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A shift in microglial β -amyloid binding in Alzheimer's disease is associated with cerebral amyloid angiopathy

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Abstract

Alzheimer's disease (AD) and cerebral amyloid angiopathy (CAA) are two common pathologies associated with β -amyloid (A β) accumulation and inflammation in the brain; neither is well understood. The objective of this study was to evaluate human post mortem brains from AD subjects with purely parenchymal pathology, and those with concomitant CAA (and age-matched controls) for differential expression of microglia-associated A β ligands thought to mediate A β clearance and the association of these receptors with complement activation. Homogenates of brain parenchyma and enriched microvessel fractions from occipital cortex were probed for levels of C3b, membrane attack complex (MAC), CD11b and α -2-macroglobulin and immunoprecipitation was used to immunoprecipitate (IP) CD11b complexed with C3b and A β . Both C3b and MAC were significantly increased in CAA compared to AD-only and controls and IP showed significantly increased CD11b/C3b complexes with A β in AD/CAA subjects. Confocal microscopy was used to visualize these interactions. MAC was remarkably associated with CAA affected blood vessels compared to AD-only and control vessels. These findings are consistent with an A β clearance mechanism via microglial CD11b that delivers A β and C3b to blood vessels in AD/CAA, which leads to A β deposition and propagation of complement to the cytolytic MAC, possibly leading to vascular fragility.

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder among people over the age of 65 and is accompanied by some degree of the microvasculopathy cerebral amyloid angiopathy (CAA) in 75–90% of cases (15). CAA is characterized by the deposition of amyloid beta (A β) in the tunica adventitia and media of leptomeningeal and

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Conflict of Interest

The authors are aware of no real or potential conflict of interest.

penetrating cortical arteries and degeneration of medial vascular smooth muscle, resulting in vascular fragility and intracerebral hemorrhage (42, 45). Evidence is mounting that CAA plays an important role in the pathogenesis of cognitive deficits associated with AD, sometimes through the mechanism of microbleeds or microinfarcts (19, 29, 39). However, the mechanisms responsible for this vascular A β accumulation, smooth muscle loss and fragility have remained speculative and defied therapeutic intervention.

Microglia are the resident immune and phagocytic cells of the nervous system and are known effectors of brain A β clearance (17, 47). There is an extensive literature describing the chemotactic and phagocytic response of microglia to A β from studies on human autopsy material, as well as animal and in vitro studies. Microglia are capable of binding and phagocytosing A β and removing its breakdown products from the brain parenchyma. In the normal brain, microglia exist in a highly ramified phenotype, constantly surveying their environment, probing for tissue architecture abnormalities and foreign infiltrates (13). After a pathological insult, microglia shift their phenotype to an “activated” amoeboid-like morphology, expressing a variety of inflammatory and receptor proteins (11, 17). Recent studies of human autopsy material suggest that microglia become dystrophic and functionally impaired with aging, resulting in immune dysfunction and improper expression of cytokine and receptor profiles (23, 40). This theme of age-related alterations in microglial function led us to examine the possible role of microglia in the pathogenesis of CAA.

In addition to the role of microglia, there is evidence for late-complement activation on the microvasculature in CAA (25, 34, 36). Complement activation involves a series of coordinated protein cleavages and interactions ultimately activating and depositing the terminal lytic Membrane Attack Complex (MAC). The MAC is a macromolecular protein complex consisting of single components of C5b, C6, C7, C8 and multiple C9 components forming a pore in target cell membranes, inducing cell lysis. MAC formation is initiated by the formation of a C5-convertase (any of a variety of complexes containing C3b). Complement C3b, a cleavage product of the central C3 component, is a high-affinity ligand for β -amyloid and can act as an opsonin of A β plaques (7, 35). This complex (C3b/A β) binds to Complement Receptor 1 (CR1) on erythrocytes, which appears to be involved in clearance of A β in the peripheral circulation (35). CD11b (CR3) a homolog of CR1, which is selectively expressed on microglia in the brain, binds to C3b and is implicated in the adhesive interactions of monocytes, macrophages, and granulocytes (17, 30) as well as complement coated particles, and binds A β in a yeast model (8). We hypothesized that CD11b may serve as a cell-surface receptor for the complex of A β and C3b and may be a mechanism of A β clearance from the parenchyma and complement activation on vascular elements. Finally, Apolipoprotein E ϵ 4 (ApoE ϵ 4), a genetic risk factor for AD, is known to activate the complement cascade (26), so we additionally asked whether or not the apoE E4/CD11b/C3b/A β complex occurred on human microglia in human postmortem tissue. This study of human, post-mortem brains with CAA has led to the identification of a microglial cell-surface complex that appears to be involved in A β clearance in CAA and may explain the late complement activation on the vessel wall at the site of A β deposition.

Methods

Tissue Selection

Post mortem tissue was obtained from the Alzheimer’s Disease Research Center Brain Bank at the University of California, Los Angeles. All patients or their surrogates consented to participate in research protocols prior to tissue donation, and the study was approved by the Institutional Review Board of Loma Linda University Medical Center (approval #54174). Patient demographics are described in Table 1. Both frozen tissue specimens and fixed tissues were available for study. Neuropathologic examination at the time of autopsy

included Braak and Braak staging of AD and Vonsattel grading of CAA pathology (6, 12). CAA staging of CAA severity is as follows: stage 1- β -amyloid deposition limited to the basement membrane of arterioles and primarily involving leptomeningeal vessels; stage 2- β -amyloid deposited between vascular smooth muscle cells and pathology extends to penetrating arterioles and stage 3- β -amyloid largely replaces vascular smooth muscle in arterioles. Specimens were graded based on the majority of vessels displaying the respective Vonsattel stage. Microaneurysms and microhemorrhages often occur with Vonsattel stage 3 CAA, however, microhemorrhages were rare in this cohort. Additionally, no patients were included in this study with fatal lobar or intracerebral hemorrhage.

Frozen tissue was isolated from the occipital lobe for five groups of patients; four samples were obtained from an aged, neurological control group (n=4), along with four AD samples without evidence of significant CAA (Vonsattel grades 0–1) and sixteen AD samples with varying degrees of CAA, grouped into mild (n=8), moderate (n=4) and severe (n=4) CAA (grade 3) (ADmiCAA, ADmodCAA and ADsevCAA, respectively).

Microvessel Enrichment

Brain microvessels were isolated from the occipital lobe of frozen postmortem brain tissue from four cases in each group. A homogenization buffer was prepared over ice consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 300 mM sucrose, 2 mM EDTA and protease inhibitor cocktail. Samples were homogenized with three to four vertical strokes of a Teflon pestle in a glass tube, the homogenate then poured over a 72 μ m mesh sieve (Gelman Sciences) with microvessels collected by flushing the sieve with ice cold homogenization buffer. Homogenates containing microvessels were then poured into 20 mL Falcon tubes, centrifuged for 15 minutes at 2,000 rpm, and vessels concentrated into homogenization buffer and verified by examining a sample fixed with 100% ethanol on charged glass slides (Fisher Scientific, Pennsylvania). Samples were sonicated 3 times to further homogenize blood vessels and protein quantification cached by the Bradford assay (Pierce Laboratories, Illinois).

Western Blot Analysis

Protein levels of complement components and regulators and α_2 M on occipital lobe vessels and occipital lobe whole brain tissue were determined by denaturing SDS-PAGE and western blot. Briefly, tissue samples were isolated containing roughly equal proportions of white and grey matter from the occipital lobe, and homogenized in an ice cold sucrose buffer containing a protease inhibitor cocktail. Tissue was further homogenized in a fitted Teflon pestle in a glass homogenization tube. Nuclear debris was cleared from the suspension by centrifugation at 1000g for 15 minutes and total protein concentration was determined in the supernatant by the Bradford assay (Pierce Laboratories, Illinois). Samples were prepared for electrophoresis by adding 25% v/v loading buffer (Invitrogen, California) and 10% v/v beta-mercaptoethanol and heating at 95°C for ten minutes. Ten micrograms of total protein were loaded into each lane of a 10% polyacrylamide gel and electrophoresed at 100V in a MiniProtean Tetra System (BioRad Laboratories, California). The proteins were then transferred to a nitrocellulose membrane at 30V for four hours. The resulting blot was blocked in an albumin-based blocking buffer (Invitrogen) and probed with either beta-actin (mouse monoclonal; 1:1000), α_2 M (rabbit polyclonal; 1:500), CD11b (rabbit polyclonal; 1:500), C3b (mouse monoclonal; 1:500), C6 (mouse monoclonal; 1:500), C5b-9 (rabbit polyclonal; 1:500), CD59 (mouse monoclonal; 1:500) and apoE E4 (mouse monoclonal; 1:500) antibodies (all from Abcam) and β -amyloid 1–16 (clone AB 10) (mouse monoclonal; 1:1000; Millipore). After an overnight incubation at 4°C, blots were thoroughly washed with TBS buffer containing 0.05% Tween-20 and a fluorescently labeled goat anti-mouse or rabbit secondary (IRDye, Licor) was applied. After two-hour incubation, blots were washed

and visualized on an Odyssey Infrared Imaging System (Licor Biosciences, Nebraska). Optical density was determined using Odyssey 2.0 software. Data was collected as the relative intensity of the band of interest compared to the corresponding loading control band of β -actin.

Immunohistochemistry

Paraffin-embedded, formalin fixed sections of occipital lobe tissue were deparaffinized in two exchanges of xylene and rehydrated in serial exchanges of ethanol. Antigen retrieval was performed by heating sections in a microwave on high power (3x at 90 seconds each) in a 10 mM citrate buffer. The sections were then treated with 1% hydrogen peroxide in PBS to block endogenous peroxidase activity, incubated with a blocking solution of 4% normal serum in PBS, and incubated with the primary antibody of choice overnight at 4°C. After washing with PBS, sections were incubated with a HRP conjugated secondary antibody for 2 h at 4°C, washed in additional exchanges of PBS and treated with diaminobenzidine/hydrogen peroxide (DAB) for 10 min (Vector Laboratories), followed by hematoxylin as a counterstain. They were then rinsed for 3 min in PBS, dehydrated through serial alcohols, cleared in xylene and coverslipped with mounting resin (Permount, Fisher). The antibody used was against C6 (mouse monoclonal 1:500) (Abcam).

Co-immunofluorescent studies were prepared on sections as previously mentioned. After blocking with 4% normal serum in PBS, slides were incubated with primary antibodies against alpha smooth muscle actin (α SMA) (mouse polyclonal; 1:200), C5b-9 (rabbit polyclonal; 1:500) (Abcam), CD11b (rabbit polyclonal; 1:500) (Abcam) and an antibody against $A\beta_{40}$ (mouse monoclonal; 1:1000) (Abiotec). Sections were similarly prepared with 0.01% Thioflavin S, but washed in two exchanges of 70% ethanol before application of DAPI. After overnight incubation at 4°C, slides were washed and incubated with secondary antibody conjugated to Dylight 550, Texas Red or FITC (all abcam) for two hours at 4°C and then washed. Sections were stained with DAPI (Vector Laboratories) and visualized by a LSM 710 confocal microscope (Zeiss). Negative controls were prepared in the same fashion, but lacked the incubation step with primary antibody.

Immunoprecipitation

Co-immunoprecipitation was done on occipital lobe whole-brain homogenates; groups consisted of controls, AD only and AD with severe CAA (n=4 in all groups). Briefly, magnetic Dynabeads (Invitrogen) were resuspended and 50 μ l was transferred to 12 tubes. Beads were separated from supernatant on a magnet and the supernatant was removed. 5 μ l of anti-CD11b antibody (1 μ g/ μ l; Abcam) was diluted in 200 μ l 1x PBS with .02% Tween-20 (pH 7.4) and each tube was incubated for 10 minutes with rotation at room temperature. Tubes were then placed on the magnet and the supernatant was removed. The bead-Antibody complex was resuspended in 200 μ l PBS with Tween-20 and washed with gentle agitation. Tubes were placed on the magnet and the supernatant was removed. Samples from control, AD-only and AD with CAA (n=4 in each group) were added to the bead-antibody complex at 150 μ l and incubated with gentle agitation at room temperature for 30 minutes. Tubes were then placed on the magnet and the supernatant was transferred to a clean tube for further analysis. The beads-antibody-antigen complex was washed three times using 200 μ l of 1x PBS with gentle agitation. The complex was resuspended in 100 μ l of 1x PBS and transferred to clean tubes to avoid co-elution of proteins bound to the tube wall. Tubes were placed on the magnet and the supernatant removed, followed by addition of 20 μ l non-denaturing elution buffer (50 mM glycine pH 2.8) and incubated for 2 minutes. Tubes were placed on the magnet and the supernatant was transferred to a clean tube and prepared for western blot as described in the Western Blot section.

Statistical Analysis

Data are reported as mean \pm standard error. Because of the small sample size, the data did not fit a normal distribution, so differences between groups were assessed by Kruskal-Wallis non-parametric ANOVA. Significant differences between groups were then reported by post-hoc analysis. Genotyping analysis was assessed with Fischer's exact test for categorical data. $\alpha < 0.05$ was taken as a significant result and statistical analysis was done on SPSS 20 software.

Results

APOE4 and CR1 genotyping analysis

Genotyping of our patient cohort did not show the usual trend for risk of AD or CAA with *APOE4*, in which even only one $\epsilon 4$ allele can significantly increase the risk of both AD and CAA. The majority of patients in all groups were homozygous for the $\epsilon 3$ allele, with at least one case harboring one $\epsilon 4$ allele in each group. *CR1* genotyping was done in light of recent findings by Biffi *et al.* (5), in which individuals homozygous for the A allele showed a higher risk for intracerebral hemorrhage (ICH) due to CAA compared to heterozygous (A/G) or homozygous neutral (G/G). The product of the *CR1* gene, CR1, binds to and cleaves C3b into inactive components iC3b and C3c (18). In our cohort, 12/16 (75%) of CAA cases were homozygous A/A (Table 1), the major risk genotype for CAA associated ICH (5). On the other hand, only one case was homozygous A/A in the AD-only group and none of the controls were homozygous A/A (Table 1). When all CAA cases were compared to all non-CAA cases, the association of CAA pathology to the A/A genotype was highly significant ($P=0.006$). Genotype data could not be obtained for patient 20.

$\alpha 2M$ and C3b levels in occipital lobe

Two different $A\beta$ receptor/ligand complexes were studied in our post-mortem brains; the well recognized LRP1/ $\alpha 2M/A\beta$ (14, 27, 28, 37) and a new cell surface complex, CD11b/C3b/ $A\beta$. A role for these adhesion molecules ($\alpha 2M$ and C3) in amyloid clearance is recognized, but yet to be fully elucidated. We report for the first time that levels of both $\alpha 2M$ and C3b, two previously indicated $A\beta$ chaperones (28, 44), differ in the AD-only and AD/CAA brain, suggesting different phenotypes between the two diseases. $\alpha 2M$ showed a significant difference between groups ($H=13.5$, 4 d.f., $P=0.009$; Figure 1a) and was significantly decreased in ADmiCAA, ADmodCAA and ADsevCAA as compared to controls ($P=0.04$, $P=0.02$ and $P=0.02$, respectively) and to AD-only ($P=0.01$, $P=0.02$ and $P=0.02$, respectively; Figure 1a). C3b also showed a significant difference between groups ($H=12.5$, 4 d.f., $P=0.01$) and was significantly increased in the same cases compared to controls ($P=0.006$, $P=0.02$ and $P=0.02$; Figure 1b) and only marginally increased in ADmodCAA and ADsevCAA compared to AD-only (Figure 1b). Changes in both $\alpha 2M$ and C3b additionally showed cross-sectional trends as CAA pathology progressed: trending decrease for $\alpha 2M$ (Figure 1a) and trending increase for C3b, although this tapered off in ADsevCAA cases (Figure 1b).

Microglial $A\beta$ binding via CD11b

Confocal fluorescent evaluations of fixed occipital lobe grey matter from each of the 12 brains (4 each of AD with severe CAA, AD-only, and control) showed a consistent pattern of CAA-specific microglial $A\beta$ protein:protein interactions. Interactions were identified by FITC conjugated secondary antibodies to localize CD11b (green) (Fig. 2a) and a Texas Red conjugated secondary antibody to localize $A\beta_{40}$ (red). Nuclei are identified by DAPI (blue). All AD/CAA CD11b+ microglia colocalized with $A\beta_{40}$ on ramified processes (Figure 2a insets) in contrast to the primarily intracellular co-localization of CD11b and $A\beta$ in the AD-

only cases. Only minimal colocalization on process-like projections in control brains was observed. Identical patterns of colocalization were found in all brains ($n = 4$ for each group). This consistent observation has led to the hypothesis that a microglial A β binding mechanism causes downstream focal A β vascular complement activation (3b) and MAC generation in AD/CAA. Representative 3D z-stack micrographs show the consistently different A β distribution found on microglial processes colocalized with CD11b in AD/CAA (arrowheads) compared to AD-only and controls (Figure 2b). Negative controls done with the same protocol, but without primary antibody validated the staining (Supplementary Figure 1).

Co-immunoprecipitation of CD11b

Co-immunoprecipitation of CD11b from occipital lobe whole brain homogenate yielded relatively equal abundance of CD11b protein on SDS-PAGE western blot analysis in all three groups (Figure 3a). C3b was significantly increased when CD11b containing supernatant was analyzed with western blot analysis in cases of AD/CAA compared to both controls and AD-only ($H=9.8$, 2 d.f., $P=0.007$; $P=0.02$ and $P=0.02$, respectively; Figure 3a, 3b).

Since the APOE $\epsilon 4$ allele is the major substantiated genetic risk factor for non-hereditary AD and CAA and the E4 isotype has been shown to potentiate complement activation *in vitro* (26), we chose to determine if it was apart of the CD11b/C3b complex. We found that apoE4 was bound to CD11b upon immunoprecipitation; however, it showed no statistical significance between groups (Figure 3b). This will need further testing in culture in order to confirm that when apoE E4 is complexed with CD11b and C3b, potentiation of complement activation occurs and that this is what causes increased vascular fragility secondary to MAC deposition.

Further analysis of CD11b containing supernatant showed increased (albeit not significant) levels of monomeric and dimeric A β bound to microglial CD11b in AD/CAA compared to AD-only and controls (Figure 3a, 3d, 3e). These data are in agreement with our confocal data, where we observed that in every case, CD11b colocalizes with A β_{40} . However, it appears that only in AD/CAA does CD11b significantly bind to A β through C3b, thus delivering active complement to the cerebral vasculature, setting up downstream MAC activation.

In order to confirm binding, we performed the reverse immunoprecipitation on the same samples for C3b, apoE4 and A β and performed western blots for CD11b (Supplementary Figure 2).

MAC on occipital lobe cerebral vasculature

The evidence for binding of A β via the CD11b/C3b complex leading to MAC activation on vascular smooth muscle cells at the vessel wall during microglial clearance in AD/CAA was further evaluated on CAA affected microvasculature by both western blot and immunofluorescence for C5b-9 deposition. C5b-9 was significantly different between groups ($H=6$, 2 d.f., $P=0.05$). C5b-9 was significantly increased when compared to both control and AD-only vessels in a microvessel enrichment preparation from the occipital lobe ($P=0.02$ and $P=0.03$; Figure 4a). Additionally, we probed the enriched microvessel fraction for CD59, an endogenous regulator of late complement activation by inhibiting the binding of C8 and C9 of the MAC (20), which has shown to be decreased in the brain parenchyma in AD (49). We found a decreasing trend in CD59 on blood vessels of AD/CAA cases compared to AD-only cases, which parallels the increased MAC deposition on the same AD/CAA vessels (Figure 4b).

Immunofluorescent studies showed C5b-9 on AD/CAA vessels colocalized with thioflavin staining, where as control and AD-only vessels lacked such staining (Figure 5). Negative controls without primary antibody were used to verify the specificity of fluorescent staining (Supplementary Figure 3). Additionally, MAC localized to the vicinity of smooth muscle cells in AD/CAA with co-immunofluorescence staining of C5b-9 and α SMA (Supplementary Figure 4). Western blot analysis for C6, an integral component of the MAC, showed significantly increased levels in the enriched microvessel fraction in AD/CAA cases compared to controls (Figure 6a). Immunohistochemical analysis showed robust staining on AD/CAA vessels (Figure 6b), which selectively localized to the muscular layer of leptomeningeal arteries (Figure 6c). This staining is almost exclusively limited to leptomeningeal arteries and cortical arterioles in the grey matter, while the white matter is almost completely spared (Figure 6d–e) -- a finding that matches the pattern of CAA involving leptomeningeal and penetrating cortical arterioles, sparing deeper white matter arterioles (43).

Discussion

In this study we present the hypothesis that A β clearance in AD subjects with CAA is distinct from that in AD subjects lacking CAA, and is mediated by microglial CD11b binding to extracellular C3b/A β complexes and delivering both active C3b and A β to the microvasculature, resulting in late complement activation. We evaluated whether observations from human *post mortem* occipital lobe tissue from subjects with varying degrees of CAA were consistent with this hypothesis. The complex CD11b/C3b/A β is found only in cases of AD/CAA and not in control and AD cases lacking CAA. This process occurs with a concomitant reduction in α 2M, a known A β chaperone and likely a major mediator of microglial A β clearance in normal brain and in AD-only (14). This CAA specific microglia shift in A β binding/clearance seems to be associated with cytolytic MAC deposition on leptomeningeal and penetrating cortical arterioles in the pattern of vascular A β deposition.

Several studies have implicated the LRP1/ α 2M complex in normal A β binding and clearance from the brain (21, 27, 28). Additionally, it has been shown that activated α 2M is capable of blocking the formation of A β fibrils *in vitro* (51). In comparison to the observation of an age-dependent decrease in LRP1 (with no change in α 2M levels) on vessels in an AD mouse model (37) and in human AD postmortem tissue (4, 9), we found a significant decrease in α 2M as the severity of CAA worsened compared to AD-only and controls. The results from these studies and ours suggest a faulty A β clearance mechanism that involves both vascular (LRP1) and parenchymal (α 2M) entities in the pathogenesis of cerebral vascular accumulation of A β .

The complement cascade and in particular, the C3b cleavage product of C3, has also been speculated to be involved in A β clearance and degradation (2). Blocking the complement cascade at C3 is thought to result in increased AD pathology, neurodegeneration and an altered microglial phenotype (24, 48). Interestingly, with a decrease in α 2M in our cohort of AD with CAA cases, we also observed a concomitant increase in C3b in the same cases compared to both controls and pure AD cases. This is mirrored by an increase in the C3b receptor, CD11b, on microglia in both human AD tissue (3) and mouse models of AD (10). This seems to be a compensatory mechanism, as if the decrease in LRP1/ α 2M is driving a shift towards increased CD11b/C3b to mediate A β clearance.

The unique mechanism implied from this human post mortem study could explain vascular A β ₄₀ deposition based on the cell surface localization of CD11b/A β ₄₀ on microglia in CAA cases rather than the intracellular localization of this complex in AD-only cases, as also

demonstrated by Adolfsson *et al.* (1). This suggests the complex may be subject to lysosomal degradation before it reaches the vascular tree. A β and C3b retained on the cell-surface are presumably transported to the vasculature intact in CAA, facilitating both amyloid deposition and late-complement activation. Control brain microglia showed an even more intriguing A β distribution. CD11b/A β colocalization seemed to appear at the ends of microglial processes, which is suggestive of unchallenged microglial phagocytosis in a healthy brain (38, 41). Although it has not been confirmed experimentally, this “ball and chain” mechanism of endocytosis by ramified microglia may represent efficient A β removal in the healthy brain (41). This observation is consistent with work by Liu *et al.* (22) who also demonstrated A β ₄₂ localizing to vesicular structures within microglial processes.

The implications of our findings of CD11b binding A β in the presence of C3b in cases with CAA may explain the visualization of MAC on penetrating cortical arterioles and correlation of CAA affected vessels and microbleeds (36). If microglia in AD cases with CAA are binding amyloid through the CD11b receptor via C3b on their cell surface, this would allow the delivery of activated complement to the vessel wall allowing for the propagation towards MAC deposition on vascular smooth muscle cells. The immune system has several mechanisms by which to check the activation of complement. CD59 is a glycosylphosphatidylinositol (GPI) anchored glycoprotein that binds the C8 component and inhibits addition of C9 and thus MAC deposition. Several studies have shown that CD59 is decreased in the AD brain (49, 50). We found a non-significant decrease in CD59 on isolated vessels from AD/CAA cases, which could explain the inability of CAA affected vessels to protect against MAC deposition and why vessels in AD cases lacking CAA are not affected despite increased parenchymal MAC loads.

Additionally, validation of our biochemical findings can be found in a recent genetic study. A variation at rs6656401 on the A allele of the *CR1* gene seems to confer risk for CAA associated ICH (5). 80% of the CAA cases in our cohort were homozygous for the risk allele, to which our protein data matches well with respect to parenchymal C3b levels and MAC levels on cerebral blood vessels. Patients with the *CR1* variant might be at an increased risk for CAA associated ICH because of the inability of CR1 to effectively inactivate C3b, thus allowing its uninhibited propagation to the terminal lytic MAC. This has been shown experimentally in a mouse model of multiple sclerosis. Ramaglia *et al.* (33) demonstrated that microglia became primed and pro-inflammatory as a result of CD11b/C3b binding after knockout of the mouse homolog of CR1. Increased levels of activated C3b could then provide a ligand for the binding of A β to CD11b on microglia. Upon clearance of this complex, microglia deposit both A β and C3b near the vascular smooth muscle cells resulting in CAA and propagation of the complement cascade to MAC and thus the generation of focal microbleeds (Figure 7).

A final point of interest is the relationship of these findings to the topic of A β immunotherapy. Immunotherapy has been tested in numerous pre-clinical studies and a clinical trial. While it appears to be successful in clearing parenchymal A β deposits, it exacerbates vascular A β pathology and has an equivocal effect on cognition (31, 32, 46). The introduction of an anti-A β antibody to the microglial A β clearance pathways discussed here would be expected to favor clearance through classical complement activation and the C3b/CD11b pathway. This may be well-tolerated in subjects who primarily clear A β through non-complement activating mechanisms, but in subjects who phenotypically accumulate A β in the vasculature, this would be expected to worsen the pathology. If this hypothesis is accurate, it is potentially relevant therapeutically, because possible CAA can be diagnosed based on the extent of Pittsburgh Compound B retention in the occipital lobe on positron emission tomography imaging (16) and subjects with evidence of significant CAA could be removed from future trials of anti-A β immunotherapy.

Although this study is observational and therefore cannot conclusively define disease mechanisms, these data point to possible explanations for: 1) complement activation on CAA affected vasculature, 2) vascular fragility in CAA, 3) immunotherapy exacerbation of the CAA phenotype, and 4) the importance of understanding and reporting pathologic phenotypes in studies of human Alzheimer's disease. These data also point to the late complement cascade as a potential therapeutic target for the sub-type of AD with CAA. More work is planned to delineate these mechanisms *in vitro* and determine how apoE4 may play a role in directing enhanced complement activation to cerebral blood vessels. Additionally, follow up studies are warranted in order to better understand the potential mechanisms described here in other types of CAA, such as the less frequent inflammatory CAA and cases of pure CAA without AD changes, and if the large lobar hemorrhages associated with CAA pathology are the result of complement-mediated vascular fragility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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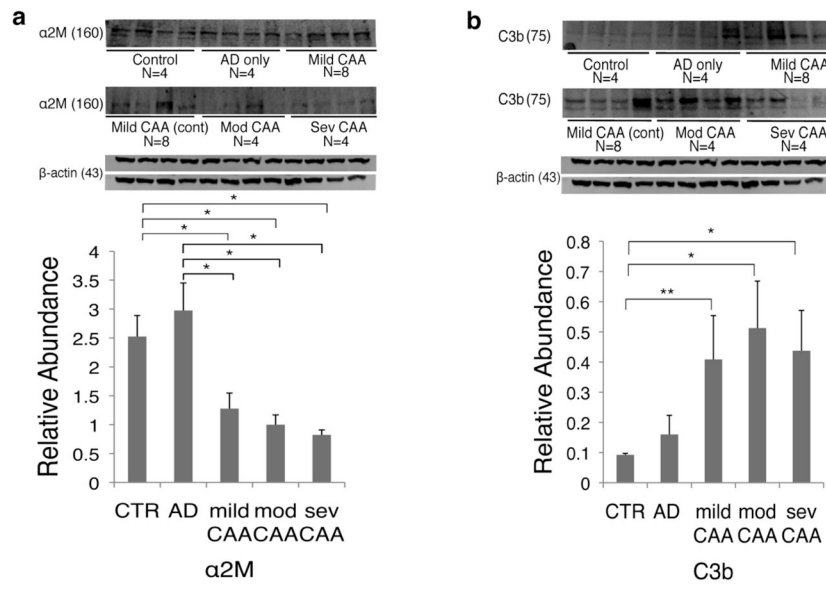


Figure 1.

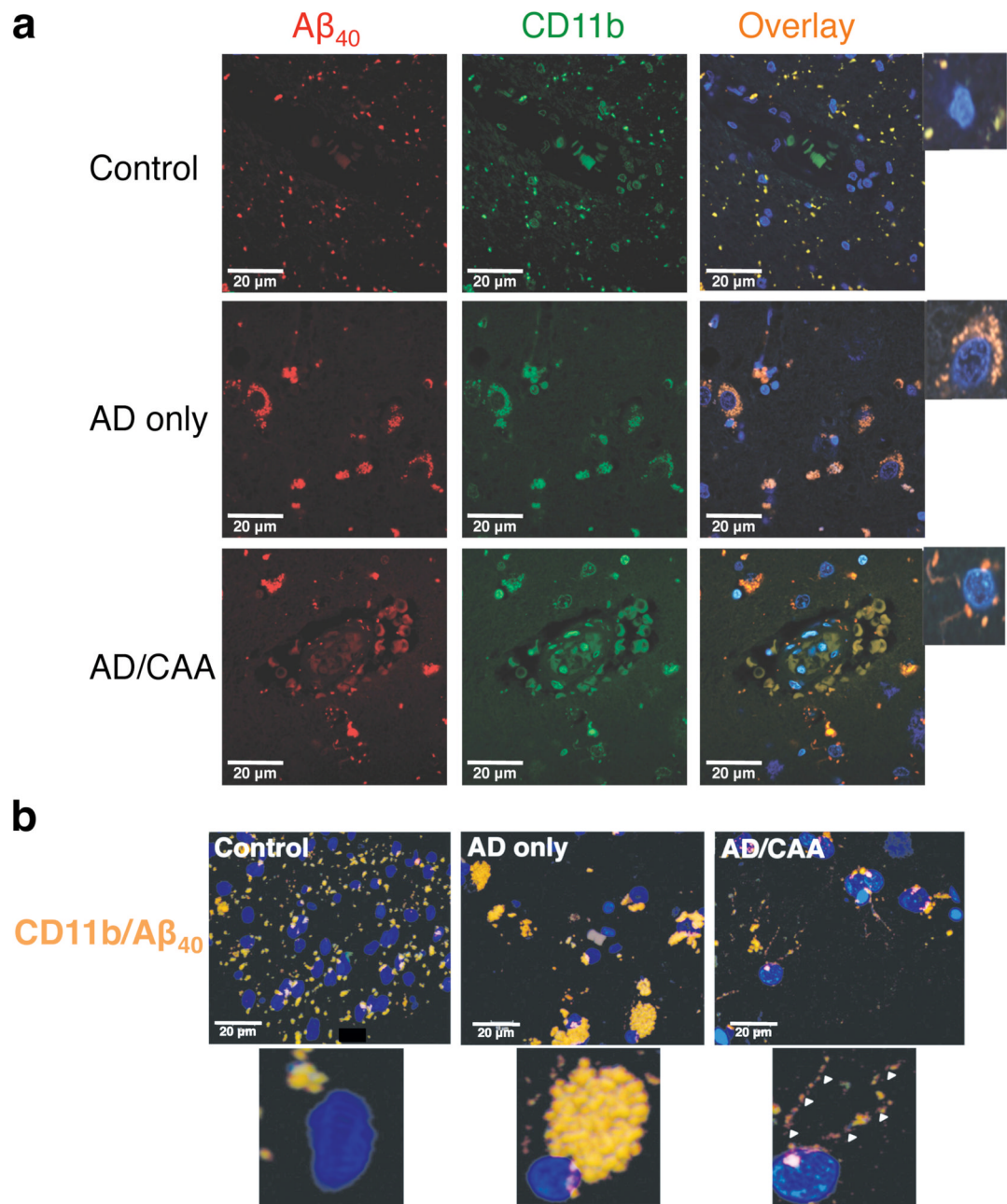


Figure 2.

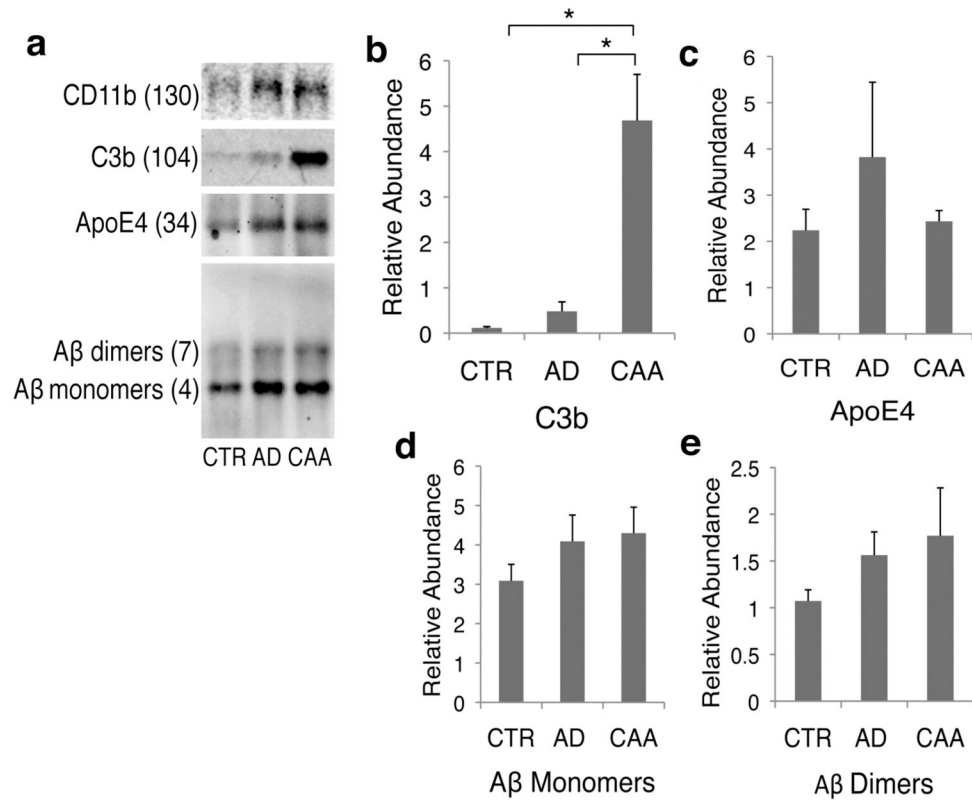


Figure 3.

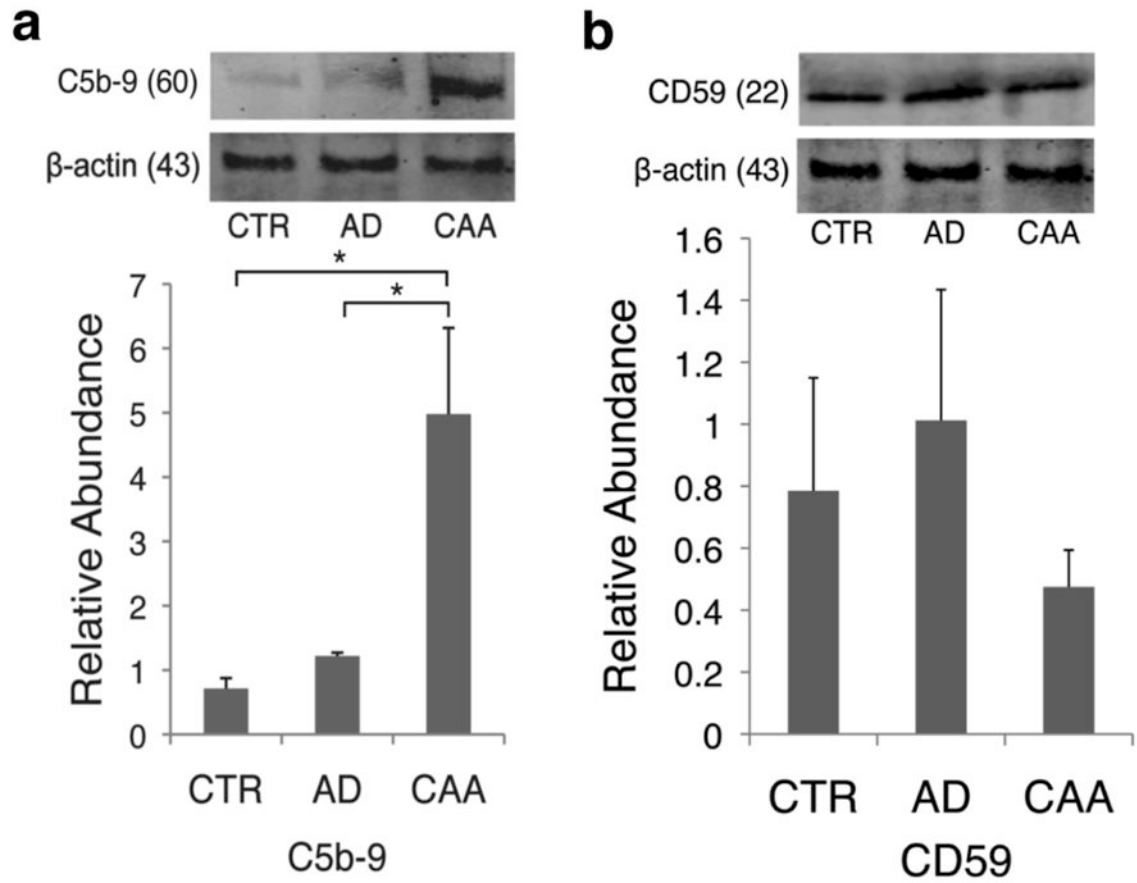


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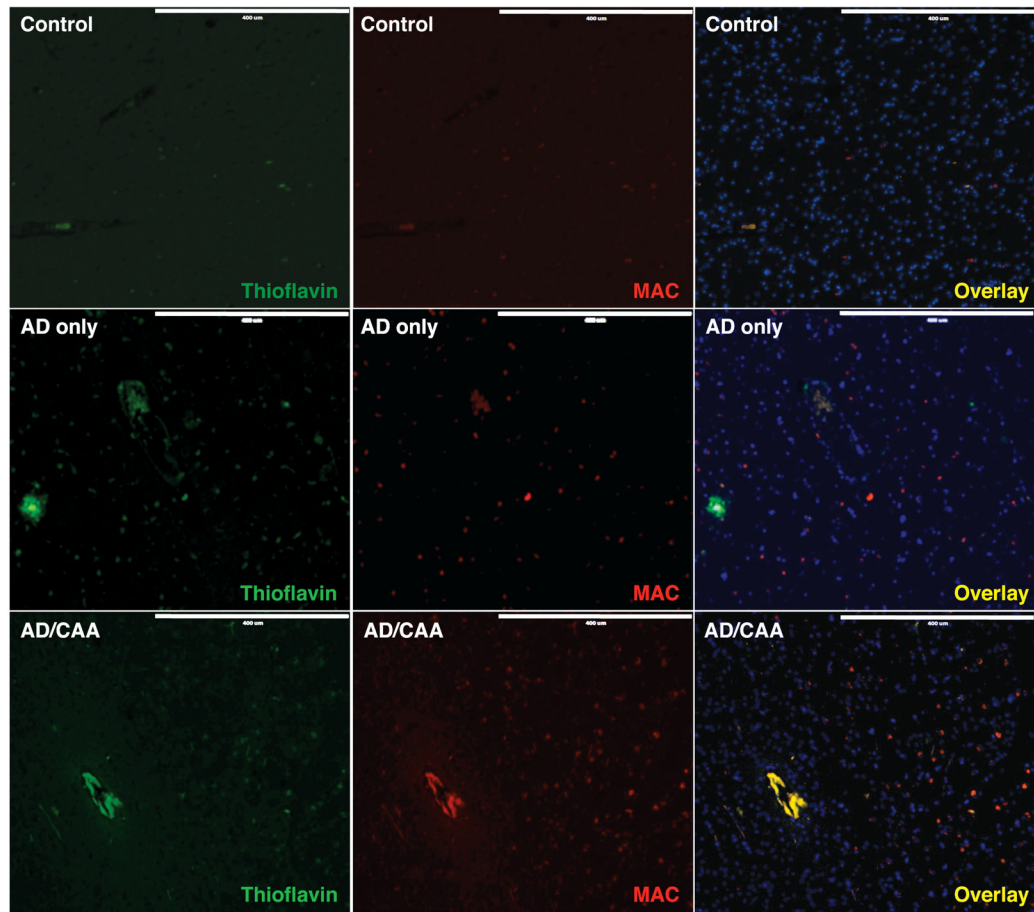


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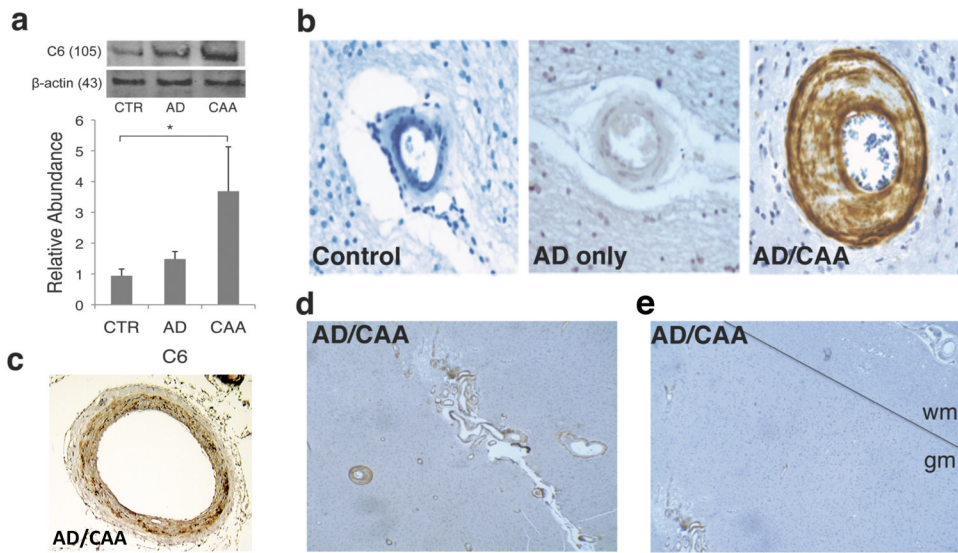


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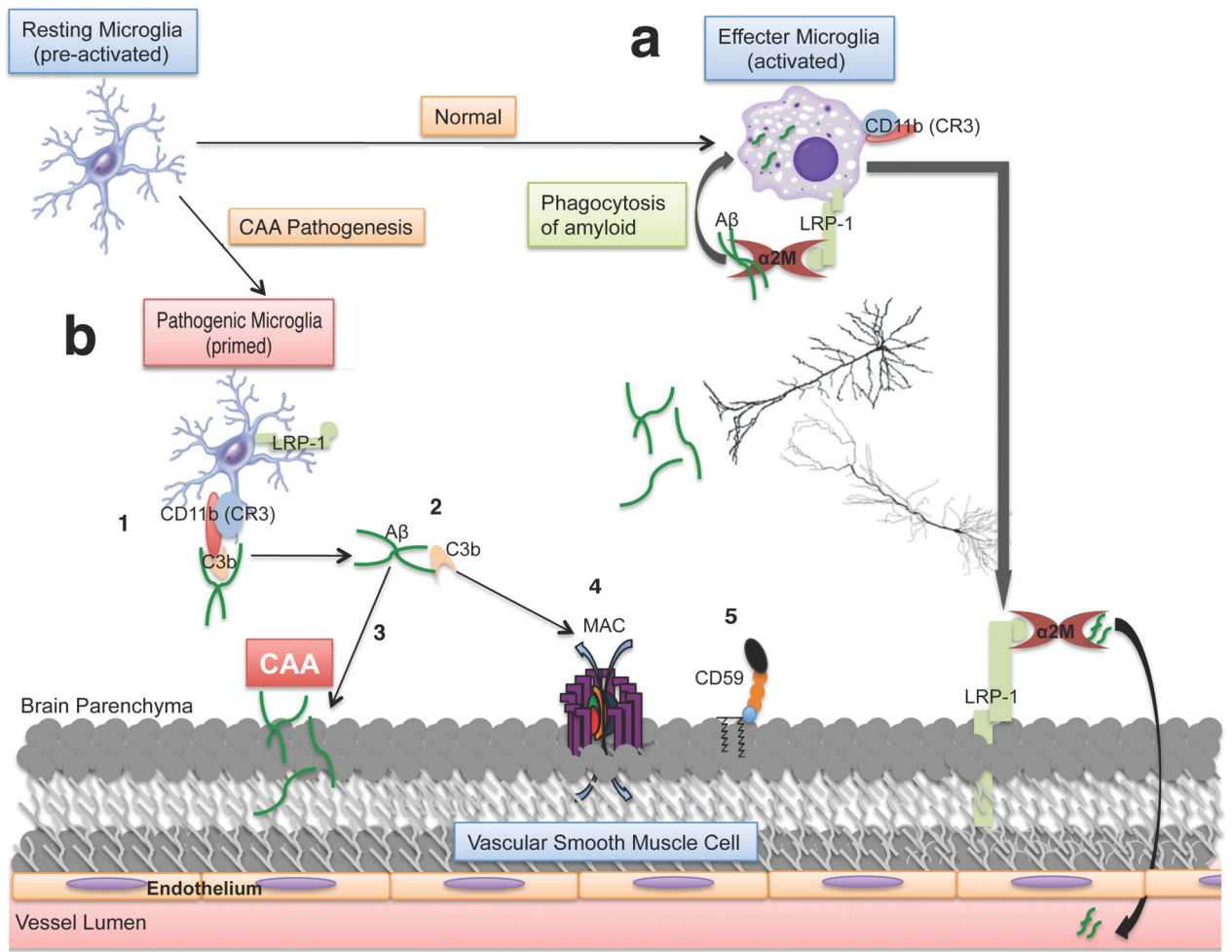


Figure 7.

Table 1

Patient Demographics

Case#	Sex	Braak & Braak Stage	APOE status	CR1 status [§]	Age (y)	PMI (h)
Controls						
1	F	NP	ε3/ε4	A/G	47	7
2	F	NP	ε3/ε3	A/G	42	22
3	F	NP	ε3/ε3	A/G	81	23
4	M	NP	ε3/ε3	A/G	70	23
AD-only						
5	M	VI	ε3/ε4	A/G	80	22
6	F	VI	ε3/ε3	A/G	81	6.5
7	M	VI	ε3/ε3	A/G	89	15
8	M	VI	ε3/ε4	A/A	81	9.5
AD mild CAA						
9	F	V	ε3/ε3	A/G	79	6
10	F	V/VI	ε3/ε3	A/A	91	3.5
11	F	VI	ε3/ε3	A/G	89	24
12	M	V/VI	ε3/ε3	A/A	82	16
13	F	VI	ε3/ε4	A/A	97	12
14	F	VI	ε3/ε3	A/A	73	13
15	F	VI	ε3/ε4	A/G	81	24
16	M	VI	ε3/ε3	A/A	95	22
AD moderate CAA						
17	M	V/VI	ε3/ε3	A/A	82	5
18	F	VI	ε3/ε3	A/A	96	7
19	F	VI	ε3/ε3	A/A	81	11
20	M	VI	--	--	83	12
AD severe CAA						
21	F	VI	ε3/ε4	A/A	88	38

Case#	Sex	Braak & Braak Stage	APOE status	CRI status [§]	Age (y)	PMI (h)
22	F	VI	$\epsilon 4/\epsilon 4$	A/A	71	20
23	F	VI	$\epsilon 3/\epsilon 3$	A/A	83	6
24	F	VI	$\epsilon 3/\epsilon 3$	A/A	69	18

NP=No Pathological Abnormality; PMI = Post mortem interval; y=Years; h=Hours; M=Male; F=Female

[§]The CRI A/A allele was significantly associated with CAA cases versus non-CAA cases, $P = 0.006$