Cell Senescence and Cerebral Small Vessel Disease in the Brains of Older People, Aged 80+

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Emma Norton: study design, data acquisition, analysis and interpretation, statistical analysis, drafting and revising the manuscript.
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Short Running Title: Senescence in Cerebral Small Vessel Disease

Abstract

Cerebral small vessel disease (cSVD) in penetrating arteries is a major cause of age-related morbidity. Cellular senescence is a molecular process targeted by novel senolytic drugs. We quantified senescence in penetrating arteries and tested whether myocyte senescence was associated with cSVD. We immunolabelled subcortical white matter of older persons (age 80-96 years, N=60) with minimal AD, using antibodies to two established senescence markers (H3K9me3, γH2AX) and a myocyte marker (hSMM). Within the walls of penetrating arteries (20-300μm), we quantified senescence-associated heterochromatic foci (SAHF)-positive nuclei, cell density (nuclei/μm^2) and sclerotic index (SI). Senescent-appearing mural cells were present in small arteries of all cases. cSVD cases exhibited a lower proportion of senescent-appearing cells and lower area fraction (AF%) of SAHF-positive nuclei compared to controls ($p=0.014$, 0.016, respectively). cSVD severity and SI both correlated negatively with AF% ($p=0.013$, 0.002). Mural cell density was lower ($p<0.001$) and SI higher ($p<0.001$) in cSVD, relative to controls. In conclusion, senescent myocyte-like cells were universal in penetrating arteries of an AD-free cohort aged 80+. Senescent-appearing nuclei were more common in persons aged 80+ without cSVD compared to cSVD cases, indicating caution in senolytic drug prescribing. Myocyte senescence and cSVD may represent alternative vessel fates in the ageing human brain.

Key Words:
Senescence; brain aging; small vessel disease; cerebrovascular disease

Abbreviations

γH2AX = phosphorylated histone-H2A, AD = Alzheimer's disease, AF% = area fraction of vessel wall occupied by SAHF-positive nuclei, BSA= bovine serum albumin, cSVD = cerebral small vessel disease, DAB= diaminobenzidine, DDF = DNA damage foci, H3K9me3= histone H3 lysine 9 tri-methylation, hSMM= human smooth muscle myosin, IQR = interquartile range, PMI= post-mortem interval, SAHF= senescence-associated heterochromatin foci, SI = sclerotic index.

Introduction

Cerebral small vessel disease (cSVD) is a highly prevalent cerebrovascular pathology in ageing populations and is a major cause of lacunar stroke, diffuse white matter lesions, deep intracerebral haemorrhages and vascular cognitive impairment (1, 2, 3). Here cSVD refers to the common, sporadic, non-amyloid vasculopathy also termed arteriolosclerosis.

A neuropathological hallmark of cSVD is fibro-hyaline thickening of deep penetrating arteries, resulting in elongated and tortuous vessels with narrowed lumina and thickened vessel walls (1, 4, 5). The pathogenesis of cSVD is not fully understood. Depletion of vascular myocytes from the tunica media, through an unknown mechanism, is reported as a qualitative feature (1, 6, 7). Depletion of myocytes is likely to underlie impaired cerebral blood flow and consequent ischaemic lesions.

Senescence is a cellular state of permanent cell-cycle arrest, following extensive telomere shortening or DNA damage (8, 9). Cellular senescence can be detected histologically via nuclear senescence-associated heterochromatic foci (SAHF) formed by the inactivation of specific transcription factors and remodelling of chromatin structure (10, 11). SAHF can be visualised by immunostaining heterochromatin markers such as tri-methylated Lys9 in histone-3 (H3K9me3) (10, 11, 12). Senescent cells also contain DNA damage foci (DDF) which can be identified via phosphorylated Ser139 on histone-2 (γH2AX) (13, 14, 15).

In ageing and age-related diseases, cell senescence is generally considered deleterious, as senescent cells accumulate and impair tissue function and regeneration (9, 16, 17). Senolytic drugs, which drive senescent cells towards a programmed cell death fate, have recently been proposed as “anti-ageing” medications, based primarily on experimental studies in mice (18).

Nevertheless, cell senescence is a physiological phenomenon that can be beneficial, by inhibiting tumour progression, curbing myocardial and liver fibrosis, and protecting against atherosclerosis in large arteries (16, 19, 20). The extent of cell senescence in vascular myocytes of small arterial vessels is unknown.

In this study, we tested the prevalence of myocyte senescence in people aged 80 or more and tested for associations between myocyte senescence, depletion, and neuropathological diagnosis of cSVD.

Materials and Methods

Human brain tissue.

Post-mortem brain tissue was obtained from the Oxford Brain Collection, John Radcliffe Infirmary, Oxford, UK (n=30) and from the autopsy tissue archive of Thomas Jefferson University Hospital, Philadelphia, USA (n=30). Informed consent for donation and patient anonymisation within the study was ensured. The study had approval from Local Research Ethics Committees and the UK National Research Ethics Service and from the Institutional Review Board of Thomas Jefferson University.

Paraffin wax-embedded sections containing subcortical frontal matter were analysed from 31 individuals with an autopsy diagnosis of "moderate/severe" cSVD and from 29 individuals without ("aged controls"). Qualitative analysis and comparison was performed on a young adult without known brain pathology (H3K9me3 staining, 20-year-old male, died of a myocardial infarction) and on four aged controls using a second senescence marker (γH2AX). All individuals had minimal Alzheimer's disease pathology (Braak stage 0-II) and no known hereditary cSVD. PMI was comparable between cSVD cases and aged controls. Further clinical data can be found in Table 1.

Neuropathological assessment of cSVD

The presence/absence of arteriolosclerosis was recorded based on autopsy diagnosis. Disease severity of cSVD was subsequently graded on neighbouring Haematoxylin and Eosin and Luxol Fast Blue stained sections using a validated semi-quantitative scale (0-6) (2) by a senior registered neuropathologist (MME). Briefly, semi-quantitative scores for SVD (0-3) were assigned to sections containing frontal and occipital subcortical white matter for each region. 0: normal appearing white or grey matter. 1: slight pallor of myelin staining in white matter, and/or slight loosening of parenchymal tissue on H&E stain and/or some mild dilatation of perivascular spaces. 2: more marked loss of myelin and/or loosening of parenchymal tissue, sometimes with a bubbly appearance to white matter and/or more markedly widened perivascular spaces. 3: regions of almost complete myelin loss in white matter, and severe loosening of parenchymal tissue extending in places to cavitation, and severely dilated perivascular spaces. White matter SVD score (0-6) was obtained as the summed scores of two sections.

Immunohistochemistry.

The primary antibody was raised against histone H3 with tri-methylated lysine-9 (anti-H3K9me3) (rabbit polyclonal IgG ab8898, Abcam, Cambridge, UK). Anti-phosphorylated-histone H2AX serine-139 (anti-γH2AX) (mouse monoclonal IgG1 JBW301, Merck-Millipore, Watford, Hertfordshire, UK) was used as a marker of DNA damage foci. Anti-human smooth muscle myosin was used as a smooth-muscle myocyte marker (hSMM, mouse monoclonal IgG1, clone hSM-V, M7786, Sigma, Poole, Dorset, UK).

All brains were fixed in 10-20 % neutral buffered formalin before wax embedding. Sections (6 μm) were dewaxed and endogenous peroxidase activity was blocked using 3% aqueous H_2O_2 for 10 minutes. High-pressure-heat-induced antigen retrieval was also performed (30 seconds, 120 ^oC, in pH 7.8 Tris-Citrate buffer). Non-specific binding was blocked with BSA (3% w/v in phosphate-buffered saline, supplemented with 0.1% Triton-X100, PBT-BSA). Primary antibodies were diluted in PBT-BSA. H3K9me3 (diluted 1:10,000), γH2AX (1:2000) and hSMM (1:400) antibodies were applied overnight at 4^oC. Labelling was visualised with a horseradish peroxidase-conjugated secondary reagent (Envision® kit, K-5007, Dako-Agilent, Glostrup, Denmark) and DAB chromogen, with a light green SF cytoplasmic counterstain. Where dual labelling was performed (n=12), H3K9me3 was visualised using DAB chromogen, hSMM with FastRed chromogen and haematoxylin as a counterstain.

Morphometric protocol.

All sections were examined by a single observer (EJN) who was blind to cSVD status and to clinical variables. For each section, in the subcortical white matter all vessels with arterial appearance and outer diameter 20-300μm were photographed and analysed. Size and area measurements were performed using ImageJ-Fiji free software.

Two measures of SAHF-positive nuclei were calculated to quantify mural cell senescence. First, the percentage of SAHF-positive nuclei within the vessel wall was recorded. Nuclei of endothelial cells were excluded (for further details see Supplementary Material, Figure S1). The criterion for SAHF-positivity was the confident identification of H3K9me3-positive dots or "stipples" across the whole nucleus, based on the documented appearance of SAHF (Figure 1L) (10, 11).

Second, the area fraction of vessel wall occupied by SAHF-positive nuclei (AF%) was calculated. The SAHF-positive nuclei were selected using a colour deconvolution Macro (Methyl Green DAB) within ImageJ to isolate the brown image components. Size criteria were then applied to exclude large-homogeneously or peripherally stained nuclei. AF% was expressed as the remaining H3K9me3-positive fraction (%) of the area within the outer circumference of the vessel (for further details see Supplementary Material, Figure S2).

To assess mural cell number, the total number of observed nuclei within the tunica media of each vessel was recorded. For all vessels with a clear, cross-sectional appearance, a nuclear density score was calculated; nuclear density = total nuclear count/vessel wall area (excluding the lumen). Agreement in the nuclear counts between two independent, blinded observers (EJN, AHH) was tested for 100 vessels using Spearman's rank-order correlation. There was a good inter-observer correlation for total nuclear counts (rho = 0.654, $p<0.001$) and number of SAHF-positive nuclei (rho = 0.747, $p<0.001$).

For all vessels with a non-inflected, circular or squat elliptical cross-section, sclerotic index (SI) was calculated as a measure of wall thickening and cSVD severity within individual vessels; SI = 1 – (inner diameter/outer diameter) as in our previous studies (5, 21).

Statistical analysis.

Percentage and AF% of SAHF-positive nuclei, mural cell number and density were tested between cSVD cases and controls using non-parametric statistics (Mann Whitney U test). To test for correlations with neuropathological cSVD score (categories 0/1/2/3/4/5/6) and SI (continuous range 0-1), Spearman's rank-order and Pearson correlation were used, respectively. Age, sex and post-mortem interval (PMI) were also compared between cSVD cases and controls. All analyses were performed using the programme Minitab (version 18.1.0). Significance was defined as $p<0.05$.

Results

Immunohistochemistry and neuropathological assessment.

Robust, strictly nuclear-delimited labelling with H3K9me3 was seen in all cases. This included dense continuous nuclear labelling, nuclear membrane-delimited labelling, or a

characteristic stippled pattern of puncta uniformly distributed across the nucleus (examples in Figure 1). In stippled nuclei, the size and density of puncta were consistent with SAHF when labelled with H3K9me3 (N=60, examples in Figure 1J, L) (10). In a small subgroup (n=4), neighbouring sections were immunolabelled with another nuclear senescence marker, γH2AX. All exhibited punctate nuclear γH2AX labelling consistent with DNA damage foci (Figure 1K) (13, 14, 15). These features were all absent in neighbouring sections treated without primary antibody, or with irrelevant primary antibody (anti-HPV, not shown). All quantitative analyses were performed using the H3K9me3 marker.

The SAHF-positive mural cells in small arterial vessels were seen in all cases, including the young control. A few severely fibrotic individual vessels lacked H3K9me3 positive mural nuclei. 12 cases were immunolabelled for both H3K9me3 and the myocyte marker hSMM. In the brains of older people without neuropathological diagnosis of cSVD (n=6) double labelling of SAHF and hSMM at a same-cell level was convincing (Figure 1G). In double stained cSVD cases (n=6), where vascular hSMM labelling was sparse, identifying convincing co-labelling of SAHF and hSMM in the same cell was problematic (Figure 1H, I). In severe cSVD cases, some vessels lacked cellular hSMM labelling.

Morphometric analysis.

At least 5 vessels were analysed per section. Among aged control cases, the median number of vessels analysed was 8 per section (range: 5-14) and for cSVD cases, the median number was 9 (range: 6-18; no significant difference between groups, p=0.675, Mann-Whitney test).

The percentage of SAHF-positive nuclei was calculated using 540 vessels from 60 individuals (29 cSVD cases, 31 aged controls). SAHF-positive AF% were calculated using 401 vessels from 48 individuals (25 cSVD cases, 23 aged controls). Complete data for mural cell number, mural cell density (nuclei/mm^2) and SI were calculated for 134 vessels (79 vessels from cSVD cases, 55 vessels from aged controls) derived from 60 individuals.

SAHF-positive mural cells were identified in both cSVD cases and aged controls. Both measures of SAHF-positive mural cells were significantly lower in cSVD cases compared to aged controls. For the percentage of SAHF-positive nuclei, the median value in cSVD cases was 17.5% (IQR = 12.3-25.0%) and in aged controls was 23.7 (IQR = 19.0-27.5%), p=0.014. For the SAHF-positive AF%, the median value in cSVD cases was 0.611 (IQR 0.379-0.738)

and in aged controls was 1.16 (IQR = 0.517-1.71), p=0.016. SAHF-positive AF% negatively correlated with neuropathological cSVD severity scores (R= -0.358, p=0.013) and with sclerotic index (R= -0.443, p=0.002). Percentage of SAHF-positive nuclei had no significant correlation with neuropathological cSVD severity scores or with SI.

Mural nuclear number and density (nuclei/ μm^2) were lower in cSVD cases. The median mural cell number in cSVD cases was 7 (IQR = 4-10) and in aged controls was 8 (IQR = 5-10), p=0.001. The median nuclear density in cases was 5.60 x 10^3 nuclei/mm^2 (IQR= 3.74-7.41 x 10^3 nuclei/mm^2) and in controls was 11.4 x 10^3 nuclei/mm^2 (IQR = 7.93-14.5 x 10^3 nuclei/mm^2), p<0.001. Nuclear number and density negatively correlated with sclerotic index at an individual vessel level (number: R=-0.345, p=0.029; density: R=-0.320, p<0.001).

Measures of mural cell senescence and number did not correlate significantly with age or sex. There was no significant correlation between the percentage of SAHF-positive nuclei and AF%. As expected, SI and neuropathological cSVD score were higher in cSVD cases diagnosed at autopsy, p<0.001 and p=0.050 respectively. Neuropathological cSVD score positively correlated with SI (Spearman's Rank-Order Correlation, rho = 0.376, p=0.008).

Discussion

Small artery mural cell senescence was a common finding in our cohort.

Senescent-appearing nuclei were demonstrated using two independent, established immunohistochemical markers, H3K9me3 (10, 11, 12) and γH2AX (13, 14, 15). Mural cell senescence was observed in all older cases. SAHF-positive mural cells were also evident in small arteries of a young adult without known brain pathology (Figure 1A, D). These observations are consistent with the view that mural cell senescence may be a feature of normal ageing in the cerebral microvasculature. The cohort studied here represents a relatively narrow age range, among persons sometimes referred to as "oldest-old" (80-96 years). This age range may be too advanced and too narrow to detect an association observed between measures of mural cell senescence and the chronological age of the tissue donor.

Small artery mural cell senescence was less common in cSVD.

Lower proportions of senescent nuclei (here defined as SAHF-positive nuclei) were found in individuals with cSVD, compared to aged controls. Similarly, the SAHF-positive AF% within small artery walls was significantly lower in cSVD cases than controls and negatively correlated with SI and neuropathological cSVD severity score. This novel finding that people of advanced age who lack cSVD exhibit more mural cell SAHF-positivity, compared to cSVD cases, implies some association between vascular cell senescence and cSVD pathophysiology.

There is little prior published work on cell senescence in ageing brain vasculature (6, 22). In other vascular beds, the impact of senescence, protective or deleterious, varies with tissue type and pathology (20). We speculate that myocyte senescence and cSVD may represent alternative vessel fates in the ageing human brain. Clearly, individuals who attain a chronological age of 80 without cSVD are unusual and it is tempting to propose that they carry some resilience factor(s). Mural cell senescence may be protective in the brain microvasculature, causing more SAHF-positive nuclei to be visible in healthy older people who have not developed cSVD despite attaining age 80. In some cellular contexts, senescent cells are more resistant to apoptosis (23). In peripheral tissues, myofibroblast senescence reduces hepatic and myocardial fibrosis (24, 25). In mice where cell senescence is abrogated (lacking the key senescence mediators p53 and p16INK4A) significantly more fibrosis is seen

around myocardial small penetrating arteries (25). These data and ours support the view that cell senescence can have a beneficial, anti-fibrotic role in some contexts. If this concept of a protective effect of vascular cell senescence is correct, then some caution is warranted in the use of so-called senolytic drugs (18).

Small artery mural cells were depleted in cSVD.

Mural cell nuclear density was significantly lower in penetrating arteries of cSVD cases compared to aged controls and negatively correlated with SI, a quantitative measure of individual vessel wall thickening (4, 5). This concurs with previous qualitative reports of myocyte depletion in cSVD (1, 7). Possible mechanisms for myocyte depletion include blood-brain-barrier dysfunction, tensile wall stress (26) or degeneration (possibly preceded by proliferation) (1, 7) and subsequent replacement by deposition of laminin and collagens I, III, V and VI (1, 6, 7, 27).

Co-localisation of SAHF and hSMM immunolabelling at a cellular level was convincing in most cases, supporting the interpretation that these mural cells are myocytes. In vessels with marked cSVD pathology, myosin staining was sparse and consequently, there was little co-localisation. Hence in vessels with severe cSVD, the phenotypic identity of these SAHF positive mural cells is unclear, whether myocytes, myofibroblasts or fibroblasts (28, 29). Endothelial cells were excluded from nuclear counts where possible, however, some may have been incidentally included.

Our study has several limitations. Firstly, in a post-mortem neuropathological analysis in individuals aged 80+ years at death, we have limited scope to assess initiating pathophysiological mechanisms. Second, not all clinical variables were controlled, in view of the limited clinical data available and the modest cohort size. For example, degree of hypertension, and medications for cardiovascular disease or dementia are not addressed. Third, we have deliberately excluded individuals with Alzheimer's disease pathology. This is an artificial constraint as "pure" pathological processes rarely occur in aged human brains.

In conclusion, we have quantified mural cell senescence and depletion in penetrating arteries of AD-free older people. We found senescent nuclear morphology to be common in both fibrotic and normal-appearing small vessels. Markers of mural cell senescence in small

penetrating arteries were significantly more abundant in individuals without cSVD pathology despite reaching age 80+ years, compared to those with a neuropathological diagnosis of cSVD. A possible protective role for brain vascular myocyte senescence may indicate caution in senolytic drug prescribing.

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Ethical Approval

The study had approval from Local Research Ethics Committees and the UK National Research Ethics Service and from the Institutional Review Board of Thomas Jefferson University. Informed consent for donation and patient anonymisation within the study was ensured.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1 Immunohistochemistry of Mural Cell Senescence in Cerebral Vessels

(a) Young control: deep penetrating vessel, haematoxylin and eosin staining. Morphology of a young cerebral vessel (20-year-old male)

(b) Aged control: deep penetrating vessel, haematoxylin and eosin staining. Morphology of an aged cerebral vessel (80-year-old female)

(c) cSVD case: deep penetrating vessel, haematoxylin and eosin staining. Morphology of a vessel with marked cSVD pathology (86-year-old female)

(d) Young control: deep penetrating vessel, H3M9me3 staining. Abundant SAHF-positive mural cells are indicated (20-year-old male)

(e) Aged control: deep penetrating vessel, H3K9me3 staining. SAHF-positive mural cell indicated (arrow) alongside non-senescent appearing nuclei (arrowheads) (88-year-old female)

(f) cSVD case: deep penetrating vessel, H3K9me3 staining. Two SAHF-positive mural cells are indicated within the fibrotic vessel wall with non-senescent appearing intimal cells (81-year-old male)

(g) Aged control: deep penetrating vessel, H3K9me3 and hSMM staining. Abundant mural cell nuclei and myosin staining visible (80-year-old male)

(h) cSVD case: deep penetrating vessel, H3K9me3 and hSMM staining. Mural cell nuclei seen with sparse myosin staining. This vessel, despite a global diagnosis of cSVD in this individual, appears to have only mild fibrotic change (81-year-old female)

(i) cSVD case: deep penetrating vessel, H3K9me3 and hSMM staining. Depleted mural cell nuclear numbers and sparse myosin staining visible (81-year-old female)

(j) Aged control: deep penetrating vessel, H3K9me3 staining. Abundant SAHF-positive nuclei seen (90-year-old male)

(k) Aged control: deep penetrating vessel, γH2AX staining. Abundant DNA damage foci-positive nuclei seen (85-year-old female)

(l) SAHF: confocal image of oncogenic H-Ras-induced senescence in a fibroblast, H3K9me3 staining. Documented appearance of SAHF (adapted from Sadaie *et al.*, 2013 (11))

Figure 2 Mural Cell Number, Mural Cell Senescence and cSVD

(a) Mural cell number of vessel wall in cSVD cases and aged controls. Box-whisker plots show median, IQR and full range. Asterisk (*) signifies outlying data points. Mann Whitney U test p=0.001

(b) Nuclear density of vessel wall (nuclei/μm^2) in cSVD cases and aged controls. Mann Whitney U test p<0.001

(c) Percentage of SAHF-positive nuclei in vessel walls in cSVD cases and aged controls. Mann Whitney U test p=0.014

(d) Area fraction of vessel wall occupied by SAHF-positive nuclei (AF%) in cSVD cases and aged controls. Mann Whitney U test p=0.016

Table 1

Table 1 Characteristics of cSVD Cases and Aged Controls			
	Aged Controls (n=29)	**cSVD Cases (n=31)**	**Total Cohort (n=60)**
Age at death (mean ± SD), years	84.5 ± 4.01	86.9 ± 4.89	85.8 ± 4.61
Male	n= 16	n= 14	n= 30
Female	n= 13	n=17	n= 30
History of hypertension	n= 14	n= 15	n= 19
History of diabetes	n= 5	n= 3	n= 8
Post-Mortem Interval (mean ± SD), hours	53.1 ± 37.6	53.3 ± 32.5	53.2 ± 33.9
cSVD score (0-6) (median, IQR)	3, 2-4	4, 3-4	4, 3-4
Sclerotic Index (mean ± SD)	0.286 ± 0.0959	0.395 ± 0.0913	0.343 ± 0.107

Figure 1

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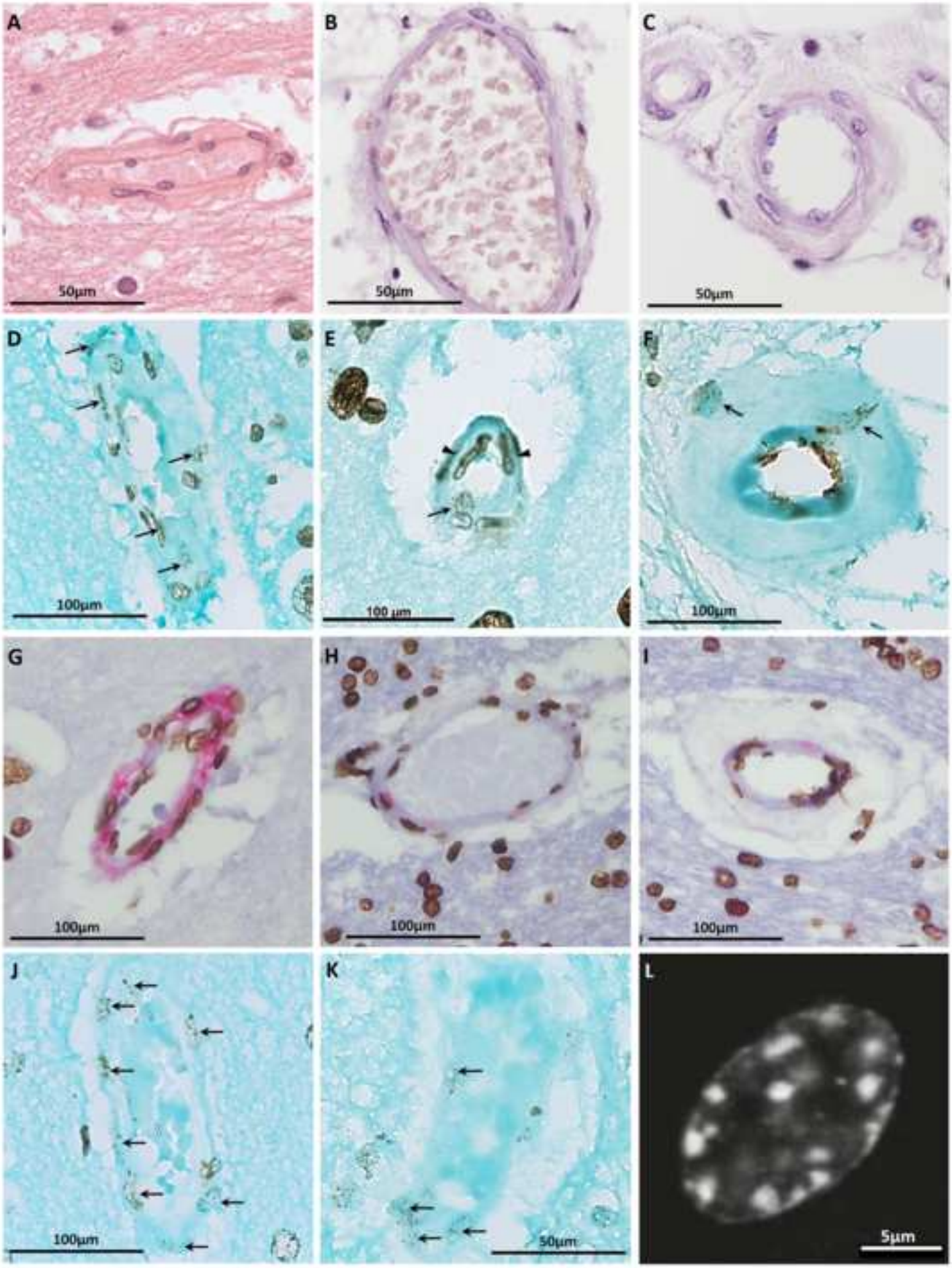

Figure 2