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Apolipoprotein E isoforms differentially regulate matrix metalloproteinase 9 function in Alzheimer's disease

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Abstract

Apolipoprotein E (APOE) has been shown to influence amyloid- β (A β) clearance from the brain in an isoform-specific manner. Our prior work showed A β transit across the blood-brain-barrier was reduced by apoE4, compared to other apoE isoforms, due to elevated lipoprotein receptor shedding in brain endothelia. Recently, we demonstrated matrix metalloproteinase 9 (MMP-9) induces lipoprotein receptor proteolysis in an apoE isoform dependent manner, which impacts A β elimination from the brain. The current studies interrogated the relationship between apoE and MMP-9 and found that apoE impacted proMMP-9 cellular secretion from brain endothelia (apoE2<apoE3=apoE4). In a cell-free assay, apoE dose-dependently reduced MMP-9 activity, with apoE4 showing a significantly weaker ability to inhibit MMP-9 function than apoE2 or apoE3. Lastly, we observed elevated MMP-9 expression and activity in the cerebrovasculature of both human and animal AD brain specimens with an apoE4 genotype. Collectively, these findings suggest a role for apoE in regulating MMP-9 disposition and may describe the effect of apoE4 on A β pathology in the AD brain.

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7. Disclosures

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1. Introduction

Alzheimer's disease (AD) is a progressive and currently irreversible neurodegenerative disorder and is the most common form of dementia. One of the key hallmarks of AD is the accumulation of amyloid- β (A β) in the brain and cerebrovasculature (Selkoe and Hardy, 2016). Increasing evidence implicates blood-brain-barrier (BBB) dysfunction in the pathogenesis of AD, due to altered A β clearance across the BBB (Clifford et al., 2007; Pluta et al., 1996). A major elimination route of A β from the brain is transport via the low-density lipoprotein receptor (LDLR) and the LDLR-related protein-1 (LRP1) receptors, which have been shown to be dysregulated in AD (Bell et al., 2007; Deane et al., 2008; Kanekiyo et al., 2014; Shibata et al., 2000; Zlokovic, 2005). Polymorphic alleles of apolipoprotein E (APOE) represent a major genetic determinant of developing AD. The prevalence of the e4 allele in the AD population is ~40% and is associated with a lower rate of A β clearance from the brain (Bachmeier et al., 2013; Bell et al., 2007; Deane et al., 2008; Martel et al., 1997). Cerebrovascular abnormalities are greater in individuals with the APOE4 isoform resulting in further impairment of BBB function (Castellano et al., 2011). Previously, we and others have found that A β elimination from the brain is influenced by APOE genotype with APOE4 being associated with reduced clearance from the brain (Bachmeier et al., 2013; Bell et al., 2007; Deane et al., 2008; Martel et al., 1997). Furthermore, we demonstrated that apoE influences lipoprotein receptor proteolysis (shedding) in an apoE isoform-specific manner, which impacts A β removal from the brain (Bachmeier et al., 2014). Our more recent work implicated matrix metalloproteinase 9 (MMP-9) in lipoprotein receptor shedding (Shackleton et al., 2019), and found that MMP-9 function was influenced by APOE genotype. However, little is known regarding the mechanisms by which the apoE isoforms impact MMP-9 disposition.

MMP-9 is a proteolytic enzyme involved in the degradation of the extracellular matrix which contributes to the structural integrity of the BBB. It is substantially upregulated in many inflammatory disease states including AD where it has been shown to be highly destructive (Backstrom et al., 1992; Bruno et al., 2009; Rosenberg, 2002). Our prior studies showed that MMP-9 induces lipoprotein receptor shedding in a dose-dependent manner in brain endothelial cells and freshly isolated mouse cerebrovessels (Shackleton et al., 2019). Moreover, treatment with SB-3CT, an MMP-9 inhibitor, diminished A β -induced lipoprotein receptor shedding in brain endothelial cells and the brains of apoE targeted replacement (apoE-TR) mice carrying the E4 allele (Shackleton et al., 2019). SB-3CT treatment also resulted in significantly greater A β clearance from the brain to the periphery following intracranial administration of A β in apoE4-TR mice (Shackleton et al., 2019). These prior studies suggest apoE influences MMP-9 function and may describe the impact of apoE isoforms on lipoprotein receptor shedding and A β clearance across the BBB.

We and others have shown that MMP-9 can influence AD pathogenesis through several mechanisms including BBB alterations, lipoprotein receptor shedding, inflammation, neurodegeneration, and cognitive dysfunction (Bell et al., 2012; Halliday et al., 2016; Mizoguchi et al., 2009; Rosenberg, 2002; Shackleton et al., 2019). Moreover, the effect of MMP-9 on these processes is apoE-isoform specific (Bell et al., 2012; Halliday et al., 2016; Shackleton et al., 2019), however, at this stage it is unclear how apoE may be influencing

MMP-9 function. Like other MMPs, MMP-9 is precisely regulated at several levels including induction and secretion of the proenzyme, activation of the proenzyme by proteolytic cleavage, and modulation of active MMP-9 function (Birkedal-Hansen et al., 1993), each of which may be impacted by APOE genotype. The purpose of the current studies was to examine each of these mechanisms in determining the relationship between apoE and MMP-9, and to further our understanding of the role of apoE in AD pathophysiology.

2. Materials and methods

2.1 Materials

Primary human brain microvascular endothelial cells (HBMEC), associated culture reagents and enzyme linked immunosorbent assay (ELISA) kits for mouse MMP-9 were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). Human A β (1–42) and ELISA kits for human MMP-9 were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Human MMP-9 activity assays were purchased from QuickZyme Biosciences (Leiden, The Netherlands). Fibronectin solution, dextran, phorbol 12-myristate 13-acetate (PMA), 4-Aminophenylmercuric acetate (APMA) and recombinant human MMP-9 were purchased from MilliporeSigma (St. Louis, MO, USA). Halt enzyme inhibitor cocktails, the bicinchoninic acid (BCA) protein assay and Hanks Balanced Salt Solution (HBSS) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Recombinant apoE was purchased from BioVision (Milpitas, CA).

2.2 Human cortex samples

Frozen human cortex samples (inferior frontal gyrus) were obtained from the autopsied brains of AD and non-demented (ND) subjects with different APOE genotypes as summarized in Table 1.

2.3 Animals

ApoE-TR mice were purchased from Taconic Biosciences (Rensselaer, NY) and allowed to adapt to the vivarium for 2 weeks prior to any experimental procedures. The apoE-TR mice were created by targeted replacement of the endogenous murine APOE gene with human APOE2, APOE3 or APOE4 (Sullivan et al., 1997). These mice retain the endogenous regulatory sequences required for apoE production and express the human apoE protein at physiological levels. The EFAD animals were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). To generate the EFAD mouse model, transgenic mice expressing 5 familial AD mutations (5xFAD, Tg6799 line) were crossed with APOE4, APOE3, and APOE2-TR mice, producing the E4FAD, E3FAD, and E2FAD mouse models respectively, as previously described (Youmans et al., 2012). 5xFAD mice are APP/PS1 double transgenic mice that co-express five FAD mutations and additively increase A β -42 production resulting in a mouse model of AD with accelerated plaque development and elevated levels of cerebral A β -42 (Oakley et al., 2006). The EFAD mice remain homozygous for the APOE allele and heterozygous of the 5xFAD mutations. Overall, the EFAD mice display less severe AD phenotypes compared to the 5xFAD line; they develop plaques in the subiculum and cortex at four months of age as opposed to two months in the 5xFAD line (Youmans et

al., 2012). EFAD mice display age-dependent A β accumulation and deficits in cognitive function, both of which are APOE-specific; E4FAD mice demonstrate the most deficits in cognitive function and developed more A β pathology compared to E3FAD and E2FAD mice (Liu et al., 2015; Youmans et al., 2012). Mice were housed under standard laboratory conditions (23 $\pm$ 1°C, 50 $\pm$ 5% humidity, and a 12-hour light/dark cycle) with free access to food and water throughout the study. All experiments using animals were performed under protocols approved by the Institutional Animal Care and Use Committee of the Roskamp Institute.

2.4 Isolation of brain fractions

Human cortex samples and whole mouse brains were homogenized and the cerebrovasculature was isolated using a stepwise density gradient extraction process as previously described (Bachmeier et al., 2014). Briefly, human or mouse brain samples were homogenized in cold HBSS using a Dounce homogenizer. The homogenates were suspended in HBSS with 20% dextran and centrifuged for 15 minutes at 6000g and 4 °C. The cerebrovascular pellet at the bottom of the tube was gently rinsed in HBSS, collected with 250 μ l lysis buffer and stored at – 80 °C before analysis with ELISA.

2.5 Measurement of active and total MMP-9 levels in mouse and human cerebrovasculature

Total MMP-9 levels in the cerebrovasculature from mouse and human brain samples were detected using an MMP-9 ELISA. Absorbance was detected using the BioTek Powerwave XS microplate apparatus at a wavelength of 450nm. Mice of both sexes were used. A quantitative substrate-based MMP-9 activity assay (QuickZyme Biosciences) was used to measure active MMP-9 levels in the human cerebrovasculature samples. This assay provides a measure of MMP-9 activity in a biological sample by capturing MMP-9 present in the sample using an antibody followed by the addition of a proenzyme as a substrate, which upon activation releases color from a chromogenic peptide substrate. Absorbance can then be detected using the BioTek Powerwave XS microplate apparatus at a wavelength of 405nm. MMP-9 levels in the cerebrovasculature fractions were normalized to total protein content, as determined by the bicinchoninic acid (BCA) assay.

2.6 Tissue processing

All mice were humanely euthanized, and their brains were collected and fixed in 4% paraformaldehyde (PFA) for 48 h as previously described (Schweig et al., 2017). The hemispheres were processed in a Sakura Tissue-Tek VIP (Leica Biosystems Inc., IL, USA) vacuum infiltration processor before being embedded in paraffin with the Sakura Tissue-Tek (Leica Biosystems Inc., IL, USA) and stored at 4 °C for 2 days for subsequent cutting with a Leica RM2235 microtome (Leica Biosystems Inc., IL, USA). All brains were cut at a thickness of 12 μ m. Sagittal slices were mounted on glass slides and dried for 48 h at 37°C for immunofluorescence staining and confocal imaging.

2.7 Immunofluorescence

Paraffin sections were washed in two baths of histoclear (National Diagnostics, USA) and progressively rehydrated with ethanol gradients and phosphate buffered saline (PBS, Sigma Aldrich, MO, USA). Brain sections were subjected to antigen retrieval for 7 min in glycine buffer (pH 3) at 100 °C. Sections were then blocked in PBS containing 100% SeaBlock (EastCoast Bio, MA, USA), 0.5mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 for 1 h. Sections were incubated in PBS containing 10% SeaBlock, 0.33mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 and the respective panel of primary antibodies overnight at 4 °C. The following antibodies were used: ab38898 (anti-MMP-9, 1:200, Abcam, Cambridge, MA, USA), ab1907 (anti-apoE [E6D7], 1:200, Abcam, Cambridge, MA, USA), AF3628 (anti-CD31/PECAM-1, 1:1000, Novus Biologicals, Centennial, CO, USA). After three washing steps in PBS for 5 min, sections were incubated in PBS containing 10% SeaBlock, 0.33mM glycine, 0.3% Triton X-100, 0.05% Tween™ 20 and the respective panel of secondary antibodies for 1 h in the dark at room temperature in a humidified chamber. The following secondary antibodies were used: donkey anti-rabbit, anti-goat, anti-mouse conjugated to Alexa 488, 568 and 647, respectively (1:1000, Life technologies). After three washing steps in PBS for 5 min, sections were mounted in Fluoroshield (Sigma Aldrich, MO, USA). All images were acquired using the confocal microscope LSM 800 (Carl Zeiss AG, Germany), the ZEN Blue 2.3 (Carl Zeiss AG, Germany) software and a 20× objective. The acquisition settings were kept the same for all genotypes within the same experiment. For the qualitative analyses, orthogonal projections of cortical brain regions from E3FAD and E4FAD mice (n=4 for each genotype, all female mice, 6 months of age) were analyzed. MMP-9 immunofluorescent intensity and area within endothelial cells (CD31/PECAM-1) were calculated and the percentage overlap of MMP-9 and apoE areas normalized to total MMP-9 area were determined. The scale bars represent 50µm.

2.8 Collection and enrichment of human lipidated apoE

The mixed glial cultures were provided by Dr. Mary Jo LaDu (University of Illinois at Chicago). Cortical glial cultures were prepared from apoE2-TR, apoE3-TR, or apoE4-TR mice as previously described (Manelli et al., 2007). Briefly, mixed glial cells from 1–2-day-old neonatal apoE-TR pups were plated in 150cm² flasks (~1½ brains per flask) in DMEM/F12 medium containing 10% FBS, L-glutamine (2mM) and 1% penicillin/streptomycin. Medium was changed every 3–5 days (Fan et al., 2011; Manelli et al., 2007). Upon confluency, cultures were collected and grown in 75cm² flasks until confluent. Cells were washed and incubated with serum-free media for 72 hours (Fan et al., 2011). Following the incubation, glial-conditioned media (GCM) was collected and centrifuged at 1,000g for 3 min to remove any residual cells before being concentrated (10x) using the Vivaspin 15 centrifugal concentrator with a molecular weight cut-off of 10,000 Da (Sartorius Mechatronics Corp., Bohemia, NY). The resulting concentrate was analyzed for apoE content using a human apoE ELISA.

2.9 Effect of APOE genotype on secreted MMP-9 *in vitro*

HBMEC were seeded at 100,000 cells per cm² onto fibronectin-coated 12-well plates in endothelial cell medium (ECM) containing 5% fetal bovine serum, 1% endothelial cell

growth supplement and 1% penicillin/streptomycin solution. When approximately 90% confluent, cells were washed twice thoroughly in PBS before being treated with serum-free ECM containing lipidated apoE2, apoE3 or apoE4 (25 ng/ml) collected from mixed glial cultures and incubated for 1h at 37 °C and 5% CO₂. Cells were then treated with PMA (1µM), human Aβ (1–42) (2µM) or DMSO (0.5%) for 18h at 37°C and 5% CO₂. After the incubation period, the conditioned extracellular media was collected, centrifuged to remove cell debris and analyzed for secreted MMP-9 using a quantitative substrate-based MMP-9 activity assay (QuickZyme Biosciences). This assay provides a measure of both active and total MMP-9 in a biological sample by capturing MMP-9 present in the sample using an antibody. To detect total MMP-9 levels, APMA is added on the plate to activate any MMP-9 present as a proenzyme. To detect active MMP-9 levels, APMA is not added. This is followed by the addition of a proenzyme as a substrate, which upon activation releases color from a chromogenic peptide substrate. Absorbance can then be detected using the BioTek Powerwave XS microplate apparatus at a wavelength of 405nm. MMP-9 levels were normalized to total protein content, as determined by the bicinchoninic acid (BCA) assay.

2.10 Artificial lipidation of apoE

Recombinant apoE was artificially lipidated as previously described using a protocol adapted from prior reporting (Hubin et al., 2019). Liposome preparation: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, Avanti Polar Lipids, Alabaster, AL) and unesterified cholesterol (Avanti Polar Lipids, Alabaster, AL) were mixed in a glass vial at a molar ratio of 90:5. This ratio was selected to mimic the physiological lipid composition of HDL-like apoE particles. Lipids were resuspended in PBS at a concentration of 5 µg lipids/µl. The solution was mixed thoroughly by vortexing intermittently for 5–10 min (with 1–2 min intervals) to produce liposomes. Complete hydration of liposomes was achieved by incubating the solution at room temperature for 30 min and occasional vortex mixing. ApoE lipidation: Sodium cholate (50 mg/ml, Sigma-Aldrich, St. Louis, MO) was slowly titrated into the liposome solution (2–3 volumes of sodium cholate for 1 volume of lipids). The solution turbidity cleared after 5 min of gentle vortex mixing (1 min interval) and the preparation was kept at room temperature for 30–60 min. Reconstituted apoE was then added to the liposome preparation (apoE:POPC:cholesterol, molar ratio of 1:90:5) and mixed gently for 5–10 min (1–2 min interval). The solution was kept at room temperature for 1 h and dialysed (10 kDa cut-off membrane) against PBS for 4 h at room temperature (to promote removal of detergents), followed by 60–72 h at 4 °C. Samples were desalted and small molecular weight solutes were removed using PD-10 Desalting Columns (Sigma-Aldrich, St. Louis, MO).

2.11 Zymographic analysis of the influence of apoE on MMP-9 conversion to the active form

Recombinant human MMP-9 (1 µg/ml) was incubated with recombinant, artificially lipidated or glia-lipidated (from GCM) apoE2, apoE3 or apoE4 (25 ng/ml) for 45 minutes at 37 °C before being incubated with APMA (1 µM) for 45 minutes at 37 °C. Samples were then analyzed by gelatin zymography to determine pro and active MMP-9 levels. For each sample, a total of 7.5 ng of total protein was loaded. The samples were separated on a 10% precast polyacrylamide gel with gelatin (Thermo Fisher Scientific). The gel was incubated in

Triton X-100 (Zymogram Renaturation Buffer, Thermo Fisher Scientific) for 30 minutes at room temperature with gentle agitation to renature the proteins. The gel was next incubated in development buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij-35 (Zymogram Development Buffer, Thermo Fisher Scientific) for 18 h at 37 °C to initiate enzyme activity. The gel was stained with Coomassie blue (Bio-Rad) for one hour and washed in destaining solution (45% deionized water, 45% methanol, 10% acetic acid) before being scanned with the universal hood II (Bio-Rad).

2.12 Binding studies

The kinetic binding of MMP-9 to each of the apoE isoforms was evaluated using an Octet RED96 instrument equipped with Streptavidin biosensors purchased from ForteBio (Menlo Park, CA). Biotinylated recombinant human MMP-9 in PBS (2 µg/ml) was immobilized to the streptavidin biosensors for 1800 seconds and washed three times for 60 seconds in PBS at 1,000 rpm using the Octet platform. The Octet analysis was performed at RT under a continuous agitation at 1,000 rpm. Each of the apoE isoforms were then loaded at multiple concentrations (10 µg/ml, 5 µg/ml, 2.5 µg/ml, 1 µg/ml, 0.5 µg/ml, 0.25 µg/ml) to separate sensors then allowed to dissociate in PBS (apoE2 association time: 1200s, dissociation time: 1200s, apoE3 association time: 1800s, dissociation time: 3800s, apoE4 association time: 1200s, dissociation time: 1200s). Individual binding affinities were assessed by measuring the dissociation constant (KD) determined from the steady state.

2.13 Cell-free activity assay

The effect of apoE isoforms on MMP-9 activity was assessed in a cell-free paradigm utilizing a fluorescent substrate as per the manufacturers' instructions (Anaspec, USA). Briefly, recombinant MMP-9 (5 nM) was incubated in the presence of lipidated apoE2, 3 and 4 (0–250 ng/ml) or SB-3CT (1 µM) and the fluorescent substrate for 1 hour at 37 °C before detection of fluorescence. To determine whether the substrate was metabolized by apoE itself, fluorescent substrate was incubated with differing concentrations of apoE alone in the absence of MMP-9. These values were used as background controls for each apoE isoform-MMP-9 treatment combination. Fluorescence was measured at 340/490 Ex/Em using BioTek Synergy HT microplate reader.

2.14 Statistical analysis

Where applicable, the investigator was blinded to the identity of the animals and samples until analysis. Data are expressed as mean ± standard error of the mean (SEM). Data was checked for normality and statistical significance was determined by analysis of variance (ANOVA) followed by the two-stage step-up method of Benjamini, Krieger and Yekutieli (BKY) unless otherwise stated. A p-value lower than 0.05 was assumed to indicate a statistically significant difference. Statistical analyses were performed with Graph Pad Prism.

3. Results

3.1 MMP-9 protein levels and activity across APOE genotype in human cerebrovasculature

Isolated cerebrovasculature from human brain tissue (AD and non-demented controls) showed MMP-9 expression was significantly higher (2-fold) in the cerebrovasculature from AD specimens, compared to control samples (Fig. 1a). Upon stratification by APOE genotype, MMP-9 expression in AD subjects was markedly elevated in $\epsilon 4$ carriers compared to non $\epsilon 4$ carriers, with the APOE3/4 genotype showing significantly higher MMP-9 expression levels compared to subjects with the APOE2/3 and APOE3/3 genotypes ($*p<0.05$), in addition to being significantly higher than the corresponding APOE3/4 control subjects ($**p<0.01$) (Fig. 1b). There was no such APOE genotype difference observed in the control samples.

To assess MMP-9 activity in relation to APOE genotype, the human brain specimens were analyzed using a quantitative substrate-based MMP-9 activity assay which measures endogenously active MMP-9 within biological samples. The levels of endogenously active MMP-9 were increased in the AD specimens compared to non-demented controls (Fig. 2a). In terms of APOE, AD subjects with the APOE3/4 and APOE4/4 genotypes exhibited significantly higher levels of active MMP-9 compared their corresponding control subjects. Furthermore, $\epsilon 4$ carriers with AD displayed significantly elevated levels of active MMP-9 compared to non-APOE4 AD subjects. Lastly, no APOE genotype differences were observed within the control population (Fig. 2b).

3.2 MMP-9 protein levels across APOE genotype in mouse cerebrovasculature

Examination of cerebrovasculature tissue from EFAD mouse brains revealed that total MMP-9 levels were increased in E3FAD and E4FAD mice compared to E2FAD mice at 40 weeks of age ($**p<0.01$) (Fig. 3b). At 70 weeks of age, representing a later-stage of the disease process, E4FAD mice demonstrated greater MMP-9 levels compared to E3FAD mice, however this effect did not reach statistical significance ($p=0.0533$) (Fig. 3c).

3.3 Effect of apoE isoform on MMP-9 secretion

To study MMP-9 secretion from cultured cells *in vitro*, HBMECs were treated with the different lipidated apoE isoforms either alone or with PMA (to stimulate MMP-9 secretion) or A β -42 (to simulate an AD-like environment). Extracellular conditioned media was examined using a quantitative substrate-based MMP-9 activity which provides a measure of endogenously active MMP-9 in addition to total MMP-9 once the remaining proMMP-9 has been activated on the plate by APMA. ApoE alone did not appear to impact total secreted MMP-9 levels in the extracellular media (Fig. 4c), however upon stimulation with PMA or insult with A β -42, apoE isoform differences were apparent (Fig. 4f, i). In both the PMA and A β -42 treatment groups, total MMP-9 levels were higher in the presence of apoE3 and apoE4 compared to apoE2 ($*p<0.05$, $**p<0.01$) (Fig. 4f, i) whereas apoE alone had no effect (Fig. 4c). A similar effect was observed with levels of proMMP-9, as treatment with apoE2 resulted in lower levels of proMMP-9 compared to apoE3 and apoE4, with apoE4 demonstrating the highest levels ($*p<0.05$, $**p<0.01$) (Fig. 4e, h). This effect was not

observed with apoE alone (Fig. 4b). ApoE isoform dependent differences in active MMP-9 levels were detected when apoE was added alone and in combination with PMA. The levels of active MMP-9 were 2-fold higher following treatment with apoE3 and apoE4 relative to apoE2 (** $p < 0.01$, *** $p < 0.001$) (Fig. 4a, d).

3.4 Effect of apoE isoform on pro MMP-9 conversion to activated MMP-9

In assessing the effect of apoE isoforms on the conversion of proMMP-9 to the active form of MMP-9, as anticipated, both proMMP-9 (92 kDa) and active MMP-9 (82 kDa) bands could be detected using zymography following treatment of recombinant proMMP-9 with APMA, which has been shown to convert proMMP-9 into its active form (Fig. 5a, c, d) (Backstrom et al., 1996; Springman et al., 1990). With respect to apoE, conversion of MMP-9 was increased by 25% and 62% in the presence of recombinant apoE4 compared to apoE2 and apoE3 respectively following APMA activation (*** $p < 0.001$) (Fig. 5b). However, following incubation of MMP-9 with apoE sourced from GCM, the greatest conversion of proMMP-9 to active MMP-9 was observed in the presence of apoE2 (Fig. 5c, d). Lastly, concerning apoE that had been artificially lipidated, no differences between apoE isoforms were identified (Fig 5e, f).

3.5 Effect of apoE isoform on MMP-9 activity

Using a cell-free MMP-9 activity assay, each lipidated apoE isoform significantly attenuated MMP-9 activity in a dose-dependent manner compared to control conditions (MMP-9 alone) (Fig. 6). Furthermore, apoE isoform-specific differences in modulating MMP-9 activity were observed at each apoE concentration on the order of apoE2>apoE3>apoE4. However, while apoE4 did impact MMP-9 function, apoE2 and apoE3 were considerably more effective than apoE4 in modulating MMP-9 activity. At 250 ng/ml apoE2 and apoE3 resulted in an 82% and 77% reduction in MMP-9 activity with respect to control, respectively, compared to a 48% reduction by apoE4, which equates to a 3-fold difference between apoE2 and apoE4 at this concentration (**** $p < 0.0001$).

3.6 Binding interactions between apoE and MMP-9

We performed kinetic binding studies using biolayer interferometry on the ForteBio Octet RED96 instrument. MMP-9 was able to bind to all isoforms of recombinant activated apoE in the association step (Fig. 7a–c), but the subsequent dissociation in PBS was limited, even when the dissociation time was extended, as shown with the apoE3 isoform (Fig. 7b). The dissociation constant (KD) was measured from the steady state analysis for each isoform (Fig. 7d–h). ApoE4 displayed a larger KD than both apoE3 and apoE2 (4.5-fold and 3.7-fold higher respectively) signifying a weaker binding interaction between MMP-9 and apoE4 compared to the other apoE isoforms (Fig. 7g, h).

3.7 Localization of apoE and MMP-9 in brain endothelia

Having shown that MMP-9 can directly bind to apoE in a cell-free environment, we next investigated this interaction in a physiological setting by using immunofluorescence and confocal microscopy. We interrogated the brain cortices of 6-month-old E3FAD and E4FAD mice and analyzed MMP-9 immunoreactivity in the cortical endothelial cells (Fig. 8a, b).

While levels of apoE or CD-31 did not differ between APOE genotypes (data not shown), there was a 56% increase in MMP-9 immunoreactivity in cortical endothelial cells from E4FAD mice compared to E3FAD mice (* $p < 0.05$) (Fig. 8c). Importantly, areas of MMP-9 and apoE predominantly overlapped, suggesting an association between MMP-9 and apoE in the cerebrovasculature *in vivo*. This interaction was significantly higher in E3FAD mouse cortices relative to E4FAD (* $p < 0.05$) (Fig. 8e) even though the total area of MMP-9 in the endothelial cells appeared to be greater in E4FAD compared to E3FAD, although this was not significant ($p = 0.06$) (Fig. 8d).

4. Discussion

Previously our group reported that MMP-9 function was influenced by APOE genotype (Shackleton et al., 2019). To continue this investigation, the current studies examined several potential mechanisms to understand the effects of APOE on MMP-9 disposition including: MMP-9 expression, MMP-9 cell secretion, proMMP-9 conversion to active MMP-9, and MMP-9 activity. As our prior work demonstrated an effect of MMP-9 on A β transit across the BBB (Shackleton et al., 2019), coupled with the known secretion of MMP-9 from brain endothelial cells (Hanemaaijer et al., 1993; Jackson and Nguyen, 1997; Tarabietti et al., 2002), we focused our investigation of MMP-9 expression to the cerebrovasculature. In the current study, we report an increase in MMP-9 in isolated cerebrovessels from human AD cortex samples compared to non-demented controls. This observation coincides with prior reporting that MMP-9 levels were increased in AD plasma and brain parenchyma (Backstrom et al., 1996; Lorenzl et al., 2008, 2003). Furthermore, we found MMP-9 activity was significantly elevated in AD subjects relative to non-demented controls, indicating the changes in MMP-9 expression levels we observed above were functionally active. MMP-9 activity levels have been previously reported to be increased in individuals with mild cognitive impairment, indicating that MMP-9 might be involved in early pathogenesis of AD (Bruno et al., 2009). Indeed, higher MMP-9 levels have been found alongside AD biomarkers in the cerebrospinal fluid of cognitively healthy individuals (Sochocka et al., 2017; Stomrud et al., 2010). The magnitude of the expression and activity of MMP-9 secreted by brain endothelia is important as elevated levels can disrupt BBB transport and alter A β clearance from the brain, further contributing to the pathogenesis of AD (Halliday et al., 2016; Shackleton et al., 2019).

In addition to global differences in cerebrovascular MMP-9 expression and activity between human AD and control specimens, we also show that MMP-9 levels in the AD cerebrovasculature are dependent on APOE genotype, as cerebrovessels from individuals carrying the APOE4 allele had significantly higher active and total MMP-9 levels than APOE4 non-carriers. Notably, the age of onset, years of dementia, or amyloid plaque load did not correlate with MMP-9 levels in these human samples. It is also important to note the limitations of the human dataset used for these experiments as 1) the sample size of the APOE2/2 AD group is small, due to the exceedingly low incidence of AD in APOE2 homozygotes (Reiman et al., 2019), and similarly 2) the mean age of the APOE4/4 non-demented control group is younger, as APOE4/4 individuals develop AD at an earlier age than other APOE genotypes (Reiman et al., 2019), making it difficult to obtain autopsied brain specimens at ages consistent with the other APOE groups. That having been said, it

does not appear these factors are driving our observations as, for example, the APOE3/4 heterozygous groups were more closely age-matched and MMP-9 levels were still significantly elevated in the APOE3/4 AD samples compared to the APOE3/4 control group and the non APOE4 groups, suggesting an effect of APOE genotype on cerebrovascular MMP-9 levels in AD, as discussed above.

In looking more closely at the data above, it appears that neither AD or APOE4 alone are driving the increase in MMP-9 levels, but only the combination of APOE4 and AD pathology results in elevated MMP-9 levels in the brain. In this regard, there was no APOE genotype effect on MMP-9 levels in the non-demented samples and, likewise, in the non APOE4 groups there was no difference in MMP-9 levels between the AD and non-demented samples. The detrimental effects of the APOE4 isoform in the progression of AD is well-defined, however the underlying mechanism has yet to be fully established. A β , proinflammatory cytokines and oxidative damage are elevated in AD (Galasko and Montine, 2010), particularly in APOE4 carriers (Murphy et al., 2013; Ramassamy et al., 2000; Zhang et al., 2011), and these factors have been previously shown to influence MMP-9 levels (Chong et al., 2001; Deb and Gottschall, 1996; Okamoto et al., 2001; Pugin et al., 1999; Shackleton et al., 2019). Hence, while MMP-9 levels may be mediated through these pathological features, only the combination of the AD environment and the APOE4 backdrop appears to be necessary to significantly elevate MMP-9 levels in the brain.

As the above analyses were performed in autopsied human brain specimens at an advanced or end stage of AD, we next evaluated MMP-9 levels in relation to age or disease progression in the context of APOE genotype. This was assessed in the EFAD mouse model with mice at 10, 40, and 70 weeks of age, which reflect different stages of AD-related pathology (Youmans et al., 2012). The E4FAD mice have previously shown greater age-dependent deficits in cognitive function and A β pathology compared to E3FAD and E2FAD mice (Liu et al., 2015; Youmans et al., 2012). Due to lack of availability of the E2FAD genotype, we could only analyze E2FAD tissue at 40 weeks of age. While we observed significantly lower MMP-9 levels in E2FAD cerebrovessels compared to other APOE genotypes, no difference was evident between the E3FAD and E4FAD animals. As APOE genotype differences often manifest over time, evaluations in these animals at more advanced ages may reveal greater differences in brain MMP-9 levels across genotypes, as we demonstrated in the human brain specimens, which were obviously collected at an advanced or late stage of disease during autopsy. There are indications of this, as we observed a greater difference in cerebrovascular MMP-9 levels between the E4FAD and E3FAD animals at 70 weeks of age than mice at 40 weeks of age or younger. In endothelial cells, differences in MMP-9 immunoreactivity between the E3FAD and E4FAD mice were detected via high-resolution laser scanning microscopy. This may be attributable to a greater expression of MMP-9 in endothelial cells specifically, as opposed to whole cerebrovasculature, which is comprised of various other cells, receptors, and proteins (Hawkins and Davis, 2005; Liu et al., 2020). Collectively, these animal studies coincide with our observations in the human brain specimens that APOE genotype can influence brain MMP-9 levels in the context of AD.

As mentioned earlier, MMP-9 is secreted as an inactive proenzyme from cells including brain endothelia (Hanemaaijer et al., 1993; Jackson and Nguyen, 1997; Taraboletti et al., 2002; Yabluchanskiy et al., 2013). Due to the prominent role of MMP-9 at the BBB and our prior reporting that MMP-9 influenced lipoprotein receptor shedding and A β -42 transit from the brain (Shackleton et al., 2019), we next investigated the release of MMP-9 from cultured brain endothelial cells. Upon stimulation with PMA or A β -42, treatment with the apoE2 isoform significantly reduced MMP-9 secretion in the extracellular media relative to apoE3 or apoE4, which correlates with our findings in the E2FAD animals where cerebrovascular MMP-9 protein levels were substantially reduced versus the other APOE genotypes. In understanding the nature our observations, the reported higher binding affinity of apoE2 for monomeric A β may explain the ability of apoE2 to mitigate the influence of A β on MMP-9 secretion, in comparison to other apoE isoforms (Aleshkov et al., 1997; Yamauchi et al., 1999). Furthermore, apoE2 has been shown to prevent the conversion of monomeric A β into oligomeric species (Fagan et al., 2002; Kim et al., 2017), which are known to induce MMP-9 levels to a greater extent than other forms of A β (Schultz et al., 2014), and may further describe our findings in the E2FAD animals. In these secretion studies, an apoE isoform-specific influence on MMP-9 levels was only observed after stimulation/insult with PMA or A β -42. These findings are consistent with the above MMP-9 expression data in the human brains which indicate APOE isoform-mediated changes in MMP-9 levels are only apparent upon insult, e.g., AD. Moreover, these observations are consistent with a “two-hit” hypothesis in which two factors are deemed necessary to propagate disease pathogenesis, in this case AD and the APOE4 genotype driving MMP-9 levels in the brain (Knudson, 1996; Zhu et al., 2007; Zlokovic, 2011).

It has been shown that lipidation status has a profound impact on apoE function (LaDu et al., 1995), which likely contributed to the diverse effects we observed between the recombinant, GCM-sourced, and artificially lipidated apoE preparations on MMP-9 conversion. This is consistent with previous studies demonstrating that various apoE preparations can yield dramatically different results (Frieden et al., 2017; Garai et al., 2014; LaDu et al., 1995; Tai et al., 2014; Tokuda et al., 2000). The role and nature of apoE lipidation in health and disease are critical topics the field has been trying to reconcile for some time, which include the extent and composition of lipidation between apoE isoforms, changes during aging and disease, and differences between central and peripheral apoE, etc. As so little is known about the makeup of apoE particles under physiological conditions, the identification of appropriate lipidated preparations to study apoE and its function has proved to be challenging. The varied responses elicited by the different apoE preparations in our studies highlight the importance of recognizing methodological differences when evaluating apoE from different sources.

Notably, in addition to apoE itself there may be other components in the GCM that could influence MMP-9 levels. For example, apoE4 is associated with increased inflammation (Zhang et al., 2011) and pro-inflammatory cytokines have been shown to modulate MMP-9 secretion in particular (Ben David et al., 2008; Strazielle et al., 2003), indicating the apoE isoforms may indirectly impact MMP-9 levels as well. However, in cells that have not been activated by lipopolysaccharide or other methods of stimulation, baseline levels of cytokines in the media are often too low to be detected (Maezawa et al., 2006; Shi et al., 2017; Vitek et

al., 2009). Therefore, while cytokines can be present, they are often in such low abundance in the extracellular media under non-stimulated conditions that their potential influence is likely negligible (Jofre-Monseny et al., 2007).

In terms of MMP9 activity, while an increased ratio of active:proMMP-9 was observed in the presence of the apoE2 isoform obtained from the GCM, this did not translate to increased activity of MMP-9. In fact, treatment with GCM-sourced apoE2 resulted in the largest inhibition of MMP-9 activity in a cell-free activity assay versus the other apoE isoforms. The apoE isoform-specific differences in modulating MMP-9 activity may be due to alterations in the binding affinities of each apoE isoform to MMP-9, i.e., the greater the binding, the greater the inhibition of MMP-9 activity. Indeed, using biolayer interferometry, we found that the binding affinity of recombinant apoE4 to MMP-9 was 3-times weaker than the other isoforms, which may not only influence MMP-9 activity, but may also describe the enhanced conversion of proMMP-9 to active MMP-9 in the presence of recombinant apoE4. In other words, recombinant apoE4 may be less able to modulate the catalytic cleavage of proMMP-9 to active MMP-9, which has previously been shown for other proteins (LaCount et al., 2000; Okada et al., 1992; Scannevin et al., 2017).

The significant and dose-dependent inhibition of MMP-9 activity that we observed in the presence of each apoE isoform suggests an interaction between apoE and MMP-9. In addition to the binding studies, further evidence for this association was identified in a more physiological setting using high-resolution laser scanning microscopy. MMP-9 and apoE immunoreactivities clearly overlapped in numerous areas of the cortex of EFAD animals. The finding that apoE4 appeared to associate with MMP-9 to a lesser extent than apoE3 as a smaller fraction of MMP-9 was found to colocalize with apoE4 compared to apoE3, could be indicative of a weaker interaction between apoE4 and MMP-9. These studies are consistent with the kinetic binding studies above, which demonstrated a lower binding affinity of apoE4 to MMP-9, compared to other apoE isoforms. Collectively, these results suggest a physiologically relevant interaction between MMP-9 and apoE in the brain cortices of E3FAD and E4FAD mice.

Lastly, while these studies have focused on the interactions of apoE and MMP-9 in the brain, the influence of apoE on MMP-9 in the periphery can also be consequential. Just as our prior work identified an apoE isoform-dependent effect of MMP-9 on A β elimination from the brain (Shackleton et al., 2019), peripheral MMP-9 could impact the clearance of A β via lipoprotein receptors in liver hepatocytes, particularly in the presence of apoE4, which would elevate A β levels in the blood, and by extension, the brain. That having been said, there are considerable physiological differences between central and peripheral apoE, e.g., glycosylation, lipidation, multimer formation (Flowers and Rebeck, 2020), which may distinguish the influence of peripheral apoE on MMP-9 from our current observations using brain-derived apoE. Certainly, further investigation is necessary to understand the impact of peripheral apoE on AD pathophysiology.

In looking more broadly at our data, MMP-9 cellular secretion in the presence of apoE2 and cerebrovascular MMP-9 levels in the E2FAD mice were substantially lower than the other apoE genotypes (apoE2<<apoE3=apoE4). The effect of apoE2 on cellular secretion and

MMP-9 levels may be similar to that reported for the secretion of A β , where apoE2 significantly attenuated the production of APP and secretion of A β compared to the other apoE isoforms (Huang et al., 2017). Conversely, the results from our MMP-9 activity and binding studies indicate apoE4 may be most relevant in the regulation of MMP-9 function. The binding affinity of apoE4 to MMP-9 and the influence of apoE4 on MMP-9 activity were considerably lower than the other isoforms (apoE2=apoE3>>apoE4). In total, while apoE can impact MMP-9 processing overall, the individual isoforms do so through different mechanisms as our findings suggest apoE2 is most effective in suppressing the production and/or cellular secretion of MMP-9, while apoE4 is least effective in binding MMP-9 and modulating its activity.

5. Conclusion

Collectively, our studies indicate that apoE influences MMP-9 disposition in the brain in an isoform-dependent manner. We show that apoE influences the levels of MMP-9 in conditioned media from brain endothelia, conversion to active MMP-9, and dose-dependently inhibits MMP-9 activity. Importantly, apoE4 was the least effective in modulating these processes compared to other apoE isoforms, which may be due to a weaker binding affinity and association with MMP-9. The net result is elevated MMP-9 levels in the cerebrovasculature when apoE4 is present, as we observed in both human and animal AD brain specimens. The elevated MMP-9 levels in APOE4 AD subjects may describe our prior work showing an effect of APOE genotype on lipoprotein receptor proteolysis and A β elimination across the BBB (Bachmeier et al., 2014, 2013), which are mediated by MMP-9 (Shackleton et al., 2019). As such, targeting MMP-9 may be an effective strategy to mitigate AD pathophysiology, particularly for individuals with an APOE4 genotype.

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Highlights

- Apolipoprotein E (apoE) inhibited matrix metalloproteinase 9 (MMP-9) activity
- Cerebrovascular MMP-9 levels in Alzheimer's disease were apoE genotype-dependent
- ApoE4 exhibited a lower binding affinity to MMP-9 compared to apoE2 and apoE3
- ApoE2 was the most effective in suppressing MMP-9 cellular secretion

Figure 1: ApoE genotype effect on total MMP-9 levels in the cerebrovasculature of human brain tissue in AD and control subjects.

Levels of total MMP-9 were found to be elevated in (A) Alzheimer's disease (AD) subjects compared to non-demented controls and (B) apoE4 carriers with AD. MMP-9 levels in the cerebrovasculature were determined using an ELISA. Values were normalized to total protein concentration and represent mean $\pm$ SEM. (A) $n = 49$ (control) $n = 55$ (AD).

*** $p < 0.001$ as determined by an unpaired t-test. (B) Control subjects: apoE2/2: $N = 10$, apoE2/3: $N = 9$, apoE3/3: $N = 10$, apoE3/4: $N = 10$, apoE4/4: $N = 10$. AD subjects: apoE2/2: $N = 3$, apoE2/3: $N = 10$, apoE3/3: $N = 13$, apoE3/4: $N = 17$, apoE4/4: $N = 12$. * $p < 0.05$, ** $p < 0.01$ as determined by two-way ANOVA and the BKY procedure.

Figure 2: ApoE genotype effect on active MMP-9 levels in the cerebrovasculature of human brain tissue in AD and control subjects.

Using a quantitative substrate-based MMP-9 activity assay, levels of endogenously active MMP-9 were found to be elevated in (A) Alzheimer's disease (AD) subjects compared to non-demented controls and (B) apoE4 carriers with AD. Values were normalized to total protein concentration and represent mean $\pm$ SEM. (A) $n = 44$ (control) $n = 48$ (AD). $*p < 0.05$ as determined by an unpaired t-test. (B) Control subjects: apoE2/2: $N = 8$, apoE2/3: $N = 8$, apoE3/3: $N = 13$, apoE3/4: $N = 8$, apoE4/4: $N = 7$. AD subjects: apoE2/2: $N = 3$, apoE2/3: $N = 9$, apoE3/3: $N = 11$, apoE3/4: $N = 15$, apoE4/4: $N = 10$. $*p < 0.05$ as determined by two-way ANOVA and the BKJ procedure.

Figure 3. MMP-9 expression in mouse cerebrovasculature tissue from EFAD mice at 10, 40 and 70 weeks of age.

Levels of MMP-9 were measured in (A) E3FAD and E4FAD mice at 10 weeks of age, (B) E2FAD, E3FAD and E4FAD mice at 40 weeks of age and (C) E3FAD and E4FAD mice at 70 weeks of age. MMP-9 levels were found to be higher in E3FAD and E4FAD mice relative to E2FAD mice at 40 weeks of age. MMP-9 levels were determined using an ELISA. Values were normalized to total protein concentration and represent mean $\pm$ SEM (E2FAD: N=4, E3FAD & E4FAD: N=8 per age group). (B) $**p < 0.01$ as determined by ANOVA and the BKJ procedure. (A, C) No significant differences as determined by an unpaired t-test.

Figure 4. ApoE isoform differences in MMP-9 secretion in conditioned media from apoE-treated HBMECs.

Using a quantitative substrate-based MMP-9 activity assay, levels of (A, D, G) endogenously active MMP-9, (B, E, H) proMMP-9 and (C, F, I) total MMP-9 were analyzed in the presence of apoE2, 3 or 4 in the conditioned extracellular media of HBMECs. Cells were stimulated with either (A, B, C) apoE alone, (D, E, F) PMA and apoE or (G, H, I) Aβ-42 and apoE. ApoE isoform effects on secreted proMMP-9 levels and total secreted MMP-9 were apparent upon stimulation/insult. Levels of active MMP-9 were elevated in an apoE isoform dependent manner (apoE2<apoE3<apoE4) when added alone and after stimulation with PMA. Values represent mean ± SEM. (A, B, C) n = 14, (D, E, F) n = 10, (G, H, I) n = 8. \$\$p<0.01, \$\$\$\$p<0.0001 indicates significant differences relative to all other conditions and *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 indicates significant differences relative to indicated bars as determined by ANOVA and the BKY procedure.

Figure 5. Effect of apoE isoform on the conversion of MMP-9 to the active form.

Recombinant human MMP-9 was incubated with (A, B) recombinant (C, D) glia-lipidated (GCM) or (E, F) artificially lipidated recombinant human apoE2, 3 or 4 or no apoE (control) before being activated with APMA. (A, C, E) Gelatin zymography demonstrating pro and active MMP-9 bands. (B, D, F) Quantification of zymogram bands (ratio of active to proMMP-9). (B) $n = 17$, (D) $n = 13$, (F) $n = 5-6$. Values represent mean $\pm$ SEM. $N=3$.

* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$ as determined by ANOVA and the BKJ procedure.

Figure 6: Differential modulation of MMP-9 activity by apoE isoforms.

MMP-9 activity was significantly modulated by apoE in an isoform and dose-dependent manner (apoE2>apoE3>apoE4). Differences between each of the apoE isoforms were statistically significant at concentrations ≥ 5 ng/ml. Treatment of 1 μ M SB-3CT was included as a positive control and showed considerable attenuation of MMP-9 activity. Values represent mean $\pm$ SEM (N = 3) and are expressed as fluorescent units. Statistical significance was determined by two-way ANOVA followed by the BKJ procedure. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Asterisk symbols above bars indicates significant differences compared to MMP-9 alone. Asterisk symbols above brackets indicates significant differences from other apoE isoforms at that concentration.

Figure 7: Kinetic binding studies of MMP-9 and apoE2, apoE3 or apoE4 using Bio-Layer Interferometry.

Biotinylated recombinant human MMP-9 was immobilized to streptavidin biosensors (1800 seconds). Recombinant apoE was then added in increasing concentrations (dark blue: 0.25 µg/ml, red: 0.5 µg/ml, light blue: 1 µg/ml, green: 2.5 µg/ml, orange: 5 µg/ml, purple: 10 µg/ml) to separate sensors then allowed to dissociate in PBS (**A**: apoE2. Association time: 1200s, Dissociation time: 1200s, **B**: apoE3. Association time: 1800s, Dissociation time: 3800s, **C**: apoE4. Association time: 1200s, Dissociation time: 1200s). Individual binding affinities were assessed by measuring the dissociation constant (K_D) (**G**, **H**) determined from the steady state (**D**: apoE2, **E**: apoE3, **F**: apoE4). ApoE4 demonstrated the weakest

binding affinity; the KD for apoE4 was greater compared to apoE3 and apoE2. Values represent mean $\pm$ SD. N=5–6.

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Figure 8: MMP-9 and apoE immunoreactivities in cortices of E3FAD and E4FAD mice.

Representative confocal images depicting 6-month-old female (A) E3FAD and (B) E4FAD mice stained with antibodies against CD31/PECAM-1 (AF3628, red), apoE [E6D7], (AB1907, purple) MMP-9 (AB38898, green). Both E3FAD and E4FAD mice exhibited a prominent MMP-9 signal that overlapped with apoE in endothelial cells surrounding blood vessels. (C) Quantification of MMP-9 immunofluorescent intensity within endothelial cells (CD31/PECAM-1). (D) Quantification of MMP-9 area within endothelial cells (CD31/PECAM-1). (E) Percentage overlap of MMP-9 and apoE areas normalized to total MMP-9 area. The scale bars represent 50µm.

Table 1.

Demographic details of human brain cortex samples.

APOE	Non-Demented		Alzheimer's Disease		
	Mean age $\pm$ StDev	N (M/F)	Mean age $\pm$ StDev	N (M/F)	Age of Onset
2/2	79.0 $\pm$ 19.49	10(3/7)	82.67 $\pm$ 21.22	3(2/1)	N/A
2/3	79.8 $\pm$ 10.84	10(4/6)	85.5 $\pm$ 10.05	10(3/7)	78.5 $\pm$ 3.45
3/3	79.8 $\pm$ 15.53	10(9/1)	84.5 $\pm$ 5.83	16(5/11)	76.9 $\pm$ 2.84
3/4	82.0 $\pm$ 9.02	10(7/3)	79.4 $\pm$ 10.18	17(10/7)	72.8 $\pm$ 3.14
4/4	51.6 $\pm$ 16.17	10(5/5)	80.8 $\pm$ 7.80	12(6/6)	72.9 $\pm$ 3.31

Abbreviations: APOE, apolipoprotein E genotype; StDev, Standard deviation; M, male; F, female; N/A, data not available.