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EXTRAVASCULAR FIBRINOGEN IN THE WHITE MATTER OF ALZHEIMER'S DISEASE AND NORMAL AGED BRAINS: IMPLICATIONS FOR FIBRINOGEN AS A BIOMARKER FOR ALZHEIMER'S DISEASE

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Abstract

The blood-brain barrier (BBB) regulates cerebrovascular permeability and leakage of blood-derived fibrinogen has been associated with cerebral arteriolosclerosis small vessel disease (SVD) and subsequent white matter lesions (WML). Furthermore, BBB-dysfunction is associated with the pathogenesis of Alzheimer's disease (AD) with the presence of CSF plasma proteins suggested to be a potential biomarker of AD. We aimed to determine if extravascular fibrinogen in the white matter was associated with the development of AD hallmark pathologies, i.e., hyperphosphorylated tau (HPτ) and amyloid-β (Aβ), SVD, cerebral amyloid angiopathy (CAA) and measures of white matter damage.

Using human *post-mortem* brains, parietal tissue from 20 AD and 22 non-demented controls was quantitatively assessed for HPτ, Aβ, white matter damage severity, axonal density, demyelination and the burden of extravascular fibrinogen in both WML and normal appearing white matter (NAWM). SVD severity was determined by calculating sclerotic indices.

WML- and NAWM fibrinogen burden was not significantly different between AD and controls nor was it associated with the burden of HPτ or Aβ pathology, or any measures of white matter damage.

Increasing severity of SVD was associated with and a predictor (both p < 0.05) of both higher WML- and NAWM fibrinogen burden (both P<0.05) in controls only. In cases with minimal SVD NAWM fibrinogen burden was significantly higher in the AD cases (p<0.05).

BBB dysfunction was present in both non-demented and AD brains and was not associated with the burden of AD-associated cortical pathologies. BBB dysfunction was strongly associated with SVD but only in the non-demented controls. In cases with minimal SVD, BBB dysfunction was significantly worse in AD cases possibly indicating the influence of CAA. In conclusions, extravascular fibrinogen is not associated with AD hallmark pathologies but indicates SVD, suggesting that the presence of fibrinogen in the CSF is not a surrogate marker for AD pathology.

Abbreviations

WML; white matter lesions; WMH, white matter hyperintensity; MRI, magnetic resonance imaging; SVD, cerebral small vessel disease; AD, Alzheimer's disease; HPτ, hyperphosphorylated tau; Aβ, amyloid-beta; CAA, cerebral amyloid angiopathy; VaD, vascular dementia; BBB, blood-brain barrier; PVS, perivascular space; LFB, luxol fast blue; IOD, integrated optical density; BiA, Bielschowsky's percentage area; NAWM, normal appearing white matter; IR, immunoreactivity; SI, Sclerotic Index

Introduction

The blood brain barrier (BBB) is an essential physical and functional barrier formed by the endothelial cell layer of the *tunica intima* and its associated tight junctions controlling the paracellular flow of water, ions and large molecules into the brain from the blood [8, 52]. Together with pericytes, which envelope endothelial cells on the abluminal side of capillaries and venules, connections with astrocytic foot processes (of which are connected to neurones) and microglia, this comprises the neurovascular unit [1, 8]. The BBB protects the central nervous system from blood-born agents and thereby plays a crucial role in the maintenance of neuronal and glial cell populations. Sporadic type 1 arteriolosclerosis cerebral small vessel disease (SVD) has been implicated in BBB dysfunction [16, 35] as degenerative changes to white matter artery/arteriole walls as a result of increased cerebral arterial pressure may lead to leakage of blood-derived plasma proteins into the vessel walls and the perivascular space (PVS). The exact neurotoxic effects of plasma proteins are still unclear but it is postulated that plasma protein leakage into the cerebral parenchyma initiates an inflammatory response mediated by the resident microglia and astrocytes [22, 40]. Plasma protein leakage into the deep white matter has been shown to be associated with worsening of SVD pathology that may lead to an increase of ischemia-associated white matter damage [37]. Histological cerebral white matter lesions (WML) are reflected by white matter hyperintensities (WMH) on *pre-* and *post-mortem* T2-weighted magnetic resonance imaging (MRI) [17] and primarily encompass white matter rarefaction due to demyelination with or without axonal loss [16]. WML are assumed to represent ischemia-associated damage due to SVD of the white matter arteries and arterioles [36]. However, we have shown recently that parietal WML can differ in their pathological and molecular signatures between Alzheimer's disease (AD) and non-demented controls suggesting different aetiologies of WML, i.e., degenerative axonal and myelin loss in AD and ischemia resulting from SVD [31]. Therefore, understanding of all mechanisms involved in WML pathogenesis is important as their presence is included in the clinical criteria for VaD/VCI [15] and mixed AD/VaD [12].

BBB dysfunction and leakage of various plasma proteins have been proposed to play a role in the onset and progression of AD via alterations of the neurovascular unit and secretion of neurotoxic substances that can promote the development of amyloid-β (Aβ) pathology [13]. Neuropathological studies have confirmed a higher prevalence of BBB dysfunction in AD compared to non-demented controls [20, 47, 59], and therefore the presence of plasma proteins in CSF has been suggested to be a potential biomarker of AD: human *in vivo* studies of BBB dysfunction, as determined by the leakage of plasma protein albumin into the CSF (i.e., albumin:CSF ratio), have revealed increased BBB permeability in demented compared to non-demented patients [14] and plasma fibrinogen γ-chain in the CSF was identified as a specific biomarker for AD over other neurodegenerative disorders [7].

Using *post-mortem* brain tissue from both AD and non-demented individuals we aimed to *i)* investigate the relationship of SVD and BBB dysfunction, as determined by extravascular fibrinogen *ii)* investigate the relationship between extravascular fibrinogen with measures of white matter damage *iii)* and to determine if the presence of extravascular fibrinogen was associated with the presence of AD hallmark cortical pathology.

Materials and methods

Study cohort

Our study cohort consisted of a 42 serially donated human *post-mortem* brains clinico-pathologically diagnosed as AD (n=20) or non-cognitively impaired controls (n=22) and contained at least one deep WML in the parietal lobe as determined and assessed for severity by *post mortem* T2 MRI as previously described [30]. Cases that exhibited any large infarcts or cerebral hemorrhages that could account for cognitive impairment were excluded.

During life, all dementia subjects underwent clinical assessments and were diagnosed by board certified Old Age Psychiatrists or Neurologists. Both dementia and control subjects had a review of clinical records after death at Newcastle Brain Tissue Resource (AJT). Control subjects showed no evidence of cognitive impairment and had normal everyday functioning. After autopsy the right hemisphere, brainstem and cerebellum were immersion fixed in 10% buffered aqueous formaldehyde solution for 6 weeks and then subsequently dissected in coronal planes at 7mm intervals and paraffin-embedded. All brains underwent neuropathological assessment according to the National Institute on Aging-Alzheimer's Association (NIA-AA) criteria [34] that included determination of Thal phases of Aβ deposition [45], Braak staging of hyperphosphorylated tau (HPτ) pathology

[4] and scoring of neuritic plaques according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) [33]. Additional neuropathological assessment of Lewy body pathology [5, 32], TDP-43 pathology [21] and of the contribution of vascular pathology to cognitive impairment (vascular impairment neuropathological guidelines (VCING) [44]), inclusive of cerebral amyloid angiopathy (CAA) scores [27], was performed. Cases did not exhibit any additional pathology including primary or secondary tumours or age-related tau astrogliopathy (ARTAG) [24]. For a summary of the demographic and neuropathological details please see table 1.

Histological and immunohistochemical procedures

Paraffin-embedded serial sections from parietal blocks (Brodmann area 40/22) were cut at 8μm thickness and mounted onto superfrost plus charged glass slides (Thermo Shandon, Cheshire, UK). Sections underwent histochemical staining with Bielschowsky silver stain (cold method) [26] for assessment of axonal density, myelin stain luxol fast blue (LFB) to assess WML area (WMLA: as a measure of WML severity) and myelin pallor (as a measure of demyelination) and haematoxylin and eosin (H&E) for visualisation of white matter artery/arteriole walls for assessment of SVD. Immunohistochemistry was performed on all sections for plasma protein fibrinogen (antibody anti- human fibrinogen), HPτ (antibody AT8) and Aβ peptide (antibody 4G8) (details on primary antibodies and antigen retrieval protocols for immunohistochemistry are presented in Table 2). Immunopositivity was detected using the Menarini X-Cell-Plus HRP Detection Kit (Menarini Diagnostics, Winnersh-Wokingham, UK) with 3,3 diaminobezidine (DAB) as a chromagen and haematoxylin as a counter stain. All histologically and immunohistochemically stained sections were subsequently dehydrated through a series of alcohols, cleared and mounted using DPX (CellPath, Powys, UK).

Quantitative image analysis

All image analysis was performed blinded to neuropathological diagnosis using a NIS elements version 3.0 (Nikon, Surrey, UK). Quantitative assessment of WMLA, the percentage area of Bielschowsky staining and the integrated optical density (IOD) in the WML area of LFB stained sections was performed as previously described [31]. The mean percentage area of Bielschowsky's stain in the WML was expressed as WML-BiA (axonal density) and the mean percentage area difference of IOD in the WML was expressed as WML-IOD (demyelination).

AT8 and 4G8 immunostained tissue sections were quantified for pathological burden using individual and standardized Red Green Blue (RGB) thresholds as previously described in [29, 31]. The percentage areas covered by AT8 and 4G8 immunoreactivity (IR) were measured and the mean values were calculated and are expressed as AT8-IR and 4G8-IR, respectively. Extravascular fibrinogen IR was measured in both the WML and normal appearing white matter (NAWM): the WML area was identified macro- and microscopically on LFB stained sections and delineated by hand using a permanent marker pen to differentiate the WML and NAWM. These sections were subsequently used to identify the corresponding areas in the adjacent fibrinogen stained sections. Separately in both the WML and NAWM areas, five 3x3 images at 100x magnification (combined to yield one large image representing an area of 1.31 mm^2) were captured. Large images were subjected to manual setting of regions of interest to include extravascular fibrinogen-IR only and exclude artery/arteriole vessel lumens (not including capillaries) as well as artifacts and regions of cortex. Standardized RGB thresholds for extravascular fibrinogen-IR were applied to the large images and the area covered by fibrinogen-IR was measured, a percentage of the total measured area calculated and expressed as WML fibrinogen-IR or NAWM fibrinogen-IR (Figure 1).

The sclerotic index (SI) calculates vessel wall thickness and is a marker of SVD severity [25] with the SI of normal arteries/arterioles between 0.2-0.29, mild SVD is a value of 0.3-0.39, moderate SVD a value of 0.4-0.49 and severe SVD is a value ≥0.5. SI measurements were performed on approximately 8 white matter arteries/arterioles as seen on H&E stained sections as previously described [31, 55]. A mean SI score was calculated for each case.

Statistics

The Statistical Package for Social Sciences software (SPSS ver. 21) was used for statistical evaluation. Variables were tested for normality using the Shapiro-Wilk test and visual inspection of variable histograms. Age and SI measurements exhibited a normal distribution, therefore parametric tests were employed; all other variables, i.e., *post mortem delay*, CAA score, AT8-IR, 4G8-IR, WML-BiA, WML-IOD, WML Fibrinogen-IR, NAWM Fibrinogen-IR and WMLA exhibited a non-normal distribution and non-parametric tests were employed. A Chi-square (χ^2) test for independence (with Yates continuity Correction) was employed to explore the relationship between categorical variables. A one-way between-groups multivariate analysis of variance (MANOVA), including preliminary assumption testing (i.e., normality, linearity, outliers, homogeneity of variance-covariance matrices and multicollinearity), was performed to investigate differences in measures of white matter and fibrinogen between AD and controls, whilst controlling for Type I error. Where appropriate, group effects were

assessed using either Mann-Whitney U (non-parametric) or independent samples t-test procedures (parametric). Spearman's (r_s; non-parametric) or Pearson's (r; parametric) correlation coefficients were used to assess associations between variables, as well as partial correlations (r_p) to control for the effects of age. Linear regression analyses were also conducted to investigate predictors of WML- and NAWM fibrinogen-IR.

Results

No significant differences were observed in mean age ($t_{(40)}$ p = 0.143) or *post-mortem* delay ($t_{(26)}$ p = 0.209) between AD and control groups. Clinicopathological diagnosis was not associated with gender ($\chi^2_{(1)}$ p = 0.763). Fibrinogen-IR was observed in the vessel walls of small arteries/arterioles of the white matter (Figure 2a), axons (Figure 2b), the endothelia cell layer of white matter capillaries (Figure 2c and ci) and PVSs (Figure 2d and di). In addition, fibrinogen positive cells that presented with a hypertrophic astroglial-like morphology were present in the white matter, the cortical-white matter border and PVS (Figure 2e and ei).

A MANOVA was performed to investigate differences in pathological measures between AD and controls. Seven dependent variables were used: CAA, SI, WMLA, WML-BiA, WML-IOD, WML fibrinogen-IR and NAWM fibrinogen-IR, and clinicopathological diagnosis (i.e., AD or control) as the independent variable, and preliminary assumptions were not violated. A statistically significant difference was observed between AD and controls on the combined dependent variables (F= 4.009, p = 0.003; Wilks Lambda = 0.525; partial eta squared = 0.375). When considered separately, using a Bonferroni's adjusted level of 0.01, CAA (F = 19.889; p = 0.0001, partial eta squared = 0.350) and WMLA (F = 12.187; p = 0.001, partial eta squared = 0.248) were significantly different and inspection of mean scores indicated that both CAA scores (M = 3.471, SD = 0.447) and WMLA scores (M = 34.616, SD = 2.706) were significantly higher in the AD group compared to the controls (CAA, M = 0.818, SD = 0.394: WMLA, M = 22.037, SD = 2.379). No difference was seen in means scores of SI (p = 0.692), WML-BiA (p = 0.559), WML-IOD (p = 0.051), WML fibrinogen-IR (p = 0.954) or NAWM fibrinogen-IR (p = 0.946).

Associations between extravascular fibrinogen, CAA, SVD, and white matter pathologies

Using correlation coefficient tests, we examined the relationships between NAWM and WML fibrinogen-IR with CAA, SI, WMLA, WML-BiA and WML-IOD (please see Table 3). Briefly, as a whole cohort, SI score both was positively correlated with WML fibrinogen-IR (r = 0.263, p = 0.048) and NAWM fibrinogen-IR (r = 0.310, p = 0.026), but when correlations were performed separately based on clinicopathological diagnosis these associations were consistent only in the control group (WML, r = 0.487, p = 0.011; NAWM, r = 0.383, p = 0.039) and not in the AD group. NAWM fibrinogen-IR was associated with CAA (r_s = 0.291, p = 0.033) with regards to the whole cohort.

To investigate whether SI severity independently predicted fibrinogen-IR, linear regression analysis was performed; the dependent variable was WML or NAWM fibrinogen-IR and the independent variable was SI. Regarding the whole cohort, SI was a significant predictor of NAWM fibrinogen-IR (model R^2= 0.195, F = 9.18; β = 0.441, p = 0.004), in contrast to WML fibrinogen-IR (model R^2= 0.085, F = 3.5; β = 0.292, p = 0.068). Linear regression was again performed dichotomized by clinicopathological diagnosis; SI was found to be a significant predictor of both WML fibrinogen-IR (model R^2= 0.237, F = 6.21; β = 0.487, p = 0.022) and NAWM fibrinogen-IR (model R^2= 0.300, F = 8.571; β = 0.548, p = 0.008) in the control group but not the AD group (WML, model R^2 = 0.067, p = 0.301; NAWM, model R^2 = 0.022, p = 0.557).

The severity of SVD and the amount of extravascular fibrinogen

To further investigate the role of SVD, cases were divided into normal-mild SVD (SI value <0.39; AD, n = 10; controls, n = 14) moderate to severe (SI value >0.39; AD, n =9; controls, n = 8). Overall, both WML fibrinogen-IR and NAWM fibrinogen-IR were higher in cases with moderate-severe SVD but this did not reach statistical significance (WML, p = 0.075; NAWM, p = 0.052) (Figure 3a-b). When separated by clinicopathological diagnosis controls had significantly higher WML fibrinogen-IR in cases with moderate-severe SVD (p = 0.019; Figure 3c). NAWM fibrinogen-IR was also higher in the moderate-severe SVD cases compared to the normal-mild, but this was not significant (p = 0.069; Figure 3d). No differences where seen in either WML or NAWM fibrinogen-IR in AD cases (WML, p = 0.823; NAWM, p = 0.532; Figure 3c-d). We then investigated the difference in extravascular fibrinogen at different SVD severities across groups: in cases with normal-mild SVD NAWM fibrinogen-IR was significantly higher in AD cases compared to controls (p = 0.045; Figure 3d), WML fibrinogen-IR was higher in the

AD cases but was not significant (p = 0.059; Figure 3c). Regarding cases with moderate-severe SVD no difference in WML or NAWM fibrinogen-IR was seen (WML, p = 0.424; NAWM, p = 0.752; Figure 3c-d). Linear regression analysis was performed to investigate if a clinicopathological diagnosis of AD was a predictor of WML- and NAWM fibrinogen-IR in cases with normal-mild SVD; no significant association was revealed in for either measure of fibrinogen-IR (WML, model $R^2 = 0.368$, p = 0.076; NAWM, model $R^2 = 0.224$, p = 0.294).

Relationships between extravascular fibrinogen and the presence of hallmark AD pathology

To determine if increasing extravascular fibrinogen was associated with the development of AD pathology a partial correlations controlled for age were employed to examine the relationships between both whole cohort values of WML and NAWM fibrinogen-IR with AT8-IR, 4G8-IR as well as Braak NFT stage and Thal Aβ Phase. No associations were revealed with either WML or NAWM fibrinogen-IR with AT8-IR (WML, r_p = 0.035, p = 0.417; NAWM, r_p = -0.039, p = 0.410), 4G8-IR (WML, r_p = 0.086, p = 0.306; NAWM, r_p = -0.114, p = 0.250), Braak NFT stage (WML, r_p = 0.03, p = 0.493; NAWM r = 0.011, p = 0.474) or Thal Aβ Phase (WML, r_p = 0.088, p = 0.301; NAWM, r_p = 0.079, p = 0.322).

Discussion

In the cerebral deep white matter, BBB dysfunction and the leakage of 'neurotoxic' blood-derived plasma proteins, is proposed to initiate inflammatory responses in the surrounding white matter parenchyma, exacerbating the development of WML. In agreement with previous human neuropathological studies [6, 18, 57], we found no associations between extravascular fibrinogen measure in the WML itself or the surrounding NAWM with WML severity or specifically axonal loss or demyelination in either AD or non-demented individuals. This supports the notion that AD related degenerative mechanisms and hypoxia, as a consequence of type 1 arteriolosclerosis SVD, have the strongest impact on the development of WML [31]. Other serum components that may leak out of blood vessel not investigated in this study, e.g., complement and cytokines, have deleterious effects, specifically causing demyelination [43] and should be included in future studies.

Although fibrinogen is currently under investigation as a biomarker for Alzheimer's disease, we found no association between extravascular fibrinogen with increasing deposition of the AD hallmark cortical pathologies HPτ and Aβ. It has been suggested that BBB impairment induced by cerebrovascular disease can influence the pathogenesis of AD [58] with both human neuropathological studies and murine AD models indicating fibrinogen deposition can contribute to neurovascular damage [20, 41], neuroinflammation [38] and neuronal degeneration [9] that may influence the pathogenesis of AD. However, our findings suggest that fibrinogen does not reflect the pathological burden of AD pathology and therefore, may not be an accurate diagnostic tool for the staging of AD progression.

A key finding from this study was the relationship between SVD and extravascular fibrinogen that differed between non-demented controls and AD groups. In general, when cases were dichotomized by the severity of SVD pathology only, a strong trend for higher WML and NAWM extravascular fibrinogen was seen in cases with moderate to severe SVD compared to those without or mild SVD. When stratified by clinicopathological diagnosis, increasing severity of SVD was associated with and a significant predictor of both increasing WML- and NAWM extravascular fibrinogen but only in the controls. Firstly, this data supports the notion that SVD is strongly associated with BBB dysfunction as previous indicated [14], and, therefore, indicates that the presence of fibrinogen is an indicator of underlying SVD. Secondly, a lack of association in the AD group suggests that, as well as SVD, another mechanism maybe impacting the BBB and leading to fibrinogen leakage. In fact, AD cases without or mild SVD had significantly higher fibrinogen in the NAWM compared to controls, and a strong trend was observed with WML fibrinogen, compared to the controls. In addition, although not significant, linear regression revealed a strong trend of a diagnosis of AD as predictor of WML fibrinogen burden in cases without or mild SVD. This indicates a potential heterogeneity in the mechanism underlying BBB dysfunction that maybe associated to the pathophysiology of AD. A potential influence is CAA; classified as type 2 SVD [31], it is characterized by Aβ deposition (predominately Aβ-40) in the vessel walls of cerebral blood vessels [44] and is prevalent in approximately 80% of AD cases [2]. Our data revealed an association between increased severity of CAA and increasing NAWM fibrinogen levels. This is in line with transgenic mouse model studies have indicated that Aβ accumulation contributes to impairment of tight junction proteins expression, therefore, compromising BBB integrity [10, 19]. Although CAA rarely affects white matter vessels, CAA impairs vasoreactivity [11] with consequential white matter hypoxia that has been shown to induce BBB disruption as a result of HIF-1α-mediated alterations of key tight junction proteins [53]. In addition, perivascular drainage (PVD), driven by the motive forces of vascular pulsations

[51], of interstitial fluid and solutes through the basement membranes of capillary endothelia cells and the *tunic media* in arteries is a key drainage pathway of CNS fluids. As a result of age and CAA, vessels can become stiff [39, 50] leading to a decrease in the amplitude and force of pulsation and, consequently, a reduction in PVD that may lead to reduced clearance of extravascular fibrinogen and contribute to the increased accumulation of fibrinogen observed in the NAWM of AD patients with minimal SVD. Further studies implementing a quantitative assessment of CAA burden are warranted to investigate further the influence of CAA on BBB permeability given is high prevalence in AD.

Numerous immunohistochemical studies have found a positive association between markers of plasma proteins and a dementia diagnosis [18, 20, 47, 48], which has led to the intensive investigation of plasma proteins as a possible biomarker of AD. Increased plasma fibrinogen γ-chain has been shown to be specific for AD [7, 23] as well as a risk factor for conversion of MCI to AD [46, 48, 54, 56]. However, our data found no significant differences in the amount of fibrinogen between AD and non-demented subjects in agreement with previous studies [2, 28, 42]. Furthermore, it is important to note that SVD is also present in normal ageing and our data indicated no significant difference in SVD severity between AD and non-demented controls. Given the strong association with type 1 SVD, and the potential link with CAA, increased levels of extravascular fibrinogen are likely associated with underlying cerebrovascular disease that is not necessarily exclusive to an AD diagnosis. However, the total variance of BBB dysfunction explained by type 1 SVD overall is 19.5% indicating other factors associated with BBB dysfunction and AD pathophysiology remains to be elucidated.

In conclusion BBB dysfunction was present in both non-demented and AD brains and was not associated with the burden of AD-associated cortical pathologies. BBB dysfunction was strongly associated with SVD but only in the non-demented controls. In cases with minimal SVD BBB dysfunction was significantly worse in AD cases indicating the influence of other cerebrovascular diseases, most likely CAA, as well as other mechanisms of BBB dysfunction possibly associated with the pathophysiology of AD that are yet to be determined. Therefore, extravascular fibrinogen likely indicates underlying age-associated cerebrovascular disease, specifically SVD that is not necessarily exclusive to an AD diagnosis, and suggests that the presence of fibrinogen in the CSF is not a surrogate marker for AD pathology. The validity of fibrinogen as a biomarker of/risk of AD requires larger longitudinal studies, in conjunction with validated biomarkers of AD and cerebrovascular disease and neuropathological confirmation.

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Figure legends

Figure 1

a) WML areas were identified on LFB stained sections of parietal tissue and delineated (red dotted line). This was used to identify the same WML and NAWM (black dotted line) area on serial sections stained with fibrinogen (b). b) The fibrinogen stained section of parietal tissue indicating the five randomly selected areas for a 3x3image capture in the WML and NAWM areas (black boxes). c) A 3x3 large image acquisition in WML area. d) Image c again with applied bespoke fibrinogen threshold including region of interest for removal of vessel and PVS (green line). Scale bar, 50mm, valid for all images in a and b; 100μm, valid for c and d. WML, white matter lesions; LFB, luxol fast blue; NAWM, normal appearing white matter; PVS, perivascular spaces.

Figure 2

Photomicrographs illustrating fibrinogen immunoreactivity in white matter and its vasculature. a) A small artery exhibiting fibrinogen in the vessel wall in both the *tunica media* (arrow) and the *tunica intima* (arrowhead) indicating leakage of fibrinogen. b) Swollen white matter axons positive for extravascular fibrinogen (arrow). c) A capillary exhibiting fibrinogen immunopositivity contained within the endothelial wall in the transverse plane. ci) A capillary exhibiting sustained leakage of fibrinogen into the endothelial wall, basement membrane and surrounding white matter parenchyma (arrowhead). d) A moderately dilated PVS (arrow) with accumulation of extraversion of fibrinogen in the white matter parenchyma (red arrowheads). di) Magnified photomicrograph of extraverted fibrinogen in the surrounding white matter parenchyma (black arrowhead). e) Fibrinogen positive cells in the deep white matter (black arrowhead) and lining a dilated PVS (red arrowhead). ei) Magnified photomicrograph of a deep white matter fibrinogen positive cell with the morphology of a hypertrophic fibrous astrocyte; immunoreactivity is present in both the cell soma (arrow) and cellular processes (arrow head). BBB, blood-brain-barrier; PVS, perivascular space. Scale bars, 200μm, valid for d and e; 100μm, valid for a and b; 20μm, valid for di and ei; 10μm valid for c and ci.

Figure 3

a, b) Regarding the whole cohort, WML and NAWM fibrinogen-IR was not significantly higher in cases with moderate to severe SVD compared to cases with none-mild SVD. c) WML fibrinogen-IR was significantly higher in control cases with moderate-severe SVD compared to control cases none-mild SVD. d) NAWM fibrinogen-IR was significantly higher in AD case with non-mild SVD compared to control cases with none-mild SVD. WML, white matter lesion; NAWM, normal appearing white matter; SVD, small vessel disease; mod-sev, moderate to severe; AD, Alzheimer's disease; IR, immunoreactivity. *, p<0.05

Table 1: Demographic and neuropathological characteristics of study cohort

	AD (n=20)	Control (n=22)	Statistic$_{(df)}$, *p*-value
Mean age, years (±SD)	83.40 (6.29)	84.41 (8.57)	$t_{(41)}$ p = 0.143
Gender M:F	9:11	11:11	χ^2 (1, n = 42) = 0.00, p = 0.988, phi = .05
Mean PMD, hours (±SD)	54.20 (19.97)	55.38 (24.13)	$t_{(26)}$ p = 0.209
Thal Aβ phase [45]	4, n = 2 5, n = 18	0, n = 6 1, n = 7 2, n = 4 3, n = 2 4, n = 2 5, n = 1	-
Braak NFT stage [4]	V, n = 2 VI, n = 18	0, n = 2 I, n = 2 II, n = 10 III, n = 7 IV, n = 1	-
CERAD [33]	C, n = 20	Negative, n = 18 A, n = 3 B, n = 1	-
NIA-AA [30]	High, n = 20	No, n = 6 Low, n = 15 Intermediate, n = 1	-
Braak LB stage [5]	0, n = 17 1, n = 0 2, n = 2 3, n = 1	0, n = 18 1, n = 1 2, n = 1 3, n = 2	-
McKeith criteria [32]	No LBD, n = 17 Brainstem, n = 3	No LBD, n = 18 Brainstem, n = 4	-
TDP-43 in Alzheimer's disease stage [21]	Absent, n = 12 1, n = 0 2, n = 2 3, n = 1 4, n = 5 5, n = 0	Absent, n = 18 1, n = 0 2, n = 1 3, n = 2 4, n = 1 5, n = 0	-
VCING criteria [44]	Low, n = 18 Medium, n = 2	Low, n = 22	-
Total CAA score* [27]	0, n = 4 1, n = 1 2, n = 1 3, n = 4 4, n = 2	0, n = 14 1, n = 4 2, n = 1 3, n = 2 6, n = 1	-
	5, n = 3 6, n = 5		
Presence of capillary CAA	Present, n = 2 Absent, n = 18	Present, n = 1 Absent, n = 21	

Abbreviations: AD, Alzheimer's disease; *df*, degrees of freedom; t, Independent samples test; X², Chi - squared test; F, female; M, Male; U, Mann-Whitney U test; PMD, *post mortem* delay; Aβ, amyloid-beta; NFT, neurofibrillary tangle; CERAD, Consortium to Establish a Registry for Alzheimer's Disease; NIA-AA, National Institute on Ageing - Alzheimer's Association criteria for AD neuropathologic change; LB, Lewy body; VCING, vascular cognitive impairment neuropathological guidelines; CAA, cerebral amyloid angiopathy; *Total CAA score was determined by the accumulation of leptomeningeal (0-3 scale) and cortical CAA (0-3 scale) scores (max score 6; please see reference 23).

Table 2: Details of primary antibodies and protocols

Primary antibody	Source	Target	Species	Primary antibody dilution	Antigen retrieval protocol
Plasma protein fibrinogen	Dako, Denmark	C-terminal	Rabbit polyclonal	1:2000	De-parraffinised sections microwaved in 0.01 mL citrate buffer for 10 min
AT8	Innogenetics, Ghent, Belgium	Phospho-PHF-tau pSer202+Thr205	Mouse monoclonal	1:4000	De-parraffinised sections microwaved in 0.01 mL citrate buffer for 10 min
4G8	Signet Labs, Dedham, MA, USA	Amyloid 17-24	Mouse monoclonal	1:15000	De-parraffinised sections were immersed in concentrated Formic acid for 1 hour

Table 3: Correlation matrix of WML- and NAWM extravascular fibrinogen-IR with SVD and white matter pathology variables

	WML Fibrinogen-IR		
	Whole cohort	**AD**	**Controls**
CAA	$r_s = 0.161$, p = 0.157	$r_s = 0.240$, p = 0.161	$r_s = -0.07$, p = 0.371
SI	r = 0.263, p = 0.048*	r = 0.073, p = 0.384	r = 0.487, p = 0.01*
WMLA	$r_s = 0.106$, p = 0.252	$r_s = -0.140$, p = 0.279	$r_s = -0.015$, p = 0.474
WML-BiA	$r_s = -0.083$, p = 0.301	$r_s = -0.171$, p = 0.236	$r_s = -0.003$, p = 0.495
WML-IOD	$r_s = -0.031$, p = 0.422	$r_s = -0.234$, p = 0.160	$r_s = 0.304$, p = 0.084

	NAWM Fibrinogen-IR		
	Whole cohort	**AD**	**Controls**
CAA	$r_s = 0.291$, p = 0.033*	$r_s = 0.149$, p = 0.271	$r_s = 0.174$, p = 0.219
SI	r = 0.310, p = 0.026*	r = 0.009, p = 0.485	r = 0.383, p = 0.039*
WMLA	$r_s = 0.191$, p = 0.116	$r_s = 0.061$, p = 0.402	$r_s = -0.172$, p = 0.222
WML-BiA	$r_s = -0.263$, p = 0.05	$r_s = -0.386$, p = 0.051	$r_s = -0.230$, p = 0.152
WML-IOD	$r_s = 0.080$, p = 0.310	$r_s = 0.070$, p = 0.388	$r_s = -0.187$, p = 0.202

Abbreviations: WML, white matter lesion; NAWM, normal appearing white matter; CAA, cerebral amyloid angiopathy; SI, sclerotic index; WMLA, white matter lesion area; WML-BiA, white matter lesion Bielschowsky's measurement for axonal density; WML-IOD, white matter lesion integrated optical density measurement for demyelination; IR, immunoreactivity; AD, Alzheimer's disease; r, Pearson's correlation coefficient; r_s, Spearman's correlation coefficient. *, p<0.05.
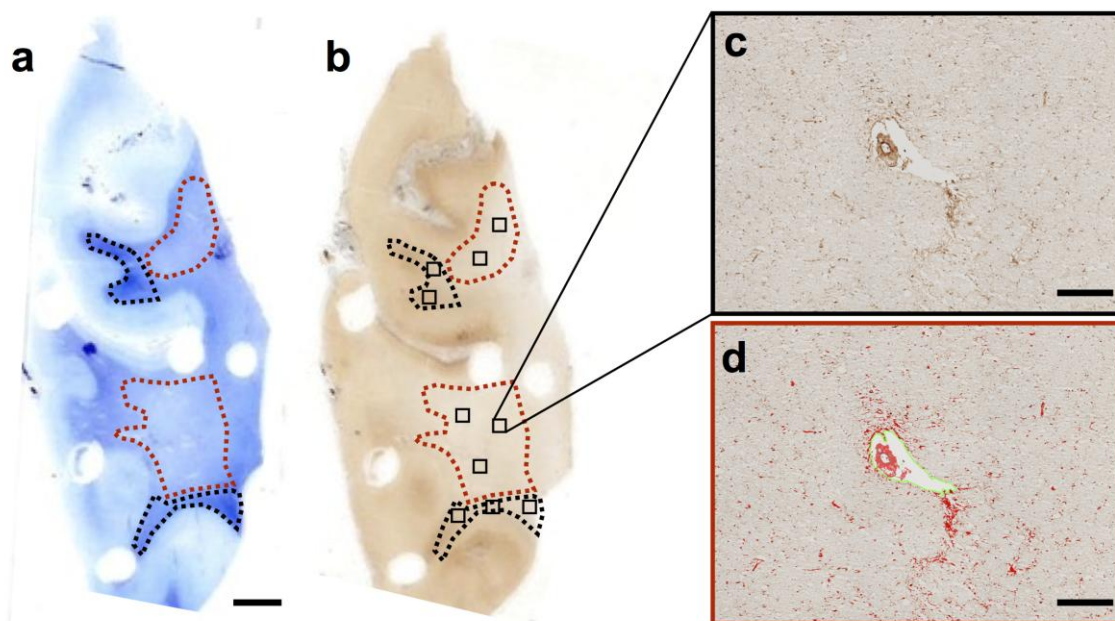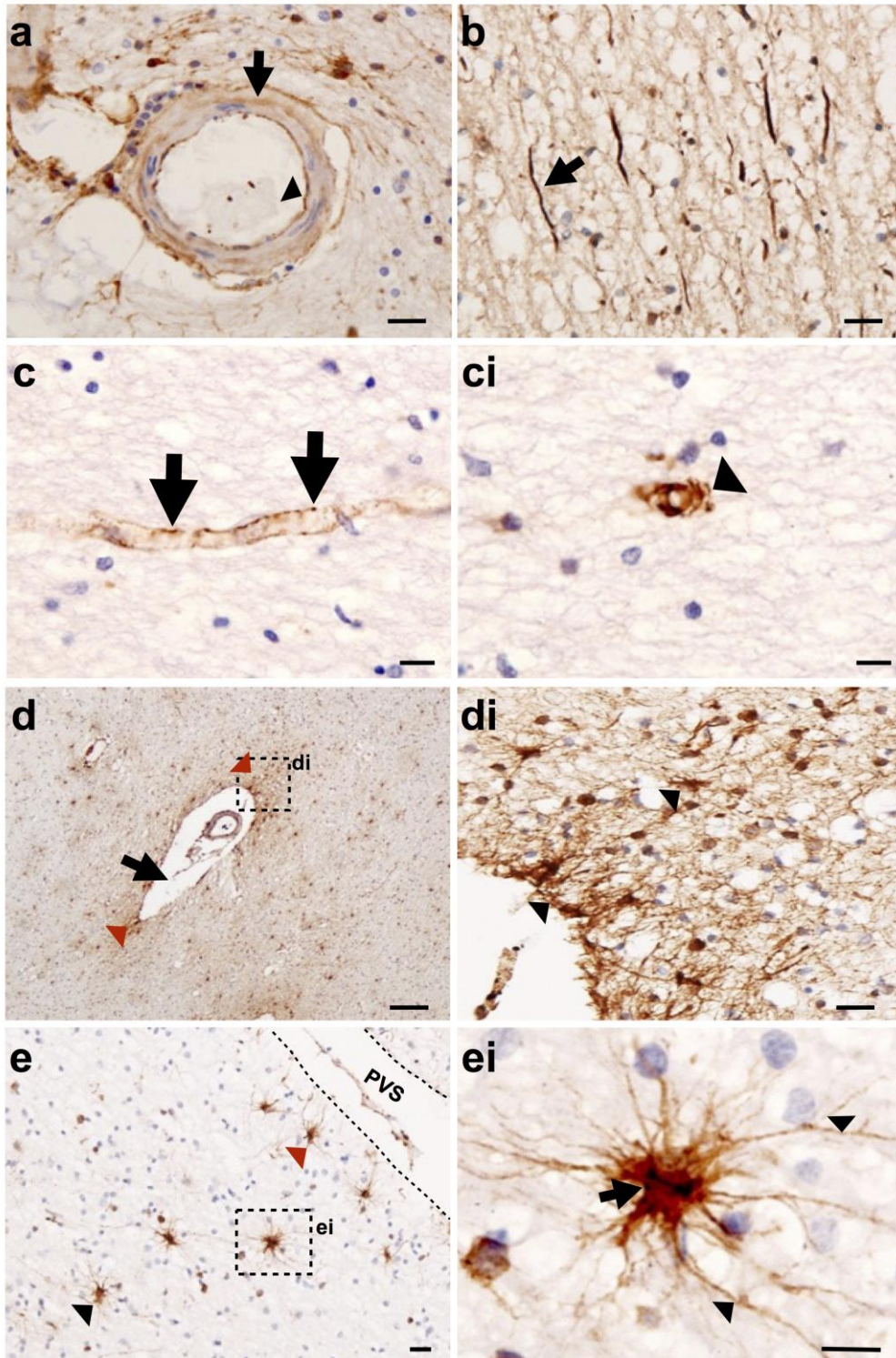
Figure 1

Figure 2

Figure 3