hcy as an independent risk factor for the presence of lacunes (OR: 1.44, 95% CI: 1.20-1.74)[186]. Hcy levels were higher in a group of 172 SVD patients compared to 172 age and sex-matched controls[290]. After controlling for traditional vascular risk factors and age, Hcy was also a strong risk factor in those with WMH lesions. Consistent with the hypothesis that Hcy mechanistically relates to SVD through endothelial dysfunction, the authors found that including other markers of endothelial dysfunction, ICAM-1 and TM, in a logistic regression model resulted in the loss of the significant association between Hcy and SVD.

Results from the Framingham Heart Study (sample size: 1,763 stroke-free participants) contradict these findings and the authors report no association

between circulating Hcy levels and several markers of SVD on MRI, including WMH lesions, lacunes and microbleeds[187].

Some studies also suggest a possible association between Hcy and clinical outcome or disease progression. For instance, in a cohort of 123 SVD patients (average age: 66.4), circulating Hcy levels correlated with WMH severity, number of lacunes, basal ganglia PVS and lobar microbleeds, and also served as an independent predictor for global cognitive impairment[291]. Hcy was also associated with radiological progression of SVD at 2-year follow-up in a longitudinal cohort study with 123 SVD patients, with higher circulating levels related to the development of new lacunes as well as an increased risk of a recurrent vascular event or death after controlling for demographic variables, vascular risk factors and disease burden[190,214]. Interestingly, a recent study reported significantly higher levels of plasma Hcy in SVD patients with cognitive impairment (n=40) compared to SVD patients without cognitive impairment (n=38) and healthy controls (n=35), respectively; however, potential confounding variables were not adjusted for in these univariate analyses[233].

2.4.1.8.2 Albumin

Microalbuminaria (urinary albumin excretion < 20-200 µg/min) is associated with systemic dysfunction of the vascular endothelium, elevated levels of fibrinogen, BBB dysfunction and an increased risk of stroke[292–294]. Though the precise mechanisms underlying these associations remain mostly unknown, it is possible that cytokines and other factors implicated in inflammation might mediate renal and glomerular damage, contributing to albuminaria and SVD[295]. Furthermore, small vessels in the brain and the kidneys share haemodynamic and physiological similarities and damage to the kidney endothelium can incite albumin leakage[296]. It is also plausible that this relationship is mediated by the involvement of relevant risk factors that might also influence renal function, such as sodium consumption, diabetes and hypertension.

In a Japanese community-based study with 651 participants (average age: 67.4), those with albuminaria had higher WMH grades and total number of

lacunes compared to those without; in addition, the total number of lacunes and WMH severity grade were significantly higher in the highest tertile of albumin-creatinine ratio[252,297]. A standard deviation increase in the albumin-creatinine ratio was associated with a nearly 2-fold increased risk of having at least one lacunar infarct and a 2.15-fold increased risk of the presence of WMH lesions, independent of age, sex and vascular risk factors.

In the longitudinal Norwegian HUNT2 population-based study with 7,261 participants, albuminaria was associated with increased overall ischaemic stroke risk over a median follow-up period of 15 years[298]. This association was modestly stronger for the lacunar subtype compared to large-artery or cardioembolic ischaemic stroke subtypes or haemorrhagic stroke, even after controlling for age, sex and vascular risk factors, perhaps providing further evidence for the role of albuminaria in diffuse endothelial and vascular dysfunction in SVD[298].

2.4.1.8.3 Haemoglobin A1c

HbA1c is a metabolite that functions as an indicator of chronically elevated glucose levels or insulin resistance and a biomarker of endothelial dysfunction. HbA1c is related to inflammation, the production of atheroma, thrombogenicity and the promotion of oxidative stress through the facilitation of ADMA synthesis[276,299]. In 809 acute ischaemic stroke patients (average age: 65.6), HbA1c levels were associated with WMH volumes on MRI and functioned as an independent predictor for WMH volume after adjusting for age, sex, race, vascular risk factors (including diabetes), coronary artery disease and prior stroke[288]. A quartile increase in HbA1c levels was associated with a 10% increase in WMH lesion burden.

2.4.1.8.4 Amyloid-beta

β-amyloid1-40 is associated with cerebral amyloid angiopathy and has been shown to alter endothelial cell-mediated cerebral blow flow regulation in mice, possibly through the formation of ROS, which can further harm the endothelium[300]. In 149 acute lacunar stroke patients and 25 age-matched controls, higher levels of plasma β-amyloid1-40 were reported in SVD patients

with WMH lesions and multiple lacunar infarcts with or without the presence of WMH, compared to those with an isolated lacunar infarct in the absence of WMH or healthy controls[301]. The authors attribute this difference to potentially distinct disease pathologies, a diffuse arteriopathy versus localised vessel atheroma, with endothelial dysfunction playing a more prominent role in the former.

2.4.1.8.5 Adiponectin

Adiponectin, a protein synthesised by adipose tissue, maintains vasoprotective properties by attenuating both inflammation and the production of ROS whilst enhancing NO generation and promoting the survival of endothelial progenitor cells[302]. The potentially protective role of adiponectin was demonstrated in a study with 133 patients with first-ever ischaemic stroke, where circulating levels of adiponectin were inversely associated with the prevalence of cerebral microbleeds after adjusting for age, sex and cardiovascular risk factors[180].

2.4.1.8.6 Klotho

Predominantly produced by the kidneys, the Klotho proteins are components of endocrine fibroblast growth factor receptor complexes that mediate metabolic processes. With circulating levels decreasing with age, Klotho is often referred to as having 'anti-ageing' properties due to its involvement in the regulation of oxidative stress, organ protection and the inhibition of cellular senescence[303]. In a prospective study with 262 first-ever ischaemic stroke patients (average age: 67.4), plasma Klotho concentration was inversely associated with the presence of WMH lesions, PVS, lacunes, total SVD burden and disease progression, though not microbleeds, possibly suggesting different pathological mechanisms underlying the protective involvement of Klotho proteins in distinct radiologic manifestations of SVD[304]. How Klotho could exert its protective qualities on the cerebrovascular system is unclear, though the regulation of NO production and bioavailability in vascular endothelial cells remains a possible mechanism.

2.4.1.8.7 Lipoprotein-associated phospholipase A_2 mass

Lipoprotein-associated phospholipase A2 mass (Lp-PLA2), an enzyme secreted by macrophages, serves to hydrolyse oxidised phospholipids and functions as a marker of vascular inflammation. Previous studies have suggested an association between high levels of Lp-PLA2 and stroke or SVD[305,306]. In addition, a relationship between Lp-PLA2 and dilation in the retinal venules was reported in the Rotterdam cohort, further substantiating its involvement in small vessel pathology[307].

In 1,763 stroke-free participants in the Framingham Heart Study, Shoamanesh *et al.* observed higher levels of circulating Lp-PLA2 in participants with lacunes and greater WMH volumes as well as a non-significant trend between higher levels of Lp-PLA2 and increasing number of microbleeds after adjusting for age, sex and vascular risk factors[187]. As part of the Northern Manhattan Study, Wright *et al.* similarly reported an association between higher levels of Lp-PLA2 and a higher WMH burden in 527 participants (average age: 71.3), with those in the highest quartile having a 1.3-fold increase in WMH volume compared to those in the lowest quartile, independent of demographic variables, the presence of risk factors, or other markers of inflammation[308].

2.5 Conclusions and future perspectives

In this scoping review, we have summarised findings from clinical neuroimaging studies investigating the relationship between radiological indications of SVD and biochemical markers of endothelial dysfunction in blood or CSF with the overarching aim of conceptualising the molecular mechanisms underlying the role of endothelial dysfunction in SVD **(Figure 2-2)**.

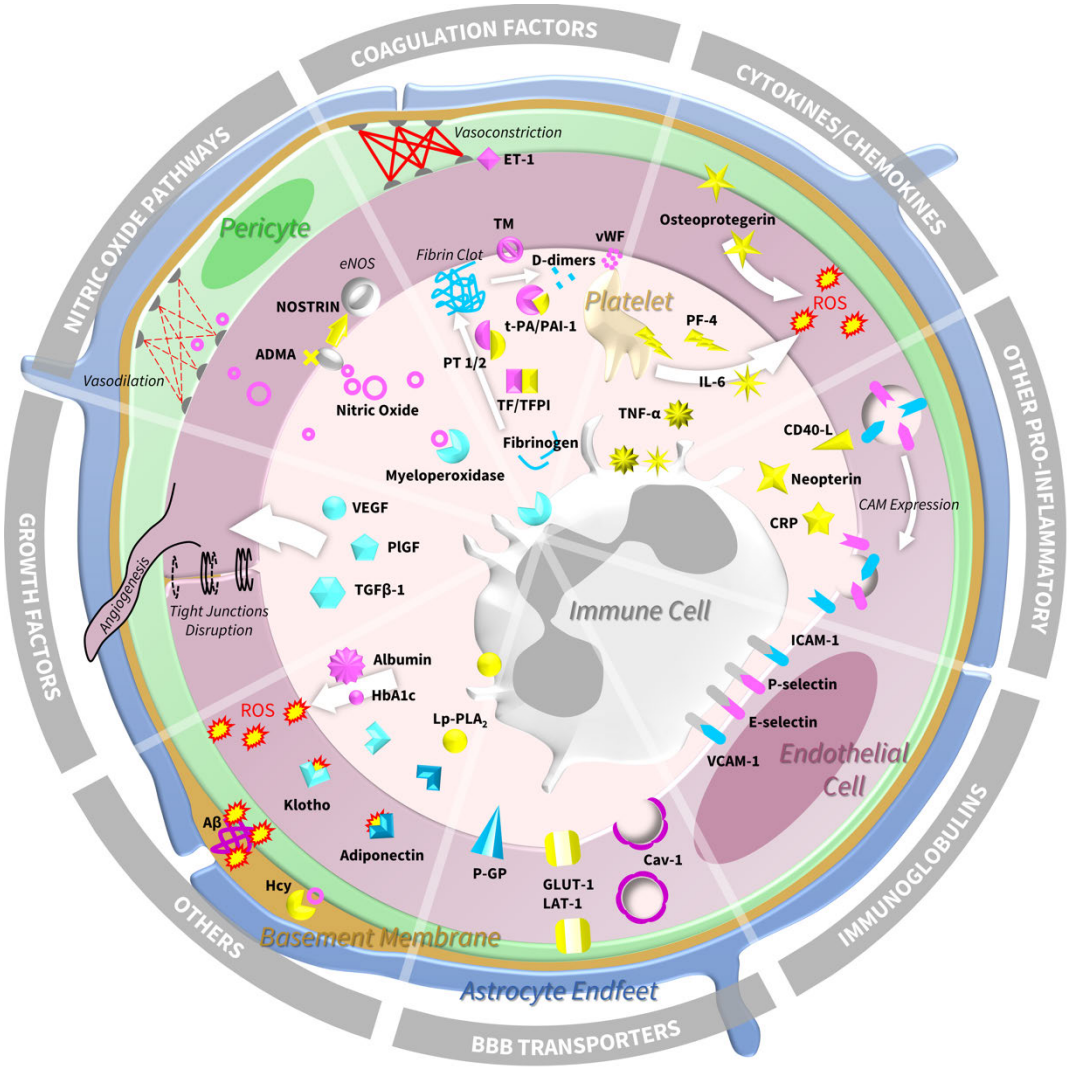


Figure 2-2. Biomarker roles in the gliovascular unit. Each octant represents a subdivision used in the review. Abbreviations per octant: (Coagulation Factors) ET-1: endothelin-1; TM: thrombomodulin; t-PA: tissue-type plasminogen activator; PAI-1: plasminogen activator inhibitor-1; PT 1/2: prothrombins 1 and 2; TF: tissue factor; TFPI: tissue factor pathway inhibitor; vWF: von willebrand factor; (Cytokines/Chemokines) ROS: reactive oxygen species; PF-4: platelet factor-4; IL-6: interleukin-6; TNF-α: tumour necrosis factor alpha; (Other Pro-Inflammatory) CD40-L: CD40-ligand; CRP: C-reactive protein; CAM: cell adhesion molecules; (Immunoglobulins) ICAM-1: intracellular cell adhesion molecule-1; P-selectin: platelet selectin; E-selectin: endothelial selectin; VCAM-1: vascular cell adhesion molecule-1; (BBB Transporters) Cav-1: caveolin-1; GLUT-1: glucose transporter-1; LAT-1: large neutral aminoacid transporter-1; P-GP: permeability-glycoprotein; (Others) Hcy: homocysteine; Aβ: amyloid beta; Lp-PLA_2: lipoprotein-associated phospholipase A_2-mass; HbA1c: haemoglobin A1c; (Growth Factors) VEGF: vascular endothelial growth factor; PlGF: placental growth factor; TGFβ-1: transforming growth factor beta-1; (Nitric Oxide Pathway) NO: nitric oxide; eNOS: endothelial nitric oxide synthase;

ADMA: asymmetric dimethylarginine; NOSTRIN: endothelial nitric oxide synthase traffic inducer (schematic is a theoretical representation and is not drawn to scale).

Overall, there is evidence of an association between E-selectin and the presence of microbleeds as well as VCAM-1, ICAM-1 and P-selectin levels with WMH lesion severity and progression stemming from large community-dwelling cohort studies as well as cross-sectional and longitudinal studies with SVD patients, even when compared to cortical stroke patient controls.

Clinical research studies investigating the role of BBB transport proteins in SVD pathology are scarce, but we describe some studies that suggest a relationship between higher levels of GLUT-1, LAT-1 and P-GP and increasing WMH lesion burden as well as lower levels of Cav-1 and the presence of microbleeds.

Results from studies linking cytokine and chemokine biomarkers to SVD pathogenesis are inconclusive, and while there is evidence of a relationship between higher IL-6 levels and SVD burden, studies comparing patients of acute lacunar stroke with patients of acute thromboembolic occlusive stroke indicate a possible mediating role of an acute endothelial response to injury. Nonetheless, there is some published evidence in support of the role of osteoprotegerin, TNF-α and PF-4 in SVD mechanisms.

The contribution of inflammatory pathways to SVD pathogenesis remains elusive. In some large population-based studies, CRP levels were independent predictors of WMH lesion burden and progression as well as number of lacunes or lacunar infarcts, though this was not the case in all the studies described. Indeed, most studies featuring acute and subacute stroke patients as controls did not observe strong associations between CRP levels and SVD burden. There is some evidence linking neopterin, though not CD40 ligand, to SVD.

Several lines of evidence also point to dysregulation in the nitric oxide pathway as being an important event in the pathogenesis of SVD. Findings from several

studies suggest associations between levels of NO, NOSTRIN, ADMA and myeloperoxidase and the presence and degree of diverse SVD lesion types.

We did not find consistent evidence advocating for the role of activation of coagulation in the development and progression of SVD, with most studies evaluating a connection between vWF and SVD reporting negative results and those focussing on TFPI, t-PA, PAI-1, prothrombin factors 1 and 2, D-dimer and fibrinogen reporting contradicting results. However, some studies did report significant associations between higher levels of circulating TM and ET-1 and SVD severity.

Growth factors have been shown to have neuroprotective and angiogenic properties, but they also can promote vascular permeability, exacerbate ischaemic tissue injury post-stroke and lead to aberrant angiogenesis. Data on their influence over SVD mechanisms are inconsistent, particularly in studies with stroke populations at different post-stroke stages and of diverse aetiologies. Larger ageing cohort studies suggest there is little to no association between VEGF levels and SVD burden; however, other studies indicate an association with higher levels of other growth factors, notably TGF-β1 and PlGF and WMH lesion burden and longitudinal disease progression.

We also found several studies supporting associations between levels of albumin, Hcy, HbA1c, β-amyloid1-40, Lp-PLA2 and adiponectin and several types of SVD lesions on MRI **(Table 2-1)**.

Table 2-1. Associations between individual biomarkers and distinct radiological manifestations of SVD present in the literature reviewed.

Class	Biomarkers	Role	Relation to SVD	Refs
Cell adhesion molecules and selectins	VCAM-1 ICAM-1 P-selectin E-selectin	• expressed by a cytokine-activated endothelium • facilitate the recruitment, rolling, adhesion and transendothelial migration of leukocytes through the endothelium of vascular walls • have been linked to ageing, BBB breakdown and age-related neurodegenerative disorders	• higher levels found in SVD patients • positively associated with disease burden and progression, worse clinical outcomes and cognitive decline • positively associated with WMH and the prevalence of lacunes and microbleeds, though not PVS	[18,22,34,37,38,40,41,42,43,44,45,46,47,48,49,50,51,52,54,70,73]
BBB Transporters	GLUT-1 P-GP LAT-1 Cav-1	• expressed by brain endothelial cells • mediate the influx and efflux of solutes from the circulation through endothelial cells and into the brain and vice-versa • implicated in BBB permeability, vessel tone and metabolic dysregulations • change with ageing, possibly disturbing neurovascular coupling processes based on metabolic demand	• higher levels found in SVD patients • positively associated with WMH and lower cognitive performance scores and inversely associated with total grey matter volume • Cav-1 levels inversely associated with the prevalence of microbleeds	[54,62]
Cytokines and chemokines	IL-6 TNF-α/TNFR2 Osteoprotegerin PF-4	• pro-inflammatory molecules that can incite platelet production and engender endothelial dysfunction through an increase in Angiotensin II type 1 (AT1) receptor activity, an interaction with tumour necrosis factor alpha (TNF-α), and the induced production of reactive oxygen species (ROS), which can cause enhanced vascular superoxide production, exacerbate oxidative stress, and impair endothelium-dependent vasodilation • associated with inflammation, stroke, atherosclerosis and cardiovascular events	• higher levels found in SVD patients • positively associated with presence of WMH, subcortical infarcts, lacunes, and microbleeds, though not PVS • contradictory results regarding the association with disease progression and worse clinical outcomes, though strong associations reported regarding adverse cardiovascular events, including recurrent stroke • some results possibly mediated by recent stroke	[43,44,45,47,48,49,68,69,70,71,72,73,81]

Class	Biomarkers	Role	Relation to SVD	Refs
Other pro-inflammatory markers	CRP Neopterin CD40-L	• these pro-inflammatory molecules can inhibit angiogenesis, promote atherosclerosis, mediate circulating cytokine levels, inhibit NO, decrease endothelial nitric oxide synthase (eNOS) activity in endothelial cells, increase Endothelin-1 production, attenuate endothelial-dependent vasodilation and increase endothelial cell adhesion molecule expression	• most studies featuring acute and sub-acute stroke patients do not report strong associations between CRP levels and SVD disease burden • contradictory results, with some showing a positive association with the prevalence of lacunar infarcts, PVS, WMH lesion burden and progression, though not microbleeds • positively associated with the risk of recurrent stroke	[34,38,43,44,47,51,68,69,70,71,72,73,90,91,92]
Coagulation factors	vWF TF TFPI t-PA PAI-1 PTF1/2 D-dimer TM ET-1 fibrinogen	• endothelial cells play a crucial role in the maintenance of physiological haemostasis and the modulation of thrombosis through the tight control of both pro-coagulant and anti-coagulant mechanisms • the endothelium provides a surface for thrombosis formation, regulates blood flow and mediates the expression of vasoactive factors relating to platelet reactivity, coagulation and fibrinolysis	• vWF levels are comparable between lacunar and cortical stroke patients at sub-acute stroke stages • there are contradicting results regarding associations between TF/TFPI, t-PA/PAI-1 and fibrinogen and SVD mechanisms • no associations reported with prothrombin factors 1 and 2 or D-dimer • levels of TM are positively associated with WMH lesion severity and number of lacunes, though not disease progression • ET-1 is positively associated with worsening WMH burden and the presence of lacunes and microbleeds	[18,38,40,46,50,52,70,71,73,99,108,109,114]
Growth factors	VEGF TGF-β1 PlGF	• potent angiogenic factors crucial for endothelial cell proliferation, migration, maturation and survival • play a key role in angiogenesis, microvascular permeability (possibly by uncoupling endothelial cell junctions),	• positively associated with the presence of lacunes and greater global SVD disease • higher levels associated with better functional outcome post-stroke • positively associated with greater WMH volume at baseline and inversely	[40,44,49,81,123,124,125,126]

Class	Biomarkers	Role	Relation to SVD	Refs
		inflammation and overall vessel function • a fundamental feature of ageing involves angiogenic signalling failure and alterations in blood flow • associated with cardiovascular disease, perhaps through the induction of hypoxia-pertinent pathways or involvement of NO production	associated with WMH lesion progression longitudinally • no associations reported with brain atrophy, white matter tract integrity or microbleeds	
Nitric oxide pathway	NO NOSTRIN ADMA Myeloperoxidase	• dysfunctional endothelial cells decrease NO bioavailability by reducing NO synthesis and secretion, which can lead to oxidative stress, inflammation, defective vasodilatory processes, impaired blood flow, platelet aggregation, proliferation of smooth muscle cells and increased blood pressure	• NO levels are inversely associated with SVD progression and WMH, lacunes and microbleeds • NOSTRIN levels are positively associated with WMH, though not grey mater volumes or cognitive performance • ADMA levels are higher in SVD patients and positively associated with WMH, though not number of lacunes • Myeloperoxidase levels are higher in SVD patients and positively associated with microbleeds, though not WMH or lacunes	[44,46,54,135,136,137]
Other relevant biomarkers	Hcy Albumin HbA1c β-a40 Adiponectin Klotho Lp-PLA2	• these molecules modulate a wide range of functions. Some exert deleterious effects on the endothelium and can facilitate oxidative stress, impair NO-mediated blood flow processes, up-regulate redox-sensitive inflammatory pathways, and promote atherogenesis and thrombogenicity, whilst others might offer protective neurovascular mechanisms	• Hcy levels are higher in SVD patients and are positively correlated with WMH severity, number of lacunes, microbleeds, PVS, disease progression and cognitive impairment, though results are inconsistent • albuminaria is associated with WMH severity, prevalence of lacunes and recurrent lacunar stroke • HbA1c is positively associated with WMH volume increase • β-amyloid1–40 levels are higher in those	[37,38,43,44,47,71,90,109,136,144,145,146,147,148,155,158,161,165]

Class	Biomarkers	Role	Relation to SVD	Refs
			with WMH lesions and multiple lacunar infarcts • levels of adiponectin are inversely associated with the prevalence of microbleeds • Klotho levels are inversely associated with WMH, PVS, lacunes and total SVD burden and progression, though not microbleeds • Lp-PLA2 levels are positively associated with WMH, lacunes and microbleeds	

Abbreviations: Cell adhesion molecules: VCAM-1: vascular cell adhesion molecule-1, ICAM-1: intracellular cell adhesion molecule-1, P-selectin: platelet selectin, E-selectin: endothelial selectin. BBB transporters: GLUT-1: glucose transporter-1, P-GP: permeability-glycoprotein, LAT-1: large neutral amino acid transporter-1, Cav-1: caveolin-1. Cytokines and Chemokines: IL-6: interleukin-6, TNF-α: tumour necrosis factor alpha, TNFR2: tumour necrosis factor receptor 2, PF-4: platelet factor-4. Other pro-inflammatory markers: CRP: C-reactive protein, CD40-L: CD40 ligand. Coagulation factors: vWF: von Willebrand Factor, TF: tissue factor, TFPI: tissue factor pathway inhibitor, t-PA: plasminogen activator, PAI-1: plasminogen activator inhibitor-1, TM: thrombomodulin, ET-1: endothelin-1. Growth factors: VEGF: vascular endothelial growth factor, TGF-β1: transforming growth factor-β1, PlGF: placental growth factor. Nitric oxide pathways: NO: nitric oxide, NOSTRIN: endothelial nitric oxide synthase traffic inducer, ADMA: asymmetric dimethylarginine. Other relevant markers: Hcy: homocysteine, HbA1c: haemoglobin A1c, β-amyloid1–40: beta-amyloid, Lp-PLA2: lipoprotein-associated phospholipase A2-mass.

Overall, the findings from this review point to a pivotal role of endothelial dysfunction in the molecular mechanisms underpinning SVD. Whilst there is no single definitive biomarker with consistent associations to specific clinical indicators of disease, promising independent predictors of disease progression include cell adhesion molecules, molecules pertaining to the nitric oxide pathway, PlGF, TGF-β1, and Hcy. It is possible that there are many ways in which endothelial dysfunction can exert influence over the development and

progression of SVD pathophysiology. Although the initial causes of endothelial dysfunction remain unclear, there is a propensity towards a pro-inflammatory and perhaps a pro-thrombotic state accompanied by aberrant angiogenesis, microvessel luminal narrowing, reduced responsiveness (vasoreactivity), altered pulsatility (vascular stiffness) and vasomotion, increased BBB permeability, and impaired blood flow regulation. It is possible to conceive of these processes as a dynamic pathological cycle resulting in a downstream cascade of dysfunction involving the entirety of the neurogliovascular system and culminating in white matter injury and ischaemia, neurodegeneration, diffuse connectivity problems and cortical thinning.

2.5.1 Implications for clinical practice

A significant challenge impeding the progress of therapeutics for SVD stems from the lack of elucidative and highly specific biomarkers associated with disease development and progression. As it stands, and despite strong evidence advocating for the involvement of endothelial dysfunction in disease pathogenesis, there is not one biomarker that is capable of defining SVD or effectively adding diagnostic or outcome predictive ability in clinical practice. In large part, this is likely due to the complexity inherent in SVD mechanisms. SVD is a diffuse, chronic, and dynamic disease process marked by fluctuating radiological lesions and symptoms[7,309–311]. It is influenced by a complex interplay between genetic factors, lifestyle variables and environmental exposures. And although it is not predominantly an atherosclerotic disease, it shares an overlap in risk factors, mechanistic pathways, and indeed biomarkers, with large-artery and cardiovascular disease. Our understanding of disease mechanisms is further hindered by the fact that human brain microvessels are not themselves visible on high-field MRI *in vivo* and the generation of SVD-relevant animal models can be conceptually challenging. It is possible, and indeed likely, that there are many distinct routes through which endothelial dysfunction can drive SVD pathophysiology, and thus, it is reasonable to consider that a panel of biomarkers, rather than a sole molecule or pathway,

is more likely to yield a comprehensive and robust diagnostic and prognostic tool in future.

2.5.2 Implications for future research

Another important reason a biomarker signature for SVD remains illusory pertains to the substantial inconsistencies in study results precipitated by heterogeneity in study design. There are several sources of methodological disparity impeding result replication and consistency. For instance, differences arise from the imaging modalities used, namely CT or MRI, and the methods and tools used to quantify radiological markers of disease. Many studies rely on diverse severity and grading scales, such as the Fazekas scale to measure WMH lesion burden, foregoing the enhanced accuracy provided by volumetric quantification in sensitive analyses such as these. Studies also vary regarding the time window between serology and MRI acquisition, or the time between stroke onset and imaging/serology (when applicable). Another factor to consider is the variation introduced by the analytes themselves; this includes taking into account their stability, the measurement techniques used to quantify them and the presence of metabolic confounders (e.g. fasting status), comorbid illness (e.g. chronic kidney disease), or acute injury, infection, or ischaemia.

Another such source of heterogeneity relates to lesion types- whilst most studies focus on WMH (which are the most commonly observed SVD lesions), other related features, including lacunes, microbleeds, siderosis, brain atrophy, and PVS remain understudied. It is possible that different lesion types arise at different disease stages, and with SVD representing a dynamic process, considering cumulative and total SVD burden, rather than individual lesion types, would likely prove insightful. However, perhaps the most critical source of variation comes from the characteristics of the study populations. Large ageing or community-dwelling cohort studies often deliberately will exclude patients with a history of stroke or MCI/dementia and tend to feature few patients with severe or even symptomatic SVD, limiting the representation

of the full spectrum of disease and lowering their statistical power to detect significant or meaningful associations between biomarkers and clinical indicators of disease. There is also considerable variation in population characteristics amongst studies with SVD patients, with some including solely hypertensive patients, or diabetic patients, or indeed excluding patients with overt clinical manifestations of SVD, including stroke or cognitive impairment. In this context, it also remains crucial to consider the characteristics of the control population and the importance of controlling for potential demographic and clinical covariates. Comparing vascular function biomarkers between recent lacunar stroke patients and healthy controls introduces the likelihood that differences identified are influenced by the recent ischaemic event (i.e. markers of acute brain injury) or a greater burden of vascular risk factors in the former group.

Finally, it is worth addressing the limitations inherent in cross-sectional studies in terms of providing conclusions regarding the causal relationship between fluid biomarkers and SVD mechanisms. Large longitudinal studies, particularly in conjunction with advanced MRI techniques that allow for insight into BBB function and vascular functionality, such as dynamic contrast-enhanced (DCE) MRI and cerebrovascular reactivity (CVR), can be advantageous by helping to provide support for a causal role or delineating predictive effects.

Here, we opted to conduct a scoping rather than a systematic research review. Scoping reviews allow for the broad exploration of key themes. Their flexible methodology can be especially useful for relatively new and emerging fields, where evidence is heterogenous or sparse, and it can help identify gaps in the literature. However, we did not assess risk of bias, and thus our results may be less reliable for evidence-based practice.

Many important questions remain. Importantly, the extent to which these biomarkers accurately reflect endothelial dysfunction confined to the brain (as opposed to systemic dysfunction), or whether endothelial dysfunction in SVD is indeed confined to the brain, remains unclear. In addition, the many ways in which endothelial dysfunction could affect other constituents in the

neurogliovascular system and how changes in these cells could, in turn, affect the endothelium, remains poorly understood. Finally, candidate catalysts for endothelial dysfunction in the context of SVD and how they relate to genetics and vascular risk factor exposure remain unknown and warrant further examination.

There is substantial evidence proposing endothelial dysfunction as a pivotal mechanism in the pathogenesis of SVD, and the identification of relevant biomarkers and their association with disease mechanisms not only provides important clues regarding the different pathways involved, but it can also provide promising avenues for therapeutic targets. Particularly exciting prospects include clinical trials targeting endothelial dysfunction, including the ongoing LACI-2 trial, which is repurposing Isosorbide Mononitrate and Cilostazol as potential treatments for patients with symptomatic SVD[161].

2.6 Concluding statements

This chapter provided a thorough examination of the existing literature regarding the molecular mechanisms of endothelial dysfunction within the context of SVD. This scoping review indicates that although there is overwhelming evidence implicating endothelial dysfunction in SVD, the specific ways through which it contributes to various manifestations of disease, and whether it can be effectively measured *in vivo*, remain unclear. Additionally, I also highlight potential sources of result inconsistency, a common issue within this subfield of SVD research.

Subsequent chapters will detail findings from my own research projects, which aim to investigate the roles of pericyte and endothelial cell dysfunction in ageing and SVD through a translational perspective. In the next chapter, **Chapter 3**, I present results from my pre-clinical investigations focusing on changes in pericyte and endothelial cell function during the ageing process.

Chapter 3 Ageing mice study: pericyte coverage and endothelial activation

3.1 Introduction

In **Chapter 2**, I discuss how various blood and CSF biomarkers of endothelial dysfunction and activation are associated with distinct manifestations of SVD, including neuroimaging markers of disease development and progression as well as cognitive decline.

In this chapter, I explore key investigations conducted to test a central premise of my hypothesis: that pericyte loss and endothelial activation are potentially interconnected processes that occur during ageing. Here, I compare pericyte coverage and brain endothelial cell activation through VCAM-1 expression between young and aged mice.

3.1.1 Ageing

Ageing remains the principle risk factor in sporadic SVD development and manifestation, and there is ample evidence suggesting the presence of BBB dysfunction with ageing, particularly in those over 60 years of age, with the cerebrovascular endothelium being nearly twice as permeable in 70-80 year-old adults compared to young controls[55]. It is possible that this observed BBB dysfunction in ageing is, at least in part, linked to pericyte and endothelial dysfunction. Genes expressed in pericytes and endothelial cells, such as *foxf2*, have been linked to SVD manifestations, including lacunar stroke and WMH[313].

3.1.2 Pericyte loss with ageing and in the context of dementia

In the young, healthy, human brain, pericyte coverage in capillaries is estimated to hover around 90%[99], however, a wealth of recently published evidence indicates that pericytes are acutely sensitive to cerebrovascular pathology and mechanisms of ageing, even in the absence of pathology. For

instance, reports of pericyte degeneration and loss have been observed in studies featuring ageing mice[117,118], rats[119], monkeys[120], and humans[53,121,125]. Human *postmortem* tissue analyses have even revealed a remarkable 45% decrease in cortical capillary pericyte coverage in aged controls (>78 years) in comparison to young controls[314].

Aside from loss and degeneration, pericytes also show structural and functional changes during ageing, including vesicular inclusions[315], larger mitochondria[316] and a foamy transformation[317]. There is also evidence to indicate that pericytes' plasticity and ability to remodel in response to injury diminishes with age[318]. In ageing mice, pericyte loss and a reduction in the expression of pericyte-induced genes in the endothelium has been observed alongside concomitant fundamental changes in transcytosis mechanisms, from ligand-specific receptor-mediated endothelial transport, towards non-specific caveolar transcytosis mechanisms, which can render the BBB more susceptible to allowing neurotoxic proteins, such as fibrinogen and thrombin, across the endothelium walls[118].

Although the precise mechanisms remain poorly understood, previous studies have indicated changes in pericyte cell count and capillary pericyte coverage in the context of several neurodegenerative diseases, including vascular dementia and Alzheimer's Disease[114,124–128] as well as acute stroke[319]. However, pericyte loss through ageing and in the context of cerebrovascular disease remains controversial. Some studies report finding no significant decrease in pericyte coverage between young and aged animals[320–323], with others even reporting an observed increase in pericyte coverage or pericyte-endothelial cell contact, including in the ageing rat[315] and ageing female mouse[324] brain. A recent study examining 124 *postmortem* brain tissue samples from young, healthy controls, aged controls, and patients with mixed vascular dementia or AD reported a preservation of frontal cortical capillary pericyte coverage in diseased cases compared to controls of similar age[314]. However, the authors suggest differential regulation of capillary pericytes in the frontal cortex compared to deep white matter regions in ageing-related

dementias, as previous investigations suggested a marked decrease in pericyte count in the frontal deep white matter of patients with AD, vascular dementia and mixed AD-vascular dementia[125].

In the context of other dementias, namely AD, pericyte loss has been observed and linked to fibrinogen leakage, hypoperfusion, and fibrillar β amyloid accumulation[114]. Investigations relying on DCE to quantify BBB permeability in AD patients revealed an association between BBB breakdown in the hippocampus in early stages of disease and pericyte injury, evidenced by CSF-derived sPDGFRβ levels[53]. Another study partly supports these findings, reporting decreased levels of pericyte marker, PDGFRβ, in the precuneus, though not the underlying white matter, of AD brain tissue, which also correlated with increased fibrinogen, a reduction in oxygenation, and with plaque load[114]. However, a recent study has refuted the notion that pericyte loss is a hallmark of AD pathology, finding no significant discernible change in pericyte density in the frontal cortex of AD patients[323].

3.1.3 Endothelial activation in ageing

One possible catalyst of pericyte dysfunction is endothelial activation. Endothelial activation refers to the increased expression of endothelial cell-surface adhesion molecules, such as VCAM-1, and leukocyte adhesion molecules induced by pro-inflammatory cytokines. As is discussed in depth in the **Introduction** and the previous **Chapter 2**, VCAM-1 is a cell adhesion molecule that serves as a marker for endothelial activation and facilitates diapedesis and the adhesion and transendothelial migration of leukocytes through the endothelium of vascular walls. The soluble form of these molecules can be released from the cell surface and into the circulation. Amongst the array of markers of endothelial cell activation and dysfunction, we have opted to focus on VCAM-1 in this instance. This decision stems from the extensive, diverse, and robust library of evidence linking VCAM-1 to vascular dysfunction in ageing, encompassing pre-clinical, proteomic, and clinical investigations[144–149,162,166,186,325–327].

Increased VCAM-1 expression has been linked to ageing and age-related neurodegenerative disorders, including various stages of SVD and AD; circulating levels of VCAM-1 have been linked to lacunar stroke, greater WMH lesion volume and lesion grade, BBB breakdown, cognitive decline and downstream signalling events associated with increased BBB permeability[145–149].

Cultured human brain endothelial cells that are exposed to VCAM-1, demonstrate increased permeability of a soluble tracer, as well as concurrent actin stress fibre formation and tight-junction decrease[145]. In plasma proteomic datasets, plasma soluble VCAM-1 emerges are one of the proteins that exhibits the most prominent increase during ageing[144].

Results from the association between circulating concentrations of VCAM-1 detectable in blood or CSF and disease burden and/or progression are discussed in detail in **Chapter 2**. Briefly, clinical studies have found associations between VCAM-1 levels and lacunar stroke[147,162], worse WMH lesion burden[147,166], the presence of lacunes[186], cognitive decline[325], and disease progression[326,327]. However, significant controversy remains, with other studies reporting no discernible differences in VCAM-1 concentration between recent lacunar stroke patients and age-matched controls[184]. Potential sources of inconsistencies in study results are discussed in-depth in **Chapter 2**, with likely candidates including differences in sample population characteristics (particularly in what constitutes as a control population), sample exclusion criteria (*e.g.* excluding those with a history of stroke or cognitive impairment), and distinct ways of quantifying disease burden (*e.g.* continuous WMH volumes versus ordinal Fazekas score versus subjective categories such as ‘mild’ and ‘severe’).

Although it is likely that pericyte loss and endothelial activation are synergistically exacerbating each other’s dysfunction, evidence of this relationship and the precise mechanisms underlying this crosstalk remains unclear.

3.1.4 Aims of the ageing mice study

There are clear discrepancies in the existing literature regarding pericyte loss and endothelial activation in ageing and in the context of SVD, and the nature of the relationship between endothelial and pericyte function remains to be elucidated. It is possible that some of the existing inconsistencies in the abovementioned results derive from relying on data from one (or few) brain regions, or even smaller sub-sections within regions, as well as using only male or female animals. In addition, due to the plastic nature of pericytes' extending and retracting cell processes, it remains conceivable that overall pericyte capillary coverage changes may be overlooked amidst a stable cell body count, highlighting a possible advantage of quantifying pericyte coverage as percent of total capillary area.

The aim of this study is thus to quantify pericyte coverage (%) and endothelial activation through VCAM-1 expression across 9 different entire grey and white matter regions in young (3-month-old) and ageing (12-month-old) mice, both male and female. Furthermore, our in-house developed software, VasOMatic, enables the segmentation of all capillaries into small micro-segments, rendering insight into the possible spatial relationship between pericyte loss and VCAM-1 expression possible.

3.2 Methods

3.2.1 Animals

16 C57BL/6J (eight 3-month-old mice (4 females) and eight 12-month-old mice (5 females)) were used in these investigations.

The C57BL/6J colony was housed in the Bioquarter University of Edinburgh animal facility. Animals were cared for in accordance with ARRIVE and UK Home Office Guidelines (Project licence number PP7610194 "Cerebrovascular and inflamm-ageing link to neurodegeneration and dementia"). All sample sizes were based according to power calculations done

prior to experimentation and both male and female animals were included. There were no cases of animals being excluded for any reason or instances where culls were inevitable due to animal suffering or injury. All animals were fed standard chow.

3.2.2 Termination and tissue processing

Animal sacrifice was performed by Dr Conor McQuaid and William Mungall. Animals were anaesthetised with 100 μL of pentobarbital administered intraperitoneally. They were perfused transcardially with 15 mL of phosphate buffer saline (PBS) with 0.005 M ethylenediaminetraacetic acid followed by 15 mL of 4% paraformaldehyde. Harvested brains were postfixed with 4% paraformaldehyde for 24 hours and subsequently washed thrice with PBS before submerging in a 20% sucrose solution for 48 hours. Following this, brains were frozen using isopentane and stored at -80 °C. The tissue was then sectioned into 40 μm thick slices in a sagittal orientation using a cryostat and stored at -20°C in wells with antifreeze solution containing 500 mL of PBS (sodium phosphate buffer, 0.1 M, pH 7.4), 85.6 g of sucrose (259 mM), 0.66 g of MgCl2 (7 mM) and 413.74 mL of glycerol).

3.2.3 Immunohistochemistry

Three sagittal brain slices per animal were placed in individual wells within 24-well plates and washed with PBS (3x 10 minutes). Sections were subsequently blocked with 10% normal donkey serum (Bio-Rad Assay, bovine serum albumin standard)/0.1% Triton-X/0.01 M PBS for two hours. Sections were then allowed to incubate overnight at 4°C under gentle agitation submerged in a solution containing the primary antibodies diluted in blocking solution. The primary antibodies utilised were: for pericytes, polyclonal goat anti-mouse aminopeptidase n/CD13 antibody (CD13; R&D Systems, AF2335, 1:100), for VCAM-1, VCAM-1 rat monoclonal antibody (VCAM-1, Origene, AM05557PU-L, 1:100) and for vasculature/cell-extra-cellular matrix junctions, mouse

monoclonal IgG β-dystroglycan antibody (β-dystroglycan; Santa Cruz Biotechnology, sc-33702, 1:1000).

Sections were then washed thrice in 0.1% Triton-X/0.01 M PBS for 10 minutes after discarding the primary antibody solution and submerged in fluorophore-conjugated secondary antibodies diluted in blocking solution for 2 hours under gentle agitation at room temperature whilst sheltered from light exposure. Secondary antibodies used were: Alexa Fluor 568 anti-goat (Invitrogen, A-11057, 1:500) for pericytes, Alexa Fluor 488 anti-rat (Invitrogen, A-21208, 1:500) for VCAM-1, and Alexa Fluor 647 anti-mouse (Invitrogen, A- 31571, 1:500) for β-dystroglycan. Tissue was subsequently washed with PBS once for five minutes before staining for cell nuclei using Hoechst (Thermo-Fisher, H370, Lot 239776) diluted in PBS (1 mg/mL) for 6 minutes. Sections were then washed with PBS (3x 10 minutes) before being mounted on slides using ProLong™ Gold antifade reagent mounting medium and cover slips.

3.2.4 Microscopy

Opera Phenix microscopy and all imaging protocols were operated by Dr Justyna Cholewa-Waclaw. Entire-brain images for this protocol were taken with an Opera Phenix High-Content Screening System Plus using a 20x resolution water objective (NA 1.0) with channels for hoescht (exposure time: 100 ms), Alexa 488 (exposure time: 400 ms), Alexa 568 (exposure time: 300 ms), and Alexa 647 (exposure time: 200 ms) and z-stack of 30 planes with step of 1μm. Analyses were conducted on maximum projection images (**Figure 3-1**).

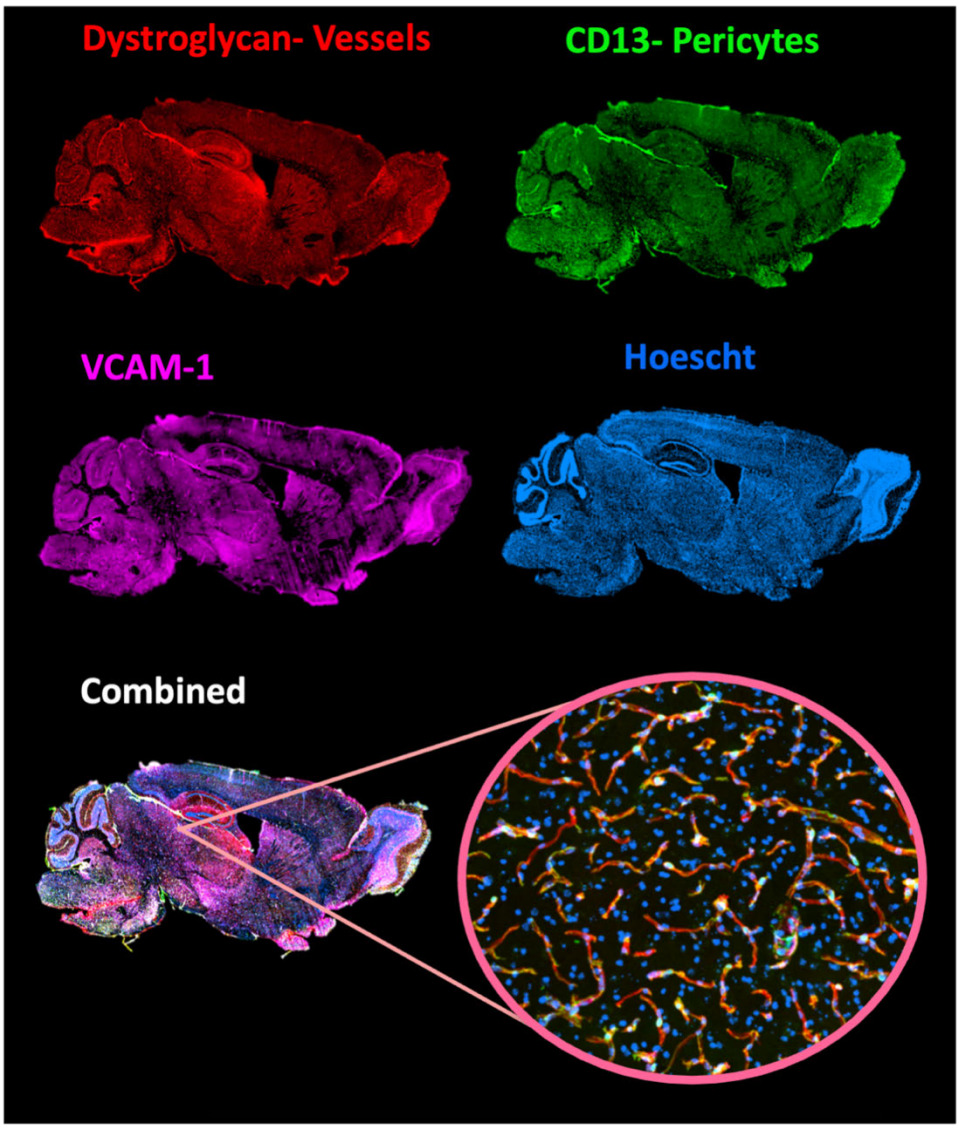


Figure 3-1.Immunohistochemistry and Opera Phenix High-Content Screening System microscopy. We stained whole-brain slices for vasculature/cell-extracellular matrix junctions using mouse monoclonal IgG β-dystroglycan antibody, pericytes using polyclonal goat anti-mouse aminopeptidase n/CD13 antibody, endothelial activation through VCAM-1 expression using VCAM-1 rat monoclonal antibody, and cell body nuclei with Hoechst.

3.2.5 Image processing and quantification

I manually segmented the following brain regions of interest: anterior corpus callosum, posterior corpus callosum, thalamus, caudate putamen, internal capsule, and from the hippocampus: Cornu Ammonis 1 (CA1), Cornu

Ammonis 2/3 (CA2/3) and the dentate gyrus) from entire-brain slices using Qupath (version 0.5.0).

All image quantification was done using the VasOMatic Macro in FIJI (FIJI is Just ImageJ, 10.1038/nmeth.2019), which was developed in-lab by Dr Audrey Chagnot in conjunction with this study[328]. Whilst blinded to mouse ID and age, I used the macro to segment the vasculature from each region of interest using median smoothing, particle analysis, and manual thresholding to refine the mask. Using this mask as an overlaying template, I then segmented the paravascular staining of interest (pericytes, VCAM-1) overlying the vasculature (**Figure 3-2**). Subsequently, the macro utilises an algorithm to split the vascular mask into segments using FIJI's watershed function. This allows for the collection of spatial metrics stemming from each individual segment, including vessel length, diameter, and paravascular signal (pericyte and VCAM-1), which are collected using skeleton measurement, distance mapping, and mask overlap, respectively.

Pericyte and VCAM-1 coverage measurements were captured as total % of vasculature covered, or signal intensity where specified. Vessel diameter was measured in μm and vessel density was converted to mm/mm^3. All mask thresholding was manually checked and adjusted as necessary. Images with unreliable vascular segmentation masks due to excessive background fluorescent noise were discarded, as paravascular masks rely on the accuracy of vascular masks to produce accurate data. Unsuitable images which would not have sustained accurate mask thresholding (*e.g.* because of tissue folding) were also discarded from the dataset.

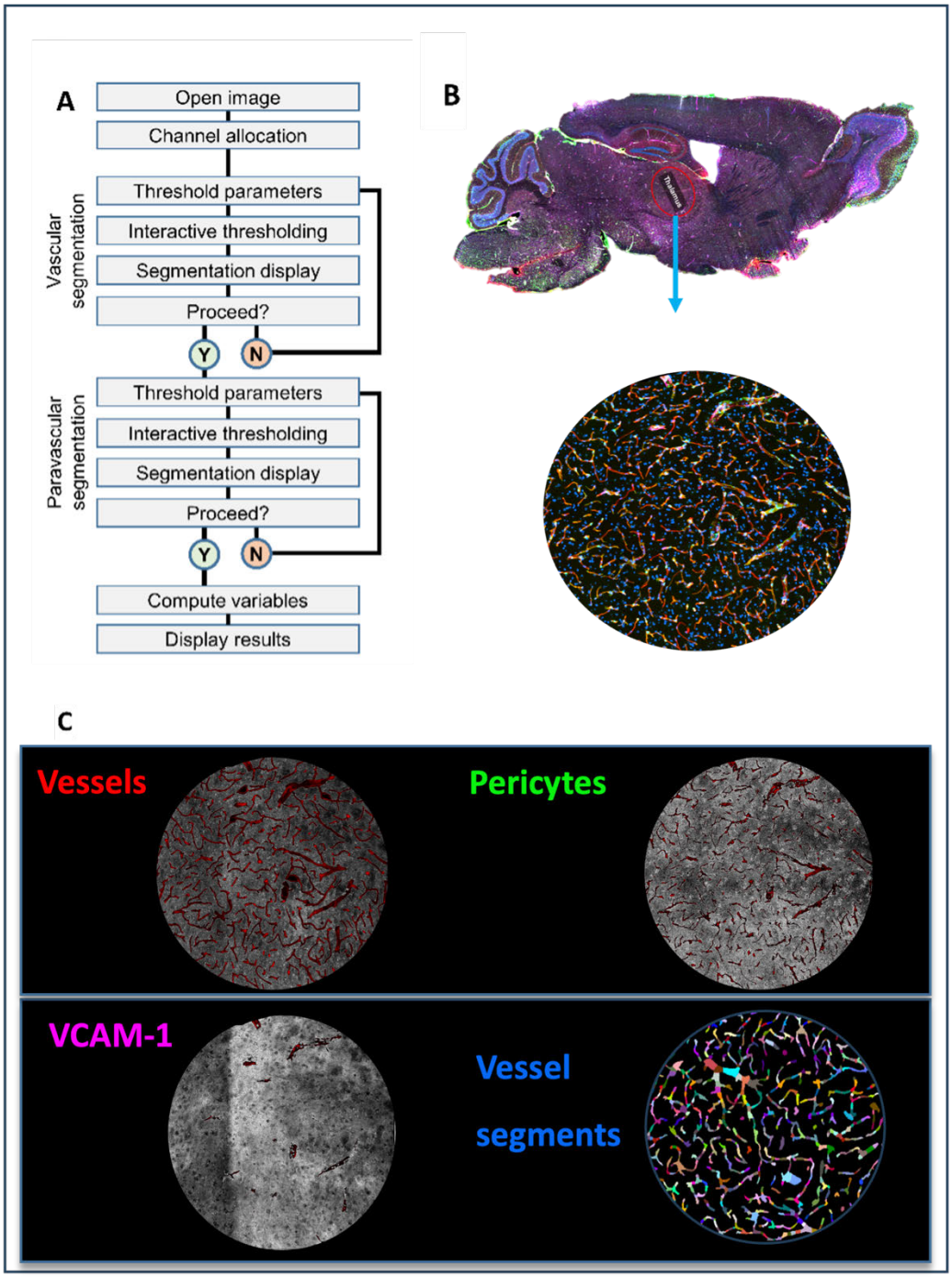


Figure 3-2. VasOMatic FIJI Macro demonstration (developed by Dr Audrey Chagnot, Montagne Lab, University of Edinburgh, 2023). A) Code structure and chronological user interface of the macro. B) Example of brain region of interest segmentation (thalamus). C) Example of VasOMatic mask segmentation (vasculature, pericytes and VCAM-1 masks) as well as a visual representation illustrating the vessel segment truncation process.

3.2.6 Statistical analysis

All statistical analyses and graphical representations were performed using R (RStudio 4.3.2) except in instances stating otherwise. Originally, a-priori power calculations were done using the 'pwr' package for a two-tailed hypothesis on a mean comparison t-test. Based on pilot results consistent with literature reports, we estimated pericyte coverage to be approximately 85% in young animals and 70% in aged animals, with a 10% variation (Cohen's w: (0.85-0.7)/0.1= 1.5). We determined at least 8 mice per group were required with alpha error (α=0.05), and power (1-beta error) (p=0.8). P-values of <0.05 were considered statistically significant. Linear mixed effects models were performed using the R 'lme4' package and modelled with random effects stemming from variance between individual animals and adjusted for key factors including age, sex, and brain region where relevant, also testing for a potential interaction between age and sex where applicable. Goodness of fit and markers of model suitability (including homogeneity of variance, distribution of residuals, and collinearity) were checked using the 'performance' and 'DHARMa' packages. Pericyte signal and VCAM-1 coverage in the microvessel segmentation dataset were log-transformed to improve model fit. Variables with a skewed distribution are reported as median (IQR). In addition, linear mixed effects models relying on data from the micro vessel segmentations (n= 93,738) were adjusted for pericyte signal, sex, and brain region to avoid collinearity.

3.3 Results

We extracted a total of 313 entire-region images suitable for analyses. In total, 71 images were discarded for the reasons outlined above (43 images from young animals and 24 from aged animals).

3.3.1 Vessel density

Young animals had an average vessel density of 516.61 mm/mm^3 (SD=192.05 mm/mm^3) compared to 526.25 mm/mm^3 (SD=185.08 mm/mm^3) in aged

animals, with the thalamus having the highest vessel density in both young and aged animals (**Figure 3-3, Figure 3-4 & Table B-1 in Appendix B**). Results from linear mixed models adjusting for brain region, an interaction between age and sex, and random effects stemming from individual animals, indicated that vessel density was 20 mm/mm^3 greater in the aged group (β=20.09, SE=39.98, t=0.50) on average and 14 mm/mm^3 lower in female animals relative to male animals (β=-14.09, SE=38.74, t=-0.36). Neither of these associations were statistically significant (p=0.6, p=0.72, respectively) and there was no significant interaction between age and sex (**Table 3-1**). A likelihood-ratio test indicated that the model including age did not provide a better fit than a model without it ($X^2(1)$= 1.02, p=0.60).

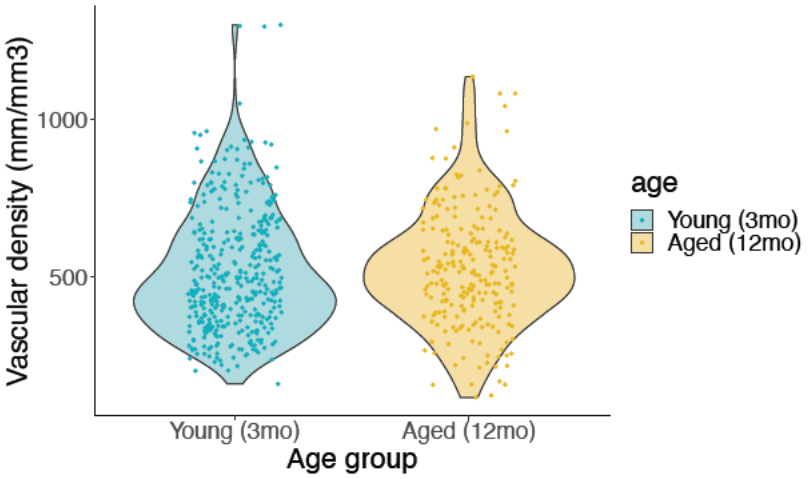


Figure 3-3. Average global vessel density (mm/mm^3) in both young and aged animals.

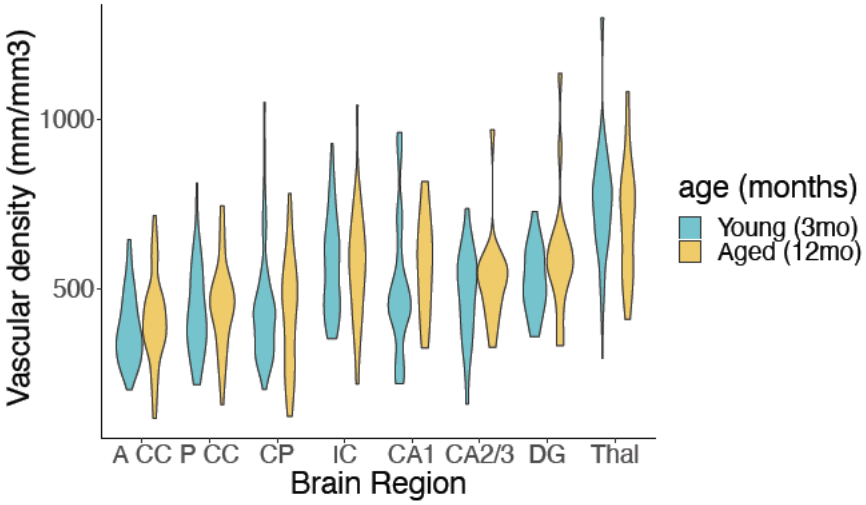


Figure 3-4. Average vessel density (mm/mm³) by brain region in both young and aged animals. *Abbreviations:* A CC: Anterior Corpus Callosum; P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Table 3-1. Linear mixed effects model results examining vessel density (mm/mm³) and age whilst controlling for sex, brain region and random effects stemming from individual animals. *Abbreviations:* P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Linear Mixed Model Results: Vessel Density

	Dependent variable:
	Vessel Density
Age (12 months)	20.092 (-58.264, 98.448)
Sex (female)	-14.086 (-90.018, 61.846)
Brain Region: PCC	52.201*** (12.785, 91.616)
Brain Region: CP	43.452** (2.355, 84.548)
Brain Region: IC	175.960*** (136.132, 215.789)
Brain Region: CA1	135.728*** (79.994, 191.462)
Brain Region: CA2/3	108.077*** (53.461, 162.693)
Brain Region: DG	163.846*** (112.152, 215.541)
Brain Region: Thal	350.623*** (311.297, 389.949)
Interaction Age(12):Sex(female)	10.936 (-99.676, 121.549)
Constant	392.633*** (333.362, 451.904)
Observations	624
Log Likelihood	-3,950.817
Akaike Inf. Crit.	7,927.635
Bayesian Inf. Crit.	7,985.305
Note:	*p<0.1; **p<0.05; ***p<0.01

3.3.2 Vessel diameter

Young animals had an average global vessel diameter of 4.08 μm (SD=0.51 μm) compared to 4.50 μm (SD=0.56 μm) in aged animals, with variations observed across different brain regions (**Figure 3-5, Figure 3-6 & Table B-2 in Appendix B**). The highest mean vessel diameter was observed in the internal capsule for all animals. Outcomes from linear mixed models fitted with age, sex, and brain region as fixed effects and individual animals as random effects showed that, on average, vessel diameter was estimated to be 0.72 μm higher in the aged group (β=0.72, SE=0.16, *t*=4.47) relative to the young group and 0.07 μm higher in female animals relative to male animals (β=0.07, SE=0.15, *t*=0.46). The effect of age was statistically significant (*p*=<0.001) whilst the effect of sex, and the interaction between age and sex, were not (*p*=0.65, *p*=0.09, respectively) (**Table 3-2**). A likelihood-ratio test indicated that including age notably enhanced the model fit compared to a model without it ($X^2(1)$= 17.63, *p*<0.001).

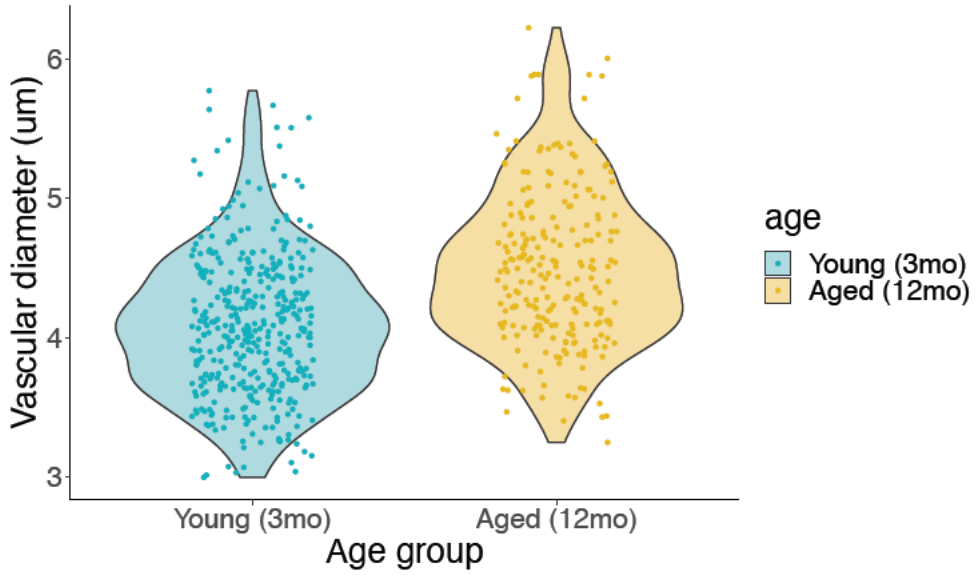


Figure 3-5. Average global vessel diameter (μm) in both young and aged animals.

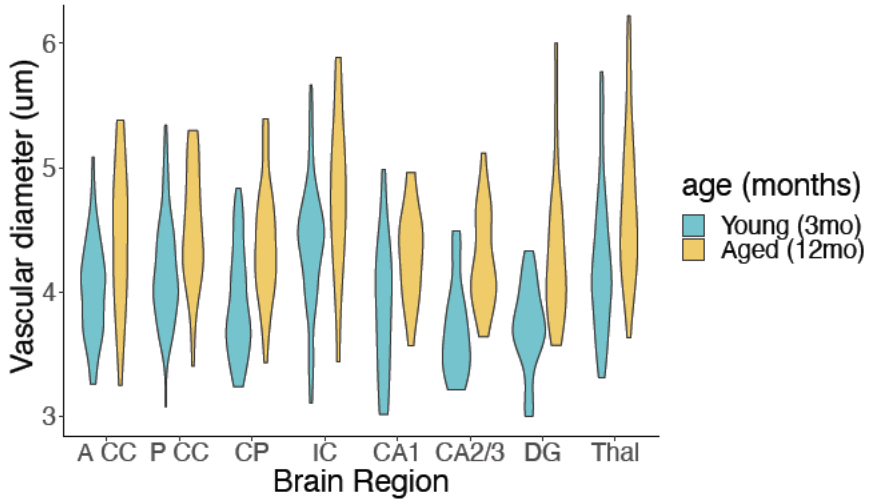


Figure 3-6. Average vessel diameter (μm) by brain region in both young and aged animals. *Abbreviations:* A CC: Anterior Corpus Callosum; P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Table 3-2. Linear mixed effects model results examining vessel diameter (μm) and age whilst controlling for sex and brain region as fixed effects as well as any random effects stemming from individual animals. *Abbreviations:* P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Linear Mixed Model Results: Vessel Diameter

	Dependent variable:
	Vessel Diameter
Age (12 months)	0.723^{***} (0.406, 1.040)
Sex (female)	0.073 (-0.238, 0.384)
Brain Region: PCC	0.125^{**} (0.0002, 0.249)
Brain Region: CP	-0.094 (-0.224, 0.036)
Brain Region: IC	0.337^{***} (0.211, 0.463)
Brain Region: CA1	-0.180^{**} (-0.357, -0.004)
Brain Region: CA2/3	-0.293^{***} (-0.466, -0.121)
Brain Region: DG	-0.243^{***} (-0.406, -0.079)
Brain Region: Thal	0.219^{***} (0.095, 0.343)
Interaction Age(12):Sex(female)	-0.411^{*} (-0.859, 0.037)
Constant	3.980^{***} (3.746, 4.214)
Observations	624
Log Likelihood	-424.962
Akaike Inf. Crit.	875.923
Bayesian Inf. Crit.	933.593
Note:	*p<0.1; **p<0.05; ***p<0.01

3.3.3 Pericyte coverage

Global pericyte coverage in older animals was reduced by an estimated 7.5% compared to young animals (β=-7.47, SE=2.15, *t*=-3.46), with 3-month-old animals having an average global pericyte coverage of 75.38% (SD= 3.79) in comparison to 67.17% (SD= 2.86) in 12-month-old animals (**Figure 3-7, Figure 3-8 & Table 3-3**). Results from linear mixed models indicated that the effect of age on pericyte coverage was statistically significant (*p=0.005)* after adjusting for sex and brain region as fixed effects and individual animals as random effects. There was no significant interaction between the effects of age and sex on pericyte coverage. Results from a likelihood-ratio test indicated that a model including age provided a better fit than a model without it ($X^2(1)$= 38.19, *p*=<0.001). Pericyte coverage was lower on average in aged animals in all the brain regions observed, with the thalamus displaying the most marked difference, where aged animals had a 16.3% reduction in pericyte coverage in comparison to young animals (**Table B-3 in Appendix B**). Pericyte coverage was approximately 1.4% lower in female animals relative to male animals (β=-1.38, SE=2.00, *t*=-0.69), although this difference was not statistically significant (*p*=0.50).

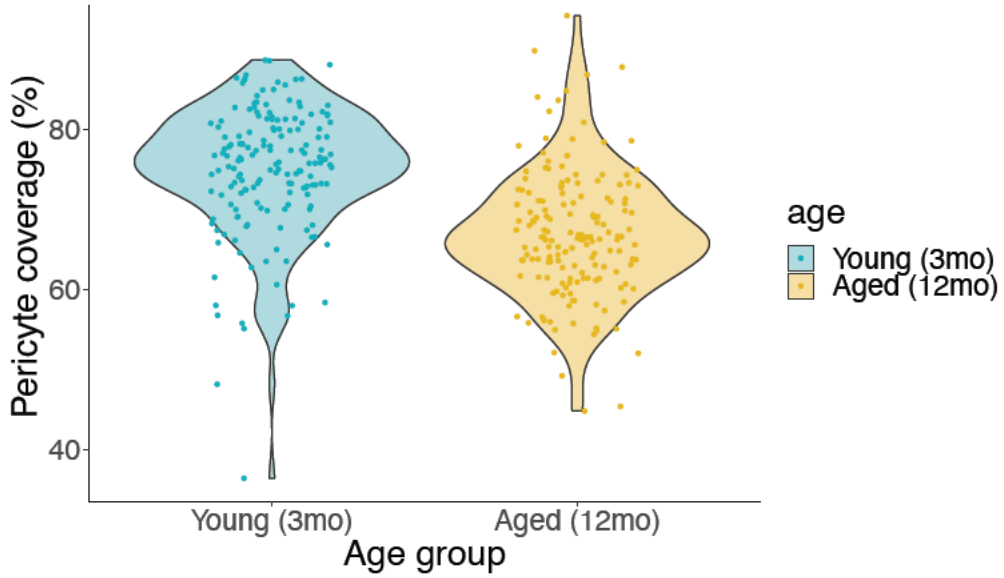


Figure 3-7. Average pericyte coverage (%) in 3-month-old mice and 12-month-old mice.

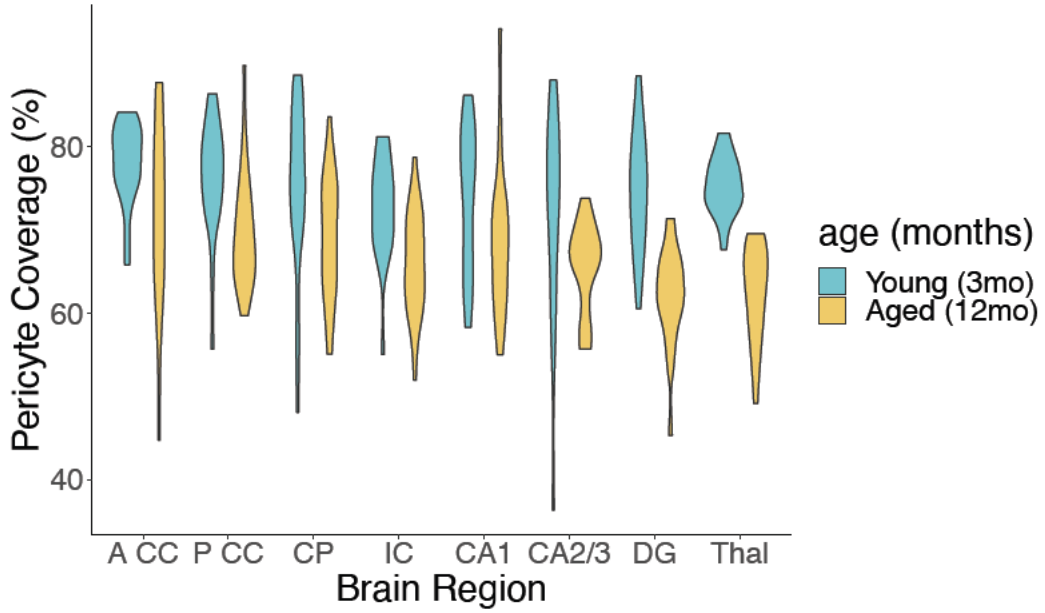


Figure 3-8. Average pericyte coverage (%) in all brain regions observed in both young and aged animals. *Abbreviations:* A CC: Anterior Corpus Callosum; P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Table 3-3. Linear mixed effects model results examining pericyte coverage (%) and age whilst controlling for sex and brain region as fixed effects as well as any random effects stemming from individual animals. *Abbreviations:* P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Linear Mixed Model Results: Pericyte Coverage

	Dependent variable:
	Pericyte Coverage
Age (12 months)	-7.472*** (-11.018, -3.926)
Sex (female)	-1.381 (-4.673, 1.911)
Brain Region: PCC	-1.509 (-4.199, 1.182)
Brain Region: CP	-2.618 (-5.279, 0.043)
Brain Region: IC	-5.327*** (-7.988, -2.666)
Brain Region: CA1	-3.572** (-6.460, -0.683)
Brain Region: CA2/3	-5.991*** (-8.810, -3.172)
Brain Region: DG	-6.111*** (-8.817, -3.404)
Brain Region: Thal	-5.906*** (-8.554, -3.259)
Interaction Age(12):Sex(female)	-1.040 (-5.749, 3.669)
Constant	79.629*** (76.647, 82.610)
Observations	330
Log Likelihood	-1,119.388
Akaike Inf. Crit.	2,264.777
Bayesian Inf. Crit.	2,314.165
Note:	*p<0.1; **p<0.05; ***p<0.01

3.3.4 VCAM-1 coverage

Median VCAM-1 coverage of the microvasculature in young animals was 3.56% (IQR= 1.67) compared to 7.62% (IQR= 5.23) in aged animals (**Figure 3-9**). VCAM-1 coverage was increased in aged animals consistently across all brain regions, with the most pronounced difference being observed in the anterior corpus callosum, where aged animals had a median VCAM-1 coverage of 11.9% (IQR= 6.19) compared to 4.37% (IQR= 1.96) in young animals **(Figure 3-10 &. Table B-4 in Appendix B).**

Results from linear mixed effects models adjusted for sex and brain region revealed a significant relationship between VCAM-1 coverage and age, with aged animals showing an estimated 99% increase in VCAM-1 coverage relative to young animals (β=0.68, SE=0.28, t=2.45, p=0.03) (**Table 3-4 & Figure 3-11**). There was no significant interaction between age and sex. A likelihood-ratio test indicated that the model including age provided a better fit than a model without it ($X^2(1)$= 11.43, p<0.001). In addition, female animals had 46.6% higher VCAM-1 coverage on average compared to male animals (β=0.32, SE=0.18, t=1.77), although this relationship did not reach statistical significance (p=0.168).

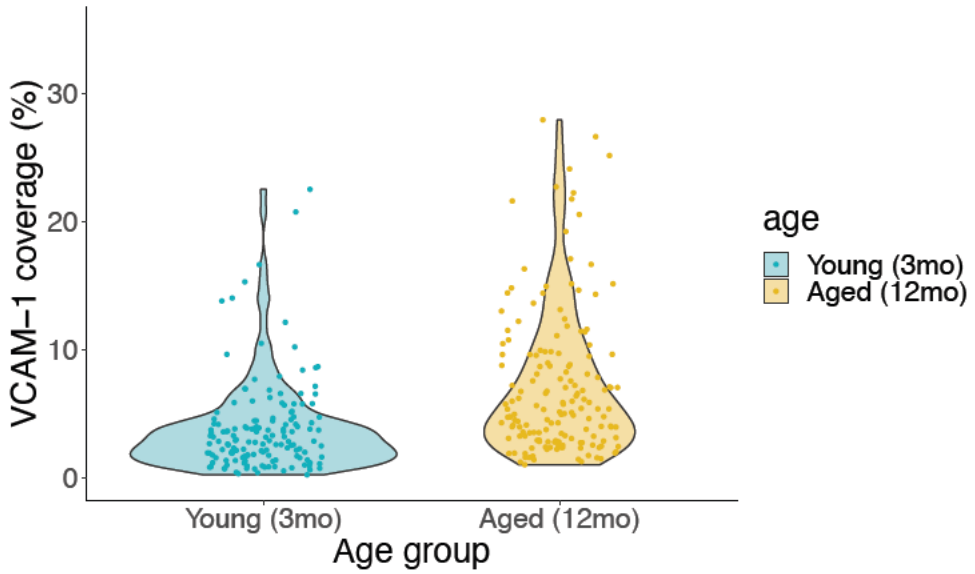


Figure 3-9. Average VCAM-1 coverage (%) in 3- and 12-month-old animals.

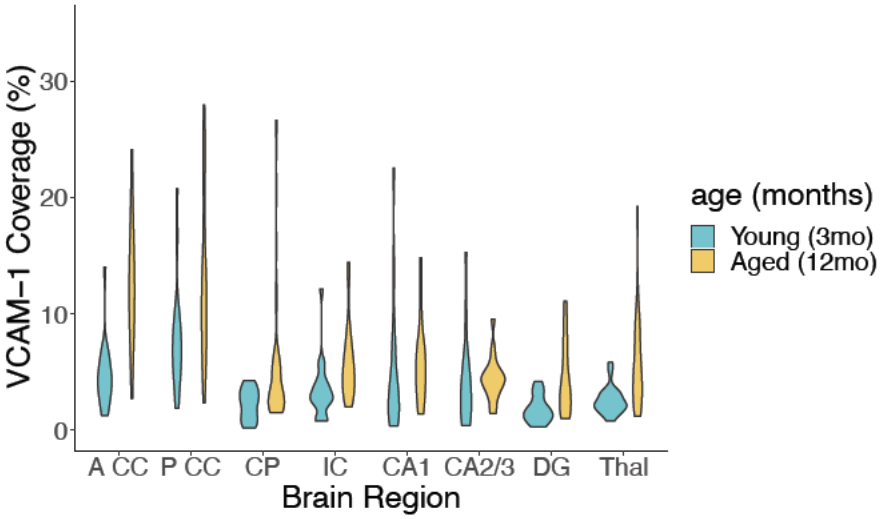


Figure 3-10. Average VCAM-1 coverage (%) in all brain regions observed in both young and aged animals. *Abbreviations:* A CC: Anterior Corpus Callosum; P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Table 3-4. Linear mixed effects model results examining VCAM-1 coverage (%) and age whilst controlling for sex and brain region as fixed effects as well as any random effects stemming from individual animals *Abbreviations:* P CC: Posterior Corpus Callosum; CP: Caudate Putamen; IC: Internal Capsule;CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; DG: Dentate Gyrus; Thal: Thalamus.

Linear Mixed Model Results: VCAM-1 Coverage

	Dependent variable:
	VCAM-1 Coverage
Age (12 months)	0.687^{**} (0.226, 1.148)
Sex (female)	0.383 (-0.047, 0.814)
Brain Region: PCC	0.191 (-0.039, 0.421)
Brain Region: CP	-1.009^{***} (-1.235, -0.782)
Brain Region: IC	-0.559^{***} (-0.783, -0.334)
Brain Region: CA1	-0.583^{***} (-0.815, -0.351)
Brain Region: CA2/3	-0.701^{***} (-0.937, -0.465)
Brain Region: DG	-1.109^{***} (-1.342, -0.875)
Brain Region: Thal	-0.740^{***} (-0.962, -0.519)
Interaction Age(12):Sex(female)	-0.093 (-0.707, 0.521)
Constant	1.414^{***} (1.073, 1.755)
Observations	313
Log Likelihood	-315.462
Akaike Inf. Crit.	656.924
Bayesian Inf. Crit.	705.625
Note:	*p<0.1; **p<0.05; ***p<0.01

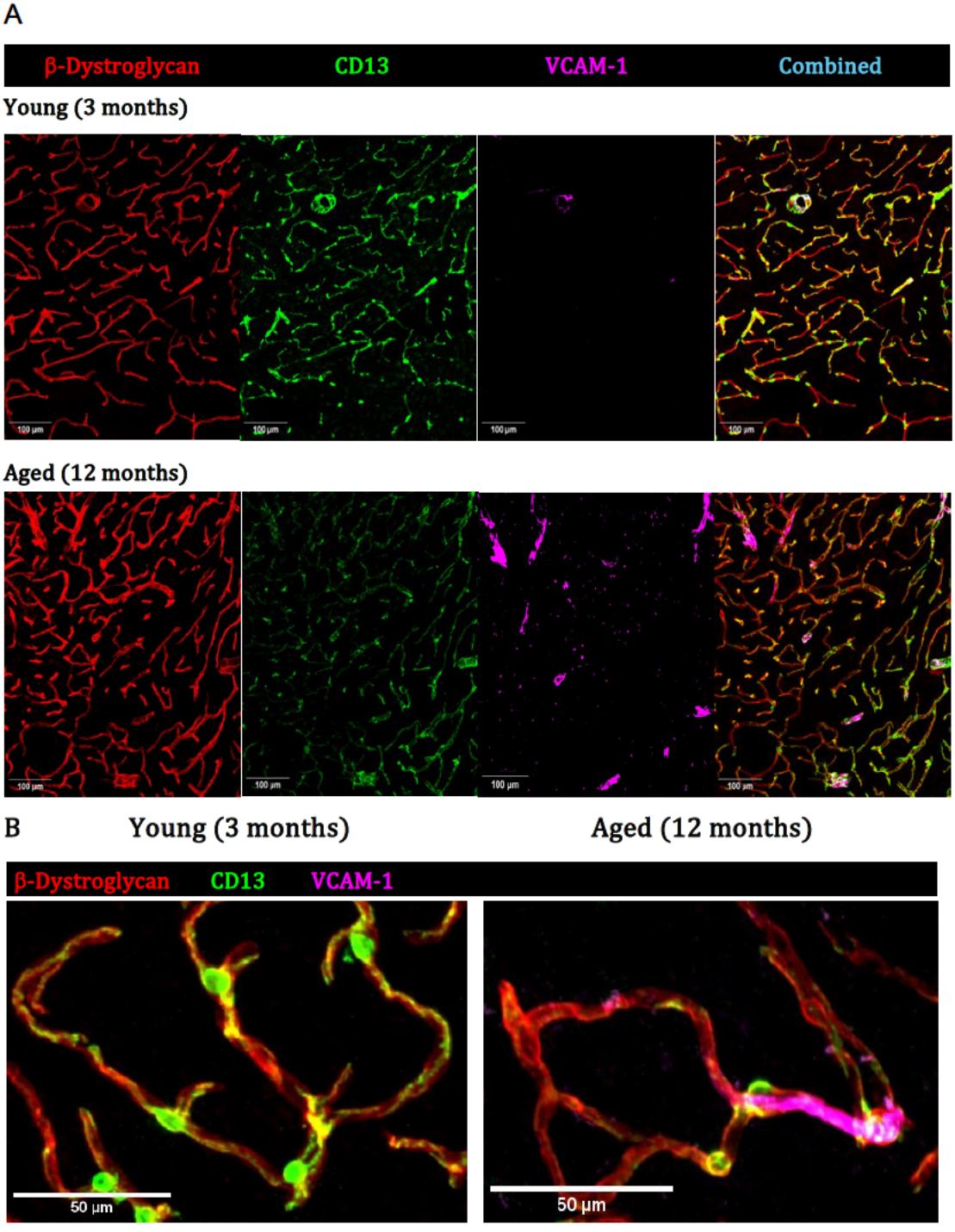


Figure 3-11. All images were stained using β-Dystroglycan (vasculature), CD13 (pericytes, green), and VCAM-1 (endothelial activation, magenta). (A) Section from the internal capsule of a young 3-month-old male mouse (top) and an aged 12-month-old male mouse (bottom) denoting an increase in vessel diameter (red), pericyte loss (green), and enhanced endothelial activation (magenta) in ageing mice. (B) Isolated capillaries from the thalamus of a young 3-month-old female mouse (left) and an aged 12-month-old female mouse (right) illustrating pericyte loss (green) and endothelial activation (magenta) in ageing.

3.3.5 Vessel segments

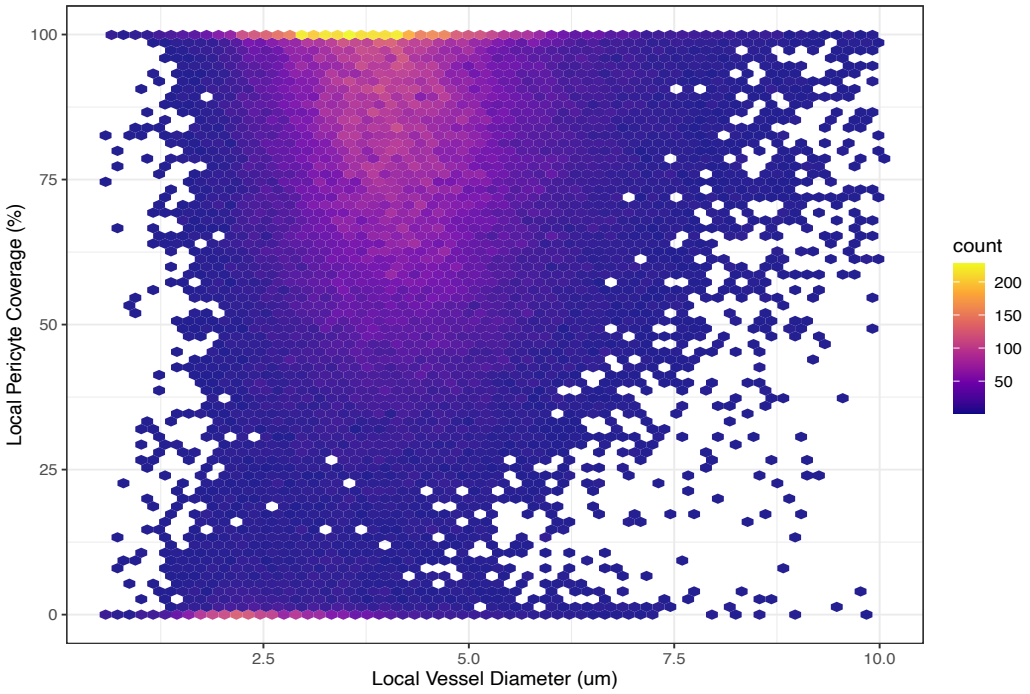
To gain a deeper understanding of the spatial relationship between pericyte coverage and VCAM-1 expression, and to specifically target capillaries rather than large vessels, I segmented each vessel across all brain regions into smaller sections, excluding any segment with a diameter greater than 10 μm[73]. I extracted 93,738 vessel segments in total with a mean length of 12.29 μm (SD=13.93) and a mean diameter of 4.43 μm (SD=1.63).

The relationship between pericyte coverage and age remained after excluding large vessels, with 3-month-old animals having significantly higher pericyte signal intensity in the vessel segments on average (mean: 1,938.07; SD=1,320.15) compared to 12-month-old animals (mean: 1,563.12; SD=876.41) (**Figure 3-12**). This pattern remained evident in all the brain regions examined, with the most pronounced difference being in the CA2/3 area within the hippocampus, where aged animals had 30% less pericyte signal intensity compared to young animals (**Figure 3-13**).

Similarly, I confirmed the presence of higher VCAM-1 coverage in capillaries of aged mice (3-month-old mice mean: 8.75% (SD=17.16); 12-month-old mice mean: 11.33% (SD=20.33)), with the starkest difference evident in the anterior corpus callosum, where aged animals had 6.4% more VCAM-1 capillary coverage on average when compared to young mice (**Figure 3-14, Figure 3-15 & Figure 3-16**).

Results from linear mixed effects models suggest an inverse relationship between log-transformed VCAM-1 coverage and log-transformed pericyte signal intensity in the capillary segments, with a 10% decrease in pericyte signal being associated with an approximate 0.20% increase in VCAM-1 capillary coverage after adjusting for sex and brain region, although this relationship was not statistically significant (β=-0.02, SE=0.01, t=-1.62, p=0.10) (**Table 3-5 & Figure 3-17**).

A. Young (3-months) vessel segments



B. Aged (12-months) vessel segments

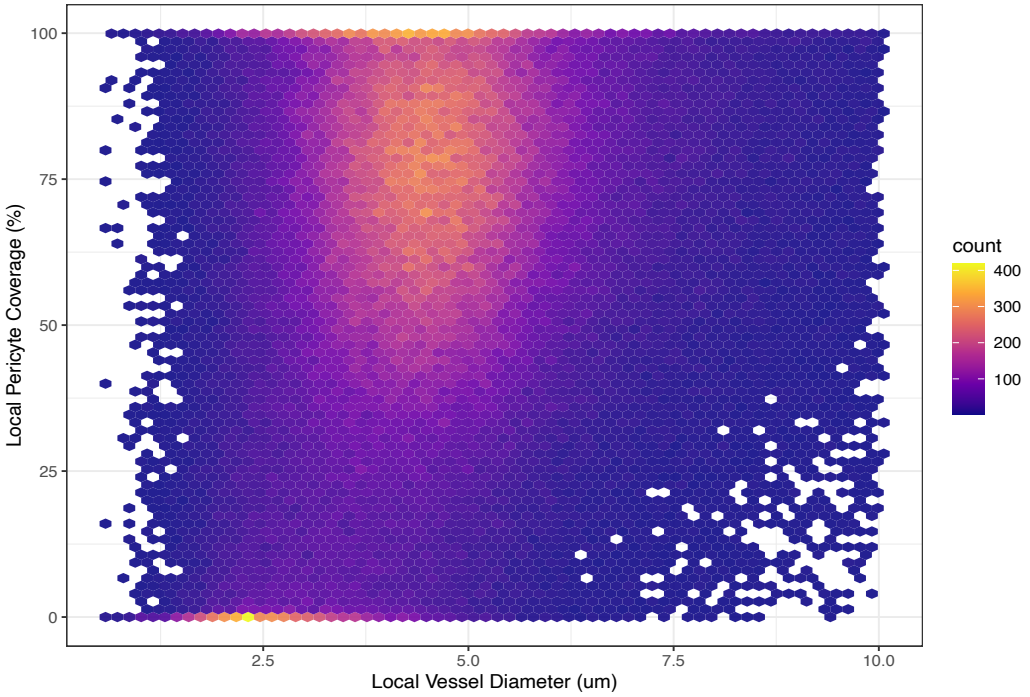


Figure 3-12. Density Hexbin plots from A) young mice (3-months) and B) Aged mice (12-months) showing the relationship between pericyte coverage (%), capillary diameter (µm), and age, in vessel segments extracted from all 8 brain regions of interest.

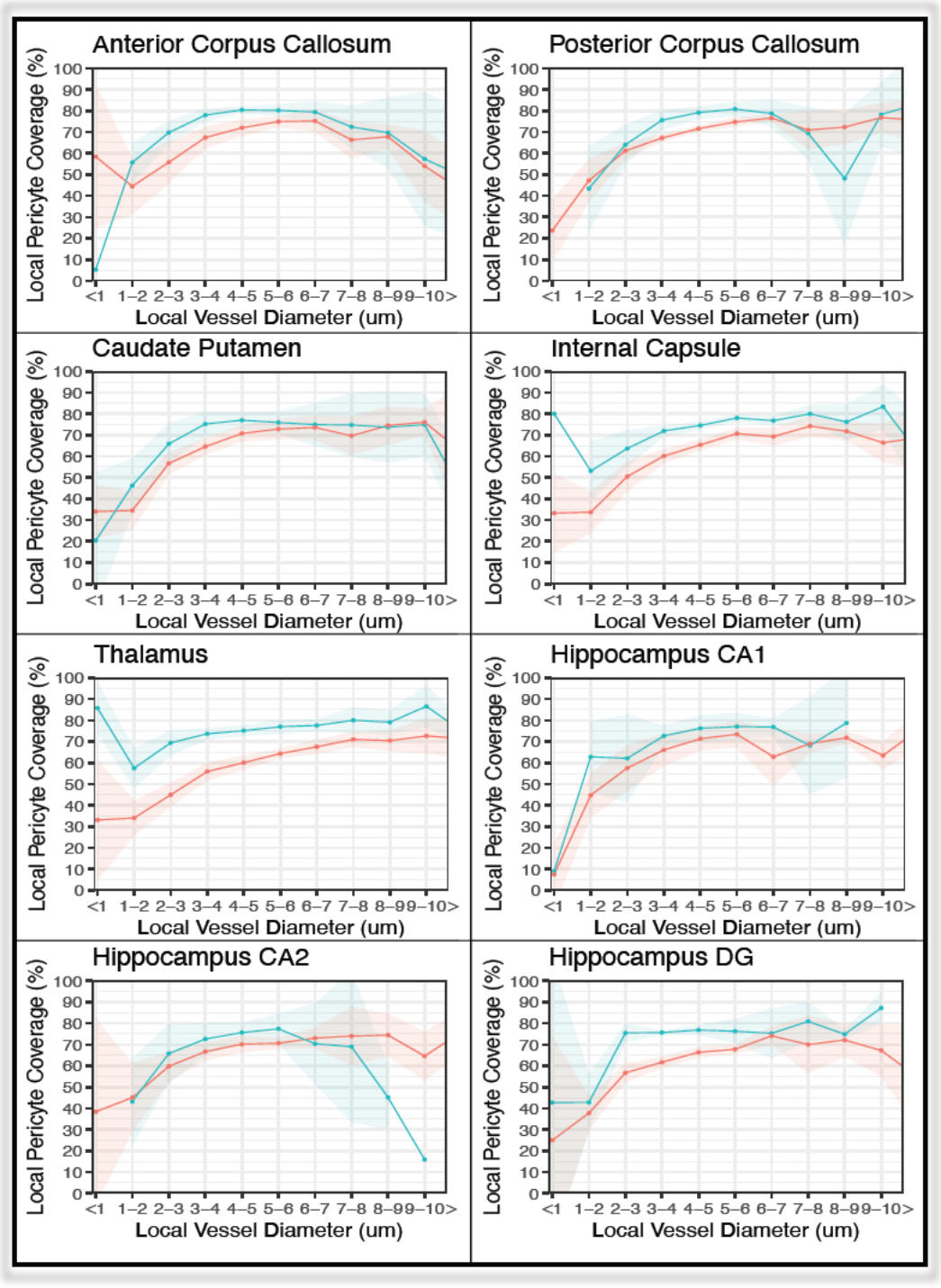
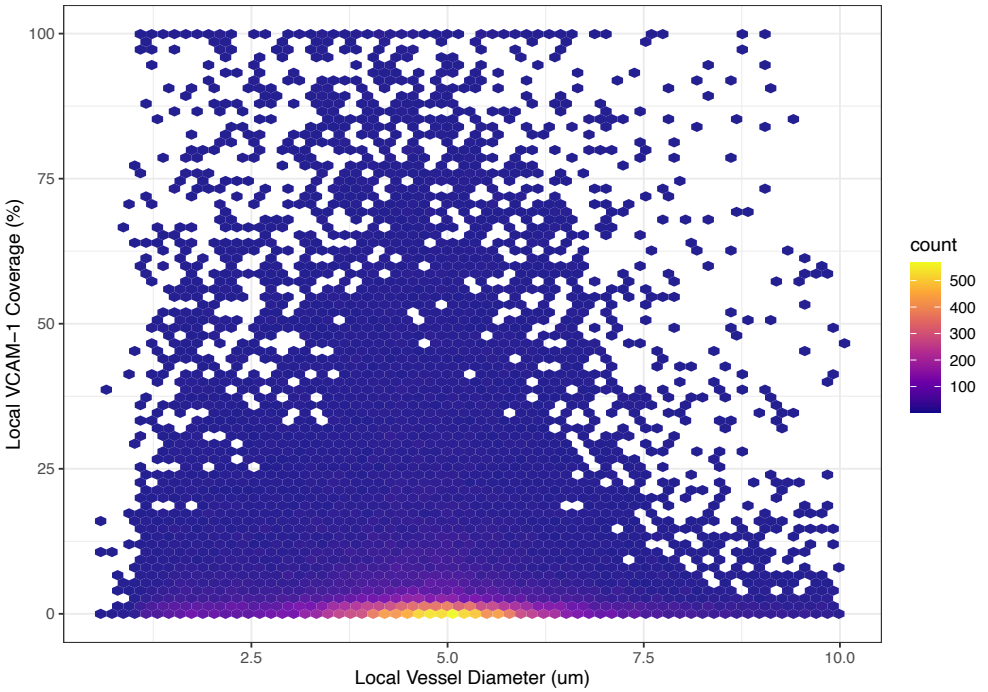


Figure 3-13. Pericyte coverage (%) and capillary vessel diameter (μm) from vessel segments from both young (3-months) and aged (12-months) animals in each of the 8 brain regions observed.

A. Young (3-months) vessel segments



B. Aged (12-months) vessel segments

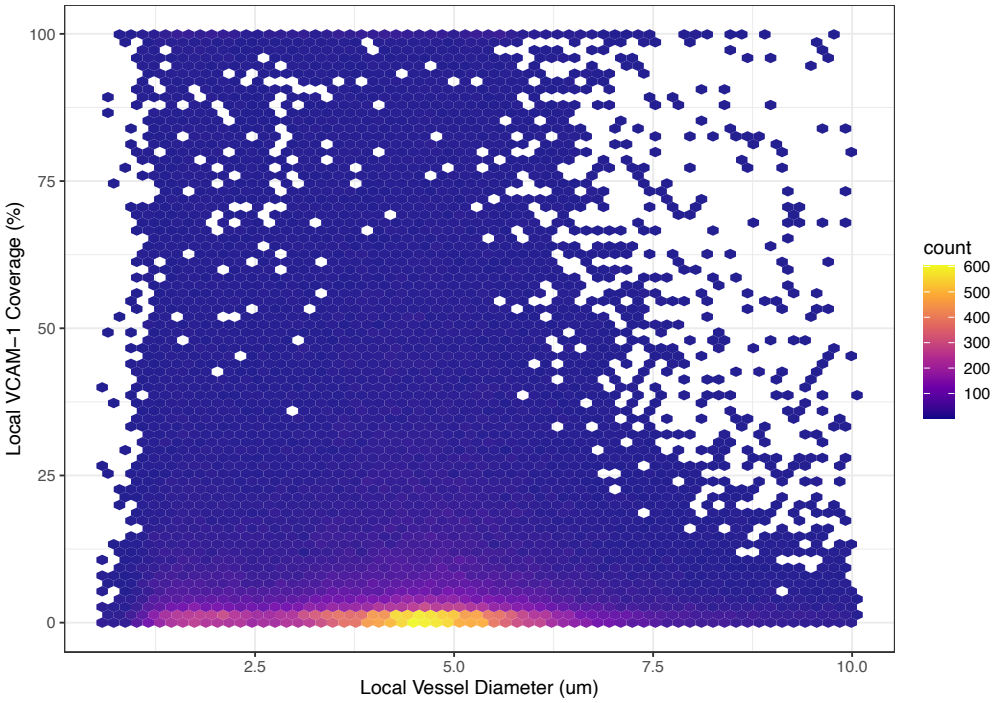


Figure 3-14. Density Hexbin plots from A) young mice (3-months) and B) Aged mice (12-months) showing the relationship between VCAM-1 coverage (%), capillary diameter (μm), and age, in vessel segments extracted from all 8 brain regions of interest.

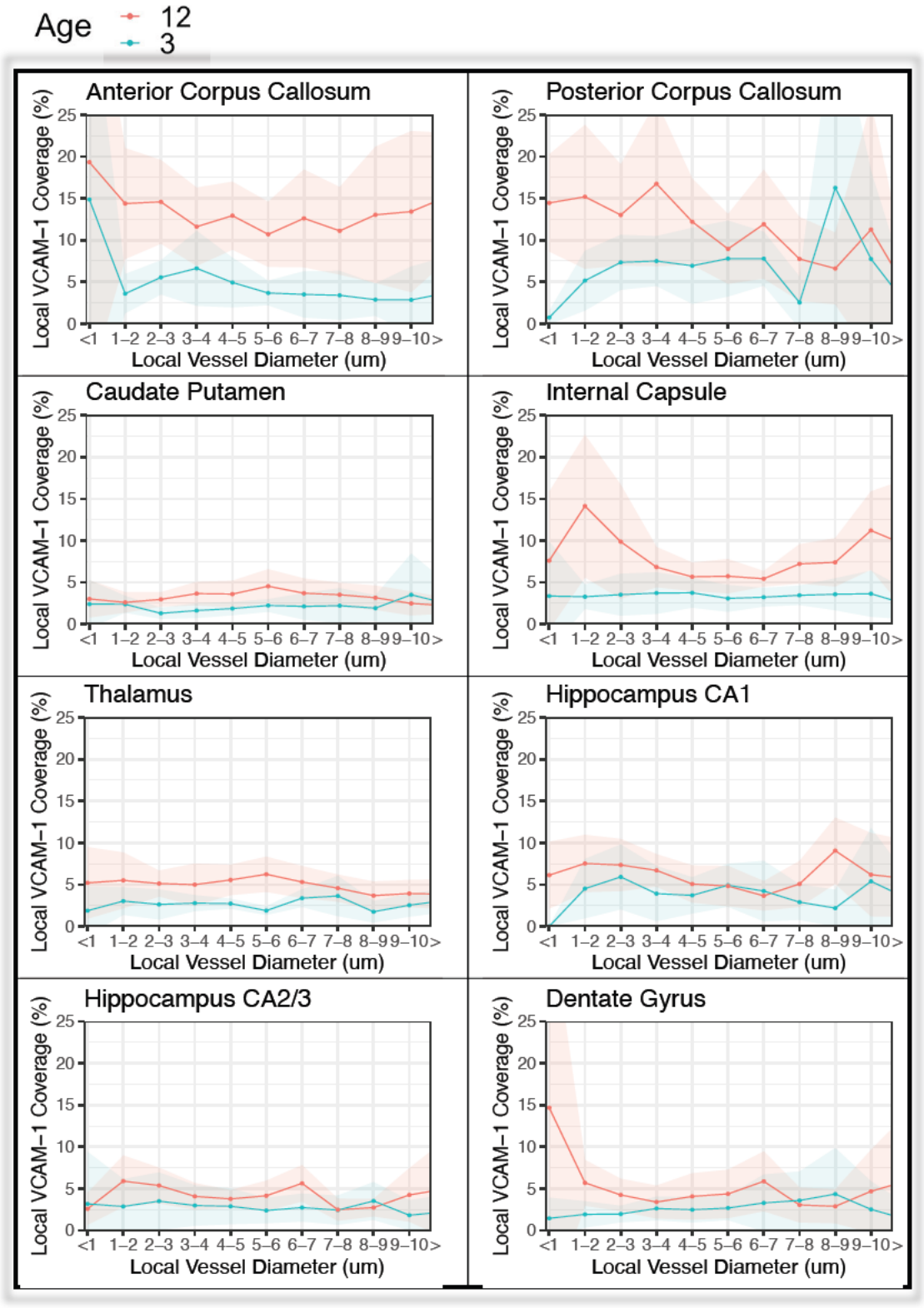


Figure 3-15. VCAM-1 coverage (%) and capillary vessel diameter (μm) taken from individual vessel segments from both young (3-months) and aged (12-months) animals in each of the 8 brain regions observed.

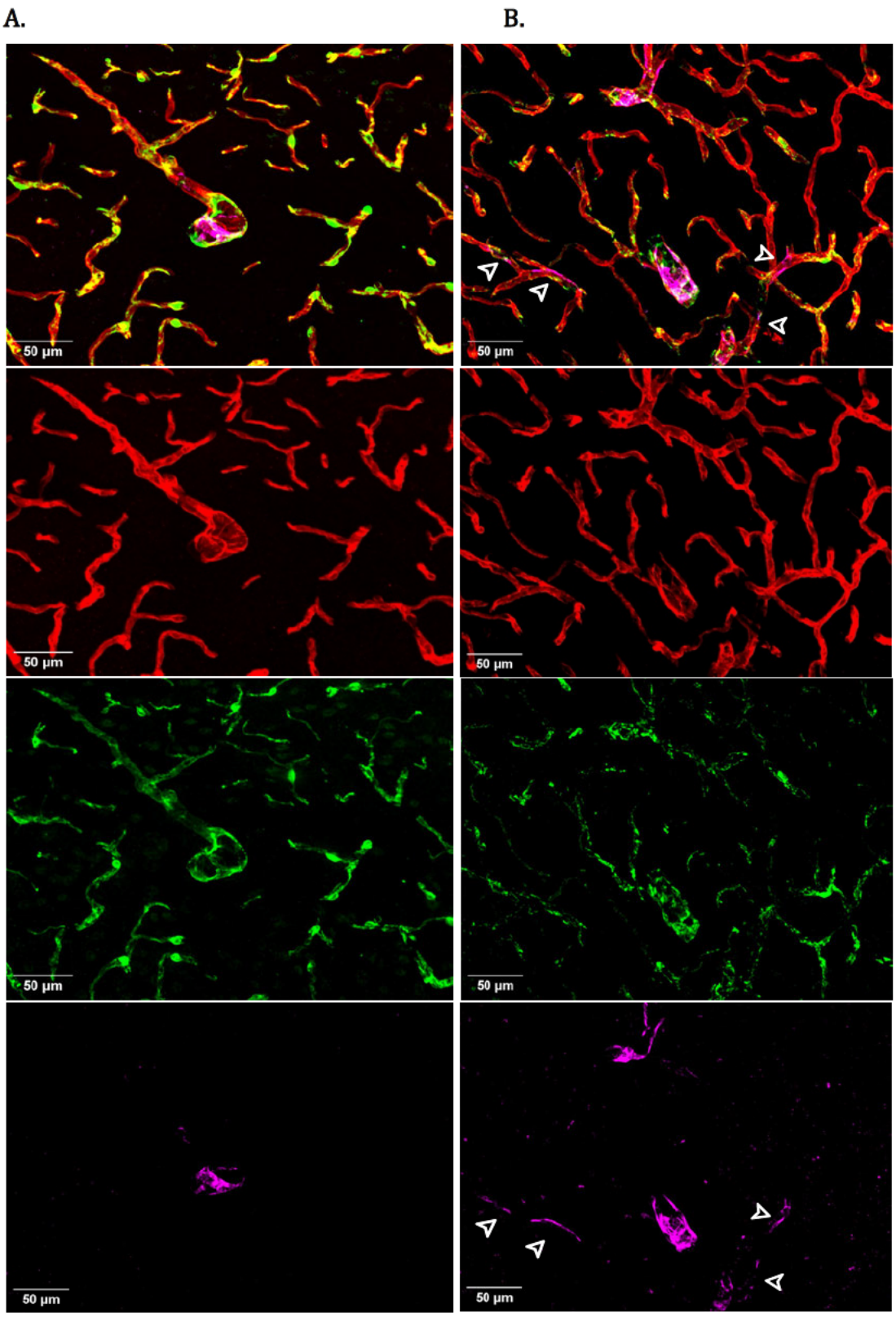


Figure 3-16. (A) Section of the thalamus from a 3-month-old female mouse (B) Section of the thalamus from a 12-month-old female mouse. All images were stained using β-Dystroglycan (vessels, red), CD13 (pericytes, green), and VCAM-1 (endothelial activation, magenta). Reduced pericyte coverage and increased VCAM-1 expression was observed in ageing capillaries (diameter <10 µm; white arrowheads).

Table 3-5. Linear mixed effects model results examining log-transformed VCAM-1 coverage and log-transformed pericyte signal adjusting for brain region and sex. *Abbreviations:* ACC: Anterior Corpus Callosum; PCC: Posterior Corpus Callosum; DG: Dentate Gyrus; CA1: Cornu Ammonis 1 in the Hippocampus; CA2/3: Cornu Ammonis 2 and 3 in the Hippocampus; IC: Internal Capsule; Thal: Thalamus.

Linear Mixed Model Results: VCAM-1 Coverage in Vessel Segments

	Dependent variable:
	VCAM-1 Coverage
Pericyte Signal	-0.019 (-0.039, 0.0003)
Brain Region: ACC	0.940^{***} (0.903, 0.976)
Brain Region: PCC	1.198^{***} (1.159, 1.238)
Brain Region: DG	0.137^{***} (0.100, 0.174)
Brain Region: CA1	0.431^{***} (0.393, 0.469)
Brain Region: CA2/3	0.395^{***} (0.351, 0.440)
Brain Region: IC	0.459^{***} (0.422, 0.496)
Brain Region: Thal	0.362^{***} (0.332, 0.392)
Sex (female)	0.392^{*} (0.038, 0.745)
Constant	0.284 (-0.018, 0.586)
Observations	93,738
Log Likelihood	-184,756.900
Akaike Inf. Crit.	369,537.800
Bayesian Inf. Crit.	369,651.200
Note:	*p<0.1; **p<0.05; ***p<0.01

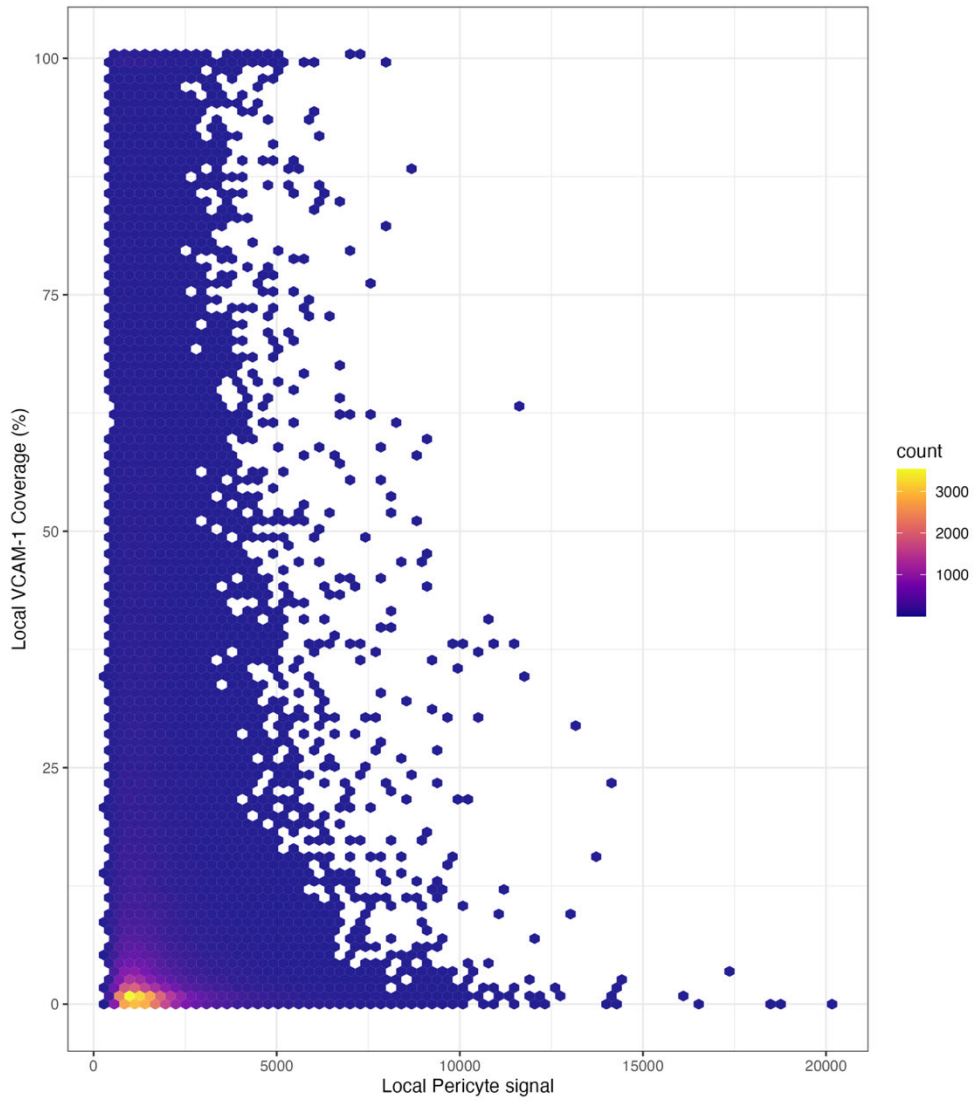


Figure 3-17. Density Hexbin plot showing the relationship between local VCAM-1 coverage (%) and local pericyte signal intensity in vessel segments extracted from all 8 brain regions of interest in young (3-month-old) and aged (12-month-old) mice.

3.4 Conclusions

Results from these investigations provide evidence of pericyte coverage loss and increased endothelial activation in normal ageing in both male and female mice and across a variety of brain regions.

Global pericyte coverage was reduced by an estimated 7.5% in 12-month-old animals compared to their younger counterparts after adjusting for sex and

brain region using linear mixed effects models. Previous studies have demonstrated a reduction in pericyte cell count and capillary pericyte coverage in the context of vascular dementia and AD, although study results can be inconsistent, and some controversy remains[114,124–128]. In our study, whilst all observed brain regions consistently showed reduced pericyte coverage in older animals, the most pronounced difference was in the thalamus. The exact reasons for this are unclear, although it may be related to the thalamus having the highest vessel density amongst the regions studied. Additionally, deep white matter structures could be particularly susceptible to small vessel damage, alterations in blood flow, or hypoxic conditions.

We also found that although vascular density remained comparable between young and aged mice, aged mice had greater vessel diameters on average. These findings mirror those discussed in previous research featuring pericyte-deficient mice, which has demonstrated that whilst endothelial cell numbers remain normal in these animals, they have sparser networks of vessels with increased diameter, possibly through the loss of pericytes' contractile abilities[150].

In the brain's small vessels, pericytes contribute to the overall integrity of the BBB as well as modulate capillary blood-flow, inflammation, and angiogenesis. In the context of SVD, it is thought chronic and acute hypoperfusion can result in pericyte death and decreased pericyte coverage, leading to downstream consequences such as increased BBB permeability, neuronal loss and white matter injury[126,128,329–332]. Reduced pericyte coverage, either through ageing or in animal models of pericyte loss or acute pericyte ablation, has been associated with neurovascular uncoupling[123], fibrinogen leakage and hypoxia[114], impaired blood flow[84,124], neuronal dysfunction[333], increased BBB permeability and disintegration[314], and cognitive decline[127]. Pericyte-deficient mice that grow into adulthood develop cerebrovascular dysfunction[85]. It is presumed that accelerated pericyte loss in ageing, particularly in the presence of cerebrovascular pathology, can contribute to cyclic repercussions which

may further harm pericytes, including BBB impairment and vascular dysfunction, inclusive of endothelial dysfunction.

In our study, VCAM-1 coverage was increased by nearly two-fold in older animals (7.62% versus 3.56% in younger animals); this difference remained statistically significant after adjusting for sex and brain region and was most pronounced in the anterior region of the corpus callosum. In addition, reduced pericyte signal was an independent predictor for increased VCAM-1 expression, with a 10% reduction in pericyte signal being associated with a 0.20% increase in VCAM-1 capillary coverage. The precise mechanism underpinning a potential crosstalk between pericytes and endothelial cells remain unclear. However, previous studies examining transcriptional and functional changes to the endothelium in pericyte-deficient mice have revealed that pericytes have the ability to regulate endothelial cell identity and are essential for the maintenance of various BBB functions inherent in endothelial cells. For instance, capillary endothelial cells lacking in direct pericyte contact tend to adopt a venous-shifted molecular pattern and can exhibit an upregulation of cell-adhesion molecules[150].

The strengths of these investigations involve the inclusion of both male and female animals, the use of the Opera Phenix High-Content Screening System to obtain high-resolution, whole-brain images, which also enables the segmentation and inclusion of entire regions of interest in analyses, and the inclusion of several brain regions. Most importantly, the development and implementation of VasOMatic enables a highly accurate, semi-automated approach to quantify these metrics, including precise vessel segmentation and spatial analyses, which facilitates insight into the relationship between pericyte and VCAM-1 signal.

There are some notable weaknesses in our study. Firstly, it is important to note that senescence in mice is defined as a minimum age of 18 months and extends to their approximate lifespan duration of 24 months; it is at this stage that biomarkers of ageing are prominently detected[334]. On balance and taking into consideration findings from previous research and ethical considerations,

we decided to include mice in their presumed ‘middle age’, although we acknowledge that it is possible, and indeed likely, that the effects of ageing on pericyte loss and endothelial activation might become more apparent as mice continue throughout the ageing process, particularly as there is some evidence suggesting that BBB permeability and endothelial dysfunction in humans may increase at an exponential rate. Based on our results and with the aim of addressing this, we are currently working on replicating these experiments whilst including a group of 18-month-old mice. Additionally, quantifying pericyte and VCAM-1 coverage using immunohistochemistry and semi-automated software may overlook important details about pericyte morphology. By focusing on coverage rather than cell body count, we may not fully capture pericyte structural changes or accurately assess their presence or loss.

Although this work gives a strong indication that these are mechanisms of ageing that might contribute to the eventual breakdown of the BBB, both in normal ageing and in the presence of pathology, this direct causality is beyond the scope of these investigations. We remain focused on addressing these questions by employing adeno-associated virus models which overexpress VCAM-1 as well as pericyte ablation models. To what extent pericyte loss contributes to the functional state of the endothelium and BBB disintegration and vice-versa, and the precise mechanisms underpinning this cyclic dysfunction remain unclear. However, these findings provide a platform to further investigate this phenomenon in humans, and in the next chapter, I delve into the translational relevance of these findings using *postmortem* brain tissue from young, healthy controls and older adults with varying degrees of SVD.

Chapter 4 Examining pericyte and endothelial cell dysfunction in SVD: Insights from *postmortem* human brain tissue analyses

4.1 Introduction

In **Chapter 3**, I highlight results from the ageing mice study, a series of investigations aimed at examining how pericyte coverage and endothelial cell activation change in ageing. Our results suggested that in ageing mice, there is evidence of a significant and consistent reduction in pericyte coverage and an increase in endothelial activation through VCAM-1 overexpression as compared to younger animals. In addition, we found a potential relationship between reduced pericyte coverage and increased VCAM-1 expression within capillary micro-segments.

Here, we have duplicated these investigations using *postmortem* human brain tissue samples to corroborate the murine findings. Our goal is to examine whether we can observe evidence of the same endothelial pro-inflammatory (activated) phenotype concurrent to pericyte loss, this time within the context of varying degrees of SVD severity in humans.

4.1.1 Pericyte loss and endothelial activation in SVD

A comprehensive summary of the available literature pertaining to the role of pericytes and endothelial cell activation in ageing and within the context of SVD is provided in the **Introduction** and in the introduction section of the previous **Chapter 3**. I will briefly recapitulate some important points here.

Pre-clinical and clinical studies have reported pericyte loss and degeneration in ageing and in SVD and AD pathology[53,117–122].These changes have been observed alongside concomitant changes in cerebrovascular function, affecting neurovascular coupling[123], plasticity mechanisms[318], increased BBB permeability[335], and cognitive decline[54]. It is hypothesised that chronic

hypoperfusion can engender pericyte death or decreased capillary coverage, which can trigger downstream consequences including the breakdown of the BBB, neuronal loss and degeneration, and white matter injury[85,126,329,330,332,336]. This loss in pericyte coverage also correlates with increased vascular dysfunction and the activation of the endothelium through the expression of specific adhesion molecules, such as VCAM-1.

A recent study of 124 *postmortem* brain tissue samples reported a significant 35-45% reduction in frontal lobe white matter capillary pericytes in vascular dementia, mixed dementia, and AD cases compared to aged controls and dementia-free post-stroke cases[335]. The same authors reported a 45% decrease in capillary pericyte numbers in the cortex of aged controls (>78 years) in comparison to young controls (56-65 years)[314]. Similarly, pericyte density was reduced between 35-40% in post-stroke dementia, vascular dementia, and AD cases compared to controls in the CA1 region of the hippocampus, an area with known pathological changes in ageing dementias[337]. However, CA1 capillary pericytes were also lost in a group of cognitively-normal stroke survivors[337].

However, pericyte loss and endothelial activation in the context of ageing and cerebrovascular disease remain controversial and *postmortem* data on structural vascular abnormalities featuring pericyte and the capillary endothelium are scarce. The aforementioned study which reported a reduction in white matter frontal lobe pericytes in vascular dementias, also reported finding preserved or even increased pericyte numbers in the frontal cerebral cortex amongst vascular, mixed, or post-stroke dementia cases and healthy controls in a separate publication, claiming a differential regulation of capillary pericytes in the frontal lobe between the cortex and white matter in ageing-related dementias[314]. Preserved pericyte populations have also been reported in the frontal cortex of AD cases[323]. Another study featuring 49 AD brain samples and 37 control cases acknowledged a marked decrease in PDGFRβ and increased fibrinogen -indicative of increased BBB permeability-, in the precuneus, though not in the underlying white matter[114]. Data from one

neuropathologic study suggested that endothelial activation in the deep penetrating arteries is not associated with SVD, as ICAM-1 was rarely detected in pathologically diagnosed SVD cases, nor in aged controls without SVD[195]. These results highlight the discrepancies in existing literature, many of which stem from a lack of pathological SVD grading standardisation, sampling heterogeneity, and other study design variables, including brain region studied and chosen outcome disease markers.

Here, we aim to understand the link between molecules involved in endothelial cell activation and pericyte loss/function and to corroborate findings from our animal model investigations. We also endeavour to address some of the aforementioned inconsistencies by utilising novel ways of quantifying pericyte and VCAM-1 coverage with multispectral imaging, incorporating varying degrees of disease severity, and examining both white and grey matter areas. Findings from these investigations could shed light on dementia disease pathology and potentially serve as a strong translational tool with which to corroborate findings from our ongoing animal and cell investigations.

4.2 Methods

4.2.1 Design and subjects

We processed a tissue request through the UK Brain Banks Network (UKBBN) [338], which was granted in January of 2023 (**Appendix C**). We requested brain tissue from healthy control cases and cases with varying degrees of SVD pathology, including 'mild', 'moderate', and 'severe'.

Tissue originated from any of the following three large research initiatives and studies in Scotland: the Lothian Birth Cohort (LBC)[339], the Lothian study of Intracerebral Haemorrhage, Pathology, Imaging and Neurological Outcome (LINCHPIN)[340], and the Edinburgh Sudden Death Brain Bank[341]. Detailed protocols, including brain tissue donation and processing, patient consent, and clinical data collection have been described in the citations provided. In brief, the LBC is a large, longitudinal community cohort study comprising of surviving

members of the Scottish Mental Survey of 1947 residing in Midlothian. Ethics permission for the study protocol was obtained from the Multi-Centre Research Ethics Committee for Scotland MREC/01/0/56, the Lothian Research Ethics Committee LREC/2003/2/29 and Scotland A Research Ethics Committee 07/MRE00/58, REC REF AM17, 07/MRE00/58/AM14. LINCHPIN is a prospective community-based study examining the causes of intracerebral haemorrhage using research autopsy in case of death. The Scotland A Research Ethics approved this study (10/MRE00/23). The Edinburgh Sudden Death Brain Bank is funded by the UK Medical Research Council and was established in 2003 as a means to increase access to younger control cases. The Procurator Fiscal and Forensic Pathology Department identify suitable cases of presumed healthy brains; the majority of sudden death cases are sudden cardiac deaths. The Medical Research Council Edinburgh Brain Bank functions within the Human Tissue (Scotland) Act of 2006 and has full ethical approval for the use of brain tissue in research (East of Scotland Research Ethics Service, ref 16/ES/0084). Informed consent is obtained from all participants in the LINCHPIN and LBC studies and post-mortem authorisation is obtained from next of kin in all cases. Exclusion criteria for cases included >5 days from time of death to autopsy and known diagnosis of neurological or neurodegenerative disease.

4.2.2 Tissue

We obtained one Formalin-fixed Paraffin-embedded section per sample (thickness: 4μm) from cortical tissue including both grey and white matter borderzone areas within the primary motor cortex/precentral gyrus, in Brodmann's area 4. Paraffin embedded blocks of brain tissue were fixed using unbuffered 10% Formalin for 48-72 hours prior to histological processing.

4.2.3 Clinical Information and post-mortem neuropathological examination

A UKBBN-designated research nurse amalgamated relevant clinical information, demographics, and medical history using routinely collected study

data in conjunction with central medical records when necessary. This included participant age, sex, known previous diagnoses for hypertension, diabetes, hypercholesterolaemia, heart disease, or atrial fibrillation (AF), previous smoking history, confirmed cause of death, post-mortem interval, and any relevant past-medical history of note, including, but not limited to: chronic disease, malignancy, stroke, inflammatory or rheumatologic disease, psychiatric illness, or cardiovascular disease.

According to Brain Bank protocols (unpublished), standard neuropathological examination was carried out by at least one experienced neuropathologist masked to clinical, radiographic and genetic features. Details on dissection protocols are published[342]. A highly experienced neuropathologist assessed tissue for mild/moderate/severe grades of SVD burden based on the presence and severity of wall sclerosis (the deposition of collagen with loss of smooth muscle), increased vessel wall thickness and reduced lumen diameter, enlarged perivascular space, perivascular haemosiderin, white matter rarefaction, lacunes, and microhaemorrhages, based on the validated vascular cognitive impairment neuropathology guidelines (VCING)[342,343]. Non-amyloid as well as amyloid-beta cerebral angiopathy (Aβ-CAA) grade and white matter (WM) pathology severity was also recorded, as well as any other abnormalities of note.

4.2.4 Immunohistochemistry

Immunohistochemistry assays were undertaken using the fully automated research stainer, Bond RX (Leica Biosystems). Samples in slides were covered with BOND Universal Covertile (Leica Biosystems, S21.4611) and placed in the machine along with a Polymer Detection Kit (Leica Biosystems, DS9800), a HRP PLEX kit (Leica Biosystems, DS9914), which includes a base platform of peroxide block, post primary, polymer reagent, DAB chromogen and haematoxylin counterstain, as well as the required chromogens, green (Leica Biosystems, DC9913) and red (Leica Biosystems, DS9390). The following primary antibodies were used: for the endothelium, CD31 (abcam,

ab134168; 1:250 dilution; Bond Epitope Retrieval Solution 1 (Leica Biosystems, AR9961) exposure for 20 minutes), for pericytes, PDGFRβ (abcam, ab69506; 1:250 dilution; Bond Epitope Retrieval Solution 1 exposure for 20 minutes), and for endothelial activation, VCAM-1 (abcam, ab134047; 1:500 dilution; Bond Epitope Retrieval Solution 1 exposure for 30 minutes). 150 μL of antibody reagents diluted in Leica diluent (Leica biosystems, AR9352) were added per slide before allowing the machine to run the sequential multiplex staining assay.

4.2.5 Multispectral Imaging and Microscopy

Multispectral imaging, microscopy, and all imaging protocols were led by Dr Justyna Cholewa-Waclaw and operated by her or myself. We used the Vectra Polaris Automated Quantitative Pathology Imaging System (Akoya Biosciences), which facilitates high speed digital whole-slide scanning and multispectral imaging to detect and quantify multiple overlapping biomarkers within a tissue section. For every individual case, we acquired eight 929.74 x 697.31 μm brightfield images at 20X from randomly segmented borderzone areas, half in the white matter and half in the grey matter (**Figure 4-1**).

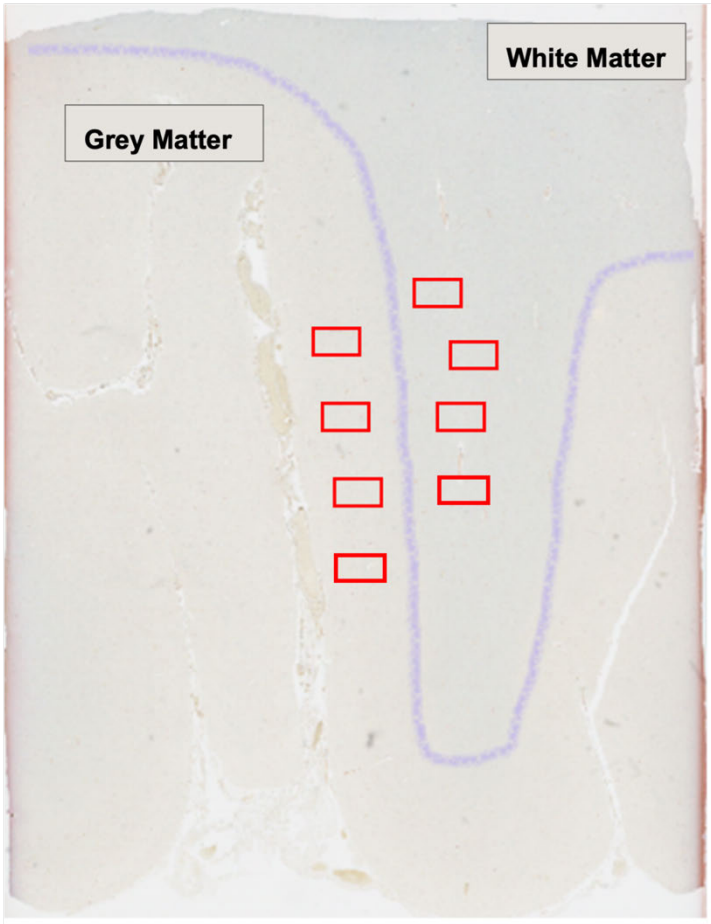


Figure 4-1. Visual representation of the segmentation of eight 929.74 x 697.31 µm images per tissue slice in borderzone grey and white matter areas (border between white and grey matter manually added for visual representation purposes).

Some limitations of the brightfield microscopy traditionally used in *postmortem* pathological analyses include low contrast, a low optical resolution due to the limitation of light's wavelength, and the need for manual and often imprecise image quantification protocols which rely on the visual extraction of data from images. To maximise the accuracy and precision of the data and to capture cellular interactions without the potential interference of autofluorescence, we performed an unmixing multispectral imaging protocol. This allows for the unmixing of multiple immunohistochemically stained tissue samples for the quantification of individual staining components by creating fluorescent-like composite images from the original brightfield images (**Figure 4-2**). Further details on the rationale and protocol can be found here[344]. Briefly, we unmixed

the brightfield images by engaging the Liquid Crystal Tuneable Filter within the Polaris system, which acts as a bandpass filter that can be tuned to step in the desired nanometre range (420-800nm). We used the spectral libraries, custom-captured, to map the spectral signal of stains at a given wavelength. If more than one dye is present on the slide/pixel we were thus able to separate out these dyes owing to their spectral signatures and contribution to the total signal. This was performed using our in-house linear unmixing algorithm. In some cases, we found this protocol produced erroneous results by confounding the haematoxylin and green chromogen (VCAM-1) and classifying both as green. As haematoxylin was not required for data quantification, we used FIJI's Image Calculator feature to eliminate instances of signal overlap in these cases.

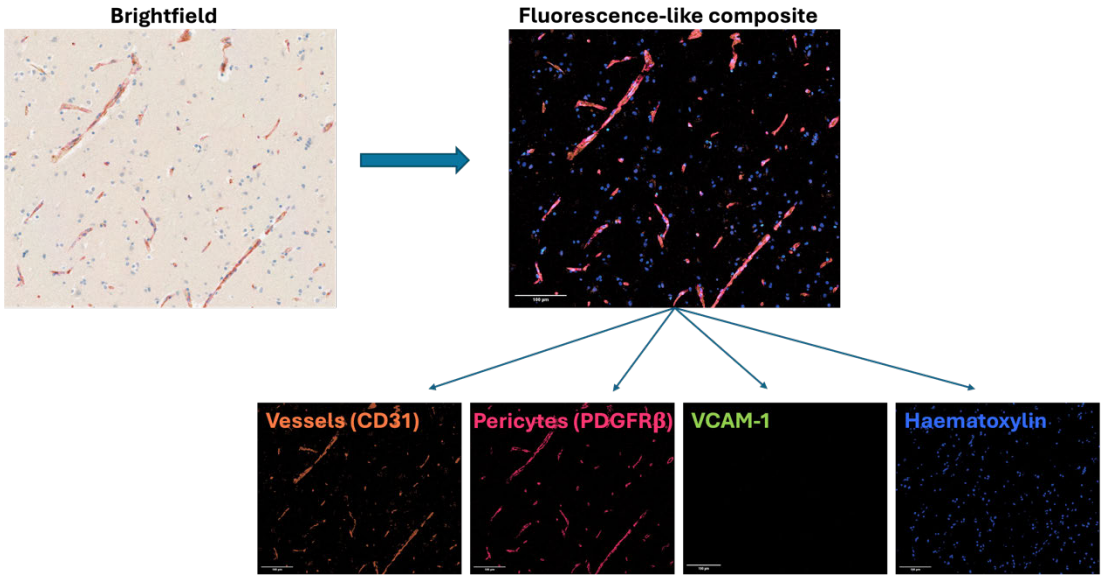


Figure 4-2. Visual representation of the unmixing of brightfield images into fluorescent-like composite images using multispectral imaging and the Vectra Polaris Automated Quantitative Pathology Imaging System for accurate data quantification.

4.2.6 Image Quantification

We used our in-house developed software, VasOMatic, to assess the relationship between vascular changes, pericyte coverage, and endothelial cell activation as they relate to varying degrees of SVD pathology[328]. All image

quantification was performed using the VasOMatic Macro in FIJI (FIJI is Just ImageJ, 10.1038/nmeth.2019), which was developed in-house by Dr Audrey Chagnot in conjunction with this study[328]. Using the macro and blinded to case ID and all clinical information, including patient age and sex, I segmented the vasculature using median smoothing, particle analysis, and manual thresholding to refine the mask. This mask served as an overlying template to then segment the paravascular staining of interest, in this case pericyte and VCAM-1 signal overlying the vasculature (**Figure 4-2 & Figure 4-3**).

Subsequently, the macro employs an algorithm to divide the vascular mask into segments using FIJI's watershed function. This enabled the collection of spatial metrics for each segment, including vessel length, diameter, and paravascular signals (pericyte and VCAM-1). These metrics are gathered using skeleton measurement, distance mapping, and mask overlap, respectively. Signal intensity, rather than vascular coverage (%) is used in the vascular micro-segments to capture subtle changes in paravascular signal change.

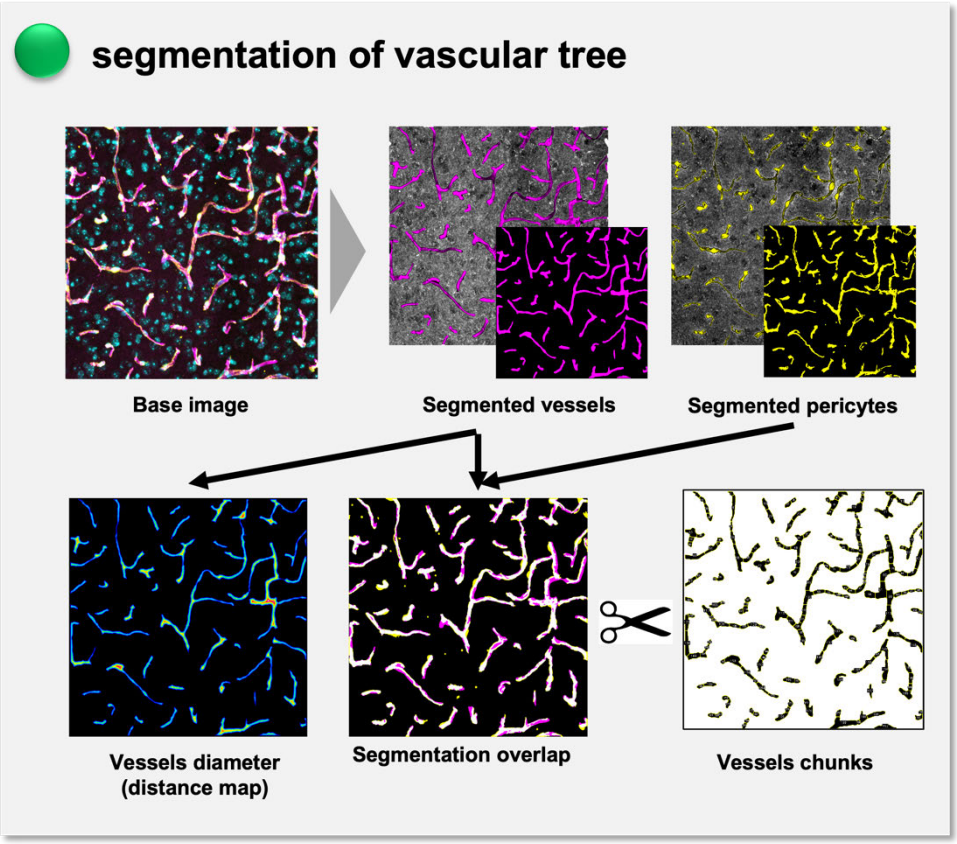


Figure 4-3. VasOMatic[328] FIJI Macro demonstration (developed by Dr Audrey Chagnot, Montagne Lab, University of Edinburgh, 2023). Example of the segmentation of the vascular tree and masks (vasculature, pericytes, and VCAM-1) as well as a visual representation of the vessel segment truncation process.

4.2.7 Statistical Analyses

All statistical analyses and respective figures were performed using R (RStudio 4.3.2), unless otherwise specified. P-values of <0.05 were considered statistically significant.

Linear mixed effects models were performed using the R 'lme4' package, with random effects accounting for variance between samples from individual participants and were adjusted for key factors such as sex, vascular risk factors, and white versus grey brain matter. To reduce the number of predictors and avoid model over-fitting, we assigned equal weight to hypertension, diabetes, hypercholesterolaemia, and smoking status (previous known history of smoking) in the creation of a vascular risk factor composite score. Age was

not adjusted for in these models to avoid collinearity. However, models were adjusted for age in analyses examining the relationship between PDGFRβ and VCAM-1 signal in the vascular micro-segments, as disease status was not considered there. In these analyses, VCAM-1 and pericyte signal were log-transformed to improve model fit and to establish normality of residuals. All models were assessed for assumptions and fit by checking the normality of residuals, heteroscedasticity, and collinearity using the 'DHARMa' and 'performance' packages. To investigate the spatial relationship between pericyte coverage and VCAM-1 expression, and to specifically target capillaries rather than large vessels, we further segmented each vessel across into smaller sections, excluding any segment with a diameter greater than 10 μm[73].

4.3 Results

We quantified pericyte and VCAM-1 coverage in 15 brain tissue samples, including 4 young, healthy control cases (mean age: 33 years (SD: 9.3), 2 females), 3 mild SVD cases (mean age: 66 years (SD:20), 1 female), 3 moderate SVD cases (mean age: 80 years (SD: 6.2), 1 female) and 5 severe SVD cases (mean age: 83 (SD: 8.8), 1 female). We quantified 120 images in total, 8 per case, half in borderzone white matter areas and half in borderzone grey matter areas. Whilst we extracted pericyte coverage from all images, 7 images were excluded from VCAM-1 analyses due to unreliable threshold masks stemming from high background noise due to pseudofluorescence. Of the excluded images, 4 were from healthy control cases, 2 from severe SVD cases and 1 from a moderate SVD case, and the vast majority (6 out of 7) were images from white matter areas.

Clinical data, including the presence of vascular risk factors and cause of death for each individual case can be found on **Table** C- 1 & **Table C-2** in supplementary materials **Appendix C**. None of the healthy controls had a known hypertension diagnosis, compared to 33% of those with mild SVD, 67% of those with moderate SVD, and 100% of severe SVD cases. One mild SVD

case and two moderate SVD cases had a known diabetes diagnosis. One mild and one severe SVD case had a hypercholesterolaemia diagnosis. In addition, one healthy control, two moderate SVD cases and one severe SVD case had known heart disease, and one mild SVD case and one moderate SVD case had a known atrial fibrillation diagnosis. Smoking history was unknown for one mild SVD case as well as three severe SVD cases. Otherwise, two healthy controls, one mild SVD case and three moderate SVD cases had a history of smoking. Cohort characteristics are summarised in **Table 4-1**.

Table 4-1. Sample Characteristics: healthy control, mild SVD, moderate SVD and severe SVD cases.
Abbreviations: **SVD: small vessel disease; VCAM-1: vascular cell adhesion molecule 1.**

	healthy control (N=4)	mild SVD (N=3)	moderate SVD (N=3)	severe SVD (N=5)	overall (N=15)
Sex, female, n (%)	2 (50 %)	1 (33 %)	1 (33 %)	1 (20 %)	5 (33 %)
Age, years, mean (SD)	33 (± 9.3)	66 (± 20)	80 (± 6.2)	83 (± 8.8)	66 (± 24)
Hypertension, yes, n (%)	0 (0 %)	1 (33 %)	2 (67 %)	5 (100 %)	8 (53 %)
Diabetes, yes, n (%)	0 (0 %)	1 (33 %)	2 (67 %)	0 (0 %)	3 (20 %)
Hypercholesterolaemia, yes, n (%)	0 (0 %)	1 (33 %)	0 (0 %)	1 (20 %)	2 (13 %)
Smoker, yes, n (%)	1 (25 %)	1 (33 %)	0 (0 %)	0 (0 %)	2 (13 %)
Risk Factor Composite Score, mean (SD)	0.25 (± 0.50)	1.0 (± 0)	1.3 (± 0.58)	1.0 (± 0)	0.82 (± 0.60)
Heart Disease, yes, n (%)	1 (25 %)	0 (0 %)	2 (67 %)	1 (20 %)	4 (27 %)
Atrial Fibrillation, yes (n%)	0 (0 %)	1 (33 %)	1 (33 %)	0 (0 %)	2 (13 %)
Post-mortem Interval, minutes, mean (SD)	100 (± 22)	79 (± 50)	44 (± 37)	61 (± 37)	72 (± 39)
Vessel Diameter, um, mean (SD)	2.1 (± 0.65)	1.9 (± 0.32)	1.9 (± 0.29)	2.2 (± 0.37)	2.1 (± 0.43)
Vessel Density, mm/mm3, mean (SD)	520 (± 38)	720 (± 170)	810 (± 79)	910 (± 260)	750 (± 220)
Pericyte Coverage, %, mean (SD)	34 (± 5.9)	25 (± 15)	5.9 (± 2.1)	7.2 (± 3.2)	18 (± 14)
VCAM-1 Coverage, %, mean (SD)	1.7 (± 0.80)	1.9 (± 0.54)	2.8 (± 0.96)	2.6 (± 0.51)	2.2 (± 0.77)

4.3.1 Vessel Density

Vessel density was lowest in young, healthy controls (mean: 520 mm/mm^3 (SD:38)), followed by those with mild SVD (mean: 720 mm/mm^3 (SD:170)), moderate SVD (mean: 810 mm/mm^3 (SD:79)), and severe SVD (mean: 910 mm/mm^3 (SD:260)), respectively (**Table 4-1 & Figure 4-4**). Results from linear mixed models adjusting for sex, brain matter type (grey versus white) and vascular risk factors, revealed that compared to healthy controls, vessel density was an estimated 187.65 mm/mm^3 greater in those with mild SVD (β=187.65, SE=208.13, *t*=0.90), 384.23 mm/mm^3 greater in those with moderate SVD (β=384.23, SE=232.01, *t*=0.76), and 540.42 mm/mm^3 greater in those with severe SVD (β=540.42, SE=207.30, *t*=2.61), with only the latter relationship having reached statistical significance (*p*=0.40, *p*=0.15, and *p*=0.04, respectively) (**Table 4-2**). A likelihood-ratio test indicated that the model including disease status and severity did not provide a better fit than a model without it ($X^2(1)$= 3.93, *p*=0.14).

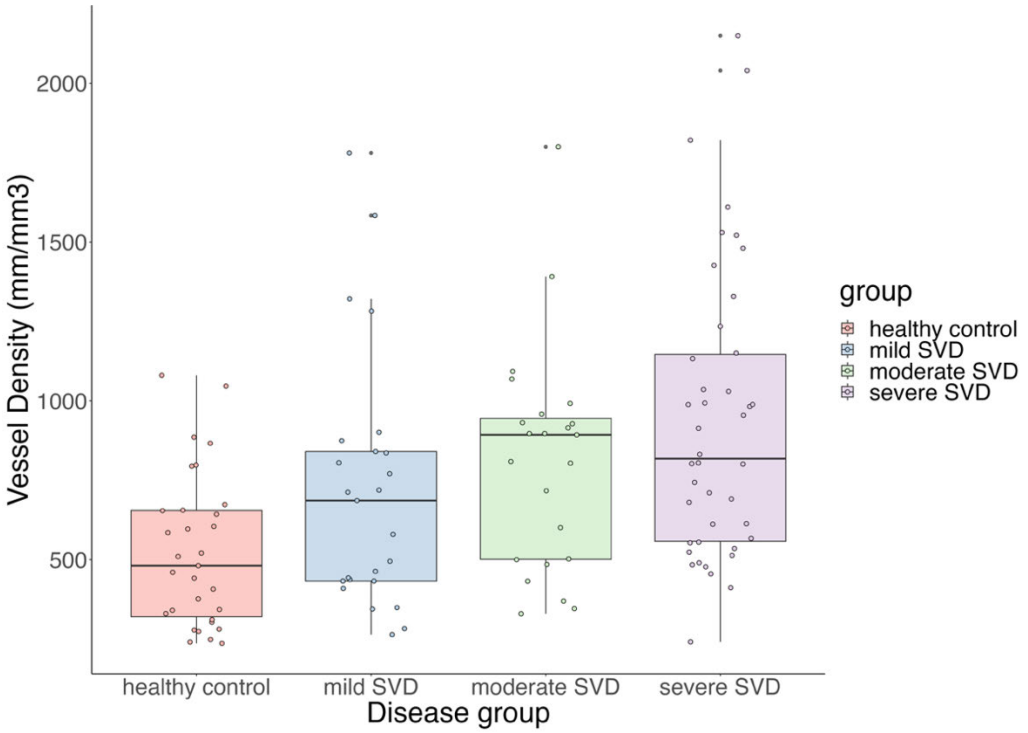


Figure 4-4. Average vessel density (mm /mm3) in healthy control, mild SVD, moderate SVD, and severe SVD cases. ***Abbreviations:*** **SVD: small vessel disease.**

Table 4-2. Linear mixed model results examining vessel density (mm/mm³) and SVD status adjusting for sex, risk factor score, brain matter type (grey versus white) and random effects stemming from repeated measures/images from each individual case. *Abbreviations:* SVD: small vessel disease.

Linear Mixed Model Results: Vessel Density and SVD

	Dependent variable:
	Vessel Density
group: Mild SVD	187.649 (-154.700, 529.998)
group: Moderate SVD	384.238* (2.502, 765.974)
group: Severe SVD	540.422*** (199.429, 881.415)
Sex (male)	132.804 (-76.370, 341.977)
Risk factor score	-107.236 (-394.717, 180.244)
Brain matter: white	-276.776*** (-379.810, -173.742)
Constant	627.665*** (435.305, 820.026)
Observations	89
Log Likelihood	-595.788
Akaike Inf. Crit.	1,209.577
Bayesian Inf. Crit.	1,231.975
Note:	*p<0.1; **p<0.05; ***p<0.01

4.3.2 Vessel Diameter

Vessel diameter was only modestly higher in those with severe SVD (mean: 2.2 μm (SD:0.37), followed by healthy controls (mean: 2.1 μm (SD:0.65), and those with mild SVD (mean: 1.9 μm (SD:0.32) and moderate SVD (mean: 1.9 μm (SD:0.29) (**Table 4-1** & **Figure 4-5**). Results from linear mixed models adjusted for sex, brain matter type and vascular risk factors, demonstrate these differences are not statistically significant (**Table 4-3**). Likelihood-ratio test results reinforced the notion that disease group is not a strong predictor for vessel diameter ($X^2(1)= 2.44$, $p=0.29$).

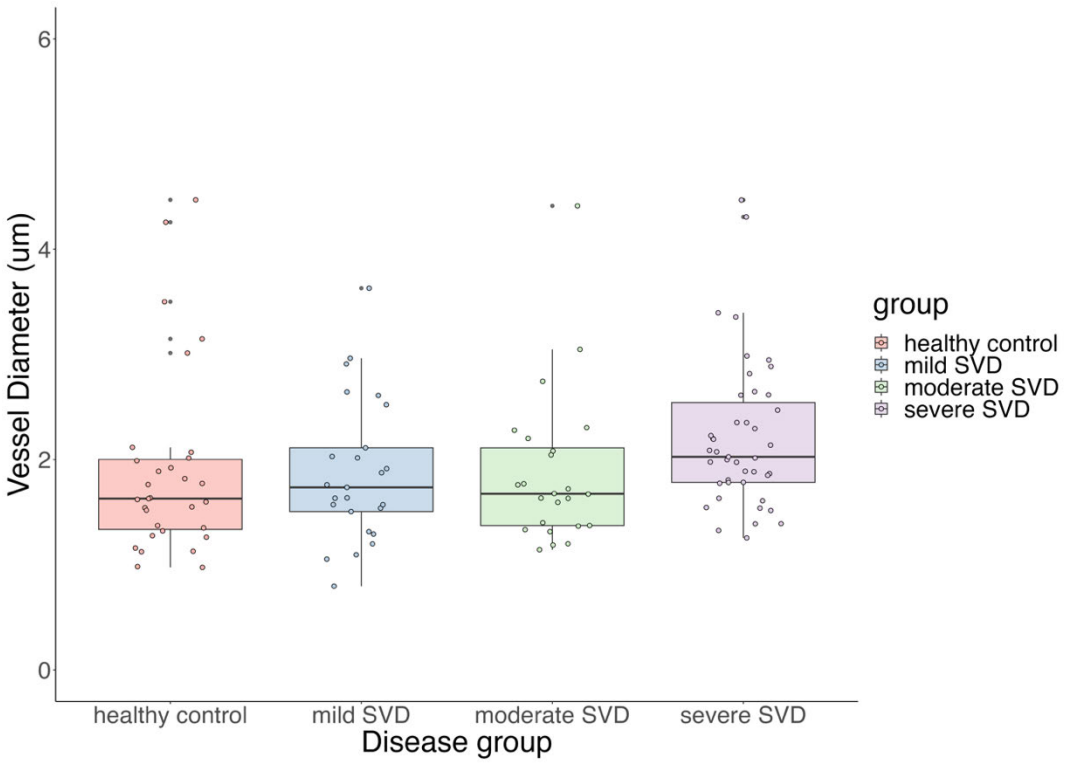


Figure 4-5. Average vessel diameter (μm) in healthy control, mild SVD, moderate SVD, and severe SVD cases. *Abbreviations:* SVD: small vessel disease.

Table 4-3. Linear mixed model results examining vessel diameter (um) and SVD status adjusting for sex, risk factor score, brain matter type (grey versus white) and random effects stemming from repeated measures/images from each individual case. *Abbreviations:* SVD: small vessel disease.

Linear Mixed Model Results: Vessel Diameter and SVD

	Dependent variable:
	Vessel Diameter
group: Mild SVD	-0.058 (-0.861, 0.745)
group: Moderate SVD	0.202 (-0.696, 1.100)
group: Severe SVD	0.591 (-0.205, 1.386)
Sex (male)	0.478 (-0.011, 0.967)
Risk factor score	-0.511 (-1.189, 0.166)
Brain matter: white	0.831^{***} (0.477, 1.185)
Constant	1.616^{***} (1.151, 2.082)
Observations	91
Log Likelihood	-132.648
Akaike Inf. Crit.	283.295
Bayesian Inf. Crit.	305.893
Note:	*p<0.1; **p<0.05; ***p<0.01

4.3.3 Pericyte Coverage

Pericyte coverage was lowest on average in moderate SVD (mean: 5.9% (SD:2.1)) and severe SVD cases (mean: 7.2% (SD: 3.2)) compared to those with a mild disease burden (mean: 25% (SD:15)) and young, healthy controls (mean: 34% (SD: 5.9)) (**Table 4-1** & **Figure 4-6**). Findings from linear mixed effects models indicated that compared to controls, moderate and severe SVD cases displayed significant reductions of 86.28% and 83% in pericyte coverage, respectively, after accounting for sex, brain matter type, and the presence of vascular risk factors (β=-1.98, SE=0.32, *t*=-6.08, *p=0.001*; β=-1.77, SE=0.29, *t*=-6.04, *p=0.001*, respectively) (**Table 4-4**). Mild SVD cases had an average reduction in pericyte coverage of 9.39% compared to young, healthy controls, although this relationship was not statistically significant (β=-

0.09, SE=0.29, *t*=-0.33, *p=0.75)*. Results from likelihood-ratio tests indicate that disease status substantially improved model fit ($X^2(1)$= 31.17, *p*<0.001). Interestingly, white matter areas had an approximate additional 21.44% reduction in pericyte coverage compared to grey matter areas independent of disease group, sex, and vascular risk factors (β=-0.24, SE=0.07, *t*=-3.31, *p=0.001*).

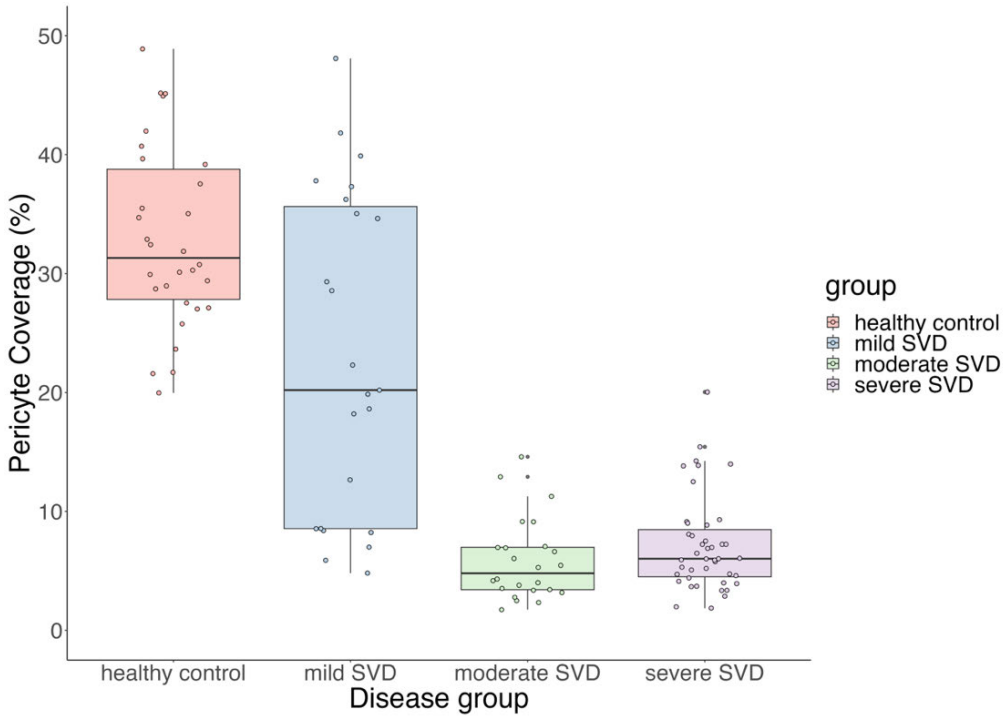


Figure 4-6. Average pericyte coverage (%) in healthy control, mild SVD, moderate SVD, and severe SVD cases. ***Abbreviations:*** **SVD: small vessel disease.**

Table 4-4. Linear mixed model results examining pericyte coverage (%) and SVD status adjusting for sex, risk factor score, brain matter type (grey versus white) and random effects stemming from repeated measures/images from each individual case. *Abbreviations:* SVD: small vessel disease.

Linear Mixed Model Results: Pericyte Coversge and SVD

	Dependent variable:
	Pericyte Coverage
group: Mild SVD	-0.099 (-0.582, 0.385)
group: Moderate SVD	-1.987*** (-2.524, -1.450)
group: Severe SVD	-1.772*** (-2.254, -1.290)
Sex (male)	0.133 (-0.162, 0.428)
Risk factor score	0.072 (-0.333, 0.478)
Brain matter: white	-0.241*** (-0.361, -0.121)
Constant	3.538*** (3.273, 3.803)
Observations	91
Log Likelihood	-44.571
Akaike Inf. Crit.	107.141
Bayesian Inf. Crit.	129.739
Note:	*p<0.1; **p<0.05; ***p<0.01

4.3.4 VCAM-1 Coverage

VCAM-1 coverage in the microvasculature was lowest amongst young, healthy controls (mean: 1.7% (SD:0.80)) and individuals with mild SVD (mean: 1.9% (SD: 0.54)), in contrast to those with a severe (mean: 2.6% (SD:0.51)) and moderate (mean: 2.8% (SD:0.96)) disease burden (**Table 4-1**, **Figure 4-7**, **Figure 4-8, Figure 4-9 & Figure C- 1 in Appendix C**). When compared to young, healthy controls, those with mild SVD showed a 54.29% increase in VCAM-1 coverage after adjusting for sex, brain matter type, and vascular risk factors (β=0.43, SE=0.59, *t*=0.72, *p=0.49*) (**Table 4-5**). Moderate SVD was associated with an approximate 3-fold increase in VCAM-1 coverage in comparison to young, healthy controls (β=0.93, SE=0.66, *t*=1.41, *p=0.21*), whilst severe SVD was associated with a 2-fold increase (β=0.78, SE=0.59,

t=1.31, *p=0.24*); however, none of these relationships were statistically significant. Likelihood-ratio tests revealed that including disease status did not significantly enhance model fit ($X^2(1)= 4.39$, *p*=0.22).

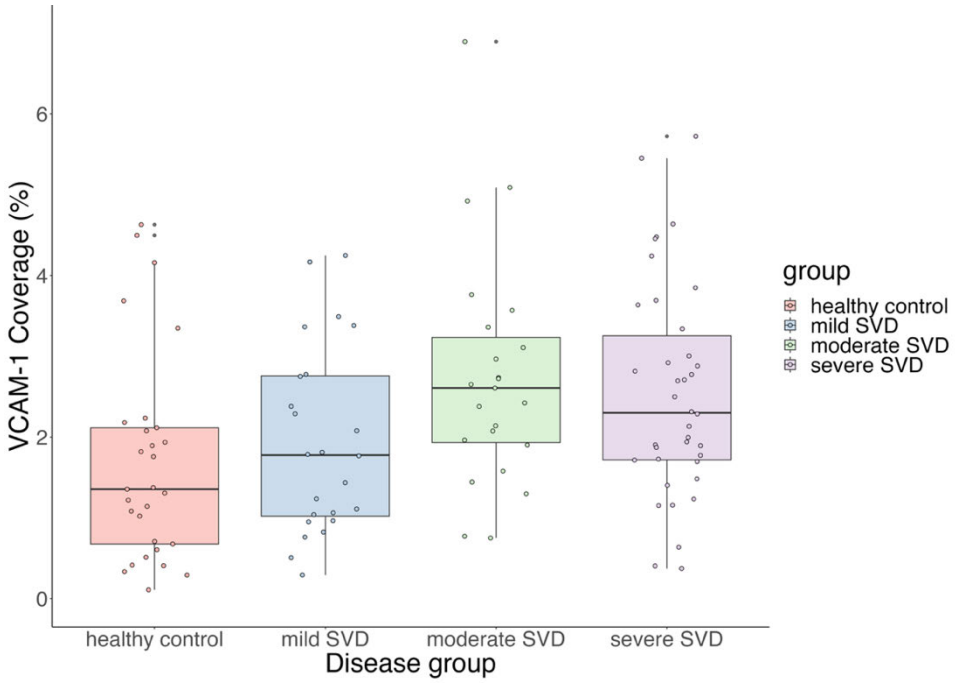


Figure 4-7. Average VCAM-1 coverage (%) in healthy control, mild SVD, moderate SVD, and severe SVD cases. *Abbreviations:* SVD: small vessel disease; VCAM-1: vascular cell adhesion molecule 1.

Figure 4-8. All images were stained using CD-31 (vasculature, DAB), PDGFRβ (pericytes, magenta), and VCAM-1 (endothelial activation, green). A 4μm section from the precentral gyrus of a healthy control (top row), a mild SVD case (second row from top), a moderate SVD case (third row from top), and a severe SVD case (bottom row) denoting pericyte loss (magenta) and enhanced endothelial activation (green, white arrowheads) in SVD.

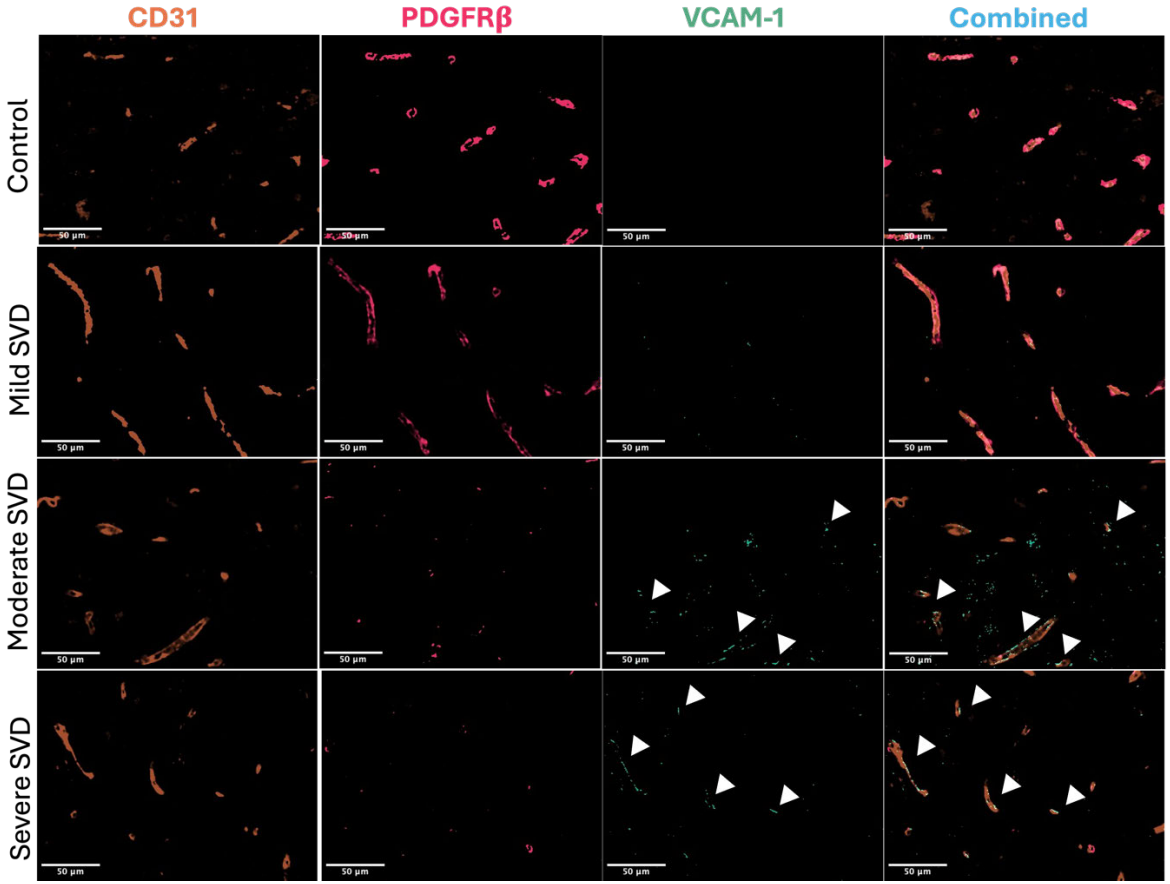


Figure 4-9. Images stained using CD-31 (vasculature, DAB), PDGFRβ (pericytes, magenta), and VCAM-1 (endothelial activation, green). Sections from the precentral gyrus of a healthy control (top row), a mild SVD case (second row from top), a moderate SVD case (third row from top), and a severe SVD case (bottom row) denoting pericyte loss and enhanced endothelial activation in SVD in individual microvessels.

Table 4-5. Linear mixed model results examining VCAM-1 coverage (%) and SVD status adjusting for sex, risk factor score, brain matter type (grey versus white) and random effects stemming from repeated measures/images from each individual case. *Abbreviations:* SVD: small vessel disease; VCAM-1: vascular cell adhesion molecule 1.

Linear Mixed Model Results: VCAM-1 Coverage and SVD

	Dependent variable:
	VCAM-1 Coverage
group: Mild SVD	0.434 (-0.547, 1.414)
group: Moderate SVD	0.935 (-0.154, 2.024)
group: Severe SVD	0.787 (-0.199, 1.772)
Sex (male)	0.062 (-0.535, 0.660)
Risk factor score	-0.222 (-1.042, 0.599)
Brain matter: white	-0.538*** (-0.767, -0.310)
Constant	0.469 (-0.068, 1.005)
Observations	82
Log Likelihood	-85.630
Akaike Inf. Crit.	189.261
Bayesian Inf. Crit.	210.921
Note:	*p<0.1; **p<0.05; ***p<0.01

4.3.5 Vessel segments

To assess the spatial relationship between PDGFRβ and VCAM-1 signal and to specifically focus on capillaries over larger vessels, I further segmented each vessel into micro-segments, excluding any segment with a diameter exceeding 10 μm[73]. In total, I analysed 18,665 segments with a mean length of 8.63 μm (SD: 8.15).

The relationship between pericyte signal and disease status remained evident within these vessel segments and after excluding larger vessels, with healthy controls having the strongest pericyte signal (mean: 0.05 (SD: 0.05)), followed by mild SVD cases (mean: 0.04 (SD: 0.05)), severe SVD cases (mean: 0.006 (SD: 0.01)), and moderate SVD cases (mean: 0.003 (SD: 0.01)), respectively.

Similarly, we also confirmed higher VCAM-1 signal intensity in those with moderate (mean: 1.57 (SD: 19.25)) and severe (mean: 1.46 (SD: 11.36)) SVD disease burden in comparison to mild SVD cases (mean: 1.03 (SD: 4.56)) and healthy controls (mean: 0.74 (SD: 3.62)) **(Figure 4-10).**

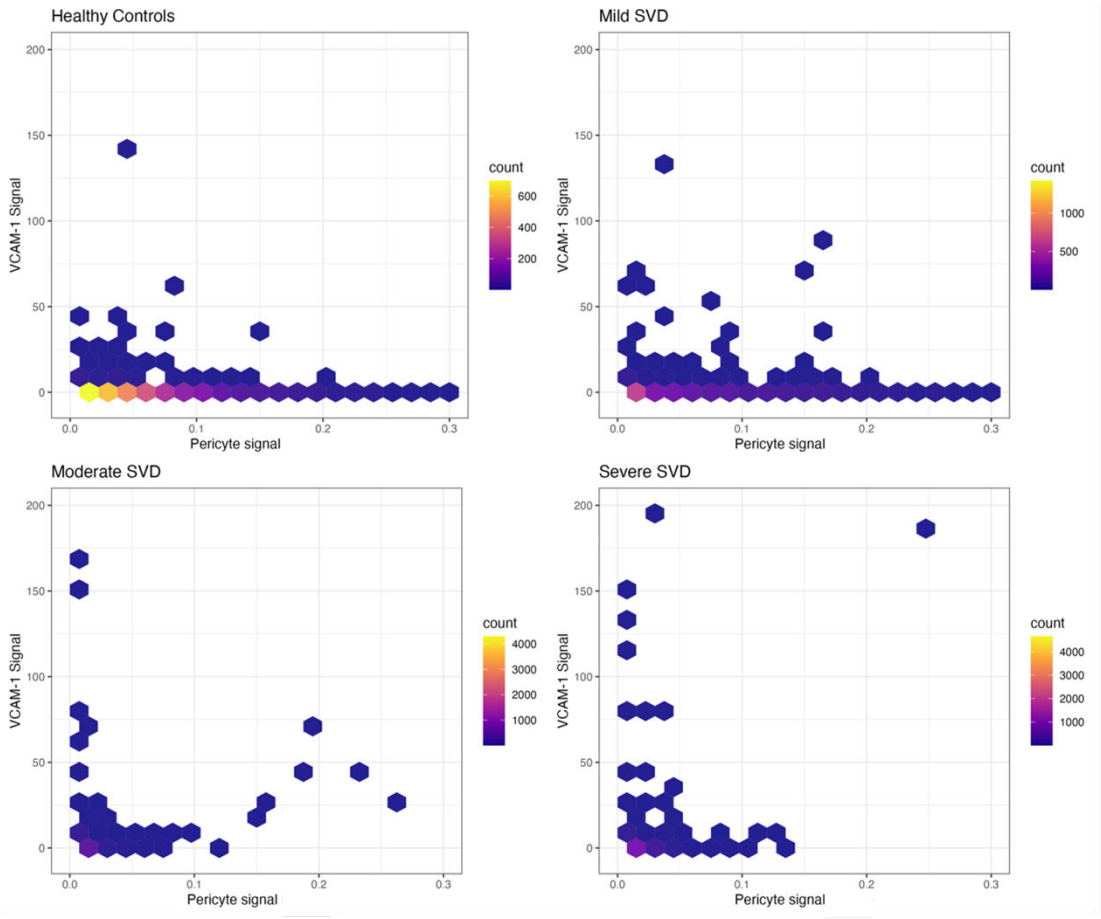


Figure 4-10. Hexbin density plots showing average VCAM-1 signal and pericyte (PDGFRβ) signal per disease group. ***Abbreviations:*** **SVD: small vessel disease; VCAM-1: vascular cell adhesion molecule 1.**

Results from linear mixed effects models adjusted for age, sex, and vascular risk factors, demonstrate a significant inverse relationship between log VCAM-1 and log pericyte immunofluorescent signal, with every 10% increase in pericyte signal being associated with an approximate 0.80% decrease in VCAM-1 signal within the same capillary micro-segment (β=-0.008, SE=0.002, *t*=-3.52, *p*<0.001) (**Table 4-6**). Results from a likelihood-ratio test suggest that including pericyte signal improves model fit ($X^2(1)$= 12.46, *p*<0.001). Unsurprisingly, results also suggested a significant relationship between

VCAM-1 signal intensity and age, with a 10% increase in age being associated with a 0.06% increase in VCAM-1 signal intensity (β=0.006, SE=0.003, *t*=2.42, *p*=0.04).

Table 4-6. Linear mixed model results examining log VCAM-1 immunofluorescent signal and log pericyte signal adjusting for age, sex and vascular risk factors as well as random effects stemming from repeated measures/images from each individual case. *Abbreviations:* SVD: small vessel disease; VCAM-1: vascular cell adhesion molecule 1.

Linear Mixed Model Results: VCAM-1 Coverage and SVD

	Dependent variable:
	VCAM-1 Coverage
Pericyte Signal	-0.084*** (-0.124, -0.045)
Age	0.006** (0.002, 0.011)
Sex (male)	-0.002 (-0.171, 0.167)
Risk factor score	-0.015 (-0.199, 0.169)
Constant	-1.505*** (-1.746, -1.265)
Observations	16,954
Log Likelihood	-16,592.780
Akaike Inf. Crit.	33,199.560
Bayesian Inf. Crit.	33,253.720
Note:	*p<0.1; **p<0.05; ***p<0.01

4.4 Conclusions

Using *postmortem* human brain tissue, we measured pericyte and VCAM-1 coverage in both grey and white matter borderzone areas within the primary motor cortex/precentral gyrus and adjacent white matter of healthy controls, as well as mild, moderate, and severe SVD cases.

Vessel density was lowest amongst young, healthy controls followed by those with mild, moderate, and severe SVD, respectively, and results from linear mixed effects models demonstrated a significant increase in vessel density in

those with severe SVD compared to controls. These results do not align with what has been previously reported in the literature. Although research focussing on vascular density in SVD is scarce, many studies report a decrease in vessel density in ageing and neurodegenerative disease, including dementia. One study with 153 histopathological samples across several dementias, including vascular dementia, reported lower microvascular densities in most ageing dementias, except in the case of 'post-stroke dementia' [345]. Studies using rodents have also shown that ageing is associated with decreased capillary density in various brain regions, with aged murine cerebrovascular networks showing a decrease in vascular length and branching density with increased vessel radii[346,347]. It is unclear why we observed an increase in vessel density in severe SVD cases compared to controls in our study. It is possible this is due to the slightly higher vessel diameters we observed in this group. In addition, we calculated vessel density as a function of vessel length by total area volume; one study reported finding a decrease in vessel density in ageing mice when using total vessel number and mean vessel length to estimate vessel density, yet found that mean vessel length alone increased with age[347]. Larger studies are required to draw more robust conclusions.

We did not detect any significant differences in vessel diameter amongst the groups, although we observed a tendency towards slightly wider vessel diameters in those with severe SVD. This could be due to a reduced presence of contractile pericytes resulting in a loss of vascular tone and an associated increase in vessel diameter. However, we did not observe an increase in diameter in the moderate SVD group, despite it having the lowest pericyte coverage. It is possible that in severe SVD cases, the prolonged reduction in pericyte coverage has led to changes in vessel diameter over time, although larger studies are required to confirm these findings. Another study reports similar results, showing a 20% increase in WM capillary diameters across varying dementias, including AD and vascular dementia compared to ageing controls; interestingly, capillary widths were also correlated with myelin loss, arteriosclerosis, and enlarged perivascular spaces[345]. The authors

hypothesise that wider vessels might reflect compensatory changes to preserve WM perfusion in ageing-related dementias.

Average pericyte coverage was highest in young, healthy controls (34%) and progressively declined in the mild (25%), severe (7.2%), and moderate (5.9%) SVD groups. Moderate and severe SVD cases had an approximate 83-86% reduction in pericyte coverage compared to young, healthy controls after adjusting for sex, brain matter type, and vascular risk factors. These results largely coincide with those reported by previous *postmortem* studies focussing on pericyte loss in ageing dementias; however, one study reports a loss of pericyte density in the deep white matter, but preserved pericyte populations in the cortex, which diverges from our results[314,337]. It is important to note that this study focussed exclusively on the white matter within the frontal lobe of cases with several types of ageing-related dementias. Interestingly, our results indicate that white matter areas had significantly lower pericyte coverage compared to grey matter areas, even whilst controlling for inter-subject variability. This possibly reinforces the concept that pericytes are more vulnerable in diffuse white matter disease.

VCAM-1 coverage of the microvasculature was higher in those with moderate (2.8%) and severe (2.6%) SVD compared to those with mild SVD (1.9%) and healthy controls (1.7%). Results from linear mixed effects models also highlight a 2-to-3-fold increase in VCAM-1 coverage between the moderate and severe SVD groups compared to healthy controls after adjusting for sex, brain matter type and vascular risk factors, though this did not reach statistical significance. *Postmortem* studies examining endothelial activation in SVD are lacking. Data from one neuropathologic study claimed endothelial activation was not a hallmark of SVD, as ICAM-1 was rarely detected in pathologically diagnosed SVD cases nor in aged controls without SVD[195]. Endothelial activation can be manifested through distinct ways and is marked by the expression of more than one molecule; it is possible that ICAM-1 and VCAM-1 could have different expression patterns in diverse brain regions or differing stages of disease severity. Notably, the aforementioned study does not include vascular risk

factors or other demographic characteristics in their analyses. In addition, they exclusively examined tissue from the caudate putamen deep grey matter areas, and used a manual gross rating system, categorising endothelial activation as being 'absent' or 'present' rather than using numeric quantification.

Our study has several limitations. Although SVD grading was performed by a highly experienced SVD neuropathologist, the lack of published and validated standardisation protocols and grading systems for non-amyloid SVD pathology may limit the generalisability of findings. An agreed, standardised system for grading histopathological assessments of vascular dementia would greatly aid the interpretation of studies and advance the field[342]. Possibly some of the differences between the current and prior studies are a consequence of differences in methods of grading the histopathology. Although novel, our use of multi-spectral imaging to transform brightfield images into fluorescent-like composites introduces a possible source of signal inaccuracy; for instance, in some cases, both green (VCAM-1) and blue (Haematoxylin) chromogens were recognised as green by our channel-unmixing algorithm. Although we remediated this by eliminating any overlap of the masks, it is possible this conservative approach might have inadvertently erased some true VCAM-1 pseudofluorescence. It is worth noting we repeated this process for all of our images, and there is no reason to believe this affects our conclusion.

Notably, four out of five cases in the severe SVD group experienced an intracerebral haemorrhage, and its impact on pathology, including in other brain regions, remains uncertain. However, we believe this is unlikely to affect our conclusions, as cases in the moderate SVD group (none of which had a previously reported intracerebral haemorrhage) exhibited the lowest pericyte coverage and the highest VCAM-1 coverage, even when compared to the severe SVD group. Cases with moderate and severe SVD were older, making it difficult to separate the effects of age and disease severity on pericyte loss and endothelial activation. Adjusting for age in our models was not feasible, as its strong correlation with disease severity could obscure or distort the true

relationship. Age may partly mediate group differences, and larger studies, ideally with age-matched controls, are needed to better distinguish the independent effects of age and disease on these outcomes. Notably, our study does not address an important remaining question: to what extent is pericyte loss/endothelial activation a consequence of ageing rather than pathology, and at what point do these dysfunctions translate to pathology or disease? Lastly, our sample size is too small to derive robust inferences at a population level and our findings require corroboration by larger and more diverse samples, ideally in tandem with high-field MRI examination.

Some of the strengths in our study include the use of semi-automatic VasOMatic software and multi-spectral imaging, despite its limitations. In contrast to manual cell counting, this approach offers a more consistent quantification of pericyte coverage as a ratio to the cerebrovasculature. This is particularly relevant in the case of pericytes, as whilst their somata can be immobile, their processes undergo extensions and retractions over prolonged periods of time, and it is possible that subtle effects from disease could have consequences for their processes prior to the cell nucleus. However, it is possible this method may not fully capture pericyte structural changes or be an accurate measure of pericyte presence/loss. We also incorporated relevant covariates, including vascular risk factors, which are usually underreported in many pathological studies and rarely addressed in statistical models of these.

The vascular contributions to dementia are understudied and under-recognised, and unfortunately, neuropathological data examining the molecular mechanisms and roles of the neurogliovascular unit in disease are scarce. These findings add to the growing knowledge on the involvement of pericyte and endothelial cell dysfunction in the early stages of vascular dementia. In the next chapter, I explore whether these dysfunctions can be detected *in vivo* and examine whether biomarkers of pericyte and endothelial function detectable in the blood associate with SVD burden and progression longitudinally in a mild-stroke population enriched for SVD.

Chapter 5 Blood biomarkers of vascular dysfunction in SVD progression: Insights from a longitudinal neuroimaging study

5.1 Context

In the previous chapter, I report findings from mouse and human postmortem studies which suggest pericyte and endothelial dysfunction in ageing and in SVD. In this chapter, I explore the potential to detect these dysfunctions in vivo and examine their associations with disease burden and longitudinal progression within a highly characterised cohort of mild stroke patients, enriched for SVD features.

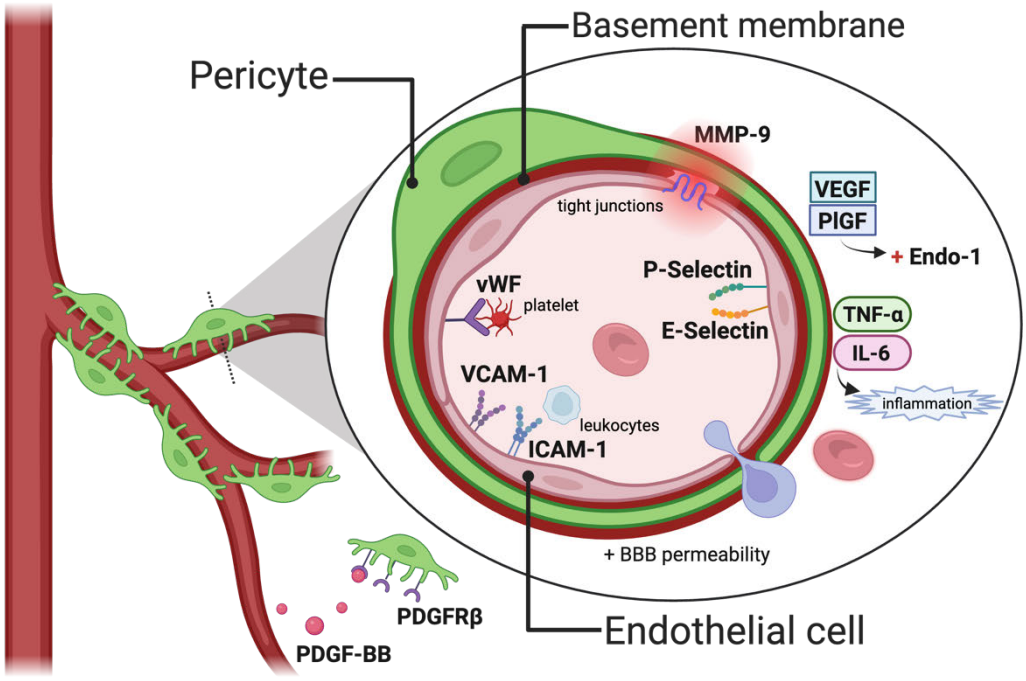
This chapter is awaiting final publication decision by a peer-reviewed journal after minor revisions suggested during peer-review.

5.2 Introduction

Cerebral small vessel disease (SVD) is a highly prevalent disorder of the small brain blood vessels and a common cause of stroke and dementia, contributing to approximately 45% of all dementia cases worldwide.[5] SVD is characterised by structural and functional alterations in cerebral arterioles, venules, capillaries, and surrounding tissue. Whilst clinical manifestations of SVD are highly varied, they can include lacunar or haemorrhagic stroke, cognitive decline, neuropsychiatric symptoms, and mobility problems.[3,6,8] SVD is distinguished by various signature lesions visible on neuroimaging, including white matter hyperintensities (WMH), lacunes, microbleeds, and enlarged perivascular spaces (PVS). Whilst many aspects of the underlying pathogenesis of SVD remain uncertain, numerous lines of evidence implicate endothelial and pericyte dysfunction, inflammation, and a compromised blood-brain barrier (BBB), which may precede visible brain lesions.[2]

Despite compelling evidence advocating for the role of endothelial and pericyte dysfunction in the pathogenesis of SVD, there are currently no blood biomarkers reliably capable of characterising the disease and results from previous studies remain inconsistent.[152] Some of these discrepancies may arise due to the inherent complexity underlying SVD mechanisms as well as variations introduced by study design and sample heterogeneity. Some results may be confounded by an acute response to ischaemic injury by comparing vascular function markers in lacunar stroke patients within the hyper-acute stroke stage to healthy controls or effects of prescribed drugs. Others exclude patients with a history of stroke or mild cognitive impairment (MCI) altogether, limiting the scope of SVD severity representation or relevance. Importantly, few studies include a wide range of SVD-relevant features, including cognitive decline, BBB permeability, and cerebrovascular reactivity (CVR), as well as diverse lesion types, because although WMH are more commonly observed, it remains possible that distinct lesions represent different stages of a dynamic disease process.[8]

We aimed to examine the relationship between various biomarkers of vascular, endothelial, and pericyte function and 1) structural and vascular functional MRI markers of SVD, including WMH, lacunes, microbleeds, summary SVD score, BBB permeability and CVR, and 2) cognitive performance (including the executive function domain) and MCI in cross-sectional and longitudinal exploratory analyses with patients with minor, non-disabling ischaemic stroke, enriched for SVD features. A concise list of the biomarkers extracted, their roles, and their putative involvement in cerebrovascular dysfunction is presented in **Figure 5-1** and includes: platelet derived growth factor receptor beta (PDGFRβ), platelet derived growth factor subunit B (PDGF-BB), vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), platelet selectin (P-Selectin), endothelial selectin (E-Selectin), endothelin-1 (Endo-1), tumour necrosis factor alpha (TNF-α), von Willebrand factor (vWF), matrix metalloproteinase-9 (MMP-9), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and placental growth factor (PlGF).



Pericyte (dys)function

- ***PDGF-BB***
  - secreted by the endothelium; serves to recruit pericytes to endothelial cells
- ***PDGFRβ***
  - PDGF-BB receptor, enriched in pericytes

Endothelial (dys)function

Activation/BBB breakdown: Cell adhesion molecules, Selectins, growth factors and enzymes

- ***VCAM-1, ICAM-1, E-Selectin, P-Selectin:***
  - facilitate the recruitment, rolling, adhesion, and transendothelial migration of leukocytes through vascular walls; associated with increased BBB permeability and endothelial activation
- ***VEGF, PIGF:***
  - angiogenic factors involved in the proliferation, migration and survival of endothelial cells; play an important role in BBB permeability
- ***MMP-9:***
  - *promotes transmigration of monocytes across the endothelium by degrading the basement membrane and disrupting tight junction proteins; implicated in atherosclerosis and vascular injury and remodeling*

Inflammation: cytokines and chemokines

- ***TNF-α, IL-6***
  - pro-inflammatory cytokines that instigate platelet production and hepatic production of C-Reactive protein; promote the secretion of endothelial cell adhesion molecules, impair vasodilation, and exacerbate oxidative stress and BBB breakdown

Haemostasis: coagulation factors

- ***vWF, Endo-1***
  - play a role in thrombus formation and facilitate the adhesion of platelets to the vessel walls; implicated in vasoconstriction and BBB permeability

Figure 5-1. Panel of serum biomarkers extracted and their putative involvement in pericyte and endothelial cell (dys)function in the cerebral

small vessels. Abbreviations: PDGF-BB: platelet derived growth factor subunit B; PDGFRβ: platelet derived growth factor receptor beta; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; P-Selectin: platelet-selectin; E-selectin: endothelial-selectin; BBB: blood-brain barrier; VEGF: vascular endothelial growth factor; PLGF: placental growth factor; MMP-9: matrix metalloproteinase 9; TNF-α: tumour necrosis factor alpha; IL-6: Interleukin 6; vWF: von Willebrand Factor; Endo-1: Endothelin-1.

5.3 Methods

5.3.1 Participants and Design

We prospectively recruited 229 patients presenting to the Lothian stroke services with acute lacunar or minor cortical ischaemic stroke from August 2018 to December 2021 into an observational longitudinal cohort study.[348] All participants provided written informed consent according to the principles expressed in the Declaration of Helsinki. This study was granted ethical approval by Lothian Ethics Medical Research Committee (REC 09/81,101/54) and NHS Lothian R&D Office (2009/W/NEU/14).

Participants were recruited between 1-3 months post- stroke. All clinical evaluations, including stroke diagnosis and subtyping, were conducted by specialised stroke physicians and neuroradiologists. Patients with MRI contraindications or severe neurological, cardiac, or respiratory conditions were excluded from the study. Minor stroke was characterised by a modified Rankin Score (mRS) of ≤ 2 at recruitment.[349,350] All patients received guideline stroke prevention treatments as clinically appropriate including antihypertensive, lipid lowering and antiplatelet drugs.

Patients attended a baseline visit that included serology, brain MRI, cognitive, and clinical assessments. Structural MRI and cognitive assessments were repeated one year later.[348] In addition, those with lacunar stroke or moderate-to-severe WMH were invited for additional follow-up scans at 2-3 months, and 4-6 months. Details of relevant clinical events occurring throughout the study period, including recurrent stroke, transient ischaemic attack, or suspected stroke, were sought.

5.3.2 MRI Acquisition

Detailed neuroimaging protocols are described elsewhere.[348] Briefly, participants underwent 3 T brain MRI (MAGNETOM Prisma, Siemens Healthcare) at baseline and one year. Images were acquired using a 32-channel head coil (Siemens Healthcare, Erlangen, Germany). The following sequences were acquired: 3D T1-weighted (1 mm^3 isotropic), T2-weighted (0.9 mm^3 isotropic), FLAIR (1 mm^3 isotropic), multi-shell diffusion weighted imaging (b=2000 (64 orientations), 1000 (64), 600 (6), 200 (3) and 0 (15) s/mm^2; 2 mm^3 isotropic voxels), susceptibility-weighted imaging (SWI/SWAN/GRE; 0.6/0.6/3.0 mm^3), and, at baseline, a 21-minute dynamic contrast-enhanced (DCE)-MRI protocol (2.0 mm^3) including a gadolinium-based contrast agent (gadobutrol) injection to assess BBB integrity, except in patients with eGFR levels of < 30 mL/min.[57,351] DCE sequence was followed by DESPOT1-HIFI sequence to determine voxel-wise pre-contrast T1 values.[352] At baseline, we also acquired 2D gradient-echo EPI images (2.5 mm^3 isotropic) in conjunction with a hypercapnic challenge to assess CVR, consisting of a 12-minute protocol alternating between medical air and hypercapnic gas (6% CO_2, 21% O_2, 73% N_2).[353] Heart rate, respiration rate, peripheral oxygen saturation levels, and end-tidal CO_2 ($EtCO_2$) were closely monitored during this procedure.

5.3.3 MRI Processing and Analysis

MRI data were processed computationally and semi-automatically using validated methods.[27,348] Intracranial volumes (ICV) were computationally generated, checked for accuracy and edited manually when required. WMH volumes were extracted from FLAIR images.[27] Index acute, old, and incident infarcts were manually segmented and excluded from total WMH volumes by an experienced image analyst.

Scans were assessed for acute ischaemic lesions, including incident small subcortical DWI-positive lesions, old infarcts, lacunes, microbleeds, summary SVD scores (assigning one point for: 1) Fazekas score of three in the

periventricular white matter or ≥ 2 in deep white matter; 2) lacunes present; 3) microbleeds present; 4) over ten counts of enlarged perivascular spaces in the basal ganglia), and Fazekas scores (periventricular white matter and deep white matter) by trained raters and checked by an experienced neuroradiologist according to STRIVE criteria using validated scales.[27,354–358] To assess BBB permeability, we derived values for permeability-surface area product (PS) and blood plasma volume fraction (V_p) for each voxel and region using validated in-house processing scripts and DCE-MRI sequences. Full details on DCE-MRI acquisition protocol are described elsewhere.[348,359] CVR was quantified as the regression coefficient associated with a time-shifted $EtCO_2$ profile divided by the baseline BOLD signal (mean BOLD intensity across the first volumes of the first medical air block) multiplied by 100. CVR corresponded to the relative change in BOLD signal per unit change in $EtCO_2$ and is reported as % /mmHg in normal-appearing white matter (NAWM), deep grey matter, and within WMH. Additional details are published.[353]

5.3.4 Blood Biomarkers

We collected a 19 mL venous blood sample from participants at the baseline visit. Within 1 hour of collection, samples were centrifuged (at 1000xg for ten minutes for serum samples and 2000xg for 15 minutes for plasma samples) to isolate serum and plasma before being stored at -80°C. Using commercially available ELISA kits, we extracted the following biomarkers from patient serum samples: PDGFRβ (Abcam-ab252357), PDGF-BB (Abcam-ab100624), VCAM-1 (Abcam-ab223591), ICAM-1 (R&D-DY720), P-Selectin (R&D-DPSE00), E-Selectin (R&D-DSLE00), Endo-1 (R&D-DET100), TNF-α (R&D-HSTA00E), vWF (Abcam-ab108918), MMP-9 (R&D-DMP900), IL-6 (R&D-HS600C), VEGF (R&D-DVE00), and PlGF (R&D-HSPG00). Plasma samples were used in lieu of serum samples for 7 participants (>4%) as the latter were unavailable. According to ELISA kit instructions, either serum or plasma can be used, with the exception for the E-Selectin and MMP-9 kits. For those two, we did not include the 7 plasma samples. Dilution testing was conducted in both serum and plasma separately. Additionally, we standardised the

biomarkers to a mean of 0 and a SD of 1, thus further minimising any extraneous variation due to processing (see the **Statistical Analyses section within the methods section and Table D- 16 & Figure D- 8 in Appendix D**. Supplemental material to Chapter 5**).** Additional details pertaining to the assay protocol, including dilutions, and calculated inter- and intra-assay %, can be found in **Appendix A** within the Supplemental Materials for this chapter, in **Appendix D**.

5.3.5 Clinical and Cognitive Assessments

Study procedures are described in detail elsewhere.[348] Participants provided medical history confirmed by a physician and supplemented by hospital medical records, including diabetes mellitus, hypertension, and hypercholesterolaemia, and provided details on smoking status and history. Stroke subtype (lacunar or cortical) was determined by reaching consensus between an expert panel of stroke physicians and neuroradiologists based on presenting symptoms and diagnostic neuroimaging or relevant investigations.[348,360] Cortical strokes acted as a control group for lacunar stroke and is more relevant than non-stroke controls since cortical strokes receive similar secondary prevention medication which can influence endothelial function and blood markers. Cognition was assessed using the Montreal Cognitive Assessment (MoCA) at baseline and again at one year. A cut-off score of 26 out of 30 (including the addition of a one-point educational adjustment for individuals with ≤ 12 years of education) was used to establish MCI classification.[361] Participants also completed the Trail Making Test (TMT) parts A and B points and time at baseline and one year. We derived the B/A ratio from this task to assess executive functioning;[362] B/A ratios of >3 indicate executive dysfunction. We also quantified premorbid/peak adult intelligence by measuring number of errors in the National Adult Reading Test (NART).[363]

5.3.6 Statistical Analyses

All analyses were performed using R (version 4.4.1).[364] We expressed WMH volumes as % ICV to adjust for variations in individual head size. WMH

volumes as % ICV were log-transformed to improve model fit and ensure normality of residuals. To reduce the number of predictors and avoid model overfitting, we assigned equal weight to hypertension, hypercholesterolaemia, diabetes, and smoking history (current smoker or ex-smoker within one year) in the calculation of a baseline vascular risk factor composite score. Models were assessed for assumptions and fit by checking the normality of residuals, heteroscedasticity, and collinearity. Blood biomarker levels were standardised (mean=0, SD=1) to improve model convergence and facilitate inter-model coefficient comparisons. The nature of this study was exploratory and hypothesis-generating, thus *p*-values were not adjusted for multiple comparisons. Findings should be interpreted as preliminary.

5.3.6.1 Cross-sectional Analyses

All models were adjusted for age, sex, and vascular risk factor composite score. Models with neuroimaging-related outcome variables also included stroke subtype and the National Institutes of Health Stroke Scale (NIHSS) at baseline as covariates, whilst models with cognition-related outcome variables were additionally adjusted for premorbid intelligence and baseline WMH volume as % ICV. Adjustment for stroke subtype (lacunar vs cortical) accounts for stroke mechanism and prescribed medication. Unadjusted model results for all analyses are also provided to test for indirect associations between biomarkers and disease outcomes.

Linear regression was used to assess the relationship between individual standardised biomarkers and log transformed WMH volume, continuous MoCA score, PS, V_p, and CVR (in NAWM, deep grey matter, and within WMH, separately) as outcome variables. Logistic regression was used to examine the relationship between the standardised biomarkers and the prevalence of lacunes and microbleeds at baseline (≥ 1), the incidence of DWI-hyperintense lesions (≥ 1) during the one-year follow-up period, and MCI prevalence. Finally, to assess the relationship between individual standardised biomarkers and Fazekas score and summary SVD score, we used ordinal regression models adjusted for the variables outlined above.

5.3.6.2 Longitudinal Analyses

Longitudinal analyses were performed using linear mixed-effects models (lme4, R, R Foundation for Statistical Computing, Vienna, Austria) with individual participants fitted as random effects to account for intraindividual trajectories.[365] Models with longitudinal WMH volumes as the outcome variable were adjusted for age, sex, vascular risk factor composite score, stroke subtype, and baseline NIHSS; models with longitudinal MoCA scores and TMT ratio as outcome variables included age, sex, vascular risk factor composite score, premorbid intelligence, and baseline WMH volume as % ICV as covariates. Longitudinal analyses using SVD summary score, Fazekas score, and lacune and microbleed prevalence were not conducted here, as these measurements might not effectively capture subtle changes occurring over a one-year period.

5.4 Results

Of the 229 participants recruited, blood biomarker data were available for 181 (mean age: 65 years (SD:11), 31% female). We acquired baseline brain MRI (n=179, including DCE-MRI (n=155) and CVR (n=140)) and cognitive data (MoCA n=174, TMT ratio n=171) and again at one year (structural brain MRI n=163; cognitive assessment (MoCA n=162, TMT ratio n=161) (**Figure 5-2 & Figure D- 1 & Figure D- 2 in Appendix D**).

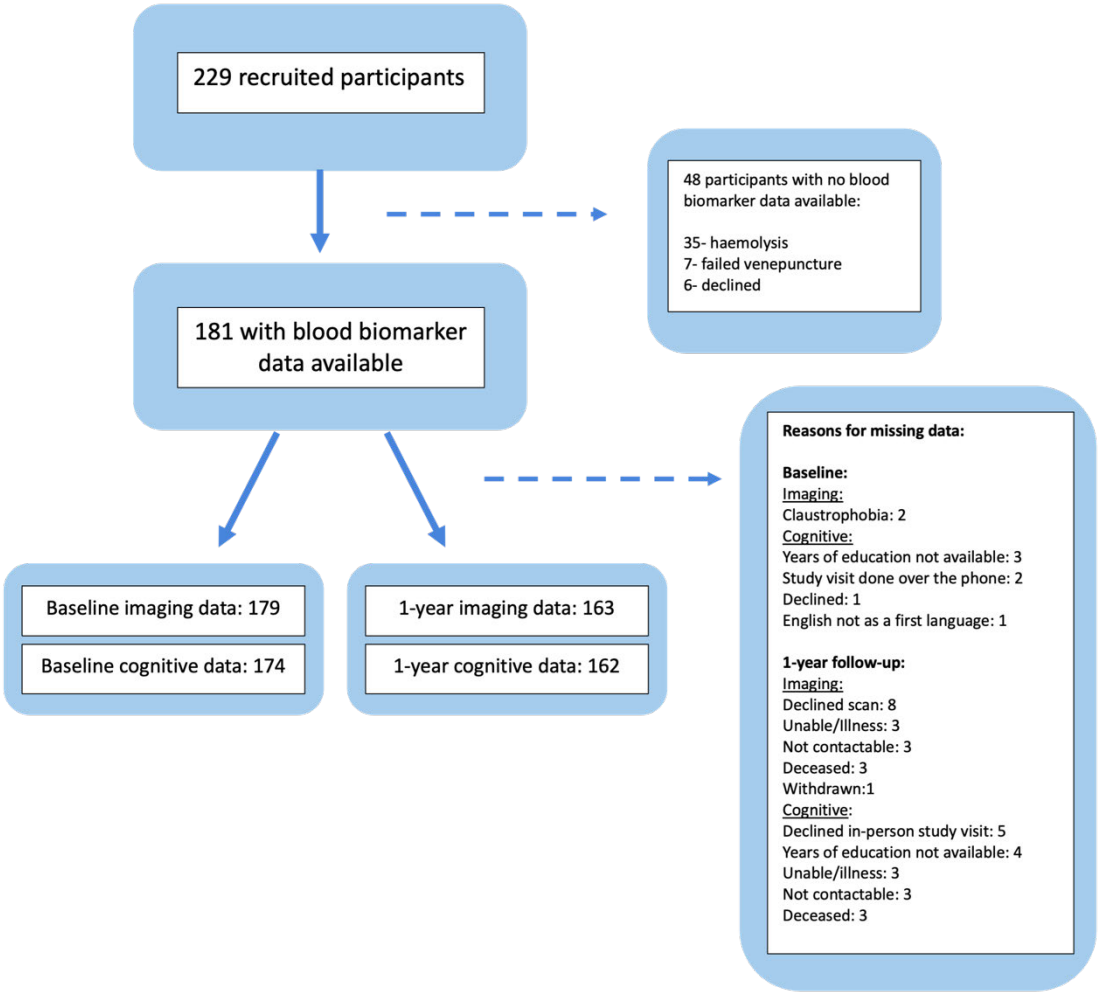


Figure 5-2. Flow diagram of study recruitment and data collection.

Stroke subtype was lacunar in 59% of our cohort. Average WMH volume (as % ICV) was 0.93% (SD:1.1) at baseline and 1.00% (SD:1.3) at one year follow-up. Participants' median Fazekas score at both time points was 3 (IQR:3,5). The prevalence of lacunes and microbleeds at baseline was 53% and 20%, respectively, and 54% and 21% at one year follow-up. At both baseline and one year, the median summary SVD score was 3 (IQR:2,4). The average adjusted MoCA score in our cohort was 25 (SD:3.5) at baseline and 26 (SD:3.5) at one year, with 63% of patients qualifying for MCI at baseline and 46% at one year follow-up. Cohort characteristics are shown in **Table 5-1**.

Table 5-1. Cohort characteristics (n=181).

	Overall N=181
Sex, female, n (%)	57 (31%)
Age, years, mean (SD)	65 (11)
Stroke subtype, lacunar, n (%)	106 (59%)
Diabetes, yes, n (%)	43 (24%)
Hypertension, yes, n (%)	126 (70%)
Hypercholesterolaemia, yes, n (%)	133 (73%)
Current or ex-smoker (<1 year), n (%)	41 (18%)
Risk factor composite score, median (IQR)	3 (2,4)
NIHSS score, median (IQR)	2 (1,3)
Baseline NART errors, mean (SD)	17 (9.5)
Baseline WMH volume (mm^3 as % ICV), mean (SD)	0.93 (1.1)
1-year follow-up WMH volume (mm^3 as % ICV), mean (SD)*	1 (1.3)
Baseline summary SVD score, median (IQR)	3 (2,4)
1-year follow-up summary SVD score, median (IQR)*	3 (2,4)
Baseline Fazekas score, median (IQR)	3 (3,5)
1-year follow-up Fazekas score, median (IQR)*	3 (3,5)
Baseline, patients with ≥1 lacune, n (%)	94 (53%)
1-year follow-up, patients with ≥1 lacune, n (%)*	88 (54%)
Baseline, patients with ≥1 microbleed, n (%)	35 (20%)
1-year follow-up, patients with ≥1 microbleed, n (%)*	34 (21%)
Patients with new DWI-positive lesions between baseline and 1-year follow-up, n (%)*	47 (26%)
Baseline MoCA score, mean (SD)	25 (3.5)
1-year follow-up MoCA score, mean (SD)*	26 (3.5)
Baseline Trail Making Test ratio (A/B), mean (SD)	2.9 (1.2)
1-year follow-up Trail Making Test ratio (A/B), mean (SD)*	2.8 (1.3)
PDGFRβ, pg/mL, mean (SD)	57,000 (23,000)
PDGF-BB, pg/mL, mean (SD)	8,900 (3,300)
VCAM-1, ng/mL, mean (SD)	2,100 (760)
ICAM-1, pg/mL, mean (SD)	200,000 (82,000)
E-Selectin, ng/mL, mean (SD)	43 (20)
P-Selectin, ng/mL, mean (SD)	120 (49)
Endo-1, pg/mL, mean (SD)	3.8 (2.8)
TNF-α, pg/mL, mean (SD)	1.2 (0.72)
vWF, mIU/mL, mean (SD)	1,300 (800)
MMP-9, pg/mL, mean (SD)	870 (440)
IL-6, pg/mL, mean (SD)	4.2 (9.5)
VEGF, pg/mL, mean (SD)	350 (200)
PLGF, pg/mL, mean (SD)	9.6 (2.9)
PS, NAWM, $x10^4$, mean (SD)	0.16 (1.0)
PS, basal ganglia, $x10^4$, mean (SD)	0.75 (1.3)
PS, WMH, $x10^4$, mean (SD)	0.84 (1.6)
V_p, NAWM, $x10^3$, mean (SD)	0.63 (1.8)
V_p, basal ganglia, $x10^3$, mean (SD)	15 (3.5)
V_p, WMH, $x10^3$, mean (SD)	8.4 (3.7)
CVR, NAWM, % /mmHg, mean (SD)	0.043 (0.017)
CVR, basal ganglia, % /mmHg, mean (SD)	0.17 (0.053)
CVR, WMH, % /mmHg, mean (SD)	0.040 (0.043)

Abbreviations: NIHSS: The National Institutes of Health Stroke Scale; **IQR:** interquartile range; **NART**: National Adult Reading Test; **WMH**: white matter hyperintensity; **ICV:** intracranial volume; **SVD**: small vessel disease; **DWI:** diffusion-weighted imaging; **MoCA:** Montreal Cognitive Assessment; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1;

ICAM-1: intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor; **PS:** permeability-surface area product ; **NAWM:** normal-appearing white matter; **V_p:** blood plasma volume fraction; **CVR:** cerebrovascular reactivity.

Compared to participants without biomarker data, those with available biomarker data were younger (mean age: 65 years (SD:11) vs 68 (SD:10)) and had more extensive WMH lesions at baseline and one year (mean WMH volume: 0.96% (SD:1.2), and 1.00% (SD:1.3)), respectively vs 0.85% (SD:0.93) and 0.99% (SD:1.2)). They also had fewer lacunes at baseline (median: 1 (IQR:1,3) vs 2 (IQR:1,3)) and one year (median: 1 (IQR:0,3) vs 2 (IQR:1,4)) as well as microbleeds, both at baseline (median: 0 (IQR:0,0) vs 1 (IQR:1,1)) and one year (median: 0 (IQR:0,0) vs 1 (IQR:1,1)) (**Table D- 1 in Appendix D**).

Of those with available biomarker data, participants who attended the one year follow-up with brain MRI (n=163 out of 181) were of comparable age, had similar WMH volumes and summary SVD and Fazekas scores at baseline, had more lacunes (2.1 (SD:3.3) vs 1.4 (SD:1.9)) and microbleeds (1.4 (SD:5.6) vs 0.71 (SD:2.4)) on average, and comparable cognitive assessment scores to those who did not attend the one year follow-up (**Table D- 2 in Appendix D**).

Compared to cortical stroke patients, lacunar stroke patients were more likely to have diabetes (28% versus 18%), hypertension (74% versus 65%), hypercholesterolaemia (79% versus 66%), be current smokers (15% versus 9%), have higher WMH volumes (1% (SD:1.2) versus 0.84% (SD:1.1)), have a higher SVD summary score (3.1 (SD:1.3) versus 2.5 (SD:1.3)), have more lacunes (2.8 (SD:3.8) versus 0.95 (SD:1.7)) and microbleeds (1.9 (SD:6.6) versus 0.58 (SD:2.7)) at baseline and one year (3.0 (SD:3.9) versus 0.98

(SD:1.8), and 2.8 (SD:8.3) versus 0.51 (SD:2.6), respectively), and more DWI-positive lesions detected throughout one year (0.73 (SD:1.4) versus 0.28 (SD:0.65)). Cohort characteristics of lacunar stroke patients compared to cortical stroke patients with blood biomarker data can be found in **Table D- 3 in Appendix D**.

Serology was performed outside the acute stroke phase, and no significant correlation was found between individual biomarkers and the time elapsed from stroke onset to serology (mean: 59.56 days (SD:21.65)) (**Table D- 4 in Appendix D**).

5.4.1 Imaging Outcomes

5.4.1.1 WMH: Cross-sectional and longitudinal WMH volumes and baseline Fazekas score

In linear mixed effects models adjusted for age, sex, vascular risk factors, stroke subtype, and baseline NIHSS, we detected trends wherein higher circulating levels of PDGF-BB (β=0.11, 95% CI:-0.04, 0.26), MMP-9 (β=0.11, 95% CI:-0.04, 0.26), and Endo-1 (β=0.06, 95% CI:-0.09, 0.21) showed possible associations with increasing WMH volumes longitudinally, as well as VCAM-1 and lower WMH volumes (β=-0.13, 95% CI:-0.29, 0.02); similar patterns were evident cross-sectionally (**Figure 5-3 & Table D- 5 in Appendix D**). In unadjusted ordinal logistic regression analyses featuring Fazekas scores at baseline, VCAM-1 (OR: 1.35, 95% CI:1.04, 1.77) and ICAM-1 (OR: 1.31, 95% CI:1.003, 1.71) showed a significant association with higher WMH burden. In adjusted analyses, PDGF-BB (OR:1.15, 95% CI:0.86, 1.55), and E-Selectin (OR:1.12, 95% CI:0.84, 1.51) showed potential associations with higher WMH burden cross-sectionally (**Figure D- 3 & Table D- 6 in Appendix D**).

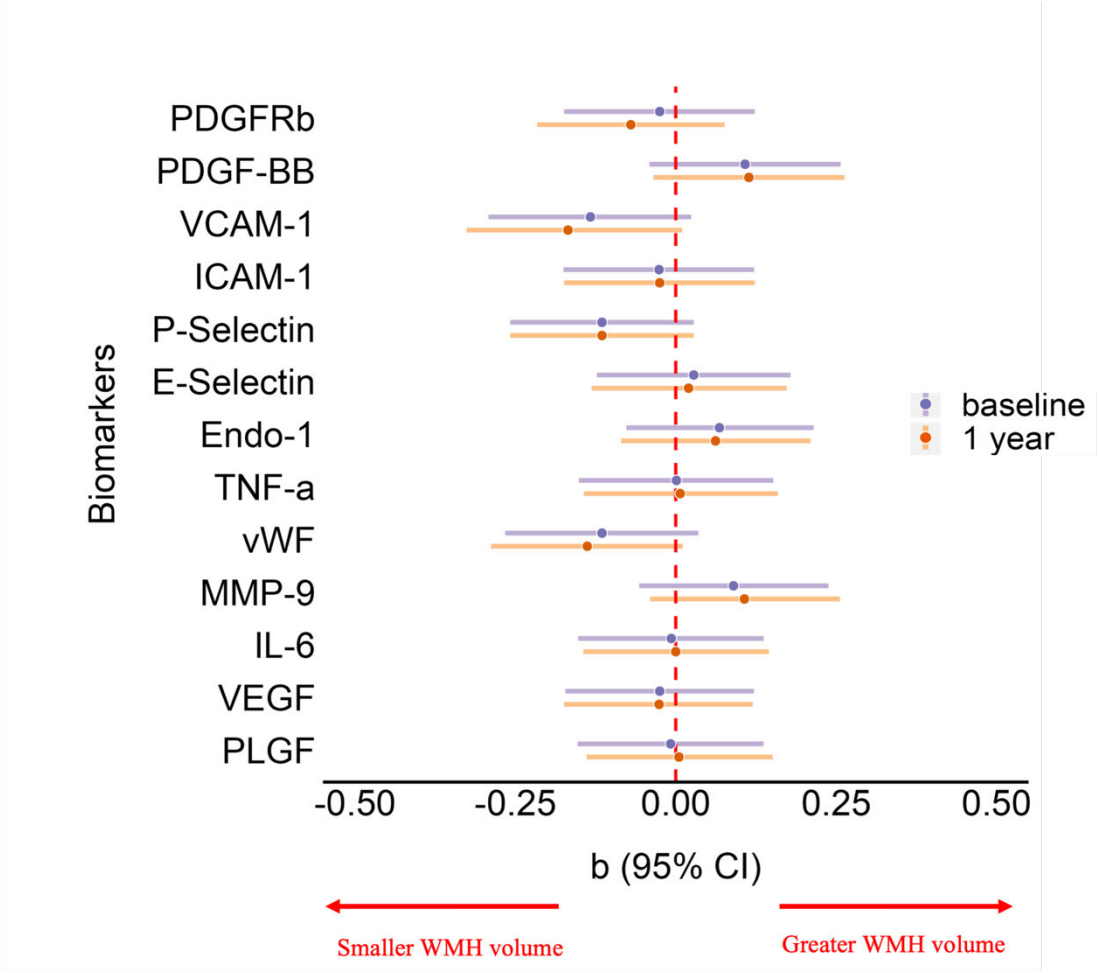


Figure 5-3. Blood biomarkers and WMH volume. Regression results for the association between distinct standardised blood biomarkers and log transformed WMH volume as % ICV at baseline (linear regression) and one year follow-up (linear mixed effects models) after adjusting for age, sex, vascular risk factors, stroke subtype, and baseline NIHSS. Abbreviations: WMH: white matter hyperintensity; ICV: intracranial volume; NIHSS: The National Institutes of Health Stroke Scale; CI: confidence interval; PDGFRβ: platelet derived growth factor receptor beta; PDGF-BB: platelet derived growth factor subunit B; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; E-selectin: endothelial-selectin; P-Selectin: platelet-selectin; Endo-1: Endothelin-1; TNF-α: tumour necrosis factor alpha; vWF: von Willebrand Factor; MMP-9: matrix metalloproteinase 9; IL-6: Interleukin 6; VEGF: vascular endothelial growth factor; PlGF: placental growth factor.

5.4.1.2 Prevalent lacunes and microbleeds at baseline

In logistic regression models adjusted for age, sex, vascular risk factors, and stroke subtype, PDGFRβ and Endo-1 were associated with decreased odds of lacunes (OR per 1 SD increase in PDGFRβ=0.71, 95% CI:0.50, 0.99; OR per 1 SD increase in Endo-1=0.71, 95% CI:0.49, 0.99). ICAM-1 (OR per 1 SD increase in ICAM-1=1.26, 95% CI:0.91, 1.77), E-Selectin (OR per 1 SD increase in E-Selectin=1.15, 95% CI:0.82, 1.67) and vWF (OR per 1 SD increase in vWF=1.14, 95% CI:0.81, 1.64), showed possible associations with more lacunes at baseline, whilst VEGF (OR per 1 SD increase in VEGF=1.27, 95% CI:0.86, 1.86), and PDGF-BB (OR per 1 SD increase in PDGF-BB=1.06, 95% CI:0.69 1.61), showed potential links to more microbleeds (**Figure D- 4 & Table D- 7 in Appendix D**).

5.4.1.3 Incident DWI-positive lesions longitudinally

Results from logistic regression adjusted for age, sex, vascular risk factors, and stroke subtype, revealed a significant association between higher VEGF concentrations and a 1.64-fold increased odds of incident DWI-positive lesions identified between baseline and one year follow up (OR per 1 SD increase in VEGF=1.64; 95% CI:1.04, 2.62). In unadjusted analyses, P-Selectin was associated with increased odds of incident lesions (OR per 1 SD increase: 1.48; 95% CI:1.006, 2.37). In adjusted analyses, we also observed possible trends between higher levels of P-Selectin (OR per 1 SD increase in P-Selectin=1.49; 95% CI:0.99, 2.35), PDGF-BB (OR per 1 SD increase in PDGF-BB =1.40; 95% CI:0.85, 2.32), PlGF (OR per 1 SD increase in PlGF =1.34; 95% CI:0.83, 2.12), and ICAM-1 (OR per 1 SD increase in ICAM-1 =1.27; 95% CI:0.84, 1.93), and increased odds of incident DWI-positive lesion detection (**Figure 5-4 & Table D- 8 in Appendix D).**

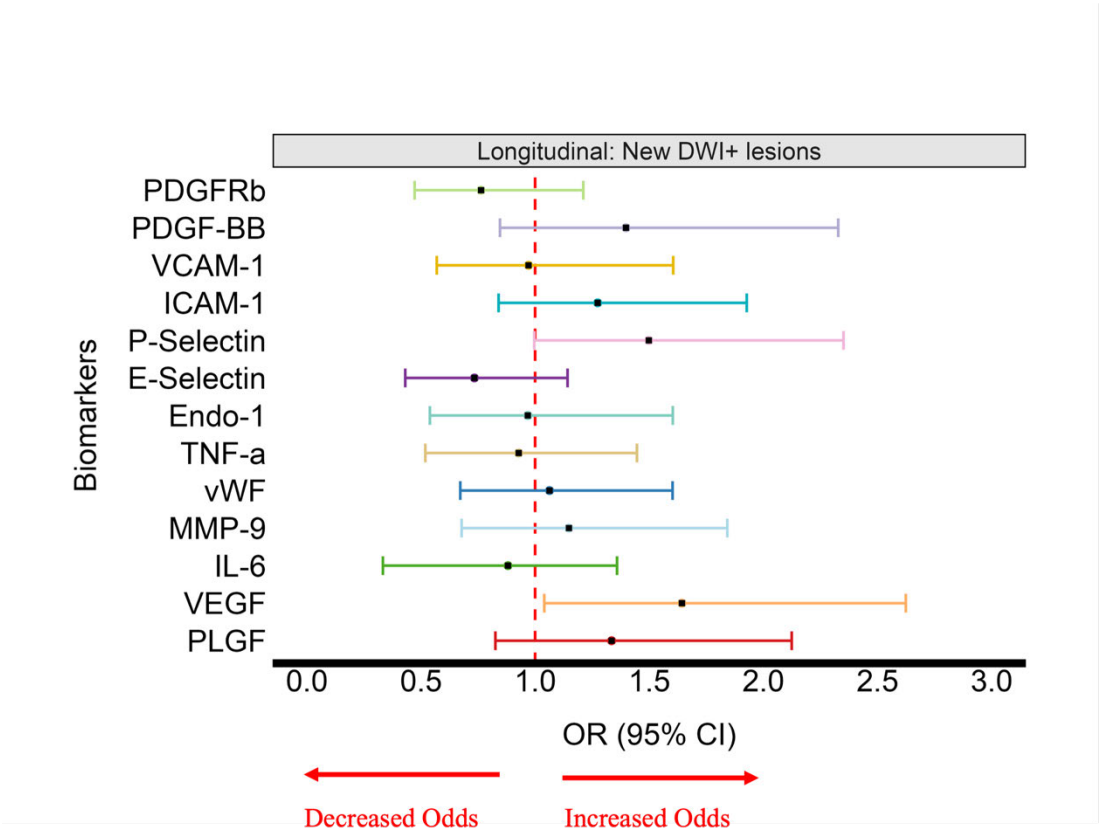


Figure 5-4. Blood biomarkers and newly appearing incident lesions (DWI-positive) over one year. Logistic regression results for the association of distinct standardised blood biomarkers to the prevalence of DWI-positive incident lesions identified throughout the one-year follow-up period after adjusting for age, sex, vascular risk factors, stroke subtype, and baseline NIHSS. Abbreviations: DWI: diffusion-weighted imaging; NIHSS: The National Institutes of Health Stroke Scale; CI: confidence interval; PDGFRβ: platelet derived growth factor receptor beta; PDGF-BB: platelet derived growth factor subunit B; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; E-selectin: endothelial-selectin; P-Selectin: platelet-selectin; Endo-1: Endothelin-1; TNF-α: tumour necrosis factor alpha; vWF: von Willebrand Factor; MMP-9: matrix metalloproteinase 9; IL-6: Interleukin 6; VEGF: vascular endothelial growth factor; PlGF: placental growth factor.

5.4.1.4 Summary SVD scores at baseline

In unadjusted analyses, both VCAM-1 (OR per 1 SD increase: 1.31; 95%CI: 1.02, 1.69) and ICAM-1 (OR per 1 SD increase: 1.32; 95%CI: 1.01, 1.72) were significantly associated with increased SVD summary score. Results from ordinal regression adjusted for age, sex, vascular risk factors, and stroke

subtype, indicate a possible trend between elevated concentrations of ICAM-1 (OR per 1 SD increase in ICAM-1=1.18; 95% CI:0.89, 1.57), PDGF-BB (OR per 1 SD increase in PDGF-BB=1.17; 95% CI:0.88, 1.57) and E-selectin (OR per 1 SD increase in E-Selectin=1.11; 95% CI:0.86, 1.47) and summary SVD burden observed on MRI, encompassing WMH, PVS, lacunes, and microbleeds (**Figure D- 5 & Table D- 9 in Appendix D**).

5.4.1.5 Blood-brain barrier permeability at baseline

In cross-sectional linear regression models adjusted for age, sex, vascular risk factors, and baseline WMH volume, we observed possible associations between higher concentrations of VCAM-1, ICAM-1, vWF, TNF-α, PlGF, and Endo-1 and greater BBB permeability (PS), with some variation dependent on tissue type (NAWM, basal ganglia, and WMH) (**Figure 5-5 & Table D- 10 in Appendix D**). For instance, in NAWM, levels of PlGF were significantly associated with increased BBB permeability (β=0.19, 95% CI:0.03, 0.35), whilst in the basal ganglia, VCAM-1 (β=0.03, 95% CI:0.04, 0.54), TNF-α (β=0.25, 95% CI:0.03, 0.47), and vWF (β=0.26, 95% CI:0.00, 0.51), levels were significantly associated with increased BBB permeability. Interestingly, we observed a possible association between higher serum levels of PDGF-BB and reduced BBB permeability in NAWM (β=-0.12, 95% CI:-0.29, 0.04), the basal ganglia (β=-0.06, 95% CI:-0.27, 0.15), and WMH (β=-0.12, 95% CI:-0.38, 0.15). Increased ICAM-1 and PDGF-BB concentrations showed possible associations with increased vascular density through V_p quantification (**Table D- 11 in Appendix D**).

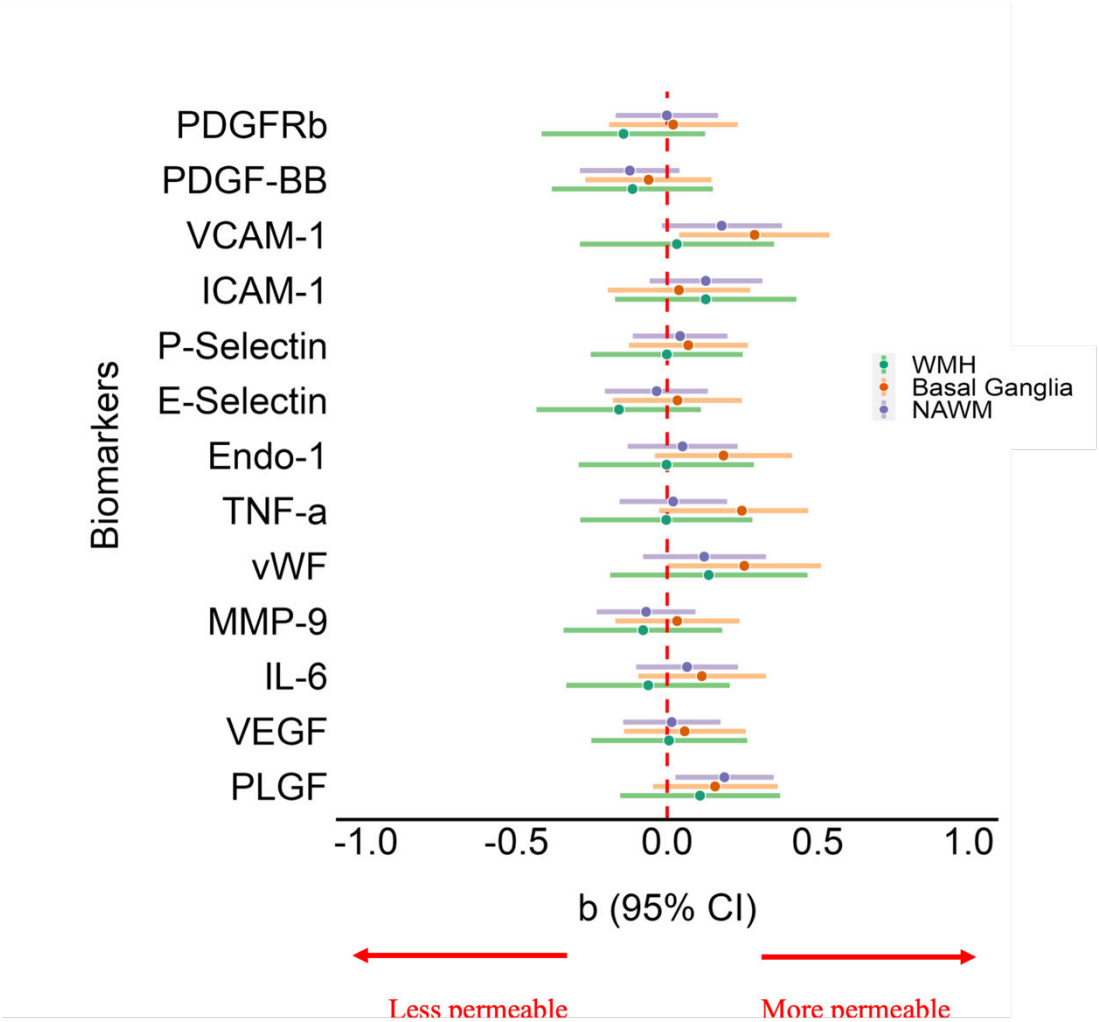


Figure 5-5. Blood biomarkers and BBB permeability (PS) from DCE-MRI. Linear regression results for the association of distinct standardised blood biomarkers to baseline DCE-MRI parameter PS (x10⁴) after adjusting for age, sex, vascular risk factors, and baseline WMH volume as % ICV. Abbreviations: BBB: blood-brain barrier; DCE-MRI: dynamic contrast-enhanced magnetic resonance imaging; PS: Permeability-Surface area product; WMH: white matter hyperintensity; ICV: intracranial volume; CI: confidence interval; PDGFRβ: platelet derived growth factor receptor beta; PDGF-BB: platelet derived growth factor subunit B; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; E-selectin: endothelial-selectin; P-Selectin: platelet-selectin; Endo-1: Endothelin-1; TNF-α: tumour necrosis factor alpha; vWF: von Willebrand Factor; MMP-9: matrix metalloproteinase 9; IL-6: Interleukin 6; VEGF: vascular endothelial growth factor; PlGF: placental growth factor.

5.4.1.6 Cerebrovascular reactivity at baseline

Higher circulating levels of ICAM-1 were significantly associated with lower CVR at baseline, particularly in the basal ganglia (β=-0.01, 95% CI:-0.02, -0.001) after adjusting for age, sex, vascular risk factors, and baseline WMH volume (**Figure 5-6 & Table D- 12 in Appendix D**). We also observed a possible association between higher levels of ICAM-1 and lower CVR in NAWM (β=-0.002, 95% CI:-0.004, 0.001) and WMH (β=-0.002, 95% CI:-0.010, 0.004), as well as increased VCAM-1 levels and lower CVR in all tissue types (NAWM: β=-0.001, 95% CI:-0.004, 0.001; basal ganglia: β=-0.01, 95% CI:-0.01, 0.004; WMH: (β=-0.01, 95% CI:-0.02, -0.001).

Conversely, higher levels of PDGF-BB and Endo-1 were significantly associated with higher CVR, particularly in the basal ganglia (β=0.01, 95% CI:0.003, 0.02; β=0.01, 95% CI:0.001, 0.02, respectively).

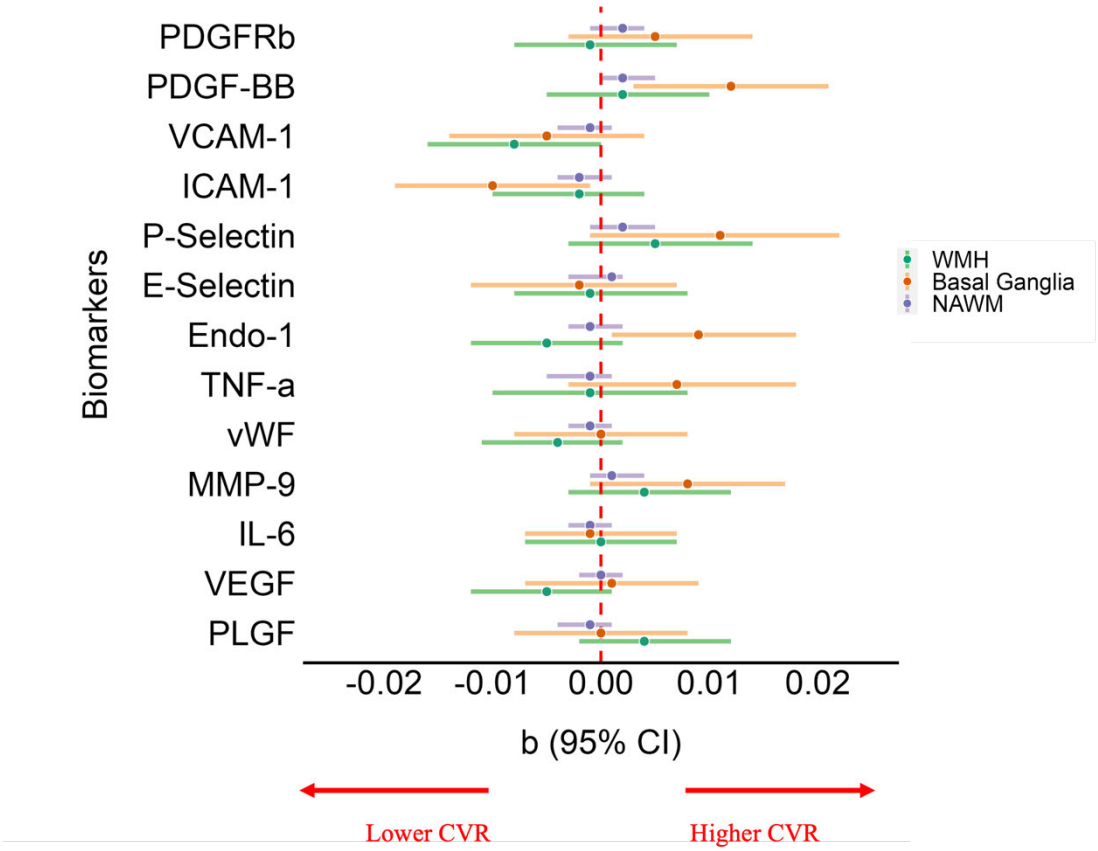


Figure 5-6, Blood biomarkers and CVR. Linear regression results for the association of distinct standardised blood biomarkers to baseline DCE-CVR in NAWM, the basal ganglia and WMH after adjusting for age, sex, vascular risk factors, and baseline WMH volume as % ICV. Abbreviations: CVR: cerebrovascular reactivity; NAWM: normal appearing white matter; WMH: white matter hyperintensity; ICV: intracranial volume; CI: confidence interval; PDGFRβ: platelet derived growth factor receptor beta; PDGF-BB: platelet derived growth factor subunit B; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; E-selectin: endothelial-selectin; P-Selectin: platelet-selectin; Endo-1: Endothelin-1; TNF-α: tumour necrosis factor alpha; vWF: von Willebrand Factor; MMP-9: matrix metalloproteinase 9; IL-6: Interleukin 6; VEGF: vascular endothelial growth factor; PlGF: placental growth factor.

5.4.2 Cognitive Outcomes

5.4.2.1 Montreal Cognitive Assessment scores and Mild Cognitive Impairment

Longitudinal linear mixed effects models adjusted for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume suggest a plausible association between higher concentrations of VCAM-1 (β=-0.32, 95% CI:-0.76, 0.12), Endo 1 (β=-0.18, 95% CI:-0.56, 0.21), VEGF (β=-0.16, 95% CI:-0.55, 0.23) and PlGF (β=-0.15, 95% CI:-0.54, 0.25) and lower MoCA scores. Cross-sectional analyses examining baseline MoCA scores showed comparable results (**Figure 5-7 & Table D- 13 in Appendix D**).

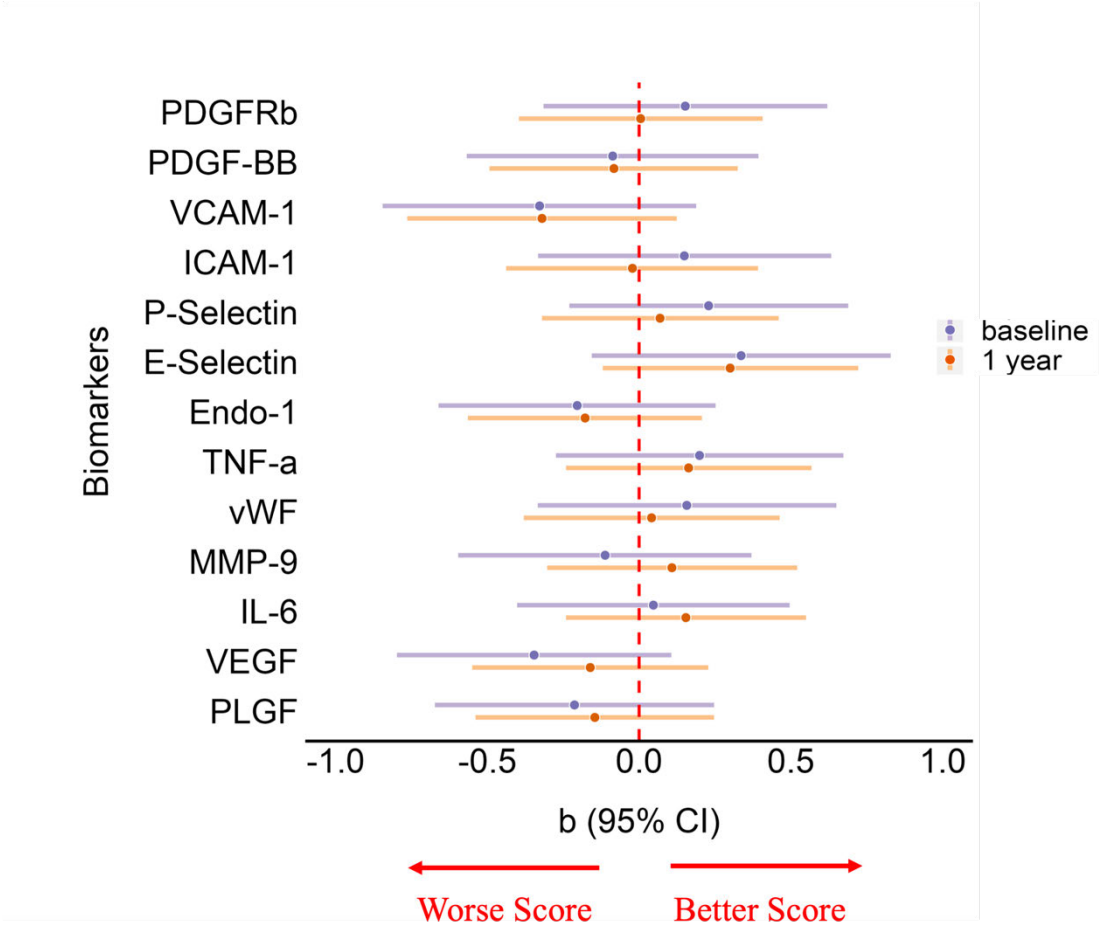


Figure 5-7. Blood biomarkers and MoCA score. Baseline cross-sectional linear regression and longitudinal linear mixed effects model results for the association of distinct standardised blood biomarkers and MoCA score after adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume as % ICV. Abbreviations: MoCA: Montreal Cognitive

Assessment; WMH: white matter hyperintensity; ICV: intracranial volume; CI: confidence interval; PDGFRβ: platelet derived growth factor receptor beta; PDGF-BB: platelet derived growth factor subunit B; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; E-selectin: endothelial-selectin; P-Selectin: platelet-selectin; Endo-1: Endothelin-1; TNF-α: tumour necrosis factor alpha; vWF: von Willebrand Factor; MMP-9: matrix metalloproteinase 9; IL-6: Interleukin 6; VEGF: vascular endothelial growth factor; PlGF: placental growth factor.

At baseline, 36% of patients had normal cognition, whilst 63% qualified for MCI. At one year follow-up, 53% of patients had normal cognition and 46% qualified for MCI or dementia. After adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume, we found a significant relationship between higher concentrations of P-Selectin (OR per 1 SD increase in P-Selectin=1.65; 95% CI:1.05, 2.72) and increased risk of MCI at 1 year as well as higher levels of IL-6 (OR per 1 SD increase in IL-6=0.39; 95% CI:0.07, 0.94) and a reduced risk of MCI at 1 year. In unadjusted analyses, both VCAM-1 and vWF were significantly associated with increased risk of MCI, both at baseline and 1-year (VCAM-1: baseline OR: 1.95; 95%CI: 1.39, 2.95; 1 year OR: 2.01; 95%CI: 1.41, 2.98 and vWF: baseline OR: 1.89; 95%CI:1.26, 3.04; 1 year OR: 1.67; 95%CI: 1.19, 2.46).

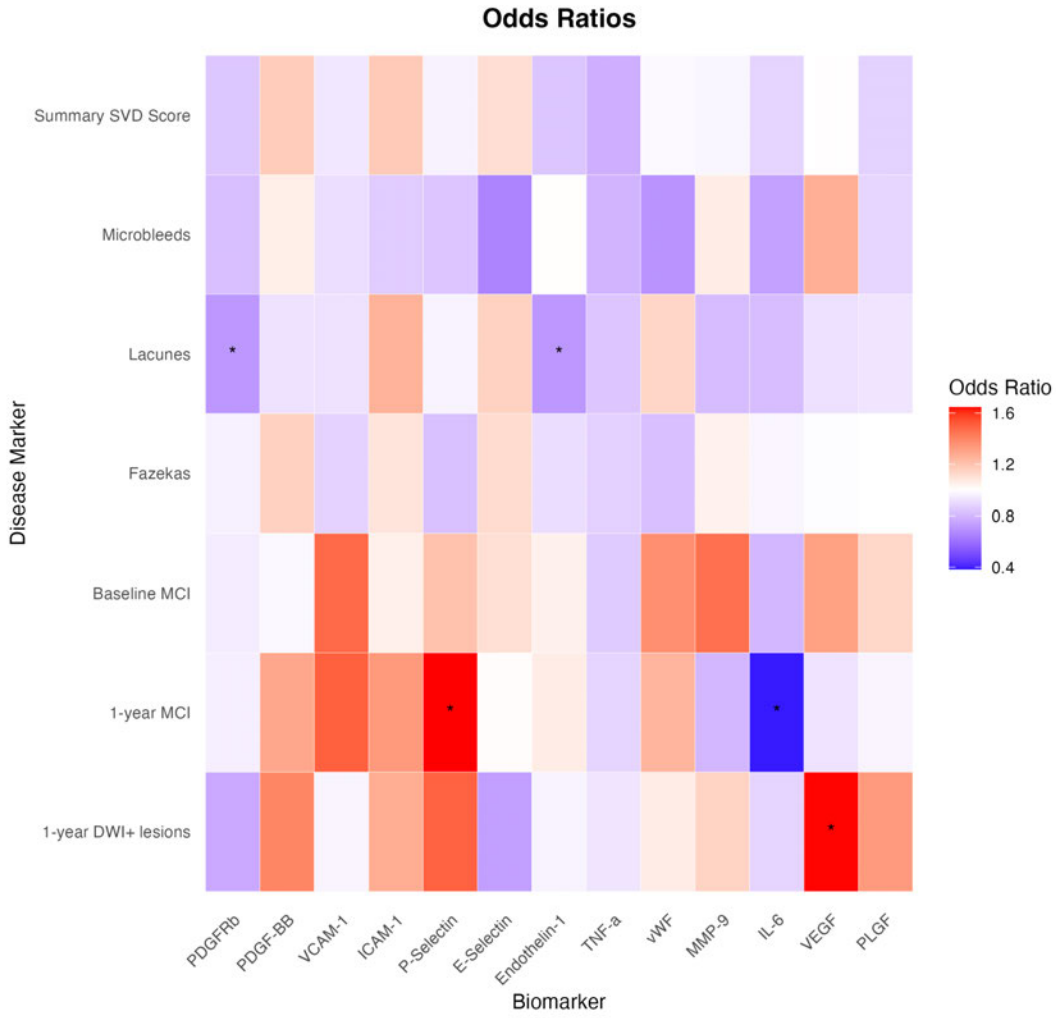
We also observed potential associations between increased risk of MCI prevalence and higher concentrations of PDGF-BB (1 year: OR per 1 SD increase in PDGF-BB=1.29; 95% CI:0.90, 1.90), VCAM-1 (baseline: OR per 1 SD increase in VCAM-1=1.48; 95% CI:0.96, 2.37 ; 1 year: OR=1.50; 95% CI:0.98, 2.40), ICAM-1 (1 year: OR per 1 SD increase in ICAM-1=1.34; 95% CI:0.90, 2.04), vWF (baseline: OR per 1 SD increase in vWF=1.37; 95% CI:0.90, 2.26; 1 year: OR=1.25; 95% CI:0.84, 21.94), MMP-9 (baseline: OR per 1 SD increase in MMP-9=1.46; 95% CI:0.98, 2.28), VEGF (baseline: OR per 1 SD increase in VEGF=1.31; 95% CI:0.93, 1.89), and PlGF (baseline: OR per 1 SD increase in PlGF=1.14; 95% CI:0.80, 1.63) (**Figure D- 6 & Table D-14 in Appendix D**).

5.4.2.2 Executive function: Trail Making Test ratio (B/A)

We examined possible associations between executive function, as measured by the TMT (B/A) ratio, and circulating biomarkers, both cross-sectionally at baseline, and longitudinally using linear mixed-effects models. After adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume, we observed possible associations between higher levels of Endo-1 (baseline: β=0.11, 95% CI:-0.06, 0.28; longitudinally: β=0.14, 95% CI:-0.01, 0.30), TNF-α (baseline: β=0.14, 95% CI:-0.04, 0.32; longitudinally: β=0.08, 95% CI:-0.07, 0.23), vWF (baseline: β=0.13, 95% CI:-0.05, 0.32; longitudinally: β=0.19, 95% CI:0.03, 0.35), and IL-6 (baseline: β=0.11, 95% CI:-0.06, 0.27; longitudinally: β=0.05, 95% CI:-0.10, 0.20), and worse executive function (**Figure D- 7 & Table D- 15 in Appendix D**). Results also suggested potential associations between higher levels of VEGF and PlGF and better executive function, both at baseline (β=-0.16, 95% CI:-0.33, 0.01 and β=-0.19, 95% CI:-0.36, -0.02, respectively) and longitudinally (β=-0.15, 95% CI:-0.30, 0.01 and β=-0.10, 95% CI:-0.25, 0.05, respectively).

5.4.3 Discussion

We performed cross-sectional and one-year longitudinal adjusted analyses relating 13 blood biomarkers of endothelial, vascular, and pericyte function, to MRI markers of SVD, including WMH, lacunes, microbleeds, summary SVD score, BBB permeability and CVR, as well as cognition and MCI prevalence in a minor stroke cohort enriched for SVD (**Figure 5-8**).


Odds Ratios
Summary SVD Score
Microbleeds
Lacunes
Fazekas
Baseline MCI
1-year MCI
1-year DWI+ lesions
Disease Marker
PDGFRb
PDGF-BB
VCAM-1
ICAM-1
P-Selectin
E-Selectin
Endothelin-1
TNF-a
vWF
MMP-9
IL-6
VEGF
PLGF
Biomarker
Odds Ratio
1.6
1.2
0.8
0.4

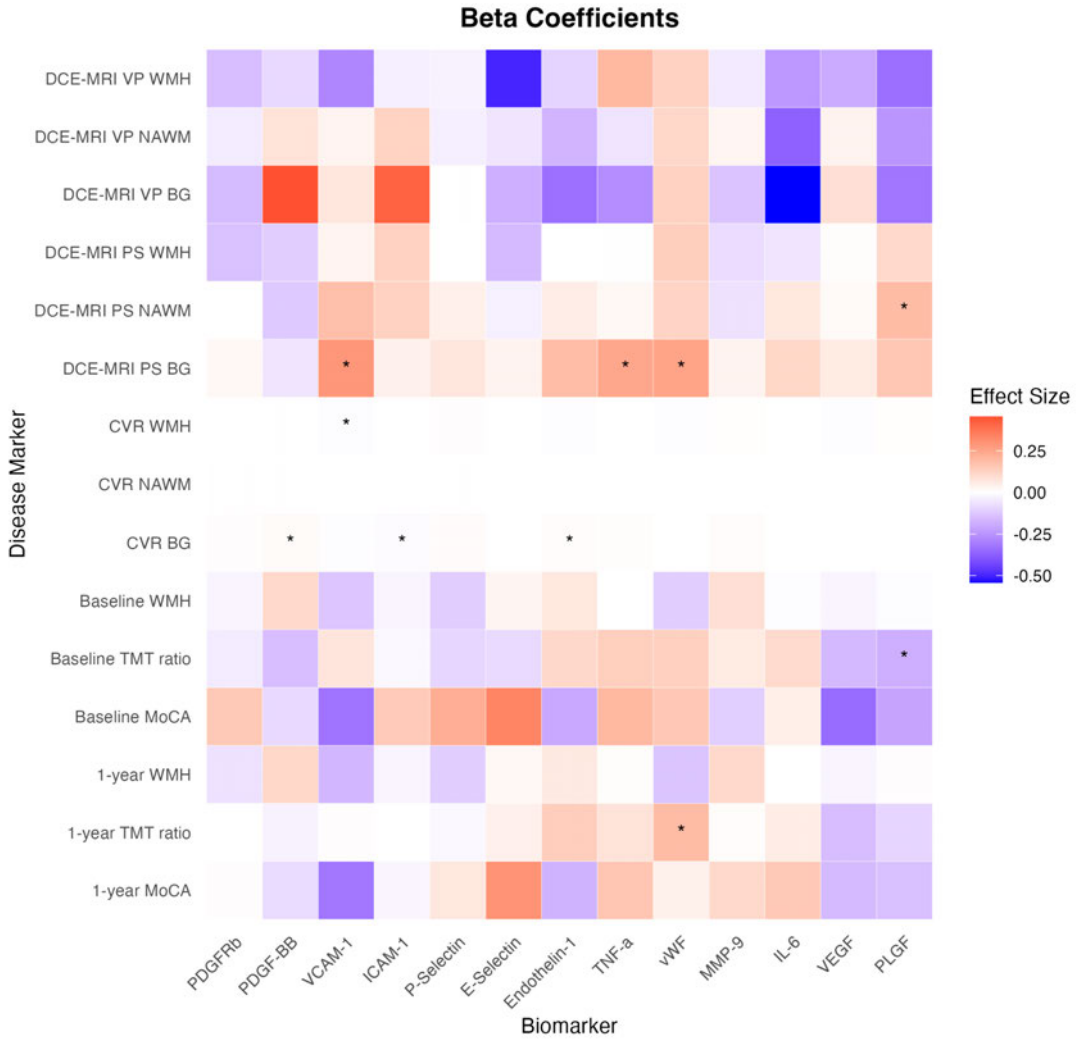


Figure 5-8. Relationship between 13 biomarkers and 24 markers of disease grouped by effect type. The first panel illustrates associations reported as odds ratios from logistic regression models, and the second shows associations reported as beta coefficients from linear regression models. intensity reflects the strength of the relationship, with darker hues representing stronger effects. Blue shades indicate negative associations, whilst red shades represent positive associations. Significance is indicted by asterisks in the case where the confidence interval excludes 0 (for beta coefficients) or 1 (for odds ratios). Abbreviations: DCE-MRI: dynamic contrast-enhanced magnetic resonance imaging; V_p: blood plasma volume fraction; WMH: white matter hyperintensity; NAWM: normal appearing white matter; BG: basal ganglia; PS: Permeability-Surface area product; CVR: cerebrovascular reactivity; TMT: Trail Making Task; MoCA: Montreal cognitive Assessment; PDGFRβ: platelet derived growth factor receptor beta; PDGF-BB: platelet derived growth factor subunit B; VCAM-1: vascular cell adhesion molecule 1; ICAM-1: intracellular adhesion molecule 1; E-selectin:

endothelial-selectin; P-Selectin: platelet-selectin; Endo-1: Endothelin-1; TNF-α: tumour necrosis factor alpha; vWF: von Willebrand Factor; MMP-9: matrix metalloproteinase 9; IL-6: Interleukin 6; VEGF: vascular endothelial growth factor; PLGF: placental growth factor; SVD: small vessel disease; MCI: mild cognitive impairment; DWI: diffusion-weighted imaging.

We found a novel significant association between circulating VEGF levels and increased odds of incident DWI-positive lesions throughout the one-year follow-up period. VEGF and PlGF are angiogenic factors implicated in endothelial cell proliferation and survival and microvascular permeability.[366] There is evidence suggesting that angiogenic signalling alterations and subsequent blood flow changes are key features of endothelial dysfunction in ageing and could contribute to inflammation and BBB permeability, possibly by uncoupling endothelial cell junctions.[367,368] Results from recent studies are inconsistent. In the Framingham offspring cohort, no associations were reported between VEGF and WMH burden, microbleeds or lacunes.[369] Other studies report links between VEGF and WMH burden, lacunes, and greater global SVD disease burden, both in acute ischaemic stroke patients and in cognitively-unimpaired elderly community dwellers.[325,370] Results from the ongoing MarkVCID consortium demonstrated strong associations between PlGF and WMH burden and cognitive impairment, although associations with executive function were modest.[371] In our study, VEGF and PlGF concentrations showed possible associations with lower MoCA scores, and increased odds of MCI at baseline and incident DWI-positive lesions, however, we found no overt relationship with WMH burden, and, interestingly, both VEGF and PlGF levels showed potential positive associations with better executive functioning. PlGF also showed an association with higher BBB permeability in NAWM whilst VEGF was possibly linked to the presence of microbleeds.

We found significant associations between higher concentrations of VCAM-1 and increased BBB permeability in the basal ganglia, ICAM-1 and lower CVR, and P-Selectin and an increased risk of MCI at 1 year. Cell adhesion molecules

and selectins, VCAM-1, ICAM-1, P-Selectin, and E-Selectin, promote the recruitment, rolling, adhesion, and trans-endothelial migration of leukocytes through vascular walls. They are constitutively expressed at low levels in the brain vasculature in a homeostatic state and their increased expression can indicate endothelial activation.[170] We observed trends between higher ICAM-1 levels and lacune and incident DWI-positive lesion prevalence as well as higher summary SVD score, BBB permeability and higher likelihood of MCI at one year. Our results support those reported in the Framingham cohort study, where ICAM-1 levels were associated with higher WMH volume and silent brain infarcts.[369] In our study, increasing VCAM-1 concentrations showed possible associations with BBB breakdown and worse cognition, yet a lower WMH burden. Previous studies report conflicting results.[152] VCAM-1 concentrations were elevated in lacunar stroke patients compared to hypertensive and healthy controls, and higher concentrations correlated with poorer cognition and higher WMH burden.[147,372] The Rotterdam cohort study reported no relationship between ICAM-1 or VCAM-1 levels and dementia diagnosis.[373] In our study, E-Selectin levels showed potential associations with worse WMH burden, higher summary SVD score, and higher baseline lacune and MCI prevalence. P-Selectin showed possible associations with DWI-positive lesion prevalence and MCI incidence, though not WMH burden. Whilst some studies report higher circulating levels of selectins in SVD compared to controls, others report no differences.[374,375] In a 10-month follow-up study, E-Selectin was associated with SVD progression.[327] Likewise, those with SVD radiological disease progression over two years had a higher compound endothelial activation score, including P-Selectin.[376]

We found possible associations between biomarkers of pericyte function and cognitive impairment and SVD MRI markers. Brain pericytes rely on PDGF-BB and PDGFRβ signalling to maintain the BBB.[86,109] PDGF-BB is secreted by the endothelium and recruits pericytes to endothelial cells, whilst its receptor, PDGFRβ, is enriched in pericytes. Increased soluble PDGFRβ secretion is thought to indicate pericyte injury and has been proposed as a BBB breakdown biomarker.[53,111–113] Increased CSF concentrations of PDGFRβ have been

implicated in MCI and BBB dysfunction in SVD and Alzheimer's Disease (AD).[53,114,115] In our study, PDGF-BB trended towards an association with higher WMH burden, increased incident DWI-positive lesions, microbleeds, higher global SVD burden, lower cognition, and a higher risk of MCI. Interestingly, circulating levels of PDGFRβ showed possible associations with lower WMH burden, lower MCI risk, and higher MoCA scores. An interaction between these molecules throughout different disease stages, perhaps even in a compensatory capacity through the attempt to increase pericyte recruitment as a response to pericyte loss, is conceivable. In animal and *in vitro* investigations, PDGF-BB was overexpressed in vascular sections with high wall shear stress and was implicated in inciting an inflammatory response and smooth muscle cell dedifferentiation via PDGFRβ signalling mechanisms.[377,378] Alternatively, it remains possible that quantifying these markers in the circulating blood does not serve as a reliable proxy for brain pericyte (dys)function; further examination, ideally with CSF samples, is warranted.

We observed a significant association between vWF and BBB permeability, and possible associations with worse cognition, increased odds of lacunes at baseline, and lower WMH burden. vWF secretion can be incited by inflammation and is involved in thrombus formation and platelet adhesion. In wild-type mice, vWF increased BBB permeability by inhibiting the expression of tight junction, claudin-5.[379] Although several studies report higher circulating levels of vWF in recent lacunar stroke patients compared to controls, others suggest these levels normalise past the acute-stroke stage or are comparable to patients of non-lacunar ischaemic stroke.[375,380,381] One study found no relationship between vWF levels and WMH or lacunes on CT, however, only a small proportion of patients presented with lacunar stroke (12%), and WMH burden was low.[370] There are possible explanations for the inverse relationship between WMH burden and vWF. Whilst high levels of vWF are associated with vascular inflammation and stroke, it has also been implicated in the promotion of endothelial integrity;[379,382] lower vWF levels have been associated with increased PVS burden.[383] Some studies suggest vWF can be differentiated

between an active and latent form, which may play diverging roles in haemostasis.[384] Regulating endothelial cell-mediated vWF secretion could serve as a potential therapeutic target, though further examination into its role in modulating endothelial and BBB integrity, particularly as it relates to an acute versus chronic response to injury or disease, is required.

The strengths of this study lie in its longitudinal aspect and the comprehensive range of disease-related markers and outcomes, which are rarely available in larger cohorts. These include vascular function MRI markers, such as CVR and BBB permeability, alongside multiple radiological indicators of disease and global SVD burden quantification to effectively capture the evolving radiological landscape of SVD. Moreover, including cortical stroke patients with varying levels of SVD, alongside conducting serology outside the acute stroke phase, serves to minimise potential confounding effects related to acute ischaemic injury. We have also accounted for clinical and vascular risk factor data within our models as well as included unadjusted analyses and results. Unadjusted analyses show the direct relationship between blood biomarkers and disease markers without the influence of covariates, which helps determine whether an association exists before considering potential confounders. Comparing unadjusted and adjusted models thus reveals the extent to which covariates may mediate or influence the relationship. For example, the significant association between elevated VCAM-1 levels and higher radiological SVD summary score becomes non-significant after adjusting for covariates. This suggests that the covariates may mediate the relationship.

Our study has several limitations. Our relatively small sample size was predominantly of European descent, limiting the generalisability of our findings. Although our study is longitudinal, a one-year period might not be sufficient to capture relationships between biomarkers and disease progression; additionally, serology was only performed at baseline, and we cannot make inferences about the stability of analytes examined over time. We adjusted for stroke subtype and included cortical strokes as a control group since they

receive the same secondary prevention drugs as do lacunar stroke patients. However, ways in which large-artery atherosclerotic disease mechanisms may influence vascular function biomarkers despite representing distinct pathological processes, remains unclear. Because all patients received routine secondary prevention medication, we did not include medication use in our models. Although effects will likely be similar across the population, how they may affect biomarkers is unknown. Participants were not excluded for concurrent infection, malignancy, or rheumatological disease, which might affect the interpretation of our results. We relied on ELISA for biomarker quantification, acknowledging that different serology techniques could produce different results with varying levels of accuracy. Notably, our approach does not always facilitate the differentiation of diverse biomarker isoforms, despite the potential for these to have opposing roles.[385] We primarily used serum samples for our analyses, with plasma substituting less than 4% of the samples; whilst testing and using separate dilutions can mitigate some variability, inherent differences between the two matrices may affect results. Importantly, the extent to which these biomarkers accurately reflect dysfunction confined to the brain rather than systemic dysfunction, and the degree to which endothelial/pericyte dysfunction in SVD is indeed confined to the brain, remains unknown. Lastly, this study was exploratory in nature, and replication in external samples will be necessary to derive more robust conclusions. Given that the field is still in early stages (compared with the Alzheimer's field for example), it is essential to disseminate comprehensive analyses and findings to foster scientific progress. Whist we agree that larger, more definitive studies are ultimately needed, incremental contributions like ours provide necessary context and direction for future work. If underpowered studies or those with nuanced findings are unpublished or relegated to abbreviated formats, there is a risk that valuable methodological insights and data may be lost, and confirmatory studies will be hampered.

Given the complexity and heterogeneity of SVD mechanisms and manifestations, it is reasonable to consider that a panel of biomarkers, rather than a sole molecule or pathway, is more likely to yield a comprehensive and

robust diagnostic and prognostic tool in future. The aim of these analyses was to examine the relationship between specific biomarkers that reflect known cells or processes and cross-sectional and longitudinal imaging and clinical variables. Here, we are using the biomarkers to explore potential disease mechanisms and not (at this stage) to perform an agnostic, hypothesis free, analysis as might be performed on a large panel of biomarkers in the hope that this might provide reliable outcome predictors for clinical diagnosis and prognosis. Whilst larger, more definitive studies are ultimately needed to corroborate our findings, incremental contributions like ours provide the necessary context and direction for future work, including determining which biomarkers are of most interest, providing data for meta-analyses, and estimates for sample size calculations.

A major challenge impeding the advancement of therapeutics in SVD arises from the absence of elucidative and specific biomarkers associated with disease. Establishing disease-specific biomarkers could facilitate efforts to uncover pathophysiology and develop potential therapeutic interventions and evaluate their effectiveness. These novel findings from a human stroke population enriched for SVD can help identify key candidate biomarkers for further investigation in SVD research.

In clinical settings, biomarkers could serve as accessible and specific diagnostic and prognostic tools. We found a novel association between circulating VEGF levels and increased incident DWI-positive lesions throughout the first year post-stroke as well as associations between higher levels of P-Selectin and increased risk for MCI at 1 year and ICAM-1 and lower CVR. If validated in larger populations, these findings could have major clinical implications in stroke clinics, emergency departments, and clinical trial design by facilitating the risk-stratification of patients who may be at a higher risk of recurrent stroke and rapid-progression SVD.

5.5 Concluding Remarks

In this chapter, I have presented findings from our study examining blood biomarkers of vascular dysfunction and SVD burden and progression in a highly phenotyped cohort of mild ischaemic stroke patients. In these exploratory analyses, I focussed on identifying patterns or trends across biologically-plausible vascular disease and function markers, and posit that there are some results worth further investigation in independent studies and highlight how some seem more promising than others.

In the next chapter, **Chapter 6**, I take a broader perspective to contextualise this research within the larger framework of SVD. Here, I will explore the burden of mild stroke and SVD by analysing 8.5-year longitudinal outcomes and their impact on patients from the cohort that preceded the one examined in the current chapter, the Mild Stroke Study II.

Chapter 6 7-9-year longitudinal outcomes of mild lacunar and cortical ischaemic stroke: dementia incidence, mortality, recurrent stroke, and functional well-being

6.1 Context

In this chapter, I broaden the scope to examine and compare the clinical and functional longitudinal outcomes of lacunar and mild cortical ischaemic stroke 7-9 years post-stroke. The content of this chapter has been prepared for publication.

6.2 Introduction

Cerebral small vessel disease (SVD) is a highly prevalent disorder of the cerebral microvessels. It is a leading cause of vascular dementia and cognitive impairment, and is associated with lacunar or haemorrhagic stroke, mobility problems, and neuropsychiatric symptoms[1,3,6,8,386]. Lacunar strokes account for a quarter of all ischaemic strokes and are distinctive in their symptomatology[387]. Lacunar infarcts are small (<15 mm in diameter) and occur in subcortical regions such as the basal ganglia, thalamus, internal capsule, and brainstem. They result from the occlusion of a single perforating artery and intrinsic small vessel pathology[388].

Compared to other stroke subtypes, lacunar stroke is generally considered to have a favourable prognosis, characterised by small infarct size, minimal risk of acute mortality, a low rate of early recurrent stroke, and typically non-disabling effects in the initial months following ischaemia. However, the long-term prognosis of lacunar infarction remains less well characterised compared to other stroke subtypes.

Research on prognostic outcomes in lacunar stroke patients is limited, with most studies featuring relatively small sample sizes and short follow-up periods, rarely extending beyond four years[389]. Additionally, most studies often lack patient-reported measures of well-being, and long-term disability and dependence, and given that lacunar strokes tend to occur at slightly younger ages, have high survival rates, and cognitive impairment is a frequent concern for these patients, it is important to consider prognosis within a broader context[9]. Notably, patients with SVD have expressed concerns regarding the long-term prognosis of their condition, often highlighting a lack of sufficient information from healthcare providers regarding their diagnosis and expected clinical trajectory[390]. Longitudinal data on the outcomes of lacunar stroke, and how they might compare to mild cortical ischaemic stroke, can offer valuable insights to help guide clinical management and inform research priorities.

Here, we report longitudinal data on clinical outcomes, including dementia, recurrent stroke, and death, and participant-reported functional and cognitive outcomes in a prospective study of participants with lacunar ischaemic stroke and those with similarly mild cortical ischaemic stroke at 7-9 years post-stroke.

6.3 Methods

6.3.1 Participants and Study Design

We prospectively recruited 264 patients who presented with acute minor and expected to be nondisabling, ischaemic stroke to stroke services in Edinburgh, UK between May 2010 and May 2012. Protocol details have been described previously[360,391]. Minor ischaemic stroke was defined as a focal onset of neurological symptoms lasting more than 24-hours with no alternative explanation, a NIH Stroke Scale (NIHSS) score <8, and an expected outcome of independence (modified Rankin Scale (mRS)) score <3. All patients were dementia-free at recruitment and received secondary stroke prevention treatment in line with UK clinical guidelines. We excluded patients who did not have a confirmed stroke diagnosis, whose symptoms resolved within 24-hours,

were unable to consent, had a disabling stroke, had a medical condition with a life expectancy of less than one year, or had contraindications to MRI. All participants provided written informed consent. This study was approved by Lothian Ethics Medical Research Committee (REC 09/81,101/54) and NHS Lothian R&D Office (2009/W/NEU/14).

6.3.2 Baseline assessments and, 1-, 3-, and 7-9-year follow-up

At baseline, we assessed sociodemographic variables, vascular risk factors, smoking status, NIHSS score, functional dependency (mRS), cognition (Addenbrooke Cognitive Examination-Revised (ACE-R))[392], premorbid intelligence (National Adult Reading Test (NART))[393], ischaemic heart disease (IHD) diagnosis, and brain MRI. Vascular risk factors included hypertension (defined as blood pressure of ≥140/90 mmHg on presentation or a previous diagnosis), hypercholesterolaemia (defined as cholesterol >5 mmol/L on presentation or a previous diagnosis), diabetes mellitus (diagnosed on admission or a previous diagnosis in accordance with the World Health Organisation criteria), and atrial fibrillation. Cognitive testing did not start until after the first 56 patients had been recruited due to delays in obtaining ethics approval

Participants were invited for a follow-up visit at 1- and 3-years post-stroke which included a face-to-face assessment where possible, and a review of hospital and medical records. We rated disability using the mRS, assessed cognitive function using the ACE-R, and collected information on recurrent vascular events or any further clinical events of note, including recurrent stroke, transient ischaemic attack (TIA), intracranial haemorrhage (ICH), myocardial infarction (MI), IHD, and new diagnosis of vascular risk factors since the previous visit. In instances where a patient was unable to attend an in-person study visit, home visits or telephone interviews were offered. Where participants did not respond, further information was sought from the GP and centralised medical records. In cases where patients had died, cause of death was obtained from hospital records and death certificates.

At 7-9 years post-stroke, we used multiple overlapping ascertainment methods, including central medical records, and self-reported and GP-derived data, to reassess adverse vascular events including dementia diagnosis, recurrent stroke incidence, deaths, cardiac events/diagnoses, and new vascular risk factor diagnoses. We sent a brief questionnaire to participants known to be alive to assess disability and dependence using the mRS and evaluate several aspects of function, cognition, subjective well-being, mobility, dexterity, and stroke recovery, and activities of daily living using items from the Stroke Impact Scale (SIS)[394]. We also collected data on participants' current living arrangement (living independently/with family/in a residential care home/assisted living facility).

6.3.3 Imaging and Stroke Diagnosis

At baseline, patients underwent brain MRI on a 1.5 Tesla MRI (Signa LX; General Electric, Milwaukee, WI). The index infarct was classified as a recent small subcortical infarct (RSSI) or a cortical infarct based on its size, location, shape, and age in relation to stroke presentation. Stroke diagnosis was confirmed and subtyped as either 'cortical' or 'lacunar' using the Oxfordshire Community Stroke Project Classification by an expert panel of neurologists, stroke physicians, and neuroradiologists based on the clinical stroke syndrome and MRI images[395]. RSSI were defined as signal characteristics consistent with a recent infarct in the deep grey or white matter of the cerebral hemispheres or brainstem with an axial diameter of <20mm. Cortical infarcts were characterised as signal characteristics consistent with a recent infarct involving the cortex or large subcortical infarcts with an axial diameter of >20 mm.

6.3.4 MRI Processing

Intracranial volumes (ICV) were computationally generated, checked for accuracy, and edited manually when required. WMH volumes were extracted from paired T2-weighted, FLAIR, and T2*-weighted images[27]. Index, acute, old, and incident infarcts were manually segmented and excluded from total

WMH volumes by an experienced image analyst. An experienced neuroradiologist assessed scans for index and prior infarcts or haemorrhages, WMH, lacunes, PVS, microbleeds, atrophy and calculated the summary SVD scores (assigning one point for: 1) Fazekas score of three in the periventricular white matter or ≥2 in the deep white matter; 2) lacunes present; 3) microbleeds present; 4) over ten counts of enlarged perivascular spaces in the basal ganglia) according to STRIVE criteria and using validated scales[27,355].

6.3.5 Death

In cases where patients had died, date and cause of death were obtained from hospital records and death certificates.

6.3.6 Statistics

All analyses were performed using R (version 4.4.1). We expressed WMH volumes as % intracranial volume (ICV) to adjust for variations in individual head size. WMH volumes as % ICV were log-transformed to improve model fit. To reduce the number of predictors and avoid model overfitting, we assigned equal weight to hypertension, hypercholesterolaemia, diabetes, and smoking history (current smoker or ex-smoker within one year) in the calculation of a baseline vascular risk factor composite score.

We conducted independent t-tests to compare continuous variables between groups, reporting 95% confidence intervals, and *p*-values. Chi-square tests (x^2) were used to compare categorical variables, with x^2 values and *p*-values presented. For ordinal or non-normally distributed variables, Mann-Whitney U tests were applied, reporting the *W* statistic and corresponding *p*-values.

Logistic regression was used to examine the relationship between stroke subtype and binary outcomes, including dementia, recurrent stroke, death, and cardiovascular events. All models were adjusted for age, sex, vascular risk factor composite score, and baseline WMH volume (as % ICV). Logistic regression models with recurrent stroke and death as outcome variables were additionally adjusted for baseline NIHSS. The *rms* (R, version 6.9-0) package

was used to assess the discrimination and calibration of logistic regression models by evaluating model performance, including using the c-statistic (area under the curve) for discrimination and calibration curves to assess how predicted outcomes align with observed outcomes.

To account for deceased participants, separate regression models were conducted for the time intervals of 0-1 year, 1-3 years, and 3-9 years for recurrent stroke outcomes. Kaplan-Meier survival curves and log-rank tests were performed using R (version 4.4.1) and the *survival* (R, version 3.2-13) and *survminer* (R, version 0.4.9) packages. Significance was defined as $p<0.05$.

6.4 Results

We recruited 264 participants (mean age at recruitment: 66.9 years (SD: 11.8), 41.7% female, 44.7% lacunar) within three months of a mild, non-disabling lacunar or cortical ischaemic stroke. Of these, clinical outcome data were available for 264 (100%) at one year, 246 (93%) at three years (16 declined, 2 relocated), and 243 (92%) at 7-9 years (3 relocated; mean follow-up time: 8.5 years (SD: 0.57)) (**Figure 6-1**). Additionally, we sent questionnaires to 153 participants known to be alive (111 excluded: 61 deceased, 27 uncontactable, 15 declined, 8 lacked capacity to consent). We received 96 (62.75%) out of 153 questionnaires sent (mean age of responders: 71 years (SD:10), 40% female, 46.9% lacunar) (**Figure 6-1**).

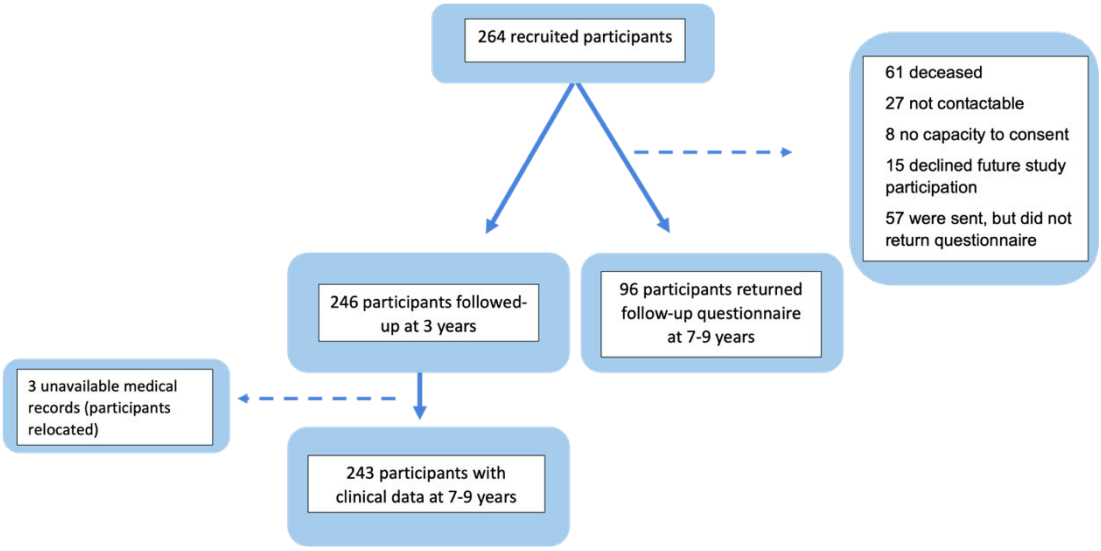


Figure 6-1. Participant flow chart for clinical outcomes (left) based on a combination of general practitioner records, hospital record data, and participant questionnaire responses and for functional and subjective well-being outcomes (right) based on a postal questionnaire sent to participants 7-9 years after initial study recruitment.

In participants with clinical outcome data available at 7-9-year follow-up (n=243), the mean age at baseline was 67 years (SD:12), 42% were female, and stroke subtype was lacunar in 43.6%. Baseline cohort characteristics are summarised in **Table 6-1**. Participants with lacunar stroke were slightly younger (65 years (SD:11) versus 68 (SD:12); *p*=0.03), had higher MRI summary SVD scores (2.53 (SD:1.25) versus 2.12 (SD:1.15); *p*=0.01) and higher median NIHSS scores (1 (IQR: 0,2) versus 0 (IQR: 0,1); *p*=0.01) compared to those with cortical stroke.

Table 6-1. Baseline cohort characteristics. Abbreviations: NIHSS: National Institutes of Health Stroke Scale; NART: National Adult Reading Test; ACE-R: Addenbrooke's Cognitive Examination; SVD: small vessel disease; WMH: white matter hyperintensity; ICV: intracranial volume.

	lacunar (N=106)	cortical (N=137)	overall (N=243)	result (p-value)
Age, years, mean (SD)	65 (±11)	68 (±12)	67 (±12)	0.249, 6.158 (0.03) †
Sex, female, n (%)	46 (43%)	55 (40%)	101 (42%)	0.143 (0.70)°
Modified Rankin Scale, n (%)				7333.5 (0.88)*
No symptoms at all	17 (16%)	28 (20%)	45 (19%)	-
No significant disability	49 (46%)	51 (37%)	100 (41%)	-
Slight disability	19 (18%)	26 (19%)	45 (19%)	-
Moderate disability	16 (15%)	28 (20 %)	44 (18%)	-
Moderate severe disability	4 (4%)	4 (3%)	8 (3%)	-
Bedridden	1 (1%)	0 (0%)	1 (0%)	-
NIHSS, median (IQR)	1 (0,2)	0 (0,1)	1 (0,2)	5875 (0.007)*
Diabetes, yes, n (%)	11 (10%)	17 (12%)	28 (12%)	0.083 (0.77)°
Hypertension, yes, n (%)	76 (72%)	101 (74%)	177 (73%)	0.04 (0.83)°
Hypercholesterolaemia, yes, n (%)	66 (62%)	82 (60%)	148 (61%)	0.06 (0.80)°
Atrial Fibrillation, yes, n (%)	6 (6%)	14 (10%)	20 (8%)	1.09 (0.23)°
Current smoker or ex-smoker <1 year ago, n (%)	46 (43%)	47 (34%)	93 (38%)	1.723 (0.18)°
NART score, mean (SD)	34 (±10)	35 (±10)	35 (±10)	-2.216, 4.690 (0.47)†
Missing, n (%)	44 (41.5%)	55 (40.1%)	99 (40.7%)	-
ACE-R score, mean (SD)	87 (±7.8)	88 (±8.6)	88 (±8.2)	-2.399, 2.962 (0.83)†
Missing, n (%)	43 (40.6%)	53 (38.7%)	96 (39.5%)	-
Summary SVD score, median (IQR)	2 (1,3)	2 (1,3)	2 (1,3)	-0.719, -0.103 (0.01)†

	lacunar (N=106)	cortical (N=137)	overall (N=243)	result (p-value)
WMH volume, mm3 as % ICV, mean (SD)	1.6 (±1.7)	1.5 (±1.6)	1.5 (±1.7)	-0.579, 0.273 (0.47)†

*:Mann-Whitney U test (W); °:Chi-square (X2); †: independent samples t-test (95% CI).

Participants with clinical outcome data at 7-9 years had lower average ACE-R scores at baseline compared to those without (88 (SD:8.2) versus 95 (SD:3.3); *p*=<0.001) (**Table E-1 in Appendix E**). Participants who returned the functional and subjective well-being outcomes questionnaire were significantly younger at baseline than those who did not (65 years (SD:11) versus 68 (SD:12); *p*=0.02). They also performed better on cognitive assessments (ACE-R score: 90 (SD:6.5) versus 87 (SD:8.8); *p*= 0.003), had lower MRI summary SVD scores (2.29 (SD:1.21) versus 2.66 (SD:1.06); *p*=<0.001), and lower WMH volumes (1mm^3 (SD:1.1) versus 1.9 (SD:1.8); *p*=<0.001) at baseline (**Table E-2 in Appendix E**).

6.4.1 Clinical outcomes: dementia, recurrent stroke, death, cardiac disease, and vascular risk factors

6.4.1.1 Dementia

Between study recruitment and 7-9-year follow-up, 9.4% (n=10/106) of lacunar and 12.4% (n=17/137) of cortical stroke participants had a dementia diagnosis. Age was significantly associated with dementia risk (OR:1.08, 95% CI 1.030-1.130) (**Table 6-2 & Figure 6-2).**

Table 6-2. Stroke subtype and clinical outcomes 7-9 years after lacunar or mild cortical ischaemic stroke. Logistic regression results for the association between dementia, recurrent stroke, death, and cardiac disease and possible predictors, including stroke subtype, age, sex, vascular risk factors, baseline WMH volume, and NIHSS where appropriate. Abbreviations: OR: Odds Ratio; CI: Confidence interval; WMH: white matter hyperintensity; ICV: intracranial volume; NIHSS: National Institutes of Health Stroke Scale.

Predictor	**OR**	**95% CI**	**p-value**	**C-statistic**
Dementia Diagnosis (n=243)				
Stroke subtype (lacunar)	0.85	0.352, 2.080	0.73	0.76
Age	1.08	1.030, 1.130	0.001	-
Sex (female)	1.05	0.448, 2.473	0.90	-
Vascular risk factor score	1.19	0.713, 2.014	0.49	-
Baseline WMH volume as % ICV	1.15	0.922, 1.456	0.20	-
Recurrent stroke years 0-1 (n=232)				0.70
Stroke subtype (lacunar)	0.89	0.371, 2.147	0.80	-
Age	1.01	0.970, 1.055	0.57	-
Sex (female)	1.87	0.801, 4.385	0.14	-
Vascular risk factor score	2.27	1.287, 4.032	0.004	-
Baseline WMH volume as % ICV	1.04	0.792, 1.365	0.77	-
Baseline NIHSS	1.16	0.835, 1.631	0.36	-
Recurrent Stroke Years 1-3 (n=220)				0.67
Stroke subtype (lacunar)	1.49	0.581, 3.854	0.40	-
Age	0.98	0.938, 1.035	0.56	-
Sex (female)	1.64	0.661, 4.101	0.28	-
Vascular risk factor score	1.48	0.837, 2.618	0.17	-
Baseline WMH volume as % ICV	1.14	0.835, 1.569	0.39	-
Baseline NIHSS	1.20	0.865, 1.675	0.27	-
Recurrent Stroke Years 3-7-9 (n=220)				0.59
Stroke subtype (lacunar)	0.76	0.349, 1.665	0.49	-
Age	1.01	0.980, 1.050	0.39	-
Sex (female)	1.28	0.615, 2.699	0.50	-

Vascular risk factor score	0.87	0.568, 1.360	0.56	-
Baseline WMH volume as % ICV	1.02	0.799, 1.312	0.84	-
Baseline NIHSS	0.92	0.670, 1.28	0.64	-
Death				0.81
(n=243)				
Stroke subtype (lacunar)	0.58	0.284, 1.185	0.13	-
Age	1.09	1.051, 1.133	<0.001	-
Sex (female)	0.40	0.196, 0.818	0.01	-
Vascular risk factor score	1.09	0.734, 1.642	0.64	-
Baseline WMH volume as % ICV	1.36	1.112. 1.664	0.002	-
Baseline NIHSS	1.10	0.838, 1.452	0.48	-
Cardiac diseases*				0.70
(n=243)				
Stroke subtype (lacunar)	0.59	0.329, 1.079	0.08	-
Age	1.04	1.019, 1.081	0.001	-
Sex (female)	0.36	0.201, 0.676	0.001	-
Vascular risk factor score	1.57	1.111, 2.236	0.01	-
Baseline WMH volume as % ICV	0.92	0.769, 1.119	0.43	-

*Cardiac disease diagnoses include: IHD/MI (n=67), heart failure (n=8), pacemaker fitted (n=1), supraventricular tachycardia (n=1), cardio ablation (n=1), left ventricular hypertrophy (LVH) (n=1), cardiovascular disease listed on death certificate/medical record (not specified) (n=2), aortic valve replacement (n=2), percutaneous coronary intervention/transcatheter aortic valve implantation (TAVI) (n=1), and fenestrated endovascular aortic repair (n=1).

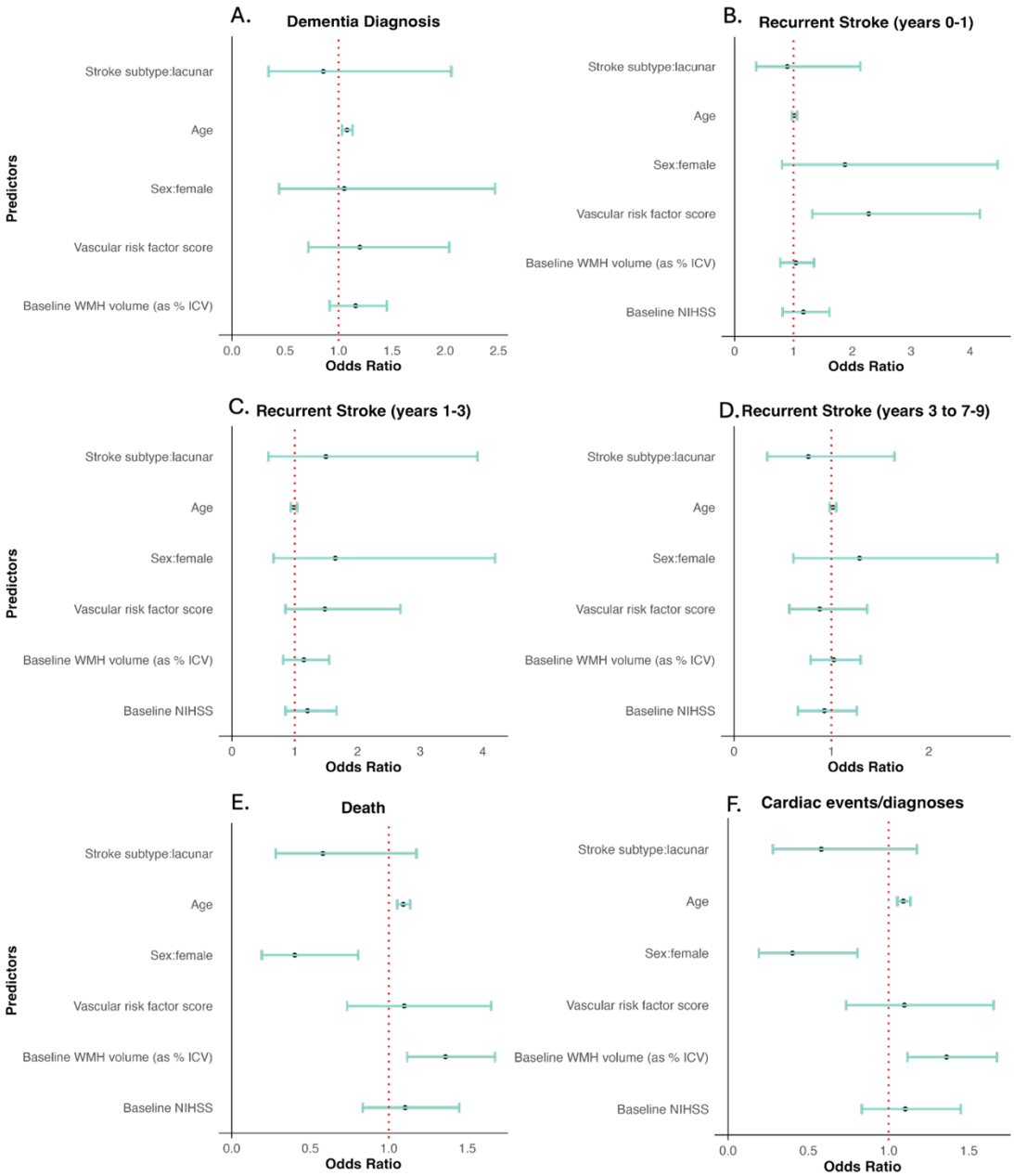


Figure 6-2. Stroke subtype and clinical outcomes at 7-9-years. Logistic regression results for the association of possible predictors (including stroke subtype) and clinical outcomes: dementia, recurrent stroke, death and cardiac diseases. Abbreviations: WMH: white matter hyperintensities; ICV: intracranial volume; NIHSS: The National Institutes of Health Stroke Scale.

6.4.1.2 Recurrent stroke

Overall, 31.7% (n=77/243) of participants had at least one recurrent stroke, including 31.1% of lacunar (n=44/106) and 32.1% (n=44/137) of cortical stroke

participants. Of these, 66 had a single recurrence, eight had two, and three had three recurrent strokes.

Within the first year post-stroke, 10.8% (n=11/102) of lacunar and 11.5% (n=15/130) of cortical stroke participants had at least one recurrent stroke. Between 1 and 3 years, 14.8% (n=12/81) of lacunar and 9.3% (n=10/108) of cortical stroke participants had at least one recurrent stroke. Between 3 and 7-9 years, 13% (n=13/100) of lacunar and 17.5% (n=21/120) of cortical stroke participants had at least one recurrent stroke (**Table 6-2 & Figure 6-2 & Table E-** 3 **& Figure E-1 in Appendix E**). Only composite vascular risk factor score was independently associated with increased risk of recurrent stroke and only within the first year post-index stroke (OR: 2.27, 95% CI 1.287-4.032) (**Table 6-2 & Figure 6-2**)

In addition, 3.8% (n=4/106) of lacunar and 4.4% (n=6/137/) of cortical stroke participants had at least one TIA diagnosis, and 1.9% (n=2/106) of lacunar and 1.5% (n=2/137) of cortical stroke participants had an ICH.

6.4.1.3 Death

Overall, 26.3% (64/243) of participants died, including 19.8% (n=21/106) of lacunar and 31.3% (n=43/137) of cortical stroke participants (**Table E-** 3 **in Appendix E**). The average age of death was 79.3 years (SD:10.3) for cortical strokes and 80.5 years (SD:8) for lacunar strokes. Age (OR:1.09, 95% CI 1.051-1.133), male sex (female sex OR:0.40, 95% CI 0.196-0.818), and baseline WMH volume (OR: 1.36, 95% CI 1.112-1.664) were significantly associated with increased risk of death (**Table 6-2 & Figure 6-2**). Cortical stroke participants were more likely to have worse survival outcomes compared to lacunar stroke patients (Kaplan-Meier curve and log-rank test on **Figure 6-3**; X^2: 8.2; p=0.004).

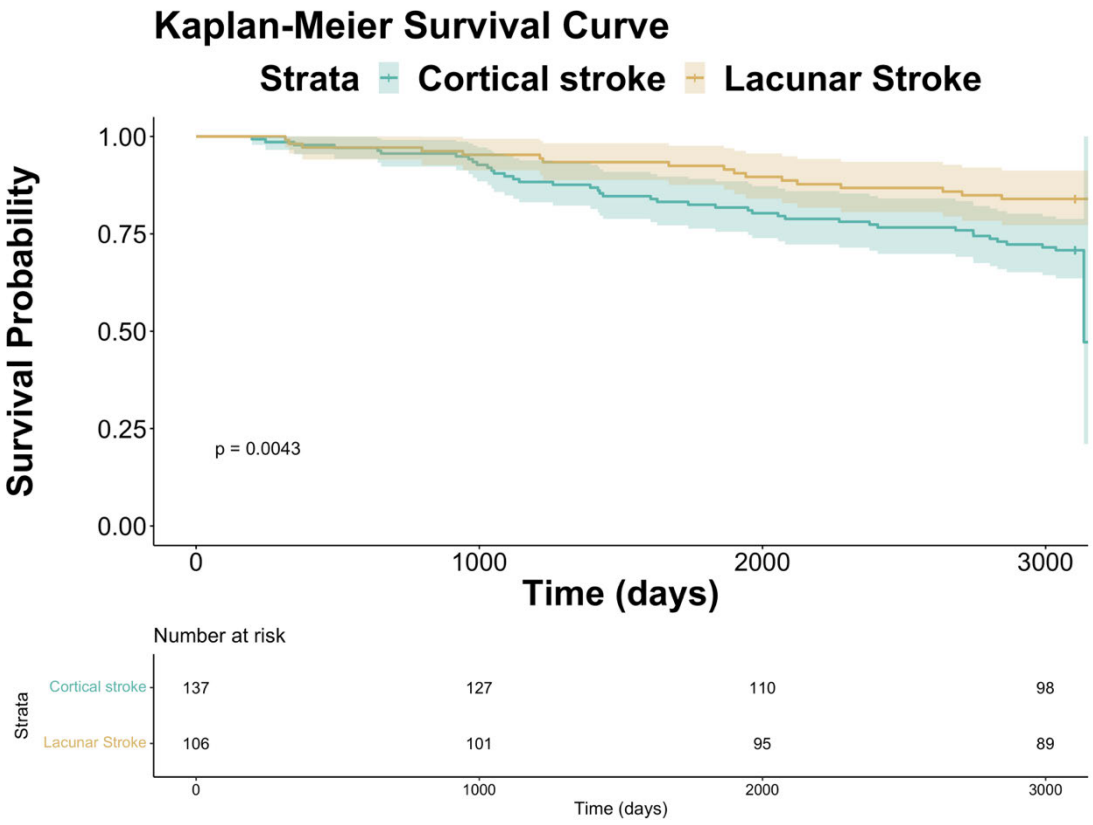


Figure 6-3. Kaplan-Meier survival curves comparing death rates between cortical and lacunar stroke 7-9 years post-stroke (n=143). The log-rank test indicated a significant difference in survival probabilities between the groups, where cortical stroke may have worse survival outcomes compared to lacunar stroke (X2=8.2; p=0.004).

In participants with lacunar stroke, malignancies were the leading cause of death (38.1%, n=8/21), followed by other/unknown causes (23.8%, n=5/21), recurrent stroke or complications from stroke (14.3%, n=3/21), and pulmonary/respiratory issues (4.8%, n=1/21). In participants with cortical stroke, the most common primary cause of death was vascular or cardiovascular disease (27.9%, n=12/43), followed by malignancies (25.6%, n=11/43), recurrent stroke or complications from stroke (18.6%, n=8/43), and pulmonary/respiratory issues or other/unknown causes (14%, n=6/43 each). Other causes of death included: pulmonary embolism (n=2), complications from Parkinson's Disease (n=1), septicaemia (n=1), and not known/not established (n=7).

6.4.1.4 Cardiac diseases

Cardiac disease diagnoses, including IHD, heart failure, and arrhythmias, were more common in cortical (40% (n=55/137)) than in lacunar (26.4% (n=28/106)) stroke.

IHD was reported in 22.6% (n=24/106) of lacunar and 31.4% (n=43/137) of cortical stroke participants. Heart failure occurred in 1.9% (n=2/106) of lacunar stroke participants, compared to 5.8% (n=8/137) of those with cortical stroke. Cardiac disease risk was significantly associated with age (OR: 1.04, 95% CI 1.019-1.079), male sex (female OR: 0.36, 95% CI 0.201-0.676), and vascular risk factor composite score (OR: 1.57, 95% CI 1.111-2.236) (**Table 6-2 & Figure 6-2**).

6.4.1.5 Vascular risk factors

At baseline, hypertension was present in 72% (n=76/106) of lacunar and 74% (n=101/137) of cortical stroke participants (X^2: 0.042, p=0.83). By the 7-9-year follow-up, hypertension incidence had increased to 89% (n=94/106) in lacunar and 83% (n=114/137) in cortical stroke participants (X^2: 1.039, p=0.30) (**Figure 6-4 & Table E-4 in Appendix E**). Hypercholesterolemia was common in both lacunar and cortical stroke participants at baseline (62% (n=66/106) and 60% (n=82/137) respectively; X^2: 0.062, p=0.80) and was only slightly more common in participants with lacunar stroke at 7-9-year follow-up (83% (n=88/106) vs 74% (n=101/137); X^2: 2.474, p=0.11). The prevalence of diabetes was greater in lacunar compared to cortical stroke participants at 7-9 years (22% (n=23/106) vs 15% (n=21/137); X^2: 1.233, p=0.26,) though not at baseline (10.4% (n=11/106) vs 12.4% (n=17/137); X^2: 0.083, p=0.77). Atrial fibrillation was consistently more prevalent amongst cortical stroke participants. At baseline, 6% (n=6/106) of lacunar stroke participants had an atrial fibrillation diagnosis compared to 10% (n=14/137) of cortical stroke participants (X^2: 1.096, p=0.29). At 7-9-year follow-up, 21% (n=22/106) of lacunar stroke participants had an atrial fibrillation diagnosis compared to 27% (n=37/137) of cortical stroke participants (X^2: 0.953, p=0.32).

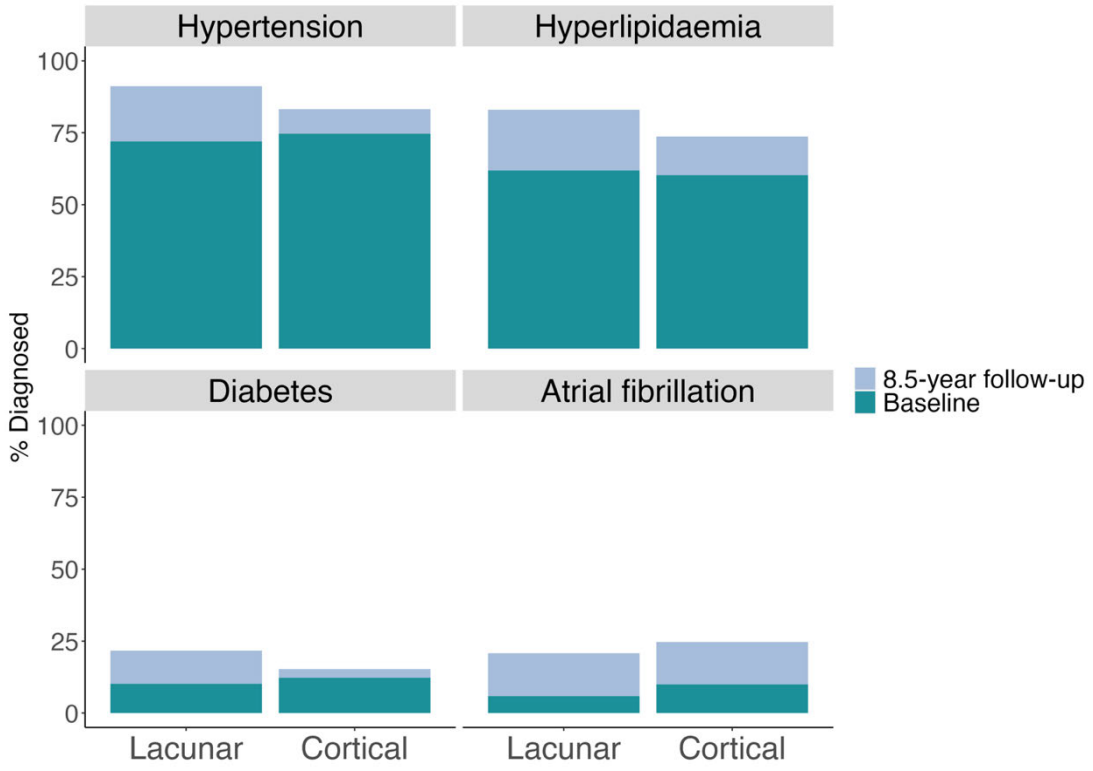


Figure 6-4. Lacunar and cortical stroke participants with diagnosed hypertension, diabetes, hypercholesterolemia, and/or atrial fibrillation at baseline, along with new diagnoses acquired between baseline and the 7-9 year follow-up.

6.4.2 Functional and subjective well-being outcomes

6.4.2.1 Disability and dependence

At baseline, 80% (n=85/106) of lacunar and 77% (n=105/137) of cortical stroke participants reported experiencing no-to-mild symptoms post-stroke and were independent in activities of daily living (mRS score 0-2). Moderate/severe disability (mRS score 3-5) was reported in 20% (n=21/106) of lacunar and 23% (n=32/137) of cortical stroke participants. At 7-9 years, most participants, both lacunar and cortical, remained independent (88% (n=38/43) and 88% (n=44/50), respectively), whilst 12% (n=5/43) of lacunar and 12% (n=6/50) of cortical stroke participants reported moderate/severe disability (**Figure 6-5**).

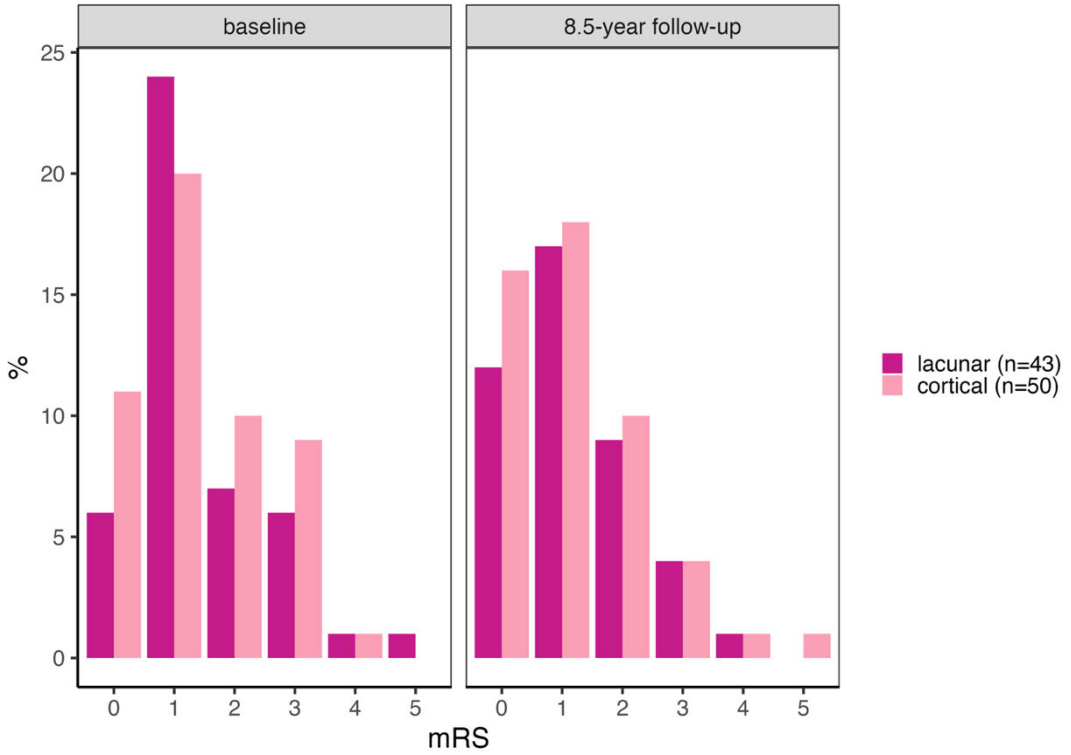


Figure 6-5. Longitudinal disability and functional independence at baseline and 7-9 years after minor ischaemic lacunar (dark pink) or cortical (light pink) stroke (n=93). Abbreviations: mRS: modified Rankin Scale.

For those who returned the postal questionnaire at 7-9-years (n=96, 47% lacunar), the median change in mRS score from baseline to 7-9 years for both lacunar and cortical stroke participants was 0 (IQR: -1, 1), with a maximum increase of 2 and a decrease of 3 score points in lacunar stroke participants and a maximum increase and decrease of 3 score points in cortical stroke participants. Between baseline and 7-9-year follow-up, 30.2% of lacunar and 28% of cortical stroke participants had a ≥1-point increase in mRS score.

6.4.2.2 Activities of daily living and subjective well-being

A substantial proportion of both lacunar and cortical stroke participants reported experiencing lingering issues from their index stroke (lacunar stroke: 38% (n=17/45); cortical stroke: 45% (n=22/49); X^2: 1.39, p=0.49) (**Table 6-3 & Figure E-2 & Figure E-3 in Appendix E**).

Table 6-3. Self-reported functional, neuropsychiatric, and subjective well-being outcomes 7-9 years post- lacunar or mild cortical ischaemic stroke (n=96).

	lacunar (N=45)	cortical (N=51)	overall (N=96)	Result (p-value)
Age, years, mean (SD)	69 (± 7)	72 (± 12)	71(± 10)	-1.719, 7.477 (0.21)†
Sex, female, n (%)	20 (44 %)	18 (35 %)	38 (40 %)	-
Modified Rankin Scale, score, n (%), (n=93)				1012 (0.73)*
No symptoms at all	12 (27 %)	16 (31 %)	28 (29 %)	-
No significant disability	17 (38 %)	18 (35 %)	35 (36 %)	-
Slight disability	9 (20 %)	10 (20 %)	19 (20 %)	-
Moderate disability	4 (9 %)	4 (8 %)	8 (8 %)	-
Moderate severe disability	1 (2 %)	1 (2 %)	2 (2 %)	-
Bedridden	0 (0 %)	1 (2 %)	1 (1 %)	-
Ongoing problems from stroke, yes, n (%)	17 (38 %)	22 (43 %)	39 (41 %)	1.399 (0.49)°
Subjective cognitive concerns, yes, n (%)	22 (49 %)	28 (55 %)	50 (52 %)	1.272 (0.52)°
Difficulty thinking quickly, n (%), (n=91)				859.5 (0.12)*
Not difficult	21 (47 %)	29 (57 %)	50 (52 %)	-
Somewhat difficult	12 (27 %)	12 (24 %)	24 (25 %)	-
Very difficult	11 (24 %)	6 (12 %)	17 (18 %)	-
Anxiety/depression, n (%), (n=94)				888.5 (0.06) *
None	23 (51 %)	33 (65 %)	56 (58 %)	-
Slight/moderate	8 (18 %)	9 (18 %)	17 (18 %)	-
Severe	14 (31 %)	7 (14 %)	21 (22 %)	-
Problems with self-care tasks, n (%), (n=93)				984.5 (0.28) *
None	34 (76 %)	41 (80 %)	75 (78 %)	-
Slight/moderate	11 (24 %)	5 (10 %)	16 (17 %)	-
Severe/unable	0 (0 %)	2 (4 %)	2 (2 %)	-
Difficulty performing household chores, n (%), (n=94)				966.5 (0.22)*
Not difficult	26 (58 %)	35 (69 %)	61 (64 %)	-
Somewhat difficult	17 (38 %)	11 (22 %)	28 (29 %)	-
Very difficult	2 (4 %)	3 (6 %)	5 (5 %)	-

	lacunar (N=45)	cortical (N=51)	overall (N=96)	Result (p-value)
How often are you limited socially?, n (%), (n=94)				996 (0.33)*
None of the time	27 (60 %)	34 (67 %)	61 (64 %)	-
A little of the time	14 (31 %)	12 (24 %)	26 (27 %)	-
All of the time	4 (9 %)	3 (6 %)	7 (7 %)	-
Mobility problems, n (%), (n=95)				1136 (0.92)*
None	25 (56 %)	28 (55 %)	53 (55 %)	-
Slight/moderate	16 (36 %)	16 (31 %)	32 (33 %)	-
Severe/unable	4 (9 %)	6 (12 %)	10 (10 %)	-
Difficulty walking without losing balance, n (%), (n=94)				1022.5 (0.48)*
Not difficult	26 (58 %)	32 (63 %)	58 (60 %)	-
Somewhat difficult	16 (36 %)	14 (27 %)	30 (31 %)	-
Very difficult	3 (7 %)	3 (6 %)	6 (6 %)	-
Regained strength of affected limb, n (%), (n=92)				705.5 (0.00)*
A lot of strength	14 (31 %)	26 (51 %)	40 (42 %)	-
Quite a bit of strength	13 (29 %)	15 (29 %)	28 (29 %)	-
A little strength	18 (40 %)	6 (12 %)	24 (25 %)	-
Problems with usual activities, n (%), (n=94)				1123.5 (0.86)*
None	24 (53 %)	27 (53 %)	51 (53 %)	-
Slight/moderate	18 (40 %)	15 (29 %)	33 (34 %)	-
Severe/unable	3 (7 %)	7 (14 %)	10 (10 %)	-
Current housing arrangement, n (%), (n=92)				-
In a care home	1 (2 %)	1 (2 %)	2 (2 %)	-
On my own	14 (31 %)	13 (25 %)	27 (28 %)	-
With my partner or relatives	29 (64 %)	34 (67 %)	63 (66 %)	-

*:Mann-Whitney U test (W); °:Chi-square (X2); †: independent samples t-test (95% CI).

Whilst a significant proportion of both lacunar (49% (n=22/45)) and cortical (55% (n=27/49)) stroke participants noted subjective cognitive concerns (X^2: 1.272, *p*=0.52), 52% (n=23/44) of lacunar stroke participants described

thinking quickly as 'somewhat' or 'very' difficult, compared to only 38% (n=18/47) of cortical stroke participants (W=859.5, *p*=0.12) (**Table 6-3**).

Reports of depression and anxiety were more prevalent amongst lacunar stroke participants (49% (n=22/45) versus 33% (n=16/49)) (X^2: 4.014, *p*=0.13). Of these, 31% (n=14/45) of lacunar strokes and 14% (n=7/49) of cortical strokes rated their anxiety/depression symptoms as 'severe' (W=888.5, *p*=0.06). Since their stroke, 40% (n=18/45) of lacunar and 31% (n=15/49) of cortical stroke participants reported feeling socially limited at least some of the time (W=996, *p*=0.33).

Difficulties with activities of daily living were more common in lacunar stroke. 42% (n=19/45) of lacunar strokes reported difficulties completing household tasks compared with 29% (n=14/49) of cortical strokes (W=966.5, *p*=0.22). 24% (n=11/45) of lacunar strokes acknowledged facing challenges with self-care tasks, including washing and dressing, compared to 10% (n=5/48) of cortical strokes (W=984.5, *p*=0.28) (**Table 6-3**).

Problems with mobility, balance and muscle strength were common: 36% (n=16/45) lacunar and 32% (n=16/50) cortical strokes had 'slight-to-moderate' problems with mobility and 9% (n=4/45) lacunar, and 12% (n=6/50) cortical strokes had severe mobility difficulties (W=1136, *p*=0.92). Impaired balance was more frequent in lacunar (42% (n=19/45)) than in cortical (35% (n=17/49)) strokes (W=1022.5, *p*=0.48). Most cortical strokes (55% (n=26/47) regained a substantial portion of strength in their affected limb post-stroke versus 31% (n=14/45) of lacunar strokes, and more lacunar strokes (40% (n=18/45)) only gained a small amount of strength compared to 13% (n=6/47) of cortical strokes (W=705.5, *p*=0.00) (**Table 6-3**).

Most participants were living with family (lacunar: 66% (n=29/44); cortical: 71% (n=34/48)), followed by those living independently (lacunar: 32% (n=14/44); cortical: 27% (n=13/48)). Only 2% (n=1/44) of lacunar and 2% (n=1/48) of cortical stroke questionnaire respondents were residing in a care home or assisted living facility.

6.4.3 Discussion

We report longitudinal data on clinical, functional, and patient-reported outcomes 7-9 years after lacunar or mild cortical ischaemic stroke. During the 7-9 years of follow-up, despite the lacunar strokes being slightly younger at baseline, a similar proportion of cortical and lacunar stroke participants were diagnosed with dementia (11%), at least one recurrent stroke (32%), or died (26%). Cortical stroke participants exhibited higher mortality rates, particularly from cardiovascular causes, compared to those with lacunar stroke. Cardiac conditions, such as ischaemic heart disease, arrhythmias, and heart failure, were also more prevalent in cortical stroke participants. Conversely, hypertension, hypercholesterolaemia, and diabetes, were more frequent amongst lacunar stroke participants. These profiles coincide with previous research results and likely reflect the differing vascular pathologies underlying the two stroke subtypes: large artery atherothrombotic disease associated with cortical strokes and small vessel disease characteristic of lacunar strokes[389,396].

The risk of dementia in patients with lacunar infarcts has been investigated in only a limited number of studies. Findings indicate that approximately 11-20% of patients develop dementia within 2-4 years following lacunar stroke, a proportion that increases to 15% after 9 years [389,397–400]. In our study, 9% of lacunar participants were diagnosed with dementia. Notably, despite lacunar strokes being slightly younger, the proportion of dementia diagnoses was similar in lacunar and cortical stokes. It is important to note that we way have underestimated dementia diagnoses due to several factors including underreporting. Patients, their relatives, or primary healthcare providers may overlook or normalise early symptoms of dementia, perhaps even attributing them to the natural ageing process. In some cases, they may perceive the pursuit of a clinical diagnosis to be unnecessary, believing it might not alter clinical management. The gradual onset of symptoms may also serve to further diminish their perceived significance and severity.

The average rate of recurrent stroke for lacunar strokes within 4-5 years is estimated to be 22%[389,398,401,402]. In our study, 32% of patients had at least one recurrent stroke within 7-9 years, with a similar incidence amongst lacunar and cortical stroke. Although some studies report lower recurrent stroke risk in lacunar infarction compared to other stroke subtypes, recent findings challenge the notion that lacunar infarcts have a more favourable prognosis for recurrent stroke[389]. When considering recurrent stroke in SVD, we perhaps must also consider the role of clinically 'silent' infarction, as in the case of most radiologically fleeting DWI+ lesions, which are often clinically covert[26,403]. Longitudinal SVD studies with frequent MRI neuroimaging are likely to provide valuable insights into the role of these lesions and their clinical outcome correlates[403].

In our study, only vascular risk factors were associated with recurrent stroke risk, although previous studies find that SVD burden severity at baseline can play crucial roles in determining the likelihood of future stroke after lacunar infarct[397].

Mortality rates following lacunar stroke are relatively low within the first year but increase significantly over time. Case fatality averages 2.8% at 1 year, rising to 27% after 5 years[389]. In our study, 26% of patients had died by 7-9 years, with cortical stroke patients having worse survival outcomes. Longer follow-up studies show that mortality rates for lacunar stroke patients can approach 60-75% within 10-14 years[401,402]. In addition to stroke and dementia, SVD progression is associated with higher risk of cognitive impairment and impaired mobility, which may lead to infections, hospitalisations, or falls; interestingly, in our study, worse WMH burden at baseline was associated with higher risk of death[404]. For patients with stroke in general, cardiovascular deaths predominate in the short- and long-term, although here, cardiovascular causes was the most common primary cause of death amongst cortical strokes only[389].

Compared to clinical outcomes such as recurrent stroke and death, global functional decline, functional status, and long-term quality of life have received

far less attention as outcomes post-lacunar infarction. Patients with lacunar infarcts generally experience better functional outcomes compared to those with other stoke subtypes[389,395,405]. However, dependency rates remain substantial, affecting over 40% of patients at 3 years post-stroke[399]. Additionally, over 10% of patients who maintain independence, experience impairments in other areas, limiting social, occupational, or recreational participation[389,399]. In our study, 40% of participants reported persistent effects from their initial stroke, with 12% also reporting moderate/severe disability. Participants with lacunar stroke were more likely to experience poorer limb recovery and report problems with balance, psychiatric symptoms, and quick thinking. These findings serve to highlight the diverse and enduring impact of mild lacunar and cortical ischaemic stroke on daily functioning and mental well-being, particularly in patients with SVD.

One of the key strengths of this study is the extended follow-up period, which stands out in comparison to many other studies, which very rarely extend beyond four years[389]. Other strengths include the use of multiple overlapping ascertainment methods, the inclusion of measures of functional status, and the high retention rate of participants. This was achieved by leveraging data collected directly from patient records, enabling long-term tracking. However, this also represents a limitation in the study, as there remains a possibility of missing patient diagnoses. This is perhaps particularly relevant in the case of dementia , which we may have underestimated. Our relatively small sample size may not fully reflect the broader ageing SVD or cortical stroke patient population. The subset of participants who were able to complete the functional outcomes questionnaire were younger and performed better in cognitive assessments and had a milder radiological SVD burden at baseline, and were likely those with the lowest morbidity, excluding patients who were most unwell or deceased, which may affect the representativeness of our findings and understate the effects of progressing disease.

These findings suggest that long-term outcomes following minor stroke are often suboptimal and are influenced by the distinct pathophysiology of each

stroke subtype. These findings also serve to highlight the significant and enduring impact of mild lacunar and cortical ischaemic stroke on patient quality of life. Considerations should be given to improving long-term outcomes in patients with mild ischaemic stroke. Recognising these long-term effects is essential for directing clinical management and guiding research priorities. Identifying possible early predictors of longitudinal SVD progression and unfavourable clinical and functional outcomes could be useful in clinical practice and aid in the development of prognostic tools.

Chapter 7 Conclusion, Discussion, and Future Perspectives

In this thesis, I have examined the potential role of pericyte and endothelial dysfunction in SVD pathogenesis through a translational perspective. My main aims were to: 1) review existing literature and identify the molecular mechanisms underlying endothelial dysfunction in SVD; 2) investigate whether ageing mice exhibit signs of pericyte loss and/or endothelial cell activation and explore the potential relationship between these two processes; 3) validate the murine findings from Aim 2 within the context of human SVD using postmortem brain tissue; 4) determine how endothelial and pericyte function blood biomarkers measured *in vivo* associate with SVD burden and progression over time and, finally, 5) to acknowledge the gravity and importance of SVD and SVD research by taking a step back from the cellular level and exploring the longitudinal outcomes and far-reaching consequences of SVD.

In this final chapter, I will summarise and critically reflect on the key findings in this work, explore potential mechanisms underlying these processes, and highlight their strengths, limitations and implications for future directions and clinical practice.

7.1 In focus: Molecular insights into endothelial dysfunction in SVD from human biomarker studies

In the published scoping literature review included in **Chapter 2**, I provide a comprehensive evaluation of available evidence from clinical biomarker studies, aiming to enhance the understanding of the molecular mechanisms underlying endothelial dysfunction in SVD[152]. Here, I identify key biomarkers associated with endothelial dysfunction in SVD, including cell adhesion molecules and selectins (e.g. VCAM-1, P-Selectin), BBB transporters (e.g. Cav-1), cytokines and chemokines (e.g. IL-6), pro-inflammatory markers (e.g. C-reactive protein), coagulation factors (e.g. vWF), growth factors (e.g. VEGF), and molecules involved in nitric oxide pathways (e.g. NO), all of which offer

unique insights into how these unseen physiological processes may contribute to underlying SVD pathophysiology.

In this review, I also highlight gaps in the current research and potential reasons a biomarker signature for SVD remains elusive, including some substantial inconsistencies in study results precipitated by heterogeneity in study design, which serve to provide barriers in the standardisation and interpretation of results. Whilst many studies focus on a single biomarker or include a limited range of SVD-associated lesions, namely WMH, few address how different biomarkers and lesions may interact or change dynamically over time in response to disease progression. The lack of large longitudinal studies, particularly those featuring participants with varying degrees of SVD and appropriate control populations, remains a significant obstacle to identifying robust biomarkers with clinical utility.

This review supports the critical role of endothelial dysfunction in SVD and identifies promising biomarkers for future investigations. The findings from these biomarker investigations may have implications for improving early detection of disease, risk stratification (both in research and clinical settings), and the treatment monitoring of SVD. Here, I also call for multi-modal, standardised, and collaborative efforts to validate biomarker investigations to materialise these molecular insights into tangible clinical applications in future.

7.2 Evidence of endothelial activation and pericyte coverage loss in ageing mice

In **Chapter 3**, I provide evidence of loss of pericyte coverage and increased endothelial activation in normal ageing in both male and female mice and across a variety of brain regions. Global pericyte coverage was reduced by an estimated 7.5% in 12-month-old animals compared to 3-month-old animals (n=16, 56% female) in linear mixed effects models after adjusting for variance introduced by sex and brain-region differences. This was also accompanied by a significant increase in vessel diameter observed in ageing animals. In

addition, VCAM-1 microvascular coverage was increased by nearly two-fold in older animals (7.62% versus 3.56%); a difference which remained statistically significant after adjusting for sex and brain region. Further segmenting brain capillaries into >90,000 micro-segments, we found evidence to suggest a potential spatial relationship between pericyte coverage and VCAM-1 expression, where lower pericyte signal was a significant and independent predictor for increased VCAM-1 expression. Our results showed an association between a 10% reduction in pericyte signal and a 0.20% increase in VCAM-1 capillary coverage.

Although this work suggests that these may be potential mechanisms of ageing that might contribute to age-related subtle increases in BBB leakage in normal ageing , whether these are accentuated in the presence of pathology, particularly that of SVD, and the direct causality remains unknown. A persistent knowledge gap in human SVD is the extent to which SVD lesions and vascular dysfunctions reflect normal ageing and the point at which they cross a boundary to become pathological. The findings in mice during normal ageing provide some underpinning information to support the concept of a spectrum of change from normality to pathology. Hence, these findings provide a platform to further investigate this phenomenon in humans, which remains my focus in subsequent chapters.

7.3 Evidence of endothelial activation and pericyte coverage loss in SVD: Findings from postmortem human brain tissue

In **Chapter 4**, I examine whether we can corroborate our murine findings and observe evidence of the same endothelial pro-inflammatory (activated) phenotype and pericyte loss, this time within the context of human SVD. Using *postmortem* human brain tissue, I measured pericyte and VCAM-1 coverage in both grey and white matter watershed areas within the primary motor cortex/precentral gyrus and adjacent white matter of healthy controls (n=4), as

well as mild (n=3), moderate (n=3), and severe (n=5) SVD cases as classified by an independent pathologist.

I did not detect any significant differences in vessel diameter between the groups, although I observed a tendency towards slightly wider vessel diameters in those with severe SVD. Average pericyte coverage was highest in young, healthy controls (34%) and progressively declined in the mild (25%), moderate (5.9%) and severe (7.2%) SVD groups. Notably, moderate and severe SVD cases had a significant 83-86% reduction in pericyte coverage compared to young, healthy controls after adjusting for sex, brain matter type, and vascular risk factors in linear mixed effects models.

VCAM-1 coverage of the microvasculature was higher in those with moderate (2.8%) and severe (2.6%) SVD compared to those with mild SVD (1.9%) and healthy controls (1.7%). Results from linear mixed effects models also highlight a 2-to-3-fold increase in VCAM-1 coverage between the moderate and severe SVD groups compared to healthy controls after adjusting for sex, brain matter type, and vascular risk factors, though this did not reach statistical significance.

Notably, results from linear mixed effects models adjusted for age, sex, and vascular risk factors, demonstrate a significant inverse relationship between log VCAM-1 and log pericyte immunofluorescent signal, with every 10% increase in pericyte signal being associated with an approximate 0.80% decrease in VCAM-1 signal within the same capillary micro-segment.

Vascular contributions to dementia remain understudied, and neuropathological data examining the molecular mechanisms and roles of the neurogliovascular unit in SVD are lacking. They provide further evidence of a progressive spectrum of pericyte loss and increased VCAM-1 binding that covers a spectrum from mild in ‘normal ageing’ to more pronounced with SVD severity, particularly when age was accounted for. These findings contribute to the growing understanding of the potential role of pericyte and endothelial cell dysfunction in the pathogenesis of SVD but leave several critical questions

unresolved. Specifically, can these dysfunctions be detected *in vivo*, and how do they relate to the burden and progression of SVD in patients?

7.4 Blood biomarkers of vascular dysfunction in SVD progression: Insights from a longitudinal neuroimaging study

In **Chapter 5**, I explore whether biomarkers of pericyte and endothelial function detectable in human blood, associate with SVD burden and progression longitudinally in patients presenting with a minor stroke (lacunar or cortical) and carefully classified and enriched for SVD. Here, I perform cross-sectional and one-year longitudinal adjusted analyses relating 13 blood biomarkers, carefully selected based on my findings in Chapter 2, to reflect endothelial, vascular, and pericyte function. I related the biomarker findings to MRI markers of SVD, including WMH, lacunes, microbleeds, summary SVD score, BBB permeability and CVR, as well as cognition and MCI prevalence and the long-term progression of SVD markers and clinical outcomes at 1 year.

I report several relationships between biomarkers of vascular function and markers of disease, including some which are novel. For instance, I report significant associations between circulating VEGF levels, a marker associated with angiogenesis and hypoxia, and increased incident DWI-positive lesions occurring in the first-year post-stroke. I also found associations between higher levels of P-Selectin, a marker of endothelial activation and thrombosis, and increased risk for MCI at 1 year. Cross-sectionally, I found associations between ICAM-1, a marker of endothelial activation associated with an immune response, and lower CVR (indicating worse SVD and vascular function), all after adjusting for relevant covariates, including vascular risk factors. If validated in larger populations, these findings could have major clinical implications in stroke and other SVD clinics, emergency departments, and possibly clinical trial design by facilitating the risk-stratification of patients who may be at a higher risk of recurrent stroke and rapid-progression SVD. They might also help to identify people with poor cerebrovascular reactivity

without having to perform an expensive brain dynamic MRI scan. One could speculate that a 'cocktail' of VEGF, P-Selectin and ICAM-1 taken in the clinic could indicate individuals with impaired CVR and high risk of worsening SVD lesions and cognitive decline within a year. This requires validation in further studies, but provides a good start point that has already accounted for interference from prescribed medications and accounted for numerous risk factors, something which many other biomarker studies in neurodegeneration have, in general, overlooked.

A major challenge impeding the advancement of therapeutics in SVD arises from the absence of elucidative and specific blood biomarkers associated with the development and progression of disease. Establishing disease-specific biomarkers could facilitate efforts to uncover pathophysiology as well as develop potential therapeutic interventions and evaluate their effectiveness. These novel findings from a human stroke population enriched for SVD can help identify key candidate biomarkers for further investigation in SVD research.

7.5 Widening the lens: Unveiling the burden of mild stroke and SVD by investigating 8.5-year longitudinal outcomes and patient impact

In **Chapter 6**, I report longitudinal data on clinical, functional, and subjective well-being outcomes 8.5 years after mild cortical and lacunar ischaemic stroke. Approximately 12% of both cortical and lacunar stroke patients were diagnosed with dementia, 23% of patients had at least one recurrent stroke, and 26% of patients were deceased. Approximately 40% of patients reported persistent effects from their initial stroke, with half expressing concerns about cognitive function and experiencing anxiety and/or depression. Whilst most patients maintained their independence, one third reported challenges with household tasks. Similarly, one third reported mobility issues, with balance impairments being more commonly noted by lacunar stroke patients.

These findings highlight the considerable long-term and life-altering impacts that mild stroke and SVD may have on patient quality of life that are far-reaching and persist for long after a stroke. Recognising the substantial longitudinal impact of mild stroke and SVD is essential for directing clinical management and research priorities.

7.6 Summary

Here, I have presented evidence through a translational perspective which supports the involvement of endothelial cell and pericyte dysfunction in the pathogenesis of SVD. I observed a loss of pericyte coverage and an instigation of a pro-inflammatory activated endothelium profile in ageing mice, as well as in moderate and severe SVD in human brain tissue compared to control or mild SVD cases. I also present evidence from blood biomarkers of endothelial and pericyte function in a mild-stroke patient cohort, where I detect many possible associations between biomarkers and distinct manifestations of disease, several of which are novel.

In this thesis, I began by focusing on the molecular mechanisms underlying endothelial and pericyte dysfunction in the context of ageing and SVD. I progressed from examining the existing literature, to investigating ageing mechanisms in mice, to exploring disease mechanisms in the human brain, and ultimately detecting these dysfunctions *in vivo* in a highly phenotyped patient cohort. I concluded by broadening the scope further and exploring the 8.5-year longitudinal outcomes of mild stroke and SVD, serving as a vital reminder of the critical importance of research into SVD pathophysiology-ultimately aimed at improving patient care and outcomes.

7.7 The role of pericytes and endothelial cells in SVD: Possible mechanisms

Our findings reveal a reduction in pericyte coverage and increased endothelial activation in both ageing and disease, observed across animal and human

studies. However, the mechanisms underlying the potential connection between these processes is not yet fully understood.

Pericyte-endothelial crosstalk plays a pivotal role in the pathophysiology of SVD, primarily through its impact on maintaining the BBB, modulating blood flow and angiogenesis, cellular transcription, transcytosis mechanisms and neuroinflammation[129,406]. Pericytes interact closely with endothelial cells to maintain the structural and functional integrity of the BBB, modulate the dilation and constriction of capillaries, and regulate angiogenesis. They are embedded within the basement membrane of capillaries and rely on gap and adherens junctions to communicate with endothelial cells at sites where the basement membrane thins; pericytes and endothelial cells are also able to communicate through basement membrane-free peg-and-socket junctions[106–108].

In our preclinical and *postmortem* investigations, reduced pericyte signal was an independent predictor for increased VCAM-1 expression in brain capillary microsegments after adjusting for potential covariates. Accelerated pericyte loss in ageing, particularly in the presence of cerebrovascular pathology, can contribute to cyclic repercussions which may further harm pericytes, including BBB impairment and vascular dysfunction, inclusive of endothelial dysfunction. Even a 20% reduction in pericyte coverage has been associated with BBB leakage[85]. To what extent pericyte loss contributes to the functional state of the endothelium and BBB disintegration and vice-versa, and the precise mechanisms underpinning this cyclic dysfunction remain unclear. Prior studies by others might suggest that pericyte detachment precedes increased VCAM1 expression and BBB breakdown or vice-versa, but I was not able to examine the sequence of changes in sufficient granularity in this study to determine a direction of effect[144,407]. It is possible that pericyte and BBB function changes that have been identified in the brain's white matter regions, also contribute to other features observed in SVD, including loss of myelin, oligodendrocytes, and axons – alterations that may also be influenced by hypoxia or changes in blood flow[408].

Previous studies examining transcriptional and functional changes to the endothelium in pericyte-deficient mice have revealed that pericytes have the ability to regulate endothelial cell identity and are essential for the maintenance of various BBB functions inherent in endothelial cells. Pericyte vascular coverage correlates to BBB strength, and capillary endothelial cells lacking in direct pericyte contact tend to adopt a 'venous-shifted' molecular pattern and can exhibit an upregulation of cell-adhesion molecules[150,409]. Another possible explanation for pericyte loss occurs through the disruption of the endothelial sphingosine-1-phosphate receptor, which is implicated in regulating vessel pericyte coverage, possibly by interfering with the transport of N-Cadherin to the membrane required for endothelial-to-pericyte adhesion[130,131]. Neuroprotective effects of S1P have been reported in mouse models of AD[410].

Interestingly, new evidence suggests that pericytes are able to proliferate, and can acquire multipotency to produce multi-lineage and progenitor cells after injury; for instance they have been shown to adopt other cell type phenotypes, including microglia, neurons, and endothelial cells, in response to ischaemic injury[411,412]. If validated in human brain pericytes, these findings could offer uniquely exciting and promising new avenues for therapeutic development and advancing target-specific treatment strategies, although they could also present unique challenges in research (e.g. finding reliable cell markers)[413].

It is possible that there are many ways in which endothelial dysfunction can exert influence over the development and progression of SVD pathophysiology. Although the initial causes of endothelial dysfunction remain unclear, there is a propensity towards an activated pro-inflammatory state accompanied by aberrant angiogenesis, microvessel luminal narrowing, reduced responsiveness (vasoreactivity), altered pulsatility (vascular stiffness) and vasomotion, increased BBB permeability, and impaired blood flow regulation. It is possible to conceive of these processes as a dynamic pathological cycle resulting in a downstream cascade of dysfunction involving the entirety of the neurogliovascular system and culminating in white matter

injury and ischaemia, neurodegeneration, diffuse connectivity problems and cortical thinning.

Many important questions remain. The exact mechanisms underlying this potential dysfunctional crosstalk between endothelial cells and pericytes, or whether dysfunction in one cell type initiates or drives the dysfunction of the other, and how other cell types in the neurogliovascular unit are involved, remains largely unclear. In addition, whether this may affect distinct subtypes of pericytes or the endothelial cells in the grey and white matter regions of the brain differently, remains unknown. Endothelial activation can be manifested through distinct ways and is marked by the expression of more than one molecule; although we have focused on VCAM-1 in our preclinical and *postmortem* investigations, it is possible that other markers of endothelial activation/dysfunction could have different expression patterns in diverse brain regions or different stages of disease severity. Importantly, candidate catalysts for endothelial dysfunction in the context of SVD and how they relate to genetics and vascular risk factor exposure remain unknown and warrants further examination. Lastly, determining whether these dysfunctions can be reversed, as well as understanding the mechanisms and optimal timing and populations for intervention, are critical questions that must be addressed before progressing toward potential treatments.

7.7.1 Strengths and limitations

Strengths and limitations for individual analyses have been discussed in each respective chapter.

This thesis has several notable strengths. Research on the role of the neurogliovascular unit in SVD remains limited, and the study of biomarkers for vascular dementia is an emerging area within the field, still in its early stages. The published scoping review presented in **Chapter 2** serves to consolidate crucial data, providing a comprehensive overview, and enhancing the understanding of the role of endothelial dysfunction in SVD[152]. It also highlights

seemingly promising, and less promising, biomarkers for future investigations, whilst identifying gaps in existing research and calling for a multi-modal, holistic and collaborative approach to standardise and validate biomarker investigations to aid in the translation of tangible clinical applications in future.

One of the key strengths of this thesis includes access to datasets like the Mild Stroke Study 2 and 3. These are vast resources of data, including clinical, cognitive, functional, and neuroimaging data in a cohort which is highly phenotyped cross-sectionally and longitudinally for key vascular measures and outcomes. This also enabled us to explore a variety of understudied topics, including the longitudinal outcomes of SVD, as well as a range of understudied SVD markers within the biomarker field, including blood-brain barrier permeability, cerebrovascular reactivity, and a broad spectrum of SVD lesions, which is a critical consideration in early SVD biomarker research.

Throughout the work presented in this thesis, I have accounted for relevant covariates in all statistical models. In animal studies, this included adjusting models for any variation introduced by sex and brain region, whilst in human *postmortem* analyses, this included demographic variables and vascular risk factors, a critical consideration that is often overlooked in SVD histopathology studies. Importantly, in the human biomarker analyses, I adjusted for several covariates generally not accounted for in earlier biomarker studies, including demographic variables, vascular risk factors, secondary stroke prevention drugs, stroke subtype and severity, and premorbid intelligence or radiological disease burden where appropriate.

In addition, for most of our analyses, we relied on linear mixed effects models. In this context, linear mixed effects models provide a powerful and advantageous framework for analysing data due to their ability to handle repeated measures and longitudinal data and account for random effects and partition variability (e.g. grouping, nesting, or clustering of data, as in the case of collecting data from several brain regions within a brain slice out of several from the same mouse). Linear mixed effects models also help improve statistical power by allowing the use of observations with incomplete or missing

data whilst reducing the likelihood of Type I errors that may arise from erroneously treating clustered or nested data as independent.

Due to the exploratory nature of some of these analyses, we have reported some results which are uncorrected for multiple comparisons. Whilst the likelihood of finding significant results due to chance increases when testing multiple comparisons, adjustments further reduce statistical power, making it difficult to detect real effects in studies with smaller sample sizes, which could increase the chances of false negatives. Whilst multiple comparisons corrections emphasise p-values for statistical significance, we highlight estimates and 95% CI, allowing for an indication of direction of association. At this stage in the development of vascular biomarkers, a neglected area, an over-specific method would be disadvantageous to the field and sensitive approaches where potential markers can be further tested in new studies are preferable. At this stage, exploratory analyses are inherently hypothesis-generating, rather than hypothesis-confirming; thus, we focus here on identifying patterns or trends across biologically-plausible vascular disease and function markers, rather than declaring definitive true effects. Instead, we posit that there are some results and hypotheses worth further investigation in independent studies and highlight how some seem more promising than others.

Lastly, throughout my PhD, I have strived to maintain a translational perspective in addressing my research questions. Access to diverse research modalities and datasets- ranging from existing literature and animal studies to human tissue samples and living patient cohorts, including one from 8.5 years ago- significantly enhanced my ability to address my research questions. This broad access also provided invaluable opportunities to approach problems from multiple perspectives through my varied roles within these frameworks- ranging from writing a literature review to learning laboratory techniques like immunohistochemistry, microscopy and animal handling, as well as patient recruitment, cognitive and clinical data collection, study management, data handling, and MRI image processing.

There are several weaknesses in the work presented in this thesis. Our ageing mouse cohort was relatively young at 12-months of age. Senescence in mice is defined as a minimum age of 18 months and extends to their approximate lifespan of 24 months. Guided by previous research and ethical considerations, we decided to include only 3- and 12-month old animals, and whilst our findings indicate early changes in pericyte and endothelial function between these age groups, findings can be strengthened by including additional age groups, which we are currently undergoing as we prepare to submit these results for publication.

Importantly, whilst we demonstrate an association between pericyte and endothelial function and disease severity or specific disease markers, this does not address the potential underlying mechanisms at play and many critical questions remain. For instance, is endothelial and pericyte dysfunction a consequence of other early disease mechanisms, or does it play a principal role in inciting downstream consequences leading to disease? Do these cell types communicate to create and exacerbate dysfunction, and if so, does one begin to malfunction first, and why? Identifying potential therapeutic avenues targeting endothelial and pericyte dysfunction would also require an understanding of how these cells influence one another, as well as other surrounding cell types.

To partially address some of these questions, we had initially planned to incorporate studies using animal models of pericyte deficiency to explore the impact on the endothelium, as well as endothelial activation models to evaluate the subsequent effects on pericytes. However, due to unforeseen issues in the establishment of the animal colonies, we were unable to perform these experiments. Ongoing investigations, particularly those utilising cell-specific animal models, *in vivo* 2-photon microscopy, and transcriptomic and proteomic analyses, will undoubtedly provide valuable insights into the previously unexplored dysfunctional crosstalk mechanisms between these cell types in disease.

Lastly, our sample sizes are relatively small, rendering most of our analyses exploratory and our findings must thus be interpreted with caution until validated in larger cohorts. Additionally, although our patient cohort biomarker analyses are longitudinal, one year is a relatively short timeframe, which limits the ability to study causal relationship between biomarkers and disease progression. The Mild Stroke Study 3 completed its 3-year patient follow-up shortly after I had completed my analyses. Longitudinal studies present unique challenges such as patient attrition, costs, and timeframes, and as such, most SVD studies are cross-sectional or have a short follow-up. However, insufficient longitudinal data remains a significant barrier in the understanding of SVD causes and trajectories, thus hindering the development of potential therapeutic avenues.

7.7.2 Research implications, clinical implications and future perspectives

Some of the findings presented in this thesis have contributed to the existing knowledge within the field. Chapter 2 has been published in a peer-reviewed journal, Chapter 5 is currently awaiting final decision after minor revisions suggested during peer review, and Chapters 3, 4, and 6 are being prepared for submission to publication at the time of writing. Additionally, I have delivered nearly 20 oral presentations at national and international meetings and conferences, sharing findings from this research.

Currently, and despite strong evidence advocating for the involvement of endothelial and pericyte dysfunction in disease pathogenesis, there is not one biomarker that is capable of defining SVD or effectively adding a diagnostic or outcome-predictive ability in clinical practice. Establishing disease-specific biomarkers could facilitate efforts to unveil pathophysiology, develop treatments, and evaluate their effectiveness.

An important reason a biomarker signature for SVD remains elusive relates to the substantial inconsistencies in study results due to methodological heterogeneity. Many studies are cross-sectional and focus on WMH as the

sole outcome of interest, and whilst WMH are the most commonly observed SVD-related lesion on MRI, SVD represents a dynamic disease, and distinct lesions might represent distinct phases of disease; indeed, even WMH can progress and regress[414]. Here, we have included a longitudinal analysis with several structural and functional radiological markers of SVD as well as cognitive impairment and decline. Findings in this thesis can help identify key candidate biomarkers for further investigation in SVD research. Additionally, I highlight the need for large longitudinal and multi-modal biomarker studies to standardise and validate findings.

In clinical settings, biomarkers have the potential to serve as accessible and specific tools for diagnosis and prognosis. Our research uncovered a novel association between elevated circulating VEGF levels and an increased incidence of DWI-positive lesions within the first-year post-stroke. Additionally, we found that higher P-Selectin levels were linked to an increased risk of MCI at one year, whilst higher ICAM-1 levels were associated with lower CVR. If these findings are validated in larger cohorts, they could have significant clinical implications in stroke clinics, emergency departments, and clinical trial design by facilitating the risk-stratification of patients who may be at a higher risk of recurrent stroke and rapid-progression SVD. In addition, our findings from an 8.5-year longitudinal cohort study demonstrate the profound and lasting impact of SVD and mild stroke on patients' lives. Many patients experience significant difficulties with daily activities, cope with the lingering consequences of their stroke, and report cognitive concerns. These results highlight the critical need for long-term and interdisciplinary care for individuals affected by SVD and mild, non-disabling ischaemic stroke and emphasise the need for further research into the broader consequences of these conditions.

The outlook for future research into biomarkers within the field of vascular dementia is promising. Efforts are increasingly focused on identifying biomarkers for early detection of disease -when interventions would likely yield the highest benefits- particularly those which can help differentiate it from other dementia subtypes, including AD. In addition, leveraging multi-omics

approaches, including transcriptomics and proteomics, will undoubtedly lead to an enhanced understanding of the molecular mechanisms driving disease, possibly by uncovering key biomarkers or aiding in the creation of diagnostic panels with improved sensitivity and specificity. The standardisation of methodologies is crucial to facilitate comparison between studies, replication, and the eventual translation of clinical findings into clinical practice. Importantly, biomarker research should continue aiming to explore the potential roles of the of the neurogliovascular unit in disease, including endothelial cells and pericytes, to further understand their role in vascular dysfunction and potentially exploit them to develop target treatments. Particularly exciting prospects include ongoing clinical trials targeting endothelial dysfunction, including the ongoing LACunar Intervention trials, which repurpose Isosorbide Mononitrate and Cilostazol as potential treatments for patients with symptomatic SVD[312].

To effectively prevent or treat SVD, further research is critical to uncover the underlying vascular abnormalities in the disease, understand their causes, and identify which pathogenic processes may be amenable to intervention.

7.7.3 Final remarks

In this thesis, I aimed to assess the role of endothelial and pericyte dysfunction in SVD through a translational perspective. A growing body of recent research highlights the critical role of endothelial and pericyte dysfunction in disease pathogenesis. Clinical studies linking biofluid biomarkers of these dysfunctions to disease demonstrate promising potential, although efforts to harmonise methodologies across studies would likely be highly beneficial to the field.

Ageing mice show a decrease in brain capillary pericyte coverage and an increase in VCAM-1 expression throughout distinct areas of the brain compared to their young counterparts. In addition, there is evidence from *postmortem* human brain tissue suggesting a proportional relationship between lower pericyte coverage, higher cell adhesion molecule expression (indicative of a pro-inflammatory activated state), and disease severity on

pathology. In both mice and humans, lower pericyte coverage was shown to be spatially related to increased endothelial activation through cell adhesion molecule expression. Several blood biomarkers of pericyte and endothelial function showed potential associations to distinct radiological and functional markers of disease, including longitudinally. Finally, results from our longitudinal observational study underscore the profound clinical impact of SVD and its significant toll on patient quality of life. Advancing research into the molecular mechanisms driving disease holds great promise for the development of effective therapeutic interventions.

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Appendix A. Supplemental material to Chapter 2

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Review

A Scoping Review on Biomarkers of Endothelial Dysfunction in Small Vessel Disease: Molecular Insights from Human Studies

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Abstract: Small vessel disease (SVD) is a highly prevalent disorder of the brain's microvessels and a common cause of dementia as well as ischaemic and haemorrhagic strokes. Though much about the underlying pathophysiology of SVD remains poorly understood, a wealth of recently published evidence strongly suggests a key role of microvessel endothelial dysfunction and a compromised blood–brain barrier (BBB) in the development and progression of the disease. Understanding the causes and downstream consequences associated with endothelial dysfunction in this pathological context could aid in the development of effective diagnostic and prognostic tools and provide promising avenues for potential therapeutic interventions. In this scoping review, we aim to summarise the findings from clinical studies examining the role of the molecular mechanisms underlying endothelial dysfunction in SVD, focussing on biochemical markers of endothelial dysfunction detectable in biofluids, including cell adhesion molecules, BBB transporters, cytokines/chemokines, inflammatory markers, coagulation factors, growth factors, and markers involved in the nitric oxide cascade.

Keywords: endothelial dysfunction; endothelial cells; endothelial activation; small vessel disease; vascular dementia; blood–brain barrier; cerebrovascular dysfunction; white matter hyperintensities; lacunar stroke; lacune; microbleeds; perivascular spaces

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1. Introduction

Small vessel disease (SVD) is a highly prevalent disorder of the brain's perforating microvessels. It is the underlying cause of a quarter of all ischaemic strokes and most haemorrhagic strokes and is the commonest cause of vascular dementia, contributing to approximately 45% of all dementias worldwide [1]. Radiological signs of SVD include lacunes, small subcortical infarcts, white matter hyperintensities (WMH), microbleeds, brain atrophy, and enlarged perivascular spaces (PVS), and are clinically associated with lacunar stroke, cognitive decline, neuropsychiatric symptoms and mobility problems [2–5]. Despite its increasing incidence and importance in the public health domain, the underlying pathophysiology of SVD remains poorly understood, and there is currently no effective therapeutic intervention beyond secondary stroke-prevention strategies.

SVD is associated with cerebral vascular dysfunction, characterised by structural and functional alterations in cerebral blood vessels, in blood flow responsiveness to changing metabolic demands, and a compromised and permeable blood–brain barrier (BBB), which precedes visible brain lesions [6]. The BBB is a highly specialised and dynamic structure referring to the set of characteristics in the vessels that vascularise the brain. It is formed by a tight monolayer of endothelial cells that line the blood vessel lumen, which uses selective permeability to closely regulate the exchange of substances between the circulating blood and the brain. Its function is also influenced by the constituents of the neurogliovascular unit, including pericytes, astrocyte endfeet, microglia, oligodendrocytes, the basal membrane, and neurons. Optimal BBB functioning is required to maintain healthy brain

function by allowing for the adequate delivery of nutrients and oxygen to neurons, promoting drainage of metabolic waste, and protecting neurons from pathogens and neurotoxic compounds [7,8].

Brain endothelial cells are joined by tight junctions and serve to maintain the integrity of the BBB as well as play a role in modulating vascular tone, blood flow, inflammation, thrombosis, and immune cell adhesion and transmigration [7–10]. Endothelial cells are responsive to changes in their environment and can sense and adapt to fluctuations in blood flow or pressure through surface mechanoreceptors as well as communicate with their cellular environments through ion channels and signalling molecules and proteins that can alter their signature and thus influence brain vessel function [11,12]. Endothelial malfunction can be detected in vivo by quantifying soluble blood and cerebrospinal fluid (CSF) markers that are released into the circulation and offer a window into the diverse facets of endothelial function that contribute to homeostasis; these include cell adhesion molecules, BBB transporters, cytokines and chemokines, pro-inflammatory markers, coagulation factors, growth factors, and markers pertaining to the nitric oxide (NO) pathway.

In the pathological context of SVD, and indeed to an extent in normal ageing, there is a loss of some of the properties that maintain BBB integrity and a propensity towards endothelial dysfunction and activation, both of which carry downstream consequences influencing the function of surrounding cells and tissue and are associated with disease burden and progression [4–6,13–23]. Within this review, the term 'endothelial dysfunction' encompasses the established definitions of both endothelial dysfunction, marked by decreased endothelium-derived nitric oxide activity, and endothelial activation, which refers to the increased expression of endothelial cell-surface adhesion molecules and leukocyte adhesion molecules induced by pro-inflammatory cytokines [10,24]. Though there is mounting evidence advocating for the role of endothelial dysfunction in SVD, the underpinning molecular mechanisms and better methods to detect dysfunction in vivo remain unclear. In this scoping review, we aim to summarise the findings from clinical studies examining the role of the molecular mechanisms underlying endothelial dysfunction and activation in SVD, focussing on biochemical markers of endothelial dysfunction detectable in blood or CSF.

2. Methods

Literature Review

We reviewed the literature examining biomarkers pertaining to endothelial dysfunction and radiological features of SVD in clinical studies. The literature search was conducted using MEDLINE and EMBASE from database inception to 1 June 2023 using key terms relating to small vessel disease (lacunes, small subcortical infarcts, white matter hyperintensities, microbleeds, and enlarged perivascular spaces) combined with terms relating to the endothelium (endotheli*). All articles were screened for eligibility criteria. We included publications relating to humans and examining biochemical markers of endothelial dysfunction detectable in blood or CSF and known radiological markers of SVD. We excluded review articles/meta-analyses, publications relating to study rationale and design, publications based exclusively on case studies or monogenic forms of SVD (including Cerebral Autosomal Dominant Arteriopathy with Sub-cortical Infarcts and Leukoencephalopathy (CADASIL)), as well as articles solely pertaining to mechanisms of cardiovascular or large-artery disease (e.g., coronary artery disease, atherosclerosis). Duplicate articles were removed. Additional studies examining the relationship between radiological signs of SVD and biomarkers of endothelial dysfunction from reference lists were also manually added to reinforce the search. We extracted summary data for each paper, including main aims, study population and sample size, methods employed, adjustment for covariates and primary findings.

3. Results

The search yielded 380 results, 135 of which were classified as reviews and were thus removed, as were two duplicate publications and four papers pertaining to excluded publication types (Figure 1). Of the 239 remaining results, 194 publications described clinical studies, of which 38 were eligible for inclusion based on the criteria outlined above. In addition, 11 papers were manually added from reference lists.

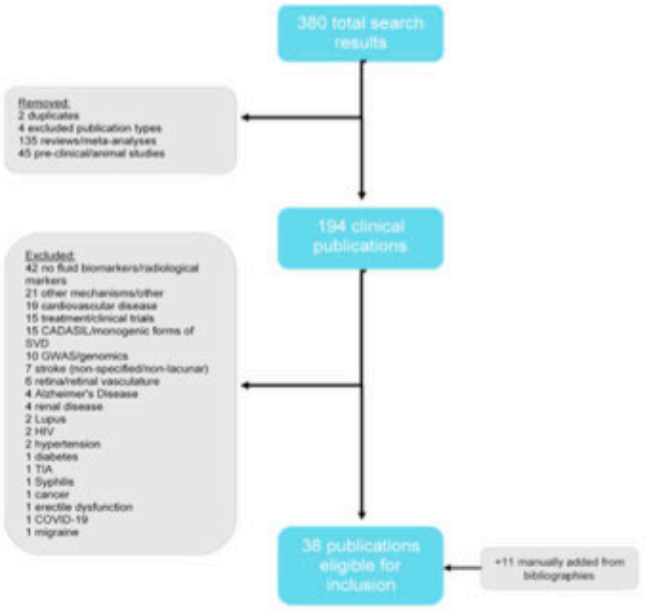

Figure 1. Study screening and selection process. A total of 49 publications were included in this review, including 11 manually selected from bibliographies.

4. Biomarkers of Endothelial Dysfunction and SVD: Molecular Insights from Human Studies

4.1. Immunoglobulin Superfamily—Cell Adhesion Molecules and Selectins

In a homeostatic state, most cell adhesion molecules are constitutively expressed at low levels in the brain vasculature, and endothelial cells have a limited adhesiveness to leukocytes. However, a cytokine-activated endothelium can express cell-surface adhesion molecules that facilitate the recruitment, rolling, adhesion and transendothelial migration of leukocytes through the endothelium of vascular walls; the soluble forms of these molecules are released from the cell surface into the circulation and can serve as biomarkers of endothelial activation [25–27]. Endothelium-leukocyte interactions engender a cyclic activation of both components, leading to the production of neopterin and cytokines, which can also instigate hepatic production of C-reactive protein (CRP), thus exacerbating an inflammatory response [28,29]. There is also evidence suggesting the involvement of other constituents of the neurogliovascular unit, particularly pericytes, in dysfunctional crosstalk, which can further aggravate the endothelium [13].

Vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) are transmembrane glycoproteins from the immunoglobulin gene superfamily which bind to integrins on the cell surface, whilst endothelial–leukocyte adhesion

molecules, Endothelial-selectin (E-selectin) and Platelet-selectin (P-selectin), interact with leukocyte surfaces via carbohydrate structures [27]. Circulating cell adhesion molecules have been linked to ageing and a series of age-related neurodegenerative disorders, including Alzheimer's Disease (AD) and SVD, and there is substantial evidence suggesting their expression can lead to downstream signalling events that are associated with increased BBB permeability [22,30–36]. For instance, cultured human brain microvascular endothelial cells exposed to soluble VCAM-1 (sVCAM-1) exhibit increased permeability of a soluble tracer accompanied by evidence of actin stress fibre formation and a decrease in tight junctions [31].

We found 19 published studies investigating the relationship between cell adhesion molecules and radiological features of SVD, featuring a total of 7364 study participants (average sample size: 368.2).

In a study with 110 lacunar stroke patients and 50 community controls with similar vascular risk-factor profiles, those with lacunar stroke had higher levels of circulating ICAM-1 [18]. These associations remained significant after controlling for conventional vascular risk factors and were not found to be associated with age, sex, blood pressure, diabetes, myocardial infarction, or time from vascular event to blood draw (median: 210 days). They also found comparable ICAM-1 concentrations amongst patients with isolated lacunar infarcts and those with WMH lesions.

Similarly, circulating levels of ICAM-1, VCAM-1 and P-selectin (though not E-selectin) were significantly higher in a group of 163 first-ever lacunar stroke patients compared to essential hypertensive patients and healthy controls [34]. Those with extensive WMH lesions had higher concentrations of sVCAM-1 than those with mild or absent WMH, and those with radiological evidence of both, a lacunar infarct and WMH lesions had the highest levels of sVCAM-1 and sICAM-1 on average. Though a marginal portion of the essential hypertensive group was found to have asymptomatic lacunes (n = 13/183) and an extensive WMH burden (n = 22/183) on magnetic resonance imaging (MRI), the distinct biomarker signatures between these groups provide further evidence in support of SVD-specific pathology beyond hypertension-related vascular injury. In the same study, multivariate analyses controlling for age, sex, pre-existing large-artery disease, and patient group revealed a positive association between higher levels of sE-selectin and the number of microbleeds, perhaps suggesting a haemorrhagic phenotype-specific role. This is further supported by another study with 126 first-ever ischaemic stroke patients, where higher levels of sE-selectin within 24 h of hospital admission were associated with the prevalence of microbleeds after adjusting for demographic and clinical variables [37]. No significant differences in P-selectin-positive platelets were found amongst 74 lacunar stroke patients and 74 controls matched for age, sex, diabetes and hypertension status with no history of stroke, though the possible presence of radiological signs of SVD in controls was not ruled out, nor was the extent of SVD radiological burden taken into account in these analyses [38].

We found few studies examining the relationship between fluid biomarkers and enlarged PVS, though, in 100 mild ischaemic stroke patients, PVS burden in the basal ganglia was not associated with ICAM-1 levels. While this might indicate different pathological processes involved in the appearance of PVS, further investigation is needed [39].

Including mild ischaemic stroke patients as controls can help parse out potential effects independent of those stemming from an acute ischaemic event or large-artery pathology. Regression analyses in a group of 263 thrombolysed acute ischaemic stroke patients showed that a combined score comprised of the number of lacunes and WMH lesion severity as detectable on computerised tomography (CT) was associated with increased levels of sICAM-1 and sVCAM-1 at 90 days post-stroke after adjusting for age, sex, and vascular risk factors [40]. Interestingly, levels of these biomarkers were comparable between patients with WMH, cerebral atrophy and/or lacunes compared to those without at the time of stroke-symptom onset, suggesting possible fluctuations in circulating cell adhesion markers during an acute stroke phase. Compared to a group of 67 age- and sex-matched controls, 64 SVD patients and 109 patients with large-artery atherosclerosis had higher concentrations

of sE-selectin and sICAM-1 (though not sVCAM-1), with the highest concentrations found in patients with intracranial macroangiopathy [41]. However, these analyses did not account for clinical covariates despite substantial group differences- for instance, nearly half of the patients with large-artery atherosclerosis were smokers, compared to 13% of SVD patients. In addition, some participants in every group had a history of stroke, including 21% of controls, which may have affected the results.

In a small study of 29 patients with SVD and 18 patients with recent ischaemic stroke on the basis of internal carotid artery stenosis, levels of sVCAM-1 and sP-selectin were significantly higher in patients in the highest quartile of periventricular WMH lesion burden compared to those in the lower quartiles [22]. This relationship did not extend to subcortical white matter lesions. To account for potential differences relating to the stroke itself, the authors performed a sub-analysis comparing cell adhesion molecule concentrations with degree of white matter lesions separately in patients with acute stroke and those with carotid atherosclerosis. Their results indicated that the increased levels of sVCAM-1 and sP-selectin in those with severe periventricular WMH lesions in the group were driven by the subgroup of patients with carotid atherosclerosis. It is plausible that this is a result of compounded chronic vascular damage, which might attenuate the relationship between WMH and cell adhesion molecules. It is important to note that cell adhesion molecule plasma concentrations in this small study sample were highly variable, and the dichotomisation of white matter lesions to 'mild' (including the lowest three quartiles of WMH lesion burden) and 'severe' (including the highest quartile), could potentially overlook a nuanced relationship between WMH volume and endothelial activation. In addition, though the study was comparing symptomatic SVD with large-artery atherosclerosis, both groups had comparable levels of WMH lesion burden, and the analyses did not account for potential confounding variables, including vascular risk factors.

Similar results are mirrored in studies with healthy ageing cohorts. In 175 healthy adults $\geq$60 years old with no history of stroke or cognitive impairment, those in the highest quartile of sICAM-1 levels had a four-fold increased risk of having WMH lesions on MRI after adjusting for age, sex and traditional vascular risk factors [42]. In a large Chinese community-based cohort study (n = 960; average age: 56.02), grouped endothelial dysfunction biomarkers, including sE-selectin, sP-selectin, sICAM-1 and sVCAM-1, were associated with WMH volume and the presence of lacunes, though not microbleeds or PVS, independent of age, sex and vascular risk factors; backward elimination regression retained sVCAM-1 as an independent predictor of WMH volume [43]. In the Framingham Offspring cohort, analyses with 1763 stroke-free individuals (average age: 60.2) showed higher levels of sICAM-1 (though not sP-selectin) in those with radiological signs of SVD, defined as the presence of lacunes and/or extensive WMH lesions on MRI. Though these results survived additional adjustment for clinical covariates, they did not withstand Bonferroni correction for multiple comparisons. It is worth noting that the mean time from blood draw to MRI was substantial, averaging 5.4 years, and thus it is possible that biomarker profiles were not representative of temporal disease progression. In addition, the study excluded those with a history of stroke, limiting the participation of individuals with symptomatic or more severe SVD; indeed, only 12–21% of participants had lacunes or extensive WMH lesions [44].

Longitudinal studies also support the involvement of cell adhesion molecules in SVD progression and adverse clinical outcomes. In a 10-month follow-up study, 35 SVD patients receiving a monthly MRI were stratified into those with SVD progression and those without, based on the appearance of incident lacunes, microbleeds or an increase in WMH volume. After adjusting for age, sex, and hypertension, circulating levels of sVCAM-1 and sE-selectin were higher in those with SVD progression compared to those without, though only the latter was statistically significant [45]. Changes in sICAM-1 and sVCAM-1 levels were positively associated with an increased risk of SVD progression in a 69-month longitudinal study with 1396 participants and were independently associated with WMH grade and the number of new appearing lacunes and microbleeds [46]. Likewise, amongst

a cohort of 123 SVD patients followed-up at 2 years, those with radiological disease progression had a higher compound z-score comprised of markers of vascular inflammation, including sICAM-1 and sP-selectin, after adjusting for age, sex, blood pressure and total SVD radiological load [47]. Circulating levels of sICAM-1 were significantly higher in 113 acute lacunar stroke patients compared to 43 healthy controls [48]. Although potential effects stemming from an acute ischaemic event cannot be discounted, they also found that within those with a lacunar stroke, sICAM-1 levels were higher in those who had neurological deterioration within 48–72 h post-stroke (defined as a $\geq$ 1 point fall in the motor items of the Canadian Stroke Scale) than those with non-progressive strokes. After adjusting for potential confounding variables, sICAM-1 levels were also independently associated with poor outcomes 3 months post-stroke, including lower functional independence and death, though infarct volume and radiological disease burden could have mediated this association. Intriguingly, in an 8-year longitudinal cohort study of 495 cognitively unimpaired elderly individuals (average age: 70.02) and 247 participants (average age: 70.89) with mild cognitive impairment (MCI), CSF levels of ICAM-1 and VCAM-1 had independent effects on declining cognition, even after adjusting for WMH volume, potentially suggesting a distinct pathway involved in cognitive decline [49].

In the Austrian Stroke Prevention Study cohort, 296 community-dwelling participants received an MRI at baseline and again at 3 and 6 years [50]. After adjusting for age, sex, CRP levels, baseline WMH volume and traditional vascular risk factors, WMH lesion progression at both follow-up time points was associated with higher levels of circulating sICAM-1. In a longitudinal study of 190 type-2 diabetes patients, baseline levels of sICAM-1 were higher in patients with radiological signs of SVD than in those without and were also associated with the increased presence of incident lacunes and WMH lesion progression at 3 and 6 years after adjusting for age, sex, hypertension, duration of diabetes, baseline MRI findings and medication use [51]. They also demonstrated a significant trend association between higher levels of sICAM-1 and lower scores in a letter-digit psychomotor task, though this could be mediated by radiological disease burden.

Examining brain-endothelial specific molecular mechanisms of SVD in patients represents a significant challenge, and while quantifying biomarkers from circulating blood can reflect possible dysregulated pathways, it provides an indirect path of examination. Studies zoning in on brain endothelial cell signatures from SVD patients provide some conflicting evidence. A post-mortem study examining vessels from 17 SVD cases and 12 aged controls found little evidence of positive endothelial ICAM-1 vessels in either group [52]. The authors hypothesise that elevated circulating levels of sICAM-1 in SVD patients are, therefore, unlikely brain-derived and may indicate inflammatory activity in distal peripheral vascular territories. However, this study limited analyses to one cell adhesion molecule and small arteries within the caudate putamen grey matter areas, excluding other vascular territories directly impacted by SVD, namely the capillaries feeding into the white matter. While it remains feasible that high sICAM-1 levels in SVD patients could stem from beyond the brain endothelium, ICAM-1 is a heterogeneous molecule and can influence endothelial function through dysfunctional crosstalk involving other cells in the brain that can also derive it, including monocytes, macrophages, smooth muscle cells and pericytes [53]. Elahi et al. [54] used plasma membrane-derived vesicles released by cells (plasma endothelial-derived exosomes (EDE)) in 26 functionally intact older adults (average age: 75.4) to compare protein cargo between those with and without WMH lesions on MRI. Though SVD cases showed higher levels of brain-expressed EDE cargo proteins, and these were associated with lower cognitive function, there were no significant group differences detected in VCAM-1 levels specifically after adjusting for age.

Although there is evidence in support of the involvement of cell adhesion molecules in diverse manifestations of SVD, the lack of clarity on the underlying mechanisms through which they might influence disease and where the molecules derive from and how they engender and interact with pathological mechanisms warrants further examination.

4.2. Blood–Brain Barrier Transporters

Membrane transport proteins at the BBB mediate the influx and efflux of solutes from the circulation through endothelial cells and into the brain and vice-versa. Glucose transporter-1 (GLUT-1) is expressed in brain vascular endothelial cells and transports glucose to the brain via facilitative diffusion. GLUT-1 levels are implicated in metabolic dysregulations and are known to decrease with ageing, possibly disturbing neurovascular coupling processes based on glucose demand [55]. Permeability-glycoprotein (P-GP) is an adenosine triphosphate binding cassette transporter expressed by the luminal surface of endothelial cells. It is known for its role in pharmacokinetics and drug-delivery to the BBB by strictly limiting the exposure of pharmaceuticals to the brain by actively transporting undesired substrates from the BBB back to the bloodstream against the concentration gradient; it has also been implicated in the accumulation of amyloid-β (Aβ) in AD pathology [39,56]. Large neutral amino acid transporter-1 (LAT-1) is expressed in both the luminal and abluminal membranes of endothelial cells and carries large essential amino acids across the BBB [57]. Caveolin-1 (Cav-1) is the principal scaffold protein comprising caveolae, which are invaginations abundant on the cell membrane of endothelial cells, facilitating vesicle trafficking and signal transduction. Cav-1 activity is related to oxidative stress, the attenuation of inflammatory pathways, and BBB permeability, perhaps through the modulation of tight junction-associated proteins [58,59]. Pro-inflammatory cytokines can also upregulate BBB transporter protein expression in endothelial cells, affecting vascular function. For instance, increases in GLUT-1 in murine smooth muscle cells can impair vascular contractility and instigate an inflammatory response to vascular injury [60]. GLUT-1 deficiency in mice overexpressing Aβ-peptide precursor protein leads to significant reductions in blood flow, BBB and microvascular breakdown, accelerated Aβ accumulation, cognitive deficits and progressive neurodegeneration [61].

In an attempt to circumvent the low specificity of fluid biomarkers for the assessment of endothelial dysfunction, a case-control study with 26 functionally intact older adults (average age: 75.4) used endothelial-derived biomarkers to examine the relationship between WMH lesions and endothelial-expressed BBB transporter proteins GLUT-1, P-GP and LAT-1 [54]. EDE cargo protein quantification offers a non-invasive way of observing cellular and molecular changes in vivo using exosomes, which are cell-released vesicles containing cargo molecules such as proteins, mRNAs and lipids from their cell of origin. Levels of GLUT-1, LAT-1 and P-GP were significantly higher in participants with WMH than in those without. Age-adjusted logistic regression analyses demonstrated a robust predictive power of GLUT-1, LAT-1 and P-GP to differentiate between participants with and without WMH, with the area under the curve for the strongest predictor, GLUT-1, being 0.89. LAT-1 levels were significantly inversely associated with total grey matter volumes after controlling for intracranial volume (ICV) and age and correcting for multiple comparisons. In addition, all brain-expressed EDE cargo proteins were associated with lower cognitive performance in a set-shifting speed task after adjusting for age. Cytokines and chronic states of inflammation could up-regulate some BBB transporter proteins in endothelial cells, and interestingly, levels of GLUT-1, P-GP and LAT-1 were associated with levels of systemic inflammatory cytokine Interleukin-6 (IL-6) in this cohort [54].

In 156 first-ever ischaemic stroke patients, circulating Cav-1 levels were lower in patients with microbleeds compared to those without and lowest in patients with multiple microbleeds or microbleeds in deep or infratentorial areas compared to those with a single microbleed or strictly lobar microbleeds [62]. Patients below their established Cav-1 concentration cut-off ($\leq$5.25 ng/mL) had a nearly four-fold increased risk of having microbleeds after adjusting for age, sex, vascular risk factors, anti-thrombotic use, glucose levels, lipids and fibrinogen. There was no relationship detected between Cav-1 levels and WMH lesions or lacunes.

Clinical research studies investigating the role of transport proteins in SVD pathogenesis are scarce. However, transporter proteins play a crucial role in BBB homeostasis and can potentially serve as promising targets to ameliorate the BBB dysfunction observed in

the early stages of the disease. The studies mentioned here suggest a relationship between transporter protein dysregulation and some radiological manifestations of SVD, though more studies are required to validate and further uncover the nature of this relationship.

4.3. Cytokines and Chemokines

4.3.1. Interleukin-6

IL-6 is a pro-inflammatory cytokine with pleiotropic effects produced by monocytes and endothelial cells. Once synthesised in a lesion site, it migrates to the liver, where it incites the release of several acute-phase proteins, including CRP and fibrinogen [63]. It also instigates platelet production and is thus involved in the development of atherosclerosis. Pre-clinical studies suggest IL-6 can engender endothelial dysfunction through an increase in Angiotensin II type 1 (AT1) receptor activity, an interaction with tumour necrosis factor alpha (TNF-α), and the induced production of reactive oxygen species (ROS), which can cause enhanced vascular superoxide production, exacerbate oxidative stress, and impair endothelium-dependent vasodilation [64,65]. Numerous clinical studies have examined the association between IL-6 and cardiovascular health, particularly in stroke, with many lines of evidence suggesting it serves as a strong predictor for stroke, atherosclerosis and adverse cardiovascular events [66,67].

In a Japanese study with 194 neurologically asymptomatic participants at a high risk of stroke, mean serum levels of IL-6 were higher in patients with lacunes visible on MRI than in those without, with one standard deviation increase in IL-6 leading to a nearly two-fold increased risk of having at least one visible lacune after adjusting for age, sex, vascular risk factors, medication use and carotid atherosclerosis [68]. In the Cardiovascular Health Study, both white (n = 3073) and black (n = 571) participants with higher IL-6 levels had a mildly increased risk of having WMH lesions and brain infarcts independent of age, sex and cardiovascular risk factors [69]. Likewise, in 247 elderly participants with MCI, though not in 495 cognitively unimpaired participants, baseline CSF IL-6 levels were associated with greater WMH volume after adjusting for age, sex, ICV and vascular risk factors [49]. Adjusting for CSF Aβ-40 attenuated the relationship. Intriguingly, lower IL-6 levels were associated with increasing WMH burden over 7 years in the same study. It is possible that the greater incidence and severity of WMH lesions in the MCI group increased the likelihood of finding significant relationships within this group due to enhanced statistical power.

In univariate analyses, plasma IL-6 concentrations were significantly higher in 113 lacunar stroke patients within 24 h of stroke onset compared to 43 healthy controls, though the possible confounding effects of an acute ischaemic event, demographic characteristics or comorbidities were not taken into account in these analyses [48]. In this same study, after adjusting for potential confounding variables, there was no association found between circulating levels of IL-6 and worsening clinical progression or poor outcome 3 months post-stroke. A study comparing 65 lacunar stroke patients with 60 cortical stroke patients reported no significant differences in circulating IL-6 levels between the groups, possibly suggesting the association between IL-6 and recent stroke might not be specific to SVD pathology [70]. It is important to consider that medication use (including aspirin treatment), glutamate and GABA levels, the presence or timing of stroke, and the degree of large-artery atherosclerosis are all elements that could influence circulating pro-inflammatory biomarker levels.

In line with previous research examining the relationship between IL-6 levels and cardiovascular event risk, IL-6 concentrations were associated with an increased risk (OR: 1.4, 95% CI: 1.1–2.2) of adverse vascular events, including stroke, cardiovascular events and/or vascular death in a 24-month longitudinal study with 130 functionally independent SVD patients [71]. This association was further confirmed by findings from the LIMITS study, which observed an association between elevated levels of circulating IL-6 and the risk of recurrent ischaemic stroke, myocardial infarction or vascular death in 1244 radiologically confirmed lacunar stroke patients [72]. However, a small 10-month

longitudinal study with 35 SVD patients reported no significant association between baseline IL-6 levels and the appearance of new lesions on diffusion-weighted MR imaging, incident lacunes and/or microbleeds or WMH progression after adjusting for age, sex and hypertension [45].

Although studies examining the relationship between IL-6 and other radiological markers of SVD were lacking, one study reported finding no association between IL-6 and the presence of basal ganglia PVS on MRI after adjusting for potential confounding variables in 100 mild ischaemic stroke patients, both lacunar and cortical [73].

4.3.2. Tumour Necrosis Factor Alpha and Its Receptors

TNF-α is a pro-inflammatory cytokine primarily secreted by macrophages and microglia that functions via cell membrane receptors, including tumour necrosis factor receptors 1 and 2 (TNFR1/TNFR2). TNF-α promotes the secretion of endothelial cell adhesion molecules, facilitating leukocyte adhesion and reduced blood flow [74]. High levels of TNF-α have been observed in patients with acute ischaemic stroke [75]. In both clinical and pre-clinical investigations, TNF-α has been associated with increased BBB permeability and dysfunction, inflammation, oligodendrocyte death and myelin damage [76–78]. In addition, increased levels of TNF-α can inhibit NO production in the endothelium, induce ROS production, and impair vasodilation [79,80].

Results from the LIMITS study provide some evidence of the relationship between TNFR1 and worse clinical outcomes in SVD patients. The risk of major vascular events, though not recurrent stroke, increased with elevated concentrations of TNFR1 (HR: 1.68, 95% CI: 0.93–3.04) after adjusting for demographics, risk factors and statin use in 1244 radiologically-confirmed lacunar stroke patients (average age: 63.3) [72]. There was no evidence that TNFR1 was associated with the risk of having a recurrent lacunar stroke.

Average plasma concentrations of TNF-α were significantly higher in 113 acute lacunar stroke patients compared to 43 healthy controls in univariate analyses [48]. In addition, within lacunar stroke patients, levels of TNF-α were higher in those who had neurological deterioration (defined as a fall of $\geq$1 point in the motor items of the Canadian Stroke Scale within inclusion and 48 h) and served as an independent predictor for worse clinical outcome (Barthel index < 85 or death). However, it is worth noting that the control group was considerably younger (average age: 55) compared to the lacunar stroke group (average age: 69.7). Moreover, comparing an acute-stroke group to a non-stroke group provides limitations in the interpretation of results, particularly as it relates to the variation of pro-inflammatory molecules that may fluctuate as a direct consequence to an acute ischaemic event. Indeed, results from another study reported no significant differences in circulating TNF-α levels between 60 cortical stroke patients and 65 lacunar stroke patients 1–3 months post-stroke [70].

Studies examining the relationship between TNF- α and radiological markers of SVD beyond WMH lesions are sparse. In a prospective study with 100 mild ischaemic stroke patients, there were no significant associations between TNF-α and PVS after adjusting for potential covariates [73]. In the Framingham Heart Study cohort (sample size: 1763, average age: 60.2), higher levels of circulating TNFR2 were associated with the presence of deep cerebral microbleeds after adjusting for age, sex, time from blood draw to MRI (average: 5 years), hypertension, WMH volume, number of lacunes, and medication use [44]. In this study, increases in TNFR2 related to a 3.3-fold increase in the odds of having more than one microbleed and a 5.7-fold increase in the odds of having more than two microbleeds, possibly suggesting distinct mechanistic pathways involved in ischaemic and haemorrhagic manifestations of SVD.

Dobrynina and colleagues [81] performed a cluster comparison in 40 SVD patients with a Fazekas score of three to categorise patients into two radiological subtypes of SVD. Patients in the MRI subgroup characterised by more severe deep WMH, lacunes in the white matter as opposed to dispersed, a lack of microbleeds or atrophy and less severe

clinical manifestations had significantly higher levels of TNF-α compared to those in the other MRI group and healthy controls.

4.3.3. Osteoprotegerin

Osteoprotegerin is a cytokine expressed by endothelial cells and smooth muscle cells involved in inflammation and vascular injury. High levels have been associated with large-artery atherosclerosis and stroke, though there is limited research regarding its relation to SVD [82]. Results from the Framingham Offspring cohort study (sample size: 1763; average age: 60.2) showed that participants with lacunes and/or extensive WMH lesions had higher levels of osteoprotegerin than those without after adjusting for age, sex, vascular risk factors and the presence of microbleeds; the relationship also withstood Bonferroni correction for multiple comparisons [44].

4.3.4. Platelet Factor-4

Platelet factor-4 (PF-4) is a chemokine stored in the alpha granules of platelets. It is released on activation, whereupon is exerts angiostatic effects by inhibiting the binding of specific growth factors, such as vascular endothelial growth factor (VEGF), to cells [83]. PF-4 has previously been associated with stroke risk, infarct size and worse clinical outcomes post-stroke [84]. A prospective study featuring 123 SVD patients aged over 60 years demonstrated that patients with radiological disease progression, as measured by an increase in WMH severity, had higher levels of PF-4 after adjusting for age, sex, blood pressure and total SVD radiological load [47]. The precise mechanisms underpinning the interaction between SVD and PF-4 remain unclear, and larger studies are required to reach more robust and definitive conclusions.

4.4. Other Pro-Inflammatory Markers

4.4.1. C-Reactive Protein

CRP is a hepatic acute-phase reactant protein known to increase in concentration as a response to IL-6 secretion and systemic inflammation. Though little is known about its direct role in SVD, there is an established connection between CRP and cardiovascular-event risk, including stroke [85,86]. CRP can influence endothelial dysfunction in conjunction with its involvement in systemic inflammation through a variety of diverse mechanisms. For instance, CRP activity can inhibit angiogenesis, promote atherosclerosis, mediate circulating cytokine levels, inhibit NO, decrease endothelial nitric oxide synthase (eNOS) activity in endothelial cells, increase Endothelin-1 production, attenuate endothelial-dependent vasodilation and increase endothelial cell adhesion molecule expression, including VCAM-1 and E-selectin [87–89].

We found 12 studies investigating the relationship between CRP levels and radiological signs of SVD, featuring a total of 9794 study participants (average sample size: 816.16).

Most studies featuring acute and sub-acute stroke patients do not report strong associations between CRP levels and SVD disease burden, though it is important to note that CRP levels are influenced by ischaemic events. For instance, baseline CRP levels were higher in 74 lacunar stroke patients within a month of symptom onset compared to 74 controls matched for age, sex, hypertension and diabetes; however, this difference was attenuated by 3 months post-stroke, possibly suggesting that the observed elevated CRP levels in lacunar stroke patients were attributable to an acute ischaemic event rather than chronic SVD disease mechanisms [38]. In a study with 65 lacunar stroke patients and 60 cortical stroke patients serving as controls, CRP levels did not vary significantly between the groups after adjusting for age, sex and the presence of traditional vascular risk factors. However, incorporating a cluster of inflammatory markers, including CRP, TNF-α and IL-6, into their base model for predicting WMH volume mildly improved model fit (from R2 = 0.289 to R2 = 0.291) [70].

Though WMH lesion burden and the presence of lacunes constituted the main outcomes in most of the publications we found, a study examining the association between

PVS in the basal ganglia and circulating CRP levels at 1–3 months post mild cortical or lacunar stroke in 100 patients reported no significant associations after controlling for age, sex and vascular risk factors [73]. In addition, a recent study reported significantly higher levels of CRP in SVD patients with cognitive impairment (n = 40) compared to SVD patients without cognitive impairment (n = 38) and healthy controls (n = 35), respectively [90]. However, these analyses did not account for potential covariates and included a relatively small sample size.

In line with existing evidence, longitudinal studies with SVD patients support the relationship between baseline CRP levels and recurrent stroke. In 1422 lacunar stroke patients with no prior history of cortical stroke or carotid stenosis, patients in the highest quartile of circulating CRP levels measured in the subacute post-stroke phase (median: 60 days) had more than twice the risk of recurrent ischaemic stroke at 3-year follow-up (OR: 2.32, 95% CI: 1.15–4.68) after adjusting for age, sex, race-ethnicity, history of cardiac disease, vascular risk factors and statin use [91]. Although approximately 70% of recurrent ischaemic strokes were of lacunar aetiology, there was no evidence of a statistically significant predictive effect of CRP on recurrent lacunar stroke specifically. In addition, a small study with 130 SVD patients (including 28 vascular Parkinsonism patients) reported a marginal association between baseline CRP levels and recurrent stroke, death or cardiovascular events at 24-month follow-up after adjusting for age, sex, vascular risk factors, statin or antiplatelet use, and radiological SVD burden (HR 1.02, 95% CI: 0.98–1.7) [71].

Larger population-based studies with stroke-free participants have yielded conflicting results. Findings from the Rotterdam cohort (n = 1033, average age: 72) showed that those in the highest quartile of CRP levels at baseline had more extensive WMH lesions compared to those in the lowest quartile after adjusting for age, sex, cardiovascular risk factors and carotid atherosclerosis [92]. In the 636 participants who had a repeat MRI at 3 years, CRP levels at baseline were also associated with WMH lesion progression and number of new lacunes, though the latter association was not statistically significant. A small study with 194 neurologically asymptomatic patients with vascular risk factors showed similar results, with circulating levels of CRP being higher in those with lacunar infarcts compared to those without and CRP levels functioning as an independent predictor for lacunar infarcts after adjusting for risk factors and carotid atherosclerotic vascular disease (OR 1.85 per SD increase in CRP) [68]. Similarly, in 3644 participants (average age: 74.65) in the Cardiovascular Health Study, both white (n = 3073) and black (n = 571) participants with higher CRP levels had a mildly increased risk of having WMH lesions (OR: 1.13, 95% CI: 1.06–1.22) independent of age, sex and cardiovascular risk factors- though statistical significance was reached only in white participants. Plasma CRP levels were only marginally associated with the presence of brain infarcts in the two groups, though the infarct subtype was not specified. Moreover, there was no association found between CRP haplotypes or genotypes and the appearance of brain infarcts or WMH lesions [69].

Contrastingly, in 1763 stroke-free individuals in the Framingham Offspring cohort (average age: 60.2), there were no significant associations found between circulating CRP levels and the presence of lacunes, extensive WMH lesions or microbleeds [44]. In a longitudinal study of 190 type-2 diabetic patients, baseline levels of CRP were comparable between patients with radiological signs of SVD and those without [51]. In addition, there was no relationship between CRP levels at baseline and SVD progression at 3 or 6 years. However, previous research has shown an association between glycaemic control and systemic inflammation in people with diabetes, with increasing levels of haemoglobin A1c (HbA1c) being significantly associated with elevated CRP levels after adjusting for covariates [93]. Therefore, it is possible that CRP levels were elevated within the control group comprised of diabetic patients, and HbA1c levels were not incorporated into these analyses.

4.4.2. Neopterin

Neopterin is a pteridine immune modulator produced by activated monocytes and macrophages and can serve as a marker for inflammation. Higher neopterin levels have been detected in patients with cardiovascular disease and are associated with an increased risk of stroke [94]. Due to endothelium-leukocyte interactions, neopterin may play a direct role in inciting endothelial activation, possibly by promoting cell adhesion molecule expression mediated by oxygen free radicals [29,95]. Research relating neopterin to SVD is lacking, though one study with 163 lacunar stroke patients and 183 essential hypertensive patients reported higher levels of circulating neopterin in patients with lacunar infarct and/or WMH lesions compared to those without [34]. In addition, neopterin levels were independently associated with a higher number of PVS in the basal ganglia after controlling for age, sex, pre-existing large-artery disease, and patient group.

4.4.3. CD40 Ligand

CD40 ligand (CD40) is a transmembrane glycoprotein expressed by endothelial cells and activated T-cells in an inflammatory state. It has been implicated in the activation of endothelial cells and leukocyte adhesion; its anchoring to the endothelium not only encourages the production of pro-inflammatory cytokines but also enhances the expression of VCAM-1, ICAM-1 and E-selectin [96]. CD40 has previously been associated with stroke risk, infarct size and worse clinical outcomes post-stroke [84].

Findings from the Framingham Heart Study suggested no association between plasma CD40 levels and WMH, lacunes or microbleeds in 1763 stroke-free participants [44]. In a prospective cohort study with 123 SVD patients aged over 60, those with radiological disease progression at 2-year follow-up had a higher z-score comprised of markers of vascular inflammation, which included CD40 ligand, after adjusting for age, sex, blood pressure, and total SVD radiological load score [47]. However, there were no reported independent associations between CD40 and radiological features of SVD or disease progression. Moreover, analyses with 1244 lacunar stroke patients in the LIMITS study demonstrated no association between circulating levels of CD40 and recurrent ischaemic stroke (including lacunar stroke), myocardial infarction, or vascular death, though analyses exploring CD40 and radiological SVD burden were not performed as part of this study [72].

Though a wealth of evidence suggests the involvement of systemic inflammation in stroke and cerebrovascular dysfunction, it is important to consider certain elements when dissecting the discrepant results from these studies. Firstly, a variety of factors can influence circulatory inflammatory markers, including comorbidities, concurrent infection, malignancy, medication use, and rheumatologic disease. Though some of these can be plausibly controlled for in certain study conditions, it remains unclear whether circulating biomarkers of systemic inflammation can serve as an adequate proxy for brain endothelial dysfunction. MRI markers of SVD reflect evidence of an accumulation of chronic disease, whereas blood biomarkers can fluctuate rapidly in response to acute events or homeostatic changes, something that bears particular relevance in the acute and subacute stages of stroke. For this reason, it is important to note the variability in the time window between stroke onset, serology and MRI acquisition in stroke studies and whether the control group was comprised of non-lacunar stroke or non-stroke participants. In addition, CRP levels have a known association with atherosclerosis and other large-artery-related pathology, which not only shares a vascular risk factor profile overlap with SVD, but might also chronically exert effects on the microvasculature. Whether SVD could be partly caused by the release of pro-inflammatory molecules into the brain microvessels and cellular milieu or whether it induces the synthesis and release of these molecules also remains unclear.

4.5. Coagulation Factors

Physiological haemostasis is preserved through blood constituents, blood flow regulation and the integrity of the vessel wall. Endothelial cells play a crucial role in the maintenance of physiological haemostasis and the modulation of thrombosis through the

tight control of both pro-coagulant and anti-coagulant mechanisms. Not only can the endothelium provide a surface for thrombosis formation, but it also regulates blood flow and mediates the expression of vasoactive factors relating to platelet reactivity, coagulation and fibrinolysis. At sites of vascular injury, platelets migrate to the endothelium and adhere to the injury site, where they form clots to effectively seal the barrier and prevent excessive blood loss. Several studies have investigated the relationship between circulating coagulation factors and radiological markers of SVD.

4.5.1. Von Willebrand Factor

Von Willebrand Factor (vWF) is a large polymeric blood glycoprotein synthesised by endothelial cells. Although it plays a role in haemostasis and thrombus formation by sensing and responding to shear blood flow changes, one of its main functions is to facilitate platelet adhesion to the endothelium in sites of vascular injury. States of chronic inflammation or endothelial activation can encourage excessive secretion of vWF from secretory granules within dysfunctional endothelial cells and increase platelet binding to vWF. Furthermore, high levels of circulating vWF are associated with prothrombotic complications, diabetes, stroke, and inflammatory cardiovascular disease [97]. Though some evidence suggests plasma vWF can function as a biomarker of endothelial dysfunction, considerable inconsistencies remain among clinical studies [98].

Average vWF levels were higher in a group of 74 recent lacunar stroke patients compared to 74 controls matched for age, sex, hypertension and diabetes; however, vWF levels had significantly decreased by 3 months post-stroke and were at comparable levels between groups, perhaps suggesting that chronic vWF elevation was not playing a central role in SVD pathophysiology [38]. A study of 263 thrombolysed acute ischaemic stroke patients further supports these findings, with vWF levels found comparable between patients with WMH lesions, cerebral atrophy or lacunes visible on CT compared to those without at the time of stroke onset [40]. Adjusted analyses at 90 days post-stroke showed similar results. Likewise, a previous study reported that vWF levels were increased to a similar extent in patients of acute-phase lacunar stroke as in patients of large-artery atherosclerotic and cardioembolic strokes [99]. There were also no significant differences in vWF levels between 65 lacunar stroke patients and 60 cortical stroke patients 1–3 months post-stroke after adjusting for age, sex and vascular risk factor profiles [70]. Collectively, these studies provide evidence for the hypothesis that although vWF might serve as a marker of endothelial dysfunction consequent to an acute endothelial response to an ischaemic insult, it might not reflect any SVD-specific pathogenic effect.

Notably, some studies suggest that an active form of vWF can be distinguished from a latent form, and though the use of total circulating vWF has yielded conflicting results, it is possible that differentiating between these subtypes could prove insightful in the context of SVD [100]. The ability to control vWF secretion from endothelial cells might serve as a future therapeutic target, though more research, particularly in the form of large longitudinal studies, is required to adequately differentiate the role of vWF from an acute response to an ischaemic insult versus a marker of chronic endothelial dysfunction pertinent to SVD disease mechanisms.

4.5.2. Tissue Factor and Tissue Factor Pathway Inhibitor

Tissue factor (TF) binds to coagulation factors and thrombin to form fibrin. TF is normally concealed within the intact vessel wall until it becomes exposed after vascular injury, wherein it initiates the clotting response with the recruitment of platelets and coagulation factors. Though it is most commonly expressed by perivascular cells and platelets, it can be secreted by the endothelium, particularly under pathophysiological conditions in which endothelial cell-secreted miR-126 inhibits thrombosis by attenuating TF expression [101]. Decreased levels of TF have been found in patients with ischaemic stroke, including lacunar stroke [102]. Tissue factor pathway inhibitor (TFPI) is an anticoagulation

protein primarily secreted by endothelial cells, which can inhibit TF activity and thereby modulate thrombosis [103].

In the 6-year longitudinal neuroimaging Austrian Stroke Prevention Study, no significant associations were found between SVD features or disease progression and TFPI in 296 community-dwelling participants after adjusting for age, sex, CRP levels, baseline WMH volume and traditional cardiovascular risk factors [50]. Importantly, as this cohort was not an SVD cohort, WMH severity grading was 'none' or 'punctate' in the vast majority (86.1%) of the sample, with only 24 participants out of 259 being classified as having SVD progression by 3 years. Another study found that TFPI levels were higher on average after adjusting for traditional vascular risk factors in a group of 110 symptomatic SVD patients compared to 50 healthy controls [18]. Patients with widespread WMH lesions had lower TFPI levels and a higher TF/TFPI ratio compared to those with isolated lacunar infarction. These results suggest that endothelial prothrombotic changes may be important in mediating an ischaemic SVD phenotype.

4.5.3. Tissue-Type Plasminogen Activator and Plasminogen Activator Inhibitor-1

Tissue-type plasminogen activator (t-PA) is a serine protease synthesised by endothelial cells that plays a critical role in fibrinolysis. It is associated with endothelial activation and increased BBB permeability [104]. Overexpression of t-PA is a known risk factor for future ischaemic stroke [105]. Plasminogen activator inhibitor-1 (PAI-1) is a serine protease inhibitor primarily produced by endothelial cells involved in the suppression of fibrinolysis through the inhibition of t-PA. Physiological levels of PAI-1 are required for haemostasis, and increased concentrations of PAI-1 are associated with atherosclerosis, thrombosis, cellular senescence and endothelial dysfunction [106,107]. Studies using in vitro BBB models provide further support regarding the involvement of t-PA in endothelial dysfunction, showing a dose-dependent effect on BBB permeability and endothelial cell morphological changes through the activation of the Rho-kinase pathway [104].

Studies investigating the relationship between t-PA, PAI-1 and SVD are limited and inconsistent. Lacunar stroke patients (n = 65) had higher levels of t-PA 1–3 months post-stroke compared to cortical stroke patients (n = 60) after adjusting for age, sex and vascular risk factors, perhaps indicating a role in the development or progression of mechanisms specific to SVD [70]. Dobrynina et al. [108] also reported a significant positive correlation between t-PA levels and WMH severity and PVS burden in 71 SVD patients and 21 healthy controls; however, they did not adjust for potential clinical covariates in this study. Conversely, a study with 100 patients of recent cortical or lacunar ischaemic stroke failed to find a significant relationship between plasma t-PA concentrations and PVS count or volume after adjusting for age, sex and vascular risk factors [73]. No association was found between PAI-1 levels and WMH volume or the presence of lacunes in 651 community-based elderly participants or in 71 SVD patients and 21 healthy controls [108,109].

4.5.4. Prothrombin Factors 1 and 2

Prothrombin factors 1 and 2 are coagulation factors that can cleave to form thrombin, which is the key enzyme that transforms fibrinogen into fibrin and facilitates platelet activation. There was no reported association between circulating levels of prothrombin factor 1 and 2 and PVS in the basal ganglia in 100 ischaemic stroke patients, including lacunar stroke patients [73]. In the aforementioned Austrian Stroke Prevention Study, no significant associations were detected between prothrombin factors 1 and 2 and SVD features or WMH lesion progression at 3- or 6-year follow-up in 296 community-dwelling participants [50]. However, as participants in this study were asymptomatic, it remains a possibility that relationships between SVD and coagulation factors could go undetected in this cohort; subsequent power analyses also revealed larger sample sizes would be necessary to detect significant differences if a subtle effect size were to be assumed.

4.5.5. D-Dimer

D-dimer is a by-product of blood clotting and fibrin degradation produced when fibrin is lysed with plasmin. High levels are indicative of plasmin activation and fibrin production and have been associated with cardiovascular disease and future atherothrombotic events [110]. There was no reported association between levels of D-dimer and PVS in the basal ganglia in 100 recent ischaemic stroke patients, including lacunar stroke patients [73]. There was also no relationship found between circulating levels of D-dimer and SVD features or progression in 296 community-dwelling participants in a 6-year longitudinal study [50]. Albeit perhaps unrelated to pathophysiological mechanisms specific to SVD, one study with 102 SVD patients did find a relationship between baseline serum concentrations of D-dimer and an increased risk of a cardiovascular event (including recurrent stroke) or death after adjusting for age, sex and radiological markers of SVD [71].

4.5.6. Thrombomodulin

Thrombomodulin (TM) is a vasoactive factor expressed on the surface of endothelial cells. It engenders a powerful anticoagulation effect by decreasing circulating levels of thrombin and inhibiting factors Va and VIIIa through the production of activated protein C [107,111]. TM can reflect systemic endothelial damage, although some studies suggest it can protect the endothelium by attenuating inflammation-inducing injury [18,107]. Increased circulating TM concentrations were significantly associated with a higher number of lacunes and more severe WMH burden in 110 lacunar stroke patients, with higher TM levels reported in SVD patients compared to 50 healthy controls after adjusting for vascular risk factors [18]. Similarly, a cross-sectional analysis with 651 community-based ageing participants (average age: 67.4) demonstrated higher average serum concentration levels of TM present in those with lacunar infarcts or moderate WMH compared to those without [109]. In contrast, no association was found between levels of TM and WMH lesion progression at 6-year follow-up after adjusting for potential confounding variables in 296 community-dwelling participants in the Austrian Stroke Prevention Cohort [50].

Intriguingly, an ex vivo pathology study examining small penetrating cerebral arteries in 17 SVD cases, 12 aged controls and 4 young controls found significantly higher levels of TM immunoreactivity in SVD cases compared to aged controls and in vessels with a higher sclerotic index; TM was entirely absent or sparse in young controls [52].

4.5.7. Endothelin-1

Endothelin-1 (ET-1) is a potent exogenous vasoconstrictor peptide produced by endothelial cells in response to hypoxia, shear stress, and exposure to cytokines, growth factors and thrombin. ET-1 can induce vasoconstriction, hypertrophy, fibrosis and increased BBB permeability and can also mediate the release of NO, implicating it in hypertension and endothelial dysfunction [107]. ET-1 has been associated with synaptic dysfunction and consequent cognitive deficits in pre-clinical investigations featuring a mouse model of transient vasoconstriction via bilateral injections of ET-1 [112]. Research examining the possible role of ET-1 in SVD is limited. A large (n = 1396) 6-year longitudinal study found baseline serum ET-1 levels to be positively associated with worsening WMH burden (Fazekas scale score $\geq$ 2) and the presence of lacunes and microbleeds after adjusting for age, sex, vascular risk factors, medication use, carotid stenosis and baseline WMH burden [46].

4.5.8. Fibrinogen

Fibrinogen is a blood plasma glycoprotein produced in the liver. It is the main structural component of a blood clot and functions as a coagulation factor. Fibrinogen can also indicate capillary leakage and can disrupt BBB integrity by converting to toxic blood-derived fibrin deposits, which disrupt blood flow and result in downstream consequences affecting other constituents of the neurogliovascular unit, eventually compromising white matter integrity [113]. Participants with elevated fibrinogen levels tended to have a more severe WMH burden and a greater number of lacunes in a Japanese community-based

neuroimaging study (n = 651) [114]. Logistic regression analyses adjusted for age, sex, vascular risk factors, CRP levels, and carotid artery atherosclerosis revealed an independent association between fibrinogen levels and WMH burden. In the same cohort, levels of fibrinogen were inversely correlated with cognitive performance, albeit only in patients without WMH. Findings from the Framingham Heart Study (n = 1763) did not support an association between circulating levels of fibrinogen and WMH volumes or the presence of lacunes or microbleeds [44]. Furthermore, there was no association between circulating levels of fibrinogen and basal ganglia PVS in 100 patients with recent ischaemic stroke [73].

4.6. Growth Factors

Vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and transforming growth factor-β1 (TGF-β1) are potent angiogenic factors crucial for endothelial cell proliferation, migration, maturation and survival; they play a key role in angiogenesis, microvascular permeability and overall vessel function [115,116]. A fundamental feature of ageing involves angiogenic signalling failure and possible subsequent alterations in blood flow [117]. Mice with a genetic deletion of VEGF display endothelial cell degeneration and death [118]. There is evidence to suggest that abnormally raised or decreased levels of VEGF and TGF-β1 are associated with cardiovascular disease, perhaps via their induction through hypoxia-pertinent pathways or through stimulation of NO production [119]. There is also evidence to suggest VEGF and PlGF encourage vascular permeability and inflammation by uncoupling endothelial cell-to-cell junctions [120,121]. Injections of VEGF can result in fibrinogen and plasma clotting, increased BBB permeability and vascular inflammation [122].

Few clinical studies have investigated the link between growth factors and SVD. We identified eight studies with a total of 5176 participants (average sample size: 739.43).

In a cross-sectional study with 293 thrombolysed acute ischaemic stroke patients, higher serum VEGF levels at 90 days post-stroke, though not baseline levels at stroke onset, were associated with the presence of lacunes and greater global SVD disease burden on CT after adjusting for age, sex, and vascular risk factors; there was no association with WMH burden specifically [40]. One study reported higher VEGF levels in a group of 91 large vessel atherosclerotic disease patients compared to 89 SVD patients within 24 h of acute ischaemic stroke onset [123]. However, serum VEGF levels were proportional to infarct volume after adjusting for potential confounding variables, which might explain the increased levels in the group with strokes of large-artery aetiology, which have larger volumes. Group differences in VEGF levels were attenuated by 3 months post-stroke, though higher serum levels of VEGF in the acute stroke phase were associated with a better functional outcome (National Institute of Health Stroke Scale (NIHSS) score decrease > 4 points) after adjusting for potential covariates including initial NIHSS and infarct volume, possibly due to their neuroprotective and angiogenic effects. However, it is important to note that the well-established roles of VEGF in acute-ischaemic stroke render the extrapolation of findings from these populations arguably unreliable in the context of SVD, as significant fluctuations in growth factor levels after an acute ischaemic event cannot be discounted.

Some large studies in stroke-free ageing populations report no significant associations between VEGF and radiological markers of SVD. For instance, no connection was reported between circulating levels of VEGF and brain atrophy, WMH volume or white matter tract integrity (fractional anisotropy via Diffusion Tensor Imaging (DTI) sequence on MRI) in a young (average age: 46) subset of 1853 participants in the Framingham Heart Study cohort after adjusting for demographic and clinical variables [124]. Likewise, Shoamanesh et al. [44] did not observe an association between circulating VEGF levels and WMH burden, microbleeds or lacunes in 1763 stroke-free adults (average age: 60.2) in the Framingham offspring cohort.

In contrast, higher baseline levels of the VEGF family and PlGF in CSF were associated with greater WMH volume after adjusting for age, sex, ICV and vascular risk factors in 247 elderly participants with MCI (average age: 70.89), though not in 495 cognitively

unimpaired participants; adjusting for CSF Aβ–40 attenuated the relationship for VEGF but not PlGF [49]. PlGF was amongst the strongest independent predictors of WMH at baseline in cognitively impaired and unimpaired individuals, independent of Aβ status, providing evidence of the overlapping implications between inflammation and cerebrovascular disease. Curiously, lower levels of VEGF and PlGF were associated with increasing WMH lesions over 7 years, leading the authors to hypothesise that vascular injury might inflict enough damage to endothelial cells to lead to a depletion in growth factor production and a subsequent decline in angiogenesis. Results from the ongoing MarkVCID consortium also indicate that in individuals at risk for cognitive impairment and dementia, PlGF may function as a candidate biomarker measuring the severity of vascular injury and WMH lesion burden and may serve as an accurate cross-sectional diagnostic biomarker and contribute as a prognostic susceptibility biomarker longitudinally [125]. In this study, Hinman et al. [125] observed a significant relationship between circulating plasma PlGF levels and WMH lesion burden (measured volumetrically and using the Fazekas scale) as well as cognitive impairment as measured by the Clinical Dementia Rating Scale (CDR) after adjusting for age and sex. Encouragingly, PlGF levels showed increasing diagnostic accuracy for all progressive disease states: WMH only (AUC: 0.66), mild cognitive impairment with WMH (AUC: 0.74) and dementia with WMH (AUC: 0.89). The authors posit that SVD mechanisms might be driving an increase in angiogenic factor circulation paired with a failure in downstream receptor signalling in cerebral microvessels, possibly involving Flt-1 receptor activity.

To determine whether the heterogeneity observed in the clinical and radiological manifestations of SVD could be attributed to different pathogeneses, Dobrynina et al. [81] categorised 40 SVD patients with a Fazekas score of three into two subgroups of radiological manifestations based on a cluster comparison and examined whether they were related to different blood biomarkers. Univariate analyses revealed lower levels of VEGF in patients in an MRI subgroup with widespread WMH and lacunes, microbleeds, atrophy and severe cognitive and gait impairments compared to 21 healthy controls or those in the other MRI subgroup, categorised by more severe deep WMH, lacunes in the white matter, no microbleeds or atrophy and less severe clinical manifestations, though the latter was not statistically significant. Whilst it is possible that VEGF could contribute to a specific SVD radiological profile amongst patients with severe WMH lesions, it is worth noting that these analyses did not include important clinical covariates that may influence blood biomarker concentrations, despite significant differences between SVD patients and controls. Notably, SVD patients were substantially more likely to be hypertensive (85.4% versus 43.5%) and diabetic (17.7% versus 0%).

In a 5-year longitudinal imaging study with 35 SVD patients, 43 age-matched participants with a history of stroke (further subdivided into lacunar and atherothrombotic) and 46 age-matched controls, TGF-β1 levels were highest among those with deep WMH lesions, which had progressed at 5-year follow-up [126]. Logistic regression analyses indicated that the odds ratio for the presence of elevated TGF-β1 was significant after adjusting for age, sex and major vascular risk factors. It is not entirely clear why this increase was not observed in the subset of participants with a history of lacunar stroke or why it was unrelated to periventricular WMH lesion presence and progression.

4.7. Nitric Oxide Pathways

4.7.1. Nitric Oxide

NO, an endothelium-derived vasodilator, inhibits platelet activation and thrombosis and limits the adhesion of inflammatory cells. Dysfunctional endothelial cells decrease NO bioavailability by reducing NO synthesis and secretion, which can lead to defective vasodilatory processes, impaired blood flow, platelet aggregation, proliferation of smooth muscle cells and increased blood pressure [127,128]. In the endothelium, NO is produced by eNOS through calcium-dependent binding of calmodulin. Hence, intracellular calcium can influence eNOS and NO production. NO can alter the calcium (Ca^{2+}) in smooth muscle cells

through second messenger and potassium (K+) channel activation pathways that facilitate contraction and relaxation. Levels of endothelial-derived factors, including NO, must be tightly regulated to allow for healthy BBB and brain function [129,130]. A population-based longitudinal study (average follow-up: 69.7 months) with 1396 stroke-free adults reported that changes in NO levels were significantly negatively associated with an increased risk of new incident WMH lesions, lacunes, and microbleeds on MRI after adjusting for age, sex, vascular risk factors, medication, carotid stenosis and baseline WMH volume [46].

4.7.2. NOSTRIN

Endothelial nitric oxide synthase traffic inducer (NOSTRIN) is involved in the regulation of perfusion and inflammation and is thought to inhibit the production of NO by sequestering and thus attenuating the activation of eNOS [131]. In addition, NOSTRIN may influence the expression of secreted cytokines and can restrict endothelial cell proliferation and adhesion [132]. Using EDE biomarkers, Elahi et al. [54] examined the cargo proteins within plasma membrane-derived vesicles of brain endothelial cells from 26 neurologically-normal older adults. They found higher levels of NOSTRIN in participants with WMH lesions compared to those without, though they did not find significant associations between NOSTRIN levels and global grey matter volumes or cognitive performance; it is important to note that only 11 patients within this small sample size had signs of SVD.

4.7.3. ADMA

Asymmetric dimethylarginine (ADMA) is a nitric oxide synthase (NOS) inhibitor associated with traditional vascular risk factors, stroke, and endothelial dysfunction, presumably via a reduction in cerebral blood flow or the promotion of oxidative stress and inflammation [133]. NOS is an endogenous enzyme involved in the conversion of substrate L-arginine to NO, the former of which is involved in various processes crucial to the maintenance of cerebrovascular health, including vasodilation, platelet aggregation inhibition, smooth muscle cell generation and vascular cell adhesion. Due to their structural affinity, ADMA and L-arginine compete for NOS binding, hence reducing the bioavailability of NO [134].

Serum ADMA levels were significantly higher in a group of 210 SVD patients compared to 207 healthy controls; however, this difference was no longer significant when adjusting for potential confounders, including age, sex, vascular risk factors and coronary heart disease [135]. ADMA levels differed significantly between SVD-associated lesion types, with higher levels reported in patients with an isolated lacune compared to those with multiple visible lacunes and significantly lower levels in those with WMH lesions compared to those with WMH lesions and accompanying cerebral atrophy. Though the study sample size is relatively small, one possible explanation for these findings relates to the potential role of ADMA in the development of atherosclerosis, which might play a more direct role in isolated lacunar infarction. Though no significant correlation was found between levels of ADMA and cognitive impairment, subgroup analyses revealed that in patients with WMH lesions specifically, levels of ADMA were significantly correlated with Montreal Cognitive Assessment (MoCA) scores. These results suggest that though ADMA may not play a key role in instigating SVD pathophysiology, it could exacerbate disease progression, conceivably through the inhibition of NO production and consequent cerebral blood flow changes.

Khan et al. [136] observed higher levels of circulating ADMA in 47 SVD patients compared to 38 controls with similar vascular risk factor profiles. ADMA levels were not correlated with the number of lacunes, though WMH severity grade was positively correlated with ADMA (OR 10.04, 95% CI: 1.70–59.27) after controlling for age, sex, vascular risk factors and creatinine clearance. Though the authors hypothesised that ADMA might mediate the relationship between homocysteine (Hcy) and endothelial activation, they found no significant associations between ADMA and Hcy levels and the relationship between ADMA and SVD was only slightly attenuated after controlling for Hcy. Along the

same vein, a study with young, asymptomatic patients (average age: 51.1) also reported significantly higher levels of ADMA amongst those with SVD compared to controls [137]. ADMA levels were positively correlated with WMH presence and severity after adjusting for potential confounding variables, further supporting the role of ADMA and endothelial dysfunction in early disease pathology.

4.7.4. Myeloperoxidase

Myeloperoxidase is an oxidizing enzyme secreted by neutrophils, leukocytes and microglia following neuronal injury. It restricts NO bioavailability by consuming it and also contains hypochlorous acid, and can thus aggravate inflammation, incite tissue damage and has been associated with BBB and endothelial dysfunction [138]. In the Framingham Heart Study, circulating levels of myeloperoxidase were measured as a proxy biomarker for oxidative stress in 1763 stroke-free participants (average age: 60.2) [44]. After controlling for age, sex, vascular risk factors and time from blood draw to MRI (average: 5 years), they observed significantly higher levels of myeloperoxidase in the presence of microbleeds (OR 1.5, 95% CI: 1.1–2.0), with each additional unit increase resulting in a 1.9-fold increased risk of having more than one microbleed. The authors also reported a non-significant association between lower myeloperoxidase levels and the presence of WMH lesions and/or lacunes. It remains unclear why lower levels of myeloperoxidase were associated with ischaemic SVD-related lesions and higher levels with haemorrhagic disease manifestations, and it remains feasible that these might have distinct pathophysiological causes. In addition, it is possible that chronic SVD could lead to an overburdened inflammatory pathway, which might eventually deplete circulating myeloperoxidase availability.

4.8. Other Relevant Biomarkers

4.8.1. Homocysteine

Hcy is a sulphur-containing protein usually excreted from the body via the kidneys. It is associated with cardiovascular events and stroke risk as well as cognitive impairment and is indicative of endothelial dysfunction [139,140]. Increased levels of Hcy are thought to exert deleterious effects on the endothelium and can facilitate oxidative stress, impair NO-mediated blood flow processes, up-regulate redox-sensitive inflammatory pathways, as well as promote atherogenesis, thrombogenicity and the aggregation of ADMA [141–143].

Higher levels of Hcy were reported in 74 lacunar stroke patients 1-month post-stroke compared to 74 healthy controls matched for age, sex, hypertension status and diabetes [38]. Though many inflammatory markers had normalised by 3 months post-stroke, Hcy levels remained unchanged, suggesting it was not a by-product of the acute ischaemic event itself. In a UK-based study of 457 black stroke patients and 179 black community controls, elevated serum Hcy concentrations were observed in SVD patients compared to controls after adjusting for age, sex, vascular risk factors and renal function [144]. In addition, Hcy levels within SVD patients were positively correlated with WMH severity and were associated with the presence of lacunes with confluent WMH lesions. Hcy levels were also found to be independently associated with a greater WMH volume burden after adjusting for demographics and vascular risk factors in 809 acute ischaemic stroke patients (average age: 65.6), with each quartile increase in plasma Hcy being associated with a WMH volume increase of 11% [145]. Furthermore, Hcy levels were positively correlated with the number of lacunes (OR 2.14, 95% CI: 1.4–3.27) in a large population-based cohort study of 1023 participants (average age: 56.7) after adjusting for age, sex and vascular risk factors [146]. Another study also reported higher circulating Hcy in 47 SVD patients compared to 38 healthy controls with similar risk factor profiles, which were positively correlated with WMH severity grade and number of lacunes after controlling for demographic and clinical covariates as well as creatinine clearance [136].

In a large Chinese community-based cohort study (n = 960, average age: 56.02), grouped endothelial dysfunction biomarkers, including Hcy, were independently associated with WMH volume and number of lacunes, though not microbleeds or PVS; backward

elimination regression retained Hcy as an independent risk factor for the presence of lacunes (OR: 1.44, 95% CI: 1.20–1.74) [43]. Hcy levels were higher in a group of 172 SVD patients compared to 172 age- and sex-matched controls [147]. After controlling for traditional vascular risk factors and age, Hcy was also a strong risk factor in those with WMH lesions. Consistent with the hypothesis that Hcy mechanistically relates to SVD through endothelial dysfunction, the authors found that including other markers of endothelial dysfunction, ICAM-1 and TM, in a logistic regression model resulted in the loss of the significant association between Hcy and SVD.

Results from the Framingham Heart Study (sample size: 1763 stroke-free participants) contradict these findings, and the authors report no association between circulating Hcy levels and several markers of SVD on MRI, including WMH lesions, lacunes and microbleeds [44].

Some studies also suggest a possible association between Hcy and clinical outcome or disease progression. For instance, in a cohort of 123 SVD patients (average age: 66.4), circulating Hcy levels correlated with WMH severity, number of lacunes, basal ganglia PVS and lobar microbleeds, and also served as an independent predictor for global cognitive impairment [148]. Hcy was also associated with radiological progression of SVD at a 2-year follow-up in a longitudinal cohort study with 123 SVD patients, with higher circulating levels related to the development of new lacunes as well as an increased risk of a recurrent vascular event or death after controlling for demographic variables, vascular risk factors and disease burden [47,71]. Interestingly, a recent study reported significantly higher levels of plasma Hcy in SVD patients with cognitive impairment (n = 40) compared to SVD patients without cognitive impairment (n = 38) and healthy controls (n = 35), respectively; however, potential confounding variables were not adjusted for in these univariate analyses [90].

4.8.2. Albumin

Microalbuminaria (urinary albumin excretion < 20–200 µg/min) is associated with systemic dysfunction of the vascular endothelium, elevated levels of fibrinogen, BBB dysfunction and an increased risk of stroke [149–151]. Though the precise mechanisms underlying these associations remain mostly unknown, it is possible that cytokines and other factors implicated in inflammation might mediate renal and glomerular damage, contributing to albuminaria and SVD [152]. Furthermore, small vessels in the brain and the kidneys share haemodynamic and physiological similarities and damage to the kidney endothelium can incite albumin leakage [153]. It is also plausible that this relationship is mediated by the involvement of relevant risk factors that might also influence renal function, such as sodium consumption, diabetes and hypertension.

In a Japanese community-based study with 651 participants (average age: 67.4), those with albuminaria had higher WMH grades and the total number of lacunes compared to those without; in addition, the total number of lacunes and WMH severity grade were significantly higher in the highest tertile of albumin–creatinine ratio [109,154]. A standard deviation increase in the albumin–creatinine ratio was associated with a nearly 2-fold increased risk of having at least one lacunar infarct and a 2.15-fold increased risk of the presence of WMH lesions, independent of age, sex and vascular risk factors.

In the longitudinal Norwegian HUNT2 population-based study with 7261 participants, albuminaria was associated with increased overall ischaemic stroke risk over a median follow-up period of 15 years [155]. This association was modestly stronger for the lacunar subtype compared to large-artery or cardioembolic ischaemic stroke subtypes or haemorrhagic stroke, even after controlling for age, sex and vascular risk factors, perhaps providing further evidence for the role of albuminaria in diffuse endothelial and vascular dysfunction in SVD [155].

4.8.3. Haemoglobin A1c

HbA1c is a metabolite that functions as an indicator of chronically elevated glucose levels or insulin resistance and a biomarker of endothelial dysfunction. HbA1c is related to inflammation, the production of atheroma, thrombogenicity and the promotion of oxidative stress through the facilitation of ADMA synthesis [133,156]. In 809 acute ischaemic stroke patients (average age: 65.6), HbA1c levels were associated with WMH volumes on MRI and functioned as an independent predictor for WMH volume after adjusting for age, sex, race, vascular risk factors (including diabetes), coronary artery disease and prior stroke [145]. A quartile increase in HbA1c levels was associated with a 10% increase in WMH lesion burden.

4.8.4. Amyloid-Beta

β-amyloid$_{1–40}$ is associated with cerebral amyloid angiopathy and has been shown to alter endothelial cell-mediated cerebral blow flow regulation in mice, possibly through the formation of ROS, which can further harm the endothelium [157]. In 149 acute lacunar stroke patients and 25 age-matched controls, higher levels of plasma β-amyloid$_{1–40}$ were reported in SVD patients with WMH lesions and multiple lacunar infarcts with or without the presence of WMH, compared to those with an isolated lacunar infarct in the absence of WMH or healthy controls [158]. The authors attribute this difference to potentially distinct disease pathologies, a diffuse arteriopathy versus localised vessel atheroma, with endothelial dysfunction playing a more prominent role in the former.

4.8.5. Adiponectin

Adiponectin, a protein synthesised by adipose tissue, maintains vasoprotective properties by attenuating both inflammation and the production of ROS whilst enhancing NO generation and promoting the survival of endothelial progenitor cells [159]. The potentially protective role of adiponectin was demonstrated in a study with 133 patients with first-ever ischaemic stroke, where circulating levels of adiponectin were inversely associated with the prevalence of cerebral microbleeds after adjusting for age, sex and cardiovascular risk factors [37].

4.8.6. Klotho

Predominantly produced by the kidneys, the Klotho proteins are components of endocrine fibroblast growth factor receptor complexes that mediate metabolic processes. With circulating levels decreasing with age, Klotho is often referred to as having 'anti-ageing' properties due to its involvement in the regulation of oxidative stress, organ protection and the inhibition of cellular senescence [160]. In a prospective study with 262 first-ever ischaemic stroke patients (average age: 67.4), plasma Klotho concentration was inversely associated with the presence of WMH lesions, PVS, lacunes, total SVD burden and disease progression, though not microbleeds, possibly suggesting different pathological mechanisms underlying the protective involvement of Klotho proteins in distinct radiologic manifestations of SVD [161]. How Klotho could exert its protective qualities on the cerebrovascular system is unclear, though the regulation of NO production and bioavailability in vascular endothelial cells remains a possible mechanism.

4.8.7. Lipoprotein-Associated Phospholipase A_2 Mass

Lipoprotein-associated phospholipase A_2 mass (Lp-PLA_2), an enzyme secreted by macrophages, serves to hydrolyse oxidised phospholipids and functions as a marker of vascular inflammation. Previous studies have suggested an association between high levels of Lp-PLA_2 and stroke or SVD [162,163]. In addition, a relationship between Lp-PLA_2 and dilation in the retinal venules was reported in the Rotterdam cohort, further substantiating its involvement in small vessel pathology [164].

In 1763 stroke-free participants in the Framingham Heart Study, Shoamanesh et al. [44] observed higher levels of circulating Lp-PLA_2 in participants with lacunes and greater

WMH volumes, as well as a non-significant trend between higher levels of Lp-PLA_2 and the increasing number of microbleeds after adjusting for age, sex and vascular risk factors. As part of the Northern Manhattan Study, Wright et al. [165] similarly reported an association between higher levels of Lp-PLA_2 and a higher WMH burden in 527 participants (average age: 71.3), with those in the highest quartile having a 1.3-fold increase in WMH volume compared to those in the lowest quartile, independent of demographic variables, the presence of risk factors, or other markers of inflammation.

5. Conclusions and Future Perspectives

In this scoping review, we have summarised findings from clinical neuroimaging studies investigating the relationship between radiological indications of SVD and biochemical markers of endothelial dysfunction in blood or CSF with the overarching aim of conceptualising the molecular mechanisms underlying the role of endothelial dysfunction in SVD (Figure 2).

Overall, there is evidence of an association between E-selectin and the presence of microbleeds as well as VCAM-1, ICAM-1 and P-selectin levels with WMH lesion severity and progression stemming from large community-dwelling cohort studies as well as cross-sectional and longitudinal studies with SVD patients, even when compared to cortical stroke patient controls.

Clinical research studies investigating the role of BBB transport proteins in SVD pathology are scarce, but we describe some studies that suggest a relationship between higher levels of GLUT-1, LAT-1 and P-GP and increasing WMH lesion burden as well as lower levels of Cav-1 and the presence of microbleeds.

Results from studies linking cytokine and chemokine biomarkers to SVD pathogenesis are inconclusive, and while there is evidence of a relationship between higher IL-6 levels and SVD burden, studies comparing patients of acute lacunar stroke with patients of acute thromboembolic occlusive stroke indicate a possible mediating role of an acute endothelial response to injury. Nonetheless, there is some published evidence in support of the role of osteoprotegerin, TNF-α and PF-4 in SVD mechanisms.

The contribution of inflammatory pathways to SVD pathogenesis remains elusive. In some large population-based studies, CRP levels were independent predictors of WMH lesion burden and progression as well as the number of lacunes or lacunar infarcts, though this was not the case in all the studies described. Indeed, most studies featuring acute and subacute stroke patients as controls did not observe strong associations between CRP levels and SVD burden. There is some evidence linking neopterin, though not CD40 ligand, to SVD.

Several lines of evidence also point to dysregulation in the nitric oxide pathway as being an important event in the pathogenesis of SVD. Findings from several studies suggest associations between levels of NO, NOSTRIN, ADMA and myeloperoxidase and the presence and degree of diverse SVD lesion types.

We did not find consistent evidence advocating for the role of activation of coagulation in the development and progression of SVD, with most studies evaluating a connection between vWF and SVD reporting negative results and those focussing on TFPI, t-PA, PAI-1, prothrombin factors 1 and 2, D-dimer and fibrinogen reporting contradicting results. However, some studies did report significant associations between higher levels of circulating TM and ET-1 and SVD severity.

Growth factors have been shown to have neuroprotective and angiogenic properties, but they also can promote vascular permeability, exacerbate ischaemic tissue injury post-stroke and lead to aberrant angiogenesis. Data on their influence over SVD mechanisms are inconsistent, particularly in studies with stroke populations at different post-stroke stages and of diverse aetiologies. Larger ageing cohort studies suggest there is little to no association between VEGF levels and SVD burden; however, other studies indicate an association with higher levels of other growth factors, notably TGF-β1 and PlGF, and WMH lesion burden and longitudinal disease progression.

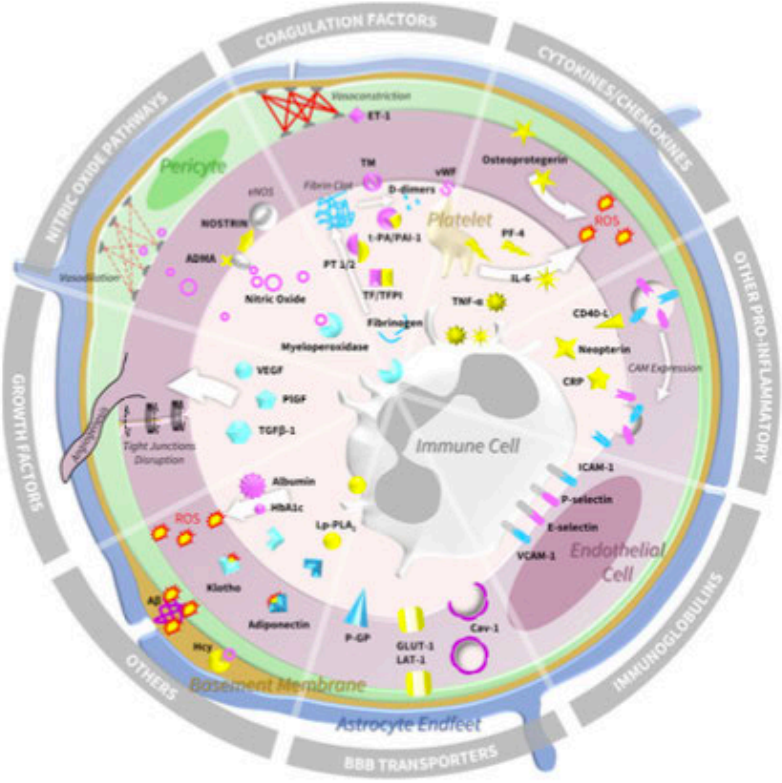

Figure 2. Biomarker roles in the gliovascular unit. Each octant represent a subdivision used in the review. Abbreviations per octant: **(Coagulation Factors)** ET-1: endothelin-1; TM: thrombomodulin; t-PA: tissue-type plasminogen activator; PAI-1: plasminogen activator inhibitor-1; PT 1/2: prothrombins 1 and 2; TF: tissue factor; TFPI: tissue factor pathway inhibitor; vWF: von willebrand factor; **(Cytokines/Chemokines)** ROS: reactive oxygen species; PF-4: platelet factor-4; IL-6: interleukin-6; TNF-α: tumour necrosis factor alpha; **(Other Pro-Inflammatory)** CD40-L: CD40-ligand; CRP: C-reactive protein; CAM: cell adhesion molecules; **(Immunoglobulins)** ICAM-1: intracellular cell adhesion molecule-1; P-selectin: platelet selectin; E-selectin: endothelial selectin; VCAM-1: vascular cell adhesion molecule-1; **(BBB Transporters)** Cav-1: caveolin-1; GLUT-1: glucose transporter-1; LAT-1: large neutral aminoacid transporter-1; P-GP: permeability-glycoprotein; **(Others)** Hcy: homocysteine; Aβ: amyloid beta; Lp-PLA_2: lipoprotein-associated phospholipase A_2-mass; HbA1c: haemoglobin A1c; **(Growth Factors)** VEGF: vascular endothelial growth factor; PlGF: placental growth factor; TGFβ-1: transforming growth factor beta-1; **(Nitric Oxide Pathway)** NO: nitric oxide; eNOS: endothelial nitric oxide synthase; ADMA: asymmetric dimethylarginine; NOSTRIN: endothelial nitric oxide synthase traffic inducer (schematic is a theoretical representation and is not drawn to scale).

We also found several studies supporting associations between levels of albumin, Hcy, HbA1c, β-amyloid$_{1–40}$, Lp-PLA_2 and adiponectin and several types of SVD lesions on MRI (Table 1).

Table 1. Associations between individual biomarkers and distinct radiological manifestations of SVD present in the literature reviewed.

Class	Biomarkers	Role in Endothelial (Dys) Function	Relation to SVD	Refs.
Cell adhesion molecules and selectins	VCAM-1 ICAM-1 P-selectin E-selectin	• expressed by a cytokine-activated endothelium • facilitate the recruitment, rolling, adhesion and transendothelial migration of leukocytes through the endothelium of vascular walls • have been linked to ageing, BBB breakdown and age-related neurodegenerative disorders	• higher levels found in SVD patients • positively associated with disease burden and progression, worse clinical outcomes and cognitive decline • positively associated with WMH and the prevalence of lacunes and microbleeds, though not PVS	[18,22,34,37,38,40–52,54,70,73]
BBB Transporters	GLUT-1 P-GP LAT-1 Cav-1	• expressed by brain endothelial cells • mediate the influx and efflux of solutes from the circulation through endothelial cells and into the brain and vice-versa • implicated in BBB permeability, vessel tone and metabolic dysregulations • change with ageing, possibly disturbing neurovascular coupling processes based on metabolic demand	• higher levels found in SVD patients • positively associated with WMH and lower cognitive performance scores and inversely associated with total grey matter volume • Cav-1 levels inversely associated with the prevalence of microbleeds	[54,62]
Cytokines and chemokines	IL-6 TNF-α/TNFR2 Osteoprotegerin PF-4	• pro-inflammatory molecules that can incite platelet production and engender endothelial dysfunction through an increase in Angiotensin II type 1 (AT1) receptor activity, an interaction with tumour necrosis factor alpha (TNF-α), and the induced production of reactive oxygen species (ROS), which can cause enhanced vascular superoxide production, exacerbate oxidative stress, and impair endothelium-dependent vasodilation • associated with inflammation, stroke, atherosclerosis and cardiovascular events	• higher levels found in SVD patients • positively associated with presence of WMH, subcortical infarcts, lacunes, and microbleeds, though not PVS • contradictory results regarding the association with disease progression and worse clinical outcomes, though strong associations reported regarding adverse cardiovascular events, including recurrent stroke • some results possibly mediated by recent stroke	[43–45,47–49,68–73,81]
Other pro-inflammatory markers	CRP Neopterin CD40-L	• these pro-inflammatory molecules can inhibit angiogenesis, promote atherosclerosis, mediate circulating cytokine levels, inhibit NO, decrease endothelial nitric oxide synthase (eNOS) activity in endothelial cells, increase Endothelin-1 production, attenuate endothelial-dependent vasodilation and increase endothelial cell adhesion molecule expression	• most studies featuring acute and sub-acute stroke patients do not report strong associations between CRP levels and SVD disease burden • contradictory results, with some showing a positive association with the prevalence of lacunar infarcts, PVS, WMH lesion burden and progression, though not microbleeds • positively associated with the risk of recurrent stroke	[34,38,43,44,47,51,68–73,90–92]

Table 1. *Cont.*

Class	Biomarkers	Role in Endothelial (Dys) Function	Relation to SVD	Refs.
Coagulation factors	vWF TF TFPI t-PA PAI-1 Prothrombin factors 1 and 2 D-dimer TM ET-1 fibrinogen	• endothelial cells play a crucial role in the maintenance of physiological haemostasis and the modulation of thrombosis through the tight control of both pro-coagulant and anti-coagulant mechanisms • the endothelium provides a surface for thrombosis formation, regulates blood flow and mediates the expression of vasoactive factors relating to platelet reactivity, coagulation and fibrinolysis	• vWF levels are comparable between lacunar and cortical stroke patients at sub-acute stroke stages • there are contradicting results regarding associations between TF/TFPI, t-PA/PAI-1 and fibrinogen and SVD mechanisms • no associations reported with prothrombin factors 1 and 2 or D-dimer • levels of TM are positively associated with WMH lesion severity and number of lacunes, though not disease progression • ET-1 is positively associated with worsening WMH burden and the presence of lacunes and microbleeds	[18,38,40,46,50,52,70,71,73,99,108,109,114]
Growth factors	VEGF TGF-β1 PIGF	• potent angiogenic factors crucial for endothelial cell proliferation, migration, maturation and survival • play a key role in angiogenesis, microvascular permeability (possibly by uncoupling endothelial cell junctions), inflammation and overall vessel function • a fundamental feature of ageing involves angiogenic signalling failure and alterations in blood flow • associated with cardiovascular disease, perhaps through the induction of hypoxia-pertinent pathways or involvement of NO production	• positively associated with the presence of lacunes and greater global SVD disease • higher levels associated with better functional outcome post-stroke • positively associated with greater WMH volume at baseline and inversely associated with WMH lesion progression longitudinally • no associations reported with brain atrophy, white matter tract integrity or microbleeds	[40,44,49,81,123–126]
Nitric oxide pathway	NO NOSTRIN ADMA Myeloperoxidase	• dysfunctional endothelial cells decrease NO bioavailability by reducing NO synthesis and secretion, which can lead to oxidative stress, inflammation, defective vasodilatory processes, impaired blood flow, platelet aggregation, proliferation of smooth muscle cells and increased blood pressure	• NO levels are inversely associated with SVD progression and WMH, lacunes and microbleeds • NOSTRIN levels are positively associated with WMH, though not grey mater volumes or cognitive performance • ADMA levels are higher in SVD patients and positively associated with WMH, though not number of lacunes • Myeloperoxidase levels are higher in SVD patients and positively associated with microbleeds, though not WMH or lacunes	[44,46,54,135–137]

Table 1. *Cont.*

Class	Biomarkers	Role in Endothelial (Dys) Function	Relation to SVD	Refs.
Other relevant biomarkers	Hcy Albumin HbA1c β-amyloid$_{1-40}$ Adiponectin Klotho Lp-PLA$_2$	• these molecules modulate a wide range of functions. Some exert deleterious effects on the endothelium and can facilitate oxidative stress, impair NO-mediated blood flow processes, up-regulate redox-sensitive inflammatory pathways, and promote atherogenesis and thrombogenicity, whilst others might offer protective neurovascular mechanisms	• Hcy levels are higher in SVD patients and are positively correlated with WMH severity, number of lacunes, microbleeds, PVS, disease progression and cognitive impairment, though results are inconsistent • albuminaria is associated with WMH severity, prevalence of lacunes and recurrent lacunar stroke • HbA1c is positively associated with WMH volume increase • β-amyloid$_{1-40}$ levels are higher in those with WMH lesions and multiple lacunar infarcts • levels of adiponectin are inversely associated with the prevalence of microbleeds • Klotho levels are inversely associated with WMH, PVS, lacunes and total SVD burden and progression, though not microbleeds • Lp-PLA$_2$ levels are positively associated with WMH, lacunes and microbleeds	[37,38,43,44,47,71,90,109,136,144–148,155,158,161,165]

Abbreviations: Cell adhesion molecules: VCAM-1: vascular cell adhesion molecule-1, ICAM-1: intracellular cell adhesion molecule-1, P-selectin: platelet selectin, E-selectin: endothelial selectin. BBB transporters: GLUT-1: glucose transporter-1, P-GP: permeability-glycoprotein, LAT-1: large neutral amino acid transporter-1, Cav-1: caveolin-1. Cytokines and Chemokines: IL-6: interleukin-6, TNF-α: tumour necrosis factor alpha, TNFR2: tumour necrosis factor receptor 2, PF-4: platelet factor-4. Other pro-inflammatory markers: CRP: C-reactive protein, CD40-L: CD40 ligand. Coagulation factors: vWF: von Willebrand Factor, TF: tissue factor, TFPI: tissue factor pathway inhibitor, t-PA: plasminogen activator, PAI-1: plasminogen activator inhibitor-1, TM: thrombomodulin, ET-1: endothelin-1. Growth factors: VEGF: vascular endothelial growth factor, TGF-β1: transforming growth factor-β1, PlGF: placental growth factor. Nitric oxide pathways: NO: nitric oxide, NOSTRIN: endothelial nitric oxide synthase traffic inducer, ADMA: asymmetric dimethylarginine. Other relevant markers: Hcy: homocysteine, HbA1c haemoglobin A1c, β-amyloid$_{1-40}$: beta-amyloid, Lp-PLA$_2$: lipoprotein-associated phospholipase A$_2$-mass.

Overall, the findings from this review point to a pivotal role of endothelial dysfunction in the molecular mechanisms underpinning SVD. Whilst there is no single definitive biomarker with consistent associations to specific clinical indicators of disease, promising independent predictors of disease progression include cell adhesion molecules, molecules pertaining to the nitric oxide pathway, PlGF, TGF-β1, and Hcy. It is possible that there are many ways in which endothelial dysfunction can exert influence over the development and progression of SVD pathophysiology. Although the initial causes of endothelial dysfunction remain unclear, there is a propensity towards a pro-inflammatory and perhaps a pro-thrombotic state accompanied by aberrant angiogenesis, microvessel luminal narrowing, reduced responsiveness (vasoreactivity), altered pulsatility (vascular stiffness) and vasomotion, increased BBB permeability, and impaired blood flow regulation. It is possible to conceive of these processes as a dynamic pathological cycle resulting in a downstream cascade of dysfunction involving the entirety of the neurogliovascular system and culminating in white matter injury and ischaemia, neurodegeneration, diffuse connectivity problems and cortical thinning.

5.1. Implications for Clinical Practice

A significant challenge impeding the progress of therapeutics for SVD stems from the lack of elucidative and highly specific biomarkers associated with disease development and progression. As it stands, and despite strong evidence advocating for the involvement of

endothelial dysfunction in disease pathogenesis, there is not one biomarker that is capable of defining SVD or effectively adding a diagnostic or outcome-predictive ability in clinical practice. In large part, this is likely due to the complexity inherent in SVD mechanisms. SVD is a diffuse, chronic, and dynamic disease process marked by fluctuating radiological lesions and symptoms [166–168]. It is influenced by a complex interplay between genetic factors, lifestyle variables and environmental exposures, and although it is not predominantly an atherosclerotic disease, it shares an overlap in risk factors, mechanistic pathways, and indeed biomarkers with large-artery and cardiovascular disease. Our understanding of disease mechanisms is further hindered by the fact that human brain microvessels are not themselves visible on high-field MRI in vivo, and the generation of SVD-relevant animal models can be conceptually challenging. It is possible, and indeed likely, that there are many distinct routes through which endothelial dysfunction can drive SVD pathophysiology, and thus, it is reasonable to consider that a panel of biomarkers, rather than a sole molecule or pathway, is more likely to yield a comprehensive and robust diagnostic and prognostic tool in future.

5.2. Implications for Future Research

Another important reason a biomarker signature for SVD remains illusory pertains to the substantial inconsistencies in study results precipitated by heterogeneity in study design. There are several sources of methodological disparity impeding result replication and consistency. For instance, differences arise from the imaging modalities used, namely CT or MRI, and the methods and tools used to quantify radiological markers of disease. Many studies rely on diverse severity and grading scales, such as the Fazekas scale, to measure WMH lesion burden, foregoing the enhanced accuracy provided by volumetric quantification in sensitive analyses such as these. Studies also vary regarding the time window between serology and MRI acquisition, or the time between stroke onset and imaging/serology (when applicable). Another factor to consider is the variation introduced by the analytes themselves; this includes taking into account their stability, the measurement techniques used to quantify them and the presence of metabolic confounders (e.g., fasting status), comorbid illness (e.g., chronic kidney disease), or acute injury, infection, or ischaemia.

Another such source of heterogeneity relates to lesion types- whilst most studies focus on WMH (which are the most commonly observed SVD lesions), other related features, including lacunes, microbleeds, siderosis, brain atrophy, and PVS, remain understudied. It is possible that different lesion types arise at different disease stages, and with SVD representing a dynamic process, considering cumulative and total SVD burden rather than individual lesion types would likely prove insightful. However, perhaps the most critical source of variation comes from the characteristics of the study populations. Large ageing or community-dwelling cohort studies often deliberately will exclude patients with a history of stroke or MCI/dementia and tend to feature few patients with severe or even symptomatic SVD, limiting the representation of the full spectrum of disease and lowering their statistical power to detect significant or meaningful associations between biomarkers and clinical indicators of disease. There is also considerable variation in population characteristics amongst studies with SVD patients, with some including solely hypertensive patients, diabetic patients or indeed excluding patients with overt clinical manifestations of SVD, including stroke or cognitive impairment. In this context, it also remains crucial to consider the characteristics of the control population and the importance of controlling for potential demographic and clinical covariates. Comparing vascular function biomarkers between recent lacunar stroke patients and healthy controls introduces the likelihood that differences identified are influenced by the recent ischaemic event (i.e., markers of acute brain injury) or a greater burden of vascular risk factors in the former group.

Finally, it is worth addressing the limitations inherent in cross-sectional studies in terms of providing conclusions regarding the causal relationship between fluid biomarkers and SVD mechanisms. Large longitudinal studies, particularly in conjunction with

advanced MRI techniques that allow for insight into BBB function and vascular functionality, such as dynamic contrast-enhanced (DCE) MRI and cerebrovascular reactivity (CVR), can be advantageous by helping to provide support for a causal role or delineating predictive effects.

Many important questions remain. Importantly, the extent to which these biomarkers accurately reflect endothelial dysfunction confined to the brain (as opposed to systemic dysfunction), or whether endothelial dysfunction in SVD is indeed confined to the brain, remains unclear. In addition, the many ways in which endothelial dysfunction could affect other constituents in the neurogliovascular system and how changes in these cells could, in turn, affect the endothelium remains poorly understood. Finally, candidate catalysts for endothelial dysfunction in the context of SVD and how they relate to genetics and vascular risk factor exposure remain unknown and warrant further examination.

There is substantial evidence proposing endothelial dysfunction as a pivotal mechanism in the pathogenesis of SVD, and the identification of relevant biomarkers and their association with disease mechanisms not only provides important clues regarding the different pathways involved, but it can also provide promising avenues for therapeutic targets. Particularly exciting prospects include clinical trials targeting endothelial dysfunction, including the ongoing LACI-2 trial, which is repurposing Isosorbide Mononitrate and Cilostazol as potential treatments for patients with symptomatic SVD [169].

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Abbreviations

AD	Alzheimer's Disease
ADMA	asymmetric dimethylarginine
AT1	angiotensin II type 1 receptor
Aβ	amyloid beta
β-amyloid$_{1–40}$	beta-amyloid$_{1–40}$
BBB	blood–brain barrier
Ca^{2+}	calcium
CADASIL	cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
Cav-1	caveolin-1
CD40	CD40 ligand
CDR	Clinical Dementia Rating Scale
CRP	C-reactive protein
CSF	cerebrospinal fluid

CT	computerised tomography
CVR	cerebrovascular reactivity
DCE-MRI	dynamic contrast-enhanced MRI
DTI	diffusion tensor imaging
EDE	endothelial-derived exosome
eNOS	endothelial nitric oxide synthase
E-selectin	endothelial selectin
ET-1	endothelin-1
GLUT-1	glucose transporter-1
Hcy	homocysteine
HbA1c	haemoglobin A1c
ICAM-1	intracellular cell adhesion molecule-1
ICV	intracranial volume
IL-6	interleukin-6
K^+	potassium
LAT-1	large neutral amino acid transporter-1
Lp-PLA_2	lipoprotein-associated phospholipase A_2-mass
MCI	mild cognitive impairment
MRI	magnetic resonance imaging
NO	nitric oxide
NOS	nitric oxide synthase
NOSTRIN	endothelial nitric oxide synthase traffic inducer
PAI-1	plasminogen activator inhibitor-1
PF-4	platelet factor-4
P-GP	permeability-glycoprotein
PlGF	placental growth factor
P-selectin	platelet selectin
PVS	perivascular spaces
ROS	reactive oxygen species
sVCAM-1	soluble vascular cell adhesion molecule-1
SVD	small vessel disease
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF-β1	transforming growth factor- β 1
TM	thrombomodulin
TNFR1	tumour necrosis factor receptor 1
TNFR2	tumour necrosis factor receptor 2
TNF-α	tumour necrosis factor alpha
t-PA	tissue-type plasminogen activator
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
vWF	von Willebrand Factor
WMH	white matter hyperintensities

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Appendix B. Supplemental material to Chapter 3

Table B-1. Average vessel density (mm/mm3) by brain region in both young and aged animals. The greatest vessel density was found in the thalamus.

Vessel Density	Young n= 8	Aged n=8
Anterior CC, mm/mm3, mean (SD)	379.59 (102)	405.17 (133)
Posterior CC, mm/mm3, mean (SD)	431.53 (131)	450.11 (131)
Caudate Putamen, mm/mm3, mean (SD)	438.48 (165)	426.84 (166)
Internal Capsule, mm/mm3, mean (SD)	565.11 (144)	564.39 (166)
Hippocampus- CA1, mm/mm3, mean (SD)	498.73 (203)	574.46 (149)
Hippocampus- CA2/3, mm/mm3, mean (SD)	483.34 (137)	528.74 (139)
Hippocampus- DG, mm/mm3, mean (SD)	526.92 (98)	594.03 (184)
Thalamus, mm/mm3, mean (SD)	757.10 (176)	701.82 (172)

Table B-2. Average vessel diameter (μm) by brain region in both young and aged animals.

Vessel Diameter	Young n= 8	Aged n=8
Anterior CC, μm, mean (SD)	4.01 (0.39)	4.44 (0.57)
Posterior CC, μm, mean (SD)	4.16 (0.42)	4.51 (0.47)
Caudate Putamen, μm, mean (SD)	3.90 (0.42)	4.39 (0.47)
Internal Capsule, μm, mean (SD)	4.37 (0.50)	4.77 (0.63)
Hippocampus- CA1, μm, mean (SD)	3.86 (0.55)	4.32 (0.38)
Hippocampus- CA2/3, μm, mean (SD)	3.69 (0.37)	4.26 (0.40)
Hippocampus- DG, μm, mean (SD)	3.72 (0.34)	4.32 (0.60)
Thalamus, μm, mean (SD)	4.22 (0.57)	4.69 (0.61)

Table B-3. Average pericyte coverage (%) by brain region in both young and aged mice.

Pericyte Coverage	Young (n= 8)	Aged (n=8)	Difference
Anterior CC, %, mean (SD)	77.964	70.251	7.713
Posterior CC, %, mean (SD)	76.780	69.206	7.574
Caudate Putamen, %, mean (SD)	74.879	63.894	10.985
Internal Capsule, %, mean (SD)	72.832	63.517	9.315
Hippocampus- CA1, %, mean (SD)	74.623	71.820	2.803
Hippocampus- CA2/3, %, mean (SD)	71.478	67.993	3.485
Hippocampus- DG, %, mean (SD)	75.110	65.367	9.117
Thalamus, %, mean (SD)	75.510	59.119	16.391

Table B-4. Median (IQR) VCAM-1 coverage in all the brain regions observed in young and aged animals

VCAM-1 Coverage	Young (n= 8)	Aged (n=8)	Difference
Anterior CC, %, median (IQR)	4.37 (1.96)	11.9 (6.19)	7.53
Posterior CC, %, median (IQR)	7.25 (5.09)	11.6 (6.27)	4.35
Caudate Putamen, %, median (IQR)	2.27 (1.70)	4.65 (3.08)	2.38
Internal Capsule, %, median (IQR)	2.98 (1.78)	5.35 (4.91)	2.37
Hippocampus- CA1, %, median (IQR)	3.14 (6.31)	5.04 (2.17)	1.90
Hippocampus- CA2/3, %, median (IQR)	2.76 (2.70)	4.81 (1.56)	2.05
Hippocampus- DG, %, median (IQR)	2.10 (1.62)	2.62 (4.55)	0.52
Thalamus, mm/mm3, %, median (IQR)	2.38 (1.35)	4.24 (2.46)	1.86

Appendix C. Supplemental material to Chapter 4

Table C- 1. Clinical and demographic data of included cases. *Abbreviations:* SVD: small vessel disease; PMI: post-mortem interval; AF: atrial fibrillation; LBC: Lothian Birth Cohort; LINCHPIN: Lothian study of Intracerebral Haemorrhage, Pathology, Imaging, and Neurological Outcome

	Study	Age	Sex	PMI	Date ICH	Hypertension	Hypercholesterolaemia	Diabetes	Smoking	Heart Disease	AF	Cause of death
Young healthy controls	Sudden Death	40	Female	72	N/A	No	No	No	Previous	No	No	Unascertained
	Sudden Death	34	Male	99	N/A	No	No	No	No	Yes	No	Ischaemic heart disease
	Sudden Death	37	Female	126	N/A	No	No	No	No	No	No	Myocardial fibrosis
	Sudden Death	19	Male	101	N/A	No	No	No	Yes	No	No	Unascertained
Mild SVD	Sudden Death	44	Male	120	N/A	No	No	No	Yes	No	No	Sudden cardiac death
	Sudden Death	71	Female	95	N/A	No	Yes	No	No	No	No	Suffocation
	Sudden Death	84	Male	23	N/A	Yes	No	Yes	N/A	No	Yes	Sepsis
Moderate SVD	LBC	85	Male	3	N/A	No	No	Yes	Previous	Yes	Yes	Myocardial infarction
	LBC	82	Male	56	N/A	Yes	No	Yes	Previous	No	No	Squamous cell carcinoma
	Sudden Death	73	Female	74	N/A	Yes	No	No	Previous	No	No	Hypertensive heart disease
Severe SVD	LINCHPIN	82	Female	114	2012	Yes	No	No	No	No	No	Myocardial infarction
	LBC	82	Male	40	N/A	Yes	No	No	No	No	No	Bronchopneumonia
	LINCHPIN	93	Male	49	2015	Yes	No	No	N/A	Yes	No	Aspiration Pneumonia
	LINCHPIN	89	Male	21	2003	Yes	No	No	N/A	No	No	Intracerebral haemorrhage
	LINCHPIN	70	male	82	2016	Yes	Yes	No	N/A	No	No	Aspiration Pneumonia

Table C-2. Clinical data of included cases, including previous medical history. *Abbreviations:* PMH: previous medical history; SVD: small vessel disease; AB-CAA: amyloid-beta cerebral amyloid angiopathy; IHD: heart disease, WM: white matter, ICH: intracerebral hemorrhage, IHD: ischaemic heart disease.

ID	group	age	sex	Other PMH	Comments
1	control	40	Female		Mild arteriolar Aβ-CAA, No significant abnormalities
2	control	34	Male		No significant abnormalities
3	control	37	Female		No significant abnormalities
4	control	19	Male		No significant abnormalities
5	Mild SVD	44	Male		Mild WM pathology, mild non-amyloid SVD, no significant abnormailities
6	Mild SVD	71	Female		braak tangle stage 1, mild WM pathology, mild non-amyloid SVD, no significant abnormalities
7	Mild SVD	84	Male		braak tangle stage 1, mild WM pathology, mild non-amyloid SVD, no significant abnormalities
8	Moderate SVD	85	Male		thal amyloid phase 1, braak tangle stage 2, moderate WM pathology, moderate non-amyloid SVD, no significant abnormalities
9	Moderate SVD	82	Male		thal amyloid phase 1, braak tangle stage 1, moderate WM pathology, moderate non-amyloid SVD, no significant abnormalities
10	Moderate SVD	73	Female		thal amyloid phase 1, moderate WM pathology, mild non-amyloid SVD, no significant abnormalities
11	Severe SVD	82	female		mild arteriolar Aβ-CAA, braak tangle stage 1, severe WM pathology, severe non-amyloid SVD
12	Severe SVD	82	male		braak tangle stage 1, severe WM pathology, severe non-amyloid SVD
13	Severe SVD	93	male		braak tangle stage 2, cerebellar haemorrhage, thal amyloid phase 1, severe non-amyloid SVD
14	Severe SVD	89	male		braak tangle stage 1, thal amyloid phase 1, severe non-amyloid SVD
15	Severe SVD	70	male		mild arteriolar Aβ-CAA, braak tangle stage 1, severe white matter pathology, severe non-amyloid SVD

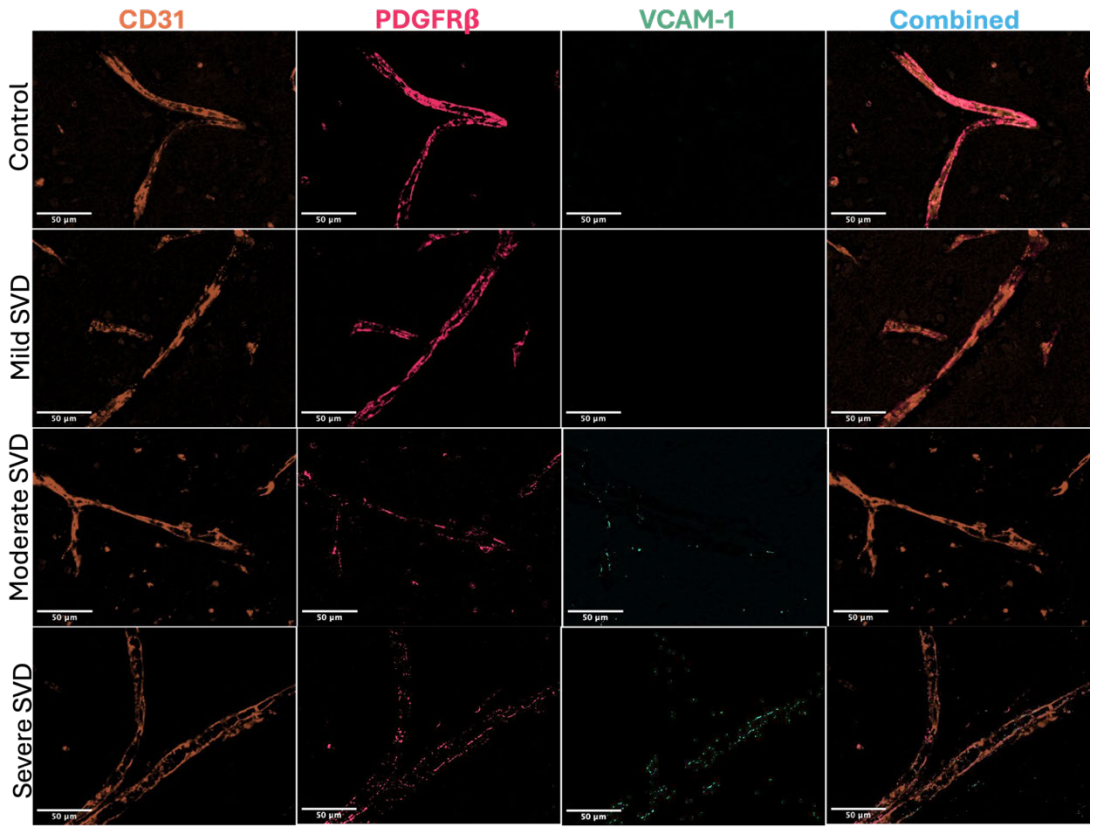

Figure C- 1. Images stained using CD-31 (vasculature, DAB), PDGFRβ (pericytes, magenta), and VCAM-1 (endothelial activation, green). Sections from the precentral gyrus of a healthy control (top row), a mild SVD case (second row from top), a moderate SVD case (third row from top), and a severe SVD case (bottom row) denoting pericyte loss and enhanced endothelial activation in SVD in individual microvessels.

UK Brain Bank Network (UKBBN) Tissue Request form

Please use this form to request human tissue from members of the UK Brain Bank network. Please note that additional cover sheets may need to be completed/signed for some of the participant banks and that individual Material Transfer Agreements will be needed before tissue can be released from each providing bank.

Requester details

Name (full name and title)	Axel Montagne, PhD Group Leader at the UK DRI, CCBS
PI/Head of Dept. (if not the requester)	
Department	Centre for Clinical Brain Sciences
Institution or Company	UK Dementia Research Institute at the University of Edinburgh
Full address	Chancellor's Building 49 Little France Crescent Edinburgh
Post code / Zip	EH16 4SB
Country	United Kingdom
Telephone	07895730745
Email	

Details of Research Project

Title:	Cerebrovascular and inflamm-ageing link to dementia
Project start date	Jan 2023
Project end date	Jun 2023

Lay Summary (approx. 250 words)

Please note this summary is for lay members on the access committees who have a non-scientific background. It is suggested that you answer the following questions remembering to avoid the use of jargon and explaining any scientific terms or techniques:

- What is already known about this topic?
- How is your project different from other projects in the area?
- What is your hypothesis and what do you hope to find out?
- How will the research improve the lives of patients and/or carers?

Small vessel disease is a neurodegenerative disorder of the small vessels in the brain. It is one of the commonest causes of dementia and is associated with the pathology underlying Alzheimer's Disease. Pericytes are cells that wrap around and support the brain microvessels, which are made up of endothelial cells. Pericytes are thought to play an integral role in maintaining the integrity between the brain and the blood healthy. There is evidence suggesting that in the early stages of small vessel disease, pericytes can become dysfunctional, an occurrence which correlates

with the activation of brain endothelial cells and the consequent disease progression.

We aim to understand the link between molecules involved in endothelial cell activation and pericyte loss/function and to corroborate findings from our animal model investigations.

Using human brain tissue from SVD disease patients as well as young and aged-match controls, we endeavor to explore the relationship between endothelial cells(CD31), a molecule known to be associated with endothelial activation(VCAM-1), and pericytes (PDGFRB). We will then use an innovative and in-house developed software, the Vas-O-Matic, to extract data on this relationship from our resulting images.

Results from these investigations could help uncover potential therapeutic avenues for small vessel disease, vascular dementia and Alzheimer's Disease.

(202 words)

Please provide a scientific summary of the project and include the necessary background, aims, justification for the type of tissue requested, techniques to be used, and expected benefits to research into neurodegenerative disease or other neurological conditions. Include enough information to enable the brain banks to check that the methodology is appropriate to meet the objectives.

Background

The blood brain barrier (BBB) helps protect the brain from harmful components found in the blood as well as selectively allowing nutrients in and waste products out. A number of cells termed the neurovascular unit, create this barrier this barrier. Among them are endothelial cells, which are held together by tight junctions and pericytes which wrap around endothelial cells in the micro-vessels and deliver vascular function support whilst helping maintain barrier integrity. There is ample recently-published evidence supporting the involvement of BBB dysfunction in the early stages of small vessel disease (SVD) and Alzheimer's Disease (ED). In addition, studies suggest there is a gradual loss of pericytes in normal ageing, which appears accelerated in both SVD and AD pathology. This loss also correlates with increased vascular dysfunction and the activation of endothelial through the expression of specific adhesion molecules.

Aim

Our main aim is to assess potential pericyte-coverage loss (PDGFRB/CD31 ratio) and corresponding endothelial activation (VCAM-1) in normal ageing and dementia disease pathology.

Techniques

Using human brain tissue from healthy young and aged controls and Alzheimer's disease patients, we will use Immunohistochemistry to stain the tissue for endothelial cells (CD31), pericytes (CD13) and endothelial cell activation (adhesion molecule VCAM-1). Using our own developed software, Vas-O-Matic, we will assess the relationship between vascular changes, pericyte coverage and endothelial cell activation as they relate to a healthy state of ageing and dementia disease pathology.

Expected benefits

Findings from these investigations would shed light on dementia disease pathology and potentially serve as a strong translational tool with which to corroborate findings from our ongoing animal and cell investigations. Not only will it help clarify the relationship between pericyte loss and endothelial activation in ageing and disease, but it will also add to the growing knowledge on the involvement of BBB breakdown in early stages of dementia, possibly leading to the development of new therapeutic avenues for an increasingly widespread and devastating condition.

Tissue requested

Number of Cases	Disease/Diagnosis	Selection criteria
5	/	Young controls
5	/	Older controls
5	SVD preferably or AD	Age- and sex-matched to the older controls

Number of Controls	Selection criteria

Type of tissue

Frozen samples (specify approximate weight/size of tissue sample)	
~~Frozen cryostat sections (specify number per area and thickness)~~	Not available
Paraffin sections (specify number per area and thickness)	3 sections per sample and 4 um thick; Preferably cortical tissue which includes

	both GM and watershed WM areas
Fixed tissue sample/FFPE (Delete as appropriate)- FFPE blocks are only given out in exceptional circumstances	
Other – e.g. CSF, DNA, tissue homogenates, whole brain for scanning (please note these may not be available for all cases)	

Other information - e.g. further constraints, mixed pathology, PM delay, and clinical information needed:

PMI as short as possible

Further details:

Ethical Approval

The participating brain banks can supply human tissue for research under their generic “Research Tissue Bank” ethical approval, provided that the respective access committees are convinced that the research proposed is ethical. However, evidence of a specific Research Ethics Committee (REC) approval is required for research taking place in the UK if:

- The tissue requested is to be used as part of a clinical trial OR
- The proposed research project involves collecting extra tissue to that required for routine diagnosis or treatment in living donors.

Please provide a copy of the ethical approval letter for your project if the research fits one of the criteria above, of if you have obtained ethical approval for your project for any other reason.

The brain banks cannot provide tissue under their generic ethics approval if the research is to be conducted outside of the UK. If the samples requested are to leave the UK, please provide a letter from the organisation where the research is to take place to confirm that the project has received ethical approval in the destination country.

My study has received specific REC approval and I have provided my REC approval letter with this application.	No
I wish to conduct my study under the brain banks’ generic ethical approval.	Yes
If the samples are to leave the UK, I have provided evidence of ethical approval from	N/A

the country of destination with this application.	
Has an ethics committee previously rejected this application?	No
If the requested Material is to be used for genetic analysis, what consideration has been given to the ethical issues surrounding the discovery of pathogenic mutations, or mutations implicated in susceptibility?	N/A

Peer-review and funding

Has the project undergone peer review? Please give details	No
Please give details of the funding bodies supporting the research	Axel Montagne PI - DRI grant (MRC, ASUK, ARUK) and MRC CDA

Collaborators

Will any part of the work or analysis be delegated to collaborators – please give details of people and their institution (N.B collaborators will also need to be party to any MTA)	**No**

Costs

Edinburgh University departments only please complete the table below with grant details.

Entity	**FUND**	**COST CENTRE**	**ACCOUNT**
110			

In line with a sustainable future for brain banking, the UKBBN implements a partial cost recovery policy, details of which can be found here:

https://brainbanknetwork.ac.uk/public/researchers/tariffs/All tissue requests are subject to an administrative fee as well as invoicing for the cost of the samples and any related consumables, packaging and transport.

I agree to pay the invoice associated with this request: yes

Declaration

The success of a brain tissue bank is gauged by the number and nature of publications arising from work on the Material. This is essential for securing future funding for human brain tissue banks.

I agree to cite the contribution made by the Edinburgh Brain Bank in the "Acknowledgements" section of all publications arising from research performed on Material and/or data that it has supplied and will send copies of such publications to the brain bank managers.

- Please refer to the agreements from each of the participating banks involved in your request for the specific acknowledgment to be used.

I understand that the Material is to be used solely for the purposes of the project detailed in this Tissue Request Form, and only by named collaborators (or research assistants) working under the direction of named individuals and that further proposed work on the same Material cannot commence until a further request has been made and permission given by the brain bank. Material must not be passed onto a third party without the prior permission of the providing brain bank.

I agree to obtain permission from the brain banks before using any Material for research that may or will be part of a commercial development or project.

The providing Brain Bank always remains the custodian of the tissue. If Material remains further to its use in named experiments, it is the responsibility of the researcher in question to dispose of the remaining tissue in accordance with all relevant legislation or return the remaining tissue to the brain bank for respectful and appropriate disposal.

Signature:

Name: Axel Montagne

Date: 12/01/23

Appendix D. Supplemental material to Chapter 5

Table D- 1. Cohort characteristics of patients with available blood biomarker data compared to patients without.

	No (N=48)	Yes (N=181)	Overall (N=229)
Sex, female, n (%)	21 (43 %)	57 (31 %)	78 (34 %)
Age, years, mean (SD)	68 (± 10)	65 (± 11)	66 (± 11)
Stroke subtype, lacunar, n (%)	25 (51 %)	106 (59 %)	131 (57 %)
Diabetes, yes, n (%)	7 (14 %)	43 (24 %)	50 (22 %)
Hypertension, yes, n (%)	31 (63 %)	126 (70 %)	157 (68 %)
Hypercholesterolaemia, yes, n (%)	38 (78 %)	133 (73 %)	171 (74 %)
Current smoker or ex-smoker < 1 year ago, n (%)	10 (20 %)	31 (17 %)	41 (18 %)
Risk factor score, mean (SD)	2.8 (± 0.97)	2.8 (± 0.96)	2.8 (± 0.96)
Baseline NIHSS, mean (SD)	2.1 (± 1.1)	2.4 (± 1.4)	2.3 (± 1.4)
Baseline NART score, number of errors, mean (SD)	17 (± 9.7)	18 (± 9.5)	17 (± 9.5)
Baseline WMH volume (mm^3 as % ICV), mean (SD)	0.85 (± 0.93)	0.96 (± 1.2)	0.93 (± 1.1)
1-year follow-up WMH volume (mm^3 as % ICV), mean (SD) *	0.99 (± 1.2)	1.0 (± 1.3)	1.0 (± 1.3)
Change in WMH volume between baseline and 1-year follow-up (mm^3 as % ICV), mean (SD)*	0.086 (± 0.29)	0.068 (± 0.20)	0.071 (± 0.22)
Baseline SVD composite score, mean (SD)	2.8 (± 1.3)	2.8 (± 1.4)	2.8 (± 1.3)
1-year follow-up SVD composite score, mean (SD)*	2.7 (± 1.2)	2.8 (± 1.3)	2.8 (± 1.3)
Baseline Fazekas score, mean (SD)	4.1 (± 1.4)	4.0 (± 1.6)	4.1 (± 1.6)
1-year follow-up Fazekas score, mean (SD)*	4.2 (± 1.5)	4.0 (± 1.7)	4.1 (± 1.6)
Lacunes at baseline, number, median (IQR)	2.0 (1,3)	1.0 (1,3)	2.0 (1,4)
Lacunes at 1-year follow-up, number, median (IQR)*	2.0 (1,4)	1.0 (0,3)	2.0 (1,4)
Microbleeds at baseline, number, median (IQR)	1.0 (1,1)	0.0 (0,0)	1.0 (1,1)
Microbleeds at 1-year follow-up, number, median (IQR)*	1.0 (1,1)	0.0 (0,0)	1.0 (1,1)
New DWI-positive lesions between baseline and 1-year follow-up detected, number, mean (SD)*	0.37 (± 0.91)	0.54 (± 1.2)	0.50 (± 1.1)
MoCA score at baseline, mean (SD)	25 (± 3.2)	25 (± 3.5)	25 (± 3.5)
MoCA score at 1-year follow-up, mean (SD)*	26 (± 3.2)	26 (± 3.6)	26 (± 3.5)
Baseline Trail Making Test ratio (A/B), mean (SD)	3 (1.6)	2.9 (1.2)	2.9 (1.3)
1-year follow-up Trail Making Test ratio (A/B), mean (SD)*	2.7 (1.6)	2.8 (1.3)	2.8 (1.4)
PS, NAWM, $x10^4$, mean (SD)	0.23 (0.98)	0.16 (1.0)	0.17 (1.0)

	No (N=48)	Yes (N=181)	Overall (N=229)
PS, basal ganglia, x10^4, mean (SD)	0.85 (1.2)	0.75 (1.3)	0.77 (1.3)
PS, WMH, x10^4, mean (SD)	0.99 (1.7)	0.84 (1.6)	0.87 (1.6)
V_p, NAWM, x10^3, mean (SD)	7.0 (2.0)	0.63 (1.8)	6.4 (1.9)
V_p, basal ganglia, x10^3, mean (SD)	16 (3.7)	15 (3.5)	15 (3.6)
V_p, WMH, x10^3, mean (SD)	9.4 (4.0)	8.4 (3.7)	8.6 (3.8)
CVR, NAWM, % /mmHg, mean (SD)	0.041 (0.014)	0.043 (0.017)	0.043 (0.016)
CVR, basal ganglia, % /mmHg, mean (SD)	0.16 (0.049)	0.17 (0.053)	0.17 (0.053)
CVR, WMH, % /mmHg, mean (SD)	0.043 (0.043)	0.040 (0.043)	0.041 (0.043)

Abbreviations: IQR: interquartile range; **NIHSS:** The National Institutes of Health Stroke Scale; **NART**: National Adult Reading Test; **WMH**: white matter hyperintensity; **ICV:** intracranial volume; **SVD**: small vessel disease; **DWI:** diffusion-weighted imaging; **MoCA:** Montreal Cognitive Assessment; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor; **PS:** permeability-surface area product ; **NAWM:** normal-appearing white matter; **V_p:** blood plasma volume fraction; **CVR:** cerebrovascular reactivity; **TMT:** trail making test.

Table D- 2. Baseline cohort characteristics of patients with blood biomarker data and one year follow-up data compared to patients without one year follow-up data.

	No (N=18)	Yes (N=163)
Sex, female, n (%)	5 (28 %)	52 (32 %)
Age, years, mean (SD)	65 (± 12)	65 (± 11)
Stroke subtype, lacunar, n (%)	9 (50 %)	97 (60 %)
Diabetes, yes, n (%)	6 (33 %)	37 (23 %)
Hypertension, yes, n (%)	12 (67 %)	114 (70 %)
Hypercholesterolaemia, yes, n (%)	13 (72 %)	120 (74 %)
Smoking status, current smoker, n (%)	5 (28 %)	18 (11 %)
Risk factor score, mean (SD)	3.0 (± 1.3)	2.8 (± 0.92)
Baseline NIHSS, mean (SD)	2.0 (± 0.91)	2.4 (± 1.5)
Baseline NART score, number of errors, mean (SD)	19 (± 9.3)	17 (± 9.5)
Baseline WMH volume (mm^3 as % ICV), mean (SD)	0.72 (± 0.56)	0.98 (± 1.2)
1-year follow-up WMH volume (mm^3 as % ICV), mean (SD)*	NA (± NA)	1.0 (± 1.3)
Change in WMH volume between baseline and 1-year follow-up (mm^3 as % ICV), mean (SD)*	NA (± NA)	0.068 (± 0.20)
Baseline SVD composite score, mean (SD)	2.6 (± 1.4)	2.9 (± 1.3)
1-year follow-up SVD composite score, mean (SD)*	18 (100%)	2 (1.2%)
Baseline Fazekas score, mean (SD)	3.8 (± 1.4)	4.1 (± 1.6)
1-year follow-up Fazekas score, mean (SD)*	NA (± NA)	4.0 (± 1.7)
Lacunes at baseline, number, median (IQR)	0 (0,2)	1 (0,3)
Lacunes at 1-year follow-up, number, median (IQR)*	NA (± NA)	1 (0,3)
Microbleeds at baseline, number, median (IQR)	0 (0,1)	0 (0,0)
Microbleeds at 1-year follow-up, number, median (IQR)*	NA (± NA)	0 (0,0)
New DWI-positive lesions between baseline and 1-year follow-up detected, number, mean (SD)*	0.33 (± 0.97)	0.56 (± 1.2)
MoCA score at baseline, mean (SD)	25 (± 2.5)	25 (± 3.6)
MoCA score at 1-year follow-up, mean (SD)*	22 (± 6.0)	26 (± 3.6)
Baseline Trail Making Test ratio (A/B), mean (SD)	2.8 (0.82)	2.9 (1.3)
1-year follow-up Trail Making Test ratio (A/B), mean (SD)*	2.3 (1.1)	2.8 (1.3)
PDGFRβ, mean (SD)	57000 (± 24000)	57000 (± 23000)
PDGF-BB, mean (SD)	9900 (± 4000)	8800 (± 3200)
VCAM-1, mean (SD)	2300 (± 720)	2100 (± 760)

	No (N=18)	Yes (N=163)
ICAM-1, mean (SD)	220000 (± 100000)	200000 (± 80000)
P-Selectin, mean (SD)	130 (± 59)	110 (± 47)
E-Selectin, mean (SD)	52 (± 22)	42 (± 19)
Endo-1, mean (SD)	5.0 (± 4.5)	3.6 (± 2.5)
TNF-α, mean (SD)	1.2 (± 0.67)	1.1 (± 0.73)
VWF, mean (SD)	1400 (± 620)	1300 (± 820)
MMP-9, mean (SD)	870 (± 350)	870 (± 450)
IL-6, mean (SD)	7.0 (± 16)	3.9 (± 8.5)
VEGF, mean (SD)	300 (± 190)	350 (± 200)
PLGF, mean (SD)	9.0 (± 3.2)	9.6 (± 2.9)
PS, NAWM, $x10^4$, mean (SD)	0.20 (± 1.1)	0.15 (± 1.0)
PS, basal ganglia, $x10^4$, mean (SD)	0.60 (± 1.4)	0.76 (± 1.3)
PS, WMH, $x10^4$, mean (SD)	1.1 (± 1.2)	0.82 (± 1.6)
V_p, NAWM, $x10^3$, mean (SD)	5.4 (± 1.6)	6.3 (± 1.8)
V_p, basal ganglia, $x10^3$, mean (SD)	13 (± 1.3)	15 (± 3.6)
V_p, WMH, $x10^3$, mean (SD)	7.3 (± 3.8)	8.5 (±3.7)
CVR, NAWM, % /mmHg, mean (SD)	0.046 (0.016)	0.043 (0.017)
CVR, basal ganglia, % /mmHg, mean (SD)	0.19 (0.050)	0.17 (0.053)
CVR, WMH, % /mmHg, mean (SD)	0.038 (0.038)	0.040 (0.044)

Abbreviations: IQR: interquartile range; **NIHSS:** The National Institutes of Health Stroke Scale; **NART**: National Adult Reading Test; **WMH**: white matter hyperintensity; **ICV:** intracranial volume; **SVD**: small vessel disease; **DWI:** diffusion-weighted imaging; **MoCA:** Montreal Cognitive Assessment; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor; **PS:** permeability-surface area product ; **NAWM:** normal-

appearing white matter; **V_p:** blood plasma volume fraction; **CVR:** cerebrovascular reactivity; **TMT:** trail making test.

Table D- 3. Baseline cohort characteristics of lacunar stroke patients compared to cortical stroke patients with blood biomarker data.

	Lacunar (N=106)	Cortical (N=74)
Sex, female, n (%)	31 (29 %)	25 (34 %)
Age, years, mean (SD)	64 (± 11)	67 (± 11)
Diabetes, yes, n (%)	30 (28 %)	13 (18 %)
Hypertension, yes, n (%)	78 (74 %)	48 (65 %)
Hypercholesterolaemia, yes, n (%)	84 (79 %)	49 (66 %)
Smoking status, current smoker, n (%)	16 (15 %)	7 (9 %)
Risk factor score, mean (SD)	3.0 (± 0.90)	2.6 (± 0.98)
Baseline NIHSS, mean (SD)	2.6 (± 1.5)	2.1 (± 1.2)
Baseline NART score, number of errors, mean (SD)	17 (± 9.3)	18 (± 9.8)
Baseline WMH volume (mm^3 as % ICV), mean (SD)	1.0 (± 1.2)	0.84 (± 1.1)
1-year follow-up WMH (mm3 as % ICV), mean (SD)	1.1 (± 1.3)	0.91 (± 1.2)
Change in WMH volume between baseline and 1-year follow-up (mm3 as % ICV), mean (SD)	0.060 (± 0.18)	0.079 (± 0.22)
Baseline SVD composite score, mean (SD)	3.1 (± 1.3)	2.5 (± 1.3)
1-year follow-up SVD composite score, mean (SD)	3.0 (± 1.3)	2.5 (± 1.2)
Baseline Fazekas score, mean (SD)	4.2 (± 1.6)	3.9 (± 1.6)
1-year follow-up Fazekas score, mean (SD)	4.2 (± 1.7)	3.8 (± 1.7)
Lacunes at baseline, number, median (IQR)	2.8 (± 3.8)	0.95 (± 1.7)
Lacunes at 1-year follow-up, number, median (IQR)	3.0 (± 3.9)	0.98 (± 1.8)
Microbleeds at baseline, number, median (IQR)	1.9 (± 6.6)	0.58 (± 2.7)
Microbleeds at 1-year follow-up, number, median (IQR)	2.8 (± 8.3)	0.51 (± 2.6)
New DWI+ lesions between baseline and 1-year follow-up detected, number, mean (SD)	0.73 (± 1.4)	0.28 (± 0.65)
MoCA score at baseline, mean (SD)	25 (± 3.4)	25 (± 3.7)
MoCA score at 1-year follow-up, mean (SD)	26 (± 3.5)	25 (± 3.7)
Baseline Trail Making Test ratio (A/B), mean (SD)	2.8 (± 1.3)	3.0 (± 1.2)
PDGFRβ, mean (SD)	55000 (± 24000)	59000 (± 22000)
PDGF_BB, mean (SD)	9100 (± 3200)	8500 (± 3300)

	Lacunar (N=106)	Cortical (N=74)
VCAM-1, mean (SD)	2100 (± 810)	2200 (± 690)
ICAM-1, mean (SD)	200000 (± 79000)	200000 (± 87000)
P-Selectin, mean (SD)	110 (± 39)	120 (± 60)
E-Selectin, mean (SD)	45 (± 22)	41 (± 16)
Endo-1, mean (SD)	3.9 (± 3.0)	3.6 (± 2.5)
TNF-α, mean (SD)	1.2 (± 0.81)	1.1 (± 0.59)
VWF, mean (SD)	1300 (± 860)	1300 (± 720)
MMP-9, mean (SD)	910 (± 400)	810 (± 480)
IL-6, mean (SD)	4.9 (± 10)	3.2 (± 8.0)
VEGF, mean (SD)	370 (± 210)	310 (± 190)
PLGF, mean (SD)	9.7 (± 3.0)	9.4 (± 2.7)
PS, NAWM, $x10^4$, mean (SD)	0.097 (± 1.0)	0.25 (± 1.0)
PS, basal ganglia, $x10^4$, mean (SD)	0.81 (± 1.3)	0.67 (± 1.2)
PS, WMH, $x10^4$, mean (SD)	0.57 (± 1.5)	1.2 (± 1.6)
V_p, NAWM, $x10^3$, mean (SD)	6.1 (± 1.8)	6.4 (± 1.9)
V_p, basal ganglia, $x10^3$, mean (SD)	14 (± 3.5)	15 (± 3.4)
V_p, WMH, $x10^3$, mean (SD)	7.9 (± 3.4)	9.2 (± 4.0)
CVR, NAWM, % /mmHg, mean (SD)	0.042 (± 0.017)	0.045 (± 0.016)
CVR, basal ganglia, % /mmHg, mean (SD)	0.17 (± 0.054)	0.18 (± 0.052)

Abbreviations: IQR: interquartile range; **NIHSS:** The National Institutes of Health Stroke Scale; **NART**: National Adult Reading Test; **WMH**: white matter hyperintensity; **ICV:** intracranial volume; **SVD**: small vessel disease; **DWI:** diffusion-weighted imaging; **MoCA:** Montreal Cognitive Assessment; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9;

IL-6: Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor; **PS:** permeability-surface area product ; **NAWM:** normal-appearing white matter; **V_p:** blood plasma volume fraction; **CVR:** cerebrovascular reactivity; **TMT:** trail making test.

Table D- 4. Pearson correlation between individual biomarkers and the time between stroke onset and serology.

	Pearson's coefficient	p-value	95% CI
PDGFRβ	-0.052	0.489	-0.197, 0.953
PDGF-BB	-0.051	0.493	-0.196, 0.095
VCAM-1	0.121	0.105	-0.025, 0.263
ICAM-1	-0.001	0.989	-0.147, 0.145
P-Selectin	0.078	0.296	-0.068, 0.222
E-Selectin	-0.139	0.067	-0.283, 0.010
Endo-1	0.104	0.173	-0.046, 0.250
TNF-α	-0.023	0.755	-0.169, 0.123
vWF	0.021	0.773	-0.125, 0.167
MMP-9	-0.044	0.559	-0.193, 0.105
IL-6	0.079	0.291	-0.068, 0.223
VEGF	-0.019	0.795	-0.165, 0.127
PLGF	0.102	0.171	-0.044, 0.245

Abbreviations: CI: confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 5. Blood biomarkers and WMH volume. Linear regression results for the association between distinct standardised blood biomarkers and log transformed WMH volume as % ICV at baseline (linear regression) and one year follow-up (linear mixed effects model) after adjusting for age, sex, vascular risk factors, stroke subtype, and baseline NIHSS.

	Baseline		*Longitudinal WMH volume*	
	Adjusted Analyses			
	β	**95% CI**	**β**	**95% CI**
PDGFRβ	-0.025	-0.174, 0.123	-0.070	-0.216, 0.076
PDGF-BB	0.108	-0.041, 0.257	0.114	-0.035, 0.263
VCAM-1	-0.133	-0.292, 0.024	-0.168	-0.326, 0.010
ICAM-1	-0.026	-0.175, 0.122	-0.025	-0.174, 0.123
P-Selectin	-0.115	-0.258, 0.028	-0.116	-0.260, 0.028
E-Selectin	0.028	-0.123, 0.179	0.020	-0.131, 0.173
Endo-1	0.068	-0.077, 0.215	0.062	-0.085, 0.210
TNF-α	0.001	-0.151, 0.152	0.007	-0.143, 0.159
vWF	-0.115	-0.266, 0.035	-0.138	-0.288, 0.011
MMP-9	0.090	-0.057, 0.238	0.107	-0.040, 0.256
IL-6	-0.007	-0.152, 0.137	0.000	-0.144, 0.145
VEGF	-0.025	-0.172, 0.122	-0.026	-0.174, 0.120
PLGF	-0.008	-0.153, 0.137	0.005	-0.139, 0.151
	Unadjusted analyses			
PDGFRβ	-0.01	-0.177, 0.158	-0.041	-0.208, 0.126
PDGF-BB	-0.033	-0.200, 0.133	-0.027	-0.196, 0.141
VCAM-1	0.131	-0.032, 0.296	0.122	-0.043, 0.290
ICAM-1	0.101	-0.063, 0.266	0.103	-0.063, 0.270
P-Selectin	-0.145	-0.310, 0.018	-0.149	-0.315, 0.017
E-Selectin	0.061	-0.104, 0.227	0.049	-0.118, 0.218
Endo-1	0.046	-0.118, 0.212	0.048	-0.119, 0.216
TNF-α	0.149	-0.014, 0.313	0.161	-0.004, 0.327
vWF	0.086	-0.079, 0.251	0.074	-0.093, 0.241
MMP-9	0.045	-0.121, 0.212	0.049	-0.119, 0.217
IL-6	0.028	-0.136, 0.194	0.034	-0.133,0.202
VEGF	-0.043	-0.209, 0.122	-0.042	-0.211, 0.125
PLGF	0.038	-0.127, 0.204	0.046	-0.122, 0.215

Abbreviations: WMH: white matter hyperintensity; **ICV:** intracranial volume; **NIHSS:** The National Institutes of Health Stroke Scale; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 6. Blood biomarkers and Fazekas score. Ordinal regression results for the association of distinct standardised blood biomarkers and Fazekas score at baseline after adjusting for age, sex, vascular risk factors, stroke subtype, and baseline NIHSS.

Baseline		
Adjusted Analyses		
	OR	**95% CI**
PDGFRβ	0.959	0.727, 1.263
PDGF-BB	1.155	0.862, 1.547
VCAM-1	0.877	0.648, 1.187
ICAM-1	1.090	0.815, 1.455
P-Selectin	0.826	0.624, 1.096
E-Selectin	1.121	0.841, 1.511
Endo-1	0.906	0.691, 1.195
TNF-α	0.872	0.641, 1.150
vWF	0.824	0.607, 1.137
MMP-9	1.045	0.786, 1.410
IL-6	0.974	0.757, 1.249
VEGF	0.991	0.751, 1.310
PLGF	1.000	0.752, 1.329
Unadjusted Analyses		
PDGFRβ	1.06	0.772, 1.312
PDGF-BB	0.912	0.699, 1.185
VCAM-1	1.355	1.040, 1.775
ICAM-1	1.312	1.003, 1.717
P-Selectin	0.821	0.631, 1.058
E-Selectin	1.135	0.881, 1.466
Endo-1	0.914	0.707, 1.185
TNF-α	1.163	0.905, 1.476
vWF	1.180	0.904, 1.542
MMP-9	0.980	0.745, 1.298
IL-6	1.067	0.835, 1.377
VEGF	0.972	0.746, 1.264
PLGF	1.098	0.833, 1.443

Abbreviations: NIHSS: The National Institutes of Health Stroke Scale; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 7. Blood biomarkers and the presence of lacunes and/or microbleeds at baseline. Logistic regression results for the association of distinct standardised blood biomarkers and the prevalence of lacunes and microbleeds at baseline after adjusting for age, sex, vascular risk factors, and stroke subtype.

	Baseline			
	Lacunes		***Microbleeds***	
Adjusted Analyses				
	OR	**95% CI (exp)**	**OR**	**95% CI (exp)**
PDGFRβ	0.715	0.508, 0.993	0.823	0.548, 1.222
PDGF-BB	0.921	0.654, 1.285	1.058	0.690, 1.607
VCAM-1	0.920	0.652, 1.307	0.910	0.587, 1.367
ICAM-1	1.259	0.909, 1.770	0.860	0.557, 1.282
P-Selectin	0.967	0.697, 1.328	0.841	0.519, 1.270
E-Selectin	1.153	0.817, 1.665	0.656	0.386, 1.023
Endo-1	0.713	0.496, 0.990	1.004	0.440, 1.989
TNF-α	0.842	0.516, 1.314	0.794	0.483, 1.178
vWF	1.140	0.812, 1.639	0.699	0.427, 1.057
MMP-9	0.814	0.565, 1.146	1.061	0.672, 1.597
IL-6	0.817	0.542, 1.118	0.739	0.251, 1.176
VEGF	0.914	0.662, 1.258	1.267	0.864, 1.856
PLGF	0.928	0.676, 1.272	0.887	0.591, 1.291
Unadjusted Analyses				
PDGFRβ	0.736	0.504, 1.00	0.771	0.520, 1.126
PDGF-BB	0.877	0.647, 1.180	0.981	0.165, 0.347
VCAM-1	1.097	0.817, 1.483	1.020	0.695, 1.458
ICAM-1	1.348	0.997, 1.857	0.954	0.642, 1.370
P-Selectin	0.918	0.672, 1.234	0.783	0.491, 1.166
E-Selectin	1.214	0.896, 1.680	0.738	0.450, 1.119
Endo-1	0.739	0.523, 1.009	0.889	0.565, 1.292
TNF-α	0.851	0.613, 1.146	0.937	0.597, 1.336
vWF	1.315	0.968, 1.844	0.798	0.494, 1.183
MMP-9	0.891	0.652, 1.205	1.028	0.687, 1.473
IL-6	0.895	0.626, 1.207	0.863	0.387, 1.265
VEGF	0.968	0.720, 1.301	1.317	0.917, 1.886
PLGF	1.020	0.758, 1.375	0.935	0.629, 1.349

Abbreviations: CI: confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 8. Blood biomarkers and newly appearing incident (DWI-positive) lesions. Logistic regression results for the association of distinct standardised blood biomarkers to the prevalence of DWI-positive incident lesions identified throughout the one-year follow-up period after adjusting for age, sex, vascular risk factors, stroke subtype, and baseline NIHSS.

	New DWI-positive lesion	
	OR	**95% CI**
Adjusted Analyses		
PDGFRβ	0.763	0.472, 1.211
PDGF-BB	1.398	0.846, 2.328
VCAM-1	0.971	0.569, 1.605
ICAM-1	1.274	0.840, 1.927
P-Selectin	1.498	0.996, 2.351
E-Selectin	0.734	0.431, 1.142
Endo-1	0.968	0.539, 1.603
TNF-α	0.928	0.519, 1.446
vWF	1.063	0.672, 1.602
MMP-9	1.148	0.678, 1.842
IL-6	0.881	0.333, 1.359
VEGF	1.643	1.040, 2.624
PLGF	1.335	0.826, 2.124
Unadjusted Analyses		
PDGFRβ	0.809	0.515, 1.248
PDGF-BB	1.427	0.930, 2.190
VCAM-1	1.034	0.655, 1.571
ICAM-1	1.431	0.975, 2.084
P-Selectin	1.486	1.006, 2.374
E-Selectin	1.062	0.683, 1.560
Endo-1	0.956	0.567, 1.480
TNF-α	1.025	0.643, 1.476
vWF	1.180	0.782, 1.702
MMP-9	1.156	0.764, 1.689
IL-6	0.899	0.404, 1.317
VEGF	1.493	0.998, 2.234
PLGF	1.259	0.828, 1.882

Abbreviations: DWI: diffusion-weighted imaging; **NIHSS:** The National Institutes of Health Stroke Scale; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 9. Blood biomarkers and summary SVD score. Ordinal regression results for the association of distinct standardised blood biomarkers to baseline summary SVD scores after adjusting for age, sex, vascular risk factors, and stroke subtype.

	Baseline	
	OR	**95% CI**
Adjusted Analyses		
PDGFRβ	0.845	0.639, 1.116
PDGF-BB	1.173	0.876, 1.568
VCAM-1	0.933	0.696, 1.251
ICAM-1	1.179	0.886, 1.571
P-Selectin	0.964	0.739, 1.251
E-Selectin	1.111	0.857, 1.473
Endo-1	0.840	0.639, 1.106
TNF-α	0.779	0.580, 1.027
vWF	0.985	0.731, 1.327
MMP-9	0.975	0.717, 1.317
IL-6	0.883	0.688, 1.134
VEGF	1.002	0.747, 1.348
PLGF	0.875	0.657, 1.160
Unadjusted Analyses		
PDGFRβ	0.859	0.658, 1.121
PDGF-BB	0.909	0.697, 1.180
VCAM-1	1.314	1.022, 1.696
ICAM-1	1.323	1.019, 1.721
P-Selectin	0.899	0.690, 1.148
E-Selectin	1.117	0.866, 1.438
Endo-1	0.854	0.665, 1.095
TNF-α	1.007	0.790, 1.286
vWF	1.253	0.976, 1.621
MMP-9	0.892	0.676, 1.183
IL-6	0.968	0.773, 1.226
VEGF	1.015	0.773, 1.336
PLGF	0.973	0.740, 1.277

Abbreviations: SVD: small vessel disease; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 10. Blood biomarkers and BBB permeability (DCE-MRI, PS). Linear regression results for the association of distinct standardised blood biomarkers to baseline DCE-MRI parameter PS (x10⁴) after adjusting for age, sex, vascular risk factors, and baseline WMH volume as % ICV.

PS	NAWM		Basal Ganglia		WMH (ventricles removed)	
	β	95% CI	β	95% CI	β	95% CI
Adjusted Analyses						
PDGFRβ	-0.001	-0.171, 0.169	0.020	-0.193, 0.234	-0.145	-0.417, 0.126
PDGF-BB	-0.124	-0.290, 0.041	-0.062	-0.272, 0.147	-0.115	-0.383, 0.152
VCAM-1	0.181	-0.018, 0.381	0.290	0.040, 0.539	0.032	-0.290, 0.355
ICAM-1	0.128	-0.058, 0.316	0.039	-0.197, 0.276	0.128	-0.173, 0.429
P-Selectin	0.043	-0.114, 0.200	0.070	-0.127, 0.268	-0.001	-0.254, 0.251
E-Selectin	-0.035	-0.207, 0.135	0.034	-0.180, 0.248	-0.160	-0.434, 0.112
Endo-1	0.051	-0.131, 0.234	0.187	-0.041, 0.415	-0.002	-0.294, 0.288
TNF-α	0.020	-0.158, 0.199	0.248	0.027, 0.468	-0.003	-0.289, 0.283
vWF	0.123	-0.080, 0.328	0.256	0.002, 0.511	0.138	-0.189, 0.466
MMP-9	-0.070	-0.234, 0.094	0.033	-0.172, 0.240	-0.080	-0.344, 0.183
IL-6	0.066	-0.103, 0.235	0.115	-0.096, 0.328	-0.063	-0.335, 0.208
VEGF	0.015	-0.146, 0.177	0.058	-0.143, 0.261	0.006	-0.252, 0.266
PLGF	0.190	0.027, 0.354	0.159	-0.047, 0.367	0.109	-0.156, 0.375
Unadjusted Analyses						
PDGFRβ	0.010	-0.153, 0.173	0.009	-0.200, 0.218	-0.107	-0.366, 0.152
PDGF-BB	-0.121	-0.281, 0.038	-0.054	-0.260, 0.151	-0.149	-0.404, 0.106
VCAM-1	0.148	-0.028, 0.325	0.258	0.032, 0.483	0.102	-0.181, 0.386
ICAM-1	0.148	-0.034, 0.330	0.102	-0.132, 0.338	0.120	-0.171, 0.413
P-Selectin	0.037	-0.117, 0.192	0.070	-0.128, 0.269	0.000	-0.246, 0.247
E-Selectin	-0.014	-0.178, 0.148	0.103	-0.105, 0.312	-0.166	-0.425, 0.092
Endo-1	0.026	-0.151, 0.204	0.198	-0.028, 0.425	-0.042	-0.324, 0.239
TNF-α	0.055	-0.111, 0.223	0.307	0.097, 0.517	0.016	-0.250, 0.283
vWF	0.127	-0.063, 0.319	0.290	0.047, 0.532	0.193	-0.111, 0.498
MMP-9	-0.076	-0.235, 0.083	0.069	-0.135, 0.273	-0.103	-0.357, 0.149
IL-6	0.059	-0.109, 0.227	0.108	-0.106, 0.323	-0.040	-0.308, 0.227
VEGF	0.025	-0.133, 0.183	0.094	-0.108, 0.296	-0.005	-0.257, 0.246
PLGF	0.207	0.045, 0.368	0.195	-0.013, 0.404	0.117	-0.144, 0.378

Abbreviations: DCE-MRI: dynamic contrast-enhanced magnetic resonance imaging; **PS:** Permeability-Surface area product; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 11. Blood biomarkers and BBB permeability (DCE-MRI, V_p). Linear regression results for the association of distinct standardised blood biomarkers to baseline DCE-MRI parameter V_p (x10^3) after adjusting for age, sex, vascular risk factors, and baseline WMH volume as % ICV.

V_P	NAWM		Basal Ganglia		WMH (ventricles removed)	
	β	95% CI	β	95% CI	β	95% CI
Adjusted Analyses						
PDGFRβ	-0.047	-0.351, 0.257	-0.159	-0.741, 0.423	-0.152	-0.717, 0.411
PDGF-BB	0.081	-0.217, 0.379	0.457	-0.110, 1.025	-0.087	-0.643, 0.467
VCAM-1	0.031	-0.328, 0.392	0.070	-0.619, 0.760	-0.283	-0.951, 0.384
ICAM-1	0.126	-0.209, 0.463	0.417	-0.224, 1.059	-0.036	-0.662, 0.588
P-Selectin	-0.037	-0.319, 0.244	0.003	-0.537, 0.543	-0.031	-0.554, 0.492
E-Selectin	-0.060	-0.365, 0.244	-0.187	-0.768, 0.394	-0.501	-1.060, 0.058
Endo-1	-0.174	-0.499, 0.150	-0.337	-0.954, 0.279	-0.102	-0.700, 0.495
TNF-α	-0.063	-0.382, 0.255	-0.268	-0.878, 0.341	0.199	-0.392, 0.791
vWF	0.113	-0.253, 0.479	0.128	-0.573, 0.830	0.128	-0.573, 0.830
MMP-9	0.026	-0.267, 0.319	-0.140	-0.700, 0.419	-0.049	-0.593, 0.494
IL-6	-0.372	-0.669, 0.074	-0.543	-1.118, 0.030	-0.242	-0.804, 0.320
VEGF	0.033	-0.255, 0.322	0.091	-0.461, 0.644	-0.197	-0.732, 0.338
PLGF	-0.249	-0.544, 0.044	-0.322	-0.889, 0.244	-0.338	-0.887, 0.211
Unadjusted Analyses						
PDGFRβ	-0.002	-0.299, 0.293	-0.074	-0.063, 0.489	-0.105	-0.711, 0.499
PDGF-BB	0.016	-0.275, 0.308	0.321	-0.232, 0.876	-0.084	-0.681, 0.512
VCAM-1	0.117	-0.205, 0.440	0.206	-0.409, 0.823	-0.251	-0.911, 0.409
ICAM-1	0.137	-0.195, 0.470	0.379	-0.253, 1.012	-0.095	-0.776, 0.586
P-Selectin	-0.011	-0.292, 0.270	0.025	-0.511, 0.562	0.183	-0.391, 0.758
E-Selectin	-0.166	-0.425, 0.092	-0.265	-0.826, 0.295	-0.399	-0.997, 0.198
Endo-1	-0.219	-0.536, 0.097	-0.393	-0.992, 0.204	-0.184	-0.825, 0.457
TNF-α	-0.006	-0.311, 0.297	-0.165	-0.745, 0.414	0.060	-0.561, 0.682
vWF	0.160	-0.187, 0.507	0.171	-0.493, 0.835	-0.142	-0.855, 0.569
MMP-9	-0.055	-0.344, 0.232	-0.299	-0.846, 0.247	-0.200	-0.787, 0.385
IL-6	-0.371	-0.670, -0.071	-0.545	-1.120, 0.030	-0.278	-0.900, 0.344
VEGF	0.050	-0.236, 0.337	0.122	-0.423, 0.669	-0.143	-0.779, 0.441
PLGF	-0.246	-0.542, 0.049	-0.327	-0.894, 0.239	-0.450	-1.056, 0.155

Abbreviations: DCE-MRI: dynamic contrast-enhanced magnetic resonance imaging; **V_p:** blood plasma volume fraction; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 12. Blood biomarkers and CVR. Linear regression results for the association of distinct standardised blood biomarkers to baseline DCE-CVR in NAWM, the basal ganglia and WMH after adjusting for age, sex, vascular risk factors, and baseline WMH volume as % ICV.

CVR	NAWM		Basal Ganglia		WMH (ventricles removed)	
	β	95% CI	β	95% CI	β	95% CI
Adjusted Analyses						
PDGFRβ	0.002	-0.001, 0.004	0.005	-0.003, 0.014	-0.001	-0.008, 0.007
PDGF-BB	0.002	-0.000, 0.005	0.012	0.003, 0.021	0.002	-0.005, 0.010
VCAM-1	-0.001	-0.004, 0.001	-0.005	-0.014, 0.004	-0.008	-0.016, -0.000
ICAM-1	-0.002	-0.004, 0.001	-0.010	-0.019, -0.001	-0.002	-0.010, 0.004
P-Selectin	0.002	-0.001, 0.005	0.011	-0.001, 0.022	0.005	-0.003, 0.014
E-Selectin	0.001	-0.003, 0.002	-0.002	-0.012, 0.007	-0.001	-0.008, 0.008
Endo-1	-0.001	-0.003, 0.002	0.009	0.001, 0.018	-0.005	-0.012, 0.002
TNF-α	-0.001	-0.005, 0.001	0.007	-0.003, 0.018	-0.001	-0.010, 0.008
vWF	-0.001	-0.003, 0.001	0.000	-0.008, 0.008	-0.004	-0.011, 0.002
MMP-9	0.001	-0.001, 0.004	0.008	-0.001, 0.017	0.004	-0.003, 0.012
IL-6	-0.001	-0.003, 0.001	-0.001	-0.007, 0.007	0.000	-0.007, 0.007
VEGF	-0.000	-0.002, 0.002	0.001	-0.007, 0.009	-0.005	-0.012, 0.001
PLGF	-0.001	-0.004, 0.001	0.000	-0.008, 0.008	0.004	-0.002, 0.012
Unadjusted Analyses						
PDGFRβ	0.001	-0.000, 0.004	0.004	-0.004, 0.013	-0.001	-0.008, 0.006
PDGF-BB	0.001	-0.001, 0.004	0.012	0.003, 0.021	0.001	-0.006, 0.008
VCAM-1	-0.001	-0.004, 0.000	-0.008	-0.017, 0.000	-0.006	-0.014, 0.000
ICAM-1	-0.001	-0.004, 0.000	-0.012	-0.020, -0.003	-0.002	-0.009, 0.004
P-Selectin	0.002	-0.000, 0.006	0.012	0.001, 0.023	0.006	-0.002, 0.016
E-Selectin	-0.000	-0.003, 0.002	-0.004	-0.014, 0.005	-0.000	-0.008, 0.007
Endo-1	-0.001	-0.004, 0.001	0.007	-0.001, 0.016	-0.004	-0.012, 0.002
TNF-α	-0.003	-0.006, 0.000	0.001	-0.009, 0.011	-0.000	-0.009, 0.008
vWF	-0.001	-0.003, 0.001	-0.003	-0.011, 0.004	-0.003	-0.010, 0.002
MMP-9	0.000	-0.002, 0.003	0.005	-0.003, 0.015	0.002	-0.005, 0.010
IL-6	-0.001	-0.003, 0.001	-0.000	-0.008, 0.007	-0.001	-0.008, 0.005
VEGF	-0.000	-0.003, 0.002	0.000	-0.008, 0.009	-0.005	-0.012, 0.001
PLGF	-0.001	-0.004, 0.000	-0.001	-0.010, 0.007	0.005	-0.002, 0.012

Abbreviations: MRI: magnetic resonance imaging; **CVR:** cerebrovascular reactivity; **NAWM:** normal appearing white matter; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 13. Blood biomarkers and MoCA score. Baseline cross-sectional linear regression and longitudinal linear mixed effects model results for the association of distinct standardised blood biomarkers and MoCA score after adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume as % ICV.

	Baseline		*One year follow-up*	
	β	**95% CI**	**β**	**95% CI**
Adjusted Analyses				
PDGFRβ	0.152	-0.315, 0.620	0.005	-0.396, 0.407
PDGF-BB	-0.087	-0.568, 0.393	-0.083	-0.493, 0.325
VCAM-1	-0.328	-0.845, 0.188	-0.320	-0.764, 0.124
ICAM-1	0.149	-0.333, 0.633	-0.022	-0.438, 0.392
P-Selectin	0.229	-0.230, 0.689	0.069	-0.320, 0.460
E-Selectin	0.336	-0.156, 0.829	0.300	-0.120, 0.722
Endo-1	-0.204	-0.661, 0.252	-0.178	-0.564, 0.207
TNF-α	0.199	-0.274, 0.673	0.163	-0.241, 0.568
vWF	0.157	-0.334, 0.650	0.041	-0.380, 0.463
MMP-9	-0.112	-0.596, 0.370	0.108	-0.303, 0.521
IL-6	0.047	-0.402, 0.496	0.154	-0.241, 0.550
VEGF	-0.346	-0.798, 0.106	-0.161	-0.550, 0.228
PLGF	-0.213	-0.673, 0.247	-0.146	-0.539, 0.247
Unadjusted Analyses				
PDGFRβ	-0.050	-0.579, 0.477	-0.134	-0.616, 0.347
PDGF-BB	0.283	-0.244, 0.812	0.265	-0.217, 0.748
VCAM-1	-1.065	-1.571, -0.559	-1.071	-1.530, -0.612
ICAM-1	-0.332	-0.856, 0.190	-0.470	-0.948, 0.008
P-Selectin	0.213	-0.314, 0.741	0.070	-0.409, 0.551
E-Selectin	0.060	-0.480, 0.601	-0.013	-0.507, 0.480
Endo-1	-0.037	-0.571, 0.496	-0.046	-0.533, 0.440
TNF-α	-0.172	-0.697, 0.353	-0.184	-0.664, 0.296
vWF	-0.524	-1.045, -0.003	-0.611	-1.082, -0.140
MMP-9	-0.175	-0.725, 0.376	0.098	-0.401, 0.599
IL-6	0.031	-0.491, 0.555	0.160	-0.327, 0.648
VEGF	-0.308	-0.832, 0.214	-0.143	-0.624, 0.338
PLGF	-0.352	-0.883, 0.178	-0.271	-0.757, 0.214

Abbreviations: MoCA: Montreal Cognitive Assessment; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 14. Blood biomarkers and MCI. Logistic regression results for the association of distinct standardised blood biomarkers and MCI prevalence at baseline and one year follow-up after adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume as % ICV. One-year cross-sectional models were also adjusted for baseline MCI status.

	Baseline		*One year follow-up*	
	OR	**95% CI**	**OR**	**95% CI**
Adjusted Analyses				
PDGFRβ	0.949	0.674, 1.334	0.955	0.653, 1.390
PDGF-BB	0.982	0.693, 1.389	1.294	0.895, 1.895
VCAM-1	1.480	0.962, 2.370	1.503	0.980, 2.402
ICAM-1	1.052	0.727, 1.549	1.336	0.897, 2.041
P-Selectin	1.206	0.816, 1.861	1.645	1.047, 2.721
E-Selectin	1.109	0.748, 1.692	1.010	0.682, 1.526
Endo-1	1.048	0.750, 1.490	1.061	0.700, 1.609
TNF-α	0.857	0.589, 1.212	0.884	0.576, 1.291
vWF	1.370	0.894, 2.260	1.247	0.844, 1.938
MMP-9	1.458	0.977, 2.278	0.802	0.540, 1.177
IL-6	0.800	0.542, 1.094	0.385	0.073, 0.942
VEGF	1.314	0.930, 1.893	0.924	0.644, 1.323
PLGF	1.136	0.804, 1.631	0.971	0.668, 1.415
Unadjusted Analyses				
PDGFRβ	1.043	0.766, 1.423	1.056	0.775, 1.440
PDGF-BB	0.820	0.599, 1.117	1.050	0.771, 1.433
VCAM-1	1.955	1.351, 2.957	2.014	1.415, 2.981
ICAM-1	1.336	0.970, 1.891	1.575	1.131, 2.245
P-Selectin	1.163	0.848, 1.664	1.388	0.997, 2.030
E-Selectin	1.224	0.887, 1.746	1.275	0.928, 1.795
Endo-1	0.967	0.710, 1.337	0.997	0.698, 1.417
TNF-α	1.019	0.752, 1.421	1.069	0.782, 1.476
vWF	1.890	1.265, 3.049	1.673	1.194, 2.463
MMP-9	1.331	0.949, 1.946	0.952	0.687, 1.308
IL-6	0.818	0.566, 1.105	0.663	0.264, 1.050
VEGF	1.205	0.884, 1.666	1.002	0.734, 1.364
PLGF	1.199	0.875, 1.678	1.072	0.782, 1.475

Abbreviations: MCI: mild cognitive impairment; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Table D- 15. Blood biomarkers and executive function (TMT ratio B/A). Baseline cross-sectional linear regression and longitudinal linear mixed effects model results for the association of distinct standardised blood biomarkers and TMT ratio (B/A) after adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume as % ICV.

	Baseline		*One year follow-up*	
	β	**95% CI**	**β**	**95% CI**
Adjusted Analyses				
PDGFRβ	-0.047	-0.225, 0.129	0.001	-0.005, 0.007
PDGF-BB	-0.151	-0.330, 0.028	-0.030	-0.077, 0.016
VCAM-1	0.073	-0.128, 0.274	0.005	-0.215, 0.227
ICAM-1	-0.016	-0.197, 0.165	0.001	-0.001, 0.002
P-Selectin	-0.095	-0.269, 0.078	-0.016	-0.164, 0.131
E-Selectin	-0.085	-0.281, 0.110	0.042	-0.121, 0.207
Endo-1	0.110	-0.062, 0.284	0.141	-0.011, 0.295
TNF-α	0.137	-0.041, 0.317	0.080	-0.072, 0.233
vWF	0.134	-0.052, 0.320	0.189	0.031, 0.347
MMP-9	0.058	-0.118, 0.234	0.008	-0.147, 0.165
IL-6	0.105	-0.062, 0.273	0.052	-0.096, 0.201
VEGF	-0.163	-0.334, 0.006	-0.153	-0.300, 0.007
PLGF	-0.187	-0.360, -0.015	-0.096	-0.245, 0.053
Unadjusted Analyses				
PDGFRβ	0.017	-0.166, 0.201	0.003	-0.003, 0.010
PDGF-BB	-0.217	-0.039, -0.034	-0.057	-0.106, -0.007
VCAM-1	0.238	0.049, 0.427	0.275	0.060, 0.490
ICAM-1	0.093	-0.089, 0.275	0.001	-0.000, 0.003
P-Selectin	-0.067	-0.250, 0.116	0.762	-2.106, 3.634
E-Selectin	-0.031	-0.226, 0.163	3.853	0.095, 7.606
Endo-1	0.071	-0.111, 0.255	0.083	-0.086, 0.252
TNF-α	0.180	0.001, 0.360	0.147	-0.013, 0.307
vWF	0.283	0.104, 0.462	0.348	0.192, 0.503
MMP-9	0.090	-0.090, 0.270	0.026	-0.140, 0.193
IL-6	0.103	-0.075, 0.283	0.052	-0.112, 0.218
VEGF	-0.153	-0.335, 0.028	-0.142	-0.305, 0.020
PLGF	-0.154	-0.338, 0.029	-0.073	-0.238, 0.091

Abbreviations: TMT: Trail Making Task; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor; **EF:** executive function.

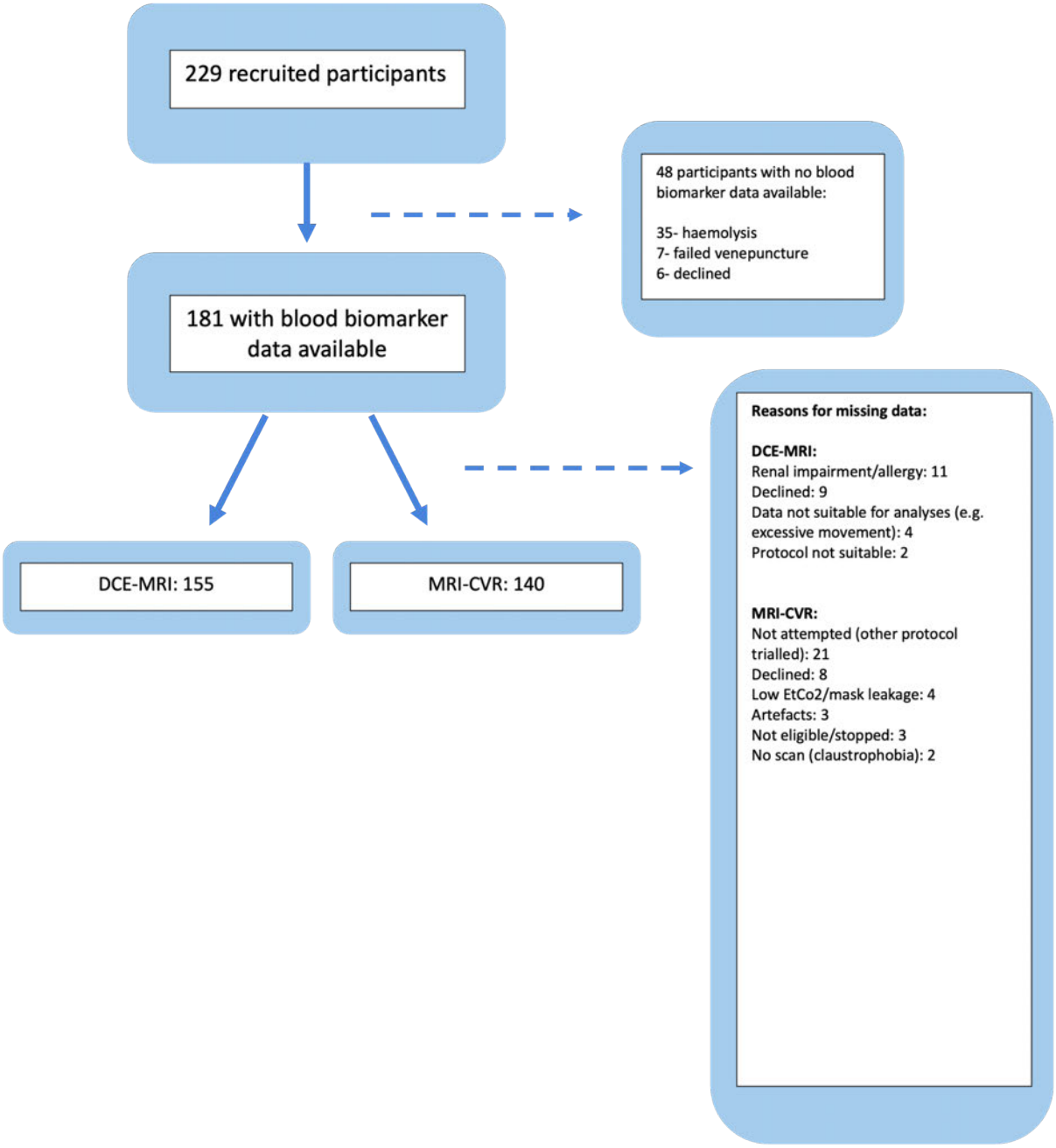

Figure D- 1. Participant flow chart for imaging data (DCE-MRI & CVR)

Abbreviations: DCE-MRI: dynamic contrast-enhanced magnetic resonance imaging; **CVR:** cerebrovascular reactivity; **$EtCO_2$:** end-tidal carbon dioxide.

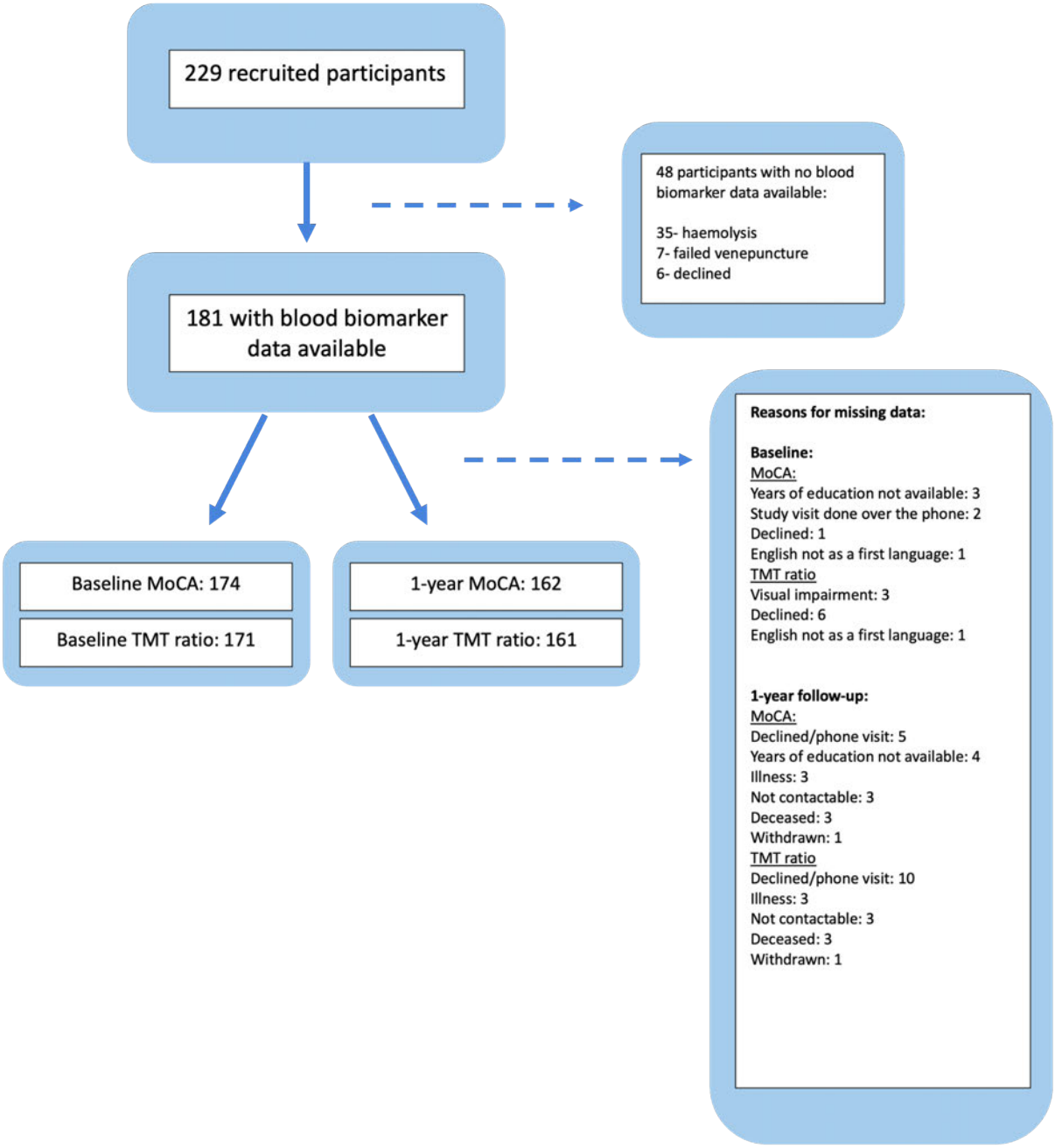

Figure D- 2. Participant flow chart for cognitive data at baseline and 1 year (MoCA & TMT).

Abbreviations: MoCA: Montreal Cognitive Assessment; **TMT:** Trail Making Test.

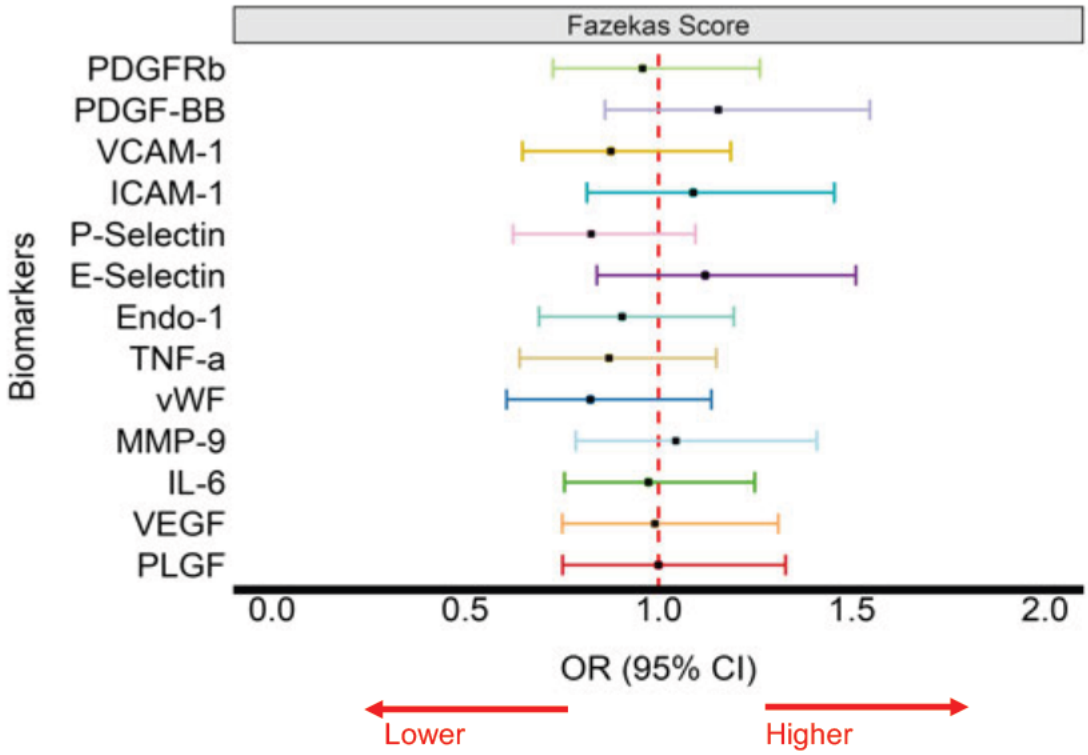

Figure D- 3. Blood biomarkers and Fazekas score. Ordinal regression results for the association of distinct standardised blood biomarkers and Fazekas score at baseline after adjusting for age, sex, vascular risk factors, stroke subtype, and baseline NIHSS.

Abbreviations: NIHSS: The National Institutes of Health Stroke Scale; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

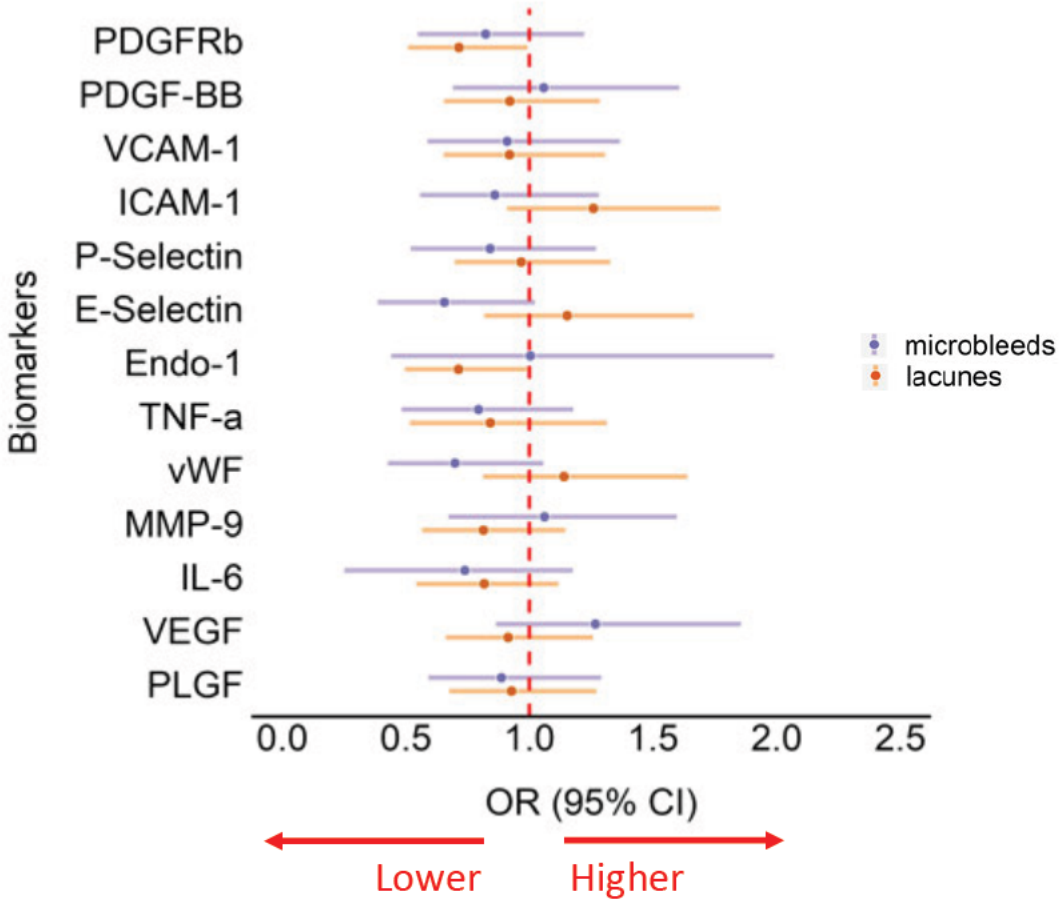

Figure D- 4. Blood biomarkers and the presence of lacunes and/or microbleeds at baseline. Logistic regression results for the association of distinct standardised blood biomarkers and the prevalence of lacunes and microbleeds at baseline after adjusting for age, sex, vascular risk factors, and stroke subtype.

Abbreviations: CI: confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

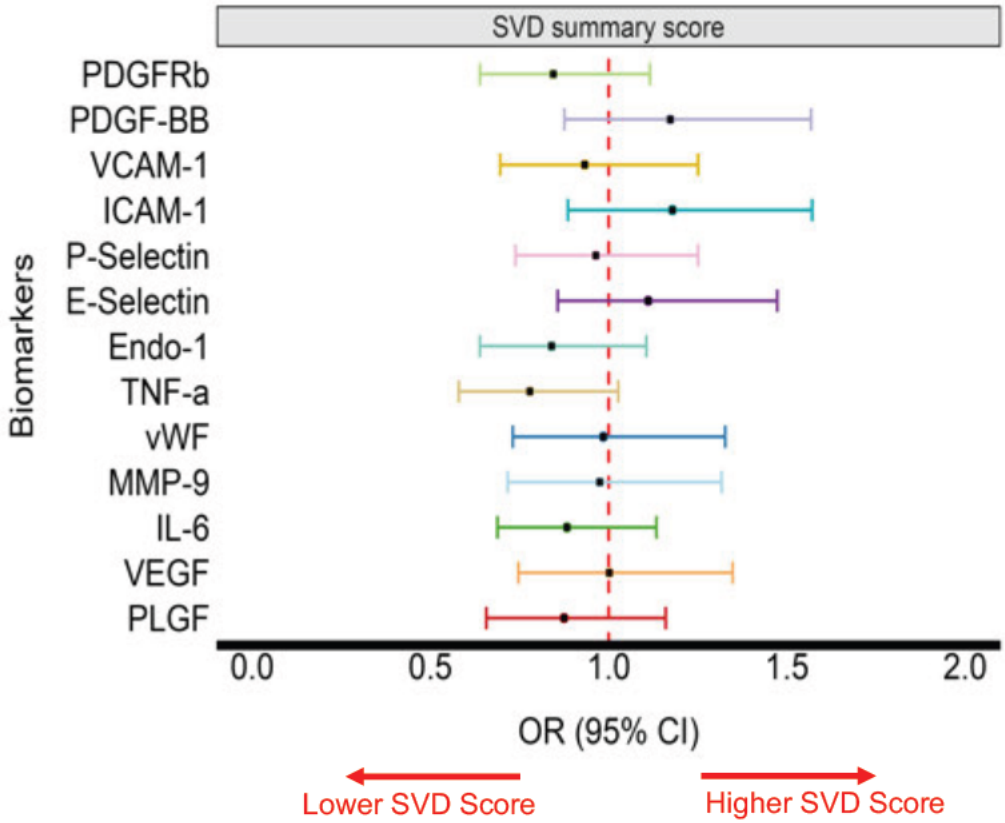

Figure D- 5. Blood biomarkers and summary SVD score. Ordinal regression results for the association of distinct standardised blood biomarkers to baseline summary SVD scores after adjusting for age, sex, vascular risk factors, and stroke subtype.

Abbreviations: SVD: small vessel disease; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

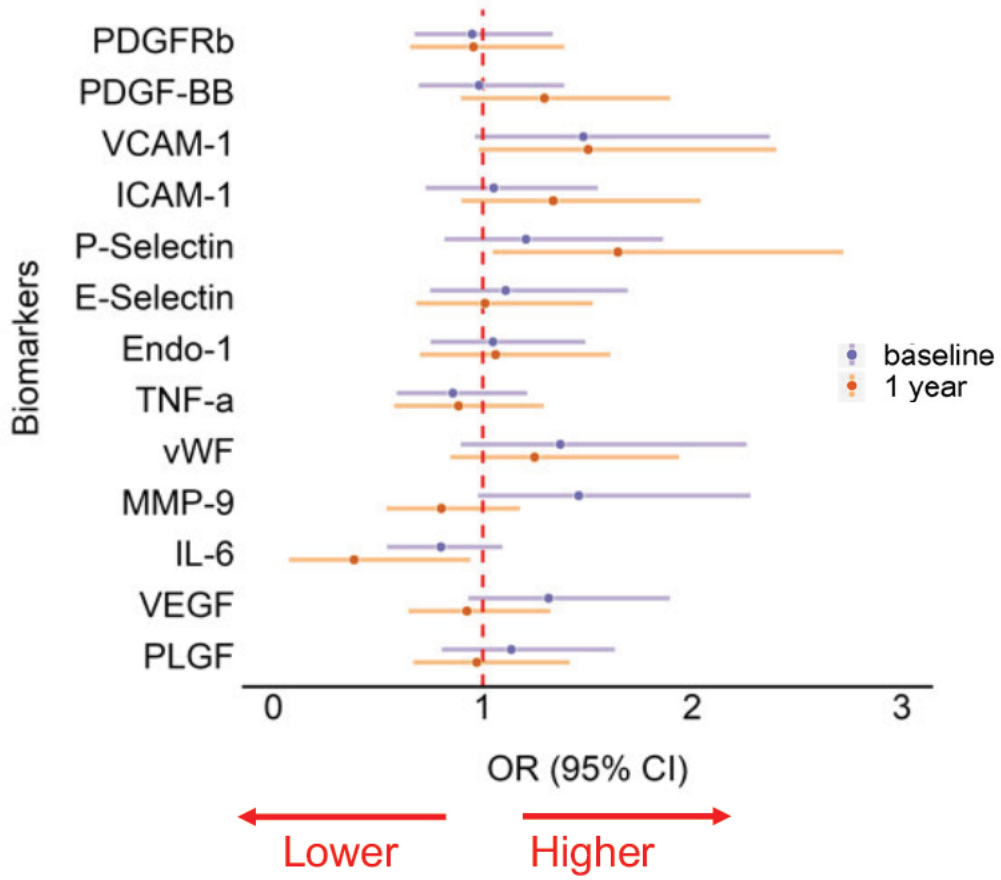

Figure D- 6. biomarkers and MCI. Logistic regression results for the association of distinct standardised blood biomarkers and MCI prevalence at baseline and one year follow-up after adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume as % ICV. One-year cross-sectional models were also adjusted for baseline MCI status.

Abbreviations: MCI: mild cognitive impairment; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

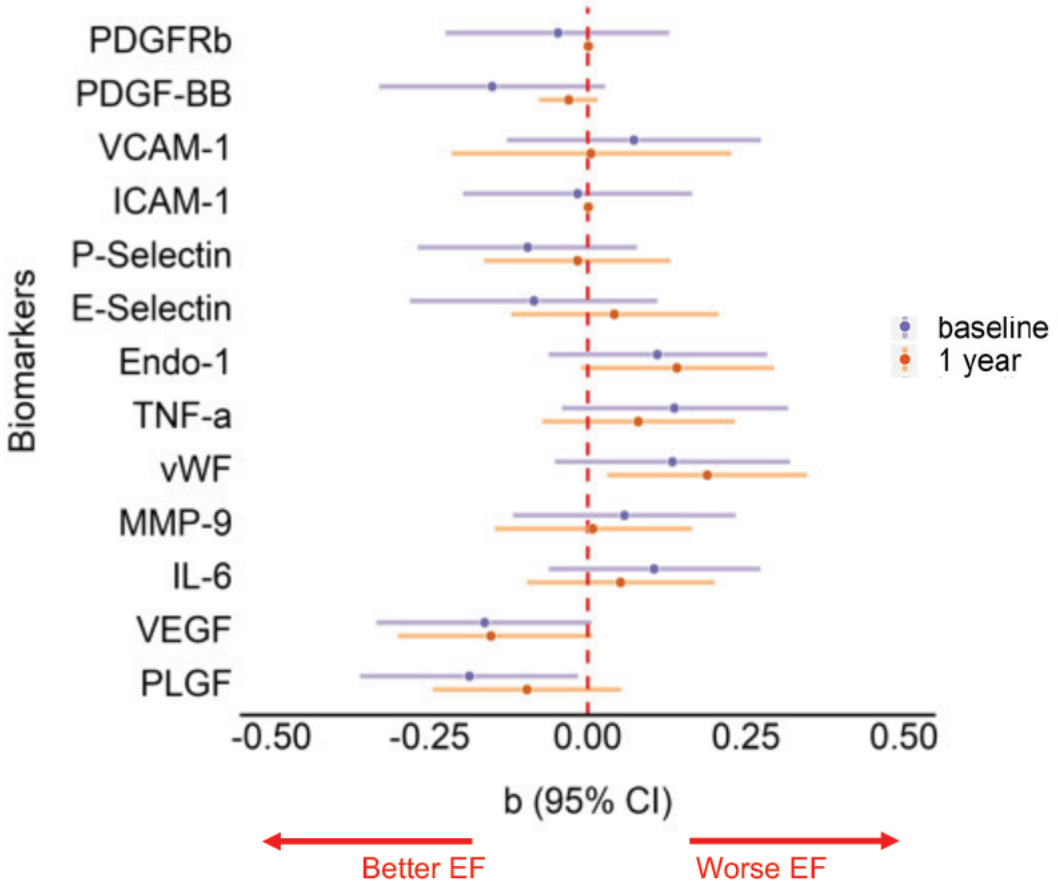

Figure D- 7. Blood biomarkers and executive function (TMT ratio B/A). Baseline cross-sectional linear regression and longitudinal linear mixed effects model results for the association of distinct standardised blood biomarkers and TMT ratio (B/A) after adjusting for age, sex, vascular risk factors, premorbid intelligence, and baseline WMH volume as % ICV.

Abbreviations: TMT: Trail Making Task; **WMH:** white matter hyperintensity; **ICV:** intracranial volume; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor; **EF:** executive function.

Table D- 16. Sensitivity analysis for DWI+ lesions with and without n=7 plasma samples.

New DWI-positive lesion				
	n=174 serum and n=7 plasma samples		***N=174 serum samples***	
	OR	**95% CI**	**OR**	**95% CI**
PDGFRβ	0.763	0.472, 1.211	0.743	0.455, 1.188
PDGF-BB	1.398	0.846, 2.328	1.252	0.723, 2.147
VCAM-1	0.971	0.569, 1.605	0.922	0.536, 1.534
ICAM-1	1.274	0.840, 1.927	1.220	0.791, 1.880
P-Selectin	1.498	0.996, 2.351	1.412	0.922, 2.212
E-Selectin	0.734	0.431, 1.142	0.734	0.431, 1.142
Endo-1	0.968	0.539, 1.603	0.935	0.519, 1.552
TNF-α	0.928	0.519, 1.446	0.934	0.529, 1.445
vWF	1.063	0.672, 1.602	1.013	0.635, 1.535
MMP-9	1.148	0.678, 1.842	1.148	0.678, 1.842
IL-6	0.881	0.333, 1.359	0.860	0.311, 1.339
VEGF	1.643	1.040, 2.624	1.560	0.99, 2.505
PLGF	1.335	0.826, 2.124	1.334	0.831, 2.114

Abbreviations: DWI: Diffusion-weighted imaging; **OR:** odds ratio; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

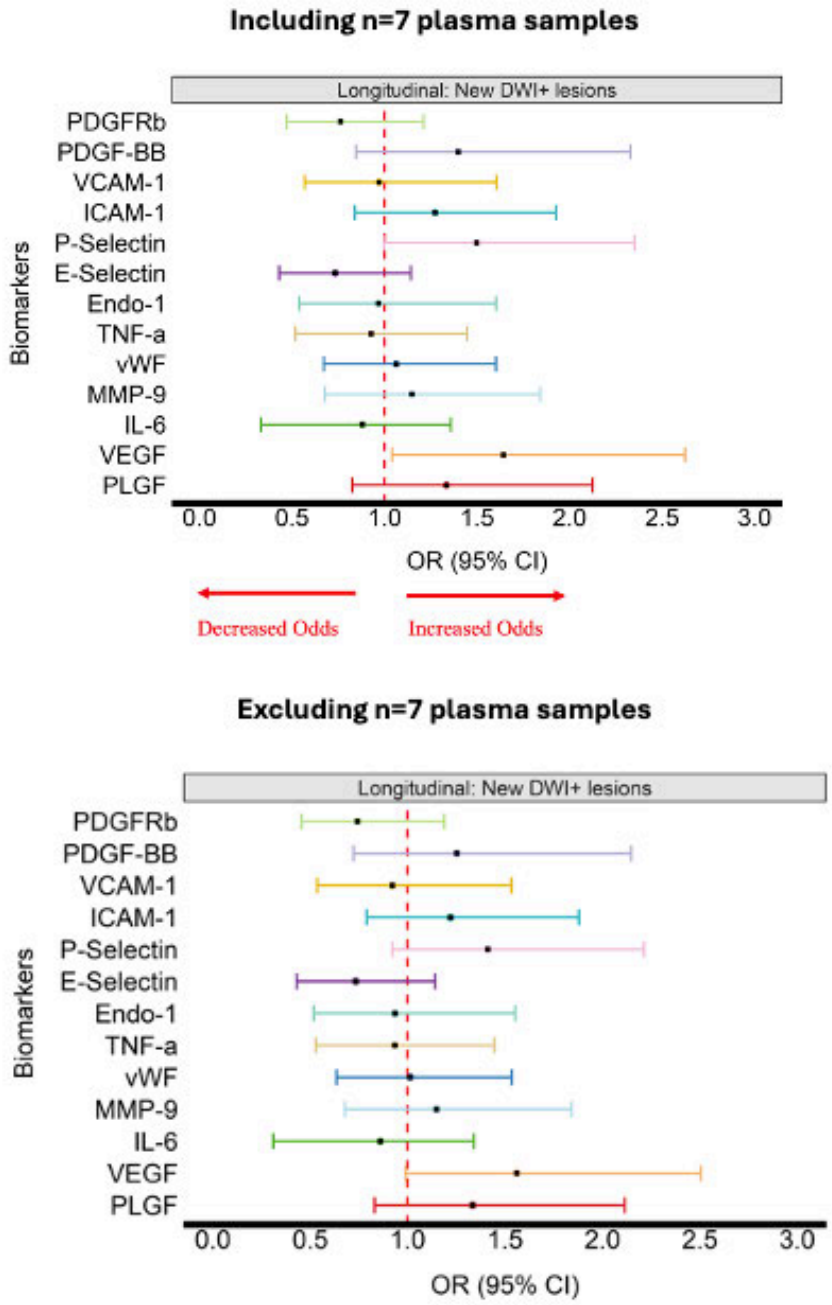

Figure D- 8. Sensitivity analysis for DWI+ lesions with and without n=7 plasma samples.

Abbreviations: DWI: Diffusion-weighted imaging; **OR:** odds ratio; **CI:** confidence interval; **PDGFRβ:** platelet derived growth factor receptor beta; **PDGF-BB:** platelet derived growth factor subunit B; **VCAM-1:** vascular cell

adhesion molecule 1; **ICAM-1:** intracellular adhesion molecule 1; **E-selectin:** endothelial-selectin; **P-Selectin:** platelet-selectin; **Endo-1:** Endothelin-1; **TNF-α:** tumour necrosis factor alpha; **vWF:** von Willebrand Factor; **MMP-9:** matrix metalloproteinase 9; **IL-6:** Interleukin 6; **VEGF:** vascular endothelial growth factor; **PLGF:** placental growth factor.

Supplemental Appendix A. Blood biomarker ELISA protocol, kits, and dilutions

PDGFRβ

Kit: Abcam ab252357. Lot number: 2101037333

Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5 and x25 dilutions. From this it was decided that serum samples would all be tested at x10 dilution, and plasma samples at x4 dilution.

Samples were tested as described; some samples required a re-test. Serum samples that were re-tested were performed at x10 and x100 dilutions, plasma samples at x20 and x50 dilutions. There was no provided control to perform an inter-assay % CV. The intra-assay % CV was calculated as 3.12%.

PDGF-BB

Kit: Abcam ab100624. Lot number: 1010637-1

Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5, x50 and x500 dilutions. From this it was decided that serum samples would all be tested at x100 dilution, and plasma samples at x20 dilution.

Samples were tested as described, and no re-tests were required. There was no provided control to perform an inter-assay % CV. The intra-assay % CV was calculated as 1.98%.

VCAM-1

Kit: Abcam ab223591. Lot number: 2101036376

Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5, x50 and x500 dilutions. From this it was decided that all samples would be tested at x500 dilution.

Samples were tested as described; some samples required a re-test. Samples that were re-tested were performed at x500, x1000, x2000 and x4000 dilutions. There was no provided control to perform an inter-assay % CV. The intra-assay % CV was calculated as 5.90%.

ICAM-1

Kit: R&D DY720. Lot number: P357734

Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5, x50 and x500 dilutions. From this it was decided that all samples would be tested at x500 dilution.

Samples were tested as described, and no re-tests were required. There was no provided control to perform an inter-assay % CV. The intra-assay % CV was calculated as 3.88%.

P-Selectin

Kit: R&D DPSE00. Lot number: P357395

Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at x20, x60 and x120 dilutions. From this it was decided that all samples would be tested at x20 dilution.

Samples were tested as described, and no re-tests were required. A control was provided with the kit, the inter-assay % CV was calculated as 15.63%. The intra-assay % CV was calculated as 3.84%.

E-Selectin

Kit: R&D DSLE00. Lot number: P367030

Controls: QC236. Lot number: P314314

Initial testing: 3 samples (3xserum; kit not suitable for plasma samples) were tested on the kit at neat, x10 and x100 dilutions. From this it was decided that all samples would be tested at x10 dilution.

Samples were tested as described; some samples required a re-test. Samples that were re-tested were performed at x10 and x100 dilutions. The inter-assay % CV was calculated as 8.08%. The intra-assay % CV was calculated as 3.75%.

Endothelin-1

Kit: R&D DET100. Lot number: P357232

Controls: QC82. Lot numbers: 1663979, 1663980, 1663982 (1 per control level)
Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5, x50 and x500 dilutions. From this it was decided that all samples would be tested neat.
Samples were tested as described, and no re-tests were required. The inter-assay % CV was calculated as 11.21%. The intra-assay % CV was calculated as 16.73%.

TNF-α

Kit: R&D HSTA00E. Lot number: P348076
Controls: QC232. Lot number: P335817
Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5, x50 and x500 dilutions. From this it was decided that all samples would be tested neat.
Samples were tested as described, and no re-tests were required. The inter-assay % CV was calculated as 13.77%. The intra-assay % CV was calculated as 7.06%.

vWF

Kit: Abcam ab108918. Lot number: 1011742-2
Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x10, x100 and x400 dilutions. From this it was decided that all samples would be tested at x100 dilution.
Samples were tested as described; some samples required a re-test. Samples that were re-tested were performed at x100, x200 and x600 dilutions. There was no provided control to perform an inter-assay % CV. The intra-assay % CV was calculated as 6.79%.

MMP-9

Kit: R&D DMP900. Lot number: P363877
Controls: QC130. Lot number: P319142

Initial testing: 3 samples (3xserum; kit not suitable for plasma samples) were tested on the kit at neat, x10 and x400 dilutions. From this it was decided that all samples would be tested at x100 dilution.
Samples were tested as described; some samples required a re-test. Samples that were re-tested were performed at x100, x200 and x600 dilutions. The inter-assay % CV was calculated as 3.68%. The intra-assay % CV was calculated as 5.48%.

IL-6

Kit: R&D HS600C. Lot number: P355671
Controls: QC246. Lot number: P357243
Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5, x50 and x500 dilutions. From this it was decided that all samples would be tested neat.
Samples were tested as described; some samples required a re-test. Samples that were re-tested were performed at x5 and x50 dilutions. The inter-assay % CV was calculated as 15.71%. The intra-assay % CV was calculated as 10.10%.

VEGF

Kit: R&D DVE00. Lot number: P350540
Controls: QC01-1. Lot number: P343594
Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x5, x50 and x500 dilutions. From this it was decided that all samples would be tested neat.
Samples were tested as described, and no re-tests were required. The inter-assay % CV was calculated as 39.68%. The intra-assay % CV was calculated as 13.26%.

PIGF

Kit: R&D HSPG00. Lot number: P382640
Controls: QC144

Initial testing: 4 samples (3xserum, 1xplasma) were tested on the kit at neat, x10 and 100 dilutions. From this it was decided that all samples would be tested neat.

Samples were tested as described, and no re-tests were required. The inter-assay % CV was calculated as 11.35%. The intra-assay % CV was calculated as 12.31%.

Appendix E. Supplemental material to Chapter 6

Table E-1. Baseline cohort characteristics: participants with and without clinical outcome follow-up at 7-9 years. *Abbreviations*: NIHSS: National Institutes of Health Stroke Scale; NART: National Adult Reading Test; ACE-R: Addenbrooke's Cognitive Examination revised; SVD: small vessel disease; WMH: white matter hyperintensity; ICV: intracranial volume.

	no (N=21)	yes (N=243)	overall (N=264)	result (p-value)
Age, years, mean (SD)	68 (±13)	67 (±12)	67 (±12)	-5.383, 6.890 (0.80)†
Sex, female, n (%)	9 (43%)	101 (42%)	110 (42%)	0 (1)°
Modified Rankin Scale, n (%)				2505.5 (0.88)*
No symptoms at all	6 (29%)	45 (19%)	51 (19%)	-
No significant disability	6 (29%)	100 (41%)	106 (40%)	-
Slight disability	3 (14%)	45 (19%)	48 (18%)	-
Moderate disability	5 (24%)	44 (18%)	49 (19%)	-
Moderate severe disability	1 (5%)	8 (3%)	9 (3%)	-
Bedridden	0 (0%)	1 (0%)	1 (0%)	-
NIHSS, median (IQR)	0 (±1)	1 (±2)	1 (±2)	1942 (0.06)*
Diabetes, yes, n (%)	2 (10%)	28 (12%)	30 (11%)	5.39e-29 (1)°
Hypertension, yes, n (%)	14 (67%)	177 (73%)	191 (72%)	0.124 (0.72)°
Hypercholesterolaemia, yes, n (%)	13 (62%)	148 (61%)	161 (61%)	5.68e-30 (1)°
Atrial Fibrillation, yes, n (%)	5 (24%)	20 (8%)	25 (9%)	3.806 (0.05)°
Current smoker or ex-smoker <1 year ago, n (%)	9 (43%)	93 (38%)	102 (39%)	0.032 (0.85)°
NART score, mean (SD)	38 (±11)	35 (±10)	35 (±10)	-4.442, 11.278 (0.35)†
Missing, n (%)	11 (52.4%)	99 (40.7%)	110 (41.7%)	-

	no (N=21)	yes (N=243)	overall (N=264)	result (p-value)
ACE-R score, mean (SD)	95 (±3.3)	88 (±8.2)	88 (±8.2)	4.206, 9.487 (<0.001)†
Missing, n (%)	11 (52.4%)	96 (39.5%)	107 (40.5%)	-
Summary SVD score, median (IQR)	3 (±2)	2 (±2)	2 (±2)	-0.134, 0.875 (0.14)†
WMH volume (mm3 as % ICV), mean (SD)	1.7 (±1.4)	1.5 (±1.7)	1.5 (±1.6)	-0.488, 0.863 (0.57)†

*:Mann-Whitney U test (W); °:Chi-square (X2); †: independent samples t-test (95% CI).

Table E-2. Baseline cohort characteristics: participants with and without available functional and subjective well-being questionnaire at 7-9 year follow-up. ***Abbreviations*****: NIHSS: National Institutes of Health Stroke Scale; NART: National Adult Reading Test; ACE-R: Addenbrooke's Cognitive Examination revised; SVD: small vessel disease; WMH: white matter hyperintensity; ICV: intracranial volume.**

	no (N=168)	yes (N=96)	overall (N=264)	result (p-value)
Age, years, mean (SD)	68 (±12)	65 (±11)	67 (±12)	0.542, 6.246 (0.02)†
Sex, female, n (%)	72 (43%)	38 (40%)	110 (42%)	0.151 (0.69)°
Modified Rankin Scale, n (%)				8426.5 (0.52)*
No symptoms at all	34 (20%)	17 (18%)	51 (19%)	-
No significant disability	62 (37%)	44 (46%)	106 (40%)	-
Slight disability	31 (18%)	17 (18%)	48 (18%)	-
Moderate disability	34 (20%)	15 (16%)	49 (19%)	-
Moderate severe disability	7 (4%)	2 (2%)	9 (3%)	-
Bedridden	0 (0%)	1 (1%)	1 (0%)	-
NIHSS, median (IQR)	1.1 (±1.3)	1.1 (±1.1)	1.1 (±1.2)	7920.5 (0.80)*
Diabetes, yes, n (%)	20 (12%)	10 (10%)	30 (11%)	0.027 (0.86)°
Hypertension, yes, n (%)	122 (73%)	69 (72%)	191 (72%)	0 (1)°
Hypercholesterolaemia, yes, n (%)	104 (62%)	57 (59%)	161 (61%)	0.075 (0.78)°
Atrial Fibrillation, yes, n (%)	20 (12%)	5 (5%)	25 (9%)	2.462 (0.11)°
Current smoker or ex-smoker <1 year ago, n (%)	72 (43%)	30 (31%)	102 (39%)	2.999 (0.08)°
NART score, mean (SD)	34 (± 10)	36 (± 10)	35 (± 10)	-5.638, 1.139 (0.191)†

	no (N=168)	yes (N=96)	overall (N=264)	result (p-value)
Missing, n (%)	73 (43.5%)	37 (38.5%)	110 (41.7 %)	-
ACE-R score, mean (SD)	87 (±8.8)	90 (±6.5)	88 (±8.2)	-6.028, -1.181 (0.003)†
Missing, n (%)	71 (42.3%)	36 (37.5%)	107 (40.5 %)	-
Summary SVD score, median (IQR)	2.5 (±1.2)	2.0 (±1.1)	2.3 (±1.2)	0.204, 0.786 (0.00)†
WMH volume (mm3 as % ICV), mean (SD)	1.9 (±1.8)	1.0 (±1.1)	1.5 (±1.6)	0.498, 1.195 (0.00)†

*:Mann-Whitney U test (W); °:Chi-square (X2); †: independent samples t-test (95% CI).

Table E- 3. Recurrent stroke and death amongst participants with lacunar and cortical stroke at 1-, 3-, and 7-9-year follow-up

	0 to 1 year *n=243*		*1 to 3 years* *n=243*		*3 to 7-9 years* *n=243*		*Total*	
	Cortical ***n=137***	***Lacunar*** ***n=106***	***Cortical*** ***n=137***	***Lacunar*** ***n=106***	***Cortical*** ***n=137***	***Lacunar*** ***n=106***	***Cortical*** ***n=137***	***Lacunar*** ***n=106***
Recurrent stroke, n (%)	16 (11.68%)	11 (10.38%)	10 (7.3%)	12 (11.32%)	28 (20.44%)	14 (13.21%)	44 (32.12%)	33 (31.13%)
Death,, n (%)	7 (5.11%)	4 (3.77%)	10 (7.3%)	2 (1.89%)	26 (18.98%)	15 (14.15%)	43 (31.39%)	21 (19.81%)

* The total column represents the total number of participants. Note that for recurrent stroke, some participants experienced additional events across multiple time points.

Table E-4. Lacunar and cortical stroke participants with diagnosed hypertension, diabetes, hypercholesterolemia, and/or atrial fibrillation at baseline, along with new diagnoses acquired between baseline and the 7-9-year follow-up.

	Baseline n=243		***1 year n=239***		***3 years n=112***		***7-9 years n=96***		***Total***		
	Cortical n=137	*Lacunar n=106*	*Cortical n=135*	*Lacunar n=104*	*Cortical n=63*	*Lacunar n=49*	*Cortical n=50*	*Lacunar n=46*	*Cortical n=137*	*Lacunar n=106*	*Result X^2 (p-value)*
Hypertension, yes, n (%)	101 (74)	76 (71.7)	12 (8.88)	14 (13.5)	0 (0)	4 (8.2)	1 (2)	0 (0)	114 (83.2)	94 (88.7)	1.039 (0.307)
Diabetes, yes, n (%)	17 (12.4)	11 (10.4)	1 (0.7)	2 (1.9)	1 (1.6)	7 (14.3)	2 (4)	3 (6.52)	21 (15)	23 (22)	1.233 (0.266)
Hypercholesterolaemia, yes, n (%)	82 (59.9)	66 (62.3)	12 (8.9)	12 (11.5)	3 (4.8)	9 (18.4)	4 (8)	2 (4.3)	101 (73.7)	89 (84)	2.474 (0.115)
Atrial Fibrillation, yes, n (%)	14 (10.2)	6 (5.6)	10 (7.4)	6 (5.8)	6 (9.5)	4 (8.2)	7(14)	6 (13)	37 (27)	22 (20.8)	0.953 (0.328)

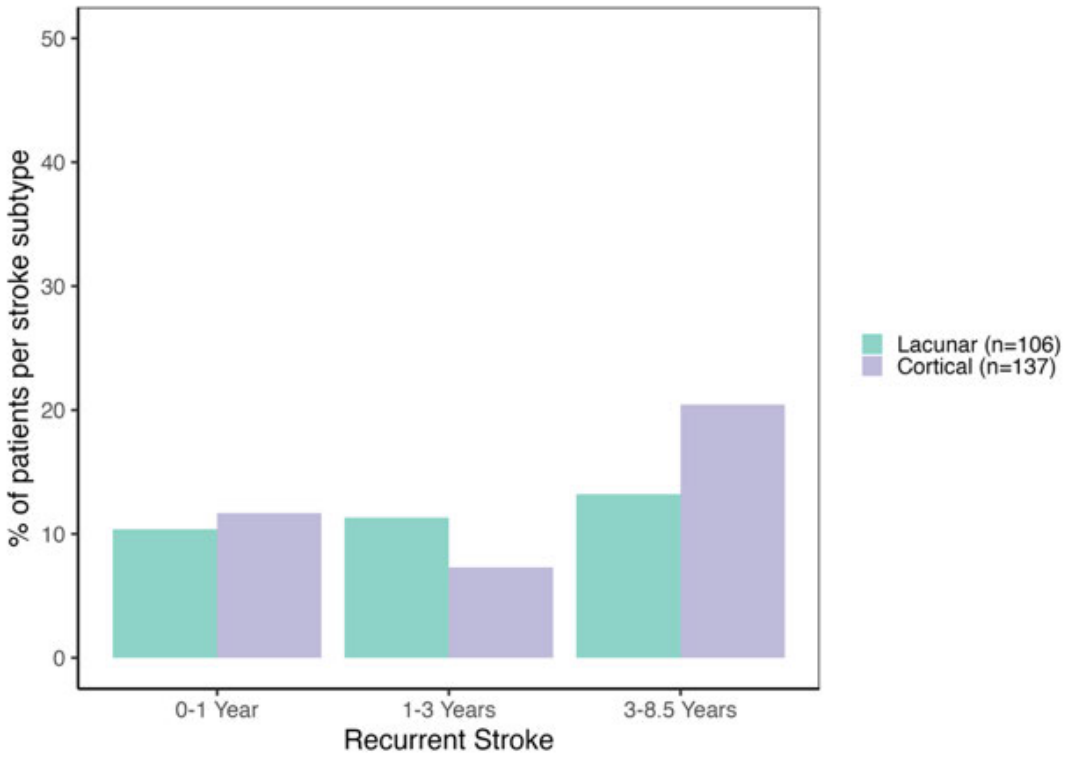

Figure E-1. Recurrent stroke incidence amongst lacunar and cortical stroke participants at 0 to 1, 1 to 3, and 3 to 7-9 years.

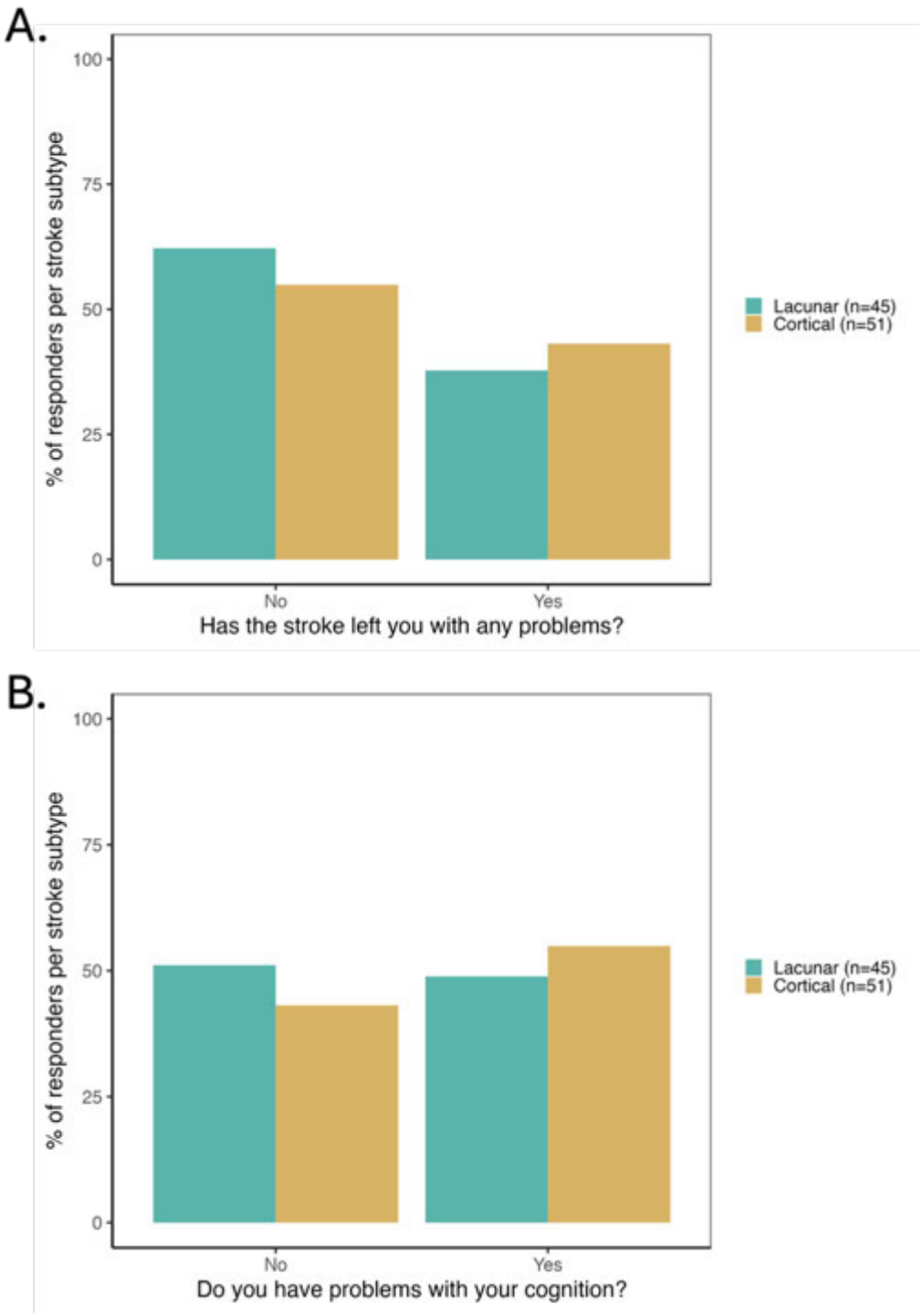

Figure E-2. Proportion of lacunar and cortical stroke participants who reported lingering problems or subjective cognitive concerns 7-9-years after their index stroke (n=96).

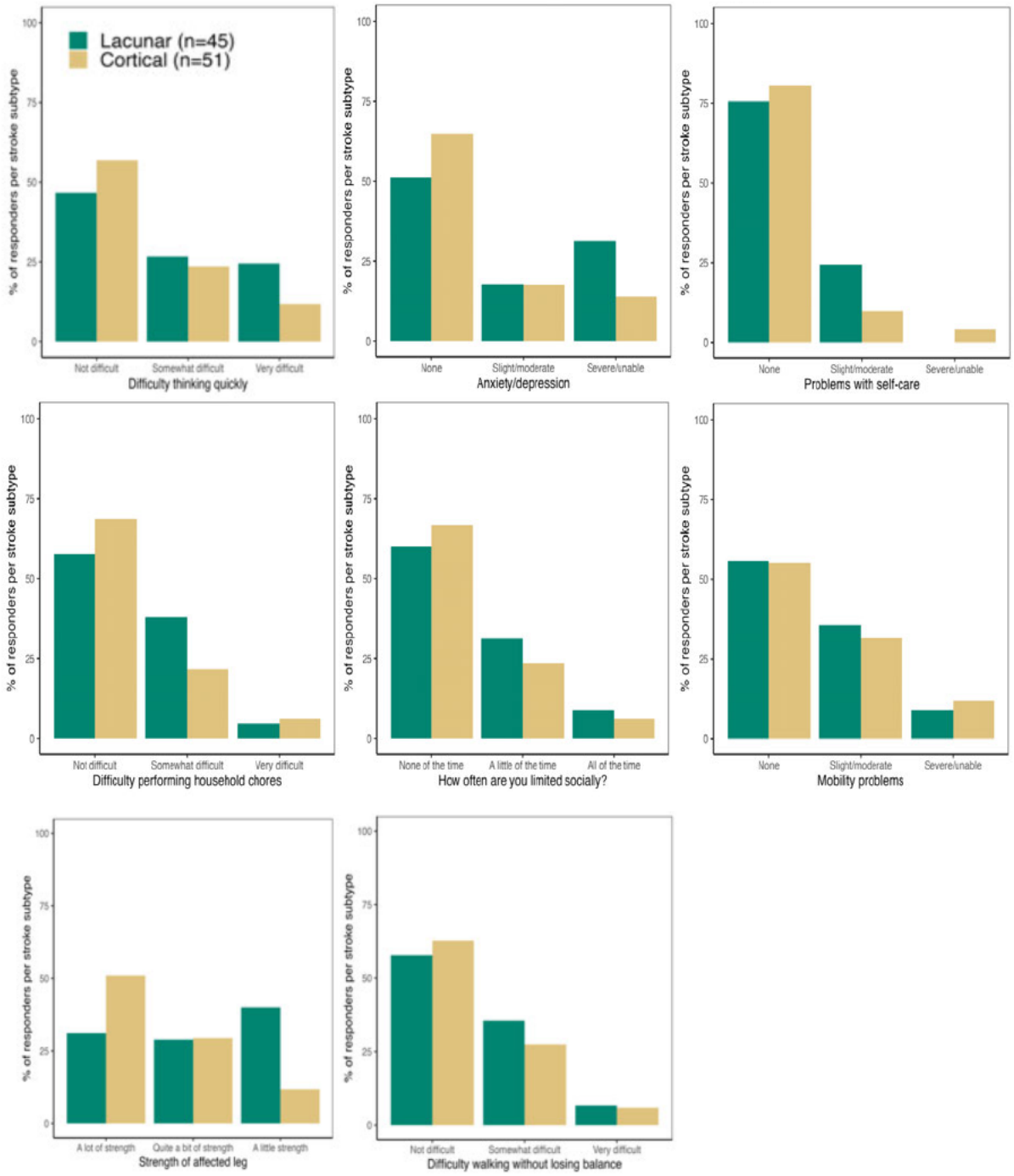

Figure E-3. Self-reported functional, neuropsychiatric, and subjective well-being outcomes at 7-9 years post- lacunar or mild cortical ischaemic stroke (n=96).