THE ANTATOMIC DISTRIBUTION AND EXPRESSION OF MATRICELLULAR PROTEINS IN THE CEREBRAL VASCULATURE OF ALZHEIMER'S DISEASE SUBJECTS

by

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Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that is characterized by the deterioration of cognitive functioning. AD is the most common form of dementia amongst older adults and currently has no cure. Through extensive research that continues to be elucidated, there are two linked factors that may contribute to AD severity. The first is the presence of the epsilon 4 allele in the apolipoprotein E gene (ApoE4 allele), and the second is severity of cerebral amyloid angiopathy (CAA). In this project, gene expression of target genes TSP1, CD36, CD47, sirpα and hif-2α were investigated in relation to ApoE allele status and the degree of cerebral amyloid angiopathy in AD patients. Immunofluorescence and immunostaining procedures were carried out on post-mortem human brain tissue samples to detect target gene expression. The experimental cohort was made up of 20 subjects with Alzheimer's disease pathology, while the non-diseased control cohort consisted of 6 subjects with minimal-to-no Alzheimer's disease pathology. Immunostaining was followed by microscopy imaging and subsequent fluorescent quantification using Image J software (NIH). Standard statistical analyses were carried out on the results using the patient demographics of each cohort. ApoE allele status and CAA grade for each cohort were obtained after immunostaining and fluorescence quantification occurred, resulting in a blinded study to prevent bias. The results of this project concluded that TSP1, sirpα, and CD36 correlate with increasing CAA grade, while sirpα, CD36 and CD47 show a correlation with ApoE allele status. When looking at ApoE4 allele and CAA grade, TSP1, sirpα, and CD36 show a correlation to both factors, suggesting ApoE4 may cause increased CAA, which may result in more severe AD. Various signaling pathways through interactions between these target genes may

contribute to severity of Alzheimer's Disease. Further research will need to be done in order to examine these mechanisms.

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PREFACE

I would like to thank Dr. Adam Straub and Dr. Jeffrey Isenberg for overseeing this project as the principal investigators. In addition, Dr. Kedar Ghimere, Dr. Jessica Cassavaugh, and Dr. Caitlin Czajka are to thank for being the overseeing post-doctoral fellows on this project. I would also like to acknowledge Dr. Straub for being the Bachelor of Philosophy thesis advisor for this project, as well as Dr. Sruti Shiva, Dr. Enrico Novelli, and Dr. Eric McDade for being thesis committee members for this defense. I'd also like to thank Dr. Julia Kofler for providing the brain tissue samples and patient data for the analysis in this project. Finally, I would like to thank the Vascular Medicine Institute at the University of Pittsburgh School of Medicine and the University of Pittsburgh Honors College for providing funding for this project.

1.0 INTRODUCTION

Alzheimer's Disease (AD) is a neurodegenerative disease that is the most common form of dementia amongst older adults. It is characterized by the deterioration of cognitive functioning, such as reasoning, thinking and remembering (Bird 2015). There is a wide spectrum of symptoms that are associated with Alzheimer's Disease, which varies from patient to patient in terms of severity and rate of progression. The clinical manifestation of AD typically begins with subtle memory failure, which progresses to a more severe state (Bird 2015). Other symptoms that accompany this are memory failure, poor judgment, confusion, agitation, withdrawal, language disturbance, and hallucinations, amongst other symptoms (Wang 2012). According to the Diagnostic and Statistical Manual of Mental Disorders V, Alzheimer's is diagnosed in patients by the following criteria:

1) New onset memory impairment.
2) Another cognitive disturbance, such as aphasia, apraxia, agnosia, or executive functioning.
3) A gradual, progressive course that results in significant functional impairment (DSM V 2017)

Though there has been extensive research done on AD, the disease and its underlying physiological mechanisms are still poorly understood. There is currently no cure for AD, only temporary treatments to subside the progressive symptoms.

AD affects about 6 million people in the United States, and this number is expected to increase to 8.4 million by 2030 (Bird 2015). A major pathologic hallmark of AD, in addition to extracellular fibrillary ß-amyloid (Aß) plaques and intracellular neurofibrillary tau tangles, is cerebral amyloid angiopathy (CAA). It is estimated that greater than 80% of AD cases exhibit CAA, which is characterized by ß-amyloid deposition within the walls of cerebral blood vessels, such as in arterioles, venules, and capillaries (Vinters 2017). ß-amyloid is recognized by the thickening of vessel walls, giving a smudge-like appearance when viewed under a microscope (Vinters 2017). Oftentimes, these CAA-affected vessels pass through the leptomeningeal region of the brain (Vinters 2017), which is one of the two layers that make up the meninges (Ilaria Decimo 2012). The leptomeninges is responsible for protecting the brain and modulating important central nervous system pathways. The buildup of ß-amyloid deposits in the leptomeningeal vessels are detrimental because they alter the structural integrity of the blood vessel. In addition, ß-amyloid is associated with the rupture of cerebral vasculature and hemorrhagic strokes, which may worsen the symptoms of AD. ß-amyloid deposition may also obstruct the lumen of cerebral blood vessels, resulting in ischemia (inadequate supply of blood to a tissue region), and cerebral infarction (narrowing or blockage of blood vessels resulting in necrosis of tissue) (Vinters 2017).

Through extensive research, there is agreement that there are strong links between certain gene expressions and AD. The interactions between the epsilon 4 allele of apolipoprotein E (ApoE4) and AD continue to be elucidated, but represents the strongest genetic risk factor in sporadic AD. ApoE4 allele expression in AD correlates with earlier onset of symptoms and increased disease severity, particularly in females (EH Corder 1993). The presence of one ApoE4 allele increased the odds ratio for developing moderate to severe amyloid by 2.9 fold, while the presence of two copies of the ApoE4 allele increased the odds ratio by 13.1 fold (Greenberg SM 1995). When the ApoE4 allele is being expressed, there is increased aggregation and decreased

clearance of amyloid proteins, which leads to increased deposition of ß-amyloid within cerebral blood vessel walls (Alonzo NC 1998). ApoE4 allele expression also correlates with increased risk of developing CAA and earlier CAA-associated hemorrhage (Alonzo NC 1998, Peter T. Nelson 2014, Greenberg SM 1995). Moreover, compared to individuals displaying the ApoE3/3 alleles, carriers of the ApoE3/4 alleles demonstrate reduced cerebral blood flow and increased neuroinflammation (Shinohara M 2016). ApoE4 allele status is strongly correlated with severity of AD, which was used as a basis for this project.

Recent research in the Vascular Medicine Institute has isolated select target genes that may be linked to CAA grade and ApoE4 allele status in AD. One of these target genes is thrombospondin-1, or TSP1. TSP1 is a secreted matricellular protein that has been reported to be upregulated in several health conditions, including peripheral vascular disease (Smadja DM 2011), pulmonary hypertension (Rogers NM 2016, Kaiser R 2016), and sickle cell disease (Novelli EM 2012). One mechanism in which TSP1 does this is by binding and activating the CD47 cell receptor, which inhibits nitric oxide signaling in vascular endothelial cells, smooth muscle cells, and platelets (Isenberg 2006). In addition to inhibiting nitric oxide signaling, this matricellular protein also activates NADPH oxidase and increases production of superoxide in vascular and non-vascular cells (Isenberg 2006, Mingyi 2014). TSP1 is increased in aging and in old animals, shown to inhibit blood flow by restricting vessel vasodilation (Pagano 2014). These findings conclude that TSP1 is a gene that may be involved in vital vascular processes.

Despite current research examining TSP1's effect on vasculopathy and vessel structure, its role in cerebral vessels remains uncertain. According to current research, TSP1 stimulates reactive oxygen species (ROS) production and induces neuronal cell death (Natasha M.Rogers 2013). In mice models, if there is an absence of TSP1-CD47 signaling, the tissue is protected from cerebral infarction and traumatic brain injury (Changhong Xing 2013). Conversely, TSP1 protein expression in brain lysates from 6 AD patients was found to be decreased, compared to samples

from non-AD individuals (Smadja, et. al 2011). Similar results were also observed and recorded on Western blot analysis of brains from AD mice, compared to non-AD control mice (Saglio 1982). One notable finding is that the C-terminus of TSP1, which binds to the CD47 cell receptor, has a structure very similar to ß-amyloid (Isenberg, et. al 2006). In vascular cells, ß-amyloid inhibits nitric oxide signaling through receptor cross-talk between CD47 and CD36, while in experimental and clinical AD, nitric oxide signaling is decreased and contributes to disease (Miller TW 2010).

Another gene of interest that was examined in this project is the CD36 membrane receptor, which interacts directly with ß-amyloid and TSP1 (Miller TW 2010). ß-amyloid interacts with CD36 to induce a CD47-dependent signal that inhibits soluble guanylate cyclase (cGMP) activation, which inhibits downstream nitric oxide signaling in AD (Miller TW 2010). This pathway relies on CD47 to inhibit cGMP nitric oxide signaling (Miller TW 2010). It should be noted that in order for nitric oxide signaling to be inhibited via this cross-talk pathway, ß-amyloid depends on the CD36 receptor to be present in order for inhibition to occur. The role of CD36 to inhibit nitric oxide signaling in AD is why this target gene was examined in this project.

Two final genes were examined to show links to AD. Sirpα is a protein in the signalregulatory protein family, which is part of an immunoglobulin umbrella family (NCBI 2018). Sirpα negatively regulates signaling processes carried out by tyrosine kinases, which is a class of enzymes that catalyzes the phosphorylation of tyrosine residues in proteins. Sirpα has also been shown to interact with TSP1, using CD47 as a ligand, to promote the production of reactive oxygen species in vascular smooth muscle cells (Pagano 2014). These reactive oxygen species contribute to the degradation of muscle and vessel integrity. The sirpα-TSP1 signaling pathway leads to the impairment of vascular relaxation, as well as enhanced vasoconstriction in ischemia reperfusion (Pagano 2014). Ischemia reperfusion occurs when blood returns to tissue after a period of hypoxia, which may be linked to the last target gene examined in this project. This gene,

hypoxia-inducible factor 2α (hif-2α), has been shown to mediate responses to hypoxic environments (David Labrousse-Arias 2016). When an environment lacks oxygen, vessels experience decreased blood flow due to vasoconstriction and increased vascular deterioration; hif-2α works to adapt the tissue to hypoxic environments via vascular remodeling (David Labrousse-Arias 2016). In relation to TSP1, research shows that chronic hypoxia increased TSP1 gene expression in lung tissue (Bauer PM 2012).

Despite this current research, the role of TSP1, CD36, CD47, hif-α, and sirpα in AD-associated cerebral vasculopathy has not been investigated. Given the data that suggests these genes negatively regulate systemic vascularity and blood flow in various vessel systems, I tested the hypothesis that TSP1, CD36, CD47, hif-2α, and sirpα are upregulated in AD-associated cerebral amyloid angiopathy.

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2.0 MATERIALS AND METHODS

2.1 Reagents

The following antibodies were used for the immunofluorescence protocol carried out in this project: goat polyclonal antibodies against TSP1 (Santa Cruz 12312), mouse polyclonal antibodies against CD47 (Thermoscientific #MA5-11895), mouse polyclonal antibodies against CD36 (Cayman Chemical Company #1000983), rat polyclonal antibodies against hif-2α (Novus Biologicals NB100-122), and goat polyclonal antibodies against sirpα (Santa Cruz 6922 C-20). 0.1% sudan black stain in 70% ethanol was used to distinguish the vascularity and structure of each brain tissue sample.

2.2 Brain Tissue Samples

Post-mortem human brain tissue was obtained within an institution-approved protocol CORID #495 (Committee for Oversight of Research and Clinical Training Involving Decedents, University of Pittsburgh). Post-mortem brain tissue from 20 individual AD cases and 6 individual non-diseased cases (normal control) was obtained from the neuropathology department tissue bank at the Department of Pathology at the University of Pittsburgh School of Medicine. The 20 AD subjects had a clinical diagnosis of mild AD and severe AD pathology (Table 1), determined at the University of Pittsburgh's Alzheimer Disease Research Center following standardized clinical criteria (McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's

Disease. Neurology. 1984;34:939–44.). The 6 control samples had no clinical diagnosis of AD and minimal-to-no AD pathology (Table 2).

2.3 Immunostaining Methods

Following 7-10 days of formalin fixation, left hemispheres of post-mortem brain tissue were coronally sectioned, embedded in paraffin, cut into thick sections, and mounted on slides. Diseased and non-diseased samples were then treated with standard immunofluorescence techniques.

First, paraffinized brain tissue samples were de-paraffinized and rehydrated in Xylene for 2x5 minutes, 100% EtOH for 2x5minutes, 90% EtOH for 2x5 minutes, 70% EtOH for 1x5 minutes, and dH2O for for 2x5 minutes. Next, an endogenous peroxidase was prepared with 100 mL MeOH and 1.5 mL H2O2 for 30 minutes. This was followed by a dH2O rinse for 5 minutes. The antigen retrieval solution was prepared using 320 mL dH2O and 3 mL of the antigen unmasking solution (Vector Labs H-3300). The slides were completely submerged in the solution and microwaved for a total of 20 minutes, ensuring the slides were completely covered in solution in 5, 4, 4, 4, and 3minute intervals. The slides were left to cool.

After the slides were completely cooled, they were rinsed with phosphate-buffered saline (PBS) for 5 minutes. Next, each slide was dried and a hydrophobic pen was used to outline each brain tissue section. The endogenous block was prepared with a 6% PBS + fish skin gelatin solution (PBS + FSG). A 10% dilution of the donkey serum (Sigma D9663 – PLUS serum) in 6% PBS+FSG solution was prepared, and 200 uL of this solution was placed on each slide and left to sit for 30 minutes. The PBS + FSG and donkey serum solution was aspirated off, and the primary antibody was added to each slide. A 1:50 dilution of the primary antibody in PBS + FSG solution was used. The primary antibody dilutions were left on the slides overnight in 4˚C.

After the primary antibodies were incubated overnight, they were aspirated off and washed with PBS/0.1% Tween and PBS. The slides were moved to a dark room for the second half of the

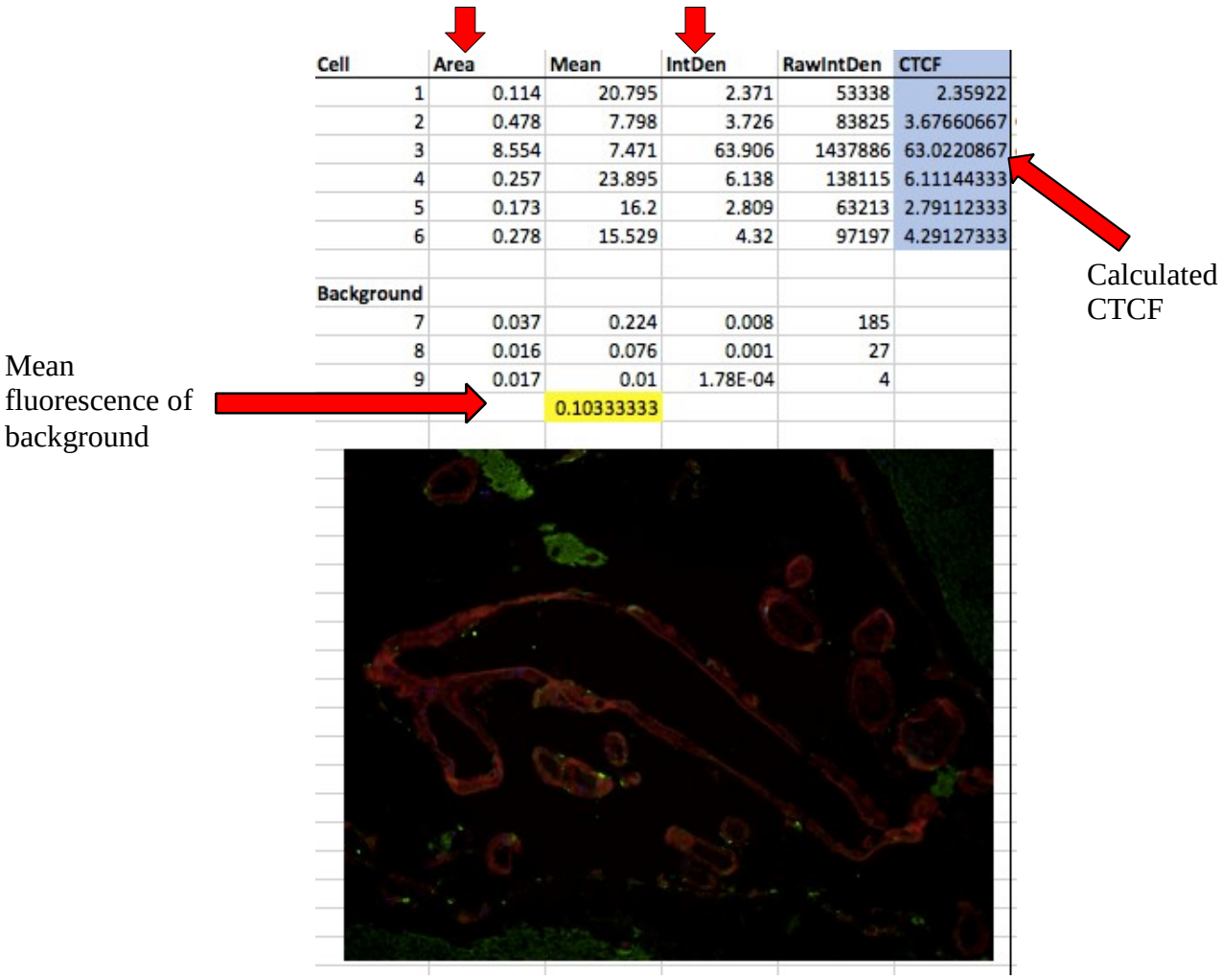
immunostaining procedure. The secondary antibodies were diluted in 6% FSG+PBS and incubated for an hour. After the hour, the secondary antibodies were aspirated off and the samples were washed 5-7 times with PBS/0.1% Tween and PBS. 0.1% Sudan Black stain in 70% ethanol was added to the samples and incubated for 10 minutes. After more PBS washes, Gelvatol with DAPI was added along with a coverslip and stored in 4˚C until it was time to image.

2.4 Immunofluorescence Microscopy and Image Quantification

Fluorescent microscopy was performed with an Olympus Provis microscope at a 20X magnification. Blood vessels in the leptomeningeal region were targeted specifically during microscopy. Once images were obtained, an outline was drawn around each blood vessel of interest using the Image J software (NIH). The area, mean fluorescence and integrated density were measured, along with several representative background measurements included for nonspecific fluorescence control. Using these values, the corrected total cellular fluorescence (CTCF) was calculated using the following equation:

CTCF = integrated density – (area of selected vessel region x mean fluorescence of background)

After calculating the CTCF, the value was normalized to CTCF/DAPI from the same region of interest. Three to five separate fields of view were captured per slide. Below is a screenshot of one field of view to show an example of CTCF calculations.



2.5 Statistical Analysis

The statistical analyses were performed using Image J software (NIH) and Microsoft Excel. Student T-tests were carried out for the quantification results.

3.0 RESULTS

Patient demographics of the diseased cohort and non-diseased cohort.

The clinical cohort of the diseased samples was comprised of 10 females and 10 males: 10 with mild CAA and 10 with severe CAA, and 10 individuals with the ApoE3 alleles and 10 with the ApoE4 allele. Amongst the females, 4 were classified as mild CAA and 6 were classified as severe CAA. Amongst the males, 6 were classified as mild CAA and 4 were classified as severe CAA (Table 1, below). CAA grade was determined by using thioflavin-S-stains conducted on the brain tissue. The CAA grade correlated to thioflavin-S positivity, which was measured against a predetermined score of numbers. Patient demographics of the diseased cohort were provided by the Department of Pathology at the University of Pittsburgh School of Medicine.

Table 1. Patient demographics for diseased cohort.

Patient #	Age	Sex	ApoE Allele status	CAA grade	Braak stage
1	85	F	33	mild	5
2	67	M	33	mild	6
3	62	M	33	mild	6
4	92	F	33	mild	5
5	93	M	33	mild	5
6	75	M	34	mild	6
7	77	F	34	mild	5
8	78	M	34	mild	6
9	81	F	34	mild	6
10	84	M	34	mild	6
11	83	F	33	severe	5
12	86	F	33	severe	6
13	83	M	33	severe	6
14	66	M	33	severe	6
15	76	F	33	severe	6

16	88	M	34	severe	5
17	89	F	34	severe	6
18	82	F	34	severe	6
19	79	F	34	severe	6
20	75	M	34	severe	5

The clinical cohort of the non-diseased samples was comprised of 6 subjects: 2 females and 4 males. All had the ApoE3 alleles and were negative for ß-amyloid angiopathy, thus negative for CAA (Table 2, below). This cohort served as the control group. Patient demographics of the control cohort were provided by the Department of Pathology at the University of Pittsburgh School of Medicine.

Table 2. Patient demographics for non-diseased cohort.

Patient #	Age	Sex	ApoE Allele status	CAA grade	Amyloid angiopathy	Braak NFT stage
21	61	M	33	negative	none	0
22	72	M	33	negative	none	1
23	62	M	33	negative	none	1
24	74	F	33	negative	none	1
25	69	M	33	negative	none	1
26	90	F	33	negative	none	3

Subsequent statistical analyses were done using the patient demographic from both tables, specifically using the CAA grade (no CAA, mild CAA, or severe CAA) and ApoE allele status (ApoE3/3 or ApoE3/4) for analysis. These columns are highlighted in both tables above.

Figure 1. Cerebral amyloid angiopathy grade effect on overall antibody fluorescence (CTCF), based on vessel size (in millimeters)

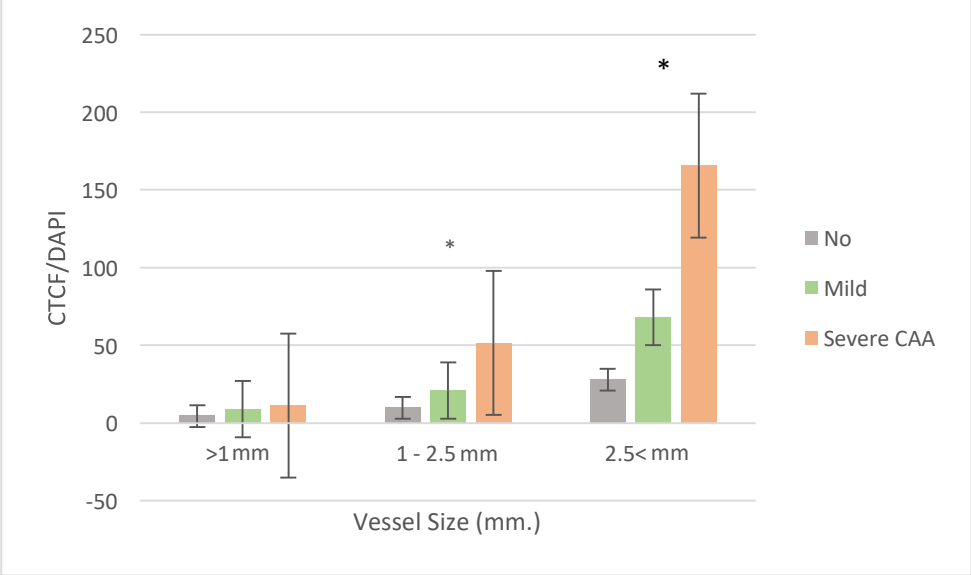


Figure 1. *Student T-tests were carried out on the overall CTCF between no CAA, mild CAA, and severe CAA. For vessel sizes between 1-2.5mm and greater than 2.5mm, the p<0.05, indicating there was a significant difference in CTCF between no CAA and severe CAA. An asterisk denotes the statistically significant results.*

Figure 2. Cerebral amyloid angiopathy grade effects on CTCF based on select target gene

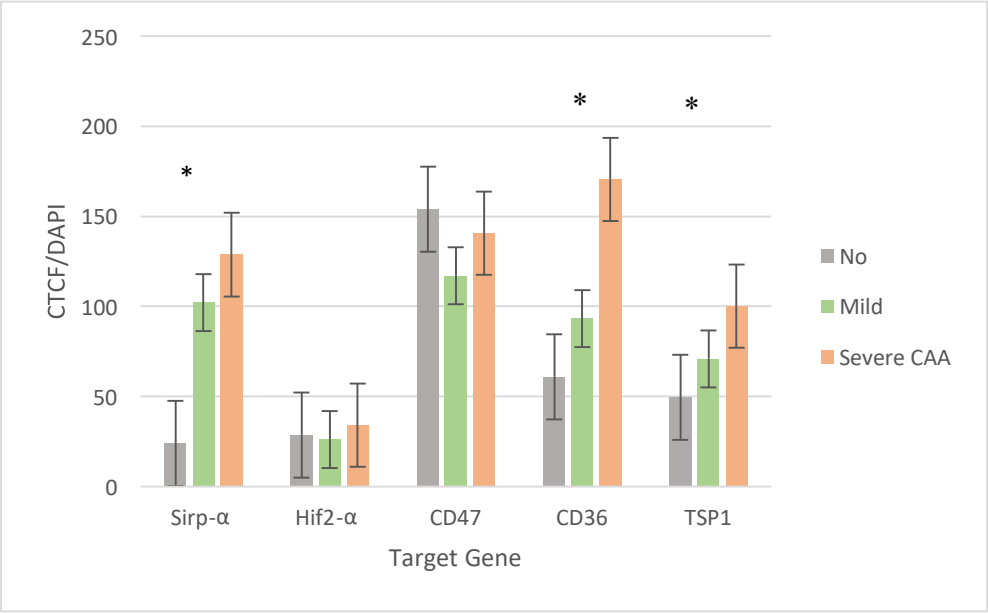


Figure 2. *Student T-tests were carried out on the CTCF between no CAA, mild CAA, and severe CAA for each target gene. For sirpα, CD36 and TSP1, p<0.05, indicating there was a significant difference in CTCF between no CAA and severe CAA. An asterisk denotes the statistically significant results.*

Figure 3. ApoE allele status effect on overall antibody fluorescence (CTCF), based on vessel size (in millimeters)

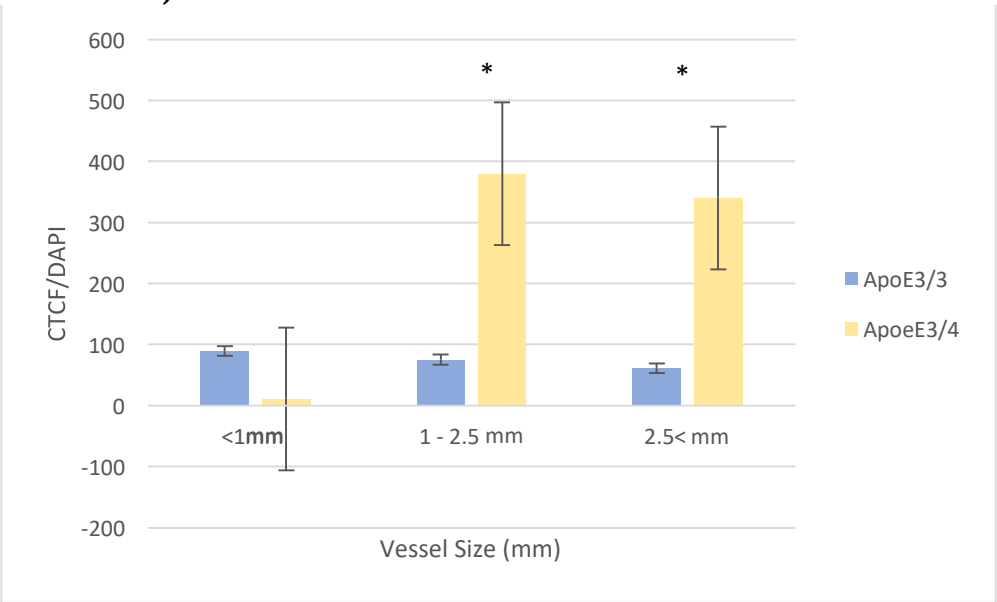


Figure 3. *Student T-tests were carried out on the CTCF between ApoE3/3 and ApoE3/4 allele status, sorted by vessel size. Vessel sizes between 1-2.5mm and above 2.5mm showed p<0.05, indicating there was a significant difference in CTCF between no CAA and severe CAA. An asterisk denotes the statistical significance.*

Figure 4. ApoE allele status effect on CTCF based on select target gene

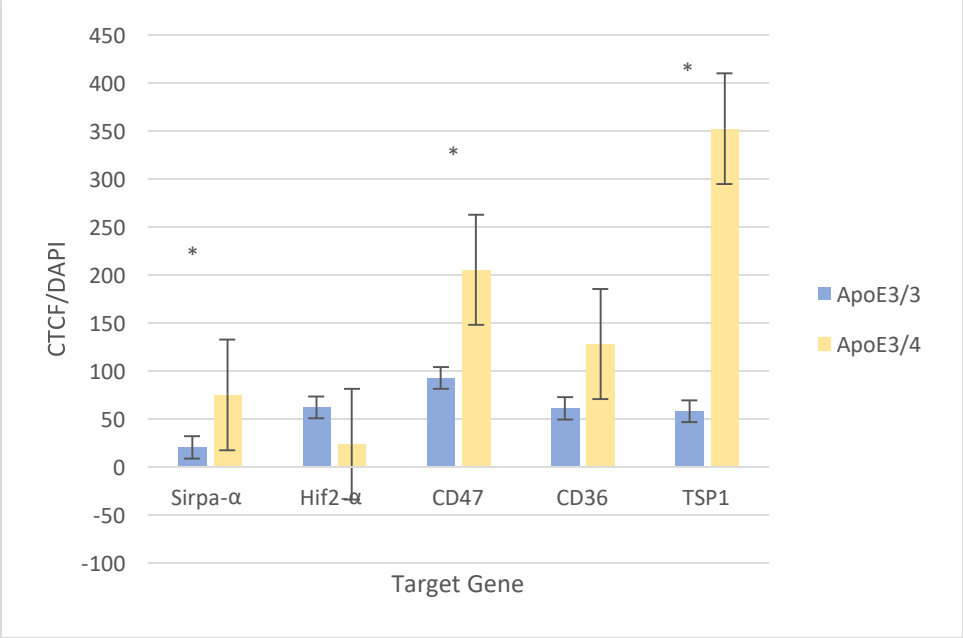


Figure 4. *Student T-tests were carried out on the CTCF between ApoE3/3 and ApoE3/4 allele status for each target gene. Sirpα, CD47 and TSP1 showed $p<0.05$, indicating there was a significant difference in CTCF between the ApoE3/3 allele and ApoE3/4 allele. An asterisk denotes the statistical significance.*

Figure 5. Immunofluorescent images of ApoE3/3 sample and ApoE3/4 sample.

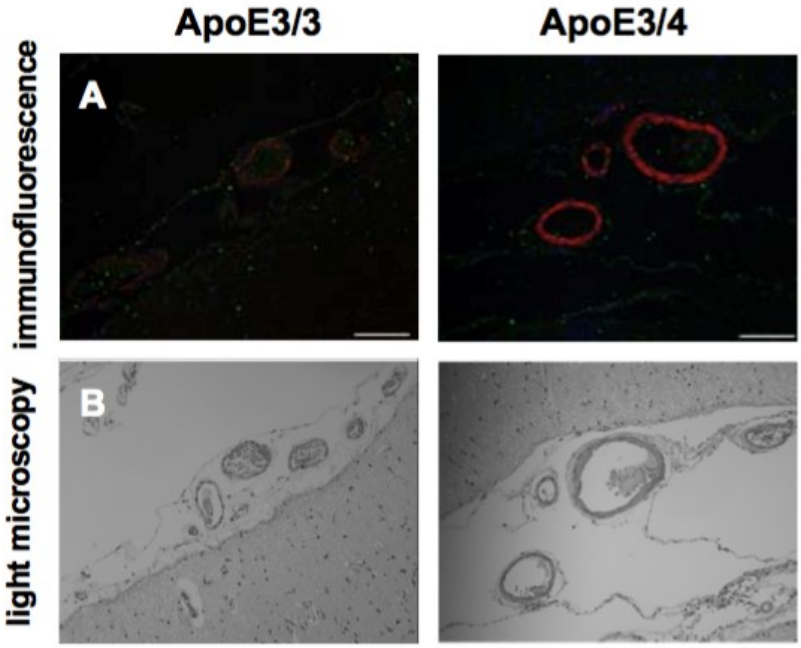


Figure 5. *The above images show a comparison between immunofluorescence samples (with corresponding light microscopy sample) on a subject with the ApoE3/3 allele status, compared to that of a subject with the ApoE3/4 allele status. The red fluorescence (cy5) indicates the TSP1 antibody. The light microscopy images show the brain vasculature and anatomy with no antibody stains.*

4.0 DISCUSSION

Current data suggests TSP1, CD36, CD47, hif-2α and sirpα negatively regulates systemic vascularity and blood flow. A decrease in blood flow within the brain is characteristic of AD, suggesting that these target genes may contribute to the onset of AD symptoms. CAA and ApoE allele status are determinants of AD pathology. The presence of ApoE4 allele expression correlates to increased risk of developing CAA, and the severity of CAA correlates to the severity of AD. However, the reason for the strong predilection of ß-amyloid for the cerebrovasculature remains uncertain. Analysis of TSP1, CD36, CD47, hif-2α and sirpα were investigated to examine correlation between these target genes with ApoE allele status and cerebral amyloid angiopathy grade and how these pathways might help to better understand CAA and AD severity.

TSP1, along with its associated genes sirpα and CD36, shows increased CTCF from no CAA to mild CAA to severe CAA

TSP1 shows the highest CTCF in severe CAA and the lowest CTCF in no CAA subjects (**Figure 2**). TSP1 is one of a small group of proteins that inhibits angiogenesis, or the formation of new blood vessels from preexisting vessels (N Bouck 1996). It also interacts directly with endothelial cells by blocking cell proliferation and migration (SD Saglio 1982). This TSP1 interaction may cause the loss of cerebral vessel integrity because the vessels may not be able to repair due to higher deposits of amyloid. Cell proliferation is necessary for repair of old vessels and regeneration of new ones, and if TSP1 is preventing these vital cell movements from occurring, then cerebral vessels will deteriorate, which may cause more severe AD through hypoxia and/or

ischemia. TSP1 signaling with sirpα and CD36 may show further evidence of these target genes contributing to AD pathology based on CAA grade.

From no CAA to mild CAA to severe CAA, sirpα shows increasing amounts of CTCF in a similar trend to that of TSP1 (**Figure 2**). This correlation between the TSP1 gene and sirpα gene may indicate that the sirpα-TSP1 signaling pathway contributes to CAA severity. Based on previous research, the sirpα-TSP1 pathway has been shown to increase reactive oxygen species in tissue and cells, as well as decrease vasodilation in vessels (Mingyi Yao 2014). Current studies have demonstrated that vessels affected by CAA develop severe oxidative stress from reactive oxygen species (Garcia-Alloza M 2009). Furthermore, when these CAA-affected vessels were treated with an oxidase peptide inhibitor, vessels were protected from severe dysfunction and deterioration (Park L, 2008, (Park L 2007). This suggests that reactive oxygen species are elevated in areas with high ß-amyloid deposition, which contributes to loss of vessel integrity. The increase in sirpα with TSP1 from mild CAA to severe CAA shows there may be an increase in reactive oxygen species in the leptomenigeal region of the brain where these vessels are located. As CAA increases with severity, the sirpα-TSP1 pathway is amplified, which increases reactive oxygen species levels. This increase in reactive oxygen species may exacerbate AD and neurovascular pathology, resulting in more severe symptoms.

Alongside sirpα and TSP1 is CD36, which also showed a progressive increase in CTCF from no CAA to mild CAA to severe CAA (**Figure 2**). This increase, along with the increase of CTCF in TSP1, suggests that the CD36-TSP1 pathway increases the amount of CAA in leptomeningeal vessels of the brain, or is upregulated in response to ß-amyloid deposition in the vessels, possibly contributing to AD. In older mice models where there was extensive amyloid deposition in the cerebral blood vessels, CD36 deletion improved cognitive function (Laibaik Park 2013). In addition, CD36 has been shown to block the activity of TSP1 by preventing its inhibitory mechanisms on its downstream targets (David W. Dawson 1997). This suggests that higher levels

of CD36, as seen in the increased amount of CTCF from low to high CAA grade, will allow TSP1 to activate its downstream proteins more readily because TSP1 activity is no longer inhibited. CD36 upregulation with TSP1 may correlate to increasing CAA grade.

CTCF of CD47 in severe CAA grade shows lower intensity than the CTCF of CD47 no CAA grade

Unlike the progressive increase of CTCF from no CAA to severe CAA observed in sirpα, CD36 and TSP1, CD47 shows the highest CTCF in the no CAA grade, the lowest CTCF in mild CAA, and severe CAA having a CTCF in between these two CAA grades (**Figure 2**). As previously-mentioned, TSP1 is known to signal through the CD47 receptor and inhibit nitric oxide signaling by mediating the CD36-TSP1 pathway (Jeff S. Isenberg 2006). CD36 engages in crosstalk interactions with CD47 when ß-amyloid binds to CD36 (Natasha Rogers 2014), which causes nitric oxide inhibition. In vascular cells, TSP1 needs CD47 for inhibition of nitric oxide signaling, (Jeff S. Isenberg 2006), which results in vessels not being able to carry out its proper physiological functions (Charriaut-Marlangue, et. al, 2013). Nitric oxide is a vasodilator that is important for maintaining the structure and integrity of vessels (Charriaut-Marlangue, et. al, 2013); if the CD47TSP1 pathway acts to inhibit this signaling, this may contribute to AD-related symptoms due to lack of vasodilation necessary for proper cerebral blood flow. The cohort with no CAA shows the most CTCF, which goes against the proposed hypothesis (**Figure 2**). This may be due to high background fluorescence that may have altered the true CTCF. What is notable about this target gene, in terms of CAA grade, is that the severe CAA shows a higher CTCF than mild CAA, which was to be expected. Increasing CD47 expression from mild to severe CAA, partnered with increased TSP1 expression from mild to severe CAA, shows that the level of ß-amyloid present in the cerebral vessels may contribute to severity of AD and may be a result of ß-amyloid activation of CD36.

Hif-2α shows a slight decrease in CTCF from no CAA to mild CAA, while severe CAA grade has the highest CTCF

There is a slight decrease in CTCF of hif-2α from no CAA to mild CAA, which may be due to inconsistencies of exposure times used during imaging. High background fluorescence may also have altered the true CTCF. However, coinciding with trends seen in the other target genes in Figure 2, severe CAA had the highest CTCF. This increased expression of hif-2α may show a signaling pathway that induces the formation of ß-amyloid in the brain. Local hypoxia, induced by the reduction of oxygen to parts of the brain, stimulates the production of ß-amyloid in endothelial cells and neurons, creating amyloid plaques (C. 2000). These hypoxic environments may increase the production of ß-amyloid peptides in neuronal cells, astrocytes, and vascular endothelial cells (C. 2000). Further studies have shown that hypoxia can inhibit Aß-degrading enzymes from functioning properly, as well as stimulate Aß-producing enzymes to synthesize more amyloid (Fisk L. 2007). TSP1 has been shown to be induced in hypoxic environments, which relies on the hif2α gene (David Labrousse-Arias 2016). The upregulation of hif-2α with TSP1 may indicate that a hypoxic environment was created in these vessel regions, inducing the formation of CAA. Increased signaling between hif-2α and TSP1 would increase CAA, thus resulting in more severe AD symptoms.

TSP1, along with its associated genes sirpα, CD36, and CD47, shows higher CTCF with ApoE3/4 allele status

TSP1 shows the highest CTCF in subjects with the ApoE3/4 allele status, compared to those with the ApoE3/3 allele **(Figure 3).** The ApoE gene, specifically the ApoE4 allele, has continued to be elucidated with research to be in association with more severe cerebral amyloid angiopathy (Yu L 2015). It is suspected that the ApoE4 allele enhances deposition of ß-amyloid protein in the walls of cerebral blood vessels (McCarron MO 2000) (D. R. Premkumar 1996), and those with the ApoE4 allele are more susceptible to developing severe symptoms of AD (D. R.

Premkumar 1996). Although current research suggests that TSP1 signaling may be independent when inhibiting nitric oxide activity via ß-amyloid interaction, it is worth noting that ß-amyloid interacts with the TSP1-receptor CD36 pathway (Miller TW 2010). CD36 is shown to be expressed more in subjects with the ApoE4 allele **(Figure 4).**

Degree of expression of the CD36 gene correlates to the presence of ß-amyloid plaques, with some studies showing ß-amyloid induces CD36 expression (RobertaRicciarelli 2004). When analyzing the CD36-Aß signaling pathway, research shows that the CD36 receptor interacts with fibrils of amyloid to induce a series of cascade events that inhibit macrophage response to inflammation (Deborah Doens, Lizmar Carreñ et al. 2017). The accumulation of amyloid in cerebral vessels may be due to the lack of macrophages necessary to suppress this Aß-induced inflammation. The upregulation of CD36 correlating to ApoE4 allele status suggests there is a link between ApoE4 inducing CAA, which is followed by ß-amyloid interacting with the CD36 receptor to cause loss of vessel integrity, structure and function. Vessel degradation may also be caused by CD47. CD47, like CD36 and TSP1, shows an increased amount of CTCF in the cohort with the ApoE4 allele status, compared to those with the ApoE3 allele (**Figure 4**). Studies on CD47 and ß-amyloid show CD47 can stimulate monocyte and microglia activity by binding directly to ß-amyloid, as well as inducing cellular binding of ß-amyloid cells (Maria E. Bamberger 2003). This induction of cell-cell adhesion via CD47 may indicate that increased expression of the CD47 gene may increase the adhesion of ß-amyloid proteins, which will lead to more severe CAA via larger ß-amyloid deposits. Because ApoE4 allele status may contribute to more severe CAA, CD47 may regulate the adhesion between ß-amyloid cells within cerebral vessels. In general, this work suggests one possible mechanism of how ApoE4 may be associated with increased CAA and a higher risk for AD.

Finally, sirpα, like CD36, CD47 and TSP1, shows increased CTCF in ApoE4 allele status (**Figure 4**). Sirpα has been shown to interact with CD47 by binding to the receptor and induces

phosphorylation of tyrosine residues (Haiyue Zhang 2016). When this phosphorylation occurs, the C-terminal site acts as a docking area for inhibitory phosphatase SH1 (SHP-1) (Haiyue Zhang 2016). SHP-1 inhibits macrophage phagocytosis, which through the sirpα-CD47 signaling pathway, may contribute to increased CAA due to lack of macrophage activity necessary to decrease ß-amyloid protein deposition. These two genes may be linked in that those with high sirpα activity may have low macrophage phagocytosis activity, which will allow for the accumulation of ß-amyloid proteins in cerebral vessels, resulting in more severe onset of AD. Furthermore, sirpα upregulation, partnered with TSP1 upregulation, may also induce AD in those with the ApoE4 allele by increasing reactive oxygen species production and promote cell death (Mingyi Yao 2014). As previously mentioned, reactive oxygen species lead to loss of vessel structure and function, which may contribute to severity of AD.

TSP1, CD36 and sirpα correlate with both ApoE4 allele status and increasing CAA grade. This suggests that these genes modulate a pathway that causes ApoE4 allele to induce more severe CAA grade, causing more severe AD.

TSP1, CD36, and sirpα correlate to both ApoE4 allele status and increasing CAA severity. Current research suggests the ApoE4 protein made by the ApoE4 allele will form a complex with a ß-amyloid monomer. This complex will aggregate onto a lipoprotein, which will assemble the sticky ß-amyloid proteins into larger ß-amyloid plaques. These ß-amyloid amyloid plaques can be transported to the vessels to induce CAA (Kline 2012). Using this pathway, the data collected may indicate that TSP1, CD36 and sirpα can modulate the Apoe4-Aß complex formation. Considering the brain is about 60% fat, this would mean there is a high volume of lipoproteins present in the tissue to allow for aggregation of ß-amyloid via ApoE4 proteins. Increased aggregation of ßamyloid proteins will lead to more severe CAA in the vessels, which may cause increased severity of AD pathology.

5.0 CONCLUSION

Based on the results of this project, TSP1, sirpα, and CD36 show a correlation with increase of severity of CAA grade and ApoE allele status. Out of the analyzed target genes, there seems to be a correlation between TSP1, sirpα, and CD36 with CAA grade alone. Additionally, sirpα, CD36 and CD47 show a correlation with ApoE allele status alone. Hif-2α only somewhat correlates with CAA grade and ApoE allele status. This project should be repeated to confirm results in a larger sample size. Furthermore, these studies should be extended to AD cases with minimal to no CAA in order to better understand if these genes are primarily associated with vacular pathology in AD. Additional research must be done to analyze the mechanisms in which these target genes contribute to AD based on CAA grade and ApoE allele status.

6.0 FUTURE DIRECTIONS

For future directions with this project, I would first repeat the immunostaining protocol on a larger control group and compare this data to the diseased cohort quantification. In addition, I would perform Western blot analyses on TSP1, sirpα, hif-2α, CD36 and CD47 proteins to obtain more specific and sensitive quantification of each target protein. To analyze these target gene effects on brain function, target genes can be deleted from mice to create knock-out mice. These knock-out mice can be analyzed for cerebral amyloid angiopathy deposition, intensity of nitric oxide signaling, presence of reactive oxygen species, development of AD symptoms and associated severity, and other markers that would indicate AD pathology. Furthermore, crosses between these knock-out mice can be performed to observe genetic implications of these target proteins and AD.

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