Blood-Brain Barrier Dysfunction and Cerebral Small Vessel Disease (Arteriolosclerosis) in Brains of Older People

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Abstract
The blood-brain barrier protects brain tissue from potentially harmful plasma components. Small vessel disease (SVD; also termed arteriolosclerosis) is common in the brains of older people and is associated with lacunar infarcts, leukoaraiosis, and vascular dementia. To determine whether plasma extravasation is associated with SVD, we immunolabeled the plasma proteins fibrinogen and immunoglobulin G, which are assumed to reflect blood-brain barrier dysfunction, in deep gray matter (DGM; anterior caudate-putamen) and deep subcortical white matter (DWM) in the brains of a well-characterized cohort of 84 elderly subjects (n = 46 years old). Morphometric measures of fibrinogen labeling were compared between people with neuropathologically defined SVD and aged control subjects. Parenchymal labeling with fibrinogen and immunoglobulin G was detectable in DGM and DWM in many subjects (>70%). Quantitative measures of fibrinogen were not associated with SVD in DGM or DWM; SVD severity was correlated between DGM and DWM (p < 0.0001). Fibrinogen in DGM showed a modest association with a history of hypertension; DWM fibrinogen was associated with dementia and cerebral amyloid angiopathy (all p < 0.05). In DWM, SVD was associated with leukoaraiosis identified in life (p < 0.05), but fibrinogen was not. Our data suggest that, in aged brains, plasma extravasation and hence local blood-brain barrier dysfunction are common but do not support an association with SVD.

Key Words: Arteriolosclerosis, Blood-brain barrier, Dementia, Fibrinogen, Leukoaraiosis, Small vessel disease.

INTRODUCTION
The blood-brain barrier (BBB) is a specialized physical and functional barrier composed of cerebral endothelial cells, with intercellular tight junctions, cell-cell signaling with astrocyte end-feet, and efflux pumps in the endothelial apical membranes (1, 2). Studies in experimental animals show that, in healthy brain tissue with a fully functional BBB, transendothelial permeability is exceedingly low and that there is minimal passive extravasation of plasma proteins, inorganic solutes, or even water molecules (1, 3, 4). The BBB is disrupted in various animal models of CNS disorders, including acute ischemia (5), multiple sclerosis (6, 7), chronic white matter ischemia (8), and acute cold injury (9).

Several lines of evidence suggest imperfect BBB function in human brain tissue that may be limited in duration and location. Quantitative magnetic resonance imaging (MRI) studies suggest penetration of circulating contrast agent into brain tissue and cerebrospinal fluid (10–12). Immunohistochemical labeling of plasma proteins (e.g., fibrinogen, immunoglobulin G [IgG], albumin, and prothrombin) has been used to assess plasma leakage (13–20). Numerous studies have demonstrated evidence of plasma extravasation in brain disease states, including multiple sclerosis (21–24), HIV encephalitis (25), cerebral malaria (26), epilepsy (27), Alzheimer disease (AD) (14, 19, 28–30), and cerebral ischemic lesions (13–18). Although some groups have reported the absence of extravascular plasma markers in healthy brain tissue (15, 23, 25), others have found evidence of paradoxical plasma leakage in “normal” control brain samples (14, 16, 21, 22, 26–28, 31).

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This study received financial support from St George’s Hospital Charity (Atticus H. Hainsworth), Neuroscience Research Foundation (Atticus H. Hainsworth), National Institute for Health Research (NIHR; Hugh S. Markus), NIHR via Oxford Biomedical Research Center (Margaret M. Esiri), and Alzheimer’s Society UK, project grant PGI46/151 (Hugh S. Markus and Atticus H. Hainsworth). Hugh S. Markus was supported by an NIHR Senior Investigator award. Funding for the University of California at Irvine Alzheimer’s Disease Research Center was provided by National Institutes of Health/National Institute on Aging Grant No. P50 AG016573. The Oxford Brain Bank was supported by the Medical Research Council (MRC, UK), Brains for Dementia Research, and the NIHR Oxford Biomedical Research Center.

The authors declare that they have no conflicts of interest.

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal’s Web site (www.jneuropath.com).

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Small vessel disease (SVD; also termed arteriolar sclerosis) is a common brain vasculopathy in older people that is associated with lacunar infarcts, vascular cognitive impairment, and diffuse white matter lesions that are identified in computed tomography and MRI scans as “leukoaraiosis” (32–35). Although age and hypertension are prominent risk factors, the pathogenesis of SVD remains obscure (33, 34, 36). Some investigators have hypothesized that BBB dysfunction is associated with SVD (37, 38).

We examined fibrinogen and IgG immunohistochemical labeling in a well-characterized cohort of donated brains of older people who had minimal AD (39). We compared those with neuropathologically-defined SVD with an age-matched control group (i.e. older people without AD, SVD, or other documented brain disease). All cases had in-life clinical and cognitive assessments (40, 41) and detailed neuropathologic examination postmortem, including assessment of SVD (39). We tested whether fibrinogen labeling was associated with age, hypertension, dementia, or SVD severity.

**MATERIALS AND METHODS**

**Human Tissue**

Paraffin-embedded tissue samples of formalin-fixed deep gray matter (DGM; anterior caudate-putamen) and deep frontal cortical white matter (DWM) from individuals aged 65 years or older (n = 84) (Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A647) were examined. This cohort was composed of all cases that were neuropathologically graded as Braak Stage II or lower for neurofibrillary tangle pathology within the Thomas Willis Oxford Brain Collection, John Radcliffe Hospital (Oxford, United Kingdom). None had more than 1 ApoE4 or ApoE2 allele. Most cases (76%) were part of the Oxford Project to Investigate Memory and Ageing cohort (www.medschl.ox.ac.uk/optimia) (41). This study was approved by the UK National Research Ethics Service. All tissues were donated after a written informed consent by donors or next of kin.

**Clinical Data**

Medical history (including a documented history of hypertension) and use of antihypertensive medication (never/ former/current) were collected from subjects and checked with family doctors’ computerized records. History of hypertension was defined as systolic blood pressure higher than 140 mm Hg, diastolic blood pressure higher than 90 mm Hg, or use of antihypertensive medication (41). Cognitive assessments were performed with the Cambridge Cognitive Examination scale (CAMCOG, part of the Cambridge Examination for Mental Disorders of the Elderly). For CAMCOG (range: 0–107) a score lower than 80 is rated as dementia. Subjects with dementia were assessed cognitively every 6 months, and others were assessed annually. In no case was the interval between cognitive assessment and death greater than 2 years.

**Rating of Leukoaraiosis Severity**

Severity of leukoaraiosis from in-life computed tomography scans was independently rated by 2 radiologists who were blinded to clinical data, as previously described (40, 41). Briefly, leukoaraiosis in the anterior frontal, posterior frontal, parietal, and occipital cortices was graded for severity (0, none; 1, mild; 2, moderate; 3, severe) and extent (0, none; 1, periventricular leukoaraiosis; 2, periventricular and deep white matter leukoaraiosis; 3, all white matter involved). Interrater agreement for these ratings was substantial (κ = 0.63–0.79 across the different regions) (40). For each area, severity × extent was summed to obtain a total leukoaraiosis score (range, 0–36). Leukoaraiosis scores were included if they were assigned within 5 years of death (n = 47).

**Antibodies**

Primary antibodies against human fibrinogen (rabbit polyclonal A-0080) and human IgG (rabbit polyclonal A-0423) were obtained from DakoCytomation (Ely, Cambridgeshire, United Kingdom). Human IgG monoclonal antibody (mouse IgG1, clone RWP49) was obtained from Novocastra-Leica Microsystems (Newcastle-upon-Tyne, United Kingdom); the immunogen was a recombinant protein corresponding to 327 residues of human IgG. Anti-fibrinogen polyclonal (1:50000), anti-human IgG polyclonal (1:120000), and anti-human IgG monoclonal (1:2000) antibodies were diluted on the day of use in phosphate-buffered saline containing 0.1% vol/vol Triton X-100 and 3% (wt/vol) bovine serum albumin.

**Immunohistochemical Methods**

Six-micrometer-thick sections were dewaxed and processed for standard immunohistochemical labeling (36, 42). Endogenous peroxidase activity was blocked by exposure to H2O2 (3% vol/vol, aqueous solution) for 8 minutes. After high-pressure heat-induced antigen retrieval (for 30 seconds at 125°C in Tris-citrate buffer, pH 7.8), nonspecific binding was blocked with phosphate-buffered saline containing 0.1% vol/vol Triton X-100 and 3% (wt/vol) bovine serum albumin for 60 minutes at room temperature, and sections were exposed to primary antibodies at 4°C overnight. Antibody labeling was visualized using a peroxidase-conjugated secondary reagent (Envision kit, K4065; Dako, Carpenteria, CA) and diaminobenzidine chromogen, and counterstained with Mayer hematoxylin. As negative control, neighboring sections were treated with irrelevant primary antibody, rabbit anti-sheep IgG (1:200; BD-Pharmingen, Oxford, United Kingdom). Sections were examined on a Zeiss Axioplan-2 microscope driven by Axiosvision version 4.7 software.

**Neuropathologic Assessment of SVD**

Assignment to “SVD” or “aged control” group was based on microscopic examination of hematoxylin and eosin (H&E)–stained sections by a registered neuropathologist (Margaret M. Esiri or Dr. Catharine Joachim FRCPath). Characteristics of the SVD and aged control groups are shown in Table 1. Small vessel disease was defined by vasculopathy-oriented criteria, as in previous studies (36, 43). These included hyaline thickening of arteriolar walls, widened perivascular spaces, and parenchymal changes caused by SVD (perivascular pallor of myelin staining, loosening with attenuation of nerve fibers with gliosis in white matter or loss of nerve cells and gliosis in DGM) in 1 or more sections (36, 43) (Figs. 1A–D).
Sections were also independently graded using a more recent SVD severity scale that is oriented to parenchymal pathology (39). Deep gray matter structures were evaluated in sections of basal ganglia and thalamus stained with H&E. White matter structures were evaluated in sections of frontal and occipital white matter stained with H&E and Luxol fast blue/cresyl violet. Semiquantitative scores for subcortical SVD (0–3) were assigned for each region as follows: 0, normal-appearing white or gray 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 dilation of perivascular spaces; 2, more marked loss of myelin and/or loosening of parenchymal tissue, sometimes with a bubbly appearance in white matter and/or more markedly widened perivascular spaces; 3, regions of almost complete myelin loss in white matter, severe loosening of parenchymal tissue extending in places to cavitation, and severely dilated perivascular spaces. White matter SVD score (0–6) and DGM SVD score (0–6) were obtained as the summed scores of 2 sections for each region.

**Neuropathologic Assessment of Cerebral Amyloid Angiopathy**

Severity of cerebral amyloid angiopathy (CAA) in cortical tissue was graded from 0 to 4 (0, vessels devoid of amyloid; 4, severe deposition accompanied by projection of...
amyloid into the adjacent parenchyma) (44). Leptomeningeal and cortical CAA were graded in 3 regions (frontal, temporal, and parietal lobes); mean leptomeningeal, cortical, and com-
posite CAA scores were then calculated (44). Composite CAA score was used for analysis.

Quantification of Fibrinogen Labeling

An unbiased protocol was used to sample images from each fibrinogen-labeled section, giving a final sampled area (~21 mm2) for each section (Figure, Supplemental Digital Content 2, http://links.lww.com/NEN/A648). Three high-resolution TIFF images (1.73 × 106 pixels/mm2) were sampled at predetermined locations, under a 2× objective lens. Examples are shown in Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A649. In DGM, these were nonoverlapping fields within the gray matter strip bounded by the ependymal lining of the lateral ventricle and the internal capsular white matter. Fields were sampled at least 100 μm distant from these boundaries and approximately equally spaced in a lateral to medial orientation. In DWM, nonoverlapping fields were equally spaced along the longest axis of the section within DWM. Mean illumination intensity was adjusted to a constant value of 225 (arbitrary units), and white balance was normalized by the imaging software. Images were sampled on a constant 1-millisecond exposure and stored as 3,840 × 3,072 8-bit TIFF files with a spatial resolution of 0.76 μm/pixel.

Fibrinogen labeling was quantified by 2 independent methods. First, categorical scores for parenchymal cellular labeling ("CELL") were assigned on visual inspection of the 3 TIFF files by a registered neuropathologist (Margaret M. Esiri) who was blinded to all clinical and experimental data. Each image file was scored "1" if it contained a minimum of 20 clearly labeled cells within parenchymal tissue and "0" otherwise, giving a range of possible CELL scores (0, 1, 2, or 3) for each section. All cells were considered (i.e. no attempt to discriminate neurons, astrocytes, oligodendrocytes, or microglia for quantitation purposes). When grading was repeated for all cases after an interval of 40 days, intrarater repeatability was high (κ > 0.80). Agreement with another independent blinded rater (Atticus H. Hainsworth) was also high (κ = 0.81). As a second independent measure of fibrinogen labeling, the fibrinogen-positive area fraction (AF) in each TIFF image was calculated using a densitometry algorithm (NIH ImageJ free software, http://imagej.nih.gov/ij). Briefly, labeled pixels were detected using a fixed threshold detection method, and AF was expressed as 100×(number of positive pixels / total pixels). This approach has no observer input (i.e. it is unbiased) but does not exclude intravascular fibrinogen. Absence of intravascular fibrinogen labeling was assumed to reflect loss of antigenicity and was used as an exclusion criterion (7 cases were excluded for this reason).

Statistical Analysis

Statistical analysis was carried out in R (version 2.14; http://www.R-project.org/). Kendall tau-b rank correlation coefficient (τ) was used to test for association, given the presence of collapsed ordinal variables.

RESULTS

Neuropathologic Assessment of Serum Markers

All cases reported were positive for intravascular fibrinogen, suggesting that antigenicity was intact (n = 84). Some degree of extravascular fibrinogen labeling was a frequent finding in DGM and DWM either as perivascular “collars” or as parenchymal cellular or axonal labeling (Figs. 1E–G; Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A649). In some instances, cellular labeling was restricted to perivascular distribution. In perivascular collars, the labeling intensity for fibrinogen was well-fitted by a decaying exponential function of distance from the outer aspect of the vessel wall (Figure, Supplemental Digital Content 4, http://links.lww.com/NEN/A650), consistent with a pattern of diffusion away from the vessel. Cellular labeling was seen in glial cells and axons (Figs. 1F, G) and rarely seen in neuronal somata (data not shown).

The patterns of human IgG labeling in neighboring sections, using either polyclonal or monoclonal IgG antibodies, were similar to that of fibrinogen (Figs. 11–K). Neighboring sections treated with no primary antibody, an irrelevant primary antibody (anti-CD34 monoclonal antibody; Fig. 1L), or polyclonal rabbit anti-sheep IgG (data not shown) did not exhibit this extravascular labeling pattern.

As positive control for fibrinogen and IgG, 2 cases of neuromyelitis optica (NMO) were also examined. Extravascular labeling with fibrinogen and IgG was a feature of NMO tissue. These were particularly evident within NMO lesional areas, defined by depletion of AQP4 and glial fibrillary acidic protein–positive astroglia (Figure, Supplemental Digital Content 5, http://links.lww.com/NEN/A651).

Postmortem processes are a potential confounding factor in exploring serum markers within brain tissue. We therefore examined tissue from a collection of aged brains with very short postmortem interval (PMI) derived from another tissue bank (Institute for Memory Impairments and Neurological Disorders, University of California at Irvine; PMI <6 hours; n = 10). Fibrinogen labeling of parenchymal cells, perivascular collars, and axonal fibers was confirmed as a frequent finding in these cases with short PMI (Figure, Supplemental Digital Content 6, http://links.lww.com/NEN/A652).

Quantitative Assessment of Fibrinogen Labeling

For the Oxford-based cohort of aged cases (n = 84) (39), we performed a semiquantitative analysis of fibrinogen labeling using 2 independent measures (CELL score and fibrinogen-positive AF). Fibrinogen was used for all quanti-
tative methods were robust (Table 2). Fibrinogen-positive AF for DGM was not associated with that for DWM (τ = 0.04, p = 0.74). Fibrinogen-derived AF and CELL score did not differ between SVD and aged control cases (Figs. 2A, B; Table 2). A recently validated regional SVD severity scale based on SVD-associated parenchymal tissue changes (39) showed no association with fibrinogen AF or CELL score...
In life, history of hypertension was associated with AF (Fig. 2C) and CELL score (Fig. 2D) in DGM, but not in DWM (Table 2). Postmortem interval and age at death did not differ between the aged control group and the SVD group (Table 1), and both showed no association with fibrinogen (Table 1). Specifically, extravascular fibrinogen was absent in some “oldest-old” cases (older than 80 years; example shown in Fig. 1H). History of dementia showed a modest association with AF in DWM (Fig. 2E), but not in DGM (Table 2). Severity of CAA based on neuropathologic assessment (44) showed a modest association with CELL score in DWM (Fig. 2F), but not in DGM (Table 2).

DISCUSSION

We assume that large plasma proteins (fibrinogen and IgG) are histologic markers for BBB dysfunction and that parenchymal cell labeling reflects cellular uptake of plasma components, as demonstrated in animal studies (45–47).

Some degree of extravascular fibrinogen and IgG labeling was a frequent finding in this study. In both DGM and DWM, no measure of fibrinogen labeling differed between aged control subjects and SVD subjects or was associated with a neuropathologic score of SVD severity (39) or with in-life leukoaraiosis severity.

Fibrinogen as a Marker of BBB Dysfunction

Fibrinogen is a large plasma glycoprotein (340 kDa) that is synthesized in the liver (48). It was assumed to be a faithful marker of BBB dysfunction for several reasons. First, many sections exhibited a clearly perivascular pattern of labeling (either intracellular or diffuse), suggesting vascular leakage. Second, in tissue from NMO cases, fibrinogen labeling overlapped with astrocytic lesions. Neuromyelitis optica is a rare condition with known molecular pathology, specifically autoantibodies to the water transporter AQP4.

TABLE 2. Test of Association With Fibrinogen-Positive Area Fraction and CELL Score for DGM and DWM

|                   | DGM       | DWM       |
|-------------------|-----------|-----------|
|                   | AF        | CELL Score|
|                   | \(\tau\)  | \(p\)     | \(\tau\) | \(p\) |
| Age at death      | \(-0.037\) | 0.65      | \(-0.037\) | 0.69 |
| History of hypertension | 0.19 | 0.047*    | 0.27    | 0.013* |
| Subgroup (SVD or AC) | \(-0.11\) | 0.25      | 0.045   | 0.68 |
| SVD severity score\(\dagger\) | \(-0.038\) | 0.66      | 0.088   | 0.36 |
| CAA severity\(\ddagger\) | \(-0.031\) | 0.73      | 0.003   | 0.98 |
| History of dementia | \(-0.07\) | 0.48      | 0.005   | 0.90 |
| PMI               | 0.121     | 0.13      | 0.022   | 0.81 |
| AF                | —         | —         | 0.52    | <0.001§ |

Kendall tau-b rank correlation coefficients (\(\tau\), a measure of association) and levels of significance (\(p\)) are presented.

*\(p < 0.001\).

**Smallwood et al (39).

\(\dagger\)Chalmers et al (44).

\(\ddagger\)Menides et al (40).

FIGURE 2. Quantitative assessment of fibrinogen immunolabeling. (A, B) Fibrinogen-positive AF (range, 0%–100%) and distribution of fibrinogen-labeled cell scores (CELL scores 0, 1, 2, or 3) did not differ between SVD subjects and aged control subjects (AC). Data are shown for DGM. (C, D) Within DGM, fibrinogen-positive AF (C) and distribution of fibrinogen-labeled CELL scores (D) were significantly higher in subjects with documented histories of hypertension (HT) versus those who were documented as being normotensive (NT). (E) Within DWM, fibrinogen-positive AF was greater in those subjects with a documented history of dementia than in subjects without dementia (non-dem). (F) Within DWM, the distribution of fibrinogen-labeled CELL scores increased with severity of neuropathologic CAA score (range, 0–24). Box-whisker plots show median, interquartile range, and full range.

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Extravascular Fibrinogen Was a Common Finding

Our data suggest that BBB abnormalities are a common feature in the DGM and DWM of older people but are possibly limited in location and duration. Although this finding conflicts with the BBB concept, numerous previous reports support some tight junction abnormality and BBB dysfunction in “healthy” human brains (14, 16, 21, 22, 26–28, 31). In a blinded quantitative study in which quantitation of cell labeling was performed, substantial IgG-positive pericymal cell numbers (~400 cells/mm²) were noted in normal nonlesional DWM (16). In pericymal vessels (>20 μm in diameter) of the frontal cortex, the fraction of vessels that were positive for intramural fibrinogen was high (~30%) in aged nondemented control subjects, further supporting the notion that plasma extravasation is common in aged brain tissue (28). These reports (and ours) on plasma leakage agree with quantitative studies of tight junction integrity. On average, 14% of small vessels with structurally abnormal tight junctions (labeled for the tight junction protein ZO-1) were detected in healthy white matter from control subjects (22).

Penetration of the BBB by circulating IgG is assumed in the concept of immunotherapy for brain disease (49–52). Brain uptake of IgG and other macromolecules may occur transcellularly or via “leaky” tight junctions (50, 51). Although brain endothelial cells express the IgG receptor FcR, brain penetration by IgG in FcR-deficient mice is similar to that in wild-type animals (53). This suggests that transjunctional entry via leaky (possibly temporarily-leaky) tight junctions is a more likely route for brain access by circulating antibodies.

Fibrinogen and its breakdown products are cleared from brain tissue by a local tissue plasminogen activator/plasminogen system (24, 54). The parenchymal cell labeling seen in our study and in those by others (13–15, 18) may be a cytotoxic process, as suggested in animal models (47, 55). Alternatively, it could reflect a protective mechanism used by long-lived brain cells to sequester potentially-harmful plasma proteins (1, 22).

BBB Dysfunction and SVD

Neuropathologic SVD severity was strongly correlated between DGM and DWM, suggesting that SVD proceeds in parallel in these 2 tissue regions. We found no association of fibrinogen with neuropathologic measures of SVD (either in DGM or in DWM) or with in-life leukoaraiosis severity in DWM. Similarly, coverage with the tight junction protein claudin-5 did not differ between the SVD group and the aged control group. Thus, our data do not support the idea that ongoing BBB dysfunction is a feature of SVD (12, 37, 56). Our findings conflict with some prior reports (14, 15) and agree with others (16, 20). The lack of association seen here does not exclude a possible role for BBB abnormality earlier in life, possibly as an SVD-initiating “trigger” event.

Comparison With Neuroimaging Data

Several radiologic studies have reported partition of intravascular contrast agent into brain tissue, interpreted as BBB dysfunction, in patient groups with SUV or vascular cognitive impairment (11, 12, 38, 56, 57). Some of these studies did not support a significant association of marker extravasation with disease (38, 56, 57). It seems most likely that brain endothelia handle MRI contrast agents (molecular weight <1,000) differently from plasma proteins (e.g. fibrinogen, IgG; molecular weight >150,000). Transgenic mice that lack claudin-5 have brain vessels that are impermeable to large plasma proteins but permit leakage of a small contrast agent molecule (58). Such imperfect tight junctions in elderly human brains might explain the conflict between MRI data suggesting an association of plasma leakage with SVD (11, 12), and neuropathologic data from our study and others (16, 20) showing no such association.

Other Clinical Variables

We observed an association of DGM fibrinogen with history of hypertension, which might reflect long-term use of antihypertensive medications. We also found an association of DWM fibrinogen with clinical history of dementia. This may reflect a neurotoxic action of fibrinogen in nerve cells, axons, and myelin, as demonstrated by in vivo studies (47, 55).

We saw a modest association of cellular fibrinogen in DWM with increasing CAA. This is in accordance with other evidence for a link between amyloid-related disease and BBB integrity (19, 28, 29). Experiments using transgenic mice clearly indicate a role for the AD-associated proteins APP and ApoE in BBB function (46, 59, 60). In studies of AD patients, the proportion of CAA-positive vessels in cortical gray matter strongly correlated with the presence of intramural fibrinogen (28), and the plasma protein prothrombin was detected in neurons, vessel walls, and perivascular tissue (19, 29). Pericyte-dependent signaling seems to be a key factor linking ApoE status with BBB function (45, 46), and pericyte degeneration was strongly associated with BBB abnormalities in the brains of AD patients (30).

The strengths of this study are as follows: 1) a well-powered cohort with detailed in-life clinical assessment; 2) use of 2 independent robust markers (fibrinogen and IgG); and 3) use of 2 independent measures of fibrinogen labeling.
(categorical CELL scoring by a blinded neuropathologist and automated AF). The study has several caveats. First, the cohort size is limited. Second, the cohort is a well-medicated middle-income group, possibly unrepresentative of populations where risk factors are less well-controlled. Third, we have not addressed membrane transporters that contribute to BBB function by exporting potential toxins from brain endothelial cells into plasma (1, 27).

In conclusion, some degree of extravascular fibrinogen and IgG, assumed to reflect plasma extravasation, is a frequent finding in the DGM and DWM of older people without AD. Measures of fibrinogen labeling are not associated with SVD.

ACKNOWLEDGMENTS

We gratefully acknowledge the tissue donors and their families for use of donated tissue. We thank our colleagues in: the Oxford Project to Investigate Memory and Ageing; Thomas Willis Oxford Brain Collection; Cellular Pathology, St George’s Healthcare NHS Trust; and Institute for Memory Impairments and Neurological Disorders, University of California at Irvine.

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