Complement activation in capillary cerebral amyloid angiopathy

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Abstract

Background: Cerebral amyloid angiopathy (CAA) is classified as type 1 with capillary amyloid β (Aβ) or type 2 without capillary Aβ. While it is known that CAA activates complement, an inflammatory mediator, there is no information on the relationship between capillary Aβ and complement activation.

Methods: We evaluated 34 autopsy brains, including 22 with CAA and 12 with other neurodegenerative diseases. We assessed the vascular density of CAA by analyzing the expression of complement (C1q, C3d, C6, C5b-9), macrophage scavenger receptor (MSR), and apolipoprotein E (ApoE).

Results: Capillary immunostaining for C1q, C3d, MSR, and ApoE was identified almost exclusively in CAA-type1 brains. There was intense expression of C1q, C3d, MSR, and ApoE, as well as weaker expression of C5b-9 and C6 in the arteries/arterioles of both CAA subtypes, but not in control brains. C5b-9 and C6 were preferentially expressed in arteries/arterioles with subcortical hemorrhage or cortical superficial siderosis. Triple-immunofluorescence revealed that C1q, C3d, and ApoE were colocalized with Aβ in CAA brain capillaries.

Conclusion: Complement, MSR, and ApoE were only co-expressed in the presence of Aβ accumulation in capillaries, suggesting a role for complement activation in the

propagation of Aβ. Additionally, C5b-9 expression may be associated with hemorrhagic brain injury in CAA.

Introduction

Cerebral amyloid angiopathy (CAA) is a disease that causes the deposition of amyloid protein in the vascular wall of the meninges and brain. Although there are multiple types of amyloid protein, the vast majority is amyloid β (Aβ); CAA due to Aβ deposition is closely related to Alzheimer's disease (AD) [1,2]. CAA is observed in more than 90% of AD cases, and approximately 25% of AD cases have severe CAA [1]. Aβ is mainly deposited in the blood vessels of the meninges and cerebral cortex, and is extremely rare in the white matter, thalamus, basal ganglia, and brainstem [3].

CAA is classified into 2 subtypes: type 1, in which CAA is recognized in the leptomeningeal arteries, cortical arteries/arterioles, and capillaries; and type 2, in which CAA is identified in the leptomeningeal arteries and cortical arteries/arterioles, but not in the capillaries [4]. CAA-type 1 is associated with a higher frequency of the apolipoprotein E (ApoE) ε4 allele than CAA-type 2, and correlates with AD pathology such as senile plaque and neurofibrillary tangle formation [4,5].

Chronic inflammation has been described as a cardinal feature of AD pathology,

partly based on the discovery of complement activation in senile plaques [6]. The

molecular weights of these complement components are relatively large and they are

charged [7], such that complement passage from the circulation through an intact

blood–brain barrier into the brain parenchyma is restricted. Instead, astrocytes and

microglia have been shown to produce complement proteins in culture, suggesting that

glial cells may be an endogenous source of complement proteins in the brain [8,9].

Resting microglia constitutively express CD11b (complement receptor; CR3), and the

intensity of CR3 expression is enhanced on reactive microglia in AD brains [10]. The

complement cascade is commonly initiated by the binding of C1 to the Fc region of

immunoglobulins; however, in AD, Aβ can directly bind C1 and activate the classical

complement pathway in the absence of antibody [11]. Moreover, other inflammatory

mediators including C-reactive protein (CRP), amyloid P (AP), and Hageman factor are

also thought to contribute to antibody-independent complement activation [12].

Previous immunohistochemical studies of AD brains have revealed the

expression of various complement proteins (C1q, C3c, C4d, C5b-9) not only in senile

plaques but also in association with CAA [13]. In one study, the degree of

immunostaining for complement (C3d, C9) was significantly correlated with CAA

severity [14]. It was recently discovered that Aβ is cleared from the brain via the

perivascular drainage pathway [15-17] or glymphatic system[18-21]. Yet, there is little information on complement activation and microglial/macrophage responses in capillary CAA.

In this study, we examined the expression of microglial/macrophage markers, complement, Aβ, and ApoE using a semi-quantitative evaluation and triple-immunofluorescence staining in autopsy CAA-type 1 and type 2 brains compared to control brains in order to inform the roles of these molecules in CAA and AD pathogenesis.

Subjects and methods

Autopsy specimens

We evaluated 34 autopsy brains including 22 from patients with CAA (CAA group) and 12 from patients with neurodegenerative disease without CAA (control group). CAA brains were obtained from 2 medical institutions; 18 were obtained from Choju Medical Institute of Fukushimura Hospital and 4 were obtained from Kyoto University Hospital. The control group included brains from 4 patients with amyotrophic lateral sclerosis, 4 with multiple system atrophy, 3 with Parkinson's disease, and 1 with frontotemporal dementia. Control brains were also obtained from 2

medical institutions; 8 were obtained from Kyoto University Hospital and 4 were obtained from Mie University Hospital. All subjects or their legal representatives provided written informed consent for the use of clinical data and tissue for scientific purposes. The present study was approved by the medical ethics committees of Mie University Hospital.

Immunohistochemistry

The parieto-temporo-occipital lobes of the cerebrum were selected for all analyses. The presence of CAA was determined by light microscopy with anti-human Aβ40 and Aβ42 immunostaining. Briefly, 6-μm sections of formalin-fixed paraffin-embedded tissues were immunostained with the following antibodies: polyclonal rabbit anti-human Aβ40 (Invitrogen: 44-348A, diluted 1/1,000, with formic acid pretreatment), polyclonal rabbit anti-human Aβ42 (Novex: 44-344, diluted 1/400, with formic acid pretreatment), monoclonal mouse anti-human ApoE (Covance: D6E10, diluted 1/1,000), monoclonal mouse anti-human ApoE4 (MBL: 1F9, diluted 1/100), polyclonal rabbit anti-human C1q (Dako: A0136, diluted 1/1,000), polyclonal rabbit anti-human C3d (Dako: A0063, diluted 1/200), monoclonal mouse anti-human C5b-9 (Abcam: ab66768, diluted 1/100, microwave and EDTA pretreatment), monoclonal mouse anti-human C6 (Quidel: A219, diluted 1/500), and polyclonal goat anti-human

macrophage scavenger receptor (MSR) (R&D Systems: AF2708, diluted 1/500). We used MSR for labeling microglia/macrophages, because it is a cell-surface marker of activated microglia/macrophages (not resting microglia/macrophages), thus allowing the visualization of cellular contours, including protrusions of microglia/macrophages, unlike CD68, an intracellular antigen [22]. Primary antibodies were visualized with biotinylated secondary antibodies as appropriate (horse anti-mouse, goat anti-rabbit, and rabbit anti-goat) and the avidin-biotinylated horseradish peroxidase (HRP) complex from Vector Laboratories. This reaction was subsequently visualized using 3,3'-diaminobenzidine and hydrogen peroxide. Immunostained paraffin sections were counterstained with hematoxylin.

Semi-quantification of immunohistochemistry

The numerical density of immunostained vessels in each cerebral cortex was measured by the grid method [23], in which the number of vascular intersections in the square is counted using a grid (100 squares, 1 mm^2) inserted into one of the eyepieces (10×). The average count from 10 representative fields was used as the vascular density in each subject.

Triple-immunofluorescence staining

Triple-immunofluorescence labeling was used to visualize the co-localization

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of complement, Aβ, and ApoE. CAA-type 1 and type 2 sections were immunostained

with the following primary antibodies: monoclonal mouse anti-human Aβ (DAKO:

6F/3D, diluted 1/100), polyclonal goat anti-human ApoE (Abcam: ab32897, diluted

1/100) and either polyclonal rabbit anti-human C1q (Dako: A0136, diluted 1/1,000) or

polyclonal rabbit anti-human C3d (Dako: A0063, diluted 1/200). Then, sections were

stained with the following secondary antibodies: goat anti-rabbit Alexa Fluor 488

(FITC), goat anti-mouse Alexa Fluor 546 (Rhod), and donkey anti-goat Alexa Fluor 647

(Cy5) diluted 1/100. Stained sections were covered with Vectashield (Vector

Laboratories) and observed with a LEICA DMi8 inverted fluorescence microscope.

Statistical analysis

Case histories and pathological findings in all groups were analyzed using chi

square tests to confirm that the selection of cases for the control group was appropriate.

Case histories and pathological findings in the CAA-type 1 and type 2 groups were also

analyzed using chi square tests. Semi-quantified measurements of immunostained

vessels are expressed as the mean ± standard deviation and were analyzed by the

Kruskal–Wallis test followed by the Mann–Whitney U test, and a Bonferroni correction

was performed. The SPSS Statistics 24 software package was used to perform

descriptive statistical analyses. A P value of < 0.05 was considered to be statistically

significant.

Results

Case histories and classical neuropathological findings

Case histories and classical neuropathological findings in the CAA and control

groups are summarized in Table 1. This study included 12 cases with CAA-type 1, 10

with CAA-type 2, and 12 controls. In the comparison of all 3 groups (Table 2), there

were significant between-group differences in the presence of cortical microinfarction

(CMI) and the level of AD neuropathological changes based on the criteria of the

National Institute on Aging-Alzheimer's Association (NIA-AA) guidelines, 2012 [24]

($P < 0.05$; chi square test). There were no significant between-group differences in other

items, indicating authenticity of the neurodegenerative disease group as a control group.

Between the CAA-type 1 and type 2 groups (Table 2), there were no significant

differences in age, gender, past medical history of hypertension or lacunar infarction, or

amyloid-related vascular changes including CMI, cortical superficial siderosis (cSS),

and subcortical hemorrhage. Yet, the incidence of high- level AD neuropathological

changes was significantly higher in the CAA-type 1 group than in the CAA-type 2

group (7 out of 12 versus 1 out of 10, respectively).

Semi-quantification of immunohistochemical results

Capillary staining with C1q, C3d, MSR, and ApoE was identified exclusively in the CAA-type1 group (Fig. 1A, Fig. 2). In large arteries, there were no significant differences in the expression of these markers between the CAA-type 1 and CAA-type 2 groups (Fig. 1B). That is, Aβ-associated molecules were only expressed when Aβ had accumulated in the capillaries, whereas Aβ-associated molecules were invariably observed in large arteries irrespective of the presence of capillary CAA. In all whole specimens, capillaries did not show any immunostaining if larger vessels were negative for Aβ and Aβ-associated molecules.

We further examined expression of the membrane attack complex (MAC; labeled by antibodies against C5b-9 or C6) in capillaries and larger vessels in some cases of CAA, regardless of subtype (Fig. 1). Immunostaining for C5b-9 or C6 revealed small numbers of immunopositive vessels (photograph not shown); however, scattered larger arteries (but not capillaries) showed intense immunostaining for C5b-9 and C6 in all 5 subjects with subcortical hemorrhage (2, 3, 12, and 20) and/or cSS (3 and 19).

Capillaries and larger vessels were often immunostained for ApoE4 in both the CAA-type 1 and CAA-type 2 groups, indicating that those cases carried the ApoE ε4 allele. However, in this study, ApoE genotypes were not determined. In some cases with

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ApoE-positive capillaries, immunostaining for ApoE4 was negative, indicating that they

were not ApoE ε4 allele carriers.

Triple-immunofluorescence staining

We found consistent co-expression of Aβ, ApoE, and complement (C1q and

C3d) in the capillaries of CAA-type1 brains (Fig. 3 A – H). We also found co-expression

of Aβ, ApoE, and complement (C1q and C3d) in the larger arteries identified in

CAA-type1 and type 2 brains (Fig. 3 I – P). There were no capillaries or arteries

expressing only 1 or 2 molecules among Aβ, ApoE, and complements C1q or C3d (data

not shown).

Discussion

This study demonstrates for the first time that capillary CAA brains selectively

express ApoE and inflammation-associated molecules including complement and MSR

in the capillary when there is capillary Aβ accumulation. In the larger arteries, these

inflammatory molecules were observed irrespective of the presence of capillary CAA. A

strong association between capillary CAA and inflammatory mediators indicate a

potential role for inflammation in capillary Aβ accumulation. Figure 4 shows a

schematic representation of the presumed mechanism for Aβ deposition and

complement activation in the basement membrane of vascular wall. Under normal

conditions, ApoE released from astrocytes binds to Aβ and this complex is transported

by way of the perivascular drainage pathway in the direction opposite to blood flow

(Fig. 4A). In contrast, under pathological conditions, the complex of ApoE and

aggregated Aβ is further bound by complement (early component), and ApoE and

Aβ-associated C3b is often bound to the perivascular microglia/macrophages.

Complexes with lower aggregability are able to reach the arteries, while those with

higher aggregability may deposit in capillaries (Fig. 4B). Our data further indicated that

AD pathology was closely associated with capillary CAA (e.g., 7 of 12 cases had a high

level of AD in the CAA-type 1 group, as compared to 1 of 10 cases in the CAA-type 2

group). Close associations among inflammatory mediators, CAA, and AD pathology

may underscore the pivotal role of inflammation in Aβ accumulation in both CAA and

AD.

Senile plaques are classified into 2 cardinal types: diffuse plaques and classical

plaques with degenerated neurites. The latter represents a later stage of plaque

formation with fibrillar Aβ and is typically immunoreactive for both C1q and C4d,

indicating activation of the classical complement pathway. C3b can label target

molecules including Aβ as opsonins and enhance microglial phagocytosis in senile

plaques [25]. Activated microglia express complement receptor CR3 (CD11b), and are

thus able to bind the C3b-Aβ complex and may relocate it to the perivascular space [26].

Hong and colleagues revealed that C1q and C3 were essential for microglial

phagocytosis of synapses with bound Aβ oligomers using a transgenic murine model of

familial AD. To this end, the complement-dependent pathway and microglia have been

postulated to be inappropriately activated and mediate synapse loss in AD [27].

Aβ clearance via the perivascular drainage pathway may be impaired in the

presence of CAA, and the disturbance of Aβ clearance may in turn contribute to AD

pathogenesis [15-17]. Within the perivascular drainage pathway, solutes containing Aβ

flow in the direction opposite to blood flow in the basement membranes of

capillaries/arterioles, ultimately draining into the lymph nodes of the neck. Alternatively,

in the glymphatic system, Aβ flows from the perivascular space of the arterioles into the

neuropil and drains into the perivenular space and veins [18-21]. In both pathways, the

basement membrane/perivascular space of capillaries/arterioles is thought to be the

location for Aβ drainage. Thus, the close association between capillary CAA and AD

pathology observed in the present study and preceding studies [4,5] may be attributable

to dysfunction of the Aβ clearance system, wherein capillary CAA has a greater

influence on brain Aβ clearance than classical CAA in larger vessels.

It remains unclear whether the complement–Aβ complex is deposited in vascular tissue or deposited aggregates of Aβ are subsequently bound by complement in vascular tissue, or both. Previously, Rogers and colleagues showed that Aβ aggregation was a strong activator of complement [11]. Additionally, complement is expressed in classical late-stage senile plaques, but not in the diffuse early-stage plaques. Therefore, it is more likely that Aβ binds to complement after aggregation, although we did not determine the order of deposition in the present study.

In the present study, C5b-9 and C6 were identified in the larger vessels of 15 of 22 CAA cases, whereas little immunostaining for these components was observed in the capillaries. In contrast, all 5 cases with subcortical hemorrhage or cSS showed immunoreactivity for either C5b-9 or C6 in larger vessels. Thus, MAC may be selectively expressed in cases with hemorrhagic brain injury. In previous studies, MAC expression was observed in CAA [13,26], but the pathological features of these cases were not specified. Alternatively, C1q and C3d were robustly observed in capillaries and arteries/arterioles in almost all cases, thereby indicating that a main role of complement may be to activate microglia/macrophages by opsonization.

The present study had some limitations as follows. First, the statistical power of this study was limited by a relatively small sample size. Second, we did not examine

ApoE genotype; therefore, we could not evaluate the effect of genotype on the expression of ApoE and complement. Third, it is impossible to exclude the possibility that complement and microglia/macrophage were activated by agonal changes.

In conclusion, we found that inflammatory mediators (complement, microglia/macrophage) and ApoE were expressed in capillaries only in the presence of Aβ accumulation, whereas Aβ-associated molecules were invariably found in large arteries irrespective of the presence of capillary CAA in CAA brains. A close association between capillary inflammation and Aβ deposition may indicate a role of complement activation in the propagation of Aβ aggregation and in the disturbance of Aβ clearance. Furthermore, the expression of C5b-9 may be involved in hemorrhagic brain injury in CAA.

Conflicts of Interest

None.

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Figure legends

Figure 1. Semi-quantitative estimation of capillaries (A) and larger vessels (B) immunopositive for Aβ40, Aβ42, ApoE, ApoE4, C1q, C3d, C5b-9, C6, and MSR by the grid method. * $P < 0.05$, ** $P < 0.1$, Kruskal–Wallis test.

Figure 2. Immunohistochemistry of capillary CAA, types 1 and 2. Capillaries immunopositive for C1q, C3d, ApoE, or MSR are found in CAA-type 1 (A, C, E, G), not in CAA-type 2 (B, D, F, H). Scale bar = 100 μm.

Figure 3. Triple-staining for complement, Aβ, and Apo E in CAA-type 1 capillaries (A–H), CAA-type 2 larger vessels (I–P), and controls (Q–X). Scale bar = 25 μm.

Figure 4. Schematic representation of the presumed mechanism for Aβ deposition and complement activation in the basement membrane of the vascular wall.

Table 1. Case histories and classical neuropathological findings in the CAA-type 1,

CAA-type 2, and control groups.

Subject	Group	Gender (M/F)	Age (y.o.)	CapCAA	Larger vessel CAA	HTN	Lacunar infarction	CMI	cSS	Subcortical hemorrhage	Neuritic plaque (CERAD)	NFT (Braak)	Level of AD neuropathologic change (NIA-AA)
A1		F	86	+	+	-	+	+	-	-	B	IV	Intermediate
A2		F	83	+	+	-	+	+	-	+	C	VI	High
A3		F	84	+	+	-	-	-	+	+	C	V	High
A4		F	88	+	+	-	-	+	-	-	C	VI	High
A5		M	85	+	+	-	-	+	-	-	A	I	Not AD
A6	CAA-type 1	M	83	+	+	+	-	+	-	-	C	V	High
A7		M	58	+	+	-	-	-	-	-	C	VI	High
A8		F	84	+	+	-	+	-	-	-	C	VI	High
A9		F	88	+	+	-	+	-	-	-	C	VI	High
A10		F	76	+	+	-	-	-	-	-	C	IV	Intermediate
A11		F	89	+	+	-	+	-	-	-	C	II	Low
A12		M	78	+	+	-	-	+	-	+	unknown	unknown	unknown
A13		F	93	-	+	+	-	+	-	-	C	III	Intermediate
A14		F	82	-	+	-	-	+	-	-	C	V	High
A15		M	98	-	+	+	-	+	-	-	C	I	Low
A16		M	79	-	+	-	-	+	-	-	B	II	Low
A17	CAA-type 2	F	87	-	+	+	-	-	-	-	unknown	IV	unknown
A18		F	90	-	+	+	-	-	-	-	B	IV	Intermediate
A19		F	83	-	+	-	-	+	+	-	C	IV	Intermediate
A20		F	74	-	+	-	+	-	-	+	unknown	III	unknown
A21		M	85	-	+	-	-	-	-	-	C	IV	Intermediate
A22		F	95	-	+	-	+	-	-	-	unknown	unknown	unknown
B1		F	72	-	-	-	-	-	-	-	A	0	Low
B2		F	66	-	-	-	-	-	-	-	0	0	Not AD
B3		M	69	-	-	-	-	-	-	-	0	0	Not AD
B4		M	60	-	-	-	-	-	-	-	0	0	Not AD
B5		M	57	-	-	-	-	-	-	-	0	0	Not AD
B6		F	61	-	-	-	-	-	-	-	0	0	Not AD
B7	Control	M	76	-	-	-	+	-	-	-	0	I	Not AD
B8		M	88	-	-	-	-	-	-	-	B	0	Low
B9		F	78	-	-	-	-	-	-	-	unknown	unknown	unknown
B10		M	77	-	-	+	-	-	-	-	0	II	Not AD
B11		M	79	-	-	-	-	-	-	-	unknown	unknown	unknown
B12		M	77	-	-	-	-	-	-	-	0	II	Not AD

AD, Alzheimer's disease; CAA, cerebral amyloid angiopathy; Cap, capillary; CERAD,

Consortium to Establish a Registry for Alzheimer's Disease; CMI, cortical

microinfarction; cSS, cortical superficial siderosis; HTN, hypertension; NFT,

neurofibrillary tangles; NIA-AA, National Institute on Aging-Alzheimer's Association.
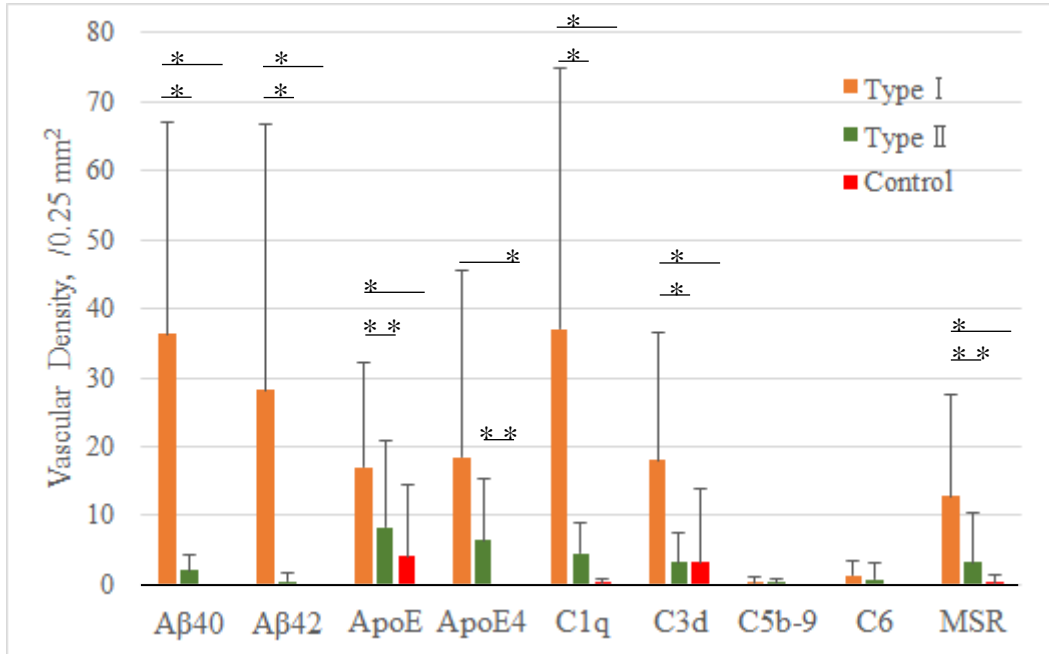
Table 2. Comparison of the CAA-type 1, CAA-type 2, and control groups.

Subject	Gender (M/F)	Age (mean ± SD)	CapCAA (%)	Larger vessel CAA (%)	HTN (%)	Lacunar infarction (%)	CMI (%)	cSS (%)	Subcortical hemorrhage (%)	High level of AD neuropathologic change (%)
CAA-type 1 (n=12)	4/8	81.8 ± 8.1	12/12 (100)	12/12 (100)	1/12 (8)	5/12 (42)	6/12 (50)	1/12 (8)	3/12 (25)	7/12 (58)
CAA-type 2 (n=10)	3/7	86.6 ± 7.1	0/10 (0)	10/10 (100)	4/10 (40)	2/10 (20)	5/10 (50)	1/10 (10)	1/10 (10)	1/10 (10)
Control (n=12)	9/3	71.7 ± 8.9	0/12 (0)	0/12 (0)	1/12 (8)	1/12 (8)	0/12 (0)	0/12 (0)	0/12 (0)	0/12 (0)
P value (comparison among 3 groups)	0.146	0.385	< 0.001*	< 0.001*	0.088	0.149	0.012*	0.553	0.161	0.002*
P value (comparison between CAA-type 1 and 2)	0.616	0.096	< 0.001*	not calculated	0.105	0.268	0.665	0.714	0.368	0.026*

Chi square test   * $P < 0.05$

AD, Alzheimer's disease; CAA, cerebral amyloid angiopathy; Cap, capillary; CMI,

cortical microinfarction; cSS, cortical superficial siderosis; HTN, hypertension.

Fig. 1
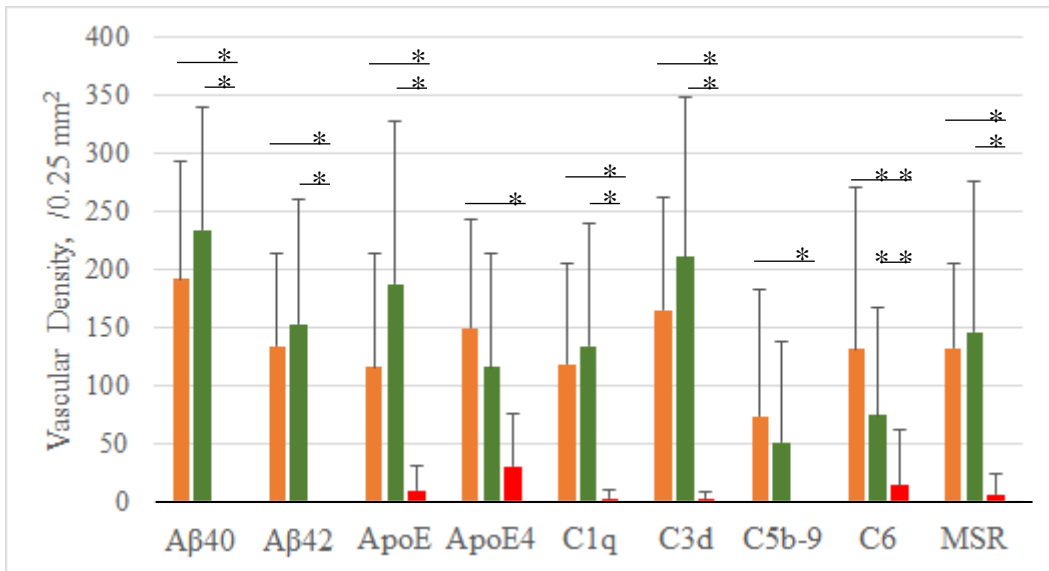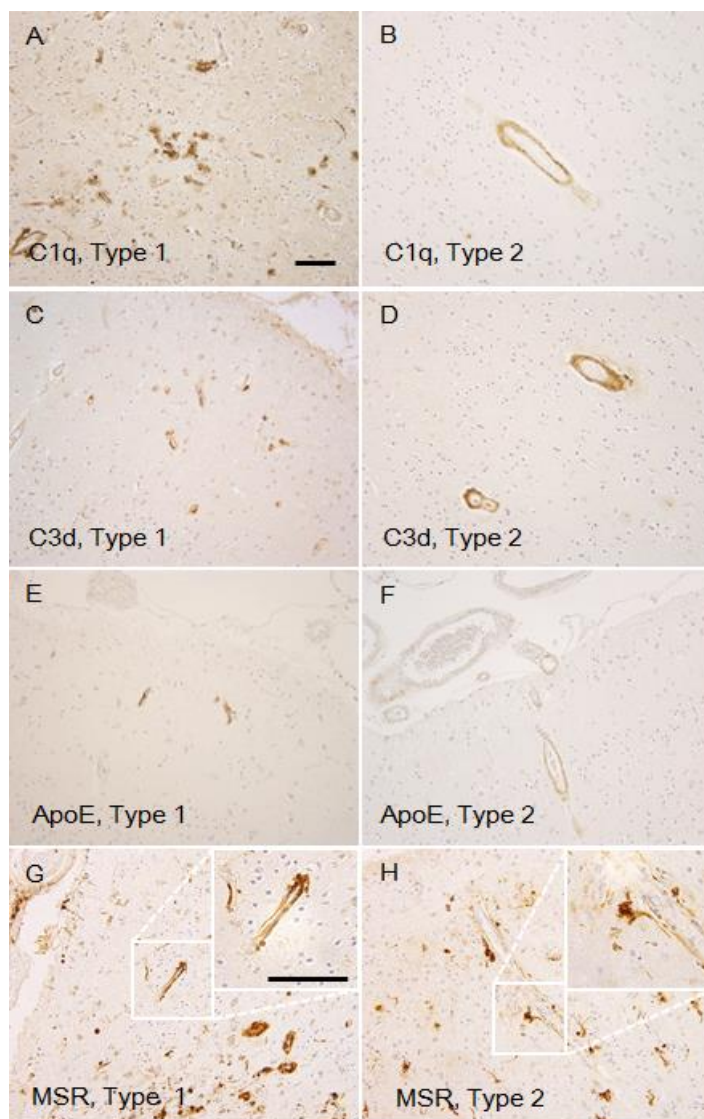
A) Capillaries

B) Larger Vessels

Fig. 2

A — C1q, Type 1
B — C1q, Type 2
C — C3d, Type 1
D — C3d, Type 2
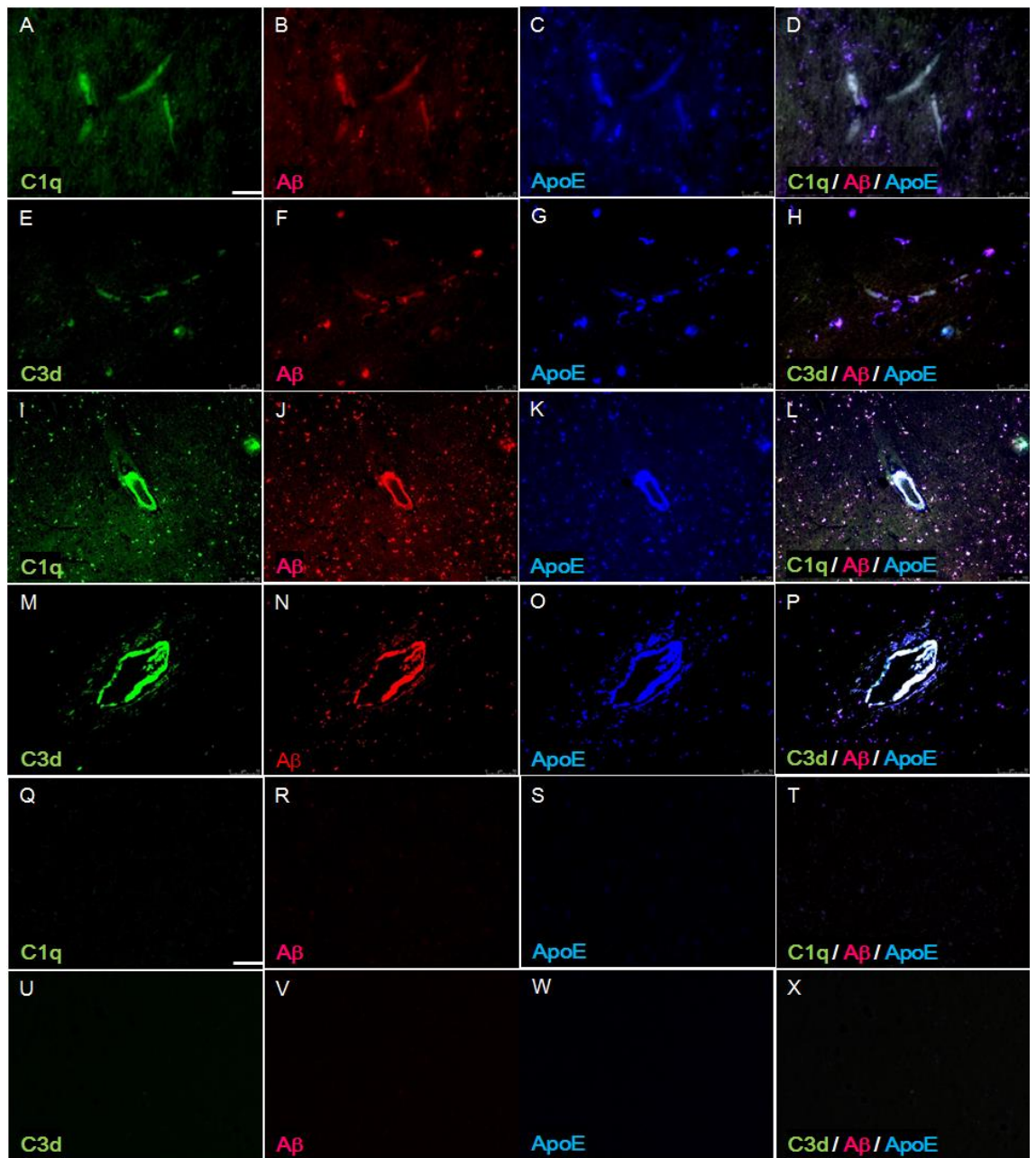E — ApoE, Type 1
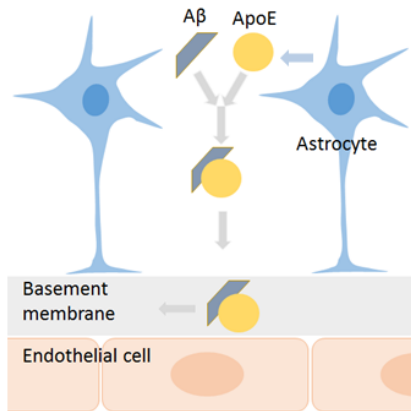F — ApoE, Type 2
G — MSR, Type 1
H — MSR, Type 2

Fig. 3

Fig. 4

a. Normal condition

b. Pathological condition