

1 **Morphometry of the hippocampal microvasculature in post-stroke and age-**
2 **related dementias**

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11 **Running title:** Neurovascular atrophy and dementia

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1 **Abstract**

2 Background: Optimal vascular function is vital for prevention of dementia. We
3 hypothesised that elderly post-stroke (PS) survivors who maintain cognitive function
4 (PSND) show unperturbed cerebral microvasculature, compared to those who become
5 demented (PSD) or acquire other dementias.

6 Methods: Using stereological spherical probe software, we compared changes in length
7 density (L_v , cumulative vessel length per unit volume of tissue) of hippocampal
8 microvasculature in post-mortem brain tissue from PS survivors, Alzheimer's disease
9 (AD), vascular dementia (VaD) and normal ageing control subjects. In addition, we
10 assessed microvessel diameter in the same subjects. Microvasculature was identified by
11 markers of endothelial cells (glucose transporter 1; GLUT1), basement membrane
12 (collagen IV; COL4) and smooth muscle cells α -actin (SMA).

13 Results: We found an increase in L_v of both GLUT1 and COL4 immunostained microvessels
14 ($p \leq 0.05$) in PSD and AD cases, compared to controls, PSND and VaD in the CA1. However,
15 no changes were apparent in the CA2 region. Additional analysis revealed a significant
16 increase in L_v in AD cases, compared to PSND and PSD, in the entorhinal cortex.
17 Microvessel mean diameter was decreased in PSD, compared to PSND, as well as in AD
18 and VaD, compared to controls. Cumulative frequency analysis of microvessel diameter
19 showed that the PSND had a significantly greater proportion of vessels with diameters,
20 ranging between 7 and 12 μm .

21 Conclusions: An increase in microvascular L_v in AD and PSD suggests either an increase in
22 angiogenesis or the formation of newer microvessel loops in response to cerebral
23 hypoperfusion. A decrease in vessel diameter was also found in AD and VaD, suggesting
24 increased vasoconstriction in dementia.

25

26 **Key words:** Alzheimer's disease, hippocampus, microvessel, post-stroke dementia, stroke,
27 vascular dementia

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1 **Introduction**

2 Previous epidemiological studies have shown that vascular disease plays a key role in the
3 progression of dementia [1, 2], inclusive of cardiovascular risk factors, such as increased
4 blood pressure [3], atrial fibrillation [4], diabetes mellitus [5] and other vascular disease-
5 related factors [6]. Studies using arterial spin labelling, have shown a reduction in cerebral
6 blood flow (CBF) in the temporal lobe associated with dementia in Alzheimer's disease (AD)
7 [7] and in post-stroke (PS) survivors [8]. The decrease in CBF was believed to have been as a
8 result of neurodegeneration in dementia, whereby the cerebral microvasculature is
9 restricted or modulated due to lower demand for glucose and oxygen. However, an
10 alternative view has also been proposed, where a decrease in CBF may be the cause or
11 instigator of neurodegeneration [9]. Consistent with this hypothesis, we recently proposed
12 there is a vascular basis for neuronal atrophy and likely neurodegeneration in post-stroke
13 dementia (PSD) subjects without significant AD pathology [10].

14 The role of the cerebral microvasculature in age-related dementias still remains unclear.
15 Using different methods of assessment, previous studies in dementia have suggested
16 microvessel density, is decreased in dementia, especially in AD, [11-13], whereas others
17 claim it is increased [14-16]. Some investigators have also demonstrated narrowing of
18 microvessels in the CA1 of the hippocampus and entorhinal cortex (EC) of AD subjects [14,
19 17]. Differences in the methods of analysis and brain regions investigated may explain the
20 variable results. However, studies in AD have indicated that there is an up-regulation or
21 modulation of pro-angiogenic proteins and vascular growth factors [18], which may increase
22 the density of the microvasculature [19-21]. Studies involving transgenic (Tg) mice models,
23 which over-express the amyloid precursor protein (APP) to simulate AD pathology, have
24 reported decreased angiogenesis in the presence of amyloid β peptides [22]. Whilst a study
25 using the Tg2576 mouse model found increased microvessel density and extensive
26 disruption to tight junctions, leading to a new hypothesis of amyloidogenesis [23].
27 Additionally, a corrosion cast model of vasculature in an AD mouse model has suggested an
28 increase in density, as well as degeneration, of vessels [24]. Conversely, other studies, in an
29 aged APP/PS1 mouse model, have shown no change in microvessel density [25].

1 To clarify these issues, we examined post-mortem tissue taken from the hippocampal
2 formation to assess whether microvascular morphology, specifically length density (L_v) and
3 diameter, was affected in different dementias, placing particular emphasis on PSD. The CA1
4 subfield was assessed due to its importance in relation to the onset of dementia and specific
5 susceptibility to both increased AD-like pathology, risk of ischaemia and hypoperfusion [26].
6 L_v measurements were conducted using a spherical probe [27] and the diameter of cerebral
7 microvessels were assessed using software that had been developed to measure vessel
8 diameter and perivascular space [28]. It is hypothesised that PSD cases will exhibit reduced
9 microvascular density and diameter, compared to post-stroke non-demented (PSND).
10 Preliminary analysis from this study was presented in abstract form at the 112th British
11 Neuropathological Society Meeting, January 2011 [29].

12

13 **Materials and methods**

14 ***Subjects and clinical features***

15 Post-mortem brain tissue was obtained from PSD and PSND subjects [30]. AD and vascular
16 dementia (VaD) subjects and similar age controls were included for comparison. The
17 demographic details of the different subjects are presented in Table 1. The post-stroke
18 subjects were enrolled in the prospective Cognitive Function After Stroke (CogFAST) study
19 [30, 31]. Stroke patients aged ≥ 75 years were selected on the basis that they were not
20 demented three months post stroke and did not exhibit disabilities that would prevent them
21 from completing cognitive testing. They received annual clinical assessments and a
22 neuropsychological test battery from baseline, including the Cognitive Drug Research (CDR)
23 battery, the Mini-Mental State Exam (MMSE) and the Cambridge Assessment of Mental
24 Disorders in the Elderly (CAMCOG), which generated sub-scores for various cognitive
25 domains, including memory and executive function [30, 32].

26 Subjects were classified as demented if they met DSM-III-R criteria for dementia.
27 Controls aged >75 years were only selected if they had not been diagnosed clinically with
28 cognitive impairment. There was no significant difference between the groups in average
29 survival time (59.4 months) post ischaemic injury event. Ethical approval was granted by

1 local research ethics committees for this study (Newcastle upon Tyne Hospitals Trust, UK)
2 and permission for post-mortem research using brain tissue was granted for this project. All
3 tissue was obtained from the Newcastle Brain Tissue Resource.

4 ***Neuropathological examination***

5 Final classification of demented subjects was assigned based on established
6 neuropathological diagnostic criteria [33]. Briefly, haematoxylin-eosin staining was used for
7 assessment of structural integrity and infarcts, Nissl and luxol fast blue staining for cellular
8 pattern and myelin loss, Bielschowsky's silver impregnation for CERAD rating of neuritic
9 plaques, and tau immunohistochemistry for Braak staging of neurofibrillary tangles. A
10 diagnosis of VaD was made when there were multiple or cystic infarcts, lacunae,
11 microinfarcts and small vessel disease, and Braak stage <III [33]. A diagnosis of AD was
12 confirmed on evidence of significant Alzheimer's- type pathology, namely a Braak stage V-VI
13 score, a moderate-severe CERAD score and an absence of significant vascular pathology.
14 Thal staging [34] was also performed: the hippocampal formation and medial temporal lobe
15 were stained for amyloid (4G8 antibody) and each case was graded, dependent on staging
16 criteria. Vascular pathology scores were derived from the presence of vascular lesions in
17 brain areas, including the frontal lobe at the level of the olfactory bulbs, temporal lobe at
18 level of the anterior hippocampus, and basal ganglia at level of mamillary body. Lesions
19 including arteriolosclerosis, cerebral amyloid angiopathy, perivascular haemosiderin
20 leakage, perivascular space dilatation in the deep and juxtacortical white matter (WM),
21 myelin loss, and cortical micro (<0.5 cm) and large (>0.5 cm) infarcts were recorded with
22 increasing severity resulting in greater scores [35]. Control subject tissue was determined
23 not to have had sufficient pathology to reach threshold to ascertain a diagnosis for
24 dementia (all pathological scores are shown in Table 1)

25 ***Immunohistochemistry***

26 Paraffin wax-embedded human hippocampal blocks were selected based upon their
27 proximity to a specific plane, adjacent to the anterior pole of the lateral geniculate body in
28 the coronal plane, within the posterior section of the hippocampus. Due to limited access to
29 tissue, as a result of working in the confines of a brain bank environment, *only one block per*
30 *case* was available for analysis. For stereological analysis, 15 30- μ m-thick serial sections

1 were cut from the front of the block and mounted onto Superfrost+ slides (Fisher Scientific,
2 UK). Every fifth section was then chosen so that three sections per case were selected for
3 uniform random sampling prior to staining with each of the markers for
4 immunohistochemistry: glucose transporter 1 (GLUT1), collagen IV (COL4) and smooth
5 muscle α -actin (SMA). Ten- μ m-thick sections were used for vessel diameter analysis and
6 mounted on 2% 3-aminopropyltriethoxysilane (APES) slides before being stained using a
7 standard immunohistochemical technique for COL4.

8 In the initial preliminary study, serially cut 10- μ m-thick hippocampal sections taken
9 from all dementia groups and controls (Table 1) were used to perform standard two-
10 dimensional analysis of the microvasculature and labelled with the same endothelial and
11 basement membrane markers.

12 Antigen heat retrieval was conducted by placing the 30- μ m-thick sections in boiling
13 0.1M citrate buffer for 10-15 min. The primary antibodies used were as follows: GLUT1
14 (ThermoScientific, UK, 1:200), COL4 (Sigma Aldrich, UK, 1:500) and SMA (Sigma Aldrich, UK,
15 1:1000). A protease antigen retrieval step was performed in the 10- μ m-thick sections
16 immunostained for COL4 by treating with 0.6% subtilisin A, type VIII bacterial protease
17 (Sigma, UK) solution for 10 mins at room temperature. Appropriate secondary antibodies
18 were used, followed by incubation with Vectastain Avidin/Biotinylated Complex (ABC) to
19 increase sensitivity of staining (Vector Labs, UK). GLUT1, SMA and COL4 (10 μ m) were
20 visualised using 3,3'-diaminobenzidine and COL4 (30 μ m) with Vector SG and counterstained
21 with haematoxylin (Figure 1).

22 ***Stereological and image analysis: length density and vessel diameter***

23 Initially, standard two-dimensional analysis was undertaken to assess trends in
24 microvascular changes within various dementia groups [36]. However, to strengthen the
25 preliminary findings and discern the three-dimensional aspect of the microvasculature, an
26 adapted stereological protocol was used.

27 The operators (MB, hippocampus and LN, EC) performed analysis blindly in order to restrict
28 operator bias. Using the Stereologer2000 software (Stereologer, WV, USA), a spherical
29 probe 'space ball' option was selected to measure L_v of microvasculature [27]. The operating

1 system was connected to a Zeiss Axiolab microscope with a motorised stage (Prior Scientific,
2 UK).

3 In this study, a spherical probe with a diameter of 18 μm was selected to allow for
4 section shrinkage and an appropriate guard volume. An outline was drawn denoting the
5 area of interest, which corresponded to the relevant hippocampal subfield or cortical region
6 at low magnification (x5). Neuronal subfields were visualised with the aid of haematoxylin
7 counter staining. A digitally generated, equally spaced grid was overlaid and used to ensure
8 random sampling within x and y axis of the reference area. A pilot study was performed to
9 determine the number of frames required to reduce the sampling coefficient of error (CE) to
10 a satisfactory level. Such calculations were based on the density of vessels in a particular
11 region, with the probe size and distance between probes altered accordingly. L_v was
12 calculated by counting the number of intersections between the probe and the parameter –
13 in this instance microvasculature (ΣQ), and the area of sampling probe (ΣA) ($L_v = 2(\Sigma Q / \Sigma A)$)
14 at x100 magnification (Mouton et al., 2002). The number of intersections was used to
15 estimate the L_v for each case. As stated earlier, a lack of tissue availability prevented the full
16 sampling of the region of interest, thus precluding the measurement of volume of the
17 hippocampal structures and the subsequent calculation of total length using L_v estimates.

18 Images for vessel diameter analysis were taken from 10- μm -thick sections stained
19 with COL4 at x40 magnification using a Zeiss AX10 research-grade microscope from the CA1
20 region. Approximately 30 images were taken at random across the area of interest in each
21 case. Analysis of the vessel diameter was determined using software developed to measure
22 vessel diameter. The software was calibrated by measuring a known length from a graticule
23 at the selected magnification of analysis [28]. Length was calculated by using digital
24 generated lines drawn by the operator across the subject matter of interest. Each vessel was
25 measured three times and the average from these measurements was recorded as the
26 mean vessel diameter (Figure 1B).

27 **Statistical analysis**

28 Statistical analysis was carried out using IBM SPSS software (version 19.0). Significance was
29 determined at $p \leq 0.05$. The Shapiro-Wilk test was used to test for normality of stereology
30 data, parametric tests were used to analyse hippocampal data, and non-parametric tests

1 were used for EC data. Group means were compared using an ANOVA and Tukey post-hoc
2 test, or Kruskal-Wallis and Mann-Whitney U test. Correlations were assessed using
3 Pearson's correlation. The Komolgrov-Smirnof test was used to assess the normality of
4 vessel diameter data, as collective data was analysed from each group. Non-parametric
5 Kruskal-Wallis test was used to analyse data and the Mann-Whitney U test was used to
6 establish significance between groups. Cumulative frequency analysis was performed of
7 increasing vessel diameter to determine if subpopulations of vessels were responsible for
8 changes in mean. Comparisons between group cumulative frequencies and power analyses
9 were carried out using Multitab 16 Statistical Software (Multitab Inc, USA).

10 **Results**

11 ***Neuropathological findings***

12 There were no significant differences in any of the pathological staging results between the
13 PSND and PSD cases. Significant increases ($P \leq 0.05$) were apparent between the AD group
14 and other disease groups for Braak stages, CERAD rating and Thal stages. The maximum Thal
15 score that could be assigned to an individual case was stage four, due to the hippocampal
16 formation and EC being the only regions assessed (Table 1).

17 ***Length density assessment***

18 Initial assessment of hippocampal microvasculature was conducted using standard two-
19 dimensional image analysis with ImagePro 4.0 software (Mediacybernetics, USA). 10- μ m-
20 thick sections were stained with GLUT1 and the data was represented as a percentage per
21 area of staining in a total of 78 samples in all groups (Table 1). Significant increases in
22 percentage per area were found in GLUT1 density in AD cases in the CA1, compared to PSND
23 and PSD ($p=0.011$ and 0.037 , respectively). A trend towards a significant decrease in GLUT1
24 expression was found in PSND cases, compared to controls ($p=0.073$). A significant increase
25 was also found in COL4 percentage per area in AD cases, compared to controls and VaD
26 ($p=0.024$ and $p=0.05$, respectively). The only significant difference in CA2 was found with
27 GLUT1, with an increase in AD compared to VaD ($p=0.04$). Based on these preliminary data,
28 we wished to address issues surrounding microvascular orientation, stemming from the use

1 of two-dimensional analysis, which may have led to inaccurate conclusions. A modified
2 stereological analysis was thus performed to estimate the microvascular L_v (Table 1).

3 The L_v results were normally distributed for all antibodies used in both the CA1 and
4 CA2 regions ($P>0.05$). GLUT1 L_v was significantly increased in the CA1 ($p \leq 0.001$, $F = 23.28$).
5 Significant increases in mean L_v in AD compared to all groups, except PSD (Figure 2A). There
6 was no significant difference in L_v in the CA2 region ($p = 0.459$, $F = 0.94$).

7 COL4 L_v followed a similar pattern to GLUT1 changes, predominantly labelling
8 capillaries (Figure 1). In the CA1, differences were apparent between groups ($p \leq 0.001$, $F =$
9 14.92). A significant increase in L_v was observed between AD, compared to controls, PSND
10 and VaD. L_v was significantly increased in PSD cases, compared to PSND and VaD. Similarly,
11 in the CA2, as with GLUT1 L_v , there were no significant differences in COL4 ($p = 0.443$, $F =$
12 0.98).

13 As expected, SMA L_v was lower in all groups, compared to other vascular markers,
14 due to its specificity for larger vessels (e.g. arterioles). Significant differences were apparent
15 between the groups in the CA1 ($p = 0.004$, $F = 5.22$), where increases were observed in AD,
16 compared to controls (AD mean $L_v = 0.118 \text{ mm/mm}^{-3}$ vs. controls mean $L_v = 0.058 \text{ mm/mm}^{-3}$,
17 $p = 0.027$) and VaD (mean $L_v = 0.0475 \text{ mm/mm}^{-3}$, $p = 0.004$). No significant difference was
18 observed in CA2 ($p = 0.959$, $F = 0.15$). SMA staining had a greater coefficient of error (CE)
19 values in all CA fields due to the relative low vessel density of arterioles. We found no
20 correlation between L_v and age of subject, post-mortem delay or length of tissue fixation.

21 The robustness of our L_v findings were verified by the strong correlation between GLUT1
22 and COL4 in the CA1 ($r^2 = 0.687$, $p = 0.000$). This indicated internal consistency of the results
23 obtained for individual microvascular L_v (Figure 2C). Thus, changes in L_v in the CA1 and lack
24 of those in CA2 were reflected in both microvascular elements and profiles across all groups.

25 We also performed analysis in the EC region within the same tissue sections to ascertain
26 microvascular density changes in the neocortical region connected to the hippocampal
27 formation. Given the similarity in expression between GLUT1 and COL4 found in the CA1,
28 only GLUT1 L_v was analysed in the EC. We observed increases in AD cases, compared to

1 PSND ($p=0.004$). Additionally, PSD cases had significantly lower L_v than AD ($p=0.016$). PSND
2 cases had significantly lower L_v , compared to controls ($p=0.015$).

3 ***Measurement of microvessel diameters***

4 A total of 4,082 microvessels were measured from all groups collectively for vessel
5 diameter analysis. However, as there were differences in L_v between groups, the first 100
6 microvessels randomly selected in each case were analysed and combined to calculate the
7 mean vessel diameter for each group (Figure 1). AD and VaD groups exhibited the narrowest
8 mean diameter (7.01 and 7.04 μm , respectively). The widest mean diameter was found in
9 the PSND (8.03 μm). However, controls and PSD had similar mean diameters (7.47 and 7.44
10 μm , respectively). After demonstrating that the data were non-parametric, we observed a
11 significant difference between PSND and all other groups ($p=0.000$ for all groups). A
12 significant difference was also found between controls and PSD, compared to AD and VaD
13 ($p=0.000$ in all cases, Figure 3A). Group data were expressed in term of increasing diameter
14 as cumulative frequency (Figure 3B). Significant differences were found between PSND
15 compared to all other groups except VaD using χ^2 distribution analysis (Table 2).

16 **Discussion**

17 Recent advances have shown that cerebral microvascular pathology is associated with age-
18 related cognitive decline [37-39]. Changes in microvessel morphology, including thinning
19 [17] and increased tortuosity [36, 40], have been described in demented cases. Our results
20 augment previous findings linking vascular pathology with cognitive decline by showing
21 increased microvascular density in the CA1 in PSD, compared to PSND, in an endothelial cell
22 (GLUT1) and basement membrane (COL4) markers. Such increases in L_v may be a reactive
23 response, whereby increases in the perfusion surface between blood and brain result in
24 remodelling of microvessels within a hypoxic environment. This interpretation does not
25 counteract the notion that microvessel structural changes reflect increased tortuosity in PSD
26 subjects.

27 We also demonstrated significant increases in L_v in AD cases when compared against
28 controls, PSND and VaD in the CA1 region, and with PSND and PSD in the EC region. This
29 may relate to the presence of different components of AD pathology (see Supplementary

1 data), including amyloid plaques and neurofibrillary tangles [41]. However, our findings are
2 consistent with previous studies showing an increase in L_v COL4 staining in the CA1 [16], as
3 well as the temporal cortex [15] of AD cases. Our observations, which indicate no significant
4 differences in the CA2 region, suggest that the increases in L_v are regionally-specific and can
5 be attributed to increased vulnerability caused by AD pathology, or as a result of
6 hypoperfusion, in the CA1 and EC. This appears to corroborate previous findings [42], which
7 have indicated a selective reduction in capillary density within the CA1, but not the CA3
8 region, albeit after acute ischaemic insults.

9 Consistent with our previous work [40, 45], GLUT1 staining was not continuous along
10 the vessel, unlike COL4 (Figure 1). This implied that the numbers of intersections recorded
11 between the probe and the vessel may not have fully corresponded with GLUT-1 expression.
12 We thus counteracted this issue by visualising vessels with a haematoxylin counterstain.
13 Nevertheless, intersections were only recorded when other portions of the vessel were
14 positively stained for GLUT1. The irregular staining of endothelial cells with GLUT1 along the
15 vessel is likely to be due to cellular damage [45]. It is possible that the GLUT1 negative
16 profiles, seen only with haematoxylin counterstaining, reflect reduced GLUT1 protein in
17 cases with dementia [46-48].

18 We assessed SMA immunoreactivity, which predominately labelled smooth muscle
19 cells within perforating branches of the hippocampal arterioles in CA1 and CA2. A significant
20 increase in SMA L_v was found in CA1 in AD compared to controls and VaD; however, no
21 difference was found between PSND and PSD.

22 Previous neuroimaging studies have suggested that medial temporal lobe atrophy
23 occurs in AD [49], as well as in PSD [50]. Thus, it could be argued that the observed increase
24 in L_v in AD and PSD is an indicator of hippocampal atrophy or even tissue shrinkage,
25 whereby loss of tissue reduces the distance between existing microvascular profiles [36].
26 Valid comparisons can be made of post-mortem tissue as all the tissues were treated in a
27 standardised manner so any difference would be disease specific. In a related study [10], we
28 found that hippocampal neuronal volume was decreased across all demented groups,
29 thereby suggesting that hippocampal atrophy is not a unique finding to AD but is also
30 apparent in PSD and VaD. Irrespective of such findings, the outcomes of the current study

1 did not concur with the notion that atrophy was the single factor leading to an increase in
2 L_v , as we found a significant decrease in L_v in PSD cases in the EC compared to AD and no
3 change in L_v in the CA1. If one hypothesises that atrophy is the only factor leading to
4 increased L_v then one would expect similar findings in both regions. Additionally, no change
5 in L_v was observed in VaD cases, compared to control or PSND subjects. As atrophy could
6 not be measured in the cases included in this study (MRI scans were not performed in all
7 subjects who came to autopsy), it cannot be ruled out as a confounding factor. However, if
8 the morphological changes were related to atrophy within the whole hippocampal
9 formation or medial temporal lobe one would predict an increase in microvessel L_v ,
10 compared to PSND subjects in all regions and including VaD cases [51].

11 A technical limitation in this study was lack of availability of the whole reference
12 volume (the hippocampus and EC) for cutting and sampling. The tissue was obtained from
13 predefined blocks and as a result only one block per case was available for sampling. As a
14 result of these inherent issues, we were unable to estimate volume of the hippocampus,
15 meaning it was not possible to convert L_v into total length per structure (i.e. the
16 hippocampus). As L_v is a measure that relies on the relationship between the numerator (in
17 this case, the number of vessel intersections) and the denominator (the background
18 neuronal tissue), this leaves open the possibility of 'reference trap' bias from tissue
19 shrinkage as, if one assumes that the density of a component within a structure acts as a
20 proxy for its total number, one must also make the assumption that the reference volume of
21 the structure itself must remain unchanged across the groups measured. Though we cannot
22 rule out the effects of shrinkage, all sections were processed identically and assessment of
23 section thickness revealed no significant differences between groups making group effects
24 unlikely. Whilst only six cases were analysed in each group, retrospective power analysis
25 showed that there is a significant number of cases per group to obtain a high level of
26 significant power.

27 Consistent with previous studies [14,17], a significant decrease in vessel diameter was
28 identified in AD and VaD. Possible string vessels [37, 45] were often observed but not
29 quantified. These results may explain the commonly associated reduced CBF previously
30 described in dementia and GLUT1 protein density [36]. A number of studies have suggested
31 that amyloid β may play a direct role in increasing vasoconstriction [52] or that nitric oxide,

1 a natural vasodilator, is altered in dementia [53]. Another study has suggested that the
2 vasoconstrictor endothelin-1 (ET-1) may play a role in both AD and VaD. However, changes
3 in the mRNA of the endothelin converting enzyme (ECE-1) were small and increases found in
4 ET-1 are most likely be caused by A β mediated up-regulation of the converting enzymes [54,
5 55]. Furthermore, inhibiting angiotensin II, a potent vasoconstrictor may improve cognitive
6 function in AD and VaD by ameliorating hypotension. However, angiotension II is also
7 involved in inhibiting the release of acetylcholine and the up-regulation of inflammatory
8 response [56, 57]. Patients with AD are less likely to have been prescribed angiotension II
9 receptor blockers compared to age matched non demented controls [58], which may affect
10 vessel diameter. Microvessel diameter was significantly increased in PSND, compared to
11 controls and no significance difference was found between PSD and controls. The results
12 outlined may thus either suggest that vessels in PSND are more vasoreactive and responsive
13 to their environment after stroke and undergo re-modelling (unlike PSD cases), or,
14 alternatively, that the vessel diameter may not be related to cognitive function post stroke.

15 Cumulative frequency analysis showed that there were significant differences in
16 distribution analysis of increasing vessel diameter between the groups, suggesting variations
17 in subpopulations of hippocampal microvessels. In the PSND group, vessel diameters were
18 significantly wider than all the other groups. Cumulative frequency analysis suggested that
19 there were greater proportions of vessels with a diameter (between approximately 7 and 12
20 μm), compared to the other groups, and that the mean diameter increase was not caused
21 by analysing a larger number of large vessels, including arterioles (Figure 3B). This could
22 mean that the PSND group had microvasculature that is more adaptable in response to
23 hypoperfusion. Distribution analysis showed that there was a significant difference between
24 the PSND group and all other groups, except VaD. This suggests a significant variation in the
25 composition of microvasculature across all groups.

26 In summary, our results show an increase in microvascular L_v in PSD and AD and,
27 furthermore, that microvessels were significantly wider in PSND cases. Moreover, there
28 were differential microvascular changes across the regions of the hippocampus, with
29 significant differences only found in the CA1 region. A significant increase was also observed
30 in AD cases compared to both PSND and PSD in the EC. Given that there is an increase in L_v
31 in PSD cases compared to PSND, but that they exhibit similar but minimal

1 neurodegenerative pathology, the microvascular changes found may be marker for
2 hypoperfusion in PS survivors. Whilst there were no significant differences in L_v between
3 PSND and VaD subjects, it is possible that the microvasculature attributes are similar in PSD
4 and AD in the hippocampus (but not EC) but that different mechanisms occur in VaD. A
5 significant increase in mean vascular diameter (between 7 and 12 μm) was also found in
6 PSND cases, compared to other groups; however, there was no significant increase in the
7 proportion of larger vessels i.e. arterioles. Furthermore, there was no significant difference
8 in vessel diameter between controls and PSD suggesting that microvessels may not possess
9 the ability to adapt to their environment and thus remained unaltered. It has been widely
10 hypothesised that the decrease in diameter in AD and VaD would reduce CBF and increase
11 brain hypoperfusion. This suggests that the increases in the proportion of small narrower
12 vessels in AD and PSD, when compared to PSND, may be an indicator of new microvascular
13 loops via angiogenic processes [23], or increased twisting of existing profiles [37]. However,
14 alternative factors, such as atrophy, cannot be completely dismissed as a reason for the
15 increases in microvascular density found in AD and PSD.

16

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30

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2 The co-authors have no disclosures with regard to this report. The study was not industry-
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4 follows:

5 Matthew JC Burke performed the experimental work and the analysis and wrote several
6 drafts.

7 Lucy Nelson contributed to collection of the data and particularly performed stereological
8 analysis of the microvasculature in other brain areas.

9 Janet Y Slade performed the initial immunocytochemical analysis and advised on the
10 analysis.

11 Arthur E Oakley advised on and interpreted the stereological analysis and correct drafts of
12 the manuscript.

13 Tuomo M Polvikoski advised on the study and performed the neuropathological analysis.

14 Ahmad Khundakar advised on and interpretation of the stereological analysis.

15 Raj N Kalaria conceived the study, performed some of the neuropathological analysis,
16 corrected several drafts and obtained the funding.

17

18 **Conflicts of Interest**

19 None declared.

20

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2 **Table 1: Demographic details**

	Controls	PSND	PSD	VaD	AD
Total number of controls or cases analysed	13	23	13	15	14
Age, y Mean (range)	80.4 (72-94)	84.3 (78-94)	86.3 (80-96)	85.1 (71-97)	83.5 (70-91)
PMD, h Mean (\pm 2SEM)	28.0 (6.1)	45 (11.2)	47.2 (14.6)	35.0 (13.4)	59 (15.8)
MMSE Score Mean (range)	N/A	27.1 (24-30)	17.3 (12-24)	N/A	N/A
CAMCOG Score (range)	N/A	89.1 (82-99)	62.6 (24-80)	N/A	N/A
Braak stage Mean (range)	2.4 (1-4)	2.5 (1-5)	2.6 (0-4)	2.0 (1-4)	5.3 (4-6)
CERAD Mean (Range)	0.5 (0-1)	1.4 (0-2)	1.0 (0-3)	1.0 (0-2)	3.0 (3-3)
	2.2 (0-4)	2.8 (1-4)	1.6 (0-4)	1.9 (0-3)	3.7 (3-4)

Vascular Pathology* (Range)	NPD	12.6 (7-16)	11.9 (8-17)	13 (12-14)	N/A
Mean time from diagnosis of dementia to death (years)	-	-	2.38	4.58	4.0

1

2 The causes of death included bronchopneumonia, cardiac arrest and carcinoma with no
3 particular distribution in any group. The time period (weeks) of tissue fixation was in range
4 8-15 weeks for all the cases.* Vascular pathology scores were derived as described
5 previously [35]. For stereological analysis, six samples representing each disease type or
6 controls were selected from the total pool and matched for age, post-mortem interval
7 (PMD) and fixation length. Abbreviations: AD, Alzheimer’s disease; CAMCOG, Cambridge
8 Assessment of Mental Disorders in the Elderly; CERAD Consortium to Establish a Registry for
9 Alzheimer’s disease; MMSE, Mini-mental state exam; NPD, no neuropathological diagnosis;
10 N/A not applicable PMD, post-mortem delay; PSND, post stroke non-demented; PSD, post-
11 stroke dementia; VaD vascular dementia.

1 **Table 2: Group differences between cumulative frequency distribution of vessel diameter**

	Controls	PSND	PSD	VaD	AD
Controls		49.11 (p≤0.001)	3.51 (N/S)	63.56 (p≤0.001)	0.535 (N/S)
PSND	49.11 (p≤0.001)		23.74 (p≤0.001)	1.49 (N/S)	55.24 (p≤0.001)
PSD	3.51 (N/S)	23.74 (p≤0.001)		34.90 (p≤0.001)	6.12 (p = 0.013)
VaD	63.56 (p≤0.001)	1.49 (N/S)	34.90 (p≤0.001)		69.89 (p≤0.001)
AD	0.535 (N/S)	55.24 (p≤0.001)	6.12 (p = 0.013)	69.89 (p≤0.001)	

2

3 Chi² (χ^2) distribution values and significant differences between the cumulative frequencies
 4 of vessel diameter. Analysis was performed using distribution analysis, with presumed
 5 distribution three parameter lognormal. N/S denotes no significant difference.

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1 **Figure legends:**

2 **Figure 1**

3 Microvasculature in the CA1 region of the hippocampal formation. (A) Microvessels
4 immunostained with GLUT1 antibodies for endothelial cells. (B) Microvessels and arterioles
5 stained for basement membranes for COL4 (grey, arrow head) and smooth muscle α -actin
6 (brown, open arrows). Note, GLUT1 stained microvessels appear discontinuous compared to
7 COL4 (cf. A vs. B). C and D, Morphometric technique used to measure vasculature diameter
8 stained with COL4 in 10 μ m sections. (C) A longitudinal cut vessel and (D) a transectionally
9 cut vessel. Magnification Bars: B=100 μ m; C and D= 10 μ m.

10

11 **Figure 2**

12 Mean L_v values of GLUT1 and COL4 immunostained microvessels. Both markers (A and B)
13 showed significant increases in L_v in AD and PSD groups in CA1 region. Significance: **
14 denotes $p \leq 0.01$ and *** $p \leq 0.001$, different means against AD (grey), PSD (black) and both
15 AD and PSD (black bold). Results for CA2 region with no significant changes are not shown.
16 C, The correlation between L_v of GLUT1 and L_v of COL4 immunostaining in CA1 showing
17 internal consistency of measurement ($r^2 = 0.687$, $p=0.000$). Key: AD, Alzheimer's disease,
18 controls, PSND, non-demented post-stroke subjects, PSD, post-stroke dementia and VaD,
19 vascular dementia.

20

21 **Figure 3**

22 Microvessel diameters in PSD and AD versus controls. To standardise comparisons between
23 disorders, due to increased microvessel densities in AD and PSD, the 1st one hundred vessels
24 measured in each case were combined to calculate the mean vessel diameter in each group.
25 *** denotes $p \leq 0.001$. Bold black asterisks indicate significance between PSND and all other
26 groups. Black asterisks indicate significant difference to PSD and grey asterisks show
27 difference to controls. Bars represent 2 x SEM. C, Cumulative frequency of increasing vessel
28 diameter between groups. The analysis allows to identify general trends in microvascular

1 changes within the population samples. Significant differences were found between PSND
2 against controls, VaD, AD and PSD subjects (Table 2). Abbreviations and key to symbols: AD,
3 Alzheimer's disease, PSND, non-demented post-stroke subjects, PSD, post-stroke dementia
4 and VaD, vascular dementia.

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1 **Supplementary data**

2 The L_v values were also related to pathology, specifically Alzheimer-type, including Braak
3 staging and Thal and CERAD scores. Braak scores were correlated with L_v in CA1 for GLUT1
4 (0.540, $p=0.004$), COL4 (0.635, $p=0.000$) and SMA (0.455, $p=0.020$). Thal staging also showed
5 a significant correlation in CA1 with GLUT1 (0.517, $p=0.010$) and COL4 (0.532 $p=0.070$).
6 However, there was no correlation between L_v and SMA. CERAD scores were correlated with
7 GLUT1 (0.501, $p=0.015$) and COL4 (0.540, $p=0.008$) in the CA1; a trend was also observed
8 with SMA staining (0.406, $p=0.054$) (Table). There was no correlation observed between L_v
9 values and any pathological substrate in the CA2.

10 **Supplementary Table: The relationship of L_v with the burden of neuropathology**

	Braak staging			Thal et al staging			CERAD scores		
	Correlation Coefficient	P value	R ²	Correlation Coefficient	P value	R ²	Correlation Coefficient	P value	R ²
GLUT1	0.54	0.004	0.295	0.517	0.01	0.235	0.501	0.015	0.234
COL4	0.635	0.000	0.403	0.532	0.007	0.283	0.540	0.008	0.292
SMA	0.455	0.02	0.207	0.257	0.225	0.066	0.406	0.054	0.165

11

12 There were significant correlations between increasing L_v and neuropathological scoring
13 criteria for Braak staging, Thal staging and CERAD scores. The L_v was not significantly related
14 to the vascular pathology scores for any of the groups (not shown). Abbreviations: COL4,
15 collagen IV; GLUT1, glucose transporter 1; SMA, α -smooth muscle actin.

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