# Morphometry of the hippocampal microvasculature in post-stroke and age-related dementias

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**Abstract**

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

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

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

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

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

## Introduction

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

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

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

## Materials and methods

### *Subjects and clinical features*

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

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

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

***Neuropathological examination***

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

***Immunohistochemistry***

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

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

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

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

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

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

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

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

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

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

**Statistical analysis**

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

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

## Results

### *Neuropathological findings*

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

### *Length density assessment*

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

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

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

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

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

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

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

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

### *Measurement of microvessel diameters*

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

## Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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## Disclosure statements for Authors

The co-authors have no disclosures with regard to this report. The study was not industry-sponsored. There are no conflicts of interest. The individual contributor statements are as follows:

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

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

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

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

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

Ahmad Khundakar advised on and interpretation of the stereological analysis.

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

## Conflicts of Interest

None declared.

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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<br><br>Mean (range) | 80.4 (72-94) | 84.3 (78-94) | 86.3 (80-96) | 85.1 (71-97) | 83.5 (70-91) |
| PMD, h<br><br>Mean (± 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<br><br>Mean (range) | 2.4 (1-4) | 2.5 (1-5) | 2.6 (0-4) | 2.0 (1-4) | 5.3 (4-6) |
| CERAD<br><br>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 |

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

**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) | |

$Chi^2$ ($\chi^2$) distribution values and significant differences between the cumulative frequencies of vessel diameter. Analysis was performed using distribution analysis, with presumed distribution three parameter lognormal. N/S denotes no significant difference.

**Figure legends:**

**Figure 1**

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

**Figure 2**

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

**Figure 3**

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

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

**Supplementary data**

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

**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 |

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