Exploring the *APOE*-specific effects of VEGF family expression and signaling in cognitive aging and Alzheimer's disease

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Dedication

I dedicate this work to my family. Without their support and sacrifices, my education and scientific career would not have been possible.

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I would like to acknowledge the Vanderbilt Department of Pharmacology for supporting me throughout my graduate career. I would also like to acknowledge the training grant which provided me with financial support as a trainee in the Vanderbilt Memory and Alzheimer's Center (T32-AG058524). I'm very grateful to have been immersed in such an exceptional, multidisciplinary training environment.

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LIST OF ABBREVIATIONS

Abbreviation

1. Alzheimer’s disease (AD)
2. Vascular endothelial growth factor (VEGF)
3. Apolipoprotein E (APOE)
4. Cerebrospinal fluid (CSF)
5. Amyloid beta (Aβ)
6. Mild cognitive impairment (MCI)
7. Neurofibrillary tangles (NFTs)
8. Low-density lipoprotein receptor (LDLR)
9. Resilience from Alzheimer’s Disease (RAD)
10. Gene set enrichment analysis (GSEA)
11. Dorsolateral prefrontal cortex (DLPFC)
12. TAR DNA binding protein 43 (TDP-43)
13. Late onset Alzheimer’s disease (LOAD)
14. Early onset Alzheimer’s disease (EOAD)
15. Positron emission tomography (PET)
16. Amyloid precursor protein (APP)

CHAPTER 1

INTRODUCTION

Alzheimer's Disease

Pathology and Progression

Alzheimer's disease (AD) is the most common major neurocognitive disorder[1] and currently affects an estimated 5.8 million people in the United States alone.[2] By the year 2050, it is expected that 13.8 million people will be affected,[2] highlighting the urgency for progress in AD-focused research. Currently available treatments are not effective in preventing the progression of cognitive decline from AD, making it the only major cause of death without effective pharmacological treatment,[3] and emphasizing the desperate need for novel approaches to target discovery and validation.

Alois Alzheimer was the first to describe Alzheimer's disease in 1906, after characterizing neuritic plaques and neurofibrillary tangles during brain autopsy of a patient who suffered from memory loss, disorientation, hallucinations and delusions.[1, 4] Many years later, seminal work by Blessed, Tomlinson and Roth was the first to show a relationship between risk for dementia and concentration of neuritic plaques in the brain, composed of aggregated amyloid-β (Aβ) protein.[5] We now appreciate that AD manifests in two distinct forms based on the age of manifestation, early and late onset AD (EOAD and LOAD, respectively).[6] EOAD can be inherited and is associated with rare, highly penetrant mutations in a small subset of genes (*APP*, *PSEN1*, *PSEN2*).[7] This

dissertation will largely focus on sporadic LOAD, which typically manifests after age 65[8] and is the most common form of AD.[9, 10] Twin studies have estimated that there is up to 79% LOAD heritability,[11] although the genetic architecture that contributes is much more complex and not as well understood as the familial form of AD. The strongest genetic risk factor for LOAD is the *APOE*-ε4 allele, which increases the risk for developing the disease by 3-fold in the presence of one copy and 12-15-fold in the presence of two copies.[8, 12] The *APOE*-ε4 allele shows a substantial effect size for its prevalence in the population when compared to other genetic risk factors (**Figure 1.1**), and will be discussed in detail later in this chapter.

Due to the estimated heritability of LOAD, genetics can provide valuable insight for the discovery of potentially actionable targets. LOAD is a heterogenous disease, with more than 70% of patients showing concomitant brain pathologies at autopsy in addition to significant amyloid and tau burden. An ongoing aim in the AD research community is to be able to use this heterogeneity for the stratification of patients enrolled in clinical trials. For example, post-hoc analyses of the phase 3 clinical trials of a humanized monoclonal antibody against amyloid-β, bapineuzumab, were conducted separately in *APOE*-ε4 carriers and non-carriers. The antibody showed significant clearance of Aβ in *APOE*-ε4 carriers but did not significantly reduce burden in non-carriers.[13] Although the primary clinical endpoint was not met in these trials, they demonstrate proof-of-principle that genomics can be leveraged to help identify patient populations who may benefit most from a given drug.

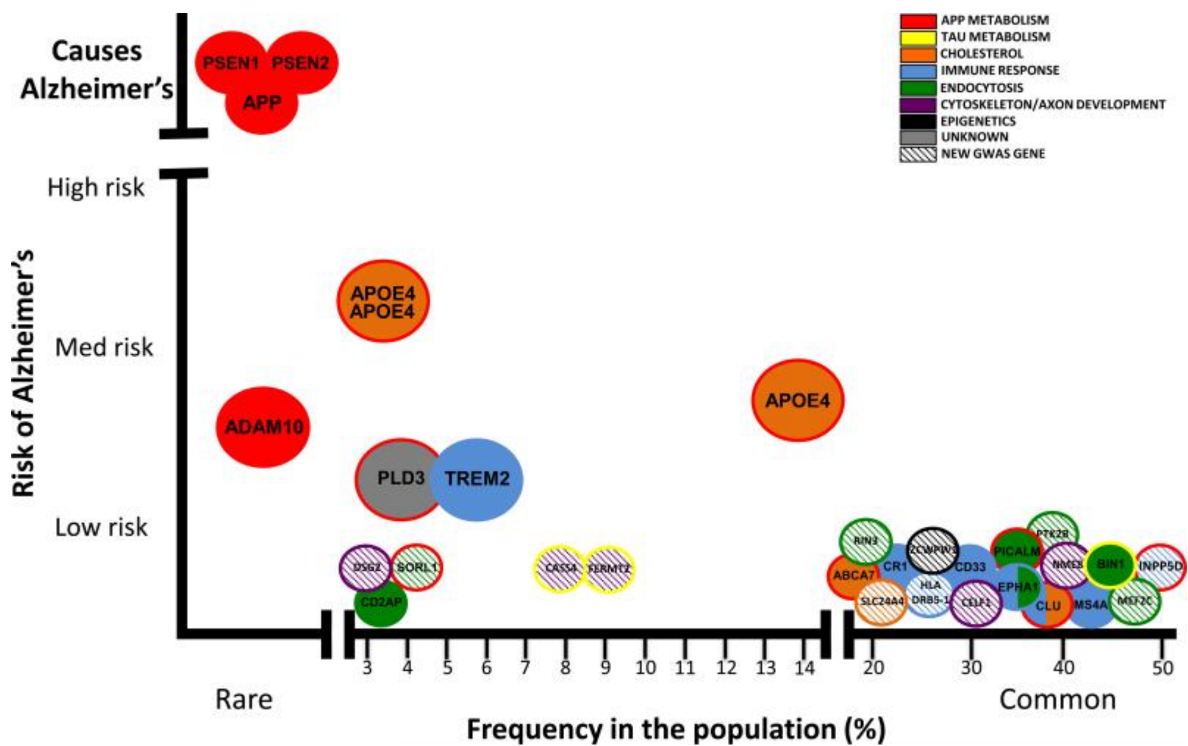


Figure 1.1. Rare and common genetic variants in relation to AD risk (Karch and Goate, 2015). It can be noted that the *APOE4* allele is the highest risk common variant.

Figure 1.2 displays a leading hypothesis of the temporal hierarchy of clinically detectable AD-related neuropathological abnormalities,[14] and this framework has been supported by additional studies.[15, 16] The first detectable AD abnormality is a decrease in Aβ protein concentration in cerebrospinal fluid (CSF), concomitant with an increased accumulation of Aβ in the brain parenchyma. Accumulation of Aβ in the brain is thought to occur during a long preclinical process which can last decades before the manifestation of clinically detectable cognitive changes.[17, 18] The pathways of

pathogenic amyloid and tau accumulation, measurement of these pathologies *in vivo* and at autopsy, as well as the potential interactions will be discussed.

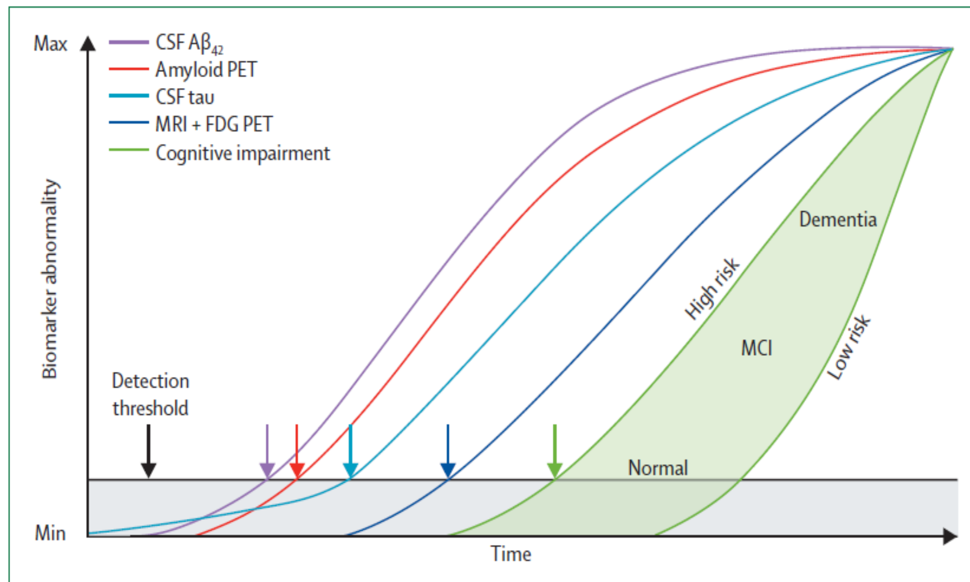


Figure 1.2. Theoretical framework that displays typical order of detectable clinical abnormalities related to Alzheimer's disease and the clinical onset of cognitive deficits (Jack *et al*, 2013). The prodromal phase of disease can span several decades.

Pathogenic Aβ accumulation can be the product of dysregulated amyloid precursor protein (APP) cleavage or an impairment in Aβ clearance. Secretases are responsible for proteolysis of APP to toxic and non-toxic forms of amyloid (**Figure 1.3**).[5] Cleavage of APP by α, then γ secretases produces the non-toxic form while cleavage by β, then γ secretases produces the toxic forms, $A\beta_{40}$ or $A\beta_{42}$. $A\beta_{42}$ is more abundant, hydrophobic and has been hypothesized to be more toxic compared to $A\beta_{40}$ because

the C-terminal alanine and isoleucine residues of $A\beta_{42}$ make this peptide species more prone to aggregation.[19]

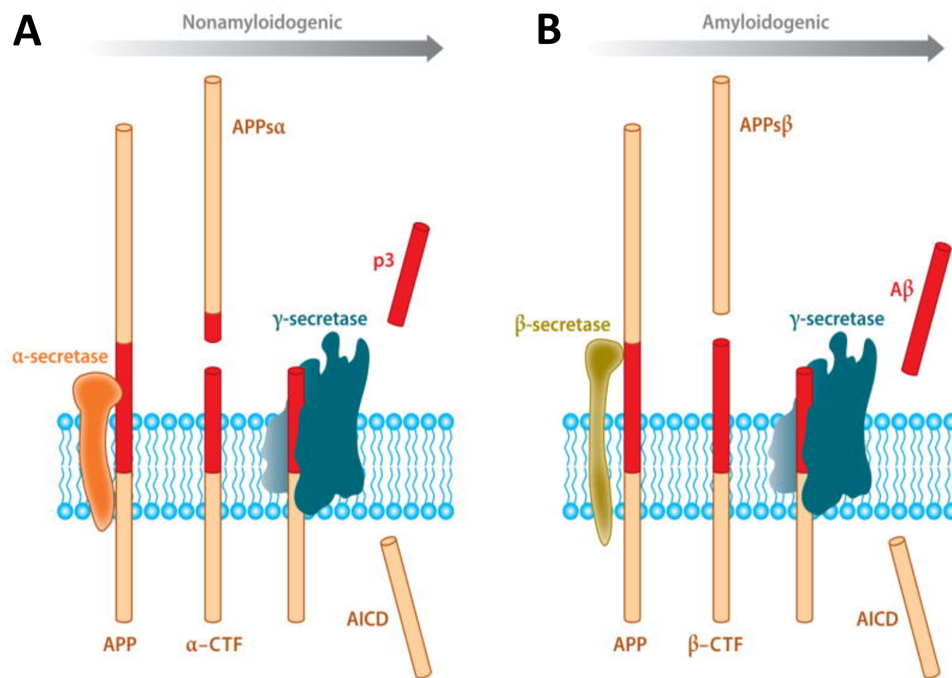


Figure 1.3. Panel **A** shows the non-pathogenic sequence of amyloid precursor protein (APP) cleavage. Full length APP is cleaved by α-secretase, generating an extracellular APP soluble alpha fragment and a transmembrane alpha C-terminal peptide which is cleaved by γ-secretase, resulting in an extracellular p3 peptide fragment and an APP intracellular domain peptide (AICD). Panel **B** displays the pathogenic processing of APP where β-secretase cleaves APP, resulting in an extracellular APP soluble β fragment and a transmembrane beta C-terminal fragment. The β C-terminal fragment cleavage by γ-secretase produces extracellular $A\beta_{40}$, or $A\beta_{42}$ (Adapted from Brien *et al*, 2011).

In 1992, the amyloid cascade hypothesis was published by Hardy and Higgins to explain the etiology of AD pathogenesis.[20] This hypothesis supported the deposition of

the amyloid protein as the causative molecular entity for downstream AD neuropathology and dementia. This hypothesis was the best fit model for the data up to that time, being consistent with genetic observations that autosomal-dominant mutations in genes in the amyloid processing pathway (APP, PSEN1/2) and overexpression APP associated with Down syndrome, all increased amyloid deposition and were associated with manifestation of AD earlier in life.[21] In later sections the amyloid cascade hypothesis will be examined in the context of anti-amyloid treatment results in clinical trials, with a discussion on current views of this hypothesis.

Amyloid burden in the brain can be estimated *in vivo* by measuring uptake of positron emission tomography (PET) radioligands, such as ^{18}F-Florbetapir.[22] Studies in cognitively normal older adults have shown that amyloid burden is associated with lower memory performance,[23] reduced cortical thickness,[24] increased neurodegeneration,[25, 26] and lower cognitive performance in attention, language and executive function.[27-29] Further, decreased CSF $A\beta_{42}$ as a biomarker of Aβ aggregation in the brain, is positively correlated with brain atrophy and CSF phosphorylated tau (p-tau) in cognitively normal older adults.[30, 31] These points support a long preclinical phase of disease, whereby amyloid deposition over the course of decades positively modulates downstream tau pathology, brain volume and cognitive deficits.

Amyloid plaques can be categorized into two subtypes, diffuse and neuritic plaques. Diffuse plaques are composed of aggregated amyloid peptides that are not fibrillar in shape but show a diffuse pattern without associated dystrophic neurites, abnormal and damaged neuronal processes. Neuritic plaques present with a dense core, are composed of fibrillar amyloid and are associated with dystrophic neurites.[32]

Neuritic plaque density at autopsy has been associated with very early cognitive changes in individuals classified as having normal cognition.[33] Both plaques are visible with Bielschowsky silver stain and are distinguished from one another based off of plaque morphology at autopsy.[34]

Neurofibrillary tangles, composed of hyperphosphorylated and aberrantly folded tau, are the next pathologic change to occur in AD. Glycogen synthase 3β kinsase (GSK3β) is the most well-characterized kinase responsible for phosphorylation of tau, and GSK3β can be activated downstream of numerous receptor tyrosine kinases. Protein phosphatase 2A (PP2A) is the principal protein for the dephosphorylation of tau and exists as a trimer composed of a regulatory (PP2 AB), catalytic (PP2A C) and scaffolding (PP2A A) subunit.[35] Under physiological conditions, tau is a microtubule associated protein that is important for cytoskeletal stabilization in neurons.[36, 37] Unlike amyloid pathology, pathogenic tau tangles begin intra-neuronally and are more highly correlated with dementia severity compared to Aβ plaque deposition, fitting with the temporal model presented previously which suggests tau pathology is downstream of Aβ pathology.[19, 38]

Measurement of tau burden in the human brain using imaging is an emerging area of research in the AD field, and several ongoing studies are investigating the physiology and specific binding of various tau PET tracers. Tau pathology is strongly correlated to both brain hypo-metabolism as well as cognitive decline. **Figure 1.2** demonstrates that abnormalities in brain metabolism, as measured by fluorodeoxyglucose (FDG) PET, typically follows CSF tau abnormalities. Neurodegeneration measured by magnetic resonance imaging (MRI) is detectable

around the same time as AD-related hypometabolism, followed by objective cognitive impairment. Similar to autopsy measures of amyloid burden, tau burden is assessed with histopathological staining at autopsy. Severity of tau pathology is classified by Braak staging, which exists as a scale from one to six, with Braak stage six showing the most extensive spread of tau pathology throughout the brain.[39]

It could be the case that pathologic amyloid accumulation in the brain acts as a trigger to initiate or exacerbate downstream tau pathology, which leads to neurodegeneration and subsequent cognitive decline. The detailed molecular mechanisms to mediate such an interaction remain unknown, but the cellular localization differences suggest an intermediate trans-membrane protein. Yet, the relative localization and path of spread between amyloid plaques and hyperphosphorylated tau also differs. AD-related amyloid pathology typically begins in the basal frontal, temporal and occipital lobes, then spreads to the hippocampus, followed by the neocortex and several subcortical regions like the striatum, thalamus and cerebellum.[19] AD-related tau pathology begins in the medial temporal cortex then spreads to the neocortex.[40] The motor, visual and primary sensory cortices show relative sparing from tau pathology.[19]

Studies on the pathologic spread of tau from rodent models have given rise to the tau propagation hypothesis, which states that the spread of hyperphosphorylated tau can be explained by interneuron transfer along neural networks.[41] Tau can exist in several distinct biochemical forms, in part due to a plethora of possible posttranslational modifications including glycation, acetylation, ubiquitination, nitration, and SUMOylation. Due to this biochemical complexity, the form of tau released into the extracellular space

from a donor neuron are not well understood.[42] The biochemical form of tau used *in vivo* to study seeding and spread patterns are not biologically identical to the pathogenic tau that forms neurofibrillary tangles in human brain, posing caveats to studies in model systems which support the tau propagation hypothesis.[42] Additionally, tau pathology in AD brains can show heterogenous origins outside of the medial temporal lobe, such as the dorsal raphe nuclei and the locus coeruleus, raising the possibility of multiple origin sites of propagation.[42]

Overall, the neuropathology of AD is characterized by a long, prodromal accumulation of amyloid in the brain that can be detected by PET imaging, with a detectable decrease in CSF amyloid concentration. The pathological burden of disease can be evaluated with staining of the brain at autopsy and is used to confirm an AD diagnosis. Although it is unclear how amyloid pathology may be molecularly connected to pathological accumulation of phosphorylated tau, the amyloid cascade hypothesis and observations from amyloid positive cognitively normal individuals suggest that tau pathology begins after amyloid accumulation and leads to cognitive decline, as suggested by the leading temporal hypothesis.

Current Pharmacological Landscape

Two classes of small molecules are currently approved for the treatment of AD and target the glutaminergic or cholinergic neurotransmitter systems. The Food and Drug Administration (FDA) approved compounds which target the cholinergic system act as cholinesterase inhibitors and include Donepezil (Eisai Co., Pfizer), Galantamine (Janssen, Takeda, Ortho-McNeil, Sanochemia, Shire) and Rivastigmine (Novartis).[43]

Cholinesterase is the metabolizing enzyme of acetylcholine, so inhibition of the enzyme results in increased acetylcholine concentration in the brain. Acetylcholine is an important neurotransmitter for the modulation of cognition, and depleted acetylcholine in the basal forebrain has been shown to decrease cognitive performance.[44] Although cholinesterase inhibitors help to maintain cognition, they are not neuroprotective, do not have an effect on disease progression or survival, and are not effective in stages of severe dementia.[45, 46]

Memantine (Forest Laboratories, Merz Pharmaceuticals), the most recent (2003) drug to be approved for the treatment of AD, acts as a low-affinity, uncompetitive NMDA receptor antagonist. Low-affinity binding and fast on/off kinetics are key to Memantine's mechanism of action. These binding properties allow the compound to bind NMDA receptors and block excessive glutamatergic transmission that can lead to excitotoxicity without preventing a lower level of physiologic activation.[46] In contrast, high-affinity NMDA antagonists such as ketamine and amantadine have neuropsychiatric side-effects and can inhibit synaptic plasticity resulting in impaired learning and memory.[46] Although these drugs can help delay cognitive decline caused by AD, they do not prevent significant cognitive decline and only show efficacy for a limited period of time.

From 1998 to 2018, there have been approximately 152 failed trials for the treatment of AD. Many drug candidates have targeted amyloid-β production and clearance. While phase 3 trials have successfully shown clearance of amyloid burden from the brain, thus far this has not significantly prevented continued cognitive decline.[47] These clinical trial results are therefore inconsistent with the amyloid cascade hypothesis, and the utility of targeting amyloid to stop the progression of AD is under

question. If pathologic amyloid were truly the root cause of AD, we would expect that clearance of the protein, especially early in the disease course, would result in significant patient improvement and prevention of further cognitive decline. Some clinical trials targeting amyloid, including a humanized monoclonal antibody against soluble Aβ known as solanezumab, have enrolled patients in the early stage of disease but have not shown cognitive efficacy after reducing amyloid burden.[21] One possibility is that amyloid-targeted therapies may only be effective before any AD symptoms emerge. An ongoing clinical trial, the Anti-Amyloid Treatment in Asymptomatic Alzheimer's disease (A4) study is targeting cognitively normal older adults with brain amyloid accumulation. This study is set to conclude in 2022 and targets the earliest possible timeframe of amyloid accumulation, as no amyloid targeted therapies have been successful by enrolling patients with even subtly detectable cognitive decline.

Small molecule inhibitors of γ-secretase, such as Semagacestat (Eli Lilly & Co.) and Avagacestat (Bristol-Myers Squibb) have failed in part due to adverse side effects, including cerebral microbleeds, nonmelanoma skin cancer, and worsening of AD symptoms. These side effects are thought to stem from γ-secretase inhibition of functions outside of Aβ production, such as the proteolysis of Notch receptors which affects widescale cell differentiation and cell fate.[43] β-secretase (BACE1) inhibitors have not gained clinical traction in part due to poor pharmacokinetic properties. First generation BACE1 inhibitors such as BI 1181181 (Boehringer Ingelheim, Vitae Pharmaceuticals Inc.) did not have adequate bioavailability, or blood-brain barrier (BBB) penetrance. Many second-generation compounds such as RG7129 (Roche) were abandoned due to liver toxicity. Currently, trials are ongoing for third generation BACE1

inhibitors which were designed to have better pharmacokinetic properties and increased potency. Anti-BACE1 antibodies have also been investigated, but low BBB penetration has also limited an immunotherapeutic approach to decreasing amyloid production.

As mentioned above, immunotherapies using monoclonal antibodies targeted to various regions of Aβ have also been tested in clinical trials, but none have been approved in a phase 3 trial for the primary endpoint. One particularly interesting human IgG1 monoclonal antibody against aggregated forms of Aβ, Aducanumab (Biogen), was re-launched in a Phase 3b open-label study in January 2020. This re-launch was prompted by findings after an interim futility analysis, which showed that a trial known as EMERGE had met its primary endpoint to show significant reduction in cognitive decline. Interestingly, this compound did show a dose-dependent reduction in amyloid beta burden in the brain and a reduction in CSF p-tau. The Phase 3b trial for Aducanumab is expected to conclude in 2023.

Additional methods to target the pathological accumulation of amyloid-β include molecular chaperones to decrease protein aggregation. New targets have also emerged, such as tau aggregation, neuroinflammation and metabolic disorders. With the staggering number of failed clinical trials for disease modifying treatments in AD, there is a distinct and urgent need to explore novel targets. Notably, an association between AD and insulin resistance resulting from type 2 diabetes have implicated the PI3K/Akt/GSK3β pathway downstream of insulin receptors and shared by vascular endothelial growth factor (VEGF) receptors as a potential therapeutic pathway.[48]

Role of Apolipoprotein E (APOE) in AD

The polymorphic apolipoprotein E (*APOE*) gene is the strongest genetic risk factor for LOAD, with the ε4 allele conferring risk and the ε2 allele conferring protection relative to the most common isoform, ε3.[49, 50] These isoforms differ at two residues, 112 and 158 of the APOE protein. The ε2 allele codes cysteine at both residues, the ε3 allele codes for cysteine at residue 112 and arginine at residue 158, while the ε4 allele codes for arginine at both residues. Carriers of the ε4 allele make up 56-65% of AD patients,[51] but the molecular mechanism by which APOE contributes to AD pathophysiology has been debated.[52] Well characterized effects of *APOE*-ε4 include compromised BBB integrity[53] and increased amyloid-beta accumulation,[54] possibly driven by a decrease in amyloid-β metabolism.[55] Recently, it was shown that BBB breakdown is caused by the *APOE*-ε4 allele and the resulting compromised vascular integrity contributes to cognitive decline.[56]

APOE is a lipid transport protein primarily produced by hepatocytes in the periphery, and acts as a high-affinity ligand for receptor-mediated clearance of very low-density lipoprotein (VLDL) and chylomicron remnant lipoprotein particles.[57] APOE containing lipoproteins do not cross the BBB and the brain is a primary source of APOE in the central nervous system, where APOE is produced by astrocytes, microglia and stressed neurons.[58, 59] These cell types, in addition to brain endothelial cells, express APOE receptors low density lipoprotein receptor (LDLR), LDLR-related protein 1 (LRP1), very low-density lipoprotein receptor (VLDLR), and the APOE receptor 2 (ApoeR2, also known as LRP8).[58] APOE also binds to heparin sulfate proteoglycans,

which can result in APOE uptake through both receptor-mediated and receptor-independent mechanisms.

The primary source of APOE in the brain are astrocytes, which secrete the APOE to deliver cholesterol and other essential lipids to neurons as well as endothelial cells. APOE4 has been strongly associated with a myriad of cerebrovascular deficits, including small vessel disease[60] and greater decline in cerebral blood flow with aging,[58] as well as enhanced risk for ischemic stroke.[61] There is evidence that the deleterious effect of APOE4 on BBB function results from decreased binding of APOE4 to ApoER2 expressed on endothelial cells.[62] Studies in *APOE*-ε4 targeted replacement (*APOE4-TR*) mice have shown enhanced perfusion deficits and neurodegeneration compared to wild-type mice.[58] APOE4 has been mechanistically tied to nearly all molecular pathways of AD pathogenesis including amyloid production and clearance, altered signaling of amyloid-β, tau phosphorylation and neuroinflammation.[58, 63-65] Additionally, APOE4 interacts synergistically with vascular risk factors, such as hypercholesterolemia and diabetes mellitus, to modulate cognitive decline over the course of aging.

Although APOE is a key driver of genetic risk for LOAD, it has also been studied as a genetic resilience factor. A rare mutation in the *APOE* gene known as the Christchurch mutation (*APOE*3*ch*) was extensively characterized in a recent case study, which documented a patient with a familial AD mutation in *PSEN1* (E280A mutation) who showed a severe amyloid burden, little tau pathology, and was not diagnosed with mild cognitive impairment (MCI) until her 70s, approximately three decades after the typical *PSEN1* mutation carrier.[66] The degree of amyloid and tau accumulation in the brain of the *APOE*3*ch* homozygote compared to an average *PSEN1* mutation carrier

can be appreciated in **Figure 1.4**. This patient was also diagnosed with hyperlipoproteinemia type III (HL-III), a condition that causes the body to metabolize lipids incorrectly and results in lipid buildup in the body.[66] The *APOE*ch mutation is an arginine to serine substitution at amino acid 136, corresponding to codon 154 which is in the low-density lipoprotein receptor (LDLR) binding region. This case presents an interesting link between amyloid accumulation, downstream build-up of tau pathology and lipid handling by APOE. Although detailed mechanisms that connect amyloid accumulation to the pathogenic spread of tau in an APOE-dependent manner are not understood, APOE remains a key mediator of AD pathogenesis and an important component when considering the genetic landscape of LOAD.

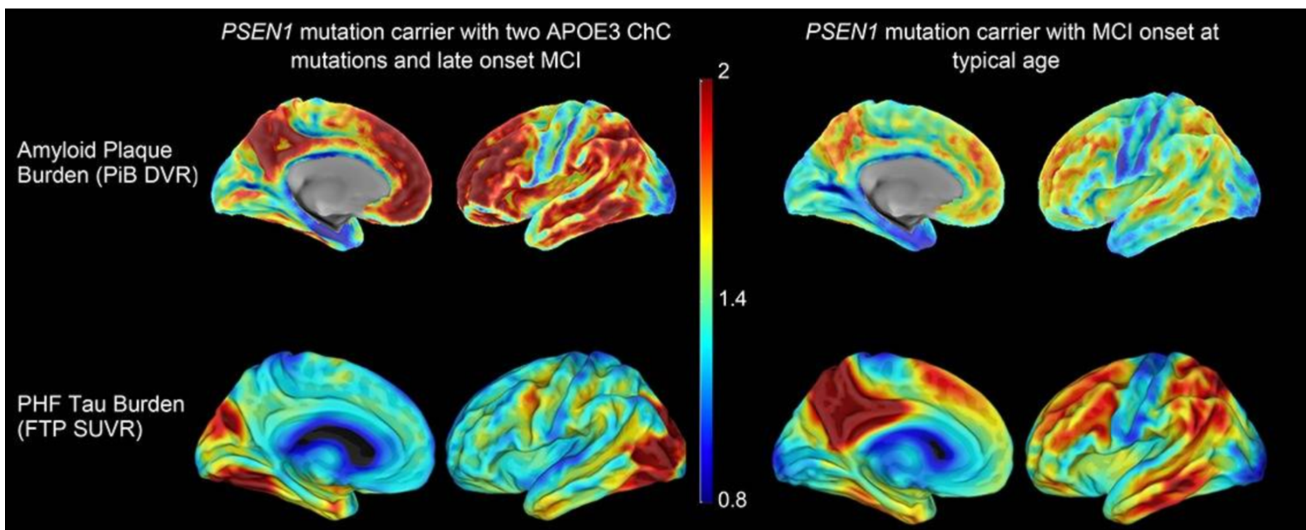


Figure 1.4. Neuroimaging showed high amyloid and low tau burden in a *PSEN1* carrier who is also homozygous carrier of the *APOE*ch mutation, compared to a typical *PSEN1* carrier. PET measurements of amyloid burden were taken using the Pittsburgh Compound-B (PiB) and distribution volume ratios (DVR) were calculated. Flortaucipir was used for tau PET and standard uptake value ratios (SUVR) were quantified. Red indicates highest radiotracer binding and blue indicates lowest binding (Arboleda-Velasquez *et al*, 2019).

Vascular Endothelial Growth Factor (VEGF) Family

Biological Functions

The vascular endothelial growth factor (VEGF) family plays a critical role in neuronal and vascular maintenance and development. The family, particularly VEGFA, was first studied in the context of angiogenesis and vascular permeability.[67-69] The most thoroughly studied member of the family remains VEGFA, which has been heavily targeted for the treatment of cancer.[70] The mammalian VEGF signaling family is large, with five genes encoding ligands (*VEGFA, VEGFB, VEGFC, VEGFD,* and *PGF*), 3 genes that encode receptor tyrosine kinases (RTKs; *FLT1, KDR* and *FLT4*), and 2 co-receptor genes which encode the neuropilins (*NRP1, NRP2).*[71] Ligands in the family function as homodimers and bind the RTKs to initiate intracellular signaling cascades including activation of a wide array of kinases such as Src, Src homology-2 domain containing protein (SHB) and Fyn kinase through scaffolding proteins such as the SH2/SH3 adaptor, Nck.

It is now appreciated that components of the VEGF family, such as *VEGFB*, are important for neuroprotection through distinct intracellular signaling cascades activated by components involving proteins such as phosphoinositide 3-kinase (PI3K) and Protein kinase B (Akt),[72] demonstrating the possibility that signaling downstream of VEGF ligands could be relevant for protection from AD-associated cognitive decline. The VEGF ligands and receptors display selective interactions (**Figure 1.5**), and the neuropilin receptors can bind to and modulate the signaling activity of the RTKs. FLT1 and KDR can also form homo- or heterodimers, which show distinct signaling

efficiencies.[73] Specifically, FLT1 receptors activate fewer intracellular kinases compared to KDR and it has been hypothesized that the biological function of FLT1/KDR heterodimers is to negatively modulate the signaling of KDR homodimers.

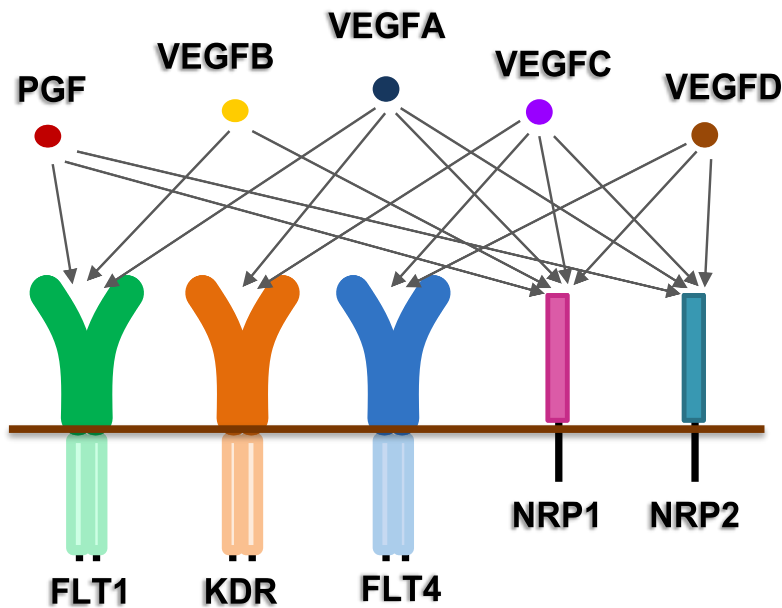
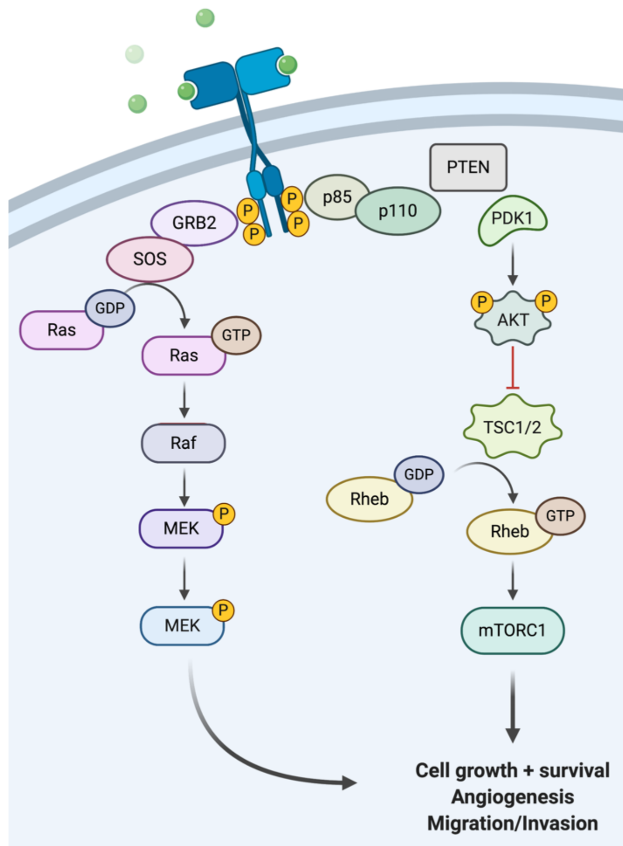


Figure 1.5. Mammalian VEGF ligand and receptor gene family. Genes encoding proteins are shown; *Gene* (Protein): *PGF* (PGF), *FLT1* (VEGFR-1), *VEGFB* (VEGFB), *KDR* (VEGFR-2), *VEGFA* (VEGFA), *FLT4* (VEGFR-3), *VEGFC* (VEGFC), *NRP1* (NRP1), *NRP2* (NRP2). Arrows represent the ability of a ligand to bind a receptor.

Studies to elucidate the distinct functional consequences of VEGF receptor activation in endothelial cells have found that VEGF signaling through KDR activates downstream mitogen-activated protein (MAP) kinases such as extracellular signal-

regulated kinases (ERK1/2), p38 MAP kinase, phospholipase C γ (PLCγ) and phosphoinositide 3-kinase (PI3K), which lead to increased DNA synthesis, endothelial cell migration, angiogenesis, and vascular permeability.[74, 75] An example schematic of receptor tyrosine kinase cascades is shown in **Figure 1.6**. Although FLT1 and KDR are both expressed by endothelial cells, FLT1 signaling in endothelial cells does not lead to activation of MAP kinases detailed above, and VEGF binding to FLT1 does not induce endothelial cell migration, angiogenesis or vascular permeability whereas VEGFA binding to KDR does.[74]

Figure 1.6. Intracellular signaling cascades downstream of receptor tyrosine kinases, such as VEGF receptors FLT1, KDR and FLT4.



FLT4 is primarily expressed by endothelial cells and signals through activation of ERK to initiate downstream lymphomagenesis.[76, 77] Interestingly, the PI3K/Akt signaling pathway is also activated downstream of VEGFC binding to FLT4, but this signaling cascade plays a role in later stages of lymph vessel development and maintenance, while ERK is an essential signaling entity for sprouting and early stages of development.[77] Lymphatic vessels are important to the absorption of lipids from the digestive tract, maintaining fluid homeostasis, as a channel for immune cells, as well as a potential channel to clear amyloid and extracellular tau from the brain.[77-79]

Many VEGF family signaling effects act in an isoform-specific manner. For example, VEGFA acts as the key regulator of blood vessel growth and can be alternatively spliced into pro or anti-angiogenic isoforms.[71] VEGFA exerts angiogenic effects primarily through binding to KDR, which can exist in either a membrane-bound form or a soluble form (sKDR), a characteristic shared by sFLT1.[71, 80] NRP1 can also exist as an extracellular, soluble receptor fragment after cleavage by ADAMs 9 and 10, which also produces an intracellular, carboxy terminal fragment that can inhibit VEGF-induced phosphorylation of KDR and decrease VEGF-induced endothelial cell migration and angiogenesis.[81] Additional isoform-specific effects will be discussed in later chapters, but these examples support in-depth study of VEGF family isoforms to fully understand how VEGF proteins modulate a given biological process.

Pharmacology

Several inhibitors of the VEGF family have been developed and approved for the treatment of multiple types of cancer and age-related macular degeneration (AMD).

Tumor cells can secrete VEGFA to recruit the growth of new vessels from existing, nearby vasculature. Access to an increased blood supply is permissive for tumor growth, so inhibiting this process has been heavily pursued for the treatment of several types of cancers. One example is glioblastoma, the most common primary brain tumor in adults, in which VEGF-mediated angiogenesis is one of the main drivers of disease.[82] The most commonly used anti-VEGF therapeutic for this indication, Bevacizumab (Avastin, Genentech) is a humanized antibody that binds all VEGFA isoforms, and was shown to significantly increase the progression-free survival of glioblastoma patients.[82-84]

Similarly, age-related macular degeneration is caused by pathologic angiogenesis in the vasculature of the eye, which damages photoreceptors and can result in blindness.[85] Several anti-VEGF therapies are used to treat AMD, including Bevacizumab and Pegaptanib sodium (Macugen, Eyetech), which is an RNA oligonucleotide that targets $VEGFA_{165}$, and these treatments have been shown to be safe and effective for AMD.[86]

VEGF family inhibitors also include antibodies which act similarly to sFLT1, to bind free VEGFA and sequester it such that it is unable to signal through FLT1 or KDR receptors.[70] Additional human monoclonal antibodies to decrease VEGFA signaling were developed to antagonize the KDR receptor to inhibit angiogenesis.[87] Several tyrosine kinase inhibitors are also approved as anti-VEGF agents for the treatment of a number of disorders such as colorectal cancer, soft tissue sarcomas and renal cell carcinoma, but these compounds do not show receptor selectivity among TKRs in the VEGF family.[87] To better understand the unique functional roles of VEGF receptors,

particularly in the CNS, more selective pharmacological tools would be impactful. Further, selective pharmacologic tools would allow for the manipulation of non-angiogenic VEGF signaling which could be relevant for AD-related neuroprotection.

Rationale and Aims

VEGF expression has been implicated to play a role in neurodegeneration, partially through its role in regulating perfusion, where increased expression positively modulates increased vasculature and profusion.[88] This regulation has been studied extensively in the hippocampus, where an increase in vasculature by increased expression of VEGFA is associated with an increase in neurogenesis.[89] Several studies in AD model mice treated with VEGFA reversed cognitive deficits, suggesting VEGFA is neuroprotective.[89-91] Increased VEGF expression by gene therapy has been proposed as a therapeutic strategy to treat amyotrophic lateral sclerosis-related neurodegeneration.[92] It is possible that a decrease in VEGFA brain expression may contribute to ischemic conditions that are unfavorable for neuronal survival. Our group has shown that high levels of CSF VEGFA is associated with less hippocampal atrophy in older individuals.[93] This study also revealed that higher CSF VEGFA is associated with slower rates of hippocampal atrophy and cognitive decline in AD biomarker-positive subjects (**Figure 1.7**).[93] Further, high CSF VEGFA was associated with less longitudinal decline in executive function performance and less hippocampal atrophy in participants with tau pathology. These findings suggest VEGFA may be especially protective among those at highest risk for AD and cognitive decline by increasing brain and cognitive

resilience from AD-related pathology. Taking findings from human and rodent studies together, we hypothesized that VEGF-mediated neuroprotection may be especially beneficial for those at highest risk for AD-related neuropathology and cognitive decline.

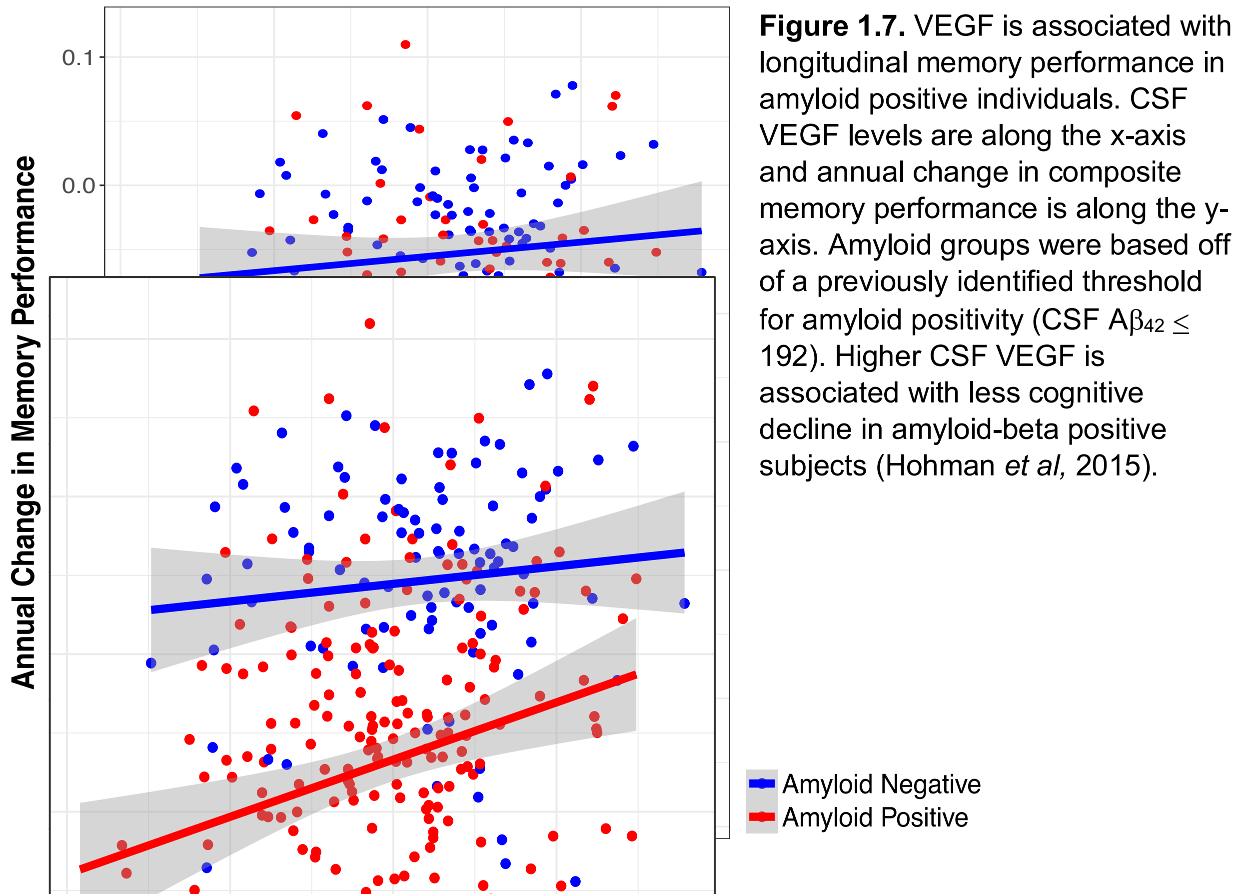


Figure 1.7. VEGF is associated with longitudinal memory performance in amyloid positive individuals. CSF VEGF levels are along the x-axis and annual change in composite memory performance is along the y-axis. Amyloid groups were based off of a previously identified threshold for amyloid positivity (CSF A$\beta_{42} \leq$ 192). Higher CSF VEGF is associated with less cognitive decline in amyloid-beta positive subjects (Hohman *et al,* 2015).

ned risk for AD, VEGF-mediated

neu ng this high-risk population. VEGF-

mediated angiogenesis could be protective against AD-mediated cognitive decline by

preventing ischemia and downstream neurodegeneration. In support of this hypothesis, VEGFA treatment in the hippocampus has been shown to rescue cognitive deficits, increase vascularization, and decrease amyloid pathology associated with aging in humanized *APOE4-TR* mice.[94] We hypothesized that high expression of angiogenesis related genes and proteins in the VEGF family will confer protection against AD and cognitive decline, particularly among *APOE*-ε4 carriers.

The VEGF family represents an exciting candidate pathway for neuroprotection, and a detailed assessment of VEGF effects based on *APOE* genotype may be a first step towards personalized medicine in AD. The complex, isoform-specific signaling of VEGF family genes results in distinct downstream molecular cascades including angiogenic, neurotrophic, lymphatic and metabolic signaling. Thus, a comprehensive characterization of *VEGF* family gene, isoform and protein expression interactions with *APOE* will provide critical information about the molecular pathways that confer neuroprotection from *APOE*-related cognitive impairment. We utilized the rich data resources of the Vanderbilt Memory and Alzheimer's Center, including harmonized human data from multiple longitudinal studies of cognitive aging and AD. Using data from the Religious Orders Study/Rush Memory and Aging Project (ROS/MAP), we determined how cortical expression of *VEGF* family genes, isoforms and proteins interact with ε4 status to modify risk for AD diagnosis, neuropathology (amyloid plaque and tau tangle burden), and cognition. We performed gene set enrichment analysis to determine if genes in the angiogenic pathway were associated with cognitive outcomes using predicted gene expression in the Resilience from AD (RAD) database. Through

the following aims, we investigated the hypothesis that *VEGF*-related angiogenic signaling modified *APOE*-ε4 associated outcomes:

Aim 1. Evaluated gene and protein expression profiles of the *VEGF* family in brain tissue to identify ligand/receptor combinations that modify the association between *APOE4* and clinical outcomes. Using cortical gene and protein expression data from the Religious Orders Study and Rush Memory and Aging Project (ROS/MAP), we tested the hypothesis that high expression of *VEGF* genes related to angiogenesis (*VEGFA, KDR, NRP1*) would modify the association between *APOE*-ε4 and related outcomes (AD diagnosis, neuropathology, cross-sectional and longitudinal cognition) in a beneficial manner.

Aim 2. Characterized transcriptional profiles of specific *VEGF* isoforms in brain tissue that modify the association between *APOE4* and clinical outcomes. Utilizing ROS/MAP data, we investigated the hypothesis that pro-angiogenic *VEGFA* isoforms, transmembrane *KDR* and *NRP1* transcripts will be the strongest modifiers of the association between *APOE*-ε4 and related outcomes, such that higher expression of these genes will confer more favorable outcomes.

Aim 3. Pathway analysis of angiogenic signaling genes investigated the potential role of angiogenesis in cognitive decline. Utilizing data from RAD, we performed an enrichment analysis to determine if predicted expression of angiogenesis-relevant genes was associated with cognitive performance. We hypothesized that decreased angiogenic pathway expression would be associated with cognitive decline. This pathway expression analysis could validate a role for angiogenesis in cognitive decline

associated with dementia and allow us to broaden the pool of preclinical targets that can be further studied in preclinical models of cognitive decline.

These studies characterized which targets along the complex VEGF signaling cascade contribute to cognitive protection in the presence and absence of the ε4 allele by integrating multi-level ‘omics data with in-depth molecular and clinical data to interrogate the functions of *VEGF* gene expression across multiple patient populations with implications for personalized medicine and targeted VEGF therapeutics for dementia to improve patient outcomes.

CHAPTER 2

EVALUATION OF *VEGF* GENE AND PROTEIN FAMILY EXPRESSION MODIFICATION OF *APOE*-ε4 RELATED OUTCOMES

Portions of this chapter are published under the title, "*APOE* ε4-specific Associations of *VEGF* Gene Family Expression with Cognitive Aging and Alzheimer's Disease" in *Neurobiology of Aging*

Introduction

Alzheimer's disease (AD) is one of the most devastating and fastest growing neurological disorders in the world. With no available treatments to halt the progression of this disease, it is of monumental importance that novel insights into the underlying biology surrounding AD-associated cognitive decline are elucidated to generate effective therapeutic targets. Vascular endothelial growth factor A (VEGFA) has been studied as an emerging therapeutic candidate for AD,[88, 93, 95, 96] however the role of VEGFA in the development and progression of AD remains debated. The VEGF family plays a critical role in neuronal as well as vascular processes and is heavily involved in angiogenic regulation, neurogenesis and neuronal survival.[67-69] Some studies have found decreased protein levels of VEGFA in serum and cerebrospinal fluid (CSF) are associated with increased risk of AD and cognitive decline, [71, 93, 97] while others have found the opposite.[98, 99] In support of VEGFA's neuroprotective role, studies have shown that AD model mice treated with VEGFA recover from cognitive deficits.[90, 91] Additionally, our group has demonstrated that higher CSF VEGFA concentration is associated with slower rates of hippocampal atrophy and cognitive decline, particularly

among AD biomarker-positive participants.[93] These studies suggest that VEGFA is especially protective among participants at highest risk for AD and cognitive decline.

The apolipoprotein E (*APOE*) gene is the strongest genetic risk factor for late-onset AD, and relative to the most common ε3 allele, the ε4 allele confers risk and the ε2 allele confers protection.[49, 50, 100] The molecular mechanism by which ApoE contributes to AD pathophysiology is still debated;[52] however, well-characterized effects of *APOE*-ε4 include compromised blood-brain barrier integrity,[53] increased amyloid-β accumulation,[54] and alterations in amyloid-β metabolism.[55] ApoE4 has been strongly associated with cerebrovascular deficits, including a greater decline in cerebral blood flow with aging[58] and a significantly enhanced risk for ischemic stroke.[101] It has been hypothesized that the decreased binding of ApoE4 to lipoprotein receptor related protein 1 (LRP1) causes an increase in matrix metallopeptidase 9 (MMP9), leading to compromised endothelial cell tight junctions and downstream decreases cerebral blood flow.[102]

Interestingly, VEGFA has also shown a neuroprotective effect in humanized *APOE*-ε4 mice, whereby treatment with VEGF results in a recovery of behavioral deficits and an increase in hippocampal neovasculartization.[94] Given that *APOE*-ε4 carriers are at heightened risk for clinical AD, it may be that VEGF-mediated neuroprotection is particularly beneficial among this high-risk population. An increase in angiogenesis through *VEGF* signaling can initiate the growth of new vessels, which may serve as a mechanism to protect against *APOE*-related cognitive decline by preventing ischemia and downstream neurodegeneration. We hypothesized that *APOE*-ε4 carriers would show protection against AD and cognitive decline as a result of high

angiogenesis-related *VEGF* gene and protein expression in the brain, which may act to compensate against the multitude of biological vulnerabilities that make this population susceptible to cognitive decline.

The present chapter investigates the interaction of prefrontal cortex *VEGF* gene and protein expression with *APOE*-ε4 allele status on clinical AD, cognition and cognitive decline, as well as AD-related neuropathology. Methods shared between gene and protein expression analyses will be presented, followed by methods and results from gene expression analyses, then corresponding sections from protein expression analyses. We hypothesized that higher expression of angiogenesis-specific *VEGF* genes and proteins would modify the association between *APOE*-ε4 status and AD-related outcomes, such that ε4 carriers would show enhanced protection compared to non-carriers. The theoretical framework of our hypothesis is depicted in **Figure 2.1.**

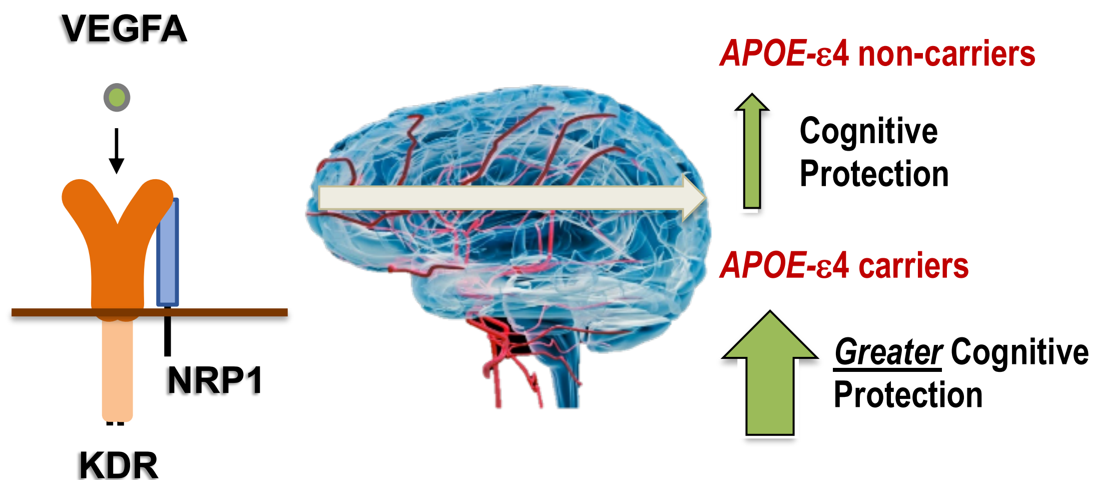


Figure 2.1. Hypothesis for the relationship between the VEGF gene family and cognition based on *APOE*-ε4 allele status.

Methods

Participants

Data collected as part of the Rush University Religious Orders Study (ROS) and Memory and Aging Project (MAP) were utilized for this study. ROS data collection began in 1994 with Catholic clergy from across the USA, and MAP data collection began in 1997 across the Chicago area.[103] In both studies, older participants were non-demented at the time of enrollment, agreed to yearly clinical evaluation, and signed an informed consent, a repository consent for resource sharing, and an Anatomical Gift Act. The goal of these studies was to identify factors important for cognitive health during aging while monitoring the development of cognitive impairment, AD, and pathology of related disorders. Both studies were approved by an Institutional Review Board of Rush University Medical Center. Data sharing was carried out within the guidelines of Institutional Review Board (IRB)-protocols, and analyses were approved by the Vanderbilt University Medical Center IRB.

Neuropsychological Composites

Neuropsychological testing details have been previously published.[104, 105] Multiple aspects of cognition and memory were assessed using established protocols.[103] Z-score composites were then calculated in the domains of episodic memory, perceptual orientation, perceptual speed, semantic memory, and working memory. An average score across all 17 neuropsychological tests was calculated to represent global cognition.

Diagnostic criteria

At each visit, a clinical cognitive diagnosis was made using a 3 stage pipeline which began with computer scoring of cognitive tests, clinical judgement by a neuropsychologist, and diagnostic classification by a clinician (neurologist, geriatrician, or geriatric nurse practitioner) as previously described.[103, 105] Clinical diagnosis of dementia followed criteria suggested by the joint working group of the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA).[106]

Genotyping

DNA was extracted from peripheral blood monocytes (PBMCs) or brain tissue and underwent quality control measures as previously described.[107] *APOE* genotyping was performed by Polymorphic DNA Technologies using high-throughput sequencing of codons 112 and 158 of *APOE* exon 4, located on chromosome 19.[108]

Neuropathological Measures

All neuropathological marker quantifications have been previously described.[104, 105] Briefly, amyloid load and paired helical filament tau density were quantified in eight brain regions.[109] Quantification of neuritic plaques and neurofibrillary tangles was based on silver staining of five brain regions (midfrontal cortex, midtemporal cortex, inferior parietal cortex, entorhinal cortex and hippocampus) to calculate the overall burden.[104]

TDP-43 immunoreactivity was assessed in the amygdala, nucleus accumbens, middle frontal gyrus, cingulate gyrus, dentate fascia, and inferior temporal cortex and scored on a graded scale (0=no pathology, 4=pathology in all regions).[110] Cerebral amyloid angiopathy (CAA) was measured by β-amyloid immunostaining in the midfrontal, midtemporal, angular, and calcarine cortices, and was scored on a scale from 0 – 4 (0=no pathology, 4=severe pathology).[111, 112] Assessment of atherosclerosis was performed by visual inspection of the vertebral, basilar, posterior cerebral, middle cerebral, and anterior cerebral arteries of the Circle of Willis, as well as proximal branches and graded based on severity (0=no pathology, 4=severe pathology).[113] Arteriolosclerosis severity was classified by a semi-quantitative grading scale (0=no pathology, 3=severe pathology) after characterization of histologic changes in the vascular lumen.[114] Gross and micro infarcts were categorized as present (1) or absent (0) based upon visual inspection in nine brain regions (midfrontal, middle temporal, entorhinal, hippocampal, inferior parietal and anterior cingulate cortices, anterior basal ganglia, midbrain, and thalamus.[115-117]

Autopsy Measures of *VEGF* and *APOE* Gene Expression

Autopsies were performed to dissect and preserve tissue blocks from discrete brain regions. RNA was extracted from prefrontal cortices using the miRNeasey mini kit (Qiagen), with the RNase free DNase Set. A Nanodrop instrument was used to quantify RNA concentration, and RNA integrity was evaluated using a Bioanalyzer (Aligent). Criteria for sample inclusion was set as an RNA integrity (RIN) score greater than five and at least 5μg of sample. Library preparation for RNA sequencing used poly(A)

selection[118] with the dUTP strand specific method.[119] The libraries were pooled using similar RIN scores to prevent an unnecessarily large spread of insert sizes during library construction, which could result in uneven coverage throughout the pool. Samples were sequenced using the Illumina HiSeq platform with 101 base paired-end reads. The first 12 samples served as a deep coverage reference and were sequenced with a coverage of 150 million reads. These 12 deep coverage references included two male and two female samples from each diagnosis of normal cognition, mild cognitive impairment and AD. The remaining samples were sequenced with a coverage target of 50 million reads, and the mean coverage was 95 million reads.

RNAseq data was trimmed of adapter sequences and rRNA reads were removed. RNAseq data was then aligned to a reference genome using Bowtie[120] and the RNA-Seq by Expectation-Maximization (RSEM) method[121] was applied to estimate expression of transcripts in fragments per kilobase million (FPKM). Details of sample processing and quality control measures have also been previously published.[122, 123] Outliers, classified as values four standard deviations in either direction from the combined sample mean, were removed.

Statistical Analyses of *VEGF* Family Gene Expression

Data were analyzed using R (version 3.5.1, https://www.r-project.org) with *APOE*-ε4 allele status categorized using a dominant model (absence or presence). Linear regression models covaried for age at death, sex, postmortem interval, and interval between final visit and death assessed *VEGF* family gene expression

associations with *APOE*-ε4 allele status and with *APOE* expression. A linear mixed effects regression model covaried for sex, age at death, postmortem interval, and interval between final visit and death, assessed *VEGF* family gene expression interactions with *APOE* expression on global cognitive change.

A binary logistic regression model assessed *APOE*-ε4 by *VEGF* family gene expression interactions on diagnosis (normal cognition [NC] compared to AD, mild cognitive impairment subjects were excluded from this analysis). Covariates included sex, age at time of death, postmortem interval, and interval between the last documented clinical visit and time of death.

A linear regression model covaried for sex, age at death, postmortem interval, and interval between final visit and death, was used to test for *APOE*-ε4 *x VEGF* family gene expression interactions on global cognition. Secondary analyses stratified by *APOE*-ε4 status investigated *VEGF* family gene expression associations with global cognition in ε4 carriers and non-carriers. This model was also used to investigate *VEGF* expression associations with *APOE* genotype and *VEGF x APOE* expression on global cognition. Additionally, a linear regression model covaried for sex, age at death, postmortem interval, and interval between final visit and death, was used to assess *APOE*-ε4 *x VEGF* family gene expression interactions on the following cognitive domains: episodic memory, perceptual orientation, perceptual speed, semantic memory, and working memory. Further, this linear regression model was also used to assess genome-wide interactions with the *APOE*-ε4 allele on cross-sectional global cognition.

A mixed effects regression model was used to analyze *APOE*-ε4 *x VEGF* family gene expression interactions on annual cognitive change. Fixed effects included age at death, *APOE*-ε4 status, sex, *VEGF* family expression, postmortem interval, years before death, and interval (years between last visit and the current visit). A three-way *APOE*-ε4 *x VEGF x* interval interaction was the term of interest. Random effects included the interval and intercept. Secondary analyses were stratified by *APOE*-ε4 status. All models were subjected to the Benjamini-Hochberg false discovery rate procedure[124] to correct for multiple comparisons (ie, correction for all 10 *VEGF* family genes).

AD-related neuropathological outcomes included amyloid load, paired helical filament tau density, neuritic plaques, and neurofibrillary tangles, all of which were square-root transformed. Linear models, covaried for age at death, postmortem interval, and sex, assessed *APOE*-ε4 *x VEGF* expression interactions on AD-related neuropathological outcomes. Non-AD neuropathological outcomes were assessed for *APOE*-ε4 *x VEGF* expression interactions using a binary logistic model for hippocampal sclerosis, gross infarcts and microinfarcts. A proportional odds logistic regression model evaluated *APOE*-ε4 *x VEGF* expression interactions on cerebral amyloid angiopathy (CAA), atherosclerosis, arteriolosclerosis, and TDP-43 reactivity. Macroinfarct count was analyzed using a Poisson regression model, and macroinfarct volume was square-root transformed and assessed using linear regression.

Sensitivity analyses were carried out for the cognitive and neuropathology models described above excluding individuals diagnosed with clinical AD to test if diagnostic status accounted for significant results.

Additional sensitivity analyses were performed to determine if cell-type marker expression, included as a covariate, would significantly alter model predictions. We first analyzed correlations between *VEGF* family expression and cell-type marker expression. Models were then re-run covarying for expression of either neuronal marker *ENO2* or expression of all other available cell-type markers (*OLIG2,* oligodendrocytes*; GFAP,* astrocytes*; CD34,* endothelial cells*; CD38,* microglia*)*. These cell-type markers have been previously validated after comparisons of expression profiles and cell population frequency in cortical tissue in this cohort[123, 125] and have been utilized to examine cell-type effects in previous analyses[126]. Additionally, we calculated adjusted *VEGF* expression by residualizing the association between each gene and cell-type marker. This adjusted expression was then used to re-run the models described above.

Replication Datasets of *VEGF* Family Gene Expression

Two additional cohorts from the AMP-AD Knowledge Portal (syn14237651) were used as replication datasets, the MayoRNAseq study (syn5550404) and the Mount Sinai Brain Bank (MSBB) study (syn3159438). For the MSBB cohort, post-mortem samples were collected from the parahippocampal gyrus, frontal pole, superior temporal gyrus, and inferior frontal gyrus, as previously described [127]. For the Mayo cohort, post-mortem samples were collected from the temporal cortex and cerebellum, as previously described [128, 129]. Clinical diagnosis was harmonized between studies based on Braak staging and cognitive scores. Binary logistic regression models covaried for age and sex assessed APOE-ε4 allele interactions with VEGF family members on diagnosis (NC

compared to AD). Only VEGF genes that were significant in the ROS/MAP cohort for interaction on diagnosis were assessed for these analyses.

Autopsy Measures of VEGF Receptor Protein Expression

Isobaric tandem mass tag (TMT) mass spectrometry of 400 human dorsolateral prefrontal cortex tissue samples was performed after random sorting of samples into batches of 8 (for 50 batches) based on demographics such as age, sex, post-mortem interval and diagnosis. A standard sample processing protocol was used,[130] and full details for this particular sample batch have been described.[131] Briefly, tissue was homogenized and centrifuged then supernatant was collected and sonicated. Protein was quantified and samples were reduced and digested. Peptides from each sample were re-suspended in buffer and labeled using the TMT 10-plex kit (ThermoFisher 90406). A high pH fractionation protocol was then used.[132] After resuspension in buffer, fractions were analyzed by liquid chromatography coupled to tandem mass spectrometry as previously described.[133] One full scan (MS1) was collected each cycle, in addition to as many MS/MS scans as possible within the time window. An m/z range of 350-1500 at 120,000 resolution (at 200 m/z) and maximum injection time of 50 milliseconds was used to perform the MS1 scan. Ions of the highest intensity were selected for higher energy collision-induced dissociation, using 0.7 m/z for isolation and 30,000 for resolution with an injection time maximum of 100 milliseconds.[131]

The Proteome Discover suite (ThermoFisher, version 2.3) and the human proteome database through UniProtKB was used to search against MS2 spectra.

Peptide spectral matches were filtered using Percolator, with a false discovery rate <1%. Peptides were assembled into proteins after spectral assignment and were filtered again based on combined constituent peptide probabilities to a 1% FDR. Batch effects were controlled for using a standard procedure.[134] Data was collected for all VEGF family receptors, but VEGF family ligands were not detectable using this methodology.

Statistical Analyses of VEGF Receptor Protein Expression

Only receptors of the VEGF family were available for protein analyses. A linear regression model covaried for sex, age at death, postmortem interval, and interval between final visit and death, was used to test for *APOE*-ε4 *x* VEGF receptor protein expression interactions on global cognition, as well as stratified cognitive domains (episodic, semantic and working memory, perceptual speed and orientation). Analyses stratified by *APOE*-ε4 status were also performed to investigate VEGF receptor protein expression associations with global cognition in ε4 carriers and non-carriers. To assess *APOE*-ε4 *x* VEGF receptor protein expression interactions on cognitive trajectory, a mixed effects linear regression model with fixed effects including age at death, *APOE*-ε4 status, sex, VEGF receptor protein expression, postmortem interval, years before death, and interval (years between last visit and the current visit). A three-way *APOE*-ε4 *x* VEGF *x* interval interaction was the term of interest. Random effects in this model were the interval and intercept.

A binary logistic regression model assessed *APOE*-ε4 by VEGF receptor protein expression interactions on diagnosis (normal cognition [NC] compared to AD, mild

cognitive impairment subjects were excluded from this analysis). Covariates included sex, age at time of death, postmortem interval, and interval between the last documented clinical visit and time of death.

Additionally, VEGF receptor protein expression x *APOE*-ε4 allele status interactions on AD-related neuropathology were assessed with linear regression models covaried for age at death, interval between final visit and death, postmortem interval and sex. All models detailed above assessed overall VEGF receptor x *APOE*-ε4 interactions on the given outcomes, followed by stratified analyses in *APOE*-ε4 carriers and non-carriers.

Lastly, VEGF receptor protein expression was tested for associations with cognitive and neuropathological outcomes. A linear regression model which covaried for sex, age at death, postmortem interval, and interval between final visit and death, was used to test for VEGF protein expression associations with cross-sectional cognition using the final time point before death, and to test for associations with AD-related neuropathology. A mixed effects linear regression model which covaried for fixed effects including age at death, sex, VEGF receptor protein expression, postmortem interval, years before death, and interval (years between last visit and the current visit). Random effects in this model were the interval and intercept. The Benjamini-Hochberg false discovery rate procedure[124] was used to correct for multiple comparisons on each outcome (ie, correction for 5 VEGF receptor proteins).

Results

VEGF Family Gene Expression Analyses

Participant Demographics, *VEGF* Family Gene Expression

Summary demographic data are presented in **Table 2.1**. This cohort was long-lived, highly educated, with the majority self-identifying as non-Hispanic White. As expected, the proportion of *APOE*-ε4 carriers was higher among AD cases (35%) compared to NC (14%), and baseline global cognition scores declined across diagnostic groups (NC highest, AD lowest). It is noteworthy that the prevalence of *APOE*-ε4 carriers among participants diagnosed with clinical AD is less than other AD cohorts[135-137], however this is likely due to enrollment criteria that required participants to be non-demented at time of enrollment and the community-based nature of studies. The average age of AD diagnosis in this cohort was 82.1 ± 6.3 years of age. Age at time of death was also significantly different across diagnostic groups, with AD cases being the oldest at time of death.

Table 2.1. Cohort demographics and summary statistics

	Clinical Diagnosis			Total (N=531)	P
	Normal Cognition (N=180)	Mild Cognitive Impairment (N=148)	Alzheimer's Disease (N=203)		
Age of death, years	86±7	89±6	91±6	89±7	**<0.001**
Male, no. (%)	70 (39)	54 (36)	70 (34)	194 (37)	0.67
Non-Hispanic white, no. (%)	177 (98)	146 (99)	195 (96)	518 (98)	0.21
Education, years	17±4	16±3	17±4	17±4	0.59
Global cognition composite z score (at last visit)	0.14±0.42	-0.49±0.45	-1.85±0.91	-0.80±1.09	**<0.001**
Average number of visits	7.12±4.04	6.93±3.65	7.55±3.69	7.23±3.8	0.26
APOE-ε4 carriers, no. (%)	25 (14)	30 (20)	72 (35)	127 (24)	**<0.001**
APOE-ε2 carriers, no. (%)	32 (18)	19 (13)	36 (18)	87 (16)	0.39

Values are presented as mean±standard deviation, unless otherwise indicated.

VEGF Gene Expression Associations with *APOE*-ε4 Allele Status and *APOE* Expression

No *VEGF* ligand or receptor genes were differentially expressed between *APOE*-ε4 carriers and non-carriers (p-values>0.09, data not shown). Additionally, no *VEGF* genes interacted with *APOE* expression on global cognition prior to death or cognitive change (p-values>0.06, **Table 2.2**).

Table 2.2. *VEGF* x *APOE* expression on cognition

	Cross-Sectional			Longitudinal		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**
VEGFC	-8.37E-04	4.67E-04	0.07	-9.40E-05	4.96E-05	0.06
NRP2	-2.13E-04	1.28E-04	0.10	-2.03E-05	1.37E-05	0.14
FLT1	-3.61E-05	2.44E-05	0.14	-2.59E-06	2.47E-06	0.29
FLT4	-1.77E-04	2.01E-04	0.38	-2.04E-05	2.13E-05	0.34
NRP1	-6.60E-05	8.76E-05	0.45	-1.31E-05	9.36E-06	0.16
VEGFB	1.89E-06	2.82E-06	0.50	1.02E-07	2.97E-07	0.73
PGF	2.82E-05	4.81E-05	0.56	2.69E-06	5.21E-06	0.61
VEGFD	-1.16E-04	2.53E-04	0.65	-6.48E-06	2.72E-05	0.81
VEGFA	-6.15E-06	1.61E-05	0.70	-1.47E-06	1.76E-06	0.40
KDR	-4.27E-05	2.52E-04	0.87	-1.62E-05	2.71E-05	0.55

Cognitive Outcomes, *VEGF* Family Gene Expression

Cross-sectional analyses revealed *NRP1* and *VEGFA* interacted with *APOE*-ε4 on global cognitive performance at the final neuropsychological assessment (*NRP1:* β=-0.287, p.fdr=0.004; *VEGFA:* β=-0.03, p.fdr=0.026; **Table 2.3, Figure 2.2**). We interpreted this interaction as evidence that *NRP1* and *VEGFA* expression associations with late life cognition differ by *APOE*-ε4 status. To clarify the nature of these interaction results on cross-sectional cognition, stratified analyses showed that in *APOE*-ε4 carriers, higher expression of *NRP1* (β=-0.176, p=0.034) and *VEGFA* (β=-0.027, p=0.019) were associated with worse global cognition scores; whereas in *APOE*-ε4 non-carriers, higher *NRP1* (β=0.112, p=0.003) expression predicted better global cognition scores. Both interaction and stratified results on cognitive performance are summarized

in **Table 2.3**. These *APOE*-ε4 interactions on global cognition did not survive a genome-wide correction in this cohort (**Figure 2.3**), however the power for genome-wide analyses was quite low given the sample size.

Models to assess *VEGF x APOE*-ε4 interactions on cognitive domains revealed that working memory, semantic memory and perceptual orientation drove the *NRP1 x APOE*-ε4 interaction on global cognition. Stratified analyses showed the same trend among *APOE*-ε4 non-carriers, whereby higher *NRP1* expression was associated with better performance in these domains (**Figure 2.4, Tables 2.4 - 2.6**).

Longitudinally, no *VEGF* genes interacted with *APOE*-ε4 on global cognitive change (**Table 2.3**). These results indicate that *VEGF* family expression associations with cognitive decline did not differ by *APOE*-ε4 status.

Clinical Diagnosis, *VEGF* Family Gene Expression

Using a binary logistic regression model, we found that *NRP1* (β=0.77, p.fdr=0.037) expression interacted with *APOE*-ε4 status on clinical diagnosis (NC compared to AD). *NRP2* expression fell just beyond the threshold for *APOE*-ε4 interaction significance after correction for multiple comparisons (p.fdr=0.060). After stratifying participants by *APOE*-ε4 status, lower *NRP1* expression was significantly associated with AD diagnosis in ε4 non-carriers. Interaction and stratified results are presented in **Table 2.7**.

Table 2.3. *VEGF* x *APOE*-ε4 interactions and stratified results on global cognition

		Interaction			***APOE*-ε4 Carriers (N=127)**			***APOE*-ε4 Non-Carriers (N=404)**		
Outcome	**Gene**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
Cross-sectional cognition	***NRP1***	-0.29	0.08	**3.58E-04**[†*]	-0.18	0.08	**0.034**	0.11	0.04	**0.003**[†]
	VEGFA	-0.03	0.01	**0.005**[†]	-0.03	0.01	**0.019**	0.004	0.01	0.416
	FLT1	-0.06	0.03	**0.035**	-0.07	0.03	**0.015**	-0.01	0.01	0.711
	FLT4	-0.24	0.16	0.148	-0.33	0.17	**0.049**	-0.11	0.09	0.196
	VEGFB	0.01	0.004	0.152	-0.003	0.004	0.522	-0.01	0.002	**3.48E-05**[†*]
	KDR	0.29	0.26	0.259	0.23	0.28	0.416	0.02	0.12	0.884
	VEGFD	0.24	0.31	0.433	0.14	0.32	0.657	-0.15	0.15	0.299
	PGF	-0.02	0.06	0.707	-0.07	0.07	0.294	-0.04	0.03	0.215
	VEGFC	-0.14	0.39	0.728	-0.27	0.42	0.531	-0.07	0.17	0.669
	NRP2	0.02	0.15	0.883	0.06	0.16	0.720	0.06	0.07	0.416
Longitudinal cognition	*NRP1*	-0.02	0.01	0.084	-0.01	0.01	0.388	0.01	0.004	0.104
	KDR	0.04	0.03	0.120	0.03	0.03	0.260	-0.02	0.01	0.211
	VEGFB	0.001	4.16E-04	0.155	-3.28E-04	4.48E-04	0.465	-0.001	1.92E-04	**1.46E-06**[†*]
	VEGFA	-0.002	0.001	0.176	-0.002	0.001	0.135	-1.95E-04	0.001	0.709
	VEGFD	0.04	0.03	0.285	0.02	0.04	0.546	-0.01	0.02	0.441
	NRP2	0.01	0.02	0.492	0.01	0.02	0.466	-7.41E-06	0.01	0.999
	VEGFC	0.02	0.04	0.608	0.01	0.05	0.909	-0.02	0.02	0.282
	FLT4	-0.01	0.02	0.723	-0.03	0.02	0.090	-0.03	0.01	**0.006**[†]
	FLT1	-4.65E-04	0.003	0.874	-0.004	0.003	0.229	-0.004	0.001	**0.011**[†]
	PGF	0.001	0.01	0.918	-0.01	0.01	0.272	-0.01	0.003	**0.004**[†]

Boldface signifies $P \leq 0.05$. [†]denotes results that were significant after adjusting for all 10 family members on outcome. *denotes results that were significant after adjusting for all models tested for main outcomes (cognition, diagnosis).

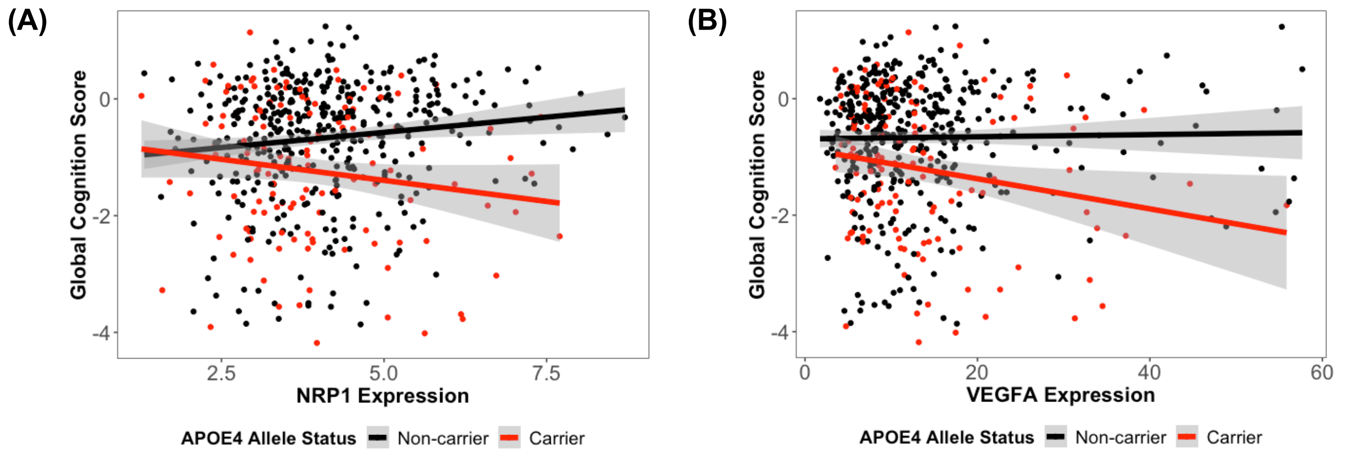


Figure 2.2. (A) *NRP1* expression associations with global cognitive performance at the final neuropsychological assessment, stratified by *APOE*-ε4 allele status. Overall interaction: *NRP1 x APOE*-ε4*,* β=-0.28*,* p.fdr=0.007; *APOE*-ε4 carriers, β=-0.17*,* p=0.038; *APOE*-ε4 non-carriers, β=0.11*,* p=0.004. **(B)** *VEGFA* expression associations with global cognitive performance at the final neuropsychological assessment, stratified by *APOE*-ε4 allele status. Overall interaction: *VEGFA x APOE*-ε4*,* β=-0.03*,* p.fdr=0.026; *APOE*-ε4 carriers, β=-0.03*,* p=0.019; *APOE*-ε4 non-carriers, β=0.004*,* p=0.4.

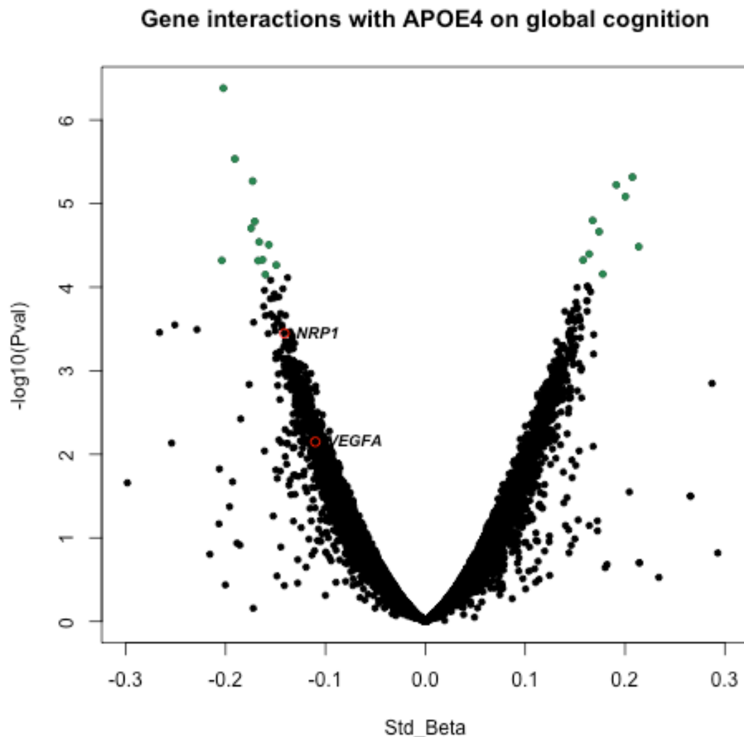
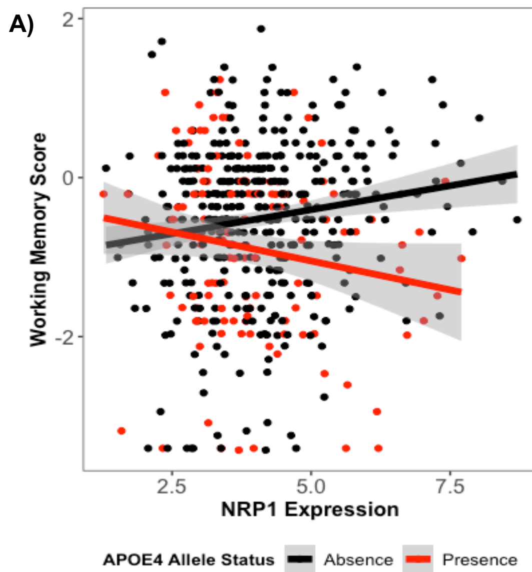
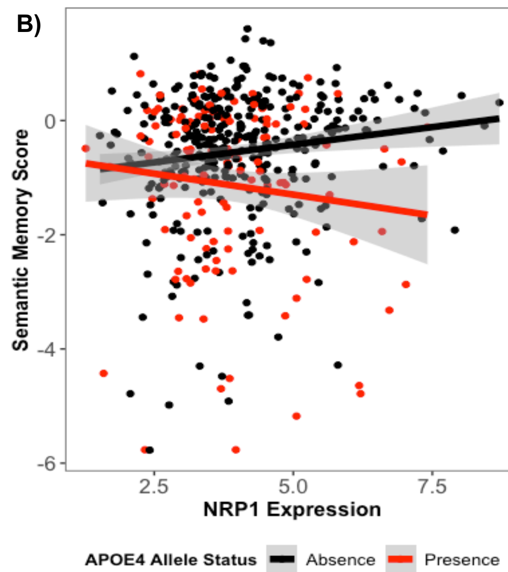


Figure 2.3. Volcano plot of gene expression x *APOE*-ε4 allele interaction results. Genes with p.fdr<0.1 are colored in green.





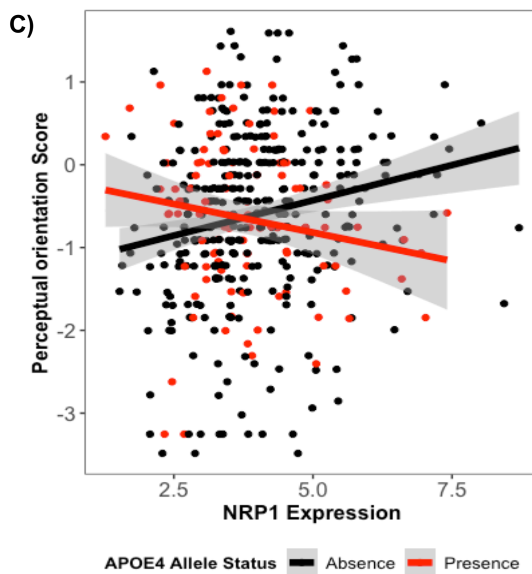


Figure 2.4. (A) *APOE*-ε4 allele stratified *NRP1* expression associations with working memory performance at the final psychological assessment. Overall interaction: *NRP1 x APOE*-ε4, β=-0.3, p.fdr=0.02; *APOE*-ε4 carriers, β=-0.3, p=0.03; *APOE*-ε4 non-carriers, β=0.1, p=7E-4. **(B)** Stratified *NRP1* expression associations with cross-sectional semantic memory. Overall interaction: *NRP1 x APOE*-ε4, β=-0.3, p.fdr=0.03; *APOE*-ε4 carriers, β=-0.3, p=0.096; *APOE*-ε4 non-carriers, β=0.1, p=0.006. **(C)** Stratified *NRP1* expression associations with endpoint perceptual orientation scores. Overall interaction: *NRP1 x APOE*-ε4, β=-0.3, p.fdr=0.03; *APOE*-ε4 carriers, β=-0.1, p=0.2; *APOE*-ε4 non-carriers, β=0.2, p=2E-4.

Table 2.4. *VEGF* x *APOE*-ε4 interactions on working and semantic memory performance

		Interaction			***APOE*-ε4 Carriers**			***APOE*-ε4 Non-Carriers**		
Outcome	**Gene**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
Working memory	***NRP1***	-0.29	0.08	**2.46E-04**†	-0.17	0.08	**0.03**	0.13	0.04	**6.87E-04**†
	VEGFA	-0.03	0.01	**0.02**	-0.02	0.01	0.05	0.01	4.73E-03	0.29
	KDR	0.49	0.26	0.06	0.42	0.27	0.12	-0.10	0.12	0.42
	VEGFB	0.01	0.00	0.16	5.93E-04	3.94E-03	0.88	-4.86E-03	1.80E-03	0.01
	FLT4	-0.16	0.16	0.31	-0.26	0.16	0.10	-0.08	0.08	0.36
	FLT1	-0.03	0.03	0.32	-0.03	0.02	0.21	-3.49E-03	0.01	0.79
	NRP2	-0.04	0.15	0.81	0.02	0.15	0.89	0.06	0.07	0.42
	PGF	-0.01	0.06	0.85	-0.04	0.06	0.57	-0.01	0.03	0.72
	VEGFD	0.12	0.30	0.68	-0.03	0.29	0.93	-0.16	0.15	0.27
	VEGFC	-0.02	0.38	0.96	-0.14	0.40	0.73	-0.06	0.17	0.72
Semantic memory	***NRP1***	-0.31	0.10	**3.35E-03**†	-0.19	0.12	0.10	0.13	0.05	**0.01**†
	VEGFA	-0.04	0.01	**0.01**†	-0.03	0.02	**0.04**	0.01	0.01	0.37
	KDR	0.74	0.34	0.03	0.81	0.39	0.04	0.10	0.15	0.48
	FLT4	-0.37	0.21	0.07	-0.40	0.22	0.08	-0.04	0.10	0.73
	VEGFD	0.48	0.37	0.20	0.36	0.41	0.38	-0.17	0.18	0.32
	VEGFB	0.01	4.98 E-03	0.19	0.00	0.01	0.97	-0.01	0.00	0.00
	FLT1	-0.04	0.03	0.25	-0.04	0.04	0.29	0.01	0.02	0.73
	NRP2	0.06	0.19	0.76	0.12	0.21	0.56	0.08	0.09	0.33
	VEGFC	0.20	0.50	0.69	0.06	0.58	0.91	-0.08	0.21	0.71
	PGF	-0.01	0.08	0.92	-0.07	0.09	0.45	-0.05	0.04	0.16

Boldface signifies P≤0.05. †denotes results that were significant after adjusting for all 10 family members on outcome.

Table 2.5. *VEGF* x *APOE*-ε4 interactions on perceptual orientation

	Interaction			***APOE*-ε4 Carriers**			***APOE*-ε4 Non-Carriers**		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	-0.28	0.09	**2.77E-03**†	-0.11	0.08	0.19	0.17	0.04	**1.58E-04**†
NRP2	-0.13	0.16	0.44	0.07	0.14	0.60	0.21	0.08	**0.01**†
FLT4	-0.24	0.19	0.20	-0.23	0.16	0.15	0.01	0.10	0.92
VEGFD	-0.15	0.33	0.65	-0.05	0.28	0.87	0.07	0.17	0.67
VEGFB	2.7E-03	4.41E-03	0.54	-3.1E-03	3.89E-03	0.43	-4.5E-03	2.12E-03	0.03
VEGFC	-0.22	0.47	0.64	-0.30	0.42	0.49	0.01	0.20	0.96
KDR	0.28	0.30	0.35	0.43	0.27	0.11	0.19	0.14	0.17
FLT1	-0.02	0.03	0.60	4.7E-03	0.03	0.86	0.02	0.02	0.11
VEGFA	-4.0E-03	0.01	0.75	3.9E-03	0.01	0.72	0.01	0.01	0.20
PGF	0.01	0.07	0.87	6.8E-04	0.06	0.99	-0.01	0.03	0.76

Boldface signifies P≤0.05. †denotes results that were significant after adjusting for all 10 family members on outcome.

Table 2.6. *VEGF* x *APOE*-ε4 interactions on episodic memory and perceptual speed

	Episodic Memory			**Perceptual Speed**		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**
VEGFA	-0.03	0.01	0.03	-0.004	0.01	0.75
NRP1	-0.22	0.10	0.03	-0.15	0.10	0.15
VEGFB	0.01	0.005	0.08	-0.001	4.32E-03	0.78
FLT1	-0.06	0.03	0.06	-0.02	0.03	0.51
KDR	0.51	0.32	0.11	0.18	0.31	0.55
NRP2	0.24	0.18	0.19	0.02	0.18	0.90
FLT4	-0.27	0.20	0.18	-0.11	0.19	0.55
PGF	0.02	0.07	0.77	0.01	0.07	0.90
VEGFD	0.01	0.37	0.98	0.64	0.35	0.07
VEGFC	0.01	0.49	0.98	0.20	0.47	0.68

Table 2.7. *APOE-ε4* stratified *VEGF* expression associations with AD Diagnosis

	Interaction			*APOE*-ε4 Carriers			*APOE*-ε4 Non-Carriers		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	0.77	0.27	**3.70E-03**†	0.45	0.24	0.06	-0.31	0.12	**0.01**†
NRP2	-1.11	0.44	**0.01**	-0.86	0.40	**0.03**	0.19	0.20	0.35
VEGFA	0.07	0.04	0.08	0.07	0.04	0.08	-0.01	0.01	0.65
PGF	-0.29	0.16	0.08	-0.07	0.14	0.59	0.18	0.09	**0.047**
VEGFB	-0.02	0.01	0.07	1.76E-03	0.01	0.84	0.02	0.01	**6.66E-04**†*
FLT1	0.08	0.09	0.38	0.14	0.08	0.10	0.02	0.04	0.52
FLT4	-0.29	0.45	0.53	0.07	0.39	0.86	0.42	0.23	0.07
VEGFD	-0.55	0.82	0.50	-0.57	0.72	0.43	0.20	0.41	0.62
KDR	-0.21	0.70	0.76	0.28	0.68	0.68	0.08	0.33	0.80
VEGFC	0.10	1.21	0.93	0.93	1.14	0.41	0.49	0.47	0.30

Boldface signifies P≤0.05. †denotes results that were significant after adjusting for all 10 family members on outcome. *denotes results that were significant after adjusting for all models tested for main outcomes (cognition, diagnosis)

Neuropathology, *VEGF* Family Gene Expression

No significant interactions were observed between *APOE*-ε4 and *VEGF* expression on AD neuropathology (**Table 2.8**). Models to assess *VEGF* x *APOE* interactions on other neuropathological measures showed no significant interaction on CAA, cerebral atherosclerosis, arteriolosclerosis, TDP-43, hippocampal sclerosis, gross infarcts, or microinfarcts (data not shown).

Table 2.8. *VEGF* x *APOE*-ε4 interactions on AD-related pathology
Note: Used square root of continuous variables (amyloid, tangles, nft, neuritic plaques)

	Amyloid burden			Tangles			NFT			Neuritic Plaques		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
FLT1	-0.04	0.03	0.17	0.02	0.03	0.58	-3.0E-3	0.01	0.74	7.3E-04	0.01	0.95
KDR	-0.24	0.28	0.38	-0.39	0.31	0.20	-0.09	0.09	0.34	-0.01	0.13	0.91
NRP1	0.06	0.09	0.53	-0.01	0.10	0.90	-0.02	0.03	0.57	0.04	0.04	0.35
VEGFA	0.01	0.01	0.58	-0.01	0.01	0.31	-3.3E-3	3.8E-3	0.40	4.6E-03	0.01	0.39
PGF	0.03	0.07	0.66	0.10	0.07	0.18	0.01	0.02	0.76	0.01	0.03	0.81
VEGFB	-2.3E-3	4.1E-3	0.58	0.01	4.4E-3	0.23	5.3E-4	1.4E-3	0.70	6.5E-04	1.8E-03	0.72
FLT4	0.07	0.18	0.70	0.19	0.19	0.32	-0.02	0.06	0.79	0.03	0.08	0.73
NRP2	-0.05	0.17	0.79	-0.19	0.18	0.30	-0.10	0.05	0.07	-0.08	0.07	0.29
VEGFC	-0.13	0.42	0.75	-0.37	0.46	0.42	-0.17	0.14	0.21	-0.01	0.19	0.97
VEGFD	0.02	0.33	0.96	0.21	0.36	0.56	0.08	0.11	0.47	3.9E-03	0.15	0.98

Sensitivity Analyses, *VEGF* Family Gene Expression

Additionally, models were run using an adjusted *VEGF* gene expression value that was calculated by residualizing the association between expression and a given cell marker on global cognition. A correlation matrix for *VEGF* family and cell-type marker expression can be found in **Figure 2.5**. Due to the fact that expression data were derived from tissue homogenate, we re-analyzed cross-sectional and longitudinal cognition interaction models to determine if significant *VEGF expression x APOE*-ε4 allele status interaction results persisted after adjusting for cell-specific effects. Cross-sectional results were generally consistent across cell-type marker adjustments and additional covariate models. Longitudinal results were consistent between the adjusted

and unadjusted expression models. Cross-sectional results can be found in **Table 2.9** and longitudinal results can be found in **Table 2.10**.

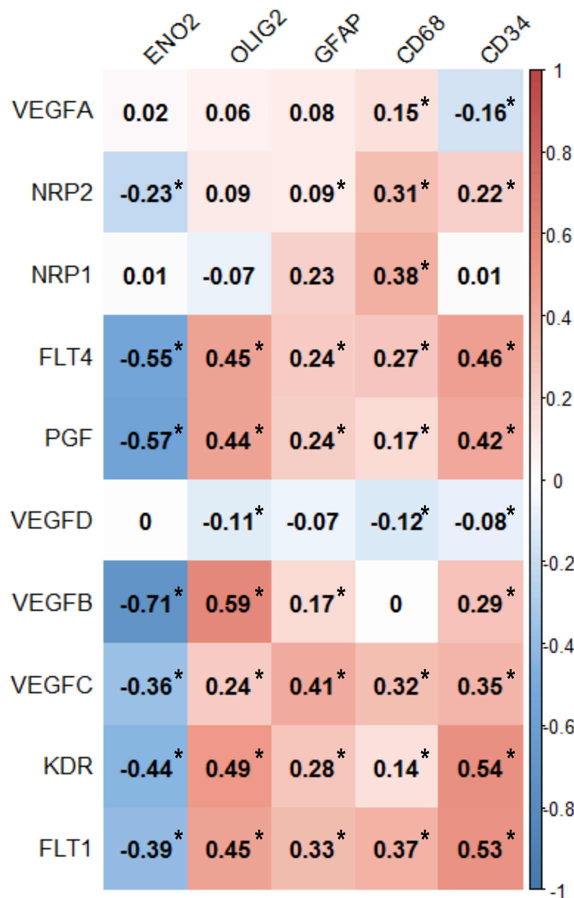


Figure 2.5. Correlation matrix for *VEGF* family gene expression and cell-type marker expression. *ENO2*, neurons; *OLIG2,* oligodendrocytes*; GFAP,* astrocytes*; CD34,* endothelial cells*; CD38,* microglia. *P<0.05, Pearson's correlation

Table 2.9. Cross-sectional *VEGF* x *APOE*-ε4 interactions on global cognition adjusted for cell-type effects Note: ENO2 was used as the marker for neurons, CD68 for microglia, OLIG2 for oligodendrocytes, GFAP for astrocytes, and CD34 for endothelial cells.

Part 1: Correction for ENO2 levels

	Cross-sectional Results			Cross-sectional Results - ENO2 Covariate			Cross-sectional Results - Adjusted for ENO2		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	-0.29	0.08	**3.58E-04**†*	-0.29	0.08	**2.59E-04**†	-0.29	0.08	**4.03E-04**†
VEGFA	-0.03	0.01	**0.005**†	-0.03	0.01	**0.003**†	-0.03	0.01	**0.005**†
FLT1	-0.06	0.03	**0.035**	-0.06	0.03	0.027	-0.07	0.03	**0.012**†
FLT4	-0.24	0.16	0.148	-0.25	0.16	0.129	-0.43	0.20	**0.028**
VEGFB	0.01	0.004	0.152	0.01	0.00	0.168	0.01	0.01	0.115
KDR	0.29	0.26	0.259	0.29	0.25	0.249	0.31	0.28	0.274
VEGFD	0.24	0.31	0.433	0.28	0.30	0.360	0.23	0.30	0.457
PGF	-0.02	0.06	0.707	-0.03	0.06	0.653	-0.07	0.08	0.335
VEGFC	-0.14	0.39	0.728	-0.13	0.39	0.728	-0.21	0.42	0.608
NRP2	0.02	0.15	0.883	0.05	0.15	0.755	0.02	0.16	0.910

Boldface signifies P≤0.05. †denotes results that were significant after adjusting for all 10 family members on outcome.

Table 2.9 *Part 2: Adjusting for expression of other cell-type markers*

	Cross-sectional Results			Cross-sectional Results - OLIG2, GFAP, CD68, and CD34 Covariates			Cross-sectional Results - Adjusted for OLIG2			Cross-sectional Results - Adjusted for GFAP		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	-0.29	0.08	**3.58E-04†**	-0.30	0.08	**3.09E-04†**	-0.29	0.08	**3.81E-04†**	-0.30	0.09	**0.001†**
VEGFA	-0.03	0.01	**0.005†**	-0.03	0.01	**0.006†**	-0.03	0.01	**0.005†**	-0.02	0.01	**0.026**
FLT1	-0.06	0.03	**0.035**	-0.07	0.03	0.017	-0.08	0.03	**0.011†**	-0.04	0.03	0.139
FLT4	-0.24	0.16	0.148	-0.22	0.17	0.214	-0.29	0.18	0.110	-0.14	0.17	0.405
VEGFB	0.01	0.004	0.152	0.01	0.00	0.199	0.01	0.00	0.120	0.01	0.00	0.157
KDR	0.29	0.26	0.259	0.19	0.27	0.480	0.34	0.28	0.235	0.39	0.26	0.134
VEGFD	0.24	0.31	0.433	0.15	0.31	0.634	0.25	0.30	0.419	0.22	0.31	0.478
PGF	-0.02	0.06	0.707	-0.04	0.07	0.510	-0.04	0.07	0.525	0.01	0.06	0.818
VEGFC	-0.14	0.39	0.728	-0.13	0.42	0.762	-0.13	0.40	0.749	0.24	0.41	0.555
NRP2	0.02	0.15	0.883	0.01	0.16	0.974	0.02	0.15	0.873	0.04	0.16	0.799

Table 2.9 *Part 2 continued.*

	Cross-sectional Results - Adjusted for CD68			Cross-sectional Results - Adjusted for CD34		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	-0.25	0.08	**0.003†**	-0.29	0.08	**3.45E-04†**
VEGFA	-0.02	0.01	**0.028**	-0.03	0.01	**0.002†**
FLT1	-0.04	0.03	0.104	-0.06	0.03	0.055
FLT4	-0.13	0.17	0.444	-0.27	0.19	0.170
VEGFB	0.01	0.00	0.121	0.01	0.00	0.088
KDR	0.32	0.26	0.220	0.45	0.29	0.116
VEGFD	0.18	0.31	0.562	0.22	0.31	0.476
PGF	0.00	0.06	0.997	-0.02	0.07	0.815
VEGFC	0.08	0.41	0.837	0.10	0.43	0.811
NRP2	0.15	0.16	0.350	0.03	0.15	0.844

Boldface signifies $P \leq 0.05$. †denotes results that were significant after adjusting for all 10 family members on outcome.

Table 2.10. Longitudinal *VEGF* x *APOE*-ε4 interactions on global cognition adjusted for cell-type effects
Note: ENO2 was used as the marker for neurons, CD68 for microglia, OLIG2 for oligodendrocytes, GFAP for astrocytes, and CD34 for endothelial cells.

Part 1: Adjusting for ENO2 expression

	Longitudinal Results			Longitudinal Results - *ENO2 Covariate*			Longitudinal Results - Adjusted for *ENO2*		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	-0.02	0.01	0.084	-0.02	0.01	0.084	-0.01	0.01	0.112
KDR	0.04	0.03	0.120	0.04	0.03	0.119	0.04	0.03	0.239
VEGFB	0.001	4.16E-04	0.155	0.001	4.16E-04	0.154	4.01E-04	0.001	0.512
VEGFA	-0.002	0.001	0.176	-0.002	0.001	0.183	-0.002	0.001	0.200
VEGFD	0.04	0.03	0.285	0.04	0.03	0.281	0.03	0.03	0.340
NRP2	0.01	0.02	0.492	0.01	0.02	0.501	0.01	0.02	0.654
VEGFC	0.02	0.04	0.608	0.02	0.04	0.601	0.001	0.05	0.981
FLT4	-0.01	0.02	0.723	-0.01	0.02	0.719	-0.03	0.02	0.166
FLT1	-4.65E-04	0.003	0.874	-4.33E-04	0.003	0.882	-0.002	0.003	0.505
PGF	0.001	0.01	0.918	0.001	0.01	0.930	-0.01	0.01	0.332

Table 2.10 *Part 2: Adjusting for expression of other cell-type markers*

	Longitudinal Results			Longitudinal Results - *OLIG2, GFAP, CD68, and CD34 Covariates*			Longitudinal Results - Adjusted for *OLIG2*		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	-0.02	0.01	0.084	-0.02	0.01	0.092	-0.01	0.01	0.096
KDR	0.04	0.03	0.120	0.04	0.03	0.129	0.05	0.03	0.111
VEGFB	0.001	4.16E-04	0.155	0.001	4.40E-04	0.199	0.001	0.001	0.160
VEGFA	-0.002	0.001	0.176	-0.001	0.001	0.297	-0.002	0.001	0.196
VEGFD	0.04	0.03	0.285	0.03	0.03	0.318	0.04	0.03	0.279
NRP2	0.01	0.02	0.492	0.01	0.02	0.459	0.01	0.02	0.473
VEGFC	0.02	0.04	0.608	0.03	0.05	0.509	0.02	0.05	0.630
FLT4	-0.01	0.02	0.723	-0.001	0.02	0.941	-0.01	0.02	0.635
FLT1	-4.65E-04	0.003	0.874	-0.001	0.003	0.795	-0.002	0.003	0.575
PGF	0.001	0.01	0.918	-0.001	0.01	0.931	-0.002	0.01	0.789

Table 2.10 *Part 2 continued.*

	Longitudinal Results - Adjusted for *GFAP*			Longitudinal Results - Adjusted for *CD68*			Longitudinal Results - Adjusted for *CD34*		
Gene	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1	-0.02	0.01	0.123	-0.01	0.01	0.190	-0.01	0.01	0.094
KDR	0.05	0.03	0.076	0.04	0.03	0.119	0.05	0.03	0.090
VEGFB	0.001	4.23E-04	0.188	0.001	4.19E-04	0.138	0.001	4.39E-04	0.128
VEGFA	-0.001	0.001	0.317	-0.001	0.001	0.305	-0.002	0.001	0.147
VEGFD	0.04	0.03	0.281	0.04	0.03	0.277	0.03	0.03	0.309
NRP2	0.01	0.02	0.432	0.02	0.02	0.198	0.01	0.02	0.500
VEGFC	0.05	0.05	0.245	0.04	0.05	0.377	0.04	0.05	0.381
FLT4	-0.001	0.02	0.947	-0.001	0.02	0.974	-0.01	0.02	0.756
FLT1	0.001	0.003	0.796	6.93E-05	0.003	0.982	-2.60E-04	0.003	0.938
PGF	0.003	0.01	0.694	0.002	0.01	0.752	0.001	0.01	0.936

Replication Results of *VEGF* Family Gene Expression

We assessed NRP1 x APOE-ε4 interactions on AD diagnosis because this was the only significant interaction that survived multiple comparisons correction. APOE-ε4 non-carriers in the Mount Sinai dataset showed higher NRP1 expression in the frontal pole of AD participants compared to controls (β=-3.95, p=0.02), which was consistent with the ROS/MAP results in pre-frontal cortex (**Table 2.11**), however the interaction was not statistically significant (p=0.06). We observed the opposite direction of effect in the parahippocampal gyrus in the same dataset, where ε4 non-carriers displayed lower NRP1 expression in AD cases compared to controls. No significant NRP1 or VEGFA x APOE-ε4 interaction results were found in the Mayo dataset (data not shown).

Table 2.11. Replication results for *NRP1* x APOE-ε4 interaction on AD diagnosis and APOE-ε4 stratified results in the Mount Sinai dataset.

Model	Tissue	β	SE	DF	P
Interaction	FP	4.47	2.46	76	0.069
Interaction	PHG	-3.12	2.30	50	0.175
Interaction	IFG	3.05	3.11	54	0.327
Interaction	STG	-0.75	1.91	66	0.694
Non-Carriers	**FP**	**-3.95**	**1.69**	**51**	**0.020**
Non-Carriers	**PHG**	**4.45**	**2.01**	**37**	**0.027**
Non-Carriers	IFG	-1.31	1.33	40	0.325
Non-Carriers	STG	0.08	1.04	44	0.938
Carriers	PHG	0.62	1.36	11	0.647
Carriers	STG	-0.58	1.81	20	0.750
Carriers	FP	0.48	1.93	23	0.805
Carriers	IFG	0.68	2.93	12	0.818

Boldface indicates P<0.05. FP=frontal pole, PHG=parahippocampal gyrus, IFG=inferior frontal gyrus, STG=superior temporal gyrus.

VEGF Receptor Protein Expression Analyses

Cognitive Outcomes, VEGF Receptor Protein Expression

NRP1 protein expression significantly interacted with *APOE*-ε4 on global cognitive trajectory (**Table 2.12, Figure 2.6**), in the same way that *NRP1* gene expression interacted with *APOE*-ε4 on global cognition. *APOE*-ε4 stratified results indicated that this interaction was driven by non-carriers, such that higher NRP1 expression in this population was associated with worse cognitive trajectories. Interestingly, NRP1 x *APOE*-ε4 on global cognitive trajectory was primarily driven by episodic memory performance (data not shown). Protein expression of VEGF receptors did not interact with *APOE*-ε4 on cross-sectional global cognition (**Table 2.12**). Additionally, FLT1 protein expression was negatively associated with cognitive trajectory and cross-sectional cognition (data not shown, longitudinal β=-0.07, p=0.006; cross-sectional β=-0.95, p=0.001), which recapitulates prior observations at the mRNA expression level previously published by our group.[126]

Clinical Diagnosis, VEGF Receptor Protein Expression

NRP1 expression interacted with *APOE*-ε4 allele status on clinical diagnosis, however stratified analyses did not show a significant association with AD diagnosis in *APOE*-ε4 stratified populations (**Table 2.12**).

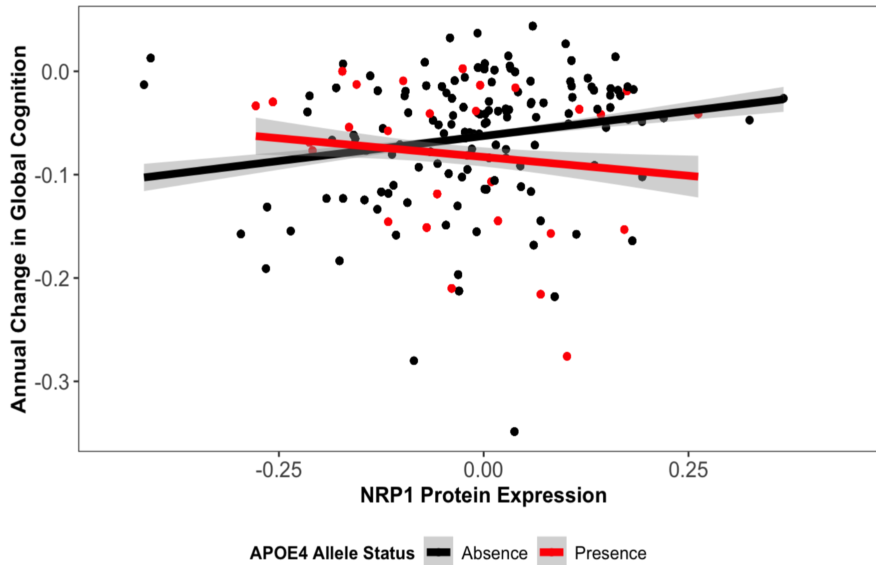


Figure 2.6. NRP1 protein expression associations with global cognitive trajectory, stratified by *APOE*-ε4 allele status. Overall interaction: NRP1 *x APOE*-ε4, *β*=-0.31, p=0.01; *APOE*-ε4 carriers, *β*=-0.18, p=0.1; *APOE*-ε4 non-carriers, *β*=0.14, p=0.01.

AD-related neuropathology, VEGF Receptor Protein Expression

KDR and FLT4 receptor expression interacted with *APOE*-ε4 allele status on tau tangle density at autopsy (KDR and FLT4, *β*=2.9, p=0.01, **Table 2.12**). No significant interactions between VEGF receptor proteins and *APOE*-ε4 allele status were found on amyloid burden, neuritic plaque, or neurofibrillary tangle pathology (**Table 2.12**). Finally, FLT1 protein expression was positively associated with amyloid pathology at autopsy (data not shown, *β*=2.2, p=4E-10), recapitulating previously published results at the mRNA level.[126]

Table 2.12. VEGF protein x *APOE-ε4* interaction on main outcomes and *APOE-ε4* stratified results

		Interaction			*APOE*-ε4 Carriers			*APOE*-ε4 Non-Carriers		
Outcome	**Protein**	**β**	**SE**	**P**	**β**	**SE**	**P**	**β**	**SE**	**P**
Longitudinal cognition	**NRP1**	-0.31	0.13	**0.015**	-0.18	0.13	0.173	0.14	0.06	**0.014**
	FLT4	-0.10	0.11	0.367	-0.17	0.12	0.162	-0.04	0.03	0.249
	KDR	-0.10	0.11	0.367	-0.17	0.12	0.162	-0.04	0.03	0.249
	FLT1	0.00	0.06	0.934	-0.08	0.05	0.142	-0.07	0.03	0.036
	NRP2	0.00	0.14	0.998	-0.01	0.14	0.957	0.00	0.06	0.979
Cross-sectional cognition	NRP1	-2.51	1.39	0.074	-1.43	1.36	0.306	1.06	0.63	0.092
	FLT4	-0.53	1.14	0.643	-1.81	1.31	0.180	-0.38	0.37	0.313
	KDR	-0.53	1.14	0.643	-1.81	1.31	0.180	-0.38	0.37	0.313
	FLT1	-0.50	0.62	0.417	-1.17	0.50	0.027	-0.67	0.37	0.068
	NRP2	0.51	1.30	0.696	0.20	1.36	0.886	-0.15	0.57	0.796
Diagnosis	NRP1	14.13	6.84	**0.039**	19.90	12.79	0.120	-3.15	1.99	0.113
	FLT4	6.77	3.77	0.072	6.09	4.53	0.179	-0.64	1.11	0.562
	KDR	6.77	3.77	0.072	6.09	4.53	0.179	-0.64	1.11	0.562
	FLT1	-0.17	1.91	0.928	2.35	2.14	0.272	1.62	1.15	0.161
	NRP2	-2.04	3.51	0.562	1.77	4.10	0.665	1.74	1.79	0.331
Tangles	NRP1	1.33	1.40	0.343	0.65	1.53	0.676	-0.76	0.60	0.202
	FLT4	2.89	1.11	**0.010**	2.54	1.40	0.083	0.01	0.35	0.984
	KDR	2.89	1.11	**0.010**	2.54	1.40	0.083	0.01	0.35	0.984
	FLT1	1.13	0.59	0.058	1.75	0.48	0.002	0.67	0.34	0.051
	NRP2	-2.23	1.29	0.086	-1.39	1.47	0.354	0.05	0.55	0.920
NFT	NRP1	0.32	0.50	0.530	0.14	0.63	0.825	-0.23	0.21	0.277
	FLT4	0.59	0.40	0.139	0.50	0.61	0.421	0.10	0.12	0.412
	KDR	0.59	0.40	0.139	0.50	0.61	0.421	0.10	0.12	0.412
	FLT1	-0.09	0.21	0.684	0.34	0.24	0.168	0.45	0.11	**1.02E-04**
	NRP2	-0.83	0.46	0.076	-0.56	0.60	0.365	0.06	0.19	0.771
Amyloid	NRP1	0.23	1.71	0.894	-0.23	1.79	0.899	-0.60	0.75	0.425
	FLT4	0.77	1.39	0.583	1.40	1.72	0.426	0.52	0.45	0.250
	KDR	0.77	1.39	0.583	1.40	1.72	0.426	0.52	0.45	0.250
	FLT1	-0.23	0.71	0.743	1.61	0.63	0.017	1.92	0.41	0.000
	NRP2	0.35	1.60	0.825	0.69	1.74	0.698	-0.37	0.69	0.590
Neuritic Plaques	NRP1	0.58	0.70	0.409	0.25	0.60	0.674	-0.43	0.31	0.170
	FLT4	0.84	0.58	0.148	0.60	0.57	0.306	0.19	0.19	0.325
	KDR	0.84	0.58	0.148	0.60	0.57	0.306	0.19	0.19	0.325
	FLT1	-0.20	0.30	0.517	0.49	0.21	0.032	0.74	0.18	0.000
	NRP2	0.05	0.67	0.944	0.52	0.57	0.378	-0.14	0.29	0.642

Discussion

We set out to determine how differences in *VEGF* gene family and protein expression might interact with one of the strongest genetic risk factors for sporadic AD, *APOE*-ε4 status, to predict age-related cognitive decline and clinical AD diagnosis. At the gene expression level, *NRP1* and *VEGFA* interacted with *APOE*-ε4 to modify the association between ε4 and the final global cognition score. Interestingly, effects of *NRP1* expression on cognition in ε4 carriers compared to non-carriers was the opposite of expectation, such that higher expression of *NRP1* was associated with worse outcomes in carriers and better outcomes in non-carriers. *VEGF* x *APOE*-ε4 interactions were not observed on AD pathology, suggesting these gene expression interactions were not driven by neuropathological changes. Further, no significant *VEGF* x *APOE*-ε4 interactions on pathological outcomes such as CAA suggest that amyloid build-up in vasculature does not drive the associations we observe on cognition. At the protein level, although we were only able to analyze expression of VEGF family receptors, we observed a significant interaction between NRP1 expression and *APOE*-ε4 on cognitive trajectory, such that higher expression in ε4 non-carriers was associated with better cognitive trajectory, consistent with gene expression effects.

At steady state, mRNA levels are typically reflective of protein levels,[138] however steady state is lost over the course of disease as we observe changes in long-term cellular processes.[139] This point makes interpretation of altered mRNA concentration and extrapolation to the protein level challenging in elderly individuals at risk of dementia. A strength of this study was the integration of multi-omic data to determine

how both mRNA and protein concentrations may affect participant outcomes. We observed a modifying effect of NRP1 protein expression on cognitive trajectory, such that higher expression was associated with better cognitive and diagnostic outcomes in *APOE*-ε4 non-carriers. Our results indicated that NRP1 protein expression mirrors the differential ε4 associations on cross-sectional cognition at the mRNA level. Together mRNA and protein results could represent temporal differences such that over the course of cognitive aging NRP1 is first upregulated at the protein level to effect cognition, then *NRP1* upregulation at the mRNA level occurs later. This temporal ordering could reflect cellular resource allocation, where a cell under age-related stressors initially decreases protein degradation as a strategy to increase the abundance of protective proteins before using a larger portion of cellular resources such as ATP and amino acids to increase the translation of protective proteins. It is notable that NRP1 protein-level interaction with *APOE*-ε4 was driven by the domain of episodic memory, while gene-level results suggested the interaction was driven by working and semantic memory, as well as perceptual orientation. Episodic memory is typically the first cognitive domain to decline in pathologic and normal aging,[140, 141] which temporally fits with protein upregulation that may begin earlier in the disease process for faster cellular compensation in response to early stressors.

The *VEGF* genes that modified the association between *APOE*-ε4 and cross-sectional cognition (*NRP1* and *VEGFA*) are positive modulators of angiogenic signaling, and NRP1 is a key angiogenic regulator at the protein level.[71, 142-144] VEGFA binds NRP1, which forms a complex with KDR on endothelial cells to initiate intracellular signaling associated with the proliferation, migration, and survival of endothelial cells.[142,

[143, 145] Cerebrovascular deficits are an early feature of AD and cognitive decline with aging, and cerebrovascular ischemic disease has been found to contribute to the severity of cognitive decline.[146, 147] The protective effects associated with high expression of angiogenesis relevant genes in ε4 non-carriers could reflect a mechanism to prevent ischemia and downstream neurodegeneration. However, angiogenic mechanisms may become damaging in the presence of the ε4 allele due to an over production of new vessels that are especially prone to leaking, as *APOE*-ε4 has been associated with increased blood-brain barrier leakiness which drives downstream cognitive decline.[56, 58, 147] It is also possible that an increase in *NRP1* expression in ε4 carriers causes an over-permeabilization of existing vessels as VEGF signaling is closely tied to vascular permeability.[148] In opposition to our original hypothesis for this study, results suggest that VEGF signaling may be beneficial in *APOE*-ε4 non-carriers but detrimental in carriers, and it seems most plausible that this effect is mediated through angiogenic or endothelial cell remodeling processes.

It is interesting to note that we did not observe significant *VEGF* x *APOE*-ε4 interactions on neuropathology. The protein level interactions between *APOE*-ε4 x KDR and FLT4 receptors are difficult to interpret because these proteins show such high homology[149] that this mass spectrometry technique was not sufficiently able to distinguish between them. However, beta-amyloid peptides have been shown to antagonize KDR,[150] suggesting amyloid accumulation could represent a potential modulator of KDR protein expression. Additionally, the build-up of amyloid plaques has been hypothesized to trap free VEGFA and contribute to an up-regulation in protein expression,[71] but our data do not suggest that the interactions between any *VEGF*

family genes and *APOE* at the gene expression level are driven primarily by alterations in amyloid, tau, or any of the other measured neuropathologies. It is notable that, as reported in earlier work from our group,[126] there are main effects of *VEGF* family genes on AD neuropathology, but these associations do not differ by ε4 status. Thus, it is likely that the *APOE*-specific vulnerability is due either to a process downstream of neuropathology, such as a unique vulnerability to repair processes highlighted above, or a process that is entirely independent of measured neuropathology. It is also possible that differences in *VEGF* expression could influence subclinical brain alterations that may not be overtly detectable upon post-mortem observation but could manifest differentially between ε4-carriers and non-carriers. Future studies which incorporate markers of angiogenesis and vascular health may help elucidate underlying brain or vascular changes which may be influenced by VEGF gene and protein expression.

While significant gene-level, cross-sectional cognition interaction results did not survive genome-wide correction for all 28,612 genes measured in the DLPFC, results of this study contribute insight for the main effects of the *VEGF* family on global cognition in this cohort.[126] Main effects results compared with *APOE*-ε4 interaction results showed that *VEGFA* and *NRP1* expression are not associated with global cognition unless the *APOE*-ε4 allele is taken into account. This is particularly interesting given the literature that connects VEGFA to cognition without consideration of *APOE*-ε4. It is notable that our observed results appear to be counter to the protective effects of VEGFA that have been reported in humanized *APOE*-ε4 mouse models.[94] It is possible that the association between high *VEGF* expression and worse cognitive trajectories in ε4 carriers is reflective of upregulation by inflammatory cytokines,[151-153] which are also

associated with AD progression,[154, 155] and that the observed *VEGF* expression effects could be a consequence of AD-related inflammation. The reparative role of angiogenesis in other conditions, such as cerebral ischemia and stroke, has also been well characterized,[156, 157] and it is possible that the upregulation in *VEGF* expression is a compensatory mechanism that fails to rescue cognitive decline. It could also be the case that transcript levels are not reflective of protein VEGFA levels. Future proteomic analyses that can capture the ligands in this family will help to shed light on the underlying expression differences we observed.

Due to the heterogeneity of cell types in brain homogenates, we considered gene expression models that covaried or residualized for expression of a neuronal-specific marker (*ENO2*) as well as cell-type markers for astrocytes (*GFAP*), microglia (*CD68*), endothelial cells (*CD34*), and oligodendrocytes (*OLIG2*). Results were not significantly altered by adjusting for these cell-type markers. Additionally, the potential for brain region heterogeneity is also reflected by the Mt. Sinai replication results, where an opposite interaction effect was observed between the frontal pole and parahippocampal gyrus. It is notable that the frontal pole results recapitulated our findings from prefrontal cortex.

Another interesting result was the lack of interaction between *APOE* expression and *VEGF* expression. Previous literature has debated the influence of genotype on *APOE* expression[55, 158, 159] and the significance of *APOE* expression in AD.[160, 161] Our study suggests that the interactions between *VEGF* genes and *APOE*-ε4 on cognition are driven by genotype-specific effects of *APOE* rather than brain *APOE* expression levels.

Several factors of this study limit generalizability, including the high level of participant education, lack of racial diversity and use of brain homogenate data which limits cell-type specific conclusions. An additional consideration is the inability to discern causal relationships given that the expression levels likely reflect a combination of cause and consequence of disease. It is also important to note the preliminary nature of the findings reported in this study, as the global cognition findings have not been replicated in another dataset. As no comparable data sets exist in the public domain, replication of this study remains a future goal.

However, this analysis also possesses several strengths, including the rich longitudinal cognitive data, measurable expression of all genes in the *VEGF* family in brain tissue, ample neuropathological data, and the comprehensive clinical characterization of the cohort. Future work should replicate these findings in other cohorts and investigate the underlying biological mechanisms driving these interactions on cognition through detailed proteomic and angiogenesis pathway analyses.

In summary, we found that *NRP1* and *VEGFA* interacted with *APOE*-ε4 on cognition and the NRP1 x *APOE*-ε4 interaction was replicated at the protein level on cognitive trajectory. Higher expression of *NRP1* was associated with beneficial outcomes in ε4 non-carriers and cognitive decline in ε4 carriers. These results suggest that angiogenic signaling may have different effects based upon an individual's *APOE*-ε4 status. Further investigation of the biological interaction between the *VEGF* family, especially components relevant to angiogenesis, and *APOE* genotype, as well as replication of cognitive associations in an independent data set, is warranted to better understand how these genes and proteins impact cognitive outcomes in older adults.

CHAPTER 3

EVALUATION OF *VEGF* ISOFORM-SPECIFIC MODIFICATION OF *APOE*-ε4 RELATED OUTCOMES

Portions of this chapter are published under the title, "*APOE* ε4-specific Associations of *VEGF* Gene Family Expression with Cognitive Aging and Alzheimer's Disease" in *Neurobiology of Aging*

Introduction

Several genes within the *VEGF* family are alternatively spliced to encode proteins with drastically different functions. For example, VEGFA is a key regulator of blood vessel growth that is alternatively spliced into pro- or anti-angiogenic isoforms that show opposing actions on endothelial cell proliferation, migration and permeability.[69, 162-165] Isoform specific studies of VEGFA have also shown that smaller isoforms, such as $VEGFA_{121}$ are important for vessel elongation while larger isoforms such as $VEGFA_{189}$ play an important role in regulating vessel branching.[71] The most abundant isoform, $VEGFA_{165}$ shows highest activation of kinases downstream of the KDR receptor, including ERK1/2 and Akt which regulate endothelial cell proliferation and permeability, respectively. While $VEGFA_{121}$ stimulation promotes cellular permeability, $VEGFA_{145}$ does not induce this functional outcome.[166] Further, $VEGFA_{165}$ binding to KDR promotes vascular sprouting while $VEGFA_{121}$ and $VEGFA_{145}$ do not. VEGFA isoforms also differentially induce intracellular phosphorylation of the KDR receptor and show different capacities to form a complex with NRP1 and KDR. For example, $VEGFA_{165}$ and $VEGFA_{121}$ can both bind to the co-receptor NRP1 *in vitro*, but the affinity of $VEGFA_{165}$

for NRP1 is approximately 10 times higher than the affinity of $VEGFA_{121}$. Further, *in vivo* studies have suggested that $VEGFA_{121}$ does not bind strongly enough to form a complex with NRP1 and KDR.[71, 166] Finally, VEGFA exerts isoform-specific effects on KDR endocytosis, intracellular trafficking into early versus late endosomes, and receptor proteolysis, all of which affect long-term endothelial cell regulation.[166]

Additionally, *FLT-1* and *KDR* mRNA can also be spliced into either transmembrane forms that signal through intracellular RTK cascades or soluble forms which act as scavengers of free ligand.[71, 167, 168] NRP1 can exist as a full-length transmembrane protein or can be pre-mRNA processed to generate two soluble isoforms which result from the use of alternate polyadenylation signals in intron 11 or 12.[169] These soluble *NRP1* isoforms, along with the transcript encoding the full protein are all expressed in the brain and can bind $VEGFA_{165}$.[169] The function of soluble NRP1 isoforms is thought to be inhibition of $VEGFA_{165}$ binding to transmembrane NRP1, similarly to the soluble forms of RTKs in the VEGF family.[169]

The isoform specific signaling and functional diversity of the VEGF family demonstrate the need for specific transcript analyses to enable detailed biological interpretation of interaction results with *APOE*-ε4. This aim will enable us to determine which isoforms drive interactions with *APOE*-ε4 at the gene level and may also uncover new interactions with *VEGF* family isoforms and *APOE*-ε4 that may be masked in gene-level analyses.

We hypothesized that transcripts coding for pro-angiogenic isoforms of VEGFA (*VEGFA-205, -209, -222, -207, -231, -218*) would show an interaction with *APOE*-ε4 on AD diagnosis and cognitive decline, such that ε4 carriers would show better outcomes

in the presence of higher *VEGFA* expression. We expected that higher expression of the transmembrane *KDR* isoform (*KDR-201*) and protein coding transcripts of the co-receptor *NRP1* will modify *APOE*-ε4 associated outcomes, such that high expression would be associated with favorable outcomes in ε4 carriers.

Methods

For details on: Participants, Neuropsychological Composites, Genotyping, Neuropathological Measures and Sensitivity Analyses, the reader is referred to Chapter 2, Methods.

Autopsy Measures of VEGF and APOE Transcript Expression

Autopsies were performed as detailed in **Chapter 2** and have been previously described.[122, 123] RNA expression of 63 collective isoforms were measured as follows: *VEGFA* (14), *VEGFB* (3), *VEGFC* (2), *VEGFD* (2), *NRP1* (12), *NRP2* (13), *FLT1* (3), *FLT4* (8), *KDR* (1), and *PGF* (5). RNA-Seq by Expectation Maximization (RSEM) was used to assign RNA sequencing reads to isoform-specific transcripts. The algorithm used by RSEM to differentiate isoforms uses expectation maximization to find the maximum posteriori estimate of the probability that a given read is derived from a particular isoform. The probability that any single read is derived from a particular isoform is proportional to the fraction of transcripts that map to that isoform out of all transcripts in a sample, multiplied by the length of the isoform because longer isoforms are expected to correspond to more reads. The expectation maximization method also

takes into account the total number of reads in a sample with the number of reads that are potentially mapped to multiple isoforms, also known as multireads, to assess isoform abundance.[170] Isoforms of very low abundance (<10% expression in the cohort) were removed. Expression values four standard deviations from the combined sample average were classified as outliers and removed.

Statistical Analyses

Models detailed in Chapter 2 were re-run using 63 unique *VEGF* isoforms across the gene family to determine if specific isoform expression interacts with *APOE*-ε4 on the proposed outcomes (AD diagnosis, cognition and cognitive trajectory, neuropathology). Additionally, models with all isoforms of genes that were found to interact with *APOE*-ε4 in Chapter 2 were investigated as a separate set of models on cognitive outcomes. Further, isoforms of genes that interacted with *APOE*-ε4 in Chapter 2 were analyzed for differential expression between carriers and non-carriers. Finally, *VEGFA* and *NRP1* isoforms that interacted with *APOE*-ε4 on cognition were tested for interaction on pathological outcomes, including AD-related pathology, and nonAD-related pathology as detailed in Chapter 2 (amyloid burden, tangle density, neurofibrillary tangles, neuritic plaques, cerebral amyloid angiopathy, cerebral atherosclerosis, arteriolosclerosis, TDP-43, hippocampal sclerosis, and gross infarcts). AD-related pathology was treated as continuous outcomes and were assessed using linear regression models covaried for age at death, interval between final visit and death, postmortem interval, and sex. Proportional odds logistic regression models using the same covariates assessed isoform x *APOE*-ε4 on cerebral amyloid angiopathy,

cerebral atherosclerosis, arteriolosclerosis, and TDP-43 pathology. Binary logistic regression models used the same covariates detailed above and assessed isoform x *APOE*-ε4 on hippocampal sclerosis and gross infarcts. Significance for all analyses was set as α = 0.05, a priori. Models were corrected for multiple comparisons using the false discovery rate (FDR) procedure based on the number of transcripts tested for a given outcome.

Results

Several *VEGF isoform x APOE-ε4* interactions on AD diagnosis were significant before FDR correction but did not meet the threshold for significance after correction, including protein coding isoforms of *NRP1*, *VEGFB*, *NRP2,* and *PGF* (**Table 3.1**).

VEGF isoform x APOE-ε4 interactions on cross-sectional cognition also did not show any significant isoforms after FDR correction, however one isoform of *VEGFA* isoform (*VEGFA-212*) approached significance after p-value adjustment (**Table 3.2**, p.fdr=0.057). Similarly, no *VEGF isoform x APOE-ε4* interactions were found on longitudinal cognition after FDR correction (**Table 3.2**).

Table 3.1. VEGF isoform x APOE-ε4 interactions on AD diagnosis

Isoform	Protein coding	β	SE	P	P.fdr
PGF-203	Yes	-0.57	0.22	0.010	0.186
NRP1-201	Yes	1.12	0.44	0.011	0.186
VEGFB-204	No	-0.03	0.01	0.012	0.186
VEGFB-201	Yes	-0.05	0.02	0.012	0.186
NRP2-202	Yes	-0.99	0.42	0.018	0.229
FLT4-208	No	-3.87	1.93	0.045	0.416
NRP2-204	Yes	0.81	0.41	0.046	0.416
VEGFA-212	No	2.51	1.32	0.057	0.451
NRP2-214	No	-1.51	0.83	0.068	0.478
NRP2-217	No	-4.84	2.91	0.097	0.509
VEGFA-211	No	1.81	1.14	0.111	0.509
NRP1-202	Yes	6.72	4.24	0.113	0.509
NRP1-210	Yes	1.78	1.13	0.113	0.509
NRP2-212	No	-7.69	4.96	0.121	0.509
VEGFA-201	Yes	1.48	0.96	0.121	0.509
NRP2-210	No	-2.38	1.65	0.149	0.568
FLT1-202	Yes	0.35	0.24	0.153	0.568
VEGFA-215	No	0.16	0.12	0.187	0.607
VEGFA-205	Yes	0.09	0.06	0.189	0.607
VEGFD-202	No	2.81	2.19	0.200	0.607
VEGFD-001	Yes	-1.25	1.00	0.210	0.607
VEGFA-216	No	0.31	0.25	0.217	0.607
PGF-205	No	-3.24	2.68	0.226	0.607
NRP2-206	Yes	-6.05	5.10	0.236	0.607
FLT4-211	No	3.39	3.02	0.262	0.607
NRP1-205	Yes	2.29	2.04	0.263	0.607
VEGFC-201	No	-6.06	5.42	0.263	0.607
VEGFA-204	Yes	0.99	0.90	0.270	0.607
FLT4-202	Yes	-4.64	4.46	0.299	0.649
VEGFA-214	No	0.42	0.44	0.341	0.679
FLT4-205	No	-1.79	1.92	0.352	0.679
NRP2-201	Yes	-3.81	4.13	0.357	0.679
NRP1-204	Yes	-2.52	2.77	0.363	0.679
FLT4-201	Yes	-0.69	0.77	0.368	0.679
NRP1-208	Yes	10.49	11.95	0.380	0.679
VEGFA-207	Yes	5.34	6.23	0.391	0.679
NRP1-212	Yes	2.97	3.61	0.411	0.679

FLT1-201	Yes	0.08	0.10	0.419	0.679
NRP2-211	No	16.60	20.77	0.424	0.679
VEGFB-202	Yes	-0.04	0.05	0.459	0.679
NRP1-009	Yes	0.83	1.13	0.460	0.679
FLT4-203	No	20.27	27.51	0.461	0.679
VEGFA-222	Yes	0.53	0.75	0.481	0.679
VEGFA-218	Yes	3.84	5.45	0.481	0.679
FLT4-207	No	1.46	2.09	0.485	0.679
NRP1-214	Yes	-0.97	1.48	0.514	0.704
NRP1-207	Yes	0.18	0.30	0.542	0.727
FLT4-210	No	-2.76	5.00	0.581	0.762
NRP2-202	Yes	-1.42	2.74	0.605	0.778
VEGFA-206	Yes	0.27	0.57	0.637	0.796
PGF-201	Yes	0.91	2.12	0.666	0.796
NRP2-209	No	-1.48	3.45	0.668	0.796
NRP1-213	Yes	-2.38	5.76	0.680	0.796
KDR-201	Yes	-0.27	0.66	0.686	0.796
FLT1-204	Yes	0.92	2.34	0.695	0.796
NRP2-215	No	2.83	9.25	0.760	0.855
PGF-206	Yes	-0.11	0.42	0.787	0.870
VEGFC-001	Yes	-0.26	1.17	0.825	0.896
NRP2-203	Yes	-0.51	4.03	0.899	0.945
VEGFA-209	Yes	0.05	0.39	0.900	0.945
NRP1-203	Yes	1.65	19.94	0.934	0.965
VEGFA-225	Yes	0.08	1.34	0.952	0.967
PGF-207	No	6945.93	350742.25	0.984	0.984

Table 3.2. VEGF isoform x APOE-ε4 interactions on cognition

		Cross-sectional cognition				Longitudinal cognition			
Isoform	**Protein coding**	**β**	**SE**	**P**	**P.fdr**	**B**	**SE**	**P**	**P.fdr**
VEGFA-212	No	-1.24	0.37	0.001	0.057	-0.10	0.04	0.018	0.144
NRP1-201	Yes	-0.38	0.13	0.004	0.078	-0.01	0.01	0.586	0.858
FLT4-207	No	-2.01	0.70	0.004	0.078	-0.12	0.08	0.130	0.481
VEGFA-211	No	-1.02	0.36	0.005	0.078	-0.04	0.04	0.338	0.752
NRP1-202	Yes	-4.17	1.54	0.007	0.089	-0.45	0.17	0.010	0.143
VEGFA-207	Yes	-3.36	1.29	0.010	0.100	-0.42	0.14	0.002	0.143

VEGFA-205	Yes	-0.05	0.02	0.012	0.108	-1.89E-03	2.05E-03	0.358	0.752
NRP1-205	Yes	-1.73	0.72	0.017	0.108	-0.12	0.08	0.139	0.485
VEGFA-216	No	-0.19	0.08	0.017	0.108	-0.01	0.01	0.326	0.752
NRP1-208	Yes	-8.54	3.57	0.017	0.108	-0.98	0.40	0.014	0.143
VEGFB-204	No	0.01	4.39E-03	0.022	0.127	1.31E-03	4.83E-04	0.007	0.143
VEGFA-214	No	-0.34	0.15	0.027	0.137	-0.01	0.02	0.461	0.765
VEGFA-215	No	-0.07	0.03	0.031	0.137	-0.01	3.86E-03	0.089	0.376
VEGFD-001	Yes	0.80	0.37	0.033	0.137	0.10	0.04	0.011	0.143
NRP1-214	Yes	1.30	0.61	0.033	0.137	0.16	0.07	0.018	0.144
NRP1-009	Yes	-0.92	0.43	0.035	0.137	-0.12	0.05	0.013	0.143
PGF-201	Yes	-1.00	0.51	0.049	0.181	-0.13	0.06	0.044	0.280
FLT1-201	Yes	-0.06	0.03	0.052	0.183	5.36E-04	3.50E-03	0.878	0.979
FLT4-208	No	1.40	0.73	0.055	0.183	0.11	0.08	0.160	0.503
NRP1-204	Yes	1.82	0.99	0.065	0.206	0.22	0.11	0.044	0.280
VEGFA-201	Yes	-0.55	0.31	0.072	0.217	-0.07	0.03	0.055	0.287
NRP2-215	No	-5.03	2.93	0.086	0.247	-0.58	0.34	0.088	0.376
VEGFB-201	Yes	0.01	0.01	0.119	0.320	1.12E-03	8.24E-04	0.176	0.503
VEGFD-202	No	-0.89	0.58	0.122	0.320	-0.08	0.07	0.237	0.645
NRP1-210	Yes	-0.58	0.40	0.146	0.362	-0.08	0.04	0.067	0.322
FLT1-202	Yes	-0.09	0.06	0.149	0.362	0.01	0.01	0.246	0.645
PGF-206	Yes	-0.23	0.17	0.170	0.396	-0.02	0.02	0.270	0.680
VEGFA-218	Yes	-2.00	1.48	0.178	0.401	-0.32	0.16	0.050	0.286
FLT1-204	Yes	-0.81	0.71	0.254	0.553	-0.06	0.08	0.450	0.765
KDR-201	Yes	0.27	0.25	0.283	0.594	0.04	0.03	0.149	0.495
FLT4-211	No	-1.00	1.01	0.325	0.643	-0.09	0.11	0.452	0.765
FLT4-201	Yes	-0.28	0.29	0.336	0.643	0.01	0.03	0.668	0.915
NRP2-211	No	-3.36	3.50	0.337	0.643	-0.52	0.38	0.168	0.503
VEGFA-222	Yes	-0.24	0.27	0.374	0.693	-0.02	0.03	0.447	0.765
VEGFA-204	Yes	-0.23	0.29	0.437	0.767	0.01	0.03	0.782	0.940
NRP2-202	Yes	0.12	0.17	0.460	0.767	0.02	0.02	0.285	0.690
NRP1-213	Yes	1.77	2.42	0.465	0.767	0.42	0.26	0.107	0.422
NRP1-212	Yes	0.88	1.25	0.481	0.767	0.02	0.14	0.886	0.979
PGF-207	No	-0.96	1.41	0.497	0.767	-0.07	0.15	0.666	0.915
FLT4-205	No	0.46	0.69	0.504	0.767	0.07	0.08	0.389	0.752
NRP2-212	No	1.28	1.95	0.513	0.767	0.19	0.22	0.394	0.752
VEGFA-209	Yes	-0.09	0.15	0.518	0.767	-0.01	0.02	0.734	0.940
NRP1-203	Yes	3.00	4.91	0.540	0.767	-0.14	0.54	0.789	0.940
NRP2-209	No	-0.71	1.16	0.541	0.767	-0.03	0.13	0.847	0.970
VEGFA-206	Yes	0.10	0.16	0.548	0.767	4.44E-03	0.02	0.806	0.940
NRP2-210	No	0.34	0.61	0.570	0.771	0.05	0.07	0.482	0.776

FLT4-203	No	5.74	10.97	0.601	0.771	-1.33	1.53	0.384	0.752
VEGFC-201	No	1.12	2.15	0.602	0.771	0.11	0.24	0.659	0.915
NRP1-207	Yes	-0.05	0.11	0.616	0.771	-7.17E-04	0.01	0.951	0.989
VEGFB-202	Yes	0.01	0.02	0.620	0.771	7.53E-04	2.05E-03	0.713	0.940
NRP2-203	Yes	-0.75	1.52	0.625	0.771	0.12	0.17	0.493	0.776
NRP2-201	Yes	0.56	1.64	0.733	0.888	-0.01	0.18	0.963	0.989
NRP2-214	No	0.08	0.32	0.798	0.949	0.01	0.04	0.772	0.940
FLT4-202	Yes	-0.32	1.68	0.851	0.983	0.16	0.18	0.369	0.752
VEGFA-225	Yes	-0.07	0.48	0.878	0.983	7.27E-04	0.05	0.989	0.989
NRP2-204	Yes	-0.02	0.15	0.879	0.983	5.70E-04	0.02	0.973	0.989
NRP2-217	No	0.15	1.14	0.897	0.983	0.01	0.13	0.944	0.989
NRP2-003	Yes	-0.11	1.04	0.916	0.983	0.04	0.12	0.731	0.940
VEGFC-001	Yes	-0.04	0.39	0.925	0.983	0.03	0.04	0.522	0.802
PGF-205	No	-0.08	1.04	0.942	0.983	0.01	0.12	0.905	0.983
PGF-203	Yes	0.01	0.09	0.952	0.983	0.01	0.01	0.562	0.843
FLT4-210	No	-0.03	2.29	0.988	0.992	0.19	0.25	0.451	0.765
NRP2-206	Yes	-0.02	2.04	0.992	0.992	0.06	0.23	0.805	0.940

Although no *VEGF* gene-level interactions with *APOE*-ε4 were observed on AD-related neuropathology in **Chapter 2**, there was one significant *VEGFD* non-protein coding isoform-level interaction with *APOE*-ε4 on neurofibrillary tangle pathology (**Table 3.3**).

Table 3.3. VEGF isoform x APOE-ε4 interactions on AD-related pathology

		Tangles				Neurofibrillary tangles (NFTs)			
Isoform	Protein coding	Beta	SE	P	P.fdr	Beta	SE	P	P.fdr
NRP1-202	Yes	5.79	1.84	0.002	0.055	1.15	0.54	0.034	0.428
VEGFD-202	No	2.15	0.67	0.001	0.055	0.81	0.20	6.37E-05	**0.004**
NRP2-209	No	3.04	1.35	0.025	0.308	0.47	0.41	0.249	0.747
NRP2-210	No	-1.42	0.70	0.044	0.308	-0.51	0.21	0.017	0.347
NRP2-215	No	7.46	3.41	0.029	0.308	2.15	1.02	0.036	0.428
NRP1-214	Yes	-1.44	0.71	0.043	0.308	-0.44	0.21	0.041	0.428
NRP1-205	Yes	1.86	0.85	0.030	0.308	0.22	0.26	0.394	0.847
NRP1-204	Yes	-2.83	1.16	0.015	0.308	-0.58	0.35	0.094	0.562
FLT4-207	No	1.74	0.83	0.037	0.308	0.17	0.25	0.510	0.847
FLT4-201	Yes	0.66	0.34	0.050	0.317	0.04	0.10	0.673	0.969
PGF-201	Yes	1.10	0.59	0.063	0.358	0.26	0.18	0.146	0.562
VEGFA-212	No	-0.63	0.44	0.153	0.514	-0.21	0.13	0.120	0.562
VEGFA-214	No	-0.27	0.18	0.130	0.514	-0.08	0.05	0.124	0.562
VEGFA-205	Yes	-0.03	0.02	0.163	0.514	-0.01	0.01	0.184	0.611
NRP2-211	No	5.79	4.11	0.160	0.514	2.16	1.23	0.080	0.562
NRP2-201	Yes	-2.84	1.92	0.139	0.514	-0.85	0.58	0.143	0.562
NRP2-203	Yes	-2.68	1.79	0.136	0.514	-0.77	0.54	0.152	0.562
FLT4-208	No	-1.25	0.86	0.145	0.514	-0.42	0.26	0.106	0.562
PGF-206	Yes	0.31	0.20	0.122	0.514	0.11	0.06	0.063	0.562
VEGFB-204	No	0.01	0.01	0.102	0.514	1.25E-03	0.00	0.421	0.847
PGF-205	No	1.65	1.21	0.173	0.520	-0.04	0.37	0.914	0.977
VEGFA-209	Yes	-0.22	0.17	0.188	0.521	-0.06	0.05	0.272	0.779
PGF-203	Yes	0.13	0.10	0.190	0.521	-3.81E-03	0.03	0.899	0.977
FLT4-202	Yes	2.55	1.98	0.199	0.522	-0.16	0.59	0.783	0.977
VEGFA-216	No	-0.11	0.09	0.217	0.526	-0.04	0.03	0.114	0.562
NRP2-202	Yes	-0.25	0.20	0.215	0.526	-0.16	0.06	0.006	0.197
NRP1-208	Yes	5.08	4.20	0.227	0.530	-0.91	1.26	0.471	0.847
VEGFA-218	Yes	1.95	1.75	0.264	0.560	0.16	0.52	0.766	0.977
FLT4-210	No	-3.01	2.67	0.260	0.560	-0.77	0.80	0.336	0.847
KDR-201	Yes	-0.32	0.29	0.267	0.560	-0.08	0.09	0.388	0.847
NRP1-207	Yes	-0.11	0.13	0.364	0.740	-0.02	0.04	0.511	0.847
VEGFA-207	Yes	1.29	1.52	0.395	0.766	-0.06	0.46	0.890	0.977
FLT4-205	No	0.68	0.81	0.401	0.766	0.20	0.24	0.401	0.847
VEGFD-001	Yes	-0.36	0.44	0.415	0.769	-0.16	0.13	0.216	0.681
VEGFA-225	Yes	-0.44	0.57	0.438	0.788	0.00	0.17	0.993	0.996
PGF-207	No	-1.22	1.65	0.457	0.800	-0.12	0.49	0.811	0.977
FLT1-201	Yes	0.03	0.04	0.476	0.810	5.60E-05	0.01	0.996	0.996

NRP1-213	Yes	-1.93	2.84	0.497	0.825	0.57	0.85	0.505	0.847
VEGFA-206	Yes	-0.12	0.18	0.516	0.834	0.02	0.06	0.694	0.969
NRP1-212	Yes	-0.89	1.50	0.556	0.876	-0.65	0.43	0.132	0.562
VEGFA-215	No	-0.01	0.04	0.726	0.914	-0.01	0.01	0.463	0.847
VEGFA-201	Yes	-0.14	0.37	0.697	0.914	0.01	0.11	0.917	0.977
NRP2-212	No	-1.03	2.31	0.657	0.914	-0.38	0.69	0.582	0.940
NRP2-214	No	0.14	0.37	0.705	0.914	-0.03	0.11	0.781	0.977
NRP2-217	No	-0.43	1.32	0.744	0.914	-0.29	0.40	0.467	0.847
NRP2-204	Yes	0.06	0.18	0.749	0.914	0.07	0.05	0.165	0.577
NRP2-206	Yes	0.63	2.38	0.792	0.914	-0.05	0.72	0.946	0.977
NRP1-009	Yes	0.19	0.51	0.716	0.914	-0.08	0.15	0.607	0.946
NRP1-201	Yes	-0.04	0.16	0.798	0.914	-0.04	0.05	0.353	0.847
NRP1-210	Yes	0.17	0.47	0.713	0.914	-0.03	0.14	0.858	0.977
NRP1-203	Yes	1.53	5.76	0.791	0.914	-0.35	1.73	0.839	0.977
VEGFB-201	Yes	3.61E-03	0.01	0.684	0.914	-7.60E-04	2.67E-03	0.776	0.977
VEGFB-202	Yes	0.01	0.02	0.639	0.914	-4.74E-04	0.01	0.942	0.977
VEGFC-001	Yes	-0.18	0.47	0.692	0.914	-0.13	0.14	0.367	0.847
FLT1-202	Yes	0.02	0.07	0.775	0.914	0.01	0.02	0.615	0.946
VEGFA-204	Yes	-0.05	0.34	0.873	0.920	-0.07	0.10	0.473	0.847
FLT4-203	No	-2.44	14.88	0.870	0.920	-4.11	3.87	0.289	0.791
FLT4-211	No	0.19	1.18	0.876	0.920	0.17	0.35	0.638	0.958
VEGFC-201	No	0.44	2.52	0.863	0.920	-0.53	0.75	0.483	0.847
FLT1-204	Yes	-0.18	0.83	0.830	0.920	0.09	0.25	0.707	0.969
VEGFA-211	No	-0.02	0.43	0.955	0.970	0.03	0.13	0.845	0.977
NRP2-003	Yes	-0.08	1.22	0.951	0.970	-0.14	0.37	0.697	0.969
VEGFA-222	Yes	-3.92E-03	0.32	0.990	0.990	-0.02	0.10	0.797	0.977

Table 3.3 cont. VEGF isoform x APOE-ε4 interactions on AD-related pathology

		Neuritic plaques				Amyloid burden			
Isoform	**Protein coding**	**Beta**	**SE**	**P**	**P.fdr**	**Beta**	**SE**	**P**	**P.fdr**
NRP1-202	Yes	2.04	0.74	0.006	0.398	2.07	1.69	0.220	0.976
VEGFD-202	No	0.49	0.28	0.075	0.911	0.60	0.62	0.331	0.976
NRP2-209	No	0.50	0.56	0.374	0.911	0.73	1.26	0.564	0.994
NRP2-210	No	-0.61	0.29	0.037	0.786	-0.57	0.65	0.381	0.976
NRP2-215	No	0.47	1.40	0.737	0.917	5.84	3.12	0.062	0.976
NRP1-214	Yes	-0.27	0.29	0.355	0.911	-0.54	0.65	0.410	0.976
NRP1-205	Yes	0.46	0.35	0.189	0.911	0.14	0.79	0.859	0.994

NRP1-204	Yes	0.23	0.48	0.625	0.915	1.31	1.07	0.220	0.976
FLT4-207	No	0.44	0.34	0.205	0.911	0.62	0.76	0.415	0.976
FLT4-201	Yes	0.19	0.14	0.177	0.911	0.17	0.31	0.578	0.994
PGF-201	Yes	0.13	0.24	0.585	0.911	0.12	0.54	0.826	0.994
VEGFA-212	No	0.18	0.18	0.320	0.911	-0.10	0.40	0.811	0.994
VEGFA-214	No	0.05	0.07	0.532	0.911	0.12	0.17	0.485	0.985
VEGFA-205	Yes	0.01	0.01	0.522	0.911	0.00	0.02	0.915	0.994
NRP2-211	No	1.48	1.68	0.381	0.911	6.10	3.74	0.104	0.976
NRP2-201	Yes	-0.48	0.79	0.541	0.911	-0.45	1.75	0.799	0.994
NRP2-203	Yes	0.40	0.74	0.586	0.911	-0.41	1.63	0.802	0.994
FLT4-208	No	-0.41	0.35	0.243	0.911	-0.09	0.79	0.912	0.994
PGF-206	Yes	0.06	0.08	0.438	0.911	0.24	0.18	0.181	0.976
VEGFB-204	No	-2.21E-03	2.12E-03	0.298	0.911	-0.01	0.00	0.260	0.976
PGF-205	No	0.09	0.50	0.852	0.942	1.58	1.11	0.155	0.976
VEGFA-209	Yes	0.06	0.07	0.368	0.911	0.03	0.16	0.867	0.994
PGF-203	Yes	-1.79E-03	0.04	0.965	0.968	0.00	0.09	0.991	0.994
FLT4-202	Yes	0.26	0.81	0.751	0.917	1.39	1.81	0.444	0.976
VEGFA-216	No	0.05	0.04	0.158	0.911	0.05	0.08	0.584	0.994
NRP2-202	Yes	-0.17	0.08	0.030	0.786	-0.14	0.18	0.429	0.976
NRP1-208	Yes	2.40	1.72	0.164	0.911	5.73	3.83	0.135	0.976
VEGFA-218	Yes	0.10	0.72	0.893	0.953	-0.65	1.58	0.682	0.994
FLT4-210	No	-1.33	1.10	0.227	0.911	-1.57	2.44	0.520	0.993
KDR-201	Yes	-0.02	0.12	0.878	0.953	-0.23	0.26	0.383	0.976
NRP1-207	Yes	-0.04	0.05	0.429	0.911	-0.02	0.11	0.886	0.994
VEGFA-207	Yes	0.46	0.63	0.462	0.911	-0.49	1.39	0.724	0.994
FLT4-205	No	0.10	0.33	0.754	0.917	0.66	0.73	0.371	0.976
VEGFD-001	Yes	-0.08	0.18	0.658	0.917	0.01	0.40	0.984	0.994
VEGFA-225	Yes	-0.06	0.23	0.800	0.917	0.62	0.51	0.223	0.976
PGF-207	No	-0.81	0.68	0.232	0.911	-0.56	1.50	0.708	0.994
FLT1-201	Yes	4.38E-03	0.02	0.772	0.917	-0.03	0.03	0.408	0.976
NRP1-213	Yes	1.80	1.16	0.123	0.911	1.60	2.59	0.537	0.994
VEGFA-206	Yes	-0.02	0.08	0.800	0.917	0.04	0.17	0.807	0.994
NRP1-212	Yes	-0.02	0.60	0.968	0.968	-1.77	1.37	0.197	0.976
VEGFA-215	No	0.02	0.02	0.189	0.911	0.04	0.04	0.327	0.976
VEGFA-201	Yes	0.06	0.15	0.671	0.917	0.24	0.33	0.465	0.976
NRP2-212	No	-0.62	0.94	0.508	0.911	-1.55	2.10	0.461	0.976
NRP2-214	No	0.14	0.15	0.361	0.911	0.35	0.34	0.299	0.976
NRP2-217	No	-0.03	0.55	0.955	0.968	0.19	1.21	0.877	0.994
NRP2-204	Yes	0.05	0.07	0.532	0.911	-0.14	0.16	0.373	0.976
NRP2-206	Yes	0.64	0.98	0.514	0.911	1.08	2.19	0.621	0.994

NRP1-009	Yes	0.04	0.21	0.840	0.942	-0.54	0.47	0.254	0.976
NRP1-201	Yes	0.07	0.06	0.304	0.911	0.19	0.14	0.196	0.976
NRP1-210	Yes	0.20	0.19	0.308	0.911	0.46	0.43	0.286	0.976
NRP1-203	Yes	1.40	2.37	0.555	0.911	1.90	5.26	0.719	0.994
VEGFB-201	Yes	1.12E-03	3.65E-03	0.759	0.917	0.00	0.01	0.962	0.994
VEGFB-202	Yes	-2.62E-03	0.01	0.770	0.917	0.00	0.02	0.912	0.994
VEGFC-001	Yes	0.01	0.19	0.939	0.968	-0.08	0.42	0.855	0.994
FLT1-202	Yes	0.03	0.03	0.292	0.911	0.00	0.07	0.961	0.994
VEGFA-204	Yes	0.07	0.14	0.607	0.911	0.00	0.31	0.994	0.994
FLT4-203	No	-3.06	5.32	0.566	0.911	-3.14	13.62	0.818	0.994
FLT4-211	No	0.25	0.49	0.604	0.911	0.48	1.08	0.658	0.994
VEGFC-201	No	0.37	1.03	0.718	0.917	-0.87	2.30	0.704	0.994
FLT1-204	Yes	0.23	0.34	0.506	0.911	-0.51	0.76	0.503	0.990
VEGFA-211	No	0.26	0.17	0.140	0.911	0.29	0.39	0.455	0.976
NRP2-003	Yes	-0.20	0.50	0.684	0.917	1.02	1.12	0.361	0.976
VEGFA-222	Yes	-0.21	0.13	0.111	0.911	-0.39	0.29	0.187	0.976

After restriction to only *NRP1* and *VEGFA* isoforms to provide interpretation of our gene-level results reported in **Chapter 2**, select transcripts of these genes showed significant interaction with *APOE*-ε4 on cross-sectional cognition (**Table 3.4**). Transcripts of *VEGFA* (*VEGFA-205, VEGFA-207*) encoding pro-angiogenic protein species VEGFA$_{165}$ and VEGFA$_{183}$ respectively, interacted with *APOE*-ε4 on cross-sectional global cognitive performance and the direction of effect was consistent with gene-level results (**Figure 3.1**). Stratified analyses showed that *APOE*-ε4 carriers drove the interaction between *VEGFA* transcripts and ε4 allele status on cross-sectional cognition, whereby higher expression was associated with lower global cognition scores. Several protein coding transcripts of *NRP1* (*NRP1-202, NRP1-201, NRP1-208,*

NRP1-209, NRP1-205) interacted with *APOE*-ε4 on cross-sectional global cognition and stratified analyses suggested that interaction between ε4 and *NRP1-202* in particular was also driven by non-carriers. No *VEGFA* or *NRP1* isoform interactions on longitudinal cognition were found after FDR correction which aligned with gene-level interaction results, however $VEGFA_{207}$ (encoding $VEGFA_{183}$) approached significance (P.fdr=0.059, **Table 3.5**).

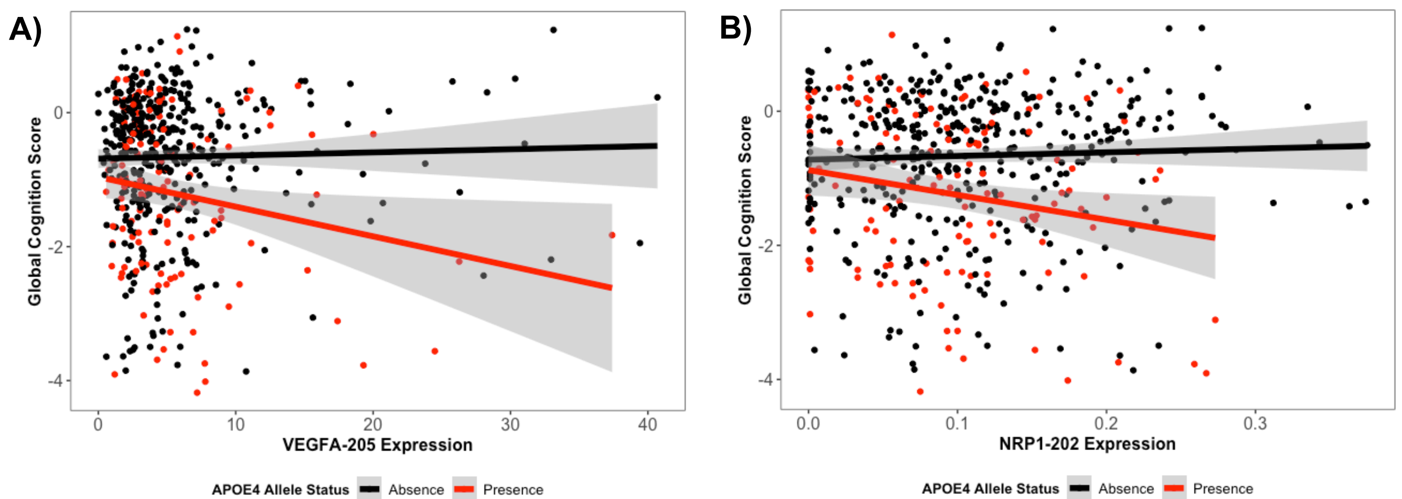


Figure 3.1. The **A)** *VEGFA-205* transcript (encoding the $VEGFA_{165}$ protein) and **B)** *NRP1-202* transcript (encoding the NRP1 canonical transmembrane protein) interacted with *APOE*-ε4 on cross-sectional global cognition and the interactions were driven by ε4 carriers.

Table 3.4. VEGFA and NRP1 isoform x APOE-ε4 interactions on cross-sectional global cognition and stratified results

	Interaction				APOE-ε4 carriers			APOE-ε4 non-carriers		
Isoform	**Protein-coding**	**β**	**SE**	**P.fdr**	**β**	**SE**	**P**	**β**	**SE**	**P**
VEGFA-212	No	-1.37	0.37	**0.007**	-1.15	0.39	**0.004**	0.24	0.16	0.131
VEGFA-211	No	-1.07	0.36	**0.028**	-0.91	0.37	**0.016**	0.17	0.17	0.320
NRP1-202	Yes	-4.42	1.53	**0.028**	-3.79	1.62	**0.021**	0.42	0.64	0.520
NRP1-201	Yes	-0.37	0.13	**0.028**	-0.25	0.14	0.087	0.14	0.05	**0.008**
VEGFA-205	Yes	-0.05	0.02	**0.028**	-0.04	0.02	**0.026**	0.01	0.01	0.418
VEGFA-216	No	-0.22	0.08	**0.030**	-0.17	0.08	**0.045**	0.05	0.04	0.185
VEGFA-207	Yes	-3.32	1.28	**0.033**	-3.57	1.40	**0.012**	0.01	0.47	0.989
NRP1-208	Yes	-9.13	3.55	**0.033**	-6.85	3.62	0.061	1.80	1.75	0.304
NRP1-209	Yes	-1.12	0.44	**0.033**	-0.93	0.48	0.056	0.19	0.16	0.249
NRP1-205	Yes	-1.79	0.72	**0.033**	-1.84	0.76	**0.016**	-0.07	0.31	0.821
VEGFA-214	No	-0.37	0.15	**0.041**	-0.26	0.16	0.108	0.11	0.07	0.114
VEGFA-215	No	-0.08	0.03	0.053	-0.06	0.04	0.099	0.02	0.01	0.225
NRP1-204	Yes	1.90	0.98	0.10	2.64	1.03	**0.012**	0.80	0.45	0.075
NRP1-214	Yes	1.17	0.61	0.10	0.76	0.67	0.259	-0.42	0.23	0.071
VEGFA-201	Yes	-0.55	0.30	0.13	-0.35	0.33	0.294	0.19	0.13	0.125
NRP1-210	Yes	-0.53	0.40	0.29	-0.21	0.43	0.628	0.40	0.17	**0.020**
VEGFA-218	Yes	-1.86	1.48	0.32	-2.28	1.66	0.172	-0.63	0.51	0.216
VEGFA-222	Yes	-0.27	0.27	0.47	-0.29	0.29	0.307	-0.07	0.12	0.564
NRP1-212	Yes	0.96	1.24	0.56	0.56	1.30	0.668	-0.50	0.56	0.381
VEGFA-204	Yes	-0.22	0.29	0.56	0.02	0.29	0.953	0.29	0.16	0.065
VEGFA-209	Yes	-0.11	0.15	0.56	-0.02	0.15	0.887	0.10	0.06	0.126
NRP1-203	Yes	3.40	4.88	0.57	1.61	5.01	0.749	-2.20	2.36	0.351
VEGFA-206	Yes	0.10	0.16	0.60	-0.001	0.16	0.997	-0.10	0.08	0.196
NRP1-207	Yes	-0.06	0.11	0.60	-0.05	0.11	0.630	0.00	0.05	0.997
VEGFA-225	Yes	-0.05	0.48	0.95	0.11	0.54	0.844	0.15	0.15	0.335
NRP1-213	Yes	-0.15	2.80	0.96	-1.61	3.01	0.595	-0.79	1.16	0.496

Boldface signifies corrected P≤0.05.
P.fdr column contains p-values corrected for 26 interaction tests using the false discovery rate (FDR)

Table 3.5. VEGFA and NRP1 isoform x APOE-ε4 interactions on longitudinal global cognition

	Interaction			
Isoform	**Protein-coding**	**β**	**SE**	**P.fdr**
VEGFA-207	Yes	-0.42	0.14	0.059
NRP1-202	Yes	-0.45	0.17	0.079
NRP1-209	Yes	-0.12	0.05	0.079
NRP1-208	Yes	-0.98	0.40	0.079
NRP1-214	Yes	0.16	0.07	0.079
VEGFA-212	No	-0.10	0.04	0.079
NRP1-204	Yes	0.22	0.11	0.158
VEGFA-218	Yes	-0.32	0.16	0.158
VEGFA-201	Yes	-0.07	0.03	0.158
NRP1-210	Yes	-0.08	0.04	0.173
VEGFA-215	No	-0.01	0.00	0.211
NRP1-213	Yes	0.42	0.26	0.232
NRP1-205	Yes	-0.12	0.08	0.277
VEGFA-216	No	-0.01	0.01	0.582
VEGFA-211	No	-0.04	0.04	0.582
VEGFA-205	Yes	0.00	0.00	0.582
VEGFA-222	Yes	-0.02	0.03	0.666
VEGFA-214	No	-0.01	0.02	0.666
NRP1-201	Yes	-0.01	0.01	0.801
VEGFA-209	Yes	-0.01	0.02	0.911
VEGFA-204	Yes	0.01	0.03	0.911
NRP1-203	Yes	-0.14	0.54	0.911
VEGFA-206	Yes	0.00	0.02	0.911
NRP1-212	Yes	0.02	0.14	0.960
NRP1-207	Yes	0.00	0.01	0.989
VEGFA-225	Yes	0.00	0.05	0.989

Boldface signifies P.fdr≤0.05.
P.fdr column shows p-values corrected for 26 interaction tests using the false discovery rate (FDR)

VEGFA and *NRP1* isoforms were not differentially expressed by *APOE*-ε4 allele status (data not shown, p>0.06). *VEGFA* and *NRP1* isoforms which interacted with *APOE*-ε4 on cognition were assessed for interactions with *APOE*-ε4 on pathology, and results showed that *NRP1-208* interacted on TDP-43 pathology (**Table 3.6**). This was the only significant interaction on neuropathology after correction for multiple comparisons for all eleven isoforms tested.

Table 3.6. Targeted VEGFA and NRP1 isoform x APOE-ε4 interactions on TDP-43 pathology and stratified results

	Interaction				APOE-ε4 carriers			APOE-ε4 non-carriers		
Isoform	**Protein-coding**	**β**	**SE**	**P.fdr**	**β**	**SE**	**P**	**β**	**SE**	**P**
NRP1-208	Yes	24.46	7.36	**0.010**	11.40	5.96	0.056	-11.89	4.54	**0.009**
NRP1-209	Yes	2.09	0.90	0.111	1.79	0.83	**0.031**	-0.19	0.36	0.602
VEGFA-207	Yes	5.66	2.75	0.145	4.82	2.54	0.057	-1.53	1.27	0.228
VEGFA-205	Yes	-0.04	0.04	0.431	-0.04	0.03	0.214	-1.0E-03	0.02	0.956
VEGFA-216	No	-0.20	0.16	0.431	-0.14	0.14	0.299	0.07	0.08	0.413
NRP1-202	Yes	3.78	3.35	0.431	4.02	3.07	0.190	1.27	1.44	0.379
NRP1-201	Yes	-0.33	0.29	0.431	-0.24	0.27	0.364	0.05	0.12	0.635
VEGFA-214	No	-0.23	0.33	0.657	-0.25	0.29	0.379	-0.03	0.15	0.843
VEGFA-211	No	-0.37	0.72	0.747	-0.46	0.64	0.472	-0.06	0.34	0.858
VEGFA-212	No	-0.24	0.77	0.760	-0.05	0.70	0.938	0.26	0.34	0.436
NRP1-205	Yes	0.52	1.48	0.760	0.70	1.30	0.593	0.45	0.67	0.504

Boldface signifies P.fdr≤0.05.
P.fdr column shows p-values corrected for 11 interaction tests using the false discovery rate (FDR)

Discussion

To determine which VEGF isoform-specific interactions with *APOE*-ε4 on cognition were driving significant gene-level results, we leveraged isoform specific data from the ROS/MAP cohort. Using data for all 63 available isoforms, we did not find any protein-coding isoforms that interacted with *APOE*-ε4 on AD diagnosis, cross-sectional or longitudinal cognition. One non-protein coding transcript of VEGFD (*VEGFD-202*) was significant for interaction with *APOE*-ε4 on neurofibrillary tangle pathology after FDR correction. This result is difficult to interpret because the small, 600 base-pair transcript does not have an open reading frame and has no known regulatory effects on other family members.

The targeted isoform subset analysis which considered only VEGF isoforms of the genes that interacted with *APOE*-ε4 on cross-sectional cognition showed that isoforms of *VEGFA* that have been characterized as pro-angiogenic interacted with *APOE*-ε4 on cross-sectional cognition. *VEGFA* gene-level results appear to be driven by transcripts that encode $VEGFA_{165}$ and $VEGFA_{183}$ proteins, both of which have associated with tumor development and progression associated with pathologic angiogenesis.[171, 172] $VEGFA_{165}$ was the first VEGFA isoform to be characterized and is a secreted protein that acts as the predominant angiogenic factor of the family. $VEGFA_{165}$ is expressed by endothelial cells, neurons and astrocytes and upregulates expression of anti-apoptotic proteins such as bcl-2.[173] Interestingly, inhibition of $VEGFA_{165}$ by an RNA oligonucleotide aptamer appears to reverse BBB breakdown associated with diabetic retinopathy,[172] and BBB breakdown in *APOE*-ε4 carriers has been shown to contribute

to cognitive decline,[56] providing further evidence that VEGF modulation could show *APOE*-ε4 specific effects. In contrast, increased $VEGFA_{165}$ expression has also been shown to protect neurons from ischemia.[174] Physiologic versus pathogenic effects of $VEGFA_{165}$ expression appear to vary by tissue environment and cell type, making its biological effects highly contextual.

$VEGFA_{183}$ is similar to $VEGFA_{189}$ as $VEGFA_{183}$ is only six amino acids shorter due to alternative splicing of exon 6A and both proteins can bind NRP1. $VEGFA_{183}$ is expressed by endothelial and glial cells, and is secreted at low levels while the majority of protein remains membrane-bound.[172] This protein species shows a decrease in expression with increasing age in rabbit tissue, and shows a longer delay in expression upregulation after exposure to hypoxia (~24-hours) compared to $VEGFA_{165}$ (~8 hours).[172] This difference in temporal upregulation is especially interesting given the trend towards significance for an interaction between VEGFA_{207} (encoding $VEGFA_{183}$) and *APOE*-ε4 on longitudinal cognition. If $VEGFA_{183}$ expression is a longer-term response to or driver of damage, we may expect to see VEGFA_{207} driving an interaction with *APOE*-ε4 on longitudinal outcomes.

NRP1 plays a key role in the regulation of angiogenesis, similarly to VEGFA. Several soluble isoforms are thought to serve as negative modulators of this process, while transmembrane isoforms increase angiogenic signaling through modulation of VEGF RTKs.[169] Results from this study showed that the NRP1 isoform *NRP1-201*, which encodes the full-length transmembrane protein, interacted with *APOE*-ε4 on cognition. Stratified analyses in *APOE*-ε4 non-carriers showed a positive association between *NRP1-201* and global cognitive performance, such that higher expression was

associated with better performance. This finding is consistent with *APOE*-ε4 interaction results at the gene-level and suggests that transmembrane NRP1 expression may have a protective effect in the context of cognition. This result is particularly interesting when assessing the potential role of angiogenic regulation, because the cytoplasmic domain of NRP1 is required for interaction with KDR after VEGFA binding.[175] The end of the short, 39 amino acid cytoplasmic domain of NRP1 binds the PDZ protein synectin and promotes endothelial cell migration *in vitro*.[175] The *NRP1-201* isoform was not differentially expressed between *APOE*-ε4 carriers and non-carriers and did not interact with *APOE*-ε4 on AD-related neuropathology. These results suggest that a neuroprotective role of NRP1 is independent of amyloid and tau pathology, fitting with gene-level findings. However, nonAD-related neuropathology results showed a significant *NRP1-208* x *APOE*-ε4 interaction on TDP-43 pathology that was driven by *APOE*-ε4 non-carriers. Stratified results indicated that higher *NRP1-208* expression was associated with lower TDP-43 pathology in ε4 non-carriers. TDP-43 is a DNA/RNA binding protein and makes up intracellular inclusions in a number of neurodegenerative disorders including amyotrophic lateral sclerosis and frontotemporal lobar dementia.[176] Literature supports a bidirectional relationship between VEGFA and TDP-43, where *VEGFA* has been identified as a target of TDP-43[176] and has also been reported to negatively modulate pathological accumulation of TDP-43.[177] A connection between *NRP1* and TDP-43 has not been previously reported, however it could be the case that *NRP1* expression contributes to alleviation of TDP-43 cytoplasmic accumulation by VEGFA.

Overall, these results suggest that angiogenic processes may play a role in the differential modulation of cognition based on *APOE*-ε4 allele status. Literature in mouse models has suggested that increased VEGFA signaling through the KDR receptor positively modulates cognitive performance and neovascularization,[178] including in *APOE*-ε4 transgenic mice.[94] We observed contrasting results in this study, such that pro-angiogenic *VEGFA* expression was negatively associated with cognitive performance in *APOE*-ε4 carriers. These findings are interesting in the context of existing literature and suggest that angiogenic properties of *VEGFA* could actually be detrimental in humans carrying an *APOE*-ε4 allele.

As discussed in chapter 2, there are several possible hypotheses to explain these isoform-level results which suggest that DLPFC angiogenesis is detrimental in *APOE*-ε4 carriers. Previous studies to elucidate VEGF effects in the context of the *APOE*-ε4 allele were performed in mice and focused on the hippocampal region.[94] It could be the case that VEGF has differential effects in the context of the *APOE*-ε4 allele based on brain region, and previously documented effects in the hippocampus may not be applicable to other brain regions. Other studies have found a crucial role of forebrain VEGF for cognition,[173, 179] but literature in this brain region has not considered effects in the context of the *APOE*-ε4 allele. Another hypothesis is that the formation of small, weak vessels formed by angiogenesis in *APOE*-ε4 carriers may decrease the strength and efficacy of parent vasculature, impeding cerebral blood flow and creating a more permissive environment for neurodegeneration.

These isoform level results suggest that VEGF expression is associated with cognition differentially based on *APOE*-ε4 allele status through angiogenic signaling

effects and vascular branching may be an important biological component, however the precise explanation for this dichotomy is not yet clear. *In vitro* experiments using cells of differing *APOE*-ε4 genetic backgrounds will be essential to unraveling the differential signaling driving these functional consequences.

There were several limitations to this study, one of which was the mapping of RNA-sequencing reads to specific isoforms. This method relies on inference and can challenging to use for genes with a large number of isoforms, as isoforms for a given gene can share a majority of their exons and spur mis-assignment of reads. This point is especially relevant to *VEGFA* isoforms, for which most protein coding isoforms that share the first five exons.[172] The size of these *VEGFA* exons are close to the average exon size of mature mRNA transcripts in the human genome (~235 base pairs), and with a read length of 101 base pairs used to collect this RNA-sequencing data, we would expect about 33-40% of reads to span two or more exons.[180] These reads are thus too short to obtain fine-detailed mapping of the beginning of many *VEGFA* isoforms. However, some VEGFA isoforms such as $VEGFA_{121}$, $VEGFA_{145}$ and $VEGFA_{165}$ also show distinct splicing of exons 6-8, so a portion of reads which mapped to these regions would be expected to show more accurate isoform distinction.[172] Additionally, lack of cell-type specificity was a major limitation, as *VEGFA* and *NRP1* isoforms are expressed by multiple cell types in the brain, including endothelial cells, neurons and astrocytes. The use of brain homogenate for RNA-sequencing is limiting for the interpretation of which cell-types are most relevant to our observations, and future cell-type specific studies are warranted to inform our findings at the isoform, gene and protein level. Cell-type specificity would also help to confirm the potential role for

angiogenesis, as *VEGF* x *APOE*-ε4 interactions would be expected to be driven by the endothelial cell population in this case.

Although there is a degree of uncertainty in RNA-sequencing read mapping, the major strength of this study was the ability to leverage the available isoform-specific data to inform our gene and protein-level results. We were able to confirm a role for pro-angiogenic *VEGFA* isoforms and transmembrane protein coding *NRP1* in our previously observed *APOE*-ε4 interactions, fitting with the data to support angiogenesis as being a primary biological mediator of these *APOE*-ε4 interactions and their relationship with cognitive performance. These isoform-specific analyses were crucial to help narrow the biological scope of our hypotheses.

CHAPTER 4

Impact of Genetically Regulated Angiogenic Gene Expression on Cognition

Introduction

Angiogenesis has been implicated as a biological pathway involved in AD,[90, 91, 181] but the role of angiogenesis in disease onset and progression remains debated.[181, 182] Our results from RNA-sequencing analyses in the ROS/MAP cohort have suggested that VEGF family isoforms, genes and proteins that are crucial for angiogenesis may be relevant to the modulation of cognitive trajectory in late life.[126, 183] Although the VEGF family is a key regulator of angiogenesis, signaling of this family is also important for neuronal health and maintenance. The neurotrophic effect of VEGF signaling protects neurons from excitotoxic or oxidative stress, potentially through PI3K/Akt or MEK/ERK intracellular pathways,[184] or through regulation of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) signaling pathways.[185] Additionally, VEGF signaling plays a role in microglial activation in response to AD-related pathology.[186] We set out to deconvolve how VEGF signaling may be playing a neuroprotective role in late life and attempted to parse apart the biological processes that VEGF is involved in by further investigation of angiogenesis as a pathway that may be driving our observations.

Gene set enrichment analysis (GSEA) is used to associate expression of genes in a defined set that serve a biological function with a phenotypic change to understand which biological processes may underlie a complex phenotypic change.[187, 188] Gene sets can be defined in a number of ways, including genes that share a Gene Ontology

annotation, genes sharing a genomic locus, and or any user-defined set. GSEA can be applied to genome wide association studies for functional interpretation of significant single nucleotide polymorphisms (SNPs), however statistical power that is needed to perform these analyses requires a very large sample size due to the number of tests across the genome. PrediXcan is a gene expression technique that can be used to overcome this challenge by integrating previously known functional information about SNPs which affect expression of a particular gene to predict gene expression from genomic data. Using this gene-based approach reduces the number of comparisons and statistical tests needed compared to single variant analyses.[189] Predicted gene expression can be functionally interrogated further by using GSEA to determine which biological pathways are genetically implicated in the manifestation of a particular phenotype.

We applied GSEA with an angiogenesis signaling pathway from the Gene Ontology (GO) database to determine if there was evidence for enrichment of predicted angiogenesis-related gene expression in association with cognitive trajectories, across three independent cohorts. We first performed GSEA with this GO defined angiogenesis gene set using RNA-sequencing data of all genes from dorsolateral prefrontal cortex (DLPFC) in ROS/MAP, to determine if there was significant enrichment of angiogenesis-related genes that interacted with *APOE*-ε4 on cross-sectional cognition. We then performed this GSEA analysis using predicted gene expression across three independent cohorts to determine if angiogenic-gene expression association with cognition may be a transcriptionally driven process. Additionally, we applied a data-driven approach to define a gene set of interest from co-expression networks of *VEGF*

family genes and tested this curated gene set for enrichment in relation to cognition and cognitive trajectories.

This study investigated genetically regulated angiogenesis in cognitive aging to highlight a potential pathway that could be involved in cognitive decline. The characterization of angiogenesis gene associations in the context of well-established biological networks could help identify novel targets for therapeutic intervention within the angiogenic pathway. Previous studies using predicted gene expression in humans has implicated angiogenesis as an important genetic resilience factor to AD,[182] and we expect that low predicted gene expression of the angiogenesis gene set will be associated with poor cognitive trajectories in late life. We also tested this hypothesis in *APOE*-ε4 carriers specifically.

There are several advantages of this study to investigate our hypothesis that angiogenic VEGF signaling impacts late life cognition, including the use of predicted gene expression which affords increased sample size compared to RNA-sequencing data because genomic information across additional cohorts can be used. Another advantage is the ability to interrogate the genetically regulated portion of gene expression, which will help narrow our scope for future mechanistic studies. It is possible that angiogenesis effects in AD are largely regulated by environmental factors or are in response to disease (rather than causing disease). In such a scenario, the results of this analysis will still be informative and suggest that a response rather than causal pathway should be further elucidated.

Methods

Participants

The Resilience from Alzheimer's Disease (RAD) database is a local resource that was built from eight longitudinal cohort studies in order to quantify an individual's resilience to cognitive decline in the presence of AD biomarkers. Cognitive data was harmonized across cohorts using a latent variable model to create universal cognitive measures of memory performance and executive function. Three cohorts [ACT (Adult Changes in Thought), ADNI (Alzheimer's Disease Neuroimaging Initiative) and ROS/MAP] were used from this database, which includes genomic and harmonized longitudinal cognitive data for 5,491 individuals. Demographics for these cohorts are shown in **Table 4.2**. ACT is a population-based study in the Seattle metropolitan area that enrolled participants aged 65 and older.[190] ADNI is a longitudinal study that enrolled participants aged 55-90 with normal cognition, mild cognitive impairment and AD.[191] The ROS/MAP cohort was described previously (**Chapter 2**). An additional analysis was performed using dorsolateral prefrontal cortex (DLPFC) RNA sequencing data from the ROS/MAP cohort detailed in **Chapter 2**.

Genotype Data

Imputed genotype data from ACT, ADNI and ROS/MAP underwent standard quality control using PLINK, including only variants with an imputation score > 0.7, MAF > 1% and in Hardy-Weinberg Equilibrium ($p>0.05$).[192]

Tissue-Specific Predicted Gene Expression

Gene expression profiles were quantified using the PrediXcan procedure,[189] which utilizes reference transcriptomic data to impute tissue-specific gene expression profiles. Scripts are publicly available (https://github.com/hakyimlab/PrediXcan/tree/master/Software). Gene expression profiles were imputed based upon previously published multi-SNP equations identified using elastic net, which are available through the PrediXcan webpage. Based on previously published power simulations using PrediXcan,[189] imputation was restricted to genes with $R^2 \geq 0.15$ in elastic net prediction models. The GTEx reference transcriptomic database (version 7) was used to impute gene expression across 48 tissues, including 13 brain regions (amygdala, anterior cingulate cortex, caudate basal ganglia, cerebellar hemisphere, cerebellum, cortex, frontal cortex, hippocampus, hypothalamus, nucleus accumbens, putamen, spinal cord cervical c-1, and the substantia nigra). Additionally, a reference transcriptomic database through the CommonMind Consortium (http://CommonMind.org) was used to impute gene expression in the dorsolateral prefrontal cortex (DLPFC).

Statistical Analyses

Analysis 1: GSEA using ROS/MAP actual gene expression and a Gene Ontology (GO) gene set. All available genes with expression in ROS/MAP (28,612) were tested for an interaction with *APOE*-ε4 allele status on cognition using a linear regression model with

the final global cognition score as the outcome and covaried for sex, age at death, postmortem interval, and the interval between the final visit and death. A mixed-effects regression tested gene expression interactions with *APOE*-ε4 on longitudinal global cognition, with fixed effects including the same covariates as cross-sectional models, and random effects including intercept and interval between a visit and death, as described in **Chapter 2**. An angiogenesis signaling gene set was downloaded from the Molecular Signatures Database (MSigDB) for the C5 category, based on GO terms. The fast gene set enrichment (fgsea) package in R was used to determine if expression interactions with *APOE*-ε4 of genes in the angiogenesis gene set were enriched for cross-sectional or longitudinal cognition. Gene set enrichment was analyzed using the t-statistic from these association tests. GSEA uses a random walk through all genes to calculate a running-sum statistic that is altered when a gene in the designated pathway (angiogenesis) is encountered or not and increases or decreases the running-sum based on the strength of association between encountered gene and phenotype. The maximum deviation from zero encountered in the random walk is the enrichment score, and this value corresponds to a weighted Kolmogorov-Smirnov-like statistic. The significance level of the enrichment score was then estimated by creating a null distribution from 1,000 (standard[193]) gene statistic permutations. Significance was set as $p<0.05$, *a priori*.

Analysis 2: GSEA using predicted gene expression and a GO gene set. The fgsea package in R was used to determine if predicted expression of genes in the angiogenesis gene set were associated with cognitive trajectories using predicted gene

expression in ACT, ADNI and ROS/MAP. First, gene expression was imputed across 48 tissues using the GTEx database, and an additional tissue using the CommonMind Consortium database. Cross-sectional cognitive performance (baseline memory and executive function) as well as longitudinal cognitive performance were tested for angiogenesis-related predicted gene expression enrichment by testing predicted gene expression for association with cognition. Cognitive phenotypes were treated as continuous outcomes. Cross-sectional models covaried for age at baseline and sex. Mixed-effects longitudinal models included fixed effects of age at baseline, sex, intercept, interval between first and current visit as well as predicted gene expression, while random effects included the intercept and interval between first and current visit. For mixed effects models, the term of interest was an interaction between predicted gene expression and interval from first visit. Gene set enrichment was analyzed using the t-statistic from these association tests using the fgsea R package.

Initial analyses using this approach included a meta-analysis of gene set enrichment in whole blood from GTEx. Gene level results were meta analyzed across all three cohorts to calculate a test-statistic. These gene-level meta-analyzed test statistics were then used to investigate overall gene set enrichment using the fgsea method detailed above. A Bonferroni procedure for multiple comparisons correction was used to account for tests in 48 tissues resulting in a statistical threshold of an enrichment $p\text{-value} < 0.001$. Remaining tissues with gene expression imputed from GTEx and CommonMind databases were analyzed at the cohort level.

Analysis 3: Gene Set Enrichment using GO gene set in *APOE*-ε4 carriers. Models detailed in Analysis 2 were tested for GO angiogenesis gene set enrichment in *APOE*-ε4 carriers for longitudinal cognition outcomes in each cohort. Gene set enrichment was tested using predicted expression imputed in the DLPFC using the CommonMind transcriptomic database because this brain region was the source of the RNA-sequencing data used in previous chapters. A Bonferroni procedure for multiple comparisons correction was used to account for the number of models tested and an enrichment p-value < 0.004 was considered significant.

Analysis 4: Gene Set Enrichment using *VEGF* co-expression networks. A similar approach to Analysis 2 was taken, but instead of using a GO set to define angiogenic genes, VEGF family gene co-expression networks were pulled from an Alzheimer's disease knowledge database (agora.ampadportal.org/genes) and used to define gene sets of interest. Initially, a combination approach was taken using co-expression networks of all family members as a single gene set of interest. A false discovery rate procedure to correct for multiple comparisons was applied, which accounted for all 6 enrichment analyses (2 per cohort) using the combined VEGF gene network. Additionally, each co-expression network of a VEGF family member was tested as its own pathway using GSEA. These gene sets were investigated for enrichment across cohorts in DLPFC using the CommonMind database for gene expression imputation for the same reason stated in Analysis 3 and using the same models for longitudinal cognition outcomes detailed in Analysis 2. A false discovery rate procedure was applied to correct for all pathways tested in a given cohort for a given outcome.

Results

Analysis 1: GSEA using a Gene Ontology (GO) gene set and gene expression in ROS/MAP.

The GO defined angiogenesis gene set showed significant enrichment of genes whose expression interacted with *APOE*-ε4 on cross-sectional cognition (**Table 4.1**). Leading edge genes, which showed the strongest interactions with *APOE*-ε4 based on the T-value of the interaction test, were ENSG00000128917 (DLL4), ENSG00000142627 (EPHA2), ENSG00000099250 (NRP1), ENSG00000143878 (RHOB), ENSG00000143125 (PROK1), ENSG00000134013 (LOXL2). No significant enrichment of the angiogenesis pathway was found on longitudinal cognition (**Table 4.1**).

Table 4.1. ROS/MAP actual gene expression angiogenesis GSEA results

Outcome	Pathway Size	Enrichment Score	P-value
Cross-sectional global cognition	279	0.38	**0.019**
Longitudinal global cognition	279	0.29	0.846

Bold indicates significance at $p<0.05$.

Analysis 2: GSEA using a Gene Ontology (GO) gene set and predicted gene expression in ROS/MAP, ACT and ADNI.

Participant demographics across cohorts used for predicted expression analyses are presented in **Table 4.2**. Meta-analyzed angiogenesis gene-set enrichment results for cognitive phenotypes are presented in **Table 4.3**. There was no significant enrichment of angiogenesis-related gene expression in whole blood in relation to longitudinal memory or executive function. Additional analyses were completed to evaluate enrichment of angiogenesis-related genes in relation to baseline cognitive measures, but no significant enrichment was observed (**Table 4.3**). It is noteworthy that although the selected set of angiogenesis-related genes was 293, the number of genes from this set whose expression could be modeled accurately was much smaller, yielding a set of only 78 genes across cohorts.

The remaining 47 tissues available for gene imputation through GTEx did not show significant angiogenesis gene set enrichment after correction for the number of tissues tested. Results that were significant before multiple comparisons correction are presented in **Table 4.4**. Additionally, no significant gene set enrichment was found after correcting for the number of tests run using the CommonMind database for imputed gene expression in DLPFC (**Table 4.5**).

Table 4.2. RAD predicted expression cohort demographics

Cohort	ROS/MAP	ADNI	ACT	Total
Sample Size	2,152	1,182	2,157	5,491
Age at baseline	78.7 ± 7.5	74.4 ± 7.1	76.1 ± 6.6	76.8 ± 7.3
Education, yrs	16.3 ± 3.5	15.9 ± 2.9	14.3 ± 3.1	15.4 ± 3.3
% Female (N)	71% (1528)	42% (499)	57% (1219)	59% (3,246)
% *APOE*-ε4 carrier (N)	25% (531)	46% (541)	25% (532)	29% (1,604)
% AD (N)	27% (588)	40% (467)	5% (116)	21% (1,171)

Table 4.3. Whole blood angiogenesis GSEA results

Outcome	Pathway Size	Enrichment Score	P-value
Baseline memory	78	0.35	0.428
Baseline executive function	78	0.32	0.696
Longitudinal memory	78	0.38	0.219
Longitudinal executive function	78	0.28	0.914

Gene set enrichment analysis of angiogenesis-related predicted gene expression revealed no genetic enrichment of angiogenic factors.

Table 4.4. GTEx database predicted gene expression GSEA results that approached significance.

Outcome	Cohort	Tissue	Pathway Size	Enrichment Score	P-value
Longitudinal Memory	ROS/MAP	Small Intestine, Terminal Ileum	23	0.56	0.013
		Esophagus Gastroesophageal Junction	47	0.44	0.043
		Coronary Artery	31	0.52	0.016
		Tibial Artery	91	0.42	0.035
Longitudinal executive function	ROS/MAP	Pituitary	35	0.52	0.010
		EBV transformed lymphocytes	30	0.50	0.026
Longitudinal Memory	ACT	Brain; Nucleus accumbens basal ganglia	30	0.52	0.011
		Brain; Hippocampus	22	0.52	0.036
		Adipose; Visceral Omentum	61	0.44	0.024
Longitudinal executive function	ACT	Brain; Spinal cord cervical c-1	32	0.52	0.013

P-value threshold for significance was 0.001 to account for models tested across 48 tissues.

Table 4.5. DLPFC predicted gene expression GSEA results.

Outcome	Cohort	Pathway size	Enrichment score	P-value
Baseline memory	ROS/MAP	112	0.32	0.57
	ADNI	130	0.33	0.50
	ACT	121	0.40	0.04
Baseline executive function	ROS/MAP	112	0.36	0.19
	ADNI	130	0.33	0.45
	ACT	121	0.37	0.14
Longitudinal memory	ROS/MAP	112	0.29	0.87
	ADNI	130	1.06	0.31
	ACT	41	0.33	0.69
Longitudinal executive function	ROS/MAP	112	0.31	0.64
	ADNI	130	0.88	0.85
	ACT	41	0.28	0.87

P-value threshold for significance was 0.004 to account for the 13 models tested.

Analysis 3: Gene Set Enrichment using GO gene set in *APOE*-ε4 carriers.

GSEA for the angiogenesis-signaling GO gene set did not show significant enrichment of predicted expression in DLPFC that was associated with longitudinal memory performance or executive function (**Table 4.6**).

Table 4.6. GSEA analysis in APOE-ε4 carriers, using DLPFC predicted expression

Outcome	Cohort	Pathway size	Enrichment score	P-value
Longitudinal memory	ROS/MAP	80	0.42	0.054
	ADNI	130	0.37	0.149
	ACT	121	0.28	0.906
Longitudinal executive function	ROS/MAP	80	0.34	0.414
	ADNI	130	0.28	0.903
	ACT	121	0.37	0.131

Analysis 4: Gene Set Enrichment using *VEGF* co-expression networks.

A detailed breakdown of the number of genes in each VEGF family member co-expression network is presented in **Table 4.7**. The combination of networks across the entire family yielded a set of 340 genes, which was used to investigate gene set enrichment in the context of longitudinal outcomes using expression that was imputed in the DLPFC. Results of analyses run with this combined gene set are presented in **Table 4.8**, and no significant VEGF co-expression gene set enrichment was found for longitudinal memory performance or executive function.

Additional analyses using this approach tested gene set enrichment in regard to longitudinal cognitive outcomes for genes co-expressed with VEGF family members separately. Results from these analyses are presented in **Table 4.9** and did not show significant gene set enrichment

Table 4.7. Co-expression network sizes of VEGF family members

Gene	Co-expression Network Size
VEGFA	20
VEGFB	34
FLT1	93
FLT4	22
KDR	53
NRP1	41
NRP2	27
PGF	30
Total	340

Table 4.8. GSEA analysis using combined VEGF co-expression networks and DLPFC predicted expression

Outcome	Cohort	Pathway Size	Enrichment Score	P-value	P.fdr
Longitudinal memory	ACT	41/340	0.31	0.762	0.762
	ADNI	157/340	0.37	0.100	0.300
	ROS/MAP	139/340	0.33	0.457	0.685
Longitudinal executive function	ACT	41/340	0.37	0.431	0.685
	ADNI	**157/340**	**0.40**	**0.018**	0.108
	ROS/MAP	139/340	0.31	0.623	0.748

Boldface indicates P<0.05. P.fdr was calculated to account for all 6 enrichment analyses run.

Table 4.9. GSEA on separated VEGF co-expression networks in DLPFC

Outcome	Cohort	Network	Size	Enrichment Score	P-value	P.fdr
Longitudinal memory	ACT	VEGFA	2/20	0.37	0.997	0.997
		VEGFB	6/34	0.27	0.983	0.997
		FLT1	11/93	0.38	0.62	0.997
		FLT4	4/22	0.45	0.727	0.997
		KDR	3/53	0.54	0.604	0.997
		NRP1	8/41	0.47	0.419	0.997
		NRP2	3/27	0.61	0.421	0.997
		PGF	4/30	0.30	0.99	0.997
	ADNI	VEGFA	8/20	0.53	0.255	0.426
		VEGFB	13/34	0.34	0.772	0.858
		VEGFC	4/10	-0.34	0.916	0.916
		VEGFD	2/10	-0.77	0.161	0.426
		FLT1	35/93	0.43	0.176	0.426
		FLT4	16/22	0.43	0.245	0.426
		KDR	30/53	0.86	0.699	0.858
		NRP1	**21/41**	**0.43**	**0.047**	0.426
		NRP2	15/27	0.81	0.569	0.812
		PGF	13/30	0.43	0.156	0.426
	ROS/MAP	VEGFA	8/20	0.58	0.131	0.659
		VEGFB	10/34	0.51	0.21	0.659
		VEGFC	4/10	0.32	0.961	0.988
		VEGFD	2/10	-0.36	0.988	0.988
		FLT1	30/93	0.39	0.33	0.659
		FLT4	15/22	0.39	0.544	0.777
		KDR	28/53	0.33	0.653	0.816
		NRP1	19/41	0.43	0.305	0.659
		NRP2	14/27	0.44	0.308	0.659
		PGF	9/30	0.45	0.431	0.719

Boldface indicates P<0.05. P.fdr was calculated to account for all pathways tested in a cohort.

Table 4.9. continued GSEA on separated VEGF co-expression networks in DLPFC

Outcome	Cohort	Network	Size	Enrichment Score	P-value	P.fdr
Longitudinal executive function	ACT	VEGFA	2/20	0.51	0.794	0.82
		VEGFB	6/34	0.41	0.71	0.82
		FLT1	11/93	0.39	0.629	0.82
		FLT4	4/22	-0.65	0.077	0.544
		KDR	3/53	0.47	0.771	0.82
		NRP1	8/41	0.59	0.136	0.544
		NRP2	3/27	0.45	0.82	0.82
		PGF	4/30	0.52	0.532	0.82
	ADNI	VEGFA	8/20	0.44	0.529	0.755
		VEGFB	**13/34**	**0.64**	**0.014**	0.144
		VEGFC	4/10	0.39	0.861	0.861
		VEGFD	2/10	-0.53	0.61	0.763
		FLT1	35/93	0.39	0.321	0.641
		FLT4	16/22	0.44	0.315	0.641
		KDR	30/53	0.37	0.484	0.755
		NRP1	21/41	0.47	0.157	0.524
		NRP2	15/27	0.33	0.77	0.856
		PGF	13/30	0.57	0.065	0.323
	ROS/MAP	**VEGFA**	**8/20**	**0.70**	**0.017**	0.172
		VEGFB	**10/34**	**0.62**	**0.038**	0.192
		VEGFC	4/10	0.38	0.862	0.862
		VEGFD	2/10	-0.86	0.061	0.204
		FLT1	30/93	0.33	0.699	0.861
		FLT4	15/22	0.32	0.787	0.861
		KDR	28/53	0.40	0.594	0.861
		NRP1	19/41	0.40	0.468	0.861
		NRP2	14/27	0.52	0.118	0.294
		PGF	9/30	0.40	0.615	0.862

Boldface indicates P<0.05. P.fdr was calculated to account for all pathways tested in a cohort.

Discussion

We tested the hypothesis that the expression of genes related to the angiogenesis pathway were significantly enriched for an interaction with *APOE*-ε4 on global cognition using RNA sequencing data from the DLFPC of participants in the ROS/MAP cohort. Interestingly, we did observe significant enrichment of angiogenic gene expression when considering interactions with *APOE*-ε4 on cross-sectional, global cognition. This result strongly supports our hypothesis that angiogenesis is the biological pathway driving VEGF gene expression interactions to mediate a differential response in *APOE*-ε4 carriers and non-carriers to impact late life cognitive performance. Leading edge genes which showed the largest magnitude of effect for an interaction with *APOE*-ε4 on cognition included NRP1, DLL4 (delta like canonical Notch ligand 4) which is a ligand for Notch receptors involved in angiogenic sprouting,[194] PROK1 which is endocrine gland-derived VEGF,[182] RHOB and LOXL2, which are a small GTPase and a secreted enzyme respectively, both involved in endothelial cell sprouting,[195, 196] and the tyrosine kinase receptor EPHA2. Interestingly, the ligand for the EPHA2 receptor, EPHA1, has been characterized as a risk gene in AD.[197, 198] The lack of a significant angiogenic gene expression enrichment on longitudinal cognition was consistent with our previous *VEGF* gene family expression observations.

To test the hypothesis that the role of angiogenesis in cognitive performance is transcriptionally driven, gene set enrichment analysis was used to determine if predicted gene expression of angiogenesis-related genes were significantly associated with cognitive trajectory. No significant angiogenesis GO gene set enrichment was found in

whole blood in individual cohorts, or after meta-analysis across all three cohorts. A substantial limitation of these analyses was the inability to impute a large number of genes in the GO angiogenesis gene set. This limitation could have been caused by a number of technical considerations, including the possibility that heritability of gene expression in the investigated set may be too low to properly model with the sample sizes available. Further, predicted gene expression relies on prior knowledge of SNPs that produce a measurable effect on gene expression. If we do not know of SNPs that have an effect on gene expression of a given gene, we would not have been able to properly impute gene expression for that gene. Thus, the use of predicted gene expression for part of this study, while increasing our sample size of participants, also limited the pool of genes that we were able to investigate with this strategy. This was reflected by the low proportion of genes in the angiogenesis gene set and VEGF co-expression networks that were available for evaluation. The resulting decrease in angiogenesis gene set size likely prevented the capture of the full biological process and decreased the power of the analysis.

Several alternative strategies were employed, and analyses were performed using additional tissues, an additional reference transcriptomic database (CommonMind) and using a data-driven approach which leveraged *VEGF* co-expression networks. No significant gene expression enrichment of angiogenesis-related or *VEGF*-coexpressed gene sets was found in relation to cognition across the three available cohorts, suggesting that genetic regulation of angiogenesis is not significantly associated with late life cognition. These results contrast previous work

which implicated angiogenesis as a relevant pathway in AD using gene set enrichment analysis with a gene set formed from a protein interaction network of *NOTCH3*.[199]

It is interesting to note that the results of Analysis 2 across all tissues available from the GTEx database, showed that a few brain regions including the nucleus accumbens, hippocampus and cervical region c-1 showed significant angiogenesis GO enrichment before multiple comparisons correction. This finding in the hippocampus is particularly interesting, given that literature from model systems has focused on characterizing the effects of angiogenic signaling in late life in this region. Studies in mouse models have suggested that enhanced cognition resulting from VEGFA treatment in the hippocampus are associated with an increase in vascularization coupled with an increase in neurogenesis.[89-91, 179] Alternative approaches for GSEA focused on predicted gene expression in DLPFC because significant associations between RNA and protein expression of the VEGF family in prior chapters of this dissertation were also focused on this region. However, it could be insightful to test *VEGF* co-expression networks for predicted gene expression enrichment in the hippocampus in future studies.

If angiogenesis is not the biological process driving the VEGF family effects on cognition, there are additional biological processes known to be regulated by VEGF that may mediate its effects. As discussed at the beginning of this chapter, VEGF signaling can enhance other neurotrophic factors, such as BDNF and NGF to protect neurons from a number of insults such as excitotoxicity and oxygen glucose depravation. In addition to neurotrophic effects, VEGF is also involved in the microglial migration and chemotaxis, which is thought to positively modulate neuroinflammation in response to

early amyloid beta deposition.[186] FLT1, as well as VEGFC and FLT4 are thought to be key players in the transition of microglia to the activated state, and interestingly activated microglia upregulate expression of VEGFA, which in turn also increases angiogenesis.[186, 200, 201]

Collectively, these GSEA results provide support that the associations observed between VEGF expression and cognition are likely driven by an angiogenic response, but that this response may not be genetically regulated, and instead may be in response to disease or aging. Associations between predicted expression of angiogenesis-related genes and cognitive decline were not enriched in *APOE*-ε4 carriers, suggesting that transcriptional differences of angiogenesis-related genes may not play a role in the differential effects of VEGF mRNA and protein expression between ε4 carriers and non-carriers. Future studies should confirm that *APOE* modifies the consequences of angiogenic signaling in the brain over the course of aging and AD. Such work will help elucidate the underlying mechanism of the differential *VEGF* associations based on *APOE*-ε4 allele status and will help push the field closer to precision medicine approaches for target identification.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

Alzheimer's disease (AD) affects one in ten people age 65 and older in the United States and no current treatments slow or stop disease progression.[2] Vascular endothelial growth factor A (VEGFA) has been proposed as an emerging therapeutic candidate for AD[88, 93, 95, 96] and VEGFA treatment in AD model mice rescues cognitive deficits;[90, 91] however, the precise role of VEGFA in both the development and progression of this neurodegenerative disease remains unclear. Our team previously showed that higher CSF VEGFA concentration is associated with slower rates of hippocampal atrophy and cognitive decline, particularly among AD biomarker-positive participants,[93] suggesting that VEGFA is especially protective among participants at highest risk for AD and cognitive decline. Given that *APOE*-ε4 carriers are at heightened risk for AD, it may be that VEGF-mediated neuroprotection is particularly beneficial among this high-risk population. Interestingly, VEGFA has a neuroprotective effect in humanized *APOE*-ε4 transgenic mice, whereby treatment with VEGF results in a recovery of behavioral deficits in parallel with an increase in hippocampal neovasculartization.[94] We hypothesized that *APOE*-ε4 carriers would show protection against AD and cognitive decline as a result of high angiogenesis-related *VEGF* gene and protein expression in the brain, which may act to compensate against the multitude of biological vulnerabilities that make *APOE*-ε4 carriers susceptible to cognitive decline. To test this hypothesis, we assessed interactions between VEGF family expression at

the transcript isoform, gene, and protein level using human autopsy samples of the dorsolateral prefrontal cortex (DLPFC) to determine which components of the family may be protective against cognitive decline, AD-related pathology and AD diagnosis in *APOE*-ε4 carriers and noncarriers. We expanded these studies to assess angiogenesis as the potential biological pathway underlying the observed differential effects of VEGF expression by *APOE*-ε4 allele status using gene set enrichment analysis.

At the protein level, we found that NRP1 expression was positively associated with cognitive trajectory in *APOE*-ε4 noncarriers, suggesting that NRP1 may be neuroprotective in this patient population. This finding was consistent with the observation that *NRP1* gene expression was associated with better cognitive performance at the final time point before death in *APOE*-ε4 noncarriers. In *APOE*-ε4 carriers, we found that *NRP1* and *VEGFA* gene expression was negatively associated with cognitive performance at the final evaluation. It is notable that the mRNA *VEGF x APOE*-ε4 interactions were significant only on cross-sectional cognition and not longitudinal cognition. Future proteomic studies should focus on capturing the ligands in the VEGF family to determine if VEGFA may be driving differential effects of NRP1 signaling at the protein level for a more complete molecular picture. Based on agreement between transcript and protein level findings in the case of NRP1, we would expect to observe a significant interaction between VEGFA protein expression and *APOE*-ε4 status on longitudinal cognition that mirrors the transcript expression findings.

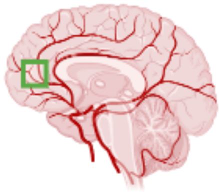
Although we cannot conclude causality from these observations, the *APOE*-ε4-dependent effects of *NRP1* and *VEGFA* do not appear to be driven by AD-related neuropathology, indicating the mechanism underlying these observations is likely

independent of pathological accumulation of amyloid and tau, and therefore may not be a specific response to AD processes but rather play a parallel role in cognitive modulation in late life. There were no differences in *NRP1* or *VEGFA* expression based on *APOE*-ε4 allele status, which demonstrates that the differential effects of VEGF expression based on *APOE*-ε4 allele status are more complex than differential expression. Our findings suggest that *APOE* gene expression and brain cell-type composition may not play a role in the interaction between *VEGF* and *APOE*-ε4 allele status on cognition.

Functionally, NRP1 and VEGFA are both key regulators in the biological process of forming new blood vessels from existing vessels, known as angiogenesis. However, signaling of the VEGF family also plays a role in other processes, including neuroprotection,[91, 202] and microglial chemotaxis.[186] We investigated the biological pathway that may mediate the differential VEGF effects on cognition based on *APOE* genetic background using *VEGF* isoform-specific expression and gene set enrichment analyses. Isoform-specific gene expression analyses suggested that angiogenic isoforms of the VEGF family drive differential cognitive outcomes in *APOE*-ε4 carriers and non-carriers. Gene set enrichment analysis using RNA sequencing data revealed that an angiogenic gene set was enriched for interaction with *APOE*-ε4 allele status on cognition. However, when we used PrediXcan to impute gene expression, we found no evidence for significant enrichment of the angiogenesis pathway, suggesting that the *VEGF* family mRNA effects we have characterized were not genetically regulated. This means that other factors which regulate VEGFA such as environmental conditions including hypoxia, cytokine concentration, and insulin-like growth factor-1 receptor (IGF-

1R) activity,[203] or repair processes in response to aging are likely driving the differential response to VEGF family protein and mRNA expression in *APOE*-ε4 carriers and noncarriers. The therapeutic potential of the VEGF family may not be as high if the observed phenomena are consequence of disease, as compared to playing a causal role. The *VEGFA* gene contains a hypoxia response element, where hypoxia inducible factor (HIF) binding can increase gene expression. Hypoxic upregulation of *VEGF*, followed by an angiogenic response that results in tissue reoxygenation in *APOE*-ε4 noncarriers could explain the beneficial association with cognition. Conversely, an angiogenic response to hypoxia could be damaging in *APOE*-ε4 carriers who have compromised blood-brain barrier integrity compared to noncarriers because this could potentiate damaging events like microbleeds in the brain.

Astrocytes are an abundant source of VEGFA within the neurovascular unit.[204] While it remains unclear which cell type most likely drives the differential NRP1 expression associations in *APOE*-ε4 carriers and noncarriers, *NRP1* is most highly expressed in endothelial cells, astrocytes and microglia.[204] Astrocytic production and release of VEGFA which then binds to receptors on endothelial cells is a mechanism by which astrocytes recruit additional vasculature to the blood brain barrier. Our working model for the mechanism underlying VEGF interactions with *APOE*-ε4 on cognition is shown in **Figure 5.1**, where increased angiogenic signaling in *APOE*-ε4 carriers leads to neurodegeneration and downstream cognitive decline as a result of compromised integrity of newly formed vessels. A recent study supported the hypothesis that *APOE*-ε4 mediated BBB breakdown contributes to cognitive decline.[56]



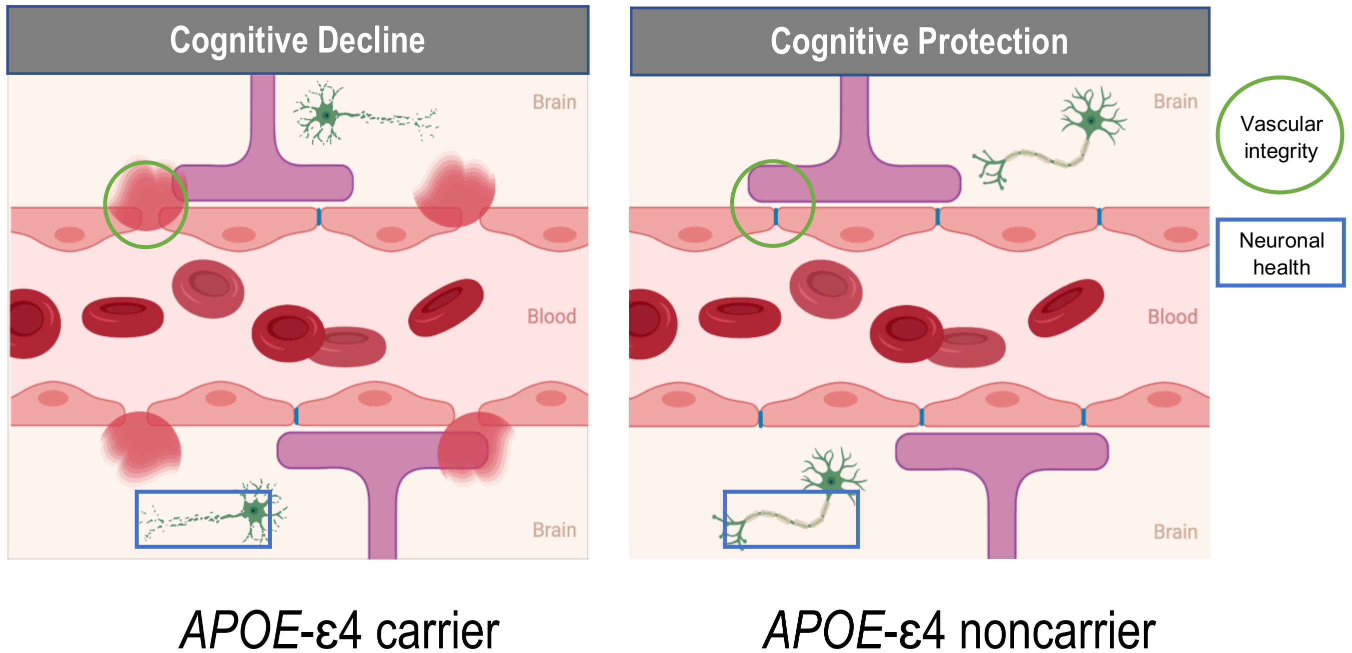


Figure 5.1. Working model for differential VEGF expression effects by *APOE*-ε4 allele status, mediated by an angiogenic process. New vessels formed in the brains of *APOE*-ε4 carriers are at higher risk for damage, potentially decreasing cerebral blood flow and resulting in neurodegeneration. Conversely, new vessels in *APOE*-ε4 noncarriers are more likely to support neurons and other cell types within the neurovascular unit.

Future work should consider how inhibition of NRP1 and VEGFA signaling may be cognitively beneficial for *APOE*-ε4 carriers, as our hypothesis moving forward is that over the course of aging and neurodegeneration, VEGFA and NRP1 drive an angiogenic response for tissue reoxygenation but this process is detrimental in the presence of the ε4 allele due to decreased integrity of new vessels and the potential for

subsequent microbleeds. Therefore, a therapeutic approach could be to inhibit this angiogenic response in ε4 carriers. This hypothesis should be broken down and tested piece by piece, where angiogenesis in response to VEGFA should be measured in ε4 carriers and noncarriers. Next, the integrity of new vasculature and potential downstream neuronal health should be assessed in each genetic background.

Several approaches have been taken to develop NRP1 inhibitors for the treatment of a variety of cancers, including peptides, small molecules, antibodies and small interfering RNA.[205-207] Antagonism of NRP1 has been shown to inhibit phosphorylation of Akt and endothelial cell migration.[205] VEGFA inhibitors have been widely utilized in the clinic for many years to inhibit angiogenesis for the treatment of different types cancers and macular degeneration, including the use of bevacizumab (a monoclonal antibody for VEGFA) for the treatment of glioblastoma.[203] Notably, bevacizumab has been shown to decrease vascular permeability[203] which could improve blood-brain barrier integrity in *APOE*-ε4 carriers. The inhibition of VEGF signaling in *APOE*-ε4 carriers is an achievable goal because FDA approved compounds exist for the inhibition this family. In contrast, the process of pharmacologically agonizing VEGF signaling in *APOE*-ε4 noncarriers for cognitive benefit is severely limited by VEGF signaling associations with cancer. No current therapeutics are approved to agonize angiogenic signaling and the effect of increased brain vasculature on glioblastoma risk would be a major concern with this strategy.

Human induced pluripotent stem cell (hiPSC) lines from *APOE*-ε4 carriers and noncarriers could be used as a model system for a number of applications to validate our findings and test our mechanistic hypothesis. The effect of VEGF expression on

brain endothelial cell phenotype could be investigated using BBB trans-well assays or 3D-hydrogel models using hiPSC lines differentiated into neurovascular unit components.[208] We could create both *APOE*-ε4 homozygote and *APOE*-ε3 homozygote neurovascular units, treat with exogenous VEGFA, then measure acute and chronic vascular phenotypes such as permeability using transendothelial electrical resistance (TEER) measurements,[209] and leakiness using fluorescent dye tracking.[210] Over the course of treatment, neuronal health could be monitored to determine if changes in brain endothelial cell properties results in prolonged neuronal health based on *APOE*-ε4 allele. The same experiment could be performed using VEGFA and NRP1 inhibitors. Based on the findings from this dissertation, we would expect to see a decrease in endothelial resistance and corresponding increase in leakiness after treatment with exogenous VEGFA in *APOE*-ε4 positive endothelial cells, with a downstream decrease in neuronal survival. We would expect to see the opposite in the *APOE*-ε4 negative neurovascular unit, including prolonged neuronal survival and corresponding increase in vascular sprouting. If the *APOE*-ε4 specific endothelial cell properties proposed in **Figure 5.1** were recapitulated using this approach, the system could be used further to interrogate specific endothelial cell signaling pathways that decrease blood-brain barrier integrity in *APOE*-ε4 carriers and this knowledge could be leveraged to develop or apply chemical modulators to correct the detrimental endothelial cell signaling in *APOE*-ε4 carriers. *In vivo* cognitive experiments using aged, humanized *APOE*-ε4 transgenic mice treated with NRP1 or VEGFA inhibitors may be used to validate the protective effect on cognition in an *APOE*-ε4 genetic background.

The PI3K/Akt pathway has been hypothesized to be the main protective VEGF signaling pathway in neurons,[211] but this hypothesis remains debated.[69] Elucidation of the signaling downstream of VEGF receptors that may be contributing to cognitive modulation should also be an aim of future studies, as pharmacologic modulators of components of the RAS/MAP kinase and PI3K/Akt/mTOR pathways are readily available. Due to the conflicting literature on the downstream pathways that are most important for neuroprotection,[69, 212] a discovery study would be best suited to assess all downstream signaling pathways. Inhibitors of RAS, each type of RAF (A-RAF, B-RAF, C-RAF), and MEK1/MEK2 selective compounds will help determine which signaling arm downstream of the RTKs in the family may be responsible for the neurovascular effects driving cognitive response. Each pathway contributes to angiogenesis through regulation of endothelial cell proliferation and migration. Some examples of clinically used compounds that inhibit VEGF signaling downstream of RTKs include a B-RAF selective inhibitor, vemurafenib, and a MEK1/2 inhibitor, trametinib, both used for the treatment of melanoma.[203] Sequential inhibition, or inhibition of multiple components simultaneously in the RAF-MEK-ERK pathway by combining treatments such as darbrafenib (B-RAF inhibitor) and trametinib (MEK1/2 inhibitor) has increased patient response and delayed the onset of drug resistance.[203] Future studies should prioritize the consideration of how clinically available tools targeting downstream VEGF signaling may be efficacious against cognitive decline in *APOE*-ε4 carriers and noncarriers. A first priority moving forward should also be replication analyses of our findings.

Previous association studies with the *VEGF* family have suggested that there are changes in the VEGFB-FLT1 signaling axis that are relevant to the cognitive

progression of AD.[126] These associations are consistent with a compensatory mechanism, where the brain upregulates protective factors in an attempt to compensate for stress from pathology but this endogenous upregulation may not be enough to prevent cognitive decline. This VEGFB-FLT1 signaling axis is not involved in angiogenesis, creating an interesting contrast between VEGF family members that are associated with AD pathology and cognitive decline, and the family members which interact with *APOE*-ε4 on cognitive decline. We interpret the differential associations of these two distinct signaling axes as evidence that different underlying biological pathways are likely driving associations between cognition and the VEGF family in different contexts.

Collectively, this work has shown that the VEGF family member NRP1 may be a neuroprotective factor during late life in individuals who do not carry the *APOE*-ε4 allele. For precision medicine, special attention should be paid to *APOE*-ε4 allele carriers for the pursuit of therapeutics that positively modulate signaling activity of the VEGF family, particularly VEGFA and its receptor NRP1, as positive modulation of these family members may be detrimental to cognition in this patient population. Future work should continue to elucidate the functional consequences of the VEGF family on late life cognition and the development of Alzheimer's disease, with consideration of how VEGF family biology may be altered by expression of the *APOE*-ε4 allele.

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