

Amyloid- β Contributes to Blood–Brain Barrier Leakage in Transgenic Human Amyloid Precursor Protein Mice and in Humans With Cerebral Amyloid Angiopathy

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Background and Purpose—Cerebral amyloid angiopathy (CAA) is a degenerative disorder characterized by amyloid- β ($A\beta$) deposition in the blood–brain barrier (BBB). CAA contributes to injuries of the neurovasculature including lobar hemorrhages, cortical microbleeds, ischemia, and superficial hemosiderosis. We postulate that CAA pathology is partially due to $A\beta$ compromising the BBB.

Methods—We characterized 19 patients with acute stroke with “probable CAA” for neurovascular pathology based on MRI and clinical findings. Also, we studied the effect of $A\beta$ on the expression of tight junction proteins and matrix metalloproteases (MMPs) in isolated rat brain microvessels.

Results—Two of 19 patients with CAA had asymptomatic BBB leakage and posterior reversible encephalopathic syndrome indicating increased BBB permeability. In addition to white matter changes, diffusion abnormality suggesting lacunar ischemia was found in 4 of 19 patients with CAA; superficial hemosiderosis was observed in 7 of 9 patients. $A\beta_{40}$ decreased expression of the tight junction proteins claudin-1 and claudin-5 and increased expression of MMP-2 and MMP-9. Analysis of brain microvessels from transgenic mice overexpressing human amyloid precursor protein revealed the same expression pattern for tight junction and MMP proteins. Consistent with reduced tight junction and increased MMP expression and activity, permeability was increased in brain microvessels from human amyloid precursor protein mice compared with microvessels from wild-type controls.

Conclusions—Our findings indicate that $A\beta$ contributes to changes in brain microvessel tight junction and MMP expression, which compromises BBB integrity. We conclude that $A\beta$ causes BBB leakage and that assessing BBB permeability could potentially help characterize CAA progression and be a surrogate marker for treatment response. (*Stroke*. 2012;43:514-523.)

Key Words: amyloid- β ■ blood–brain barrier ■ cerebral amyloid angiopathy ■ neuroimaging

Cerebral amyloid angiopathy (CAA) is a degenerative neurovascular disease that affects most patients with Alzheimer’s disease (AD) and approximately 30% of elderly people.¹ The main characteristic of CAA is deposition of amyloid- β ($A\beta$) proteins in leptomeningeal and cortical blood vessels in the brain that contribute to cortical cerebral brain hemorrhage. Because CAA is a disease of the elderly, it overlaps with a variety of cardiovascular risk factors (eg, hypertension, diabetes, hypercholesterolemia) that could contribute to CAA pathology, in particular white matter lesions.^{2,3} Recent findings suggest that elevated levels of 2 transcription factors, serum response factor and myocardin, contribute to CAA phenotype. Both serum response factor and myocardin regulate differentiation of mural vascular cells in response to hypoxia and lead to the development of CAA

and hypercontractile arterial phenotype with brain hypoperfusion in patients with AD and mouse models, thus aggravating white matter lesions.^{3,4} Cortical cerebral brain hemorrhage is the main criterion for CAA diagnosis and causes and/or contributes to neurodegeneration with dementia and cognitive impairment that overlaps with AD. However, CAA also exhibits acute phases with neurovascular injury that show symptoms ranging from headache, weakness, sensory loss, visual changes, or speech problems.²

CAA is diagnosed according to the Boston criteria that distinguish 4 categories.⁵ Diagnoses of “definite CAA” and “probable CAA with supporting pathological evidence” require histopathologic evidence from postmortem tissue or evidence of amyloid deposition in pathological tissue in addition to clinical criteria. Diagnosis of “probable CAA”

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requires clinical and radiological evidence showing at least 2 lobar hemorrhagic lesions due to CAA. The same criteria apply to “possible CAA,” but radiological evidence of only 1 hemorrhage is required.

A reliable CAA diagnosis solely based on neurological signs and cognition is difficult. However, MRI with gradient-echo sequences now allows detection of small cortical and subcortical hemorrhages and thus, diagnosis of different CAA phenotypes including hemosiderosis (SH), acute cerebral ischemia, and superficial and posterior reversible encephalopathic syndrome (PRES). For the diagnosis of the latter, diffusion-weighted imaging and fluid-attenuated inversion recovery imaging (FLAIR)^{6–9} are required.

In the present study, we used MRI to classify CAA in 19 patients who were admitted to our stroke unit with acute stroke symptoms, and phenotyped these patients with CAA based on symptoms of neurovascular damage, that is, blood–brain barrier (BBB) leakage. Recent evidence suggests that BBB leakage observed in patients with CAA is, at least in part, due to A β accumulation in cerebral microvessels.^{10,11} To test this hypothesis, we performed additional experiments in which we exposed isolated rat brain microvessels to human A β_{40} and determined the effect of A β on proteins of the tight junction (TJ) complex and on matrix metalloproteases (MMPs) that are responsible for degradation of the microvessel basal membrane. We also measured barrier integrity in isolated brain microvessels from 9-month-old transgenic human amyloid precursor protein (hAPP)-overexpressing mice (Tg2576) that are a well-established CAA animal model.^{12,13}

Methods

Animals

Animal protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee and were in accordance with Association for Assessment and Accreditation of Laboratory Animal Care International regulations and the US Department of Agriculture Animal Welfare Act. Male CD IGS Sprague-Dawley rats (275–300 g) were from Charles River Laboratories (Wilmington, MA). Male transgenic hAPP mice (Tg2576 strain, 129S6.Cg-Tg[APP^{SWE}]2576Kha) and wild-type mice from Taconic Farms (Germantown, NY) were 9 months old at the time of the experiments. Animals were housed under controlled conditions (23°C, 35% relative humidity, 12-hour dark/light cycle) with free access to water and food.

Chemicals

ZO-1, occludin, claudin-1, and claudin-5 antibodies were from Invitrogen (Carlsbad, CA). MMP-2, MMP-9, β -actin antibodies, and MMP-9 protein were from Abcam (Cambridge, MA). Human A β_{40} was from EMD Chemicals (Gibbstown, NJ).

Brain Microvessel Isolation

Brain microvessels were isolated as described previously.^{14,15} Animals were euthanized with CO₂ and decapitated. Brains were removed, dissected, and homogenized in phosphate-buffered saline. Ficoll (Sigma, St Louis, MO) was added to a final concentration of 15% and the homogenate was centrifuged (5800 g, 20 minutes, 4°C). After resuspending the pellet in 1% bovine serum albumin, the suspension was passed over a glass bead column. Microvessels were collected in 1% bovine serum albumin, washed with phosphate-buffered saline, and used for experiments.

Brain Microvessel Membrane Isolation

Brain microvessels were homogenized in lysis buffer (Sigma) containing Complete protease inhibitor (Roche, Mannheim, Germany).^{14,15} Samples were centrifuged at 10 000 g (15 minutes, 4°C) and the resulting supernatants were centrifuged at 100 000 g (90 minutes, 4°C). Brain microvessel membranes were resuspended and stored at –80°C.

Western Blotting

Brain microvessel membranes were analyzed by Western blotting as described previously.^{14,16} Western blots were performed with the Invitrogen NuPage Bis-Tris electrophoresis/blotting system (Invitrogen, Carlsbad, CA). After protein transfer, membranes were blocked and incubated with primary antibody. Membranes were washed and incubated with horseradish peroxidase-conjugated ImmunoPure secondary IgG (1:15 000; Pierce, Rockford, IL). Proteins were detected with SuperSignal West Pico Chemoluminescent Substrate (Pierce) and visualized using a BioRad Gel Doc 2000 system (BioRad, Hercules, CA). Western blots were quantitated by densitometric analysis using QuantityOne 1-D Version 4.6.5 software (BioRad Laboratories). Data were normalized to β -actin loading controls.

Texas Red Efflux

Microvessels were transferred to glass cover slips and loaded with 2 μ mol/L Texas red (sulforhodamin101-free acid, 1 hour, 25°C). After washing with phosphate-buffered saline, Texas red efflux from microvessels was monitored with confocal microscopy (Nikon C1, Nikon TE2000 inverted microscope, 40 \times oil objective, NA 1.3, 543 nm HeNe laser; Nikon Instruments, Melville, NY). Mannitol (100 mmol/L) was used as a positive control for barrier opening and maximum permeability marker. For each treatment, 7 confocal images were acquired and luminal fluorescence was measured using Image J (National Institutes of Health, Bethesda, MD).¹⁷ First-order Texas red efflux rate constants were calculated by nonlinear regression (Prism 4; GraphPad Software, San Diego, CA).

Zymography

Brain capillary lysate was incubated with Gelatin Sepharose 4B (GE Healthcare, Piscataway, NJ) for 90 minutes. After centrifugation (500 g, 2 minutes) and washing with phosphate-buffered saline/Tween20, MMPs were eluted with Novex Tris-Glycine SDS sample buffer (1 \times). The eluate was loaded on Novex 10% Zymogram gelatin gels (Invitrogen, Carlsbad, CA) and electrophoresis was performed. Gels were stained with BioRad Coomassie Brilliant Blue R-250 staining solution and clear bands indicating protease activity were visualized using a BioRad Gel Doc 2000 system (BioRad, Hercules, CA). Band intensity representing MMP activity was determined by densitometric analysis.

Patient Selection and Phenotyping

Our patient study (study period: June 2010 to March 2011) was approved by the University of Regensburg ethics committee according to the declarations of Helsinki (1995) and Tokyo (1998). All patients admitted to the study were identified in the Stroke Unit of the Bezirksklinikum Regensburg, University of Regensburg, Germany, and underwent routine cerebral CT. Patients presented with atypical intracerebral hemorrhage or SH with symptoms attributed to either direct brain damage or secondary symptoms such as focal epileptic seizures. Patients >55 years with atypical or multiple hemorrhages were screened for CAA. Risk factors for white matter disease, particularly hypertension and diabetes, were documented and classified.¹⁸

Magnetic Resonance Imaging

MRI was performed using a 1.5-T MR system (Siemens Symphony, Erlangen, Germany). The following MR sequences were applied: axial T2, T1, FLAIR (repetition time 7530, inversion time 2300, echo time 110), diffusion-weighted imaging, apparent diffusion coefficient, high-resolution sagittal magnetization prepared rapid gradient echo-T1 (MPRAGE-T1), time-of-flight MR angiography, and axial T2* fast low-angle shot sequences (FLASH). The MRI

sequences had 21 slices of 5-mm thickness and an interslice gap of 1 mm with an in-plane voxel size of 1×1 and field of view of 250 mm. Sequence details included: axial T2* FLASH=800, echo time=26 ms, and flip angle=20°; FLAIR echo time=110 ms, repetition time=7530 ms, inversion time=2300 ms, and flip angle=180°; diffusion-weighted imaging repetition time=4900 ms, echo time=108 ms, b-value=1000 and flip angle=90° T1 pre-/postcontrast repetition time=511 ms, echo time=12 ms, and flip angle=90°. Additional sequences included venous time-of-flight MR angiography and postcontrast MRI (0.01 mmol/kg, Magnevist; Schering, Berlin, Germany) and were applied in cases of contrast agent contraindications or when considered appropriate by the neuroradiologist.

The following criteria were used for MRI image analysis. Using a single reader (F.S.) from FLAIR sequences, white matter changes (WMCs) were categorized into 3 classes according to Verdello et al¹⁹: “mild” WMCs were defined as single lesions <10 mm and/or areas of grouped lesions <20 mm in diameter; “moderate” WMCs were defined as single lesions between 10 and 20 mm and/or areas of grouped lesions >20 mm in diameter; “severe” WMCs were defined as single lesions or confluent areas of hyperintensity ≥20 mm in diameter. Microbleeds were only diagnosed when >2 mm and <10 mm in diameter and localized in cortical/corticosubcortical regions excluding hemorrhage in basal ganglia, thalamus, pons, or cerebellum. SH was diagnosed when located distal to lobar hemorrhage. PRES was diagnosed from initial FLAIR images when lesions had disappeared on follow-up examinations. Contrast-enhancing lesions suggesting BBB leakage were diagnosed using T1 and postcontrast T1 sequences.

Statistical Analysis

Data are presented as mean±SEM. A 2-tailed unpaired Student *t* test was used to evaluate differences between groups. Two-way analysis of variance was performed for Texas red efflux rates. Differences were considered statistically significant when *P*<0.05. Due to the lack of a standardized design and a control patient group, descriptive analysis was performed for the clinical study.

Results

CAA and BBB Dysfunction

Over a period of 1 year, 19 patients fulfilled the criteria for “probable CAA” (9 female, 10 male, average age: 75 years; age range: 61–84 years; 19 patients represent approximately 3% of all admissions to our unit within 12 months). No patient was diagnosed with “definitive or probable CAA with supporting evidence.” Details of patient data are summarized in the Table.

Twelve of 19 patients (63%) had a history of hypertension. At the time of discharge, 18 out of 19 patients (95%) were on antihypertensive medication to treat blood pressure-induced cerebral hemorrhage or to minimize the risk of hemorrhage recurrence. Four patients were diabetic. Six patients were diagnosed with severe WMCs, 2 of which were identified with PRES and reversible contrast-enhancing lesions due to BBB leakage. Five other patients were diagnosed with moderate WMCs; the remaining 8 patients had mild WMCs.

The most common findings within the patient population included lobar hemorrhage in 13 of 19 (68%) patients, cortico/corticosubcortical microbleeds in 17 of 19 (89%) patients, SHs in 7 of 19 (37%) patients, acute lacunar ischemic infarcts at atypical sites in 4 of 19 (21%) patients, and both PRES and reversible BBB leakage in 2 of 19 (10%) patients (Figures 1 and 2). In these 2 patients we also observed severe WMCs.

Human Aβ₄₀ Decreases TJ Proteins and Increases MMPs

To test the hypothesis that Aβ compromises BBB integrity, we exposed isolated rat brain microvessels to human Aβ₄₀ (hAβ₄₀) for 6 hours and determined protein expression of 4 major TJ proteins and 2 matrix-degrading MMPs by Western blotting of microvessel membranes. Figure 3A shows that hAβ₄₀ exposure decreased claudin-1 and claudin-5 protein expression in brain microvessel membranes in a concentration-dependent manner (claudin-1: control: 100%, 10 nmol/L Aβ: 115%, 50 nmol/L Aβ: 96%, 100 nmol/L Aβ: 69%; claudin-5: control: 100%, 10 nmol/L Aβ: 118%, 50 nmol/L Aβ: 92%, 100 nmol/L Aβ: 81%; determined by densitometric analysis; data are normalized to β-actin-loading controls). Protein expression of ZO-1 and occludin remained unchanged. In contrast, expression of MMP-2 and MMP-9 was elevated with increasing hAβ₄₀ concentration (Figure 3B; MMP-2: control: 100%, 10 nmol/L Aβ: 175%, 50 nmol/L Aβ: 224%, 100 nmol/L Aβ: 194%; MMP-9: control: 100%, 10 nmol/L Aβ: 120%, 50 nmol/L Aβ: 158%, 100 nmol/L Aβ: 163%). Consistent with this, zymography revealed a 2.4-fold increase of MMP-9 activity in brain microvessels treated with hAβ₄₀ (Figure 3C; control: 100%, hAβ₄₀: 239%±72%); MMP-2 activity was undetectable by zymography.

We also analyzed TJ protein and MMP expression in brain microvessels from 9-month-old transgenic hAPP mice (Tg2576 strain; hAPP gene with Swedish mutation). hAPP mice overexpress hAPP resulting in Aβ overproduction. At 9 months, hAPP mice exhibit substantial cerebrovascular hAβ accumulation and are therefore a model for CAA.²⁰ Consistent with our data from rat brain microvessels, claudin-1 and claudin-5 expression was decreased in microvessels from hAPP mice compared with microvessels from wild-type mice; ZO-1 and occludin expression were unchanged (Figure 4). Densitometric analysis revealed that claudin-1 expression was decreased by 84%±10% and claudin-5 expression was decreased by 43%±1%. As in our Aβ experiments with microvessels, MMP-2 and MMP-9 expression was increased in microvessels from transgenic hAPP mice compared with those from wild-type mice by 90%±12% and 145%±7%, respectively.

Our results demonstrate that hAβ₄₀ reduces expression of 2 major TJ proteins and increases expression of 2 matrix-degrading enzymes in brain microvessels *ex vivo* and in brain microvessels of hAPP mice *in vivo*. These findings suggest that Aβ triggers BBB leakage observed in CAA.

Increased BBB Permeability in Transgenic hAPP Mice

To test the extent to which decreased claudin-1 and claudin-5 expression and increased MMP-2 and MMP-9 expression affect BBB integrity, we measured the permeability of isolated brain microvessels from wild-type and hAPP mice using a previously established assay.¹⁵ In this assay, isolated brain microvessels are loaded in a solution of fluorescent Texas red dye (641 Da, 2 μmol/L, 1 hour). After washing with buffer, Texas red efflux from microvessel lumens is monitored by live cell imaging using confocal microscopy; afterward, luminal Texas red fluorescence is quantitated using image analysis software.

Table. Summary of Patient Data

Patient No.	Age, y	Sex	Hypertension	Diabetes Mellitus	Microbleeds	Lobar Bleeds	Additional MRI Findings	WMC	Apolipoprotein E	Additional Information
1*	84	F	–	+	>5	>3	SH, BBB leakage	Severe	N/P	Rapid-onset dementia, malignant course, angiography negative
2	61	M	+	+	11	2	2 acute lacunar ischemia	Severe	N/P	...
3	74	M	+	–	3	1	Acute lacunar ischemia	Mild	ε3/ε4	Focal seizures
4	73	F	+	–	3	0	SH, PRES	Severe	ε3/ε4	Angiography negative, focal seizures,
5	78	M	+	–	>20	1	SDH	Mild	N/P	Mild cognitive impairment, incidental cerebral and abdominal aneurysm
6*	71	M	+	+	>20	>2	PRES, BBB leakage	Severe	ε3/ε3	PRES, BBB leakage Asymptomatic, occipital bleeds/WMC, angiography negative
7	75	M	–	–	>7	1	SH	Severe	N/P	Acute and old severe SH, mild cognitive impairment, complex partial seizures
8	78	F	–	+	5	0	SH	Mild	ε3/ε3	Mild cognitive impairment
9	85	F	–	–	>20	1	...	Mild	ε3/ε4	Occipital bleeds/WMC
10	70	M	+	–	>40	0	...	Severe	N/P	Microbleeds cortical and subcortical, mild hypertension, dementia >7 y
11	83	F	–	–	0	2	SDH	Moderate	N/P	...
12	78	M	–	–	2	0	SH	Mild	ε2/ε3	Focal seizures, additional old basal ganglia hemorrhage
13	84	F	+	–	0	2	...	Mild	N/P	SH due to severe lobar cortical hemorrhage
14	74	M	+	–	>20	3	...	Moderate	ε3/ε4	Generalized seizure, status postembolic ischemia due to AF
15	66	M	+	–	8	0	Acute lacunar infarct	Moderate	ε2/ε4	Mild cognitive impairment, angiography negative
16	80	F	+	–	1	3	...	Moderate	N/P	SH due to severe lobar cortical hemorrhage
17	65	F	+	–	8	...	Acute lacunar infarct	Mild	N/P	Chemotherapy due to mamma carcinoma
18	81	M	–	–	7	2	SH	Moderate	N/P	...
19	74	F	+	–	12	2	SH	Mild	N/P	AF, malignant melanoma, rapid CAA

WMCs indicates white matter changes; F, female; M, male; SH, superficial hemosiderosis; BBB, blood–brain barrier; PRES, posterior reversible encephalopathic syndrome; SDH, subdural hematoma; N/P, not performed; CAA, cerebral amyloid angiopathy.

*Patient 1 is represented in Figure 1; Patient 6 is represented in Figure 2.

As previously described, we used a 100-mmol/L hypertonic mannitol solution as a positive control to osmotically increase microvessel permeability.¹⁷ Figure 5 shows that Texas red efflux from microvessels from hAPP mice was faster compared with efflux from microvessels from wild-type mice; addition of mannitol increased Texas red efflux to the maximum effect. Texas red efflux rates over the entire time course were calculated as 0.265 ± 0.04 minutes⁻¹ (wild-type), 0.397 ± 0.05 minutes⁻¹ (hAPP; $P < 0.001$ compared with wild-type), 0.336 ± 0.05 minutes⁻¹ (wild-type with mannitol; $P < 0.001$ compared with wild-type), and 0.356 ± 0.03 minutes⁻¹ (hAPP with mannitol; $P < 0.001$ compared with

wild-type). Thus, Texas red efflux in microvessels from hAPP mice was 1.5-fold that of microvessels from wild-type mice indicating higher permeability. Note that luminal Texas red fluorescence in microvessels from hAPP mice did not reach the same levels as in microvessels treated with mannitol (positive control for maximum barrier opening). This observation argues for barrier leakage rather than full disruption.

Discussion

CAA is a multifaceted disorder resulting from Aβ deposition in brain microvessels that causes injury to the neurovascular unit especially in the elderly. Here, we demonstrate in brain

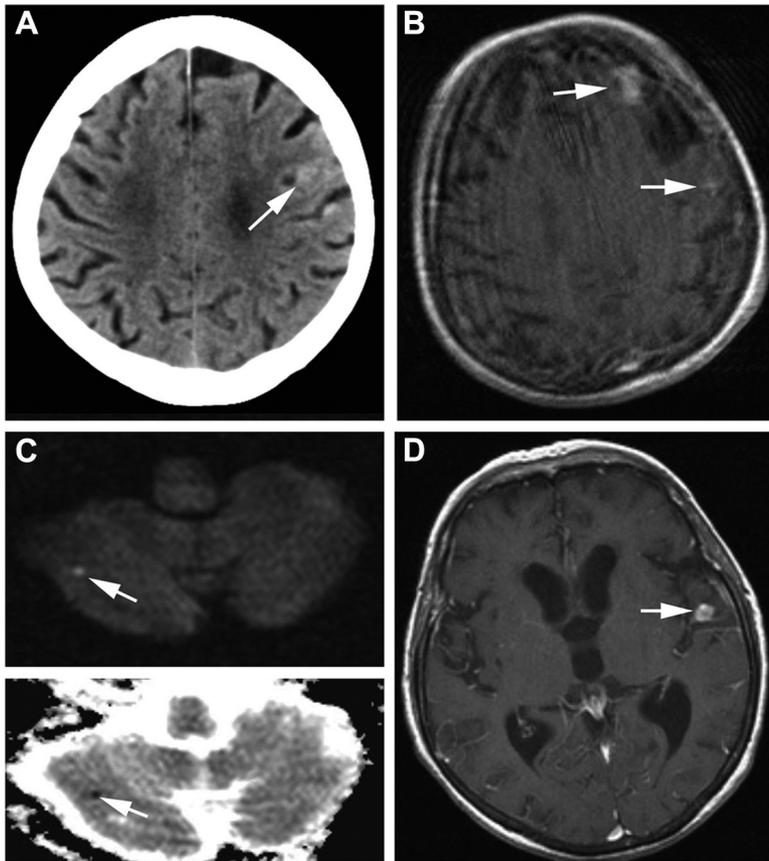


Figure 1. Imaging data of Patient 1. **A**, Day 19 after initial presentation: cranial CT showing left frontal small subarachnoid hemorrhage. **B**, Day 22: postcontrast T1-MRI showing left frontal and small left parietal area of contrast enhancement. **C**, Day 28: diffusion-weighted imaging (upper) and apparent diffusion coefficient (lower) showing small right cerebellar infarct. **D**, Day 28: postcontrast T1-MRI showing new area of contrast enhancement, whereas left frontal lesion disappeared.

microvessels from rats that $hA\beta_{40}$ decreases expression of the TJ proteins claudin-1 and claudin-5 and increases expression and activity of the matrix-degrading enzymes MMP-2 and MMP-9. We confirmed our results in microvessels from transgenic hAPP mice that overproduce human $A\beta$. Consistent with these findings, we show increased Texas red efflux in brain microvessels from hAPP mice compared with microvessels from wild-type mice.

Our findings suggest that $A\beta$ contributes to loss of BBB integrity that could be responsible for BBB dysfunction, cognitive decline observed in patients with CAA, and additional cerebrovascular pathology including SH, PRES, lacunar cerebral ischemia, and BBB leakage. Indeed, in a group of 19 patients with “probable CAA,” we identified 2 patients with BBB leakage for MRI contrast agents, and 1 of the 2 patients already presented with PRES 10 months earlier. Both contrast leakage and PRES are likely due to increased BBB permeability, which underscores the relevance of our animal data.

$A\beta$ Contributes to Loss of BBB Integrity

The effect of $A\beta$ on BBB integrity has been studied in several cell culture models. Gonzalez-Velasquez et al showed that treatment of cultured human brain endothelial cells with 2.5 to 10 $\mu\text{mol/L}$ $A\beta_{40}$ triggered the TJ protein ZO-1 to retreat from the plasma membrane, which was accompanied by decreased transendothelial electric resistance.¹¹ Marco and Skaper demonstrated that exposing rat brain endothelial cells to 20 $\mu\text{mol/L}$ $A\beta_{42}$ triggered ZO-1 and claudin-5 relocation from the plasma membrane, a decrease in occludin expres-

sion, and an increase in claudin-1 expression.²¹ Tai et al demonstrated that $A\beta_{40}$ activated microtubule-associated protein kinase, which decreased occludin expression and increased permeability in human brain endothelial cell cultures, whereas claudin-5 and ZO-1 remained unchanged.²² Carrano et al analyzed postmortem CAA patient brain slices for TJ protein and observed a loss of occludin, claudin-5, and ZO-1 in brain microvessels.²³ We show that $hA\beta_{40}$ decreased rat brain microvessel expression of claudin-1 and claudin-5, whereas ZO-1 and occludin expression was not affected.

The discrepancies in our observations and those mentioned are likely due to several factors. First, the $A\beta$ species were different ($hA\beta_{40}$ versus $A\beta_{40}$ aggregates versus $A\beta_{42}$). We used monomeric $hA\beta_{40}$, which is 1 main $A\beta$ species in AD and CAA. Second, the $A\beta$ concentrations were different (nanomolar versus micromolar). We used $hA\beta_{40}$ at 100 nmol/L, which is comparable to $A\beta_{40}$ levels found in brain tissue from patients with AD, whereas micromolar $A\beta$ concentrations do not reflect AD or CAA and probably lead to acute toxicity.²⁴ Third, the experimental models in the mentioned studies were in vitro cultures of rat, mouse, or immortalized human endothelial cells. In contrast, we used intact, living brain capillaries ex vivo that were isolated from rats and confirmed our data with microvessels from hAPP mice (Tg2576). These mice overexpress the Swedish mutation of the human APP gene (*APP^{Swe}*) and show progressive $A\beta$ accumulation in brain and brain microvessels, $A\beta$ brain aggregates similar to “senile plaques” in human AD, and cognitive deficits that correlate with $A\beta$ brain load.²⁰ In hAPP

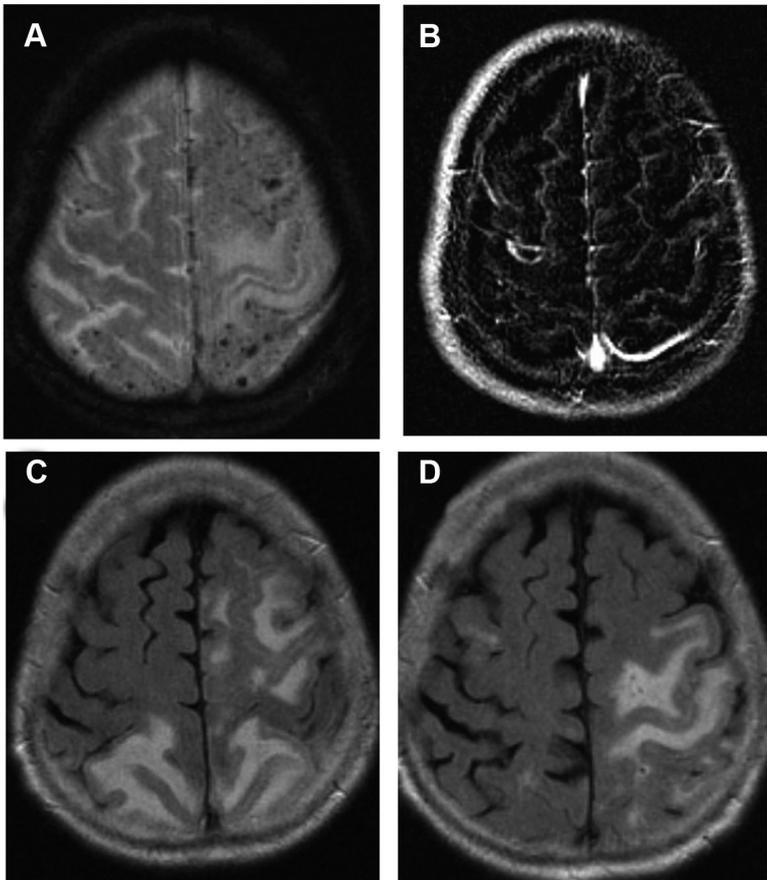


Figure 2. Imaging data for Patient 6 with CAA and PRES. **A**, MRI with T2* FLASH sequences showing multiple microhemorrhages. **B**, Subtraction image demonstrating BBB leakage around a small microhemorrhage. **C**, FLAIR image with occipital edema. **D**, FLAIR image 2 months later with new left parietal and no occipital edema. CAA indicates cerebral amyloid angiopathy; PRES, posterior reversible encephalopathic syndrome; FLASH, fast low-angle shot; BBB, blood–brain barrier; FLAIR, fluid-attenuated inversion recovery.

mice, A β also accumulates in brain microvessels, impairs vascular smooth muscle function, and causes cell death, all of which are CAA characteristics.²⁵ Therefore, hAPP mice are a well-established CAA model to study the A β effect on the neurovasculature and the mechanisms of these effects.¹³

Reports also show that expression of matrix-degrading MMPs is changed in the CAA brain and that increased MMPs contribute to BBB dysfunction. Lee et al found A β -mediated upregulation of MMP-9 in mouse endothelial cells and a mouse CAA model (APPsw mice).²⁶ These data are consistent with our findings in microvessels exposed to A β and microvessels from hAPP mice showing increased MMP-2

expression and increased MMP-9 expression and activity. In this regard, Taskanen et al performed microarrays with human brain tissue and found an association between increased MMP-19 expression and CAA with intracerebral hemorrhage as well as an association between increased MMP-26 expression and CAA alone.²⁷

Together, our data suggest that A β contributes to the loss of barrier integrity in CAA by decreasing TJ protein expression and increasing MMP expression and activity. Likely consequences are barrier leakage and cortical cerebral hemorrhage, which are consistent with our observations in patients with CAA.

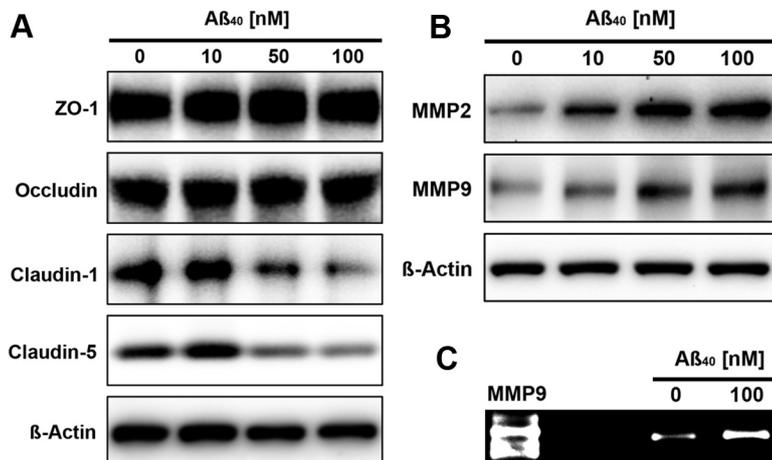


Figure 3. A β effects on tight junction (TJ) proteins and MMPs in brain microvessels. Western blots showing concentration-dependent **(A)** reduction of the TJ proteins claudin-1 and claudin-5 and **(B)** increase of the matrix metalloproteases MMP-2 and MMP-9 in rat brain microvessels exposed to human A β_{40} for 6 hours. Expression of the TJ proteins ZO-1 and occludin remained unchanged. **C**, Zymogram showing increased MMP-9 activity in isolated brain microvessels that were exposed to 100 nmol/L A β_{40} for 6 hours. A β indicates amyloid- β .

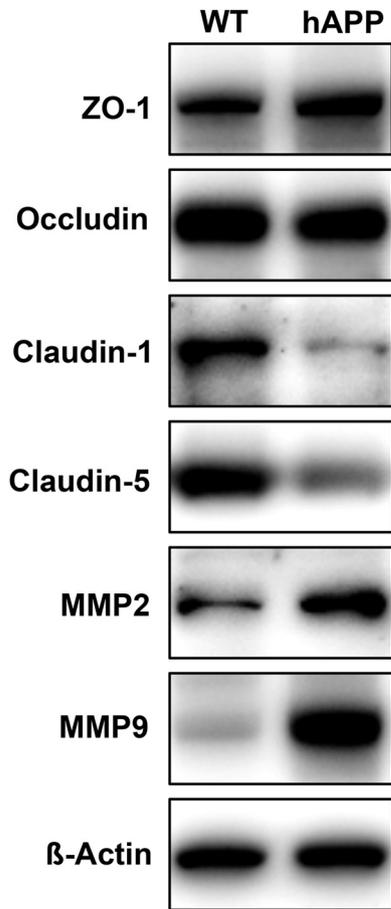


Figure 4. TJ proteins and MMPs in brain microvessels from hAPP mice. Western blots showing reduction of claudin-1 and claudin-5 and increase of MMP-2 and MMP-9 in brain microvessels from hAPP mice. Expression of ZO-1 and occludin remained unchanged. TJ indicates tight junction; MMP, matrix metalloproteinase; hAPP, human amyloid precursor protein.

Amyloid- β Brain Clearance

In the diseased brain, $A\beta$ accumulates in the pericapillary space or in capillary membranes, which is a CAA hallmark. $A\beta$ is mainly produced in neurons and astrocytes and then released into brain interstitial fluid.²⁸ Recent evidence suggests that the BBB is critical for $A\beta$ brain clearance and that this process involves 2 steps.²⁹ Studies indicate that low-density lipoprotein receptor-related protein 1 is responsible for the first step— $A\beta$ uptake from the brain into endothelial cells.^{30–32} In CAA, lipoprotein receptor-related protein 1 is downregulated by serum response factor and myocardin, which reduces lipoprotein receptor-related protein-mediated clearance of $A\beta$.³³ Both transcription factors, serum response factor and myocardin, have been shown to be overexpressed in patients with AD and mouse models and are thought to be involved in the development of CAA and the hypercontractile arterial phenotype that is associated with brain hypoperfusion.⁴ The second step— $A\beta$ efflux from the endothelium into blood—seems to be mediated by the efflux transporter P-glycoprotein (P-gp, *ABCB1*).^{34,35} This was first demonstrated by Cirrito et al who showed reduced $A\beta$ brain clearance in P-gp-null mice.³⁶ With regard to CAA, Vogelgesang et al reported P-gp upregulation in human brain

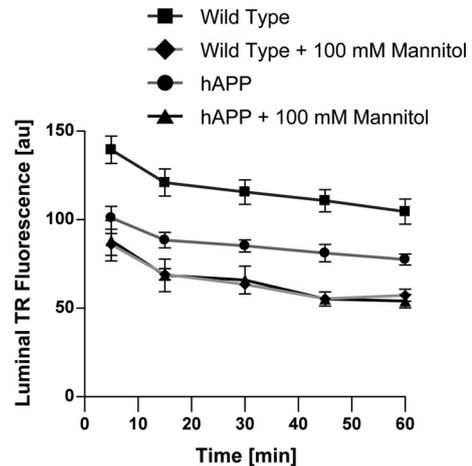


Figure 5. Permeability of brain microvessels from hAPP mice. Efflux of the fluorescence dye Texas red (TR) was monitored over 60 minutes in microvessels from wild-type and hAPP mice with/without mannitol used as positive control for barrier opening. Data are mean \pm SEM for 7 brain microvessels per time point from 1 microvessel isolation from 20 mice; SEM are in the range 3.1 to 11.4. Shown are arbitrary units (0–255). First-order efflux rates were calculated using nonlinear regression. hAPP indicates human amyloid precursor protein.

microvessels in early CAA suggesting a compensatory mechanism to increase $A\beta$ brain clearance. However, in later stages of CAA, vessels with high $A\beta$ load showed no P-gp expression.³⁷ We recently demonstrated that P-gp is decreased in brain microvessels from hAPP mice. Treating these mice with pregnenolone–carbonitrile restored P-gp, decreased $A\beta$ brain levels, and significantly reduced $A\beta$ deposition in microvessels.¹⁴

Our data from brain microvessels suggest that $A\beta_{40}$ contributes to BBB dysfunction and our patient data indicate that CAA is accompanied by multiple injuries, of which only lobar hemorrhage and cognitive decline attributed to AD are clinically relevant. It has been shown that neurovascular injury in CAA results from amyloid aggregation when cerebrovascular occlusive disease and inflammation are also present.⁷ In this regard, it has been shown that soluble $A\beta_{40}$ can cause inflammation with monocyte transmigration through endothelial cells, decreased transendothelial electric resistance, and TJ redistribution, all of which contribute to BBB dysfunction.¹¹ Moreover, it has been demonstrated that soluble $A\beta_{40}$ generates reactive oxygen species and proinflammatory cytokines, which can induce MMPs that promote cerebral hemorrhage through basal membrane degradation.¹⁰

Finally, $A\beta_{40}$ -mediated injury to the neurovasculature unit could have profound effects on neuroendocrine crosstalk among neurons, astrocytes, microglia, pericytes, and endothelial cells ultimately resulting in BBB breakdown. Postmortem analysis of brains from patients with advanced AD demonstrated that thinning and discontinuities of the vascular basal membrane are associated with BBB leakage and brain penetration of the plasma protein prothrombin.³⁸ However, BBB leakage, especially in the elderly, could also be due to other diseases such as hypertension, diabetes, and inflammation.^{39,40}

Clinical Findings

Noninvasive neuroimaging is critical to diagnose CAA. Imaging can be indirect, for example, cerebral CT and MRI,

to visualize hemorrhage indicative of CAA, or direct, for example, positron emission tomography, to visualize deposition of $A\beta$ labeled with the Pittsburgh Compound B.^{41,42} We used MRI to characterize 19 patients with “probable CAA” for neurovascular pathology. We found the following 3 main characteristics.

Superficial Hemosiderosis

SH in the context of CAA appears to be more frequent than previously described and occurred in 7 of 19 patients with CAA, who became symptomatic with acute cephalgia. SH is characterized by distinct symptoms such as absent headache, focal seizures mimicking transient ischemic attacks, and migraine aura-like symptoms.⁴³ SH can only be diagnosed in patients with at least “probable CAA” and after exclusion of vasculitis, arterial dissection, dural arteriovenous fistulas, or cortical venous thrombosis. Finelli reported of 4 patients with CAA with SH and included a summary of 7 previously published cases in the report.⁴¹ Raposo et al reanalyzed MRI data from 10 patients with SH according to the Boston criteria and reclassified 8 of 10 patients as SH with probable CAA and 2 of 10 patients as SH with “possible CAA.”⁸ Note that at this point we cannot rule out if additional factors such as hypertension, diabetes, and hypercholesterolemia could have potentially contributed to CAA in these patients.

Ischemic Lacunar Infarction, WMCs, and CAA

Menon and Kidwell reported on 1 patient with evolving small vessel ischemic injury detected by diffusion-weighted imaging.⁷ We observed this phenomenon in 4 of 19 patients, none of whom displayed symptoms such as transitory ischemic attacks or stroke. Without autopsy data, one could speculate that endothelial dysfunction leads to small vessel occlusion and silent brain infarcts that could contribute to WMC and cognitive decline, which may contribute to the development of dementia.⁴⁴ Again, given our patient collective with acute neurological symptoms, the true incidence of these ischemic lesions may be overestimated.

PRES and CAA-Induced Inflammation

Symptoms of PRES are diverse and include headache, confusion, seizures, and vision loss. In 1 patient, Kofler et al linked PRES to CAA based on MRI evidence FLAIR. Additional evidence from a brain biopsy included endothelial cell activation, T-cell trafficking, and increased expression of vascular endothelial growth factor, all indicating inflammation.⁶ The authors concluded that PRES was triggered by inflammation from an upper respiratory infection the patient had earlier. In contrast, Bernstein and colleagues interpreted a similar finding as CAA-induced inflammation (CAA-I) and stated that the resolution of symptoms was due to successful steroid treatment.⁴⁵ In a recent review, Chung et al described overlapping CAA-I symptoms with those of PRES, including headaches, cognitive and behavioral changes as well as seizures and focal neurological deficits.⁹ In addition, histopathologic evidence of CAA-I includes either focal spots with inflammatory cells such as multinucleated giant cells and macrophages or infiltration by epithelioid cells adjacent to amyloid-loaded microvessels. The majority of reports de-

scribe CAA-I diagnosis using FLAIR-MRI and only rarely using postcontrast-enhanced MRI.

In our study, PRES was diagnosed in 2 of 19 patients (10%) and reversible BBB leakage was also diagnosed in 2 of 19 patients. With the proposed criteria, all 4 of these patients would be classified as probable CAA-I. In our patients, PRES was found in brain areas with microbleeds that are a potential trigger for edema due to reduced expression of the TJ proteins claudin-1 and claudin-5.

Classic PRES etiology is not fully understood but could result from vasogenic edema with decreased serum albumin levels and increased BBB permeability.⁴⁶ PRES is often associated with hypertension and medically induced immunosuppression. Note that CAA-I has a similar pathophysiological background and that hemorrhage can also be caused by immunosuppression.⁴⁷ However, PRES and CAA-I clearly differ in their inflammatory response and endothelial activation. In addition, increased BBB permeability seen in both states could differ and be due to paracellular or transcellular barrier opening. For example, reduced expression of TJ proteins could increase paracellular BBB permeability allowing MRI contrast agents to enter the brain.⁴⁸ Indeed, Carrano et al recently showed that $A\beta$ reduced claudin-5, occludin, and ZO-1 expression in human brain microvessels.²³ We did not observe reduced occludin and ZO-1 expression but found reduced claudin-1 and claudin-5 expression in $A\beta$ -treated rat brain microvessels and in microvessels from hAPP mice. Consistent with this, paracellular BBB permeability was increased in brain microvessels from hAPP mice. Alternatively, edema could be caused by increased transcellular permeability due to changes in plasma membrane proteins that are involved in maintaining central nervous system water/electrolyte homeostasis. Whether or not this is the case in CAA is unclear.

Together, it remains to be shown if PRES and CAA-I are linked and contribute to overall CAA pathology.

Study Limitations

This study has 3 limitations: (1) recruitment of patients with CAA was performed in the stroke unit setting as opposed to a memory clinic with focus on acute therapy and diagnostics. Therefore, a standard stroke MRI protocol was used and contrast agents were only given when deemed necessary by the attending neuroradiologist. Neuropsychological testing or analysis of cerebrospinal fluid was not performed, which is important for dementia work-up but not acute stroke therapy. Patient control groups with other underlying factors (eg, PRES or SH) were not included in our study and brain biopsy or histopathologic verification was not performed. Therefore, the patients with CAA included in our study may differ significantly from patients in other studies. Future prospective and longitudinal studies should link neurological, neuro-radiological, and neuropsychological findings in acute stroke and neurodegenerative neurology to better characterize CAA; (2) the sensitivity of gadolinium-based MRI contrast agents (approximately 600 Da) to detect BBB leakage is dependent on the MRI field strength, the MRI sequence, and the timing after administration of contrast agents; and (3) the animal model used here (hAPP mice) resembles the genetic variants

of CAA, and thus, our findings likely do not apply to patients with spontaneous CAA. Small animal MRI with high field strength to visualize cortical microbleeds may further help to characterize hAPP mice with respect to CAA.

Experimental Therapy of AD/CAA

Two strategies that increase A β brain clearance and lower A β brain levels have been proposed to treat CAA/AD.^{49,50} One approach, A β immunotherapy, capitalizes on antibodies that bind and clear plasma A β , thereby creating a peripheral “sink” that accelerates removal of soluble A β from the brain.⁵¹ In a recent study, passive immunization was used in patients with AD who were treated with the A β antibody bapineuzumab,³⁸ and treatment with this antibody at the highest dose led to mild symptomatic and reversible vasogenic edema in 2 of 19 patients (10%). Clinical symptoms were similar to those observed in PRES and CAA-I indicating that the cerebral endothelium is the target of this therapy and thus, likely an interesting target for AD and CAA. This approach—passive immunization with an A β antibody—represents a case of “life imitating art” in which therapeutically administered antibodies mimic naturally occurring anti-A β antibodies.⁵² A similar approach is based on using recombinant, soluble lipoprotein receptor-related protein that also binds A β has thus far only been tested in mice.⁴⁹ Another strategy to treat CAA and AD targets the efflux transporter P-gp at the BBB.⁴⁹ P-gp has been shown to be involved in A β brain clearance but its expression is reduced in AD.^{14,44,53,54} Therefore, in this approach, P-gp is restored through activation of the nuclear receptor PXR to improve A β brain clearance. Given that a large number of drugs are P-gp substrates that could act as competitive P-gp inhibitors, modulation of P-gp could result in drug–drug interactions. Such complications should be considered when treating elderly patients with AD using multiple medications and could limit the therapeutic benefit of BBB P-gp induction.

Conclusions

Reduced A β brain-to-blood clearance through the BBB, compromised BBB integrity, and increased BBB permeability could all contribute to WMCs and mental decline in CAA/AD. Indeed, our patient and animal data suggest amyloid deposition in the neurovasculature contributes to dynamic multifocal endothelial dysfunction in CAA. Our study further suggests that the Boston criteria for the diagnosis of CAA might need to be refined to include SH, ischemia, and CAA-I with MRI findings of PRES and BBB leakage.

Future studies should focus on serial neuroimaging, BBB and inflammatory biomarkers, other cerebrovascular risk factors, and treatment to identify the events that trigger BBB dysfunction. A prospective and longitudinal study should be conducted to investigate the overall incidence of SH, ischemia, and CAA-I with MRI findings of PRES and BBB leakage in acute and chronic phases of the disease; the findings should be correlated with the Boston criteria.

Together, our findings suggest that the BBB plays a crucial role in CAA/AD pathology. Novel therapeutic strategies that prevent A β -mediated BBB leakage and restore BBB function could help improve CAA/AD treatment.

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