CEREBROSPINAL FLUID CALCIUM, APOE PROTEIN AND GENOTYPE IN INDIVIDUALS WITH AND WITHOUT CEREBRAL VASOSPASM AFTER SUBARACHNOID HEMORRHAGE

by

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ABSTRACT

Subarachnoid hemorrhage (SAH) is a hemorrhagic stroke subtype affecting 30,000 Americans per year. The most common cause of secondary injury after SAH is cerebral vasospasm (CV). Currently, there are no biomarkers to identify risk for CV. Apolipoprotein E (apoE) is a protein with potential to alter tone of cerebral vessels by influencing intracellular calcium homeostasis. ApoE has isoform specific effects on intracellular calcium (Ca^{++}) level, cerebral vessel tone, and potentially CV. The overall objective of this project was to describe the relationship between apoE genotype (APOE), apoE protein level, calcium level and presence of CV after SAH. This study included individuals age 18-75 with a diagnosis of severe SAH from an aneurysmal source. Daily cerebrospinal fluid samples were drawn from a drainage catheter. APOE genotyping was performed using standard restriction fragment length polymorphism techniques. The sample was dichotomized based on APOE ε4 allele presence. Daily apoE protein levels were analyzed using an enzyme linked immunoassay. Ca^{++} level was analyzed using potentiometry. CV was verified using cerebral angiogram, computed tomographic angiogram, or elevated transcranial dopplers the entire group were lower than normal and associated with one another. Overall and daily apoE protein levels were higher in individuals with an APOE ε4 allele but there was no association with CV. Overall Ca^{++} level was higher in the individuals without CV, however daily mean was not significantly different between the two CV groups.

Overall and daily mean Ca^{++} levels were higher in individuals with an APOE ε4 allele. This project further describes the relationship between apoE, Ca^{++} and neurologic disease, however more work is required before either CSF apoE or Ca^{++} can be used as a predictor of CV.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

1.1. SUBARACHNOID HEMORRHAGE

Subarachnoid hemorrhage (SAH) is an emergent condition characterized by the presence of blood in the subarachnoid space, primarily resulting from a ruptured cerebral aneurysm or arterio-venous malformation. Approximately 50% of individuals surviving the initial hemorrhage will succumb to a secondary insult (Saito, Ueda et al. 1977). The most common condition resulting in secondary insult is cerebral vasospasm (CV), with 30 to 60 percent of people with SAH developing secondary CV (Kassell 1985; Ohman 1991; Solenski 1995). CV is a narrowing of the lumen of the cerebral blood vessels that causes a decrease in cerebral blood flow to the associated brain tissue. As CV progresses, there is a decrease in the delivery of oxygen and nutrients to the brain resulting in increased risk of cerebral ischemia. Occurrence and severity of CV has been associated with amount and location of blood on initial computed tomography (CT) scan after SAH (Fisher, Kistler et al. 1980). The breakdown of blood in the subarachnoid space following SAH releases oxyhemoglobin-derived free radicals which can inhibit the adenosine triphosphate (ATP)-dependent calcium pump (Fujii and Fujitsu 1988; Macdonald 1991; Macdonald, Weir et al. 1991) and lead to CV.

1.2. CALCIUM HOMEOSTASIS

1.2.1. Primary Pathway

The ATP-dependent calcium (Ca^{++}) pump is the primary pathway that maintains intracellular Ca^{++} homeostasis in vascular smooth muscle cells. Nerve cell stimulation and subsequent muscle cell membrane depolarization results in the movement of charge down the muscle cells' membrane and stimulation of the transverse tubules, a series of folds in the membrane. The potential then moves across the cytosol in the cell to the sarcoplasmic reticulum where large Ca^{++} channels are stimulated to open and release Ca^{++} into the cytosol. When the cytosolic Ca^{++} elevates to a level >10-5 M low-affinity, high-capacity Ca^{++} pumps in the inner mitochondrial membrane and/or the sarcoplasmic reticulum take up additional Ca^{++} (Alberts, Bray et al. 1994). This ATP-dependent calcium pump is the primary method of decreasing cytosolic Ca^{++} in vascular smooth muscle cells (Alberts, Bray et al. 1994). The contractile activity of vascular smooth muscle is regulated by the concentration of cytosolic Ca^{++} (Alberts, Bray et al. 1994). An increase in cytosolic Ca^{++} leads to the shortening of myofibrils within the cell and subsequent cellular contraction (Alberts, Bray et al. 1994). Contraction of adjacent cells within a section of an artery decreases the internal lumen of the vessel and is known as vasospasm. When this primary method of cytosolic Ca^{++} maintenance is impaired, secondary pathways take over much of the responsibility of Ca^{++} maintenance.

1.2.2. Secondary Pathways

There are three main secondary cytosolic Ca^{++} pathways: 1) the Na^{+}/Ca^{++} exchanger, 2) ligand gated Ca^{++} channels, and 3) G-protein linked receptors (Alberts, Bray et al. 1994). The Na^{+}/Ca^{++} exchanger is an antiporter system that requires a protein located in cell plasma membranes to export Ca^{++} in exchange for Na^{+}. This protein is a Ca^{++} pump driven by the Na^{+} electrochemical

gradient. The pump requires that Na^{+} move into the cell as the Ca^{++} is pumped out (Alberts, Bray et al. 1994).

There are over 100 different ligands that open Ca^{++} channels (Alberts, Bray et al. 1994). The receptor on the Ca^{++} channel of the plasma membrane has a series of amino acids on its binding site that form weak bonds with the ligand(s), which change the shape of the original Ca^{++} channel. This causes the Ca^{++} channel to open (Alberts, Bray et al. 1994) and move extracellular Ca^{++} inward increasing cytosolic Ca^{++} (Tolar, Keller et al. 1999).

The third secondary pathway, the trimeric guanisine triphosphate-binding regulatory protein (G-protein) linked-receptors, indirectly regulates plasma membrane activities. A variety of molecules (i.e. acetylcholine) interact with their receptors, triggering activation of a mediating protein, a G-protein (Alberts, Bray et al. 1994). Activation of G-protein can alter ion permeability of the plasma membrane or stimulate other intracellular mediators that alter the behavior of subsequent proteins (Alberts, Bray et al. 1994). The end result is opening of Ca^{++} channels in the plasma membrane and in the sarcoplasmic reticulum allowing for increasing cytosolic Ca^{++} level (Alberts, Bray et al. 1994).

1.2.3. Apolipoprotein E and Calcium

Apolipoprotein E (apoE) influences intracellular Ca^{++} through a G-protein pathway. ApoE protein attachment to a G-protein linked receptor on the cell surface results in an influx of extracellular Ca^{++} into the cytosol (Veinbergs, Everson et al. 2002). When the primary method of cytosolic homeostasis (the ATP-dependent Ca^{++} pump) is inhibited, the role of secondary pathways on Ca^{++} homeostasis, including that of the apoE associated G-protein pathway, becomes more dominant.

1.3. APOE FUNCTION

Historically, apoE has been described primarily as a lipid clearance and metabolic protein. ApoE also has been associated with maintenance of cellular membranes (Hayashi, Igbavboa et al. 2002), (Mahley 1988; Weisgraber, Roses et al. 1994), control of enzyme activity (Reyland, Gwynne et al. 1991), immunoregulation (Pepe and Curtiss 1986), neuronal plasticity (Nathan, Bellosta et al. 1994; Nathan, Jiang et al. 2002) and cytosolic calcium level maintenance (Muller, Meske et al. 1998; Tolar, Keller et al. 1999; Veinbergs, Everson et al. 2002). ApoE is present in highest concentration in the liver where the majority of lipid maintenance activities occur, however the second highest concentration of apoE is in the brain.

There are three apoE protein isoforms (apoE2, apoE3, and apoE4) that differ in their amino acid sequences and structure that reportedly can impact lipid clearance (Utermann, Kindermann et al. 1984). ApoE4 protein is less effective at lipid clearance resulting in increased plasma cholesterol while the apoE2 protein is more effective at clearing lipids and thereby decreasing plasma cholesterol (Utermann, Kindermann et al. 1984). Differential effects of the apoE2 protein, as compared to the E3 or E4 proteins, in the nervous system are not well documented. The apoE4 protein, when compared to the E3 isoform, is linked with increased lipid peroxidation and increased oxidative injury (Nathan, Bellosta et al. 1994; Holtzman, Pitas et al. 1995; Fagan, Bu et al. 1996; Ma, Brewer et al. 1996; Miyata and Smith 1996; Laskowitz, Sheng et al. 1997; DeMattos, Curtiss et al. 1998; Jordan, Galindo et al. 1998; Sun, Wu et al. 1998; Horsburgh, Kelly et al. 1999; Montine, Olson et al. 1999; Sheng, Laskowitz et al. 1999; Teter, Xu et al. 1999), decreased neuronal plasticity (Ignatius, Gebicke-Harter et al. 1986; Poirier, Hess et al. 1991; Poirier, Baccichet et al. 1993), and interacts with amyloid beta in a manner that is associated with a less favorable outcome in neurons (Namba, Tomonaga et al. 1991; LaDu, Falduto et al. 1994; LaDu, Pederson et al. 1995; Beffert, Danik et al. 1998). In

addition, the apoE4 protein also is associated with increased neurotoxicity (Marques, Tolar et al. 1996; Michikawa and Yanagisawa 1998; Moulder, Narita et al. 1999). This neurotoxicity is associated with a G-protein linked influx of Ca^{++} from the extracellular space. The Ca^{++} influx associated with apoE is dose dependent, with increased influx associated with increased apoE level (Veinbergs, Everson et al. 2002). Further, the influx is greater when the apoE 4 isoform is present in the extracellular space as compared to apoE3 (Veinbergs, Everson et al. 2002). This influx of Ca^{++} is sustained and nonreversible (Veinbergs, Everson et al. 2002). Given that the contractile activity of vascular smooth muscle is associated with increased neurotoxicity (Crutcher, Clay et al. 1994; Marques, Tolar et al. 1996), regulated by cytosolic Ca^{++} concentration, Ca^{++} influx associated with apoE, particularly the apoE4 isoform, could lead to CV.

1.4. PURPOSE

The purpose of this study was to examine the patterns of apolipoprotein E total and apoE4 isoform specific protein expression relative to Ca^{++} levels in individuals *with and without CV* after SAH.

1.5. SPECIFIC AIMS

1. Describe the total and apoE4 isoform-specific CSF apoE protein expression, in relation to APOE genotype, of individuals with and without CV over time for the first 1-14 days after SAH.
2. Compare CSF Ca^{++} levels, in relation to APOE genotype, of individuals with and without CV over time for the first 1-14 days after SAH.
3. Compare the relationship between Ca^{++} levels and apoE (genotype and protein) in CSF of individuals with and without CV over time for the first 1-14 days after SAH.

1.6. RESEARCH QUESTIONS/HYPOTHESIS

1. Is there a difference in total apoE and apoE4 isoform-specific protein expression, in relation to APOE4 genotype, in CSF 1-14 days after SAH in individuals with and without CV?
2. Is there a difference in Ca^{++} levels, in relation to APOE4 genotype, in the CSF 1-14 days after SAH in individuals with and without CV?
3. Is there a difference in the relationship between total apoE and apoE4 isoform-specific protein expression and Ca^{++} level in the CSF 1-14 days after SAH in individuals with and without CV after controlling for APOE genotype?

1.7. DEFINITION OF TERMS

1.7.1. Independent variables

1.7.1.1. Cerebral vasospasm. A decrease in external lumen size of the cerebral blood vessels will be determined via angiogram or computed tomography angiogram. A neurosurgeon or

neuroradiologist coded the cerebral artery narrowing. CV group status was based on presence: CV positive and CV negative.

1.7.1.2. APOE genotype. Based on their genetic code, each individual was classified into one of 6 possible genotypes: ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4. Genotype was dichotomized into two groups based on APOE ε4 allele presence: APOE4 positive and APOE4 negative.

1.7.2. Dependent Variables

1.7.2.1. ApoE protein expression. When an individual is homozygous (i.e. ε2/ε2, ε3/ε3, ε4/ε4), the isoform specific apoE protein produced by the gene is 100% representative of the associated allele. However, when an individual is heterozygous (i.e. ε2/ε3 or ε2/ε4), two different proteins are produced representing the two alleles in an unknown ratio. ApoE protein levels were quantified daily by total apoE protein expressed and by the isoform ratio relative to the APOE allele pair.

1.7.2.2. Calcium level. The level of Ca^{++} in the CSF as measured in meq/L. The average CSF calcium level in humans is about 2.1 meq/L (Kandel and Schwartz 1991). Currently, CSF Ca^{++} level after SAH or associated with CV is unknown.

1.8. CONCEPTUALIZATION

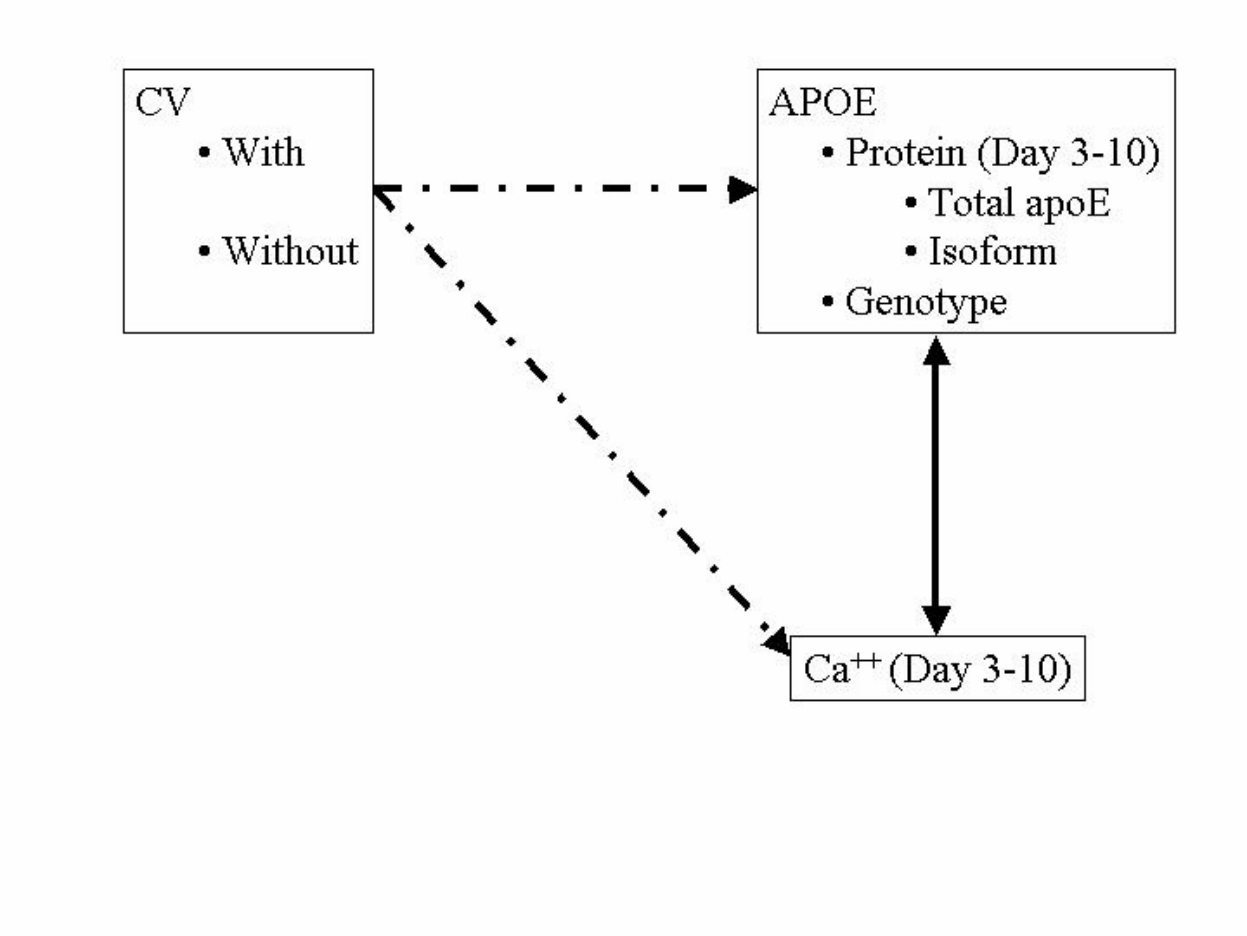


Figure 1. Model of study variables

2. BACKGROUND AND SIGNIFICANCE

2.1. SUBARACHNOID HEMORRHAGE

Aneurysmal SAH afflicts 30,000 Americans per year (Mayberg, Batjer et al. 1994) with only 50% surviving the initial hemorrhage (Newell, Grady et al. 1990). SAH most commonly occurs when a cerebral aneurysm, a weakened and bulging (ballooning) wall of a blood vessel, ruptures releasing blood into the subarachnoid space. Despite progress in standardizing treatment of SAH over the last 3 decades, the outcome of patients experiencing SAH remains poor (Ronkainen 1999) with a disability rate of 50-70% (Dorsch and King 1994). Less than one-half of survivors of the initial SAH recover to their pre-hemorrhage neurological state by the first year after insult (Dorsch and King 1994). Secondary injuries from complications of SAH include cerebral edema, increased intracranial pressure (ICP), clot, mass effect, and CV. They affect over 50% of the patients (Saito, Ueda et al. 1977) and can lead to hypoxia and ischemia increasing the risk of morbidity and mortality.

2.1.1. Primary Complication of SAH: Cerebral Vasospasm

CV, the prolonged contraction of cerebral vascular smooth muscle cells, is the most common complication of SAH occurring in 50% of patients (Newell, Grady et al. 1990). It can decrease delivery of blood to the brain and is associated with increased risk of cerebral ischemia, leading to increased morbidity and mortality (Kassell and Torner 1983; Weir 1995). The onset of CV occurs most often between day 3 and day 9 post-SAH (Dorsch and King 1994; Cook 1995; Weir 1995). The primary risk factor for CV is increased blood volume in the ventricles and

subarachnoid space (Fisher, Kistler et al. 1980). Investigations of agents responsible for CV focus primarily on molecular constituents released from the clot after SAH that may directly or indirectly alter vasoconstriction and/or dilation regulatory mechanisms of the cerebral vasculature (Dorsch and King 1994; Cook 1995).

2.1.2. Pathophysiology of CV

Following rupture of a cerebral aneurysm, blood enters the cerebrospinal fluid (CSF) in the subarachnoid space surrounding the cerebral vessels. As red blood cells of the resultant clot lyse and breakdown, the oxygen-bound hemoglobin contained in those cells is released into the CSF (Fujii and Fujitsu 1988; Macdonald, Weir et al. 1991). The unbound hemoglobin generates other reactive oxygen species (Fujii and Fujitsu 1988; Macdonald, Weir et al. 1991). The presence of oxyhemoglobin-derived free radicals disrupts the primary intracellular Ca^{++} homoeostasis mechanism (Fujii and Fujitsu 1988; Macdonald 1991; Macdonald, Weir et al. 1991). Wang and associates (1994) found that in a canine model of experimentally induced SAH, the activity of the adenosine triphosphatase (ATP)-dependent Ca^{++} pump (the primary method of intracellular Ca^{++} homoeostasis) initially increased, but this activity significantly decreased within 24 hours (Wang, Ohta et al. 1994). Although the mechanism for the relationship between this process of elevating intracellular Ca^{++} and CV remains uncertain, there is evidence that CV begins with smooth muscle contraction initiated by increased and sustained intracellular Ca^{++} levels (Vollrath, Weir et al. 1990; Kim, Kim et al. 1999). Kim et al, 1996, suggested from their animal model work that increased intracellular Ca^{++} levels were linked to CV (Kim, Wier et al. 1996).

2.2. The Role of Intracellular Ca^{++} in Vascular Contraction

The contractile activity of vascular smooth muscle is regulated by cytosolic Ca^{++} concentration (Alberts, Bray et al. 1994). An increase in cytosolic Ca^{++} shortens myofibrils within the cell causing cellular contraction (Alberts, Bray et al. 1994). Contraction of adjacent cells within a section of an artery decreases the internal lumen of the vessel resulting in vasospasm.

2.2.1. Primary Ca^{++} Homeostasis Pathway

The ATP-dependent Ca^{++} pump is the primary pathway maintaining intracellular Ca^{++} homeostasis in vascular smooth muscle cells (Pyne, Cadoux-Hudson et al. 2001). Nerve cell stimulation depolarizes the membrane of a muscle cell, which stimulates the transverse tubules, a series of folds in the membrane. The action potential stimulates the sarcoplasmic reticulum to open large Ca^{++} channels, which release Ca^{++} into the cytosol. When cytosolic Ca^{++} elevates to a level greater than 10-5 M, low-affinity, high-capacity Ca^{++} pumps in the inner mitochondrial membrane and/or the sarcoplasmic reticulum are activated to remove additional Ca^{++} from the cytosol (Alberts, Bray et al. 1994). (See figure 2.) This ATP-dependent Ca^{++} pump, while being the primary method of maintaining cytosolic Ca^{++} level, is not believed to play a major role in the initiation or maintenance of CV after SAH (Cook 1995). Using patch clamp techniques in smooth muscle cells from rat basilar artery, Kim et al (1996) found that hemolysate reduced the amplitude of the L-type Ca^{++} current (ATP-dependent Ca^{++} channels) (Kim, Wier et al. 1996). This lowering of intracellular Ca^{++} levels indicates that the increased intracellular Ca^{++} associated with CV is related to a mechanism other than the ATP-dependent Ca^{++} channels.

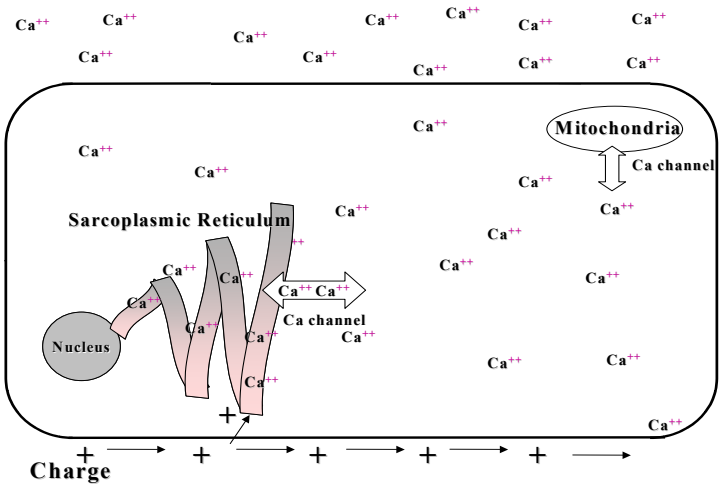


Figure 2. Intracellular calcium homeostasis

2.2.2. Secondary Ca^{++} Homeostasis Pathways

When this primary method of cytosolic Ca^{++} maintenance is impaired, the role of secondary pathways in cytosolic Ca^{++} maintenance is more influential. The three principal secondary cytosolic Ca^{++} pathways are a) the Na^{+}/Ca^{++} exchanger, b) ligand-gated Ca^{++} channels and c) G-protein linked receptors (Alberts, Bray et al. 1994).

The Na^{+}/Ca^{++} exchanger is an antiporter system that requires a plasma membrane protein to export Ca^{++} in exchange for Na^{+}. This Ca^{++} pump is driven by the Na^{+} electrochemical gradient and requires that Na^{+} moves into the cell as Ca^{++} is pumped out, expending one to two-thirds of a cells energy (Alberts, Bray et al. 1994).

A series of amino acids on the cellular plasma membrane serve as the Ca^{++} channel receptor. When a second molecule (the ligand) forms weak bonds with these amino acids, the shape of the original Ca^{++} channel changes causing it to open (Alberts, Bray et al. 1994), move extracellular Ca^{++} inward and increase cytosolic Ca^{++} (Tolar, Keller et al. 1999). Nimodipine (Nimotop), a Ca^{++} channel blocker, is routinely administered to patients after SAH to mediate the specific effects of these ligands and prevent CV (Barker and Ogilvy 1996; Dorsch 1998; van

Gijn and Rinkel 2001). Despite the routine administration of this medication, CV persists as the most common cause of morbidity and mortality after SAH (Dorsch 1998). There are over 100 ligands that reportedly open Ca^{++} channels (Alberts, Bray et al. 1994) yet no single ligand related medication has successfully eliminated the risk of CV after SAH.

Trimeric GTP-binding regulatory protein (G-protein) linked-receptors indirectly regulate plasma membrane activities. A variety of molecules (i.e. acetylcholine) interact with their receptors, triggering activation of a mediating protein called a G-protein. Activation of G-protein can alter ion permeability of the plasma membrane directly or stimulate other intracellular mediators. This sets off a chain reaction that opens Ca^{++} channels in both the plasma membrane and sarcoplasmic reticulum and increases cytosolic Ca^{++} level (Alberts, Bray et al. 1994).

One such G-protein linked pathway is activated by apolipoprotein E (apoE). ApoE attaches to a receptor on the cell surface that activates a G-protein. (See figure 3.) This stimulates the opening of Ca^{++} channels and the influx of extracellular Ca^{++} into the cytosol (Wang, Gruenstein et al. 1997; Veinbergs, Everson et al. 2002). Veinbergs and associates (2002) found that when mouse hippocampal cell lines were exposed to extracellular apoE, intracellular Ca^{++} increased from an extracellular source and intracellular Ca^{++} clearance was impaired (Veinbergs, Everson et al. 2002). This suggests that when the ATP-dependent Ca^{++} pump is inhibited the influence of the apoE G-protein pathway, as well as other secondary pathways, on Ca^{++} homeostasis and vascular muscle tone becomes more dominant. Minimal investigation of this secondary pathway in relation to the development of CV has been conducted.

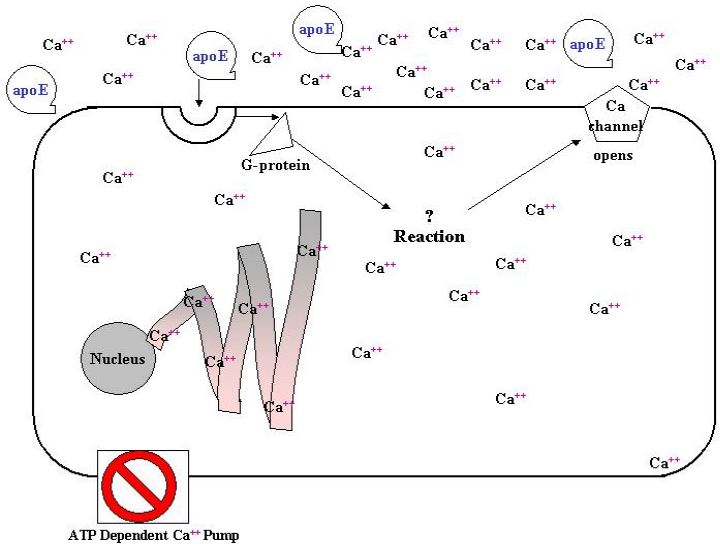


Figure 3. Hypothesized role of apoE in intracellular calcium homeostasis

2.3. APOE GENOTYPE-PHENOTYPE

Evidence shows that one's genetic profile predisposes individuals to variations in both the development and response to treatment for neurologic diseases (Mahley, Nathan et al. 1996). Potential genetic variants have been identified in the development of Alzheimer's disease, Parkinson's disease and other neurodegenerative processes (Saunders, Pericak-Vance et al. 1995; Kamboh, Ferrell et al. 1998; Becker 2001). Kojima and associates (1998) found the odds of developing aneurysmal SAH are significantly increased (42.1%) in individuals with a positive family history (Kojima, Nagasawa et al. 1998). The little work that has been done to study the impact of genetic influences on cerebral aneurysm formation supports a genetic susceptibility to aneurysm development (Schievink 1997; Schievink 1998; Peters, Kassam et al. 1999; Peters, Kassam et al. 2001).

Genetic variation also has been implicated in response to recovery from other acute cerebral injuries such as traumatic brain injury (Strittmatter, Schmechel et al. 1993; Jordan, Relkin et al. 1997) (Friedman, Froom et al. 1999), and stroke (Laskowitz, Horsburgh et al. 1998; Sheng, Laskowitz et al. 1998; Sheng, Laskowitz et al. 1999). For example, variation in APOE genotype has been associated with poorer prognosis after brain injury (McCarron, Muir et al. 2000).

Only two base changes in the APOE gene result in 3 common alleles with 6 associated genotypes (APOE ε2/2, ε2/3, ε2/4, ε3/3, ε3/4, ε4/4). The base changes (codon change from CGC to TGC at position 112 in the ε2 allele versus the ε3 allele; codon change from TGC to CGC at codon 158 in the ε4 allele versus the ε3 allele) lead to amino acid alterations at positions 112 and 158 respectively when the gene is translated into protein (Utermann, Kindermann et al. 1984). The APOE ε3 allele, the most common allele, is translated into a protein with cysteine at position 112 and arginine at position 158 (Utermann, Kindermann et al. 1984). As compared to the APOE ε4 allele that translates arginine rather than cysteine at position 112, the APOE ε2 allele translates cysteine rather than arginine at position 158 (Utermann, Kindermann et al. 1984; Weisgraber 1994). Allele frequencies in the general population of the United States are approximately 8% for ε2, 75% for ε3, and 15% for ε4 (Weisgraber 1994), although distribution frequencies differ based on ethnicity.

The APOE ε4 allele also has been associated with increased risk for Alzheimer's disease (Saunders, Pericak-Vance et al. 1995; Kamboh, Ferrell et al. 1998), schizophrenia (Martorell, Virgos et al. 2001; Hata, Kunugi et al. 2002), poorer recovery from traumatic brain injury (Jordan, Relkin et al. 1997; Friedman, Froom et al. 1999), cerebral ischemia (Laskowitz, Horsburgh et al. 1998; Sheng, Laskowitz et al. 1998; Sheng, Laskowitz et al. 1999), and

increased cognitive impairment related to time spent on cardio-pulmonary bypass machine during cardiac surgery (Chey, Kim et al. 2000; Kutner, Erlanger et al. 2000). In contrast the APOE ε2 allele has been associated with increased risk for cerebral amyloid angiopathy-related hemorrhage- a specific type of intracerebral hemorrhage (Nicoll and McCarron 2001).

ApoE is primarily viewed as a lipid clearance and metabolism protein. Its functions include maintenance of cellular membranes (Hayashi, Igbavboa et al. 2002),(Mahley 1988; Weisgraber, Roses et al. 1994), control of enzyme activity (Reyland, Gwynne et al. 1991), immunoregulation (Pepe and Curtiss 1986), neuronal plasticity (Nathan, Bellosta et al. 1994; Nathan, Jiang et al. 2002) and cytosolic calcium level maintenance (Muller, Meske et al. 1998; Tolar, Keller et al. 1999; Veinbergs, Everson et al. 2002). The highest concentrations of apoE are found in the liver where the majority of lipid maintenance activities occur, with the second highest levels in the brain. In the brain, the production and secretion of apoE by astrocytes increases in response to central nervous system injury (Lin, Duan et al. 1999; Duan, Gu et al. 2000).

Little work has been done to study the impact of genetic variance on recovery after SAH. Two studies independently found that presence of at least one copy of the APOE ε4 allele (i.e. APOE genotypes ε2/4, ε3/4, and ε4/4) was associated with poorer outcomes at 3 months after SAH (Niskakangas 2001; Leung, Poon et al. 2002). Alternatively, Dunn et al (2001) did not find a significant association between APOE genotype and outcome from SAH, although the allelic distribution of the sample did not match that of the general population (Dunn, Stewart et al. 2001). No other work known to this investigator has been done to identify genetic biomarkers associated with development of CV or recovery from SAH.

2.4. APOE PROTEIN ISOFORM AND NEUROLOGIC DISEASE PROCESS

Each allele codes for a single isoform of the apoE protein (apoE2, 3, or 4) resulting in structurally related differences in function. For example, the apoE4 protein isoform is less effective at lipid clearance and increases plasma cholesterol (Utermann, Kindermann et al. 1984). Differential effects of the apoE2 protein, as compared to the E3 or E4 proteins, in the nervous system continue to emerge. It is unclear whether the variations in neurologic disease development, progression and response to cerebral injury are related to differences in the structure, function, or amount of the protein expressed. The apoE4 protein isoform has been linked with increased lipid peroxidation and increased oxidative injury (Ma, Brewer et al. 1996; Miyata and Smith 1996; Laskowitz, Sheng et al. 1997; Jordan, Galindo et al. 1998; Horsburgh, Kelly et al. 1999; Montine, Olson et al. 1999; Sheng, Laskowitz et al. 1999), increased cytoskeletal protein binding, decreased neurite outgrowth (Bellosta, Mahley et al. 1995) (Nathan, Bellosta et al. 1994; Holtzman, Pitas et al. 1995; Fagan, Bu et al. 1996; DeMattos, Curtiss et al. 1998; Sun, Wu et al. 1998; Teter, Xu et al. 1999), decreased neuronal plasticity (Ignatius, Gebicke-Harter et al. 1986; Poirier, Hess et al. 1991; Poirier, Baccichet et al. 1993), increased neurotoxicity (Crutcher, Clay et al. 1994; Marques, Tolar et al. 1996; Tolar, Marques et al. 1997; Michikawa and Yanagisawa 1998; Moulder, Narita et al. 1999), and interaction with amyloid beta in a manner that is associated with a less favorable outcome in neurons (Namba, Tomonaga et al. 1991; LaDu, Falduto et al. 1994; LaDu, Pederson et al. 1995; Beffert, Danik et al. 1998).

2.5. APOE GENE/PROTEIN: REGULATION OF CEREBRAL VESSELS

There is little direct evidence to link variation in APOE genotype and protein to CV. The apoE4 protein is associated with increased neurotoxicity (Crutcher, Clay et al. 1994; Marques, Tolar et al. 1996; Tolar, Marques et al. 1997; Michikawa and Yanagisawa 1998; Moulder, Narita et al. 1999). This neurotoxicity has been associated with an influx of Ca^{++} from the extracellular space related to the secondary Ca^{++} homeostasis pathway involving G-protein activation and is apoE dose dependent, with increased influx associated with increased apoE level (Veinbergs, Everson et al. 2002). Further, the influx occurs at when less apoE4 is available and is greater when the apoE4 isoform is present in the extracellular space as opposed to the apoE3 isoform (Muller, Meske et al. 1998; Veinbergs, Everson et al. 2002). The apoE4 associated influx of Ca^{++} is sustained and nonreversible (Muller, Meske et al. 1998). Given that the contractile activity of vascular smooth muscle is regulated by cytosolic Ca^{++} concentration, the Ca^{++} influx associated with apoE, specifically the apoE4 isoform could lead to complications following SAH, such as CV. d'Uscio et al., developed a mouse model of human atherosclerosis, specifically an apoE knockout mouse. They found that lack of apoE is associated with increased impairment of endothelial function related to inactivation of nitric oxide (NO) by superoxide ions in smooth muscle cells of apoE knockout mice (d'Uscio, Baker et al. 2001). These results have been supported by the work of others (d'Uscio, Smith et al. 2001; Brown, Wright et al. 2002). Sacre and associates found that the presence apoE3 stimulates more NO release in cultured human endothelial cells than apoE2 or apoE4 (Sacre, Stannard et al. 2003). Vascular relaxation is dependent upon NO and a decrease in NO associated with apoE2 or apoE4 could lead to ineffective vascular reactivity and CV. Kay and associates (2003) found that in humans apoE

CSF concentration was lower after SAH when compared to that of controls (Kay, Petzold et al. 2003) suggesting that apoE may be utilized at an increased rate after SAH.

2.6. SIGNIFICANCE

The premise of this study is that after SAH cytosolic Ca^{++} homeostasis shifts from primary to secondary pathways. Exposure of the vessel walls to increased available apoE protein and Ca^{++} in the CSF, via SAH, results in an increase in apoE attachment to the receptors in the cells of the vessel wall. This phenomenon can in turn lead to a G-protein linked increase in cytosolic Ca^{++} level, vascular smooth muscle cell contraction and hence vessel contractility (i.e. CV).

Nurses, as the caregivers that monitor neurologic status of patients following SAH, identify changes indicative of hypoxia and ischemia caused by CV. The ability to a priori identify subsets of individuals at greatest risk for CV and the associated poorer outcomes using genetic biomarkers would lead to better utilization of nursing resources in the form of increased monitoring of these patients. It would also encourage the development apoE-Ca^{++} based treatment options tailored to an individuals genetic background aimed at increasing odds of survival and improving function of survivors.

While APOE genotype has been implicated in many neurodegenerative disease processes and recovery from acute neurologic injury, it has not been thoroughly explored in the SAH population. ApoE protein expression has rarely been considered, and isoform specific effects in relation to cytosolic Ca^{++} level and CV has not been investigated in this population. Knowledge of how genetic differences impact recovery from SAH could advance our knowledge of the biology of secondary injury following SAH and aid in efforts at designing individualized treatment.

3. PILOT STUDIES

This series of pilot studies were conducted as a component of an independent study to acquire skill in measuring apoE protein level and Ca^{++} level in CSF. The overall purpose of these studies was to pilot test the laboratory techniques involved in the measurement of these variables and to collect data for effects size calculations for the larger study.

3.1. PRELIMINARY STUDY #1: APOE GENOTYPE AND OUTCOME AFTER SAH

3.1.1. Introduction

Apolipoprotein E (APOE) genotype has been associated with outcomes after many neurologic injuries, most commonly with presence of the ε4 allele associated with poorer outcome after both severe TBI and stroke. The association between APOE genotype and outcome after SAH is inconclusive with some researchers finding the APOE ε4 allele associated with poorer outcomes (Niskakangas 2001; Leung, Poon et al. 2002) while others did not find any significant association (Dunn, Stewart et al. 2001).

3.1.2. Specific Aims

The purpose of this project was to examine outcome after SAH in relation to APOE genotype.

3.1.3. Methods

This study used a nonequivalent two-group design to investigate the relationship between APOE genotype and outcome, as measured by the Glasgow Outcome Score (GOS) and mortality at 6 months after injury, in patients recovering from an aneurysmal SAH. Subjects for this study were taken from a larger population of individuals entered into a larger study after informed

consent was obtained. Criteria for this study included 1) age 18-75, 2) admitted with a diagnosis of severe aneurysmal SAH (Fisher grade ≥2 or Hunt and Hess grade ≥3), 3) without previously existing static neurologic disease, 4) CSF available for genotyping and 5) 6 month GOS available. Age and severity of injury measures (Fisher grade, Hunt and Hess grade, and Glasgow coma score) were extracted from the medical record prospectively as part of the parent study. CSF samples were drawn from an extraventricular drainage bag on the day of consent. APOE genotype was assessed using standard restriction fragment length polymorphism analysis. Patients were classified by APOE ε4 allele presence (APOE4+) or APOE ε4 allele absence (APOE4-). GOS and mortality were obtained by a trained researcher during telephone interview with the subject, their significant other, or physician office. GOS was dichotomized into 'good' (GOS = 4 or 5) and 'poor' (GOS =1, 2, or 3). Descriptive statistics, t-tests and Chi square analysis were conducted using SPSS v11.0.

3.1.4. Results

Forty-five subjects (mean age of 53.4 years) were recruited into the parent study, had complete outcome data at 6 months post-SAH and samples available for genotyping. The sample was 73.3% female, and 93.3% Caucasian. Thirty-one (68.9%) patients had APOE genotype =3/3, 11 (24.5%) patients had APOE 3/4 genotype, 2 (4.4%) patients had APOE 2/3 genotype and 1 (2.2%) patient had APOE 2/4 genotype. The sample was divided into two groups based on APOE ε4 allele presence, the APOE 4+ group (n=12; 26.7%) and the APOE 4- group (n=33; 73.3%).

Based on t-test analysis, there were no significant differences in age or admission Glasgow coma score. Using Chi-square analysis, there were no significant differences in Fisher scores or race between the two groups. A significant association was found between APOE ε4

allele presence and Hunt and Hess scores, with individuals with the APOE ε4 allele having a lower Hunt and Hess score (indicating less severe injury) ($X^2 = 11.29$; $p = 0.023$). Using Chi-square analysis there were no significant differences in good versus poor outcome or mortality based on genotype.

3.1.5. Conclusions

While individuals with an APOE ε4 allele presented with less severe initial injuries, there was no significant difference in outcome. This suggests that individuals with the APOE ε4 allele have a response to the initial hemorrhage that is different from those without an APOE ε4 allele.

3.2. PRELIMINARY STUDY #2:APOE PROTEIN LEVEL IN CSF AFTER SAH

3.2.1. Introduction

CV occurs when cerebral blood vessel diameter decreases causing decreased delivery of blood to brain tissue. The contractility of vascular smooth muscle is regulated by cytosolic calcium (Ca^{++}). Following SAH, primary pathways of Ca^{++} homeostasis are disrupted and secondary pathways, such as the apolipoprotein E (apoE) pathway may play a greater role in regulating Ca^{++} influx. The role of the apoE protein, a secondary Ca^{++} homeostasis pathway has never been examined in the development of CV after SAH.

3.2.2. Purpose

The purpose of this project was to 1) examine CSF apoE protein level over time in relation to CV status after SAH and 2) determine whether differences exist in apoE protein level over time in individuals with and without an APOE 4 allele.

3.2.3. Methods

This study used a matched cohort design. Individuals between the ages of 18-75 admitted with a diagnosis of severe aneurysmal SAH (Fisher grade >=2 or Hunt and Hess grade >=3) entered into a larger study after informed consent was obtained were considered for entry into this study. Only individuals who had cerebral angiography and an EVD (with CSF sampling) were considered for entry into this study. Six patients with CV were matched on severity of hemorrhage, age, race and gender to 6 patients without CV (N=12). Hunt and Hess and Fisher grades were matched within 1 level of categorical assignment. Presence/absence of CV was verified by cerebral angiography. Subjects were dichotomized into two groups based on CV status (CV+ versus CV-). Daily CSF samples were drawn from day of consent to day 12 after SAH. APOE genotype was assessed using standard restriction fragment length polymorphism analysis. While subjects were not matched on genotype, they were dichotomized into two groups based on APOE ε4 allele presence (APOE4+ versus APOE4-). ApoE protein levels were measured using enzyme linked immunoassay analysis. Descriptive statistics, t-tests and graph analysis were conducted using SPSS v11.0.

3.2.4. Results

The overall mean total apoE protein level across all patients at all time points was .577 ng/ml (SD=.441). Baseline apoE protein levels were .624 ng/ml (SD=.56) in patients with CV and .529 ng/ml (SD=.33) in patients without CV (t=-.358; p=.09) (See figure 1). Individuals without CV had a greater increase in apoE protein level over time as compared to those with CV (t=2.26; p=.047).

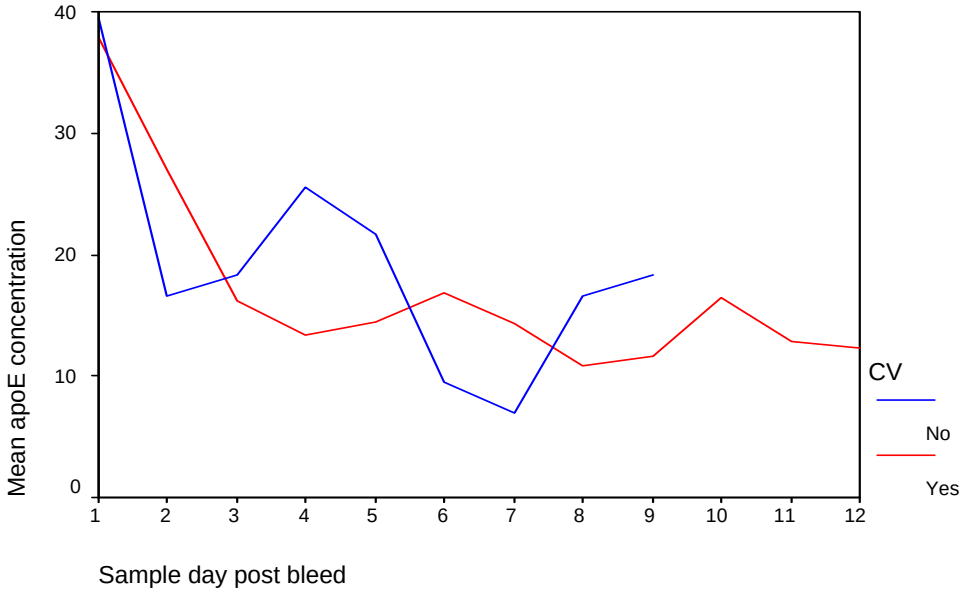
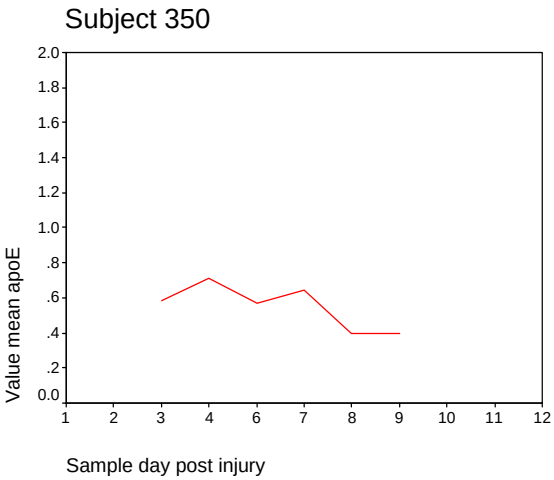
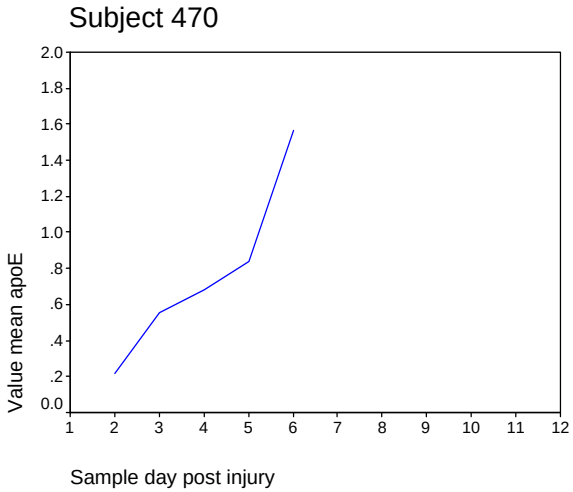


Figure 4. Mean apoE level by sample day by CV

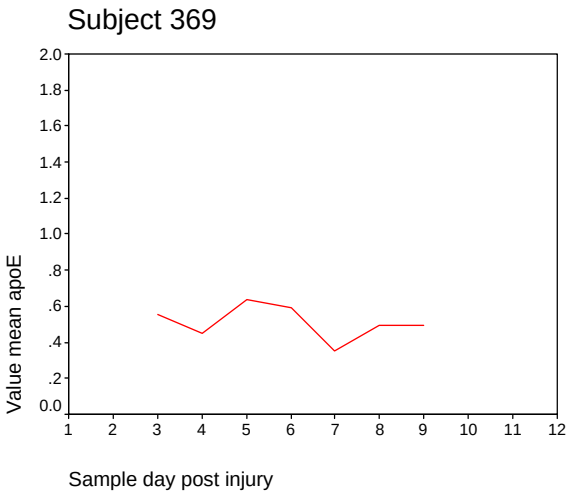
CV+



CV-



CV+


Subject 369
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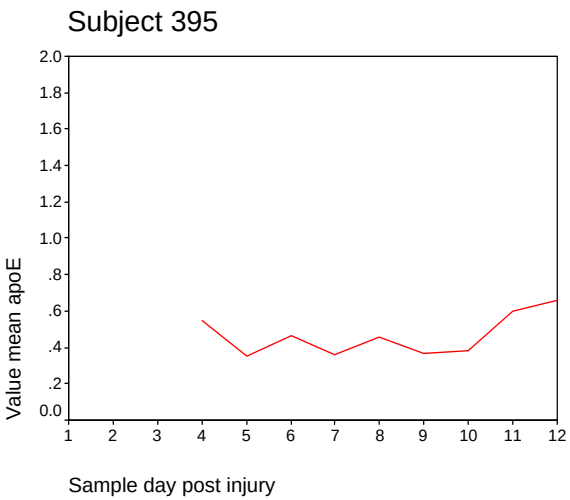
1.8
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Sample day post injury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1.6
1.4
1.2
1.0
.8
.6
.4
.2
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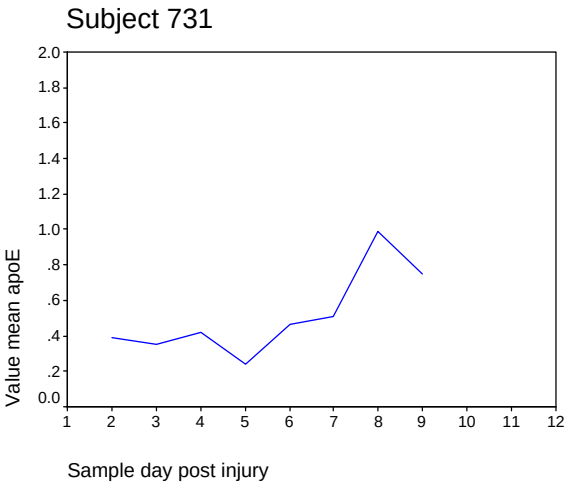
1 2 3 4 5 6 7 8 9 10 11 12
Sample day post injury

CV-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1.6
1.4
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1.0
.8
.6
.4
.2
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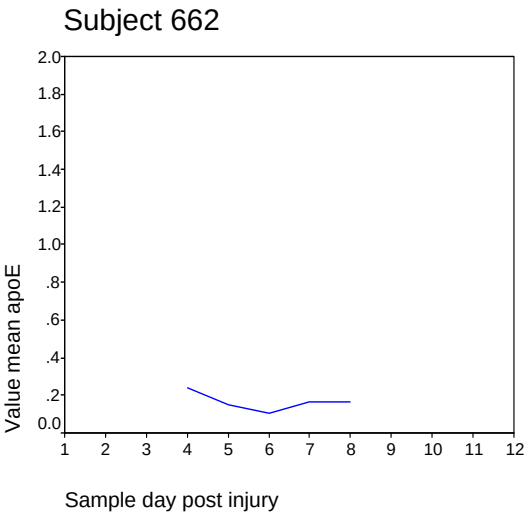
Sample day post injury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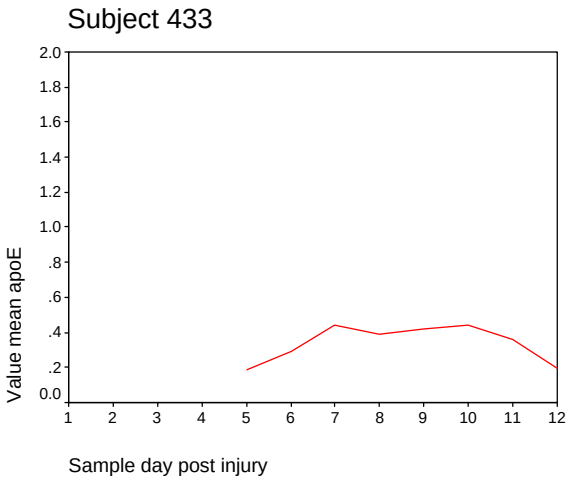
Sample day post injury

CV+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1.0
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.6
.4
.2
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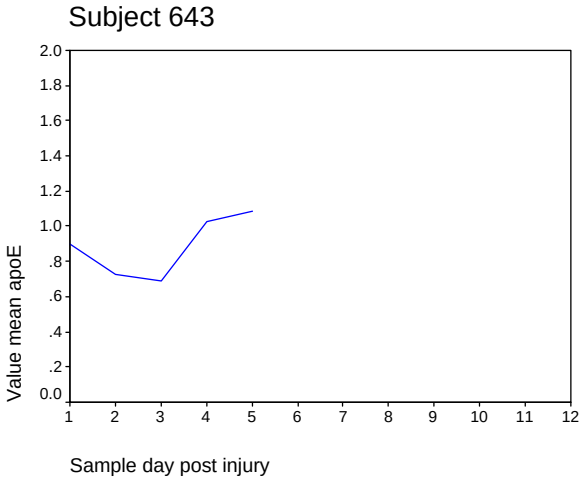
1 2 3 4 5 6 7 8 9 10 11 12
Sample day post injury

CV-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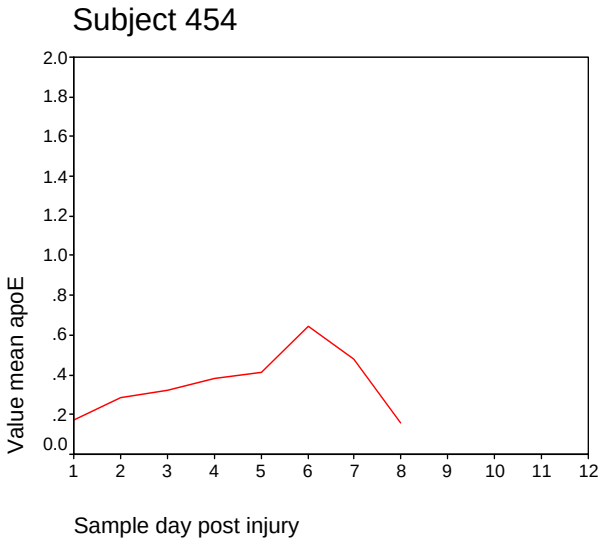
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Sample day post injury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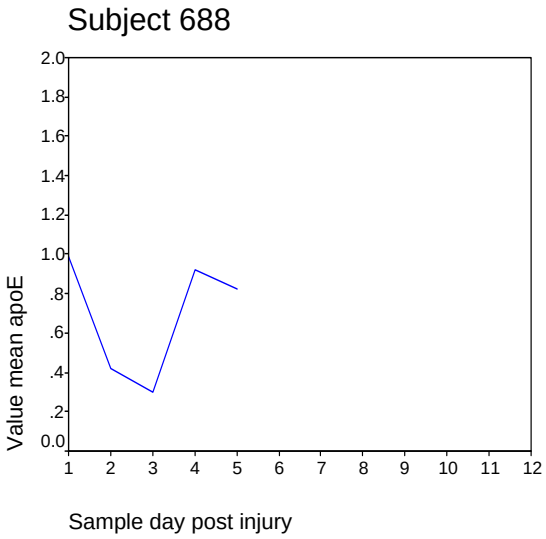
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Sample day post injury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1.4
1.2
1.0
.8
.6
.4
.2
0.0
1 2 3 4 5 6 7 8 9 10 11 12
Sample day post injury

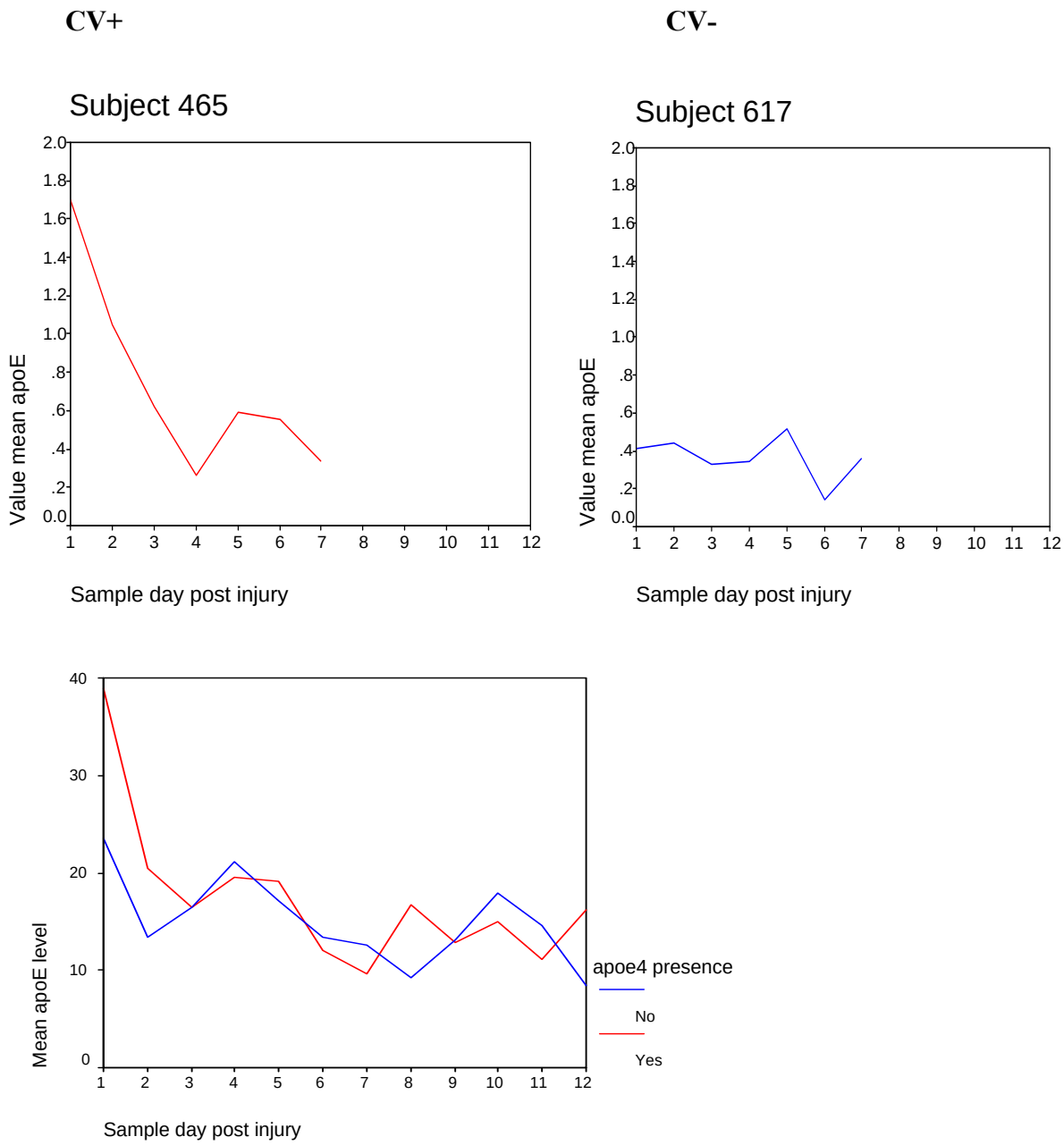

Figure 5. Mean apoE level over time by APOE ε4 allele presence

3.2.5. Conclusions

Sustained lower apoE protein levels are associated with CV after SAH and APOE genotype may influence apoE protein level.

3.3. SUMMARY OF PRELIMINARY STUDIES

These preliminary studies show that the PI has the ability to perform apoE protein quantification using ELISA methodology, and Ca^{++} level quantification using potentiometry. Results of preliminary study #1 investigating outcome by APOE genotype, while not statistically significant, will provide data for sample size estimation for the proposed project. Preliminary study #2 showed that not only are there differences in apoE protein level in groups based on presence/absence of CV, there are also differences based on genotype. This pilot study shows that further exploration of this question is necessary to determine long-term effects of apoE on CV and outcome after SAH. This preliminary work shows that it is feasible for this PI to carry out the larger proposed study.

4. METHODS

4.1. DESIGN

This descriptive, prospective study used a between-group, within-subject design to examine daily apoE protein and Ca^{++} levels in CSF over time classified by presence of at least one copy of the APOE ε4 allele (APOE ε4 allele presence) in 97 individuals (with and without CV) admitted to University of Pittsburgh Medical Center.

4.2. SAMPLE

The population presenting to the University of Pittsburgh Medical Center for treatment of severe SAH is approximately 60% women and 9% African American, which is consistent with the general SAH population. All patients admitted to the Neurovascular Intensive Care Unit were screened for eligibility based on the following criteria.

Inclusion criteria:

1. 21-75 years of age
2. Diagnosis of subarachnoid hemorrhage (Hunt and Hess grade ≥3 or Fisher grade ≥2) verified by CT scan
3. Subsequent verification of a cerebral aneurysm as the probable cause of SAH
4. Placement of a CSF drainage catheter within 3 days of SAH

Exclusion criteria:

1. Any pre-existing, uncontrolled or nonstatic chronic neurologic disease or deficit
2. SAH resulting from a traumatic injury, mycotic aneurysm, or arterio-venous malformation
3. Died within 72 hours after hospital admission, precluding evaluation of CV.

Children were not included in this study because the incidence of aneurysmal SAH in children is <.001% (Anderson, Jamrozik et al. 1993). Patients with a diagnosis of subarachnoid hemorrhage graded as Hunt and Hess ≥ 3 and Fisher grade ≥ 2 have an increased risk of CV. Individuals with a pre-existing, static neurological condition were excluded to avoid confounding of the initial neurologic severity of injury assessment. Patients with SAH from non-aneurysmal sources have a different course of recovery and a decreased risk of CV and were therefore omitted from this project.

4.3. RATIONALE FOR SAMPLE SIZE

The rationale for the sample size was based on previous recruitment/enrollment history and Power Analysis and Sample Size software [(PASS) NCCS Statistical Software, Kaysville, Utah] analysis. In this population, the mean and variance of apoE protein levels are unknown, making effect size difficult to predict. Using PASS analysis and a sample size determined to be feasible, the minimum effect size that could be detected for each specific aim is identified below.

Given a sample size of 97 subjects:

1. Specific aim #1 would detect an effect size of 0.30 for differences in apoE protein expression in CSF of individuals with and without an APOE ε4 allele over the first 1-14 days after SAH at α=.05.

2. Specific aim #2 would detect an effect size of 0.30 for differences in CSF Ca^{++} levels, in relation to APOE genotype of individuals with and without CV over the first 1-14 days after SAH at α=.05.

3. Specific aim #3 would detect an effect size of 0.20 for differences in CSF Ca^{++} levels and apoE protein expression in CSF of individuals with and without CV while controlling for APOE ε4 allele presence over the first 1-14 days after SAH at α=.05.

Samples were collected from July, 2001 through November, 2003. A total of 97 subjects were recruited and had daily specimens were available for analysis.

4.4. SETTING

Subjects were recruited from the Neurovascular Intensive Care Unit (NVICU), Presbyterian University Hospital, University of Pittsburgh Medical Center (UPMC), Pittsburgh Pennsylvania. This 10 bed unit is designed to care for individuals in the acute phase of neurologic injury.

4.5. RECRUITMENT

This project is linked to two prospective studies involving patients that experienced a SAH (NIH/NINR RO1 NR04339, PI- Dr. Kerr and Copeland Foundation Grant, PI- Dr. Kassam). Approximately 2 patients per month were recruited into the study. Project personnel made daily rounds on the NVICU with the nursing staff. Potential patients, with a diagnosis of SAH were reviewed for eligibility criteria. Once eligibility was established, the bedside nurse reviewed the study briefly with the patient or the patients' family and asked if study personnel could present the study to them. If the patient/representative was willing, the nurse would introduce the study

personnel. Project personnel would present the study, and obtain informed consent. Both studies were reviewed and approved by the University of Pittsburgh Institutional Review Board. In order to assure subject confidentiality, all identifiers were removed from medical records and samples. A nonidentifying study number was given to link the sample and medical record data.

4.6. STANDARD MEDICAL CARE

All individuals presenting to the UPMC PUH with SAH were admitted to the NVICU. A CT scan of the head is done upon admission. Hunt and Hess grade and Fisher grade were assigned by the neurosurgeon or a neuroradiologist upon admission. Cerebral angiography was conducted to identify aneurysm and perform coiling and embolization of the aneurysm if that was the preferred treatment for the patient (as determined by attending neurosurgeon). If coiling and embolization were not performed, surgical clipping of the aneurysm was conducted as soon as possible. Continuous arterial blood pressure (ABP), central venous pressure (CVP), pulse oximetry, respiratory rate and cardiac rate and rhythm were monitored. Extraventricular drain or lumbar drain was placed if necessary (for ICP maintenance) and ICP was monitored continuously. Temperature was monitored rectally or orally every two hours. Complete neurologic exam was conducted every 1 hour for the first 24 hours after admission and then every 2 hours until discharge from the NVICU. Normovolemia was maintained with fluid bolus. Mean arterial blood pressure was maintained with anti-hypertensives or vasopressors. The head of the bed was elevated 30 degrees. Nimodipine (Nimotop) was given at a dose of 60mg every 4 hours for fourteen days as tolerated. Anti-convulsants were administered. Sedatives were administered as needed for agitation.

4.7. DATA COLLECTION

Demographic and medical condition information was recorded onto data collection sheets by project personnel. Data collection sheets were electronically transmitted directly into an automated data entry and verification system (Teleform). Data was stored in a locked office of the PI of one of the parent studies (Kerr). Cerebrospinal fluid (CSF) samples were drawn at least once every day using the University of Pittsburgh Medical Center standard procedure for ventriculostomy or lumbar drain bag changes. Specimens were drawn only while the drainage catheter was in place for routine medical care. Upon bag exchange, CSF was sampled from the bag and aliquoted into three-1 ml tubes and placed into a -80°C freezer located on the 2nd floor lab at Victoria Building, School of Nursing, University of Pittsburgh. All analysis of specimens for the proposed project was conducted by the principal investigator of this study in the laboratory under the supervision and guidance of Dr. Conley. Equipment was available in Dr. Conley's laboratory to perform: Deoxyribonucleic Acid (DNA) extraction and amplification (using polymerase chain reaction), electrophoresis, UV light visualization, quantitative Enzyme-Linked Immunosorbent Assay (ELISA) and potentiometry.

4.8. MEASUREMENT

The study independent variable was the presence and severity of CV. APOE ε4 allele presence was treated as an independent variable in analysis of specific aim #3 and as a covariate for analysis of specific aim #1, #2. Dependent variables were the levels of apoE protein and Ca^{++} in the CSF. Data on the severity of injury and demographic characteristics (age, gender, race) was collected for investigation as potential covariates.

4.8.1. Independent variable: cerebral vasospasm

CV was determined in one of three ways: report or direct view of cerebral angiogram (CA), report or direct view of computed topographic angiogram (CTA) or elevated transcranial dopplers (TCD) with clinical neurologic deterioration (CND). For CA and CTA's, vasospasm in the anterior cerebral and anterior communicating, middle cerebral, internal carotid, posterior cerebral and posterior communicating, and vertebral and basilar arteries was classified by a neurosurgeon or neuroradiologist. Cerebral vessels with 0-25% constriction were coded as 'none to minimal vasospasm (CV-). Segments of cerebral vessels with ≥ 26% constriction were coded as moderate to severe vasospasm (CV+). If the absence of CV was documented in the radiology report, the subject was coded as none to minimal vasospasm (CV-). If the presence of moderate or severe CV was documented in the radiology report, the CA or CTA, the subject was coded as positive for moderate to severe vasospasm (CV+).

Transcranial Dopplers (TCDs) were obtained daily. A middle cerebral artery systolic velocity greater than 200mmHg, a mean velocity greater than 120 mmHg and/or a Lindegaard ratio greater than 2.5 was considered elevated. Clinical neurologic deterioration (CND), defined as a decrease of >2 points in GCS or the National Institutes of Health Stroke Scale or computerized neurological assessment sheet, or a deterioration in papillary or motor response, or a documented focal or global neurologic change by the bedside nurse. Presence of elevated TCDs and CND was coded as CV; no elevation in TCDs or CND was coded as CV-.

4.8.2. Independent variable: APOE genotyping

APOE genotype was detected from the CSF samples using the following DNA extraction, amplification, digestion and genotyping procedures.

4.8.2.1. DNA extraction. The CSF specimens were quick thawed in small batches. Deoxyribonucleic acid (DNA) was extracted from the CSF using the following procedure based on the QiaAmp directions (Qiagen, Valencia, CA). Twenty-five µl of each CSF sample was mixed with 200µl of buffer AL and 20µl Proteinase K and vortexed for 15 seconds. An additional 200µl of buffer AL was added and samples were incubated at 70° C for ten minutes and spun down via micro-centrifuge. Two hundred and ten µl's of ethanol (96-100%) was added to each sample before vortexing for 10 seconds to precipitate the DNA. The sample was poured into a spin column and centrifuged at 6000xg for 1 minute before being transferred to a new tube. A 500 µl of buffer AW was added and centrifuged at 6000xg for 1 minute. Excess buffer was poured off, leaving the DNA in the bottom of the tube. Five hundred µl of buffer AW was added and the sample was centrifuged at full speed for 1 minute. Excess buffer was poured off and the sample was placed into a clean tube and centrifuged for 1-minute before 400 µl of buffer AE was added. Samples were then incubated at 70° C for 10 minutes, then centrifuged at 6000xg for 1 minute. The pellet was then resuspended in 1X TE buffer and stored at 4°C.

4.8.2.2. DNA amplification. DNA was amplified using a polymerase chain reaction (PCR) procedure defined by QIAGEN for use with 'Proofstart DNA Polymerase' (Qiagen, Valencia, CA). A stock solution of 'mastermix' was created containing: 50µl 0X Proofstart buffer, 2.5mM

$MgSO_4$, 15µl dNTP, 15µl forward primer with sequence: 5' - TAA GCT TGG CAC GGC TGT CCA AGG A -3', 15µl reverse primer with sequence: 5' - ACA GAA TTC GCC CCG GCC TGG TAC AC - 3', 15µl Proofstart DNA polymerase, and distilled water to a total volume of 500µl. Ten µl of the mastermix solution and 1µl of the extracted DNA solution (for each patient) was placed into a microtube for each sample. Microtubes were placed into a thermal

cycler and heated to 95° C for 5 minutes to activate Proofstart DNA polymerase. The thermal cycler was programmed to heat to 94° C for 1 minute (to break the bonds between the two DNA strands), 50-68° C for 1 minute (to allow annealing), 72° C for 1 minute per 1 kilobase (to allow the nucleotide bases to link to the primers and replicate the region of DNA between them), and repeat 45 times (to produce more copies). The thermal cycler then returned to a temperature of 4° C (to stop the reaction and store the samples).

4.8.2.3. Restriction length fragment polymorphism digestion. The DNA digestion (or 'cut') for the APOE gene was based on polymorphism (Gioia, Vogt et al. 1998). The following procedure was used: 15 µl of amplified DNA, 7.5 U (0.75 l) of the Hha I restriction enzyme (New England Biolabs, MA) and 2µl of 10X buffer was incubated for 1.5 hours at 37°C. Seven and a half U was added to the mixture and digestion continued for 1.5 hours. At the end of the incubation, the reactions were terminated by the addition of EDTA and electrophoresis dyes (R-stop buffer).

4.8.2.4. Genotyping procedure. A polyacrilamide gel was prepared and placed into an electrophoresis machine. Ten µl of orange dye was added to each of five of the 19µl of digested DNA product (sample), and then loaded into the first five wells of the 6 well gel. Ten µl of 1000kb DNA ladder was added to the final well. The electrophoresis machine was turned on and run at 100mv for 2 to 2 1/2 hours until the dye had run 3/4ths of the way down the gel. The electrophoresis machine was disconnected from the power source and the gel removed from the machine. The gel was soaked in a solution of 5µl Ethidium Bromide dye in 200 cc's 1X TBE for 20 minutes, rinsed and viewed under UV light. A computer generated picture of the gel was recorded. Two independent researchers reviewed the pictures and assigned an APOE genotype

based on appropriate banding. Genotyping was repeated in 10% of the samples to assure reliability. The genotype mismatch was not greater than 10%, so the entire process did not need to be rerun on all samples.

4.8.3. Dependent variable: total apoE protein and apoE4 protein quantification

One ml of each CSF sample (each patient days 1-14) was thawed at room temperature and analyzed in batch using enzyme-linked immunosorbent assay (ELISA) analysis (MBL International Corp., Watertown, MA). The quantity of total apoE protein in subject samples was compared against the quantity of apoE in a known standard concentration. The apoE standard was prepared by diluting 2400 nanograms (ng) of lyophilized human apoE with 0.1ml of distilled water. From that standard, seven standards of apoE at concentrations of 240 mg/L, 120 mg/L, 60 mg/L, 30 mg/L, 15 mg/L, 7.5 mg/L and 3.78 mg/L were created. Subject CSF samples were prepared by adding 5 microliters (µl) of CSF and 245µl of a diluent solution that is a 1:4 concentration of assay diluent taken from the kit and distilled water. One hundred µl of this diluted sample and each of the standards was put into two adjacent wells of a ninety-six well plate coated with anti-Human Pan-ApoE antibody. The plate was covered and incubated at 37°C for 60 minutes. Wells were then washed 4 times in an autowasher with 25°C temperature solution of 1:9 concentration of wash concentrate (Phosphate buffer solution). One hundred µl of a 1:100 concentration of apoE conjugate solution (peroxidase conjugated apoE monoclonal antibody) in a conjugate diluent solution consisting of 1:20 concentration of conjugate diluent2 to conjugate diluent1 was added to each well. The plate was again covered and incubated at 37°C for 60 minutes before being washed. One hundred µl of substrate reagent (TMB/H_2O_2) was added to each well and the plate was covered and incubated at 37°C for 30 minutes. One hundred µl of the stop reagent (0.5mol/L H_2SO_4) was added to each well and the plate was

placed into the plate reader. Absorbance was determined using a 450nm wavelength to measure substrate catalysis by transmitting light through the well and measuring the amount of absorption of that light (Crowther 1995). The mean absorbance value of each well was calculated and means of the duplicate wells were averaged.

For subjects with the APOE ε4 allele, a second ELISA was performed using the above methodology but with the following substitutions. The quantity of apoE4 protein in subject samples was compared against the quantity of apoE4 in a known standard concentration. Standards for the apoE4 at concentrations of 120 mg/L, 60 mg/L, 30 mg/L, 15mg/L, 7.5 mg/L, 3.78 mg/L, and 1.89 mg/L were created using the same methodology as above, but beginning with 1200 ng of lyophilized human apoE4 protein. Subject CSF samples were prepared by adding 5 microliters (µl) of CSF and 245µl of a diluent solution that is a 1:4 concentration of assay diluent taken from the kit and distilled water. One hundred µl of this diluted sample and each of the standards was put into two adjacent wells of a ninety-six well plate coated with anti-Human Pan-ApoE antibody. The plate was covered and incubated at 37°C for 60 minutes. Wells were then washed 4 times in an autowasher with 25°C temperature solution of 1:9 concentration of wash concentrate (Phosphate buffer solution). One hundred µl of a 1:100 concentration of apoE4 conjugate solution (peroxidase conjugated apoE4 monoclonal antibody) in a conjugate diluent solution consisting of 1:20 concentration of conjugate diluent2 to conjugate diluent1 was added to each well. The plate was again covered and incubated at 37°C for 60 minutes before being washed. One hundred µl of substrate reagent (TMB/H_2O_2) was added to each well and the plate was covered and incubated at 37°C for 30 minutes. One hundred µl of the stop reagent (0.5mol/L H_2SO_4) was added to each well and the plate was placed into the plate reader. Absorbance was determined using a 450nm wavelength to measure

substrate catalysis by transmitting light through the well and measuring the amount of absorption of that light (Crowther 1995). The mean absorbance value of each well was calculated and means of the duplicate wells were averaged.

4.8.4. Dependent variable: Ca^{++} quantification

The level of total Ca^{++} was quantified from the daily sample of CSF using potentiometry. Potentiometry is a method that uses electrical potential to determine concentration. CSF sample (0.25 ml) was mechanically inserted into a partial electrical circuit completing the circuit. Given that all other components of the electrical circuit are known, the Ca^{++} level can be computed as:

$$Ca^{++} = \text{total potential (measured)} - \text{known potential.}$$

Ca^{++} level measured with potentiometry has a detection precision (or degree of variation) of .008-.01 and accuracy is 98-99% (Covington et al. 1989).

4.8.5. Covariate: severity of injury

Severity of SAH was determined using two indices: the Hunt and Hess grade and Fisher Scores. The Hunt and Hess grading system is based on clinical symptoms, with scores ranging from zero (0), indicating no clinical symptoms, to five (5), indicating a comatose state (Hunt 1983). Oshiro et al. (1997) found the Hunt and Hess scale interrater reliability to be good with a kappa value of 0.41 (p=.0005) (Oshiro, Walter et al. 1997). Gruber et al. (1998) found higher Hunt and Hess scores to be correlated with infarct related to CV (Gruber, Ungersbock et al. 1998).

The Fisher scoring system is a tool that measures severity of neurologic injury based on amount and distribution of blood on CT scan. Fisher scores range from zero (0) indicating no blood noted on CT scan, to four (4) indicating intracerebral or intraventricular clot with diffuse or no blood in the subarachnoid space (Fisher, 1980). Ogilvy and associates found Fisher grade to have excellent interrater reliability with kappa value of 0.9 (Ogilvy and Carter 1998). Higher

Fisher scores have been correlated with CV after SAH (Lasner and Raps 1997; McGirt, Mavropoulos et al. 2003).

4.8.6. Covariate: demographic characteristics

Demographic information (age, gender, and race) was collected from the medical record upon entry into the study.

4.9. DATA MANAGEMENT

Subjects were assigned a unique identification code upon admission to the parent studies. Data were linked by a unique identification code. Data were entered into SPSS package [SPSS Inc, Chicago, Ill.]. Demographic data and severity of injury were entered electronically. CV status and APOE genotype were hand entered into SPSS databases and verified. Demographic data, severity of injury, and APOE genotype databases were merged and apoE protein levels, apoE4 protein levels, and Ca^{++} levels, were hand entered and verified.

4.10. DATA ANALYSIS PLAN

4.10.1. Preliminary data analysis

4.10.1.1. Accuracy. Accuracy of the data was assessed by screening for missing or out of range data. Frequency analyses were performed for all categorical data (gender, race, genotype, CV and APOE ε4 allele presence). There were no missing categorical or descriptive data. Missing data on serial samples (apoE protein, apoE4 protein, and Ca level) were controlled for using hierarchical linear modeling for the analysis.

4.10.1.2. Normality. Normality was assessed by examining histograms, skewness and kurtosis of each variable by research question. No gross non-normality was found in these variables except for race, which was controlled for as a covariate in analysis.

4.10.2. Primary data analysis

Descriptive analyses were described using frequencies, percentages for categorical variables, means standard deviations and ranges for continuous variables. Group comparisons were conducted using t-tests for continuous variables and Chi-square analysis for categorical variables. Protein (apoE and apoE4) levels and Ca^{++} levels were described using mean, range and standard deviation of the entire sample and by group based on the presence or absence of CV and presence or absence of at least one copy of the APOE ε4 allele. The trajectory of protein (apoE and apoE4) and Ca^{++} levels were examined using hierarchical linear modeling (HLM) and results presented as t-ratio and p value. Criteria for level of significance was set at p=.05, criteria for trends was p=.10.

4.10.3. Analysis of specific aims

Specific aim #1. Describe the total and apoE4 isoform-specific CSF apoE protein expression, in relation to APOE genotype, of individuals with and without CV over time for the first 1-14 days after SAH.

RQ1. Is there a difference in total and apoE4 isoform-specific protein expression, in relation to APOE4 genotype, in CSF 1-14 days after SAH in individuals with and without CV?

Specific aim #1 was analyzed using HLM to compare daily CSF apoE level in patients with and without CV while controlling for APOE ε4 allele presence and covariates (i.e. race, age, severity of injury). HLM analysis is an excellent technique to examine serial samples across time and between groups because it is not necessary for all subjects to have the same number of CSF specimens available for analysis. HLM controls for inconsistencies in the number of repeat measures by creating individual regression lines for each subject and computing an average regression line for the entire sample or by group. This analysis examined apoE levels over time using the within subject repeated measures component (sample day after injury) as the level 1 predictor. Level 2 is the between subject component based on APOE4 group status, CV group status, and potential covariates (Hunt and Hess score, Fisher score, age, and race). This model estimated the intercept and slope and by group and tested whether the intercept and slope differed by group based on APOE and CV group status. The variances for the intercept and slope were estimated and tested to determine whether there is substantial group variability around both the mean starting and mean rate of change over time.

The same analysis was applied to compare the daily CSF apoE4 protein levels while controlling for CV and covariates in individuals in the APOE4+ group.

Specific aim #2. Compare CSF Ca^{++} levels, in relation to APOE genotype, of individuals with and without CV over time for the first 1-14 days after SAH.

RQ2. Is there a difference in Ca^{++} levels, in relation to APOE4 genotype, in the CSF 1-14 days after SAH in individuals with and without CV?

Specific aim #2 was assessed using HLM analysis as described in detail under specific aim #1. HLM was used to compare daily CSF Ca^{++} level in patients with and without CV while controlling for APOE ε4 allele presence and the influence of covariates (i.e. race, age, severity of injury). This analysis examined Ca^{++} levels over time with the within subject repeated measures component (sample day after injury) as the level 1 predictor variable. Level 2 is the between subject component based on APOE4 group status, CV group status, and potential covariates (Hunt and Hess score, Fisher score, age, and race). This model estimated intercept and slope by group and tested whether the intercept and slope differ by group based on APOE4 and CV. The variances for the intercept and slope were estimated and tested to determine whether there is substantial group variability around both the mean starting and mean rate of change over time.

Specific aim #3. Compare the relationship between Ca^{++} levels and apoE (genotype and protein) in CSF of acutely ill individuals with and without CV over time for the first 1-14 days after SAH.

RQ3. Is there a difference in the relationship between total apoE versus apoE4 isoform-specific protein expression and Ca^{++} level in the CSF 1-14 days after SAH in individuals with and without CV after controlling for APOE genotype?

Specific aim #3 was assessed using HLM. HLM was used to assess the relationship between daily CSF Ca^{++} and apoE protein levels while controlling for APOE ε4 allele presence, CV presence and the influence of covariates (i.e. age, severity of injury). This analysis examined apoE protein level over time with both the within subject repeated measures component (sample day after injury) and Ca^{++} level as level 1 predictor variables. The level 2 between subject component was based on APOE4 group status, CV group status, and potential covariates (Hunt and Hess score, Fisher score, age, and race). This model estimated means for the initial value and slope and tested whether the intercept and slope are significantly between groups based on APOE4 and CV group status over time. The variances for the intercept and slope were estimated and tested to determine whether there is substantial group variability around both the mean starting and mean rate of change over time.

5. RESULTS

5.1. SAMPLE DESCRIPTION

The mean age of the sample of 97 subjects was 53.7 years old (range 18 to 75; SD±12.8). The sample was primarily Caucasian (n=89; 91.8%) and female (n=65; 67.0%). Refer to table 1 for sample description. Severity of hemorrhage as measured by the Fisher grade ranged from 2-4 with a mode of 3 (n=65; 67.0%) (See table 2): the Hunt and Hess score ranged from 1-5 with a mode of 2 (n=31; 31.0) (See table 3).

	Mean	SD	Range
Age	53.7	12.8	18-75
	N	Percent	
Race (caucasian)	89	91.8	
Gender (female)	65	67.0	

Table 1. Demographic characteristics of sample.

Fisher (*Mode)	n	Percent
0- No CT scan	0	0
1- No blood detected on CT scan	0	0
2- Diffuse or vertical layers of blood <1mm	14	14.4
3- Localized clot &/or vertical layers of blood >1mm*	65 *	67.0 *
4- Intracerebral or intraventricular clot with diffuse or no SAH	18	18.6

Table 2. Severity of injury characteristics of sample: Fisher grade.

Hunt & Hess (*Mode)	n	Percent
1- Asymptomatic, mild headache, nuchal rigidity	15	15.5
2- Mod-severe HA, nuchal rigidity	31 *	32.0 *
3- Other than cranial nerve palsy, drowsy	27	27.8
4- Stupor, moderate to severe hemiparesis, decerebrate	15	15.5
5- Deep coma	9	9.3

Table 3. Severity of injury characteristics of sample: Hunt and Hess grade.

5.2. VARIABLES

5.2.1. Independent variable: APOE genotype

Figure 6 provides the genotype distribution of this sample. See table 4 for a comparison of genotype distribution in this population to other North American populations. Twenty-four subjects (24.0%) had an APOE ε4 allele present (see figure 7). There were no significant differences between individuals with and without an APOE ε4 allele in categories of race, age, gender or severity of hemorrhage (Hunt and Hess or Fisher grade). See table 5 for comparison of demographic and severity of hemorrhage scores by the presence/absence of an APOE ε4 allele.

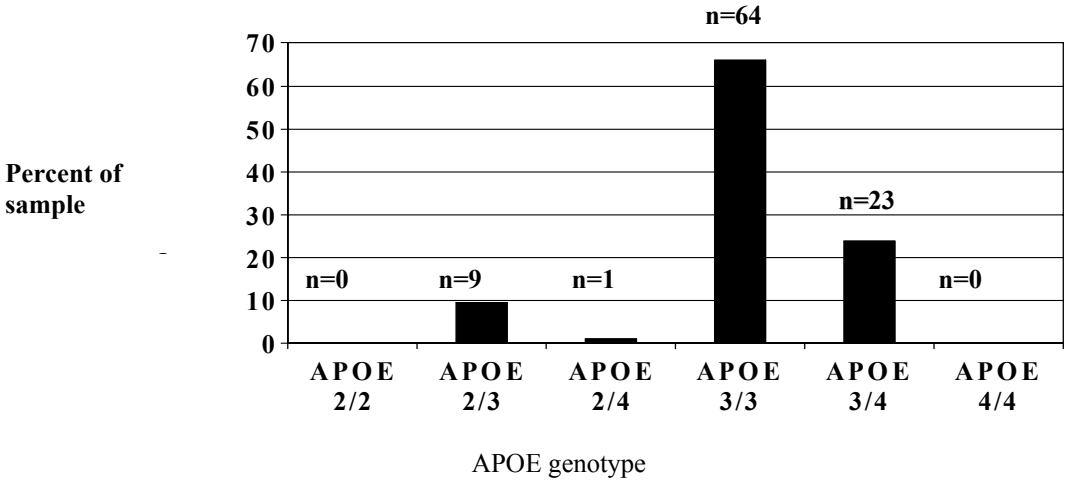

Figure 6. APOE genotype distribution of the sample.

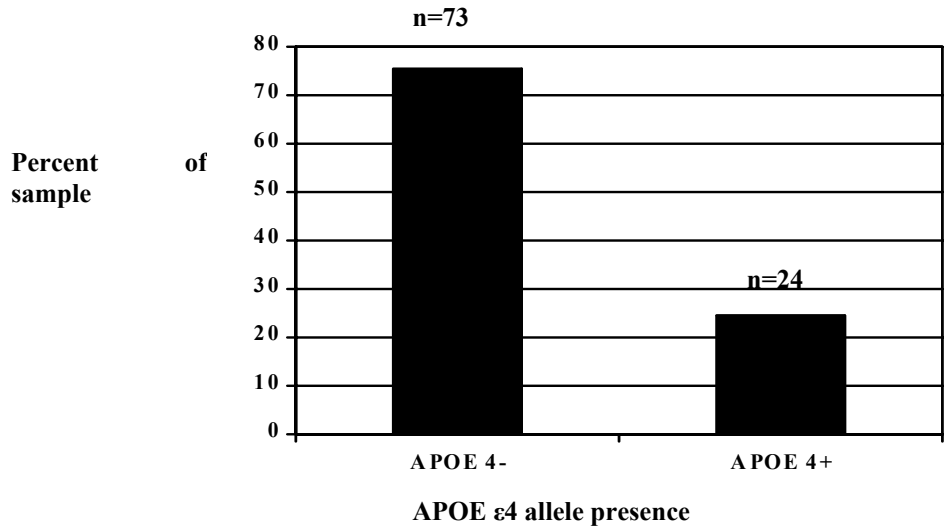

Figure 7. APOE4 allele presence in the sample.

Population	ε2	ε3	ε4	Reference
All (male, twins) N=396	.09	.76	.15	DeCarli et al. (1999)
All N=61	.11	.72	.17	Zannis et al. (1981)
SAH N=97	.05	.83	.12	Current study

Table 4. APOE allele frequencies of different North American populations

	Age Mean (SD)	Race % white (n)	Gender % female (n)	HH mode (%)	Fisher mode (%)
APOE4+	54.3 (12.9)	87.5 (21)	62.5 (15)	2 (41.7)	3 (58.3)
APOE 4-	52.0 (12.6)	93.2 (68)	68.5 (50)	2/3 (28.8/30)1)	3 (69.9)
Statistic	t=.77	F=1.22	F=.29	F=.34	F=.169
p	.45	.27	.59	.56	.68

Table 5. Demographic and severity of injury characteristics by APOE4 allele presence

5.2.2. Independent variable: presence of CV

The presence or absence of CV was determined utilizing three methods. Twenty-eight (28.9%) subjects had CV status verified by a cerebral angiogram reviewed by a project physician; an additional twenty-nine subjects (29.9%) had documentation of a cerebral angiogram by medical record review. Five (5.2%) subjects had CV status verified by a CTA reviewed by a project physician; an additional 9 (9.3%) subjects had documentation of a CTA by medical record review. The presence of CV determined by an elevation in TCDs associated with clinical neurologic deterioration was used to determine CV status of 26 (25.7%) subjects. See figure 8 and table 6 for diagnostic breakdown. Fifty-three (54.6%) subjects were categorized as CV+ (see figure 9). There was not a statistically significant difference in source of identification of vasospasm by vasospasm presence (χ^2=4.7; p=.33). The number of hours from hemorrhage to CV identification ranged from 30 to 360, with a mean of 129.83 hours (SD±67.63). There were no significant differences between individuals with and without CV in categories of race, age, gender, Hunt and Hess score or Fisher grade (see table 7).

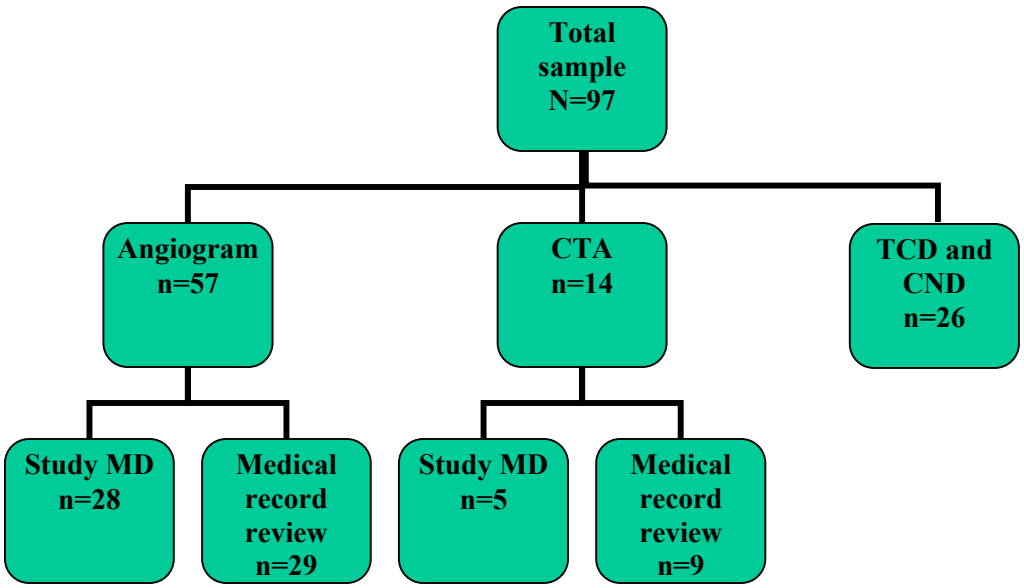

Figure 8. CV diagnosis

	Cerebral Angiogram		CTA		Elevated TCDs with CND n (%)
	Project MD read n (%)	MR review n (%)	Project MD read n (%)	MR review n (%)	
CV+	14 (14.4)	18 (18.6)	4 (4.1)	2 (2.1)	15 (15.5)
CV-	14 (14.4)	11 (11.3)	1 (1.0)	7 (7.2)	11 (11.3)
Total	28 (28.9)	29 (29.9)	5 (5.2)	9 (9.3)	26 (26.8)

Table 6. CV diagnostic method by group.

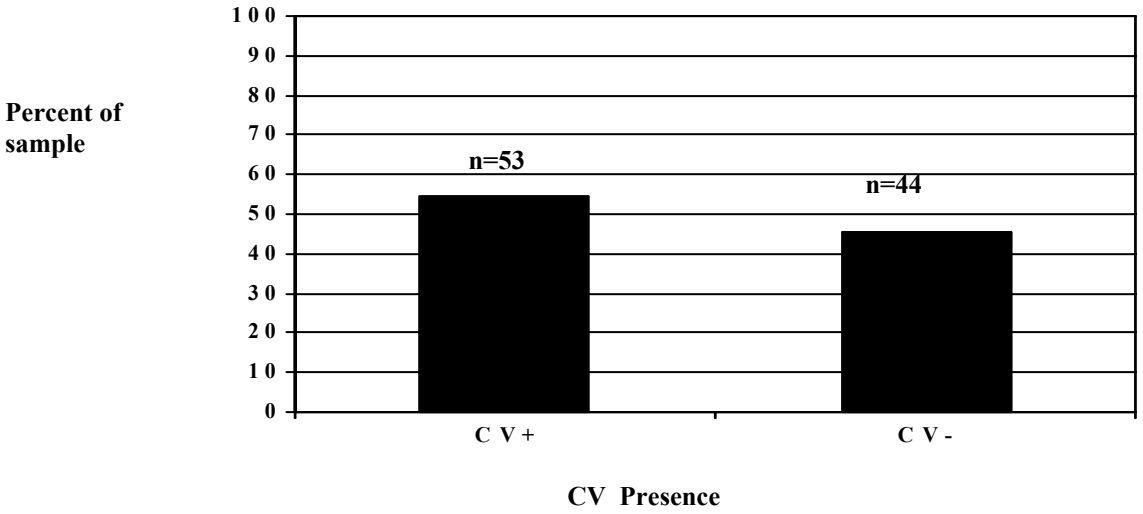

Figure 9. CV distribution of the sample

	CV+			CV-			t	p
	M	**SD**		**M**	**SD**			
Age	52.5	12.7		55.1	12.9		1.01	.32
	n	**%**		**n**	**%**		**F**	**p**
Female	37	69.8		28	63.6		.41	.53
Caucasian	49	92.5		40	90.9		.01	.92
	Mode	**n**	**%**	**Mode**	**n**	**%**		
Hunt & Hess	3	17	32.1	2	16	36.4	2.08	.15
Fisher	3	34	64.2	3	31	70.5	2.97	.09

Table 7. Demographic and severity of injury by CV group

5.2.3. APOE ε4 allele presence and CV

Of the 24 subjects with an APOE ε4 allele, 54.2% (n=13) had CV compared to 54.8% (n=40) of the 73 subjects without an APOE ε4 allele. There were three subjects without CV data, none of whom had an APOE ε4 allele. See table 8 for CV and CV diagnostic method by APOE ε4 allele presence. There was not a significant association between CV presence and APOE ε4 allele presence (χ^2=.003; p=.96).

	CV n (%)	Cerebral Angiogram		CTA		Elevated TCDs with CND n (%)
		Project MD read n (%)	MR review n (%)	Project MD read n (%)	MR review n (%)	
APOE4+						
CV+	**13**	**5 (20.8)**	**4 (16.7)**	**0 (0)**	**0 (0)**	**4 (16.7)**
CV-	**11**	**6 (25.0)**	**4 (16.7)**	**1 (4.2)**	**0 (0)**	**0 (0)**
APOE4-						
CV+	**40**	**9 (12.3)**	**14 (19.2)**	**4 (5.5)**	**3 (4.1)**	**10 (13.7)**
CV-	**33**	**8 (11.0)**	**7 (9.6)**	**0 (0)**	**7 (9.6)**	**11 (15.1)**
Total	**97**	**28 (28.9)**	**29 (29.9)**	**5 (5.2)**	**10 (10.3)**	**25 (25.8)**

Table 8. CV and CV diagnostic method by APOE ε4 allele presence

5.2.4. Dependent variable: total apoE protein level

There were 703 samples available over time for analysis in 97 subjects. Individuals had between 1 and 13 samples available for analysis with an average of 7.6 samples per subject (SD±3.49). The total apoE protein level of all samples at all timepoints was a mean of 10.06 mg/L (Range 0-68.8, SD±8.14). See table 9 for daily mean total apoE protein levels.

5.2.4.1. Total apoE protein level and APOE ε4 allele presence. The total apoE protein level of all samples at all timepoints in the APOE4+ group was a mean of 11.7 mg/L (range 0.25-42.93, SD±7.59) as compared to the APOE4- group mean of 9.5 mg/L (range 0-68.76, SD±8.26). The APOE4+ group had a significantly higher mean total apoE protein level (t=-3.168; p=.002). See table 9 for daily mean total apoE protein levels by APOE4 group.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
n	5	16	19	20	20	19	17	15	14	13	11	9	8	4
APOE4+ Mean apoE (SD)	14.4 (14.1)	11.3 (7.5)	9.9 (5.1)	11.4 (10.1)	8.7 (4.2)	10.1 (5.0)	11.7 (6.7)	11.4 (8.3)	12.1 (5.2)	12.5 (5.3)	17.4 (8.7)	16.5 (8.7)	13.4 (8.8)	13.6 (8.1)
n	24	47	56	62	63	52	45	40	36	30	26	23	15	6
APOE4- Mean apoE (SD)	9.9 (8.2)	8.3 (6.2)	9.4 (7.8)	9.5 (8.2)	9.1 (9.0)	9.6 (11.2)	7.9 (5.8)	8.4 (5.9)	10.1 (6.2)	12.2 (12.3)	11.7 (7.8)	12.7 (6.2)	9.0 (6.0)	11.8 (8.5)
N	29	63	75	82	83	71	62	55	50	43	37	32	23	10
Mean apoE (SD)	10.7 (9.3)	9.1 (6.6)	9.5 (7.2)	10.0 (8.9)	9.0 (8.1)	9.7 (9.9)	8.9 (6.3)	9.2 (6.7)	10.7 (6.0)	12.3 (10.7)	13.4 (8.4)	13.8 (7.1)	10.6 (7.2)	12.5 (7.9)

Table 9. Mean total apoE protein level (in mg/L) by day by APOE ε4 allele presence.

5.2.4.2. Total apoE protein level and CV. The total apoE protein level of all samples at all timepoints in the CV+ group was a mean of 11.7 mg/L (range .01-58.69, SD±8.43) as compared

to the CV- group mean of 9.5 mg/L (range 0-68.76, SD±7.69). There was no significant difference in the overall average ApoE protein level between these two groups (t= 1.008; p=.32). See table 10 for daily mean total apoE protein levels by CV group.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
n	18	35	43	45	47	42	39	35	32	28	26	23	16	6
CV+ Mean apoE (SD)	10.6 (9.9)	8.3 (5.2)	8.9 (7.7)	9.4 (9.3)	8.4 (8.4)	10.2 (11.3)	9.0 (5.3)	9.0 (6.4)	9.9 (5.3)	10.6 (4.9)	13.2 (7.7)	14.4 (7.0)	10.6 (7.2)	13.4 (7.4)
n	11	28	32	37	36	29	23	20	18	15	11	9	7	4
CV- Mean apoE (SD)	10.8 (8.6)	10.0 (7.4)	10.3 (6.5)	10.6 (8.5)	9.8 (7.6)	9.1 (7.5)	8.9 (7.8)	9.6 (7.3)	12.0 (7.0)	15.4 (16.0)	13.9 (10.3)	12.2 (7.5)	10.5 (7.8)	11.3 (9.7)
N	29	63	75	82	83	71	62	55	50	43	37	32	23	10
ApoE level (SD)	10.7 (9.3)	9.1 (6.6)	9.5 (7.2)	10.0 (8.9)	9.0 (8.1)	9.7 (9.9)	8.9 (6.3)	9.2 (6.7)	10.7 (6.0)	12.3 (10.7)	13.4 (8.4)	13.8 (7.1)	10.6 (7.2)	12.5 (7.9)

Table 10. Mean total apoE protein level (in mg/L) by day by CV group.

5.2.5. ApoE4 protein level

The overall apoE4 protein level of all samples in the APOE ε4 allele present group was a mean of 8.5 mg/L (Range 6.0-23.8, SD±6.6). On average, apoE4 protein accounted for 62% of the total apoE protein in CSF. See table 11 for mean apoE4 protein by day.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
N	4	15	16	18	19	17	15	13	13	11	8	8	6	4
Mean apoE4 (SD)	9.2 (7.5)	6.8 (4.8)	5.5 (3.4)	5.2 (3.6)	5.0 (3.7)	8.8 (9.5)	8.5 (9.4)	10.8 (13.2)	8.8 (9.2)	6.4 (3.7)	7.8 (4.1)	8.3 (5.5)	16.3 (12.8)	13.1 (11.0)

Table 11. Mean apoE4 protein level (in mg/L) by day.

5.2.5.1. ApoE4 level and CV. The apoE4 protein level of all samples at all timepoints in the CV+ group was a mean of 8.6 mg/L (range 1.9-20.8, SD±5.8) as compared to the CV- group mean of 8.4 mg/L (range 6.0-23.8, SD±7.8). There was no significant difference in the mean apoE4 protein level of all samples at all timepoints of the two groups (t=-6.83; p=.50). See table 12 for mean apoE4 protein level by day by CV group.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
n	3	9	9	10	11	9	9	8	8	7	5	5	4	4
CV+ Mean apoE4 (SD)	11.2 (7.8)	6.1 (2.8)	5.1 (3.4)	5.7 (3.1)	5.6 (4.3)	6.2 (4.6)	7.3 (2.8)	7.4 (5.1)	6.8 (3.6)	6.2 (2.6)	7.8 (4.1)	10.6 (5.9)	9.3 (7.7)	15.3 (12.3)
n	1	6	7	8	8	8	7	6	5	4	3	3	2	1
CV- Mean apoE4 (SD)	32.3 n/a	7.8 (7.1)	5.9 (3.5)	4.5 (4.1)	4.1 (2.5)	11.8 (12.8)	12.5 (13.8)	10.2 (15.1)	12.0 (14.5)	6.9 (5.6)	7.7 (5.1)	4.6 (12.7)	4.6 (3.8)	6.3 n/a
N	4	15	16	18	19	17	15	13	13	11	8	8	6	4
Mean apoE4 (SD)	9.2 (7.5)	6.8 (4.8)	5.5 (3.4)	5.2 (3.6)	5.0 (3.7)	8.8 (9.5)	8.5 (9.4)	10.8 (13.2)	8.8 (9.2)	6.4 (3.7)	7.8 (4.1)	8.3 (5.5)	16.3 (12.8)	13.1 (11.0)

Table 12. Mean apoE4 protein level (in mg/L) by day by CV group.

5.2.6. Total apoE and apoE4 ratio by CV

See table 13 for total apoE to apoE4 ratio by day by CV.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
n	4	15	16	18	19	17	15	13	13	11	8	8	6	4
ratio (SD)	9.2 (7.5)	6.8 (4.8)	5.5 (3.4)	5.2 (3.6)	5.0 (3.7)	8.8 (9.5)	8.5 (9.4)	10.8 (13.2)	8.8 (9.2)	6.4 (3.7)	7.8 (4.1)	8.3 (5.5)	16.3 (12.8)	13.1 (11.0)

Table 13. Total apoE to apoE4 ratio by day

5.2.7. Dependent variable: Ca^{++} level

The Ca^{++} level of all samples at all timepoints was a mean of 0.97 meq/L (Range 0.2-1.79, SD±.26).

5.2.7.1. Ca^{++} level and APOE ε4 allele presence. The Ca^{++} level of all samples at all timepoints in subjects with an APOE ε4 allele present was a mean of .89 meq/L (range .2-1.68, SD±.23) as compared to those without an APOE ε4 allele with a mean of 1.00 meq/L (range .41-1.79, SD±.26). There was a significant difference in the mean Ca^{++} level of all samples at all timepoints in subjects with and without an APOE ε4 allele present (t=4.78; p=.00). See table 14 for mean Ca^{++} by day by APOE ε4 allele presence.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
n	**5**	**15**	**18**	**20**	**20**	**19**	**17**	**15**	**13**	**13**	**11**	**9**	**8**	**5**
APOE4+ Mean Ca^{++} (SD)	**.80 (.2)**	**.79 (.21)**	**.84 (.21)**	**.90 (.21)**	**.91 (.26)**	**.97 (.29)**	**1.03 (.26)**	**.99 (.23)**	**.86 (.18)**	**.85 (.28)**	**.83 (.19)**	**.83 (.15)**	**.86 (.18)**	**.90 (.24)**
n	**23**	**46**	**57**	**61**	**61**	**51**	**44**	**40**	**36**	**29**	**25**	**21**	**15**	**5**
APOE4- Mean Ca^{++} (SD)	**1.04 (.28)**	**1.03 (.29)**	**.98 (.28)**	**1.01 (.23)**	**.97 (.24)**	**.96 (.26)**	**.99 (.28)**	**.99 (.28)**	**.98 (.25)**	**1.00 (.23)**	**1.02 (.27)**	**1.03 (.25)**	**1.02 (.30)**	**.94 (.32)**
N	**28**	**61**	**75**	**81**	**81**	**70**	**61**	**55**	**49**	**42**	**36**	**30**	**23**	**10**
Mean Ca^{++} (SD)	**1.00 (.27)**	**.98 (.28)**	**.95 (.27)**	**.97 (.27)**	**.95 (.25)**	**9.6 (.28)**	**1.00 (.27)**	**.99 (.26)**	**.95 (.24)**	**1.00 (.23)**	**.96 (.26)**	**.97 (.24)**	**.97 (.27)**	**.92 (.27)**

Table 14. Mean Ca^{++} level (in meq/L) by day by APOE ε4 allele presence.

5.2.7.2. **Ca^{++} level and CV.** The Ca^{++} level of all samples at all timepoints in the CV+ group was a mean of .96 meq/L (range .29-1.79, SD±.26) as compared to the CV- group was a mean of .99 meq/L (range .2-1.75, SD±.26). There were trends suggesting a possible difference in Ca^{++} level of all samples at all timepoints in subjects with and without CV (t=1.66; p=.098). See table 15 for mean Ca^{++} level by CV group.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
n	**16**	**33**	**42**	**44**	**45**	**41**	**38**	**35**	**32**	**26**	**25**	**21**	**16**	**7**
CV+ Mean Ca^{++} (SD)	**.88 (.15)**	**.92 (.24)**	**.93 (.26)**	**.96 (.24)**	**.96 (.26)**	**.99 (.28)**	**1.03 (.30)**	**1.00 (.29)**	**.93 (.26)**	**.92 (.24)**	**.95 (.27)**	**.95 (.24)**	**.96 (.30)**	**.97 (.29)**
n	**12**	**28**	**33**	**37**	**36**	**29**	**23**	**20**	**17**	**16**	**11**	**9**	**7**	**3**
CV- Mean Ca^{++} (SD)	**1.17 (.34)**	**1.04 (.33)**	**.98 (.28)**	**.99 (.22)**	**.95 (.24)**	**.93 (.25)**	**.96 (.23)**	**.97 (.20)**	**.98 (.19)**	**1.01 (.22)**	**.98 (.28)**	**1.00 (.25)**	**.98 (.23)**	**.81 (.22)**
N	**28**	**61**	**75**	**81**	**81**	**70**	**61**	**55**	**49**	**42**	**36**	**30**	**23**	**10**
Mean Ca^{++} (SD)	**1.00 (.27)**	**.98 (.28)**	**.95 (.27)**	**.97 (.23)**	**.95 (.25)**	**9.6 (.28)**	**1.00 (.27)**	**.99 (.26)**	**.95 (.24)**	**.95 (.24)**	**.96 (.26)**	**.97 (.24)**	**.97 (.27)**	**.92 (.27)**

Table 15. Mean Ca^{++} level (in meq/L) by day by CV group.

5.3. RESEARCH QUESTIONS

5.3.1. Research question #1

1. Is there a difference in total apoE and apoE4 isoform-specific protein expression, in relation to APOE4 genotype, in CSF 1-14 days after SAH in individuals with and without CV?

5.3.1.1. Total apoE level over time. There were significant changes in total apoE protein level over time (t-ratio=.091; p=.003), total apoE protein levels started out lower than normal and continued to decrease over time until day 10 post-bleed when they rose only to drop again on day 13 post-bleed. See figure 10 for predicted mean total apoE protein level over time.

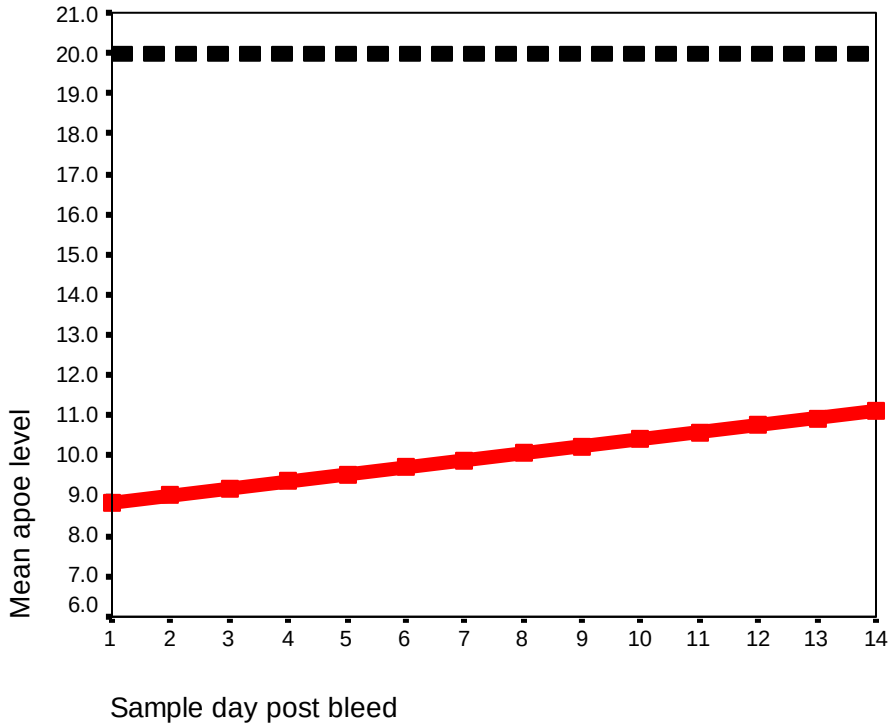

Figure 10. Predicted mean total apoE protein level (in mg/L) over time.

5.3.1.2. Total apoE level over time by APOE ε4 allele presence. There were no significant differences in the daily total CSF apoE levels (t-ratio=1.30; p=.195) or in the trajectory of those levels in subjects with and without an APOE ε4 allele present (t-ratio=1.42; p=.157) (See figure 11). See table 9 for mean total apoE protein level by day by APOE ε4 allele presence.

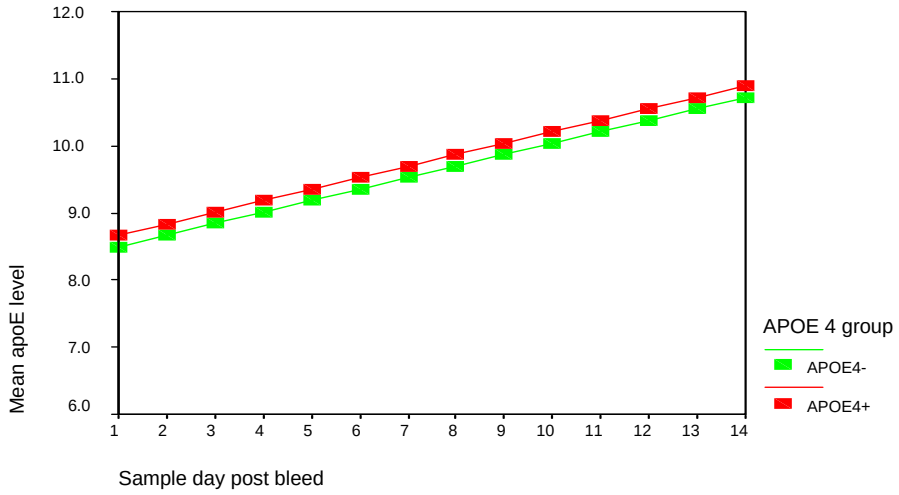

Figure 11. Predicted mean total apoE protein level (in mg/L) by day by APOE ε4 allele presence.

5.3.1.3. Total apoE level over time by CV presence. There were no statistically significant differences in daily CSF total apoE protein level (t-ratio= -.08; p=.415) or trajectory of mean total apoE protein level by CV presence (t-ratio=.1.46; p=.143) (See figure 12). See table 10 for mean total apoE protein level by day by CV.

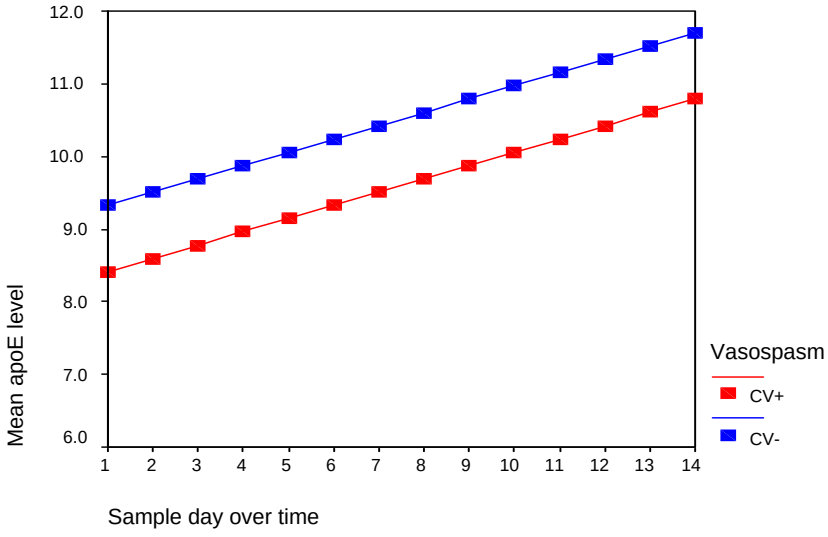

Figure 12. Predicted mean total apoE protein level (in mg/L) by day by CV group.

5.3.1.4. Total apoE level by APOE ε4 allele presence and CV. The total apoE protein level of all samples at all timepoints for subjects with an APOE ε4 allele and with CV (n=13) was a mean of 11.7 mg/L (range .25-42.9, SD±7.7 mg/L). The total apoE protein level of all samples at all timepoints for subjects without an APOE ε4 allele and with CV (n=40) was a mean of 9.1 mg/L (range .9-16.8, SD±3.4 mg/L). The total apoE protein level of all samples at all timepoints for subjects with an APOE ε4 allele but without CV (n=11) was 11.6 mg/L (range .5-23.0, SD±5.7 mg/L). The total apoE protein level of all samples at all timepoints for subjects without an APOE ε4 allele or CV (n=33) was 10.1 mg/L (range 0-68.7, SD±8.6 mg/L). See figure 13 for graph of total apoE mean by subgroups.

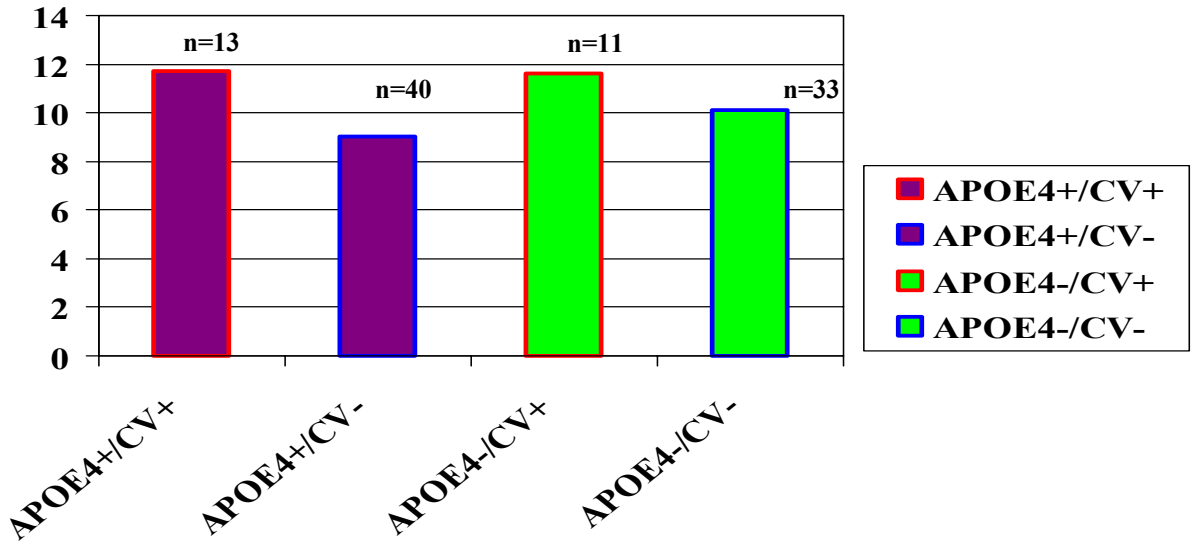

Figure 13. Mean total apoE protein level (in mg/L) by subgroups.

5.3.1.5. Total apoE over time with covariates, APOE ε4 allele presence and CV. There was not a significant association between daily CSF total apoE protein level and any covariates, APOE ε4 allele presence (t-ratio=.752; p=.452), or CV presence (t-ratio=-.705; p=.480).

5.3.1.6. Total apoE levels and apoE4 levels. There was a significant association between mean total apoE protein level and mean apoE4 protein level (t-ratio=.526 p=.000), although this association did not change over time (t-ratio=.386, p=.702). CV did not impact this relationship when added to the model (t-ratio=-.691; p=.497). See table 16 for mean total apoE to apoE4 ratio by day by CV.

	day 1	day 2	day 3	day 4	day 5	day 6	day 7	day 8	day 9	day 10	day 11	day 12	day 13	day 14
n	1	6	7	6	8	6	4	2	3	3	2	1	1	1
CV+ ratio (SD)	.56 n/a	.56 (.11)	.47 (.09)	.41 (.08)	.51 (.09)	.63 (.08)	.62 (.07)	.66 (.07)	.54 (.17)	.48 (.08)	.48 (.11)	.39 n/a	.30 n/a	.44 n/a
n	3	7	7	8	9	7	8	6	7	6	4	5	4	2
CV- ratio (SD)	.61 (.05)	.56 (.02)	.63 (.05)	.53 (.09)	.63 (.08)	.65 (.07)	.65 (.04)	.65 (.04)	.55 (.06)	.65 (.10)	.65 (.11)	.68 (.11)	.74 (.12)	.90 (.01)
N	4	15	16	18	19	17	15	13	13	11	8	8	6	4
ratio (SD)	9.2 (7.5)	6.8 (4.8)	5.5 (3.4)	5.2 (3.6)	5.0 (3.7)	8.8 (9.5)	8.5 (9.4)	10.8 (13.2)	8.8 (9.2)	6.4 (3.7)	7.8 (4.1)	8.3 (5.5)	16.3 (12.8)	13.1 (11.0)

Table 16. Total apoE to apoE4 ratio by day by CV

5.3.1.7. ApoE4 levels over time by CV. There was no statistically significant difference in daily mean apoE4 levels (t-ratio=1.16; p=.259) or trajectory of daily mean apoE4 protein levels by CV (t-ratio=1.412; p=.171). See table 12 for mean apoE4 protein level by day by CV.

5.3.2. Research question #2

2. Is there a difference in Ca^{++} levels, in relation to APOE4 genotype, in the CSF 1-14 days after SAH in individuals with and without CV?

5.3.2.1. Ca^{++} level over time. Ca^{++} level did not change over time (t-ratio= -.549; p=.582). See figure 14 for modeled mean Ca^{++} level over time.

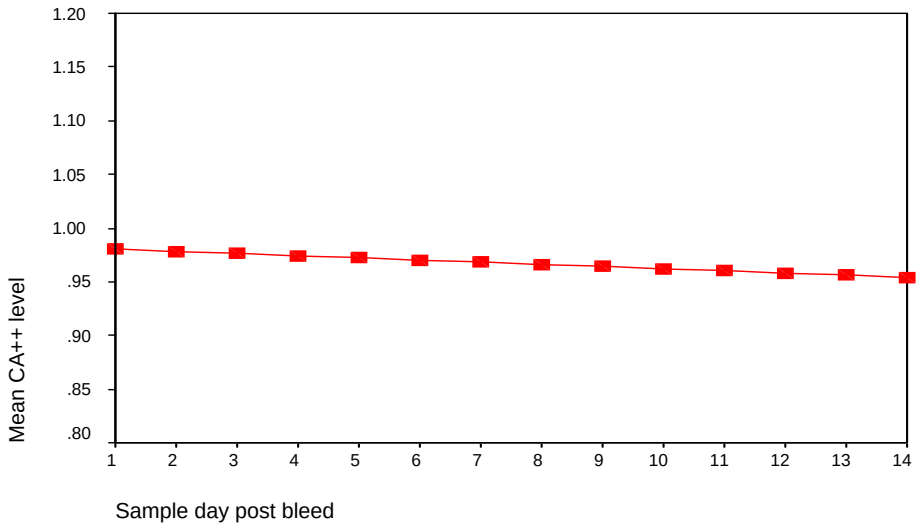

Figure 14. Predicted mean Ca^{++} level (in meq/L) over time.

5.3.2.2. **Ca^{++} level over time and APOE ε4 allele presence.** There was a significant difference in the daily Ca^{++} level of subjects with and without an APOE ε4 allele (t-ratio=-4.738; p=.000). Individuals with an APOE ε4 allele had lower Ca^{++} levels, but there was not a difference in the trajectory across time (t-ratio=-0.265; p=.791). See figure 15 for graph of modeled Ca^{++} levels by APOE ε4 allele presence. See table 14 for mean Ca^{++} level by day by APOE ε4 allele presence.

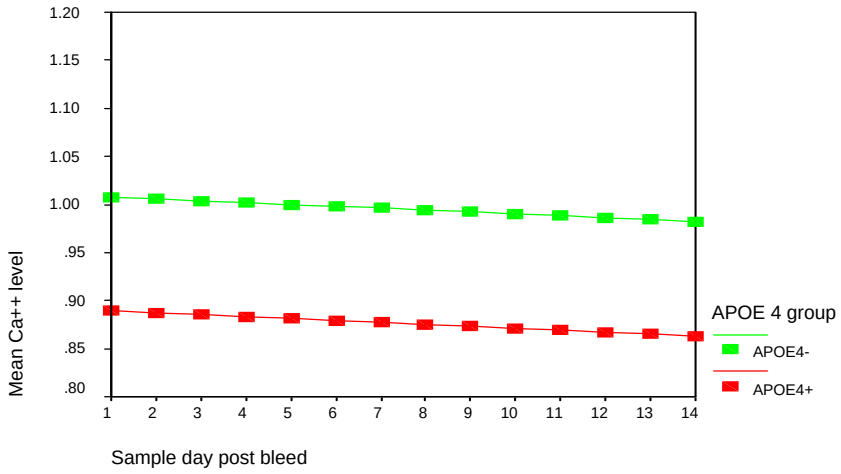

Figure 15. Predicted mean Ca^{++} level (in meq/L) by day by APOE ε4 allele presence.

5.3.2.3. Ca^{++} level over time and CV. There was no significant difference in daily Ca^{++} levels in subjects with and without CV (t-ratio=-.322, p=.740) nor was there a significant difference in the trajectory of Ca^{++} levels in subjects with and without CV (t=-.535; p=.592). See figure 16 for modeled Ca^{++} level by CV. See table 15 for mean Ca^{++} level by day by CV group.

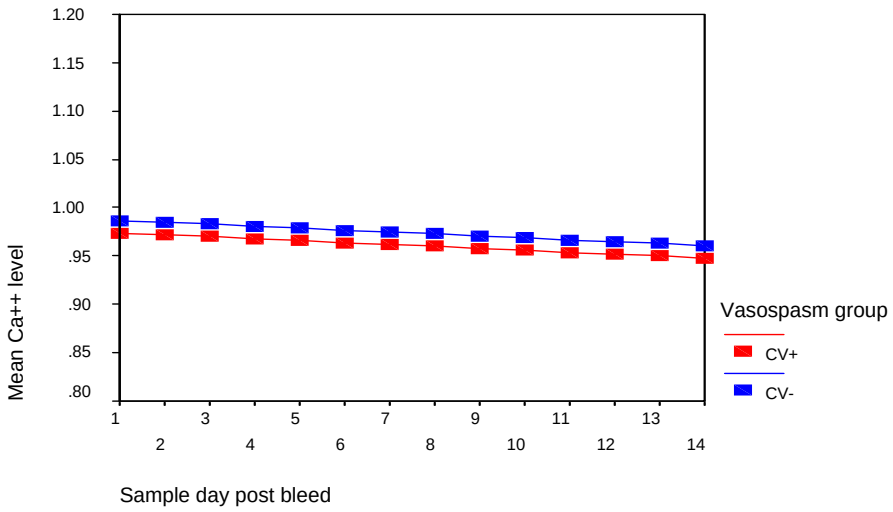

Figure 16. Predicted mean Ca^{++} level (in meq/L) by day by CV group.

5.3.2.4. Ca^{++} level and APOE ε4 allele presence and CV. The Ca^{++} level of all samples at all timepoints for subjects with an APOE ε4 allele and CV (n=13) was a mean of .96 meq/L (range .29-1.68, SD± .24 meq/L). The Ca^{++} level in individuals without an APOE ε4 allele and with CV (n=40) was a mean of .96 meq/L (range .64-1.79, SD±.29 meq/L). The Ca^{++} level of all samples at all timepoints for subjects with an APOE ε4 allele but without CV (n=11) was .78 meq/L (range .2-1.14, SD±.21 meq/L). The Ca^{++} level of all samples at all timepoints for subjects without an APOE ε4 allele or CV (n=33) was 1.05 meq/L (range .54-1.67, SD±.22 meq/L). See figure 17 for mean Ca^{++} level of subgroups.

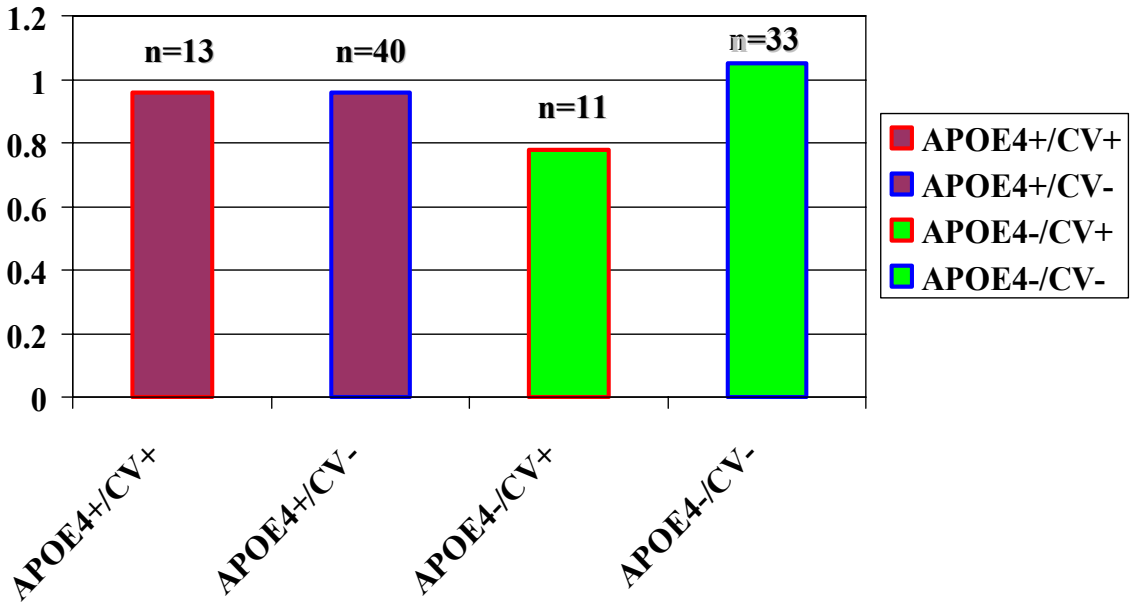

Figure 17. Mean Ca^{++} level (in meq/L) by subgroups

5.3.2.5. Ca^{++} level over time with covariates, APOE ε4 allele presence and CV. There was a significant association between daily Ca^{++} level and APOE ε4 allele presence (t-ratio=-4.738; p=.000). Ca^{++} levels were lower in subjects with an APOE ε4 allele, but there were no differences in the trajectory across time between the two groups (t-ratio=-.265; p=.791). There was no significant association between daily Ca^{++} level and CV presence (t-ratio=-1.576; p=.115).

5.3.3. Research question #3

3. Is there a difference in the relationship between total apoE and apoE4 isoform-specific protein expression and Ca^{++} level in the CSF 1-14 days after SAH in individuals with and without CV after controlling for APOE genotype?

5.3.3.1. Total apoE protein and Ca^{++} levels. There was a statistically significant association between daily total apoE protein level and Ca^{++} level (t-ratio=3.458, p=.001), and this association did not change over time (t-ratio=-.098, p=.327).

5.3.3.2. Total apoE protein and Ca++ levels and APOE ε4 allele presence. When APOE ε4 allele presence was entered into the model, it was found to be a significant predictor (t-ratio=-3.272, p=.001) however the association between daily total apoE protein level and daily mean Ca^{++} level remained essentially unchanged (t-ratio=3.586; p=.001). Further, the fit of this model (indicated by a covariance component deviance of -328.54 vs –316.7) remained essentially unchanged.

5.3.3.3. Total apoE protein and Ca^{++} levels and CV. There was no significant change in the relationship between total apoE protein and Ca^{++} level in the presence or absence of CV (t-ratio=-.217, p=.829).

5.3.3.4. Total apoE protein and Ca^{++} levels, covariates, APOE ε4 allele presence and CV. The presence of the APOE ε4 allele was predictive (t-ratio=-3.272, p=.001) of the association between total apoE protein level and Ca^{++} level, with individuals without the APOE ε4 allele having a slightly stronger association. There were no changes in the model when covariates age (t-ratio=-.353, p=.724), race (t-ratio=.500, p=.616), gender (t-ratio=-1.282, p=.20), Hunt and Hess grade (t-ratio=.582, p=.56), Fisher grade (t-ratio=-.573, p=.566), or CV (t-ratio=-.204, p.838) were added to the model.

5.3.3.5. ApoE4 protein and Ca^{++} levels. There was no significant association between daily apoE4 protein and Ca^{++} levels (t-ratio=1.317, p=.201).

5.3.3.6. ApoE4 protein and Ca^{++} levels and CV. Controlling for the presence of CV, there was no change in the association between daily apoE4 protein and Ca^{++} levels (t-ratio=1.145, p=.264).

6. DISCUSSION

This study examined the relationship between APOE genotype, daily CSF total apoE protein level, and daily CSF Ca^{++} levels in the presence and absence of CV after SAH. We hypothesized that individuals with an APOE ε4 allele would have lower CSF Ca^{++} and total apoE protein levels and that individuals with CV would have lower CSF Ca^{++} and total apoE protein levels.

6.1. APOE ε4 ALLELE PRESENCE

Twenty-four percent of this population had an APOE ε4 allele as compared to approximately 30-35% obtained in other studies of North American populations (Zannis 1986) (Decarli, Reed et al. 1999). While this difference was not significant, some suggest that the presence of the APOE ε4 allele may predispose an individual to a more severe initial injury making survival to the hospital (and subsequent entry into study) less likely (McCarron and Nicoll 1998). McCarron and Nicoll found that individuals with SAH had a higher than expected frequency of the APOE ε4 allele at autopsy (McCarron and Nicoll 1998). Dunn and associates also found that the APOE ε4 allele was under represented in their SAH patient population (Dunn, Stewart et al. 2001). Niskangas and associates found APOE ε4 allele representation of their SAH population similar to that of the general population in the area (Heilsinki) (Niskakangas 2001). Tang and associates found a statistically insignificant higher representation of the APOE ε4 allele in their Chinese population as compared with controls (Tang, Zhao et al. 2003).

This study did not find APOE ε4 allele specific differences in admission severity of injury scores, however there were fewer individuals with an APOE ε4 allele than anticipated.

Other groups also found a lack of APOE ε4 allele specific differences in Hunt and Hess scores (Niskakangas 2001; Tang, Zhao et al. 2003) or Glasgow coma scores on admission (Dunn, Stewart et al. 2001). Leung and associates found contrary results in that in their Chinese population, individuals with an APOE ε4 allele had higher World Federation neurologic scale scores, indicating worse clinical presentation on admission than individuals without an APOE ε4 allele (Leung, Poon et al. 2002). Others did not collect clinical presentation measures on admission (Morris, Wilson et al. 2004). This evidence suggests that in non-Asian populations, individuals with an APOE ε4 allele had similar or less severe clinical presentations on admission.

6.2. APOE ε4 ALLELE PRESENCE AND CV

This study found a relationship between APOE ε4 allele presence and Ca^{++} levels but no association between APOE ε4 allele presence and CV after SAH. To our knowledge, this is the first study to examine APOE ε4 allele presence and possible mechanisms for CV.

While individuals with CV and with an APOE ε4 allele had the highest apoE levels, individuals without CV and without the APOE ε4 allele had the highest CSF Ca^{++} levels. In the brain, the production and secretion of apoE by astrocytes increases in response to central nervous system injury (Messmer-Joudrier, Sagot et al. 1996; Lin, Duan et al. 1999; Duan, Gu et al. 2000). Intracellular Ca^{++} in both astrocytes and neurons increases in an apoE4 dose dependent manner, with apoE4 associated with greater levels of intracellular Ca^{++} (Muller, Meske et al. 1998). The rise in Ca^{++} is associated with neurotoxicity and neuronal death (Tolar, Keller et al. 1999). Recent work of Ohkubo and associates suggests that the apoE induced increase in intracellular

Ca^{++} may trigger a further increase in Ca^{++} from other sources, including the endoplasmic reticulum but that these sources are not affected by the extracellular levels (Ohkubo, Mitsuda et al. 2001). This may provide an explanation for the insignificant differences in daily Ca^{++} levels found in this study. First, the initial apoE4-mediated increase in intracellular Ca^{++} triggers additional release of Ca^{++} from the endoplasmic reticulum in individuals with the APOE ε4 allele. The increase in CSF Ca^{++} in patients without the APOE ε4 allele may represent an increase in extracellular Ca^{++} from other sources independent of intracellular Ca^{++}. The shift of extracellular Ca^{++} into the intracellular space combined with a decrease in intracellular Ca^{++} clearance, increase vascular contraction contributing to CV. The decrease in coupling we found between apoE protein and Ca^{++} level in APOE ε4 allele carriers suggests that as intracellular Ca^{++} is sequestered from other sources, the apoE- Ca^{++} association changes.

APOE ε4 allele carriers exhibit higher release of apoE protein after injury, and an increase in available extracellular apoE could translate to an increase in apoE binding to cell receptors causing an increased influx of Ca^{++} from extracellular to intracellular space (Veinbergs, Everson et al. 2002). As expected, individuals with the APOE ε4 allele had an increase in apoE protein in the CSF. We also found that individuals with the APOE ε4 allele had lower CSF Ca^{++} levels. In that increased apoE from an extracellular source is known to increase cytosolic Ca^{++} and the apoE4 isoform also increases the influx of Ca^{++} from the extracellular space to the cytosol, these results suggest an isoform differential effect of Ca^{++} moving from the extracellular to intracellular space. This may be seen as a lower CSF Ca^{++} levels. It is also shown that increased intracellular Ca^{++} level may contribute to CV. We found trends to support increased apoE and CV, but there was no increase CV seen in individuals with an APOE ε4 allele.

There are potential alternative mechanisms influencing vascular reactivity via an apoE mediated pathway. L-arginine, which stimulates production of nitric oxide (NO), a potent vasodilator, has been shown to increase cerebral blood flow and decrease infarct volume in rats (Morikawa, Moskowitz et al. 1994). Work by Colton and associates (Colton, Czapiga et al. 2001) showed that apoE increases microglial and neuronal arginine uptake resulting in increased production of nitric oxide (NO). Using a mouse model, they found that apoE4 presence was associated with a greater increase in cellular arginine uptake and NO production as compared to apoE3 mice or apoE deficient mice (apoE4>apoE3>apoE deficient) (Colton, Czapiga et al. 2001). Others have reproduced these findings. Czapiga and Colton also found this relationship and that the apoE isoform specific increased arginine uptake and NO production is further enhanced in the presence of immune activation (Czapiga and Colton 2003). NO mediated arterial relaxation is impaired in atherosclerosis (Zeiher, Drexler et al. 1991), a condition associated with presence of the APOE ε4 allele. ApoE deficient mice have been found to have increased atherosclerosis and associated decreased NO-related endothelial relaxation. An apoE-mediated production of NO could account for decreased apoE in the CSF found in this study. The production of NO specifically requires Ca^{++} (Colton, Czapiga et al. 2001). Reduced Ca^{++}-dependent NOS activity is an important component responsible for endothelial relaxation in blood vessels (d'Uscio, Baker et al. 2001). If there were an increase in NO related to apoE induced cellular uptake, an associated decrease in extracellular Ca^{++}, and hence CSF Ca^{++}, as seen in the CV- group in this project would be anticipated.

While this study did not examine long-term outcome, CV presence is associated with poorer long-term outcome (Weir 1995) and this study provides some clarification to the relationship between APOE ε4 allele presence and outcome after SAH. Work by Tang (Tang,

Zhao et al. 2003), Leung (Leung, Poon et al. 2002), and Niskangas (Niskakangas 2001) found that presence of the APOE ε4 allele was associated with poor long term outcomes after SAH, but our work suggests that this does not occur as a result of CV. Our work does provide some support for the work of Dunn and associates (Dunn, Stewart et al. 2001) who found that presence of the APOE ε4 allele was not associated with initial rebleed or outcome, good or poor, 6 months after SAH. To some extent, this project also provides support for the work of Morris et al (Morris, Wilson et al. 2004) who considered APOE ε4 allele presence and broader long-term outcome measures including cognitive tests and global measures of function. They found that while presence of the APOE ε4 allele was not directly associated with improved outcome after SAH, there were trends to indicate that individuals with at least one copy of the APOE ε4 allele and a more severe hemorrhage (i.e. Fisher=4) had better outcomes than their counterparts without an APOE ε4 allele (Morris, Wilson et al. 2004).

6.3. TOTAL APOE PROTEIN LEVEL

CSF total apoE protein levels were lower than normal [normal CSF total apoE protein level ~20mg/L(Kay, Petzold et al. 2003)] after SAH, regardless of CV status, and continued to decrease over time until day 10. Total apoE protein levels were higher in subjects with at least one copy of the APOE ε4 allele. This finding agrees with the work of Kay et al. (2003) in that they also found CSF total apoE protein levels in the SAH population to be lower than normal (Kay, Petzold et al. 2003). However, the mechanism for this finding is unclear. The apoE protein may be utilized within the parenchyma as suggested by Kay, or within the cerebral vasculature as suggested by Paris and associates (Paris, Town et al. 1998). While it is known

that apoE protein production is upregulated in the parenchyma after acute neurologic injury (Skene and Shooter 1983; Stoll and Muller 1986; Stoll, Meuller et al. 1989; Lin, Duan et al. 1999; Duan, Gu et al. 2000) the increase in apoE production at the tissue level does not support the decrease noted in CSF. One would speculate that apoE protein levels would be increased in the CSF after SAH due to the increase in apoE protein entering the CSF from plasma during the initial hemorrhage. However, the decrease in total apoE level over time may also reflect increased uptake of apoE by neurons as they recover from this brain injury. Other possible mechanisms for the decrease in CSF total apoE protein that occurs after SAH include an increase in apoE-bound lipoproteins (Gregg and Brewer 1986), very low density lipids (VLDL) and chylomicron bound apoE (Gregg and Brewer 1986) (Steinmetz, Jakobs et al. 1989), none of which would be detected by ELISA methodology used in this project.

Kay also found that amyloid ß level was decreased in CSF after SAH, and that CSF total apoE protein and amyloid ß levels correlated. Kay et al. suggested that when apoE protein interacts with other molecules, such as amyloid ß, the brain tissue indirectly sequesters more apoE protein from the CSF (Kay, Petzold et al. 2003). Bound apoE, regardless of whether it is bound to lipids, a receptor, or some other molecule, would not be detected by ELISA methodology. An increase in bound apoE could be responsible for the decrease in CSF apoE levels found by this study as well as that of Kay and associates.

Alternatively, it is possible that apoE protein is utilized at a greater rate or that the protein itself is being broken down at an increased rate after injury. It is known that apoE protein is produced by astrocytes and microglial after cerebral injury and incorporated into the neurons for use(Mahley, Huang et al. 1999; Sheng, Laskowitz et al. 1999; DeMattos, Brendza et al. 2001). While 60-80% of apoE protein internalized by hepatic cells is secreted for re-use (Swift, Farkas

et al. 2001), the potential for recycling of apoE in the central nervous system is not clear (Dekroon and Armati 2001). It appears that this phenomenon occurs, although the mechanism differs for astrocytes and neurons and is also different for the different isoforms (Dekroon and Armati 2001). This project was not designed to explore the utilization or break down of apoE protein. Certainly, crossing of apoE protein from the CSF into the extracellular space and then into neurons for use is also an explanation for the finding of decreased levels in this study.

The finding of lower apoE protein levels after SAH, indicate that apoE protein is either being broken down at rate higher than it is being produced, or it is being sequestered from the CSF for use in either the cerebral vasculature or brain tissue. ApoE could be used in the cerebral vasculature to sequester Ca^{++} into the cell. However, there were not significant differences in apoE protein level by CV group, indicating that these levels are not directly influencing CV presence. Ca^{++} levels were not different based on CV group. Interaction between apoE protein and Ca^{++} levels is evident from this project, however there was no significance based on CV group, indicating that there is some other mechanism influencing CV. The apoE- Ca^{++} interaction could influence CV by increasing intracellular Ca^{++} levels while other mechanisms are also exerting influences. For instance, if Ca^{++} is drawn into the cell via an apoE-mediated pathway, there could be additional influence on cerebral vascular response via another apoE pathway such as the NO pathway. It is known that apoE can induce vascular relaxation by stimulating NO expression (Wang, Gruenstein et al. 1997; Colton, Needham et al. 2004).

Alternatively, Paris and associates found apoE-induced vasoconstriction occurred in conjunction with beta-amyloid peptide, another common molecule that interacts with apoE in an isoform specific manner (E4>E3)(Paris, Town et al. 1998). Binding of apoE with beta-amyloid could account for decreased level of apoE protein level in the CSF.

While this project provides valuable information about apoE isoforms, their presence in the CSF after SAH and apoE-Ca^{++} interaction, it did not find evidence to support this mechanism as the major influence on CV. The additional hypotheses presented regarding apoE interactions with NO and beta-amyloid, while not supported by our data, are of sufficient interest to support further investigation.

6.4. TOTAL APOE/APOE4 PROTEIN RATIO

There was a significant association between total apoE protein level and apoE4 protein level. ApoE4 protein accounted for approximately 62% of the total apoE protein present in individuals with an APOE ε4 allele, and the total apoE protein level was higher in this group. APOE genotype has not been associated with differences in CSF apoE levels (Kiunicki, Richardson et al. 1998). The work of Kiunicki and associates did not measure isoform specific apoE protein but they did not find differences in total apoE protein level based on genotype (Kiunicki, Richardson et al. 1998).

Of the 3 major apoE isoforms, apoE4 is cleared faster than the apoE2 or apoE3 from plasma (Gregg and Brewer 1986). ApoE4 binds to the low density lipid receptor with equal affinity as apoE3, and binds to the low density lipid receptor related protein with equal affinity as apoE3 and apoE2. However, apoE4 binds to VLDL with greater affinity (Dong and Weisgraber 1996). VLDL is cleared more rapidly from plasma and may be associated with apoE4 clearance as well (Gregg and Brewer 1986).

ApoE is recycled in the liver, but the processing and potential recycling of different apoE isoforms is not as well defined in the CNS. ApoE protein is processed and secreted for re-use by astrocytes more than neurons (Dekroon and Armati 2001). This processing and release of apoE4

in higher amounts than apoE3 could account for apoE4 being more than half of the total apoE protein in CSF of this study. If similar binding and clearance rates apply and the apoE is endocytosed back into the extracellular space, the slightly higher apoE4 in the CSF found by this project is not surprising.

Possibly, the apoE4 protein is either not as likely to maintain the bond to the cellular receptor or is bound to lipids less often/for a shorter period of time. The apoE4 protein is more susceptible to proteolysis (Brecht, Harris et al. 2004), so it is more likely that it would be broken down at an increased rate resulting in lower rather than higher levels. ApoE3 protein may be bound at a higher percentage (due to affinity for select lipids and binding at cellular membrane) than apoE4 and would therefore not be detected using ELISA methodology, which measures free protein.

It is possible that the APOE ε4 allele is in disequilibrium with a gene or promoter region that increases apoE protein production, however one would anticipate that only the apoE4 isoform (being produced as a direct result of the chromosome with the APOE ε4 allele) would be elevated. The finding that approximately 62% of total apoE protein is apoE 4 isoform supports this mechanism as having a role in this study.

6.5. Ca^{++}, TOTAL APOE PROTEIN AND APOE4 PROTEIN LEVELS

Daily Ca^{++} and total apoE protein levels were correlated, however daily Ca^{++} and apoE4 protein levels were not correlated. If apoE 4 protein is broken down at a higher rate than apoE 3 protein, as suggested by Brecht and associates (Brecht, Harris et al. 2004), there may be less apoE4 protein available to stimulate uptake of intracellular Ca^{++}. An additional theory to explain this finding is related to sample size and the power to identify such an association. ApoE4 protein is

only present in individuals with an APOE ε4 allele, therefore the sample size for analysis of the association between apoE4 protein and Ca^{++} levels is 24. While there was not a significant association or a trend to indicate an association (p=.2), an increase in sample size may improve the association.

6.6. Ca^{++} LEVELS

Daily CSF Ca^{++} level was lower than normal (Normal CSF Ca^{++} level ~2.1 meq/L) after SAH. While Ca^{++} moves across the blood-brain barrier slowly (Tai, Smith et al. 1986), it can move freely between CSF and the extracellular space (Fenstermacher, Patlak et al. 1974) via the choroid plexus (Tai, Smith et al. 1986). This free flow of Ca^{++} in conjunction with the findings of this study (low levels of CSF Ca^{++}) suggest that the Ca^{++} exists either in a bound form in CSF, or a bound or unbound form in the CNS tissue or extracellular space.

Individuals with CV had higher overall CSF Ca^{++} levels (all samples at all timepoints) than those without, but there was not a significant difference in the daily levels, suggesting a possible differential utilization of Ca^{++} in individuals with and without CV. It is known that intracellular Ca^{++} of the cerebral blood vessels increases after SAH, and that this increase is more severe and prolonged with vasospastic vessels (Zuccarello, Boccaletti et al. 1996). The higher concentration of CSF Ca^{++} levels of the CV+ group may be directly related to an increase in Ca^{++} availability to both neurons and cerebral blood vessels, contributing to vasospasm.

6.7. CONCLUSIONS

The results of this study show that presence an APOE ε4 allele is not directly associated with CV, although there were trends to suggest differences in clinical presentation on admission. Total apoE protein levels were lower than normal after SAH, decreased for the first 10 days and then began to gradually increase. Trends suggested that total apoE protein levels may be higher in individuals with an APOE ε4 allele; however there were no significant group differences in daily apoE protein levels across time. Ca^{++} was linked to total apoE levels, and this relationship was strengthened in the presence of CV, suggesting that apoE may influence CV via Ca^{++} regulation. There may be an allele differential role of apoE in intracellular Ca^{++} regulation. ApoE4 protein may encourage Ca^{++} uptake by cells and possibly increase vascular reactivity. Individuals with an APOE ε4 allele in the presence of CV had higher CSF Ca^{++} levels, suggesting that the increase in intracellular Ca^{++} level may be related to not only the initial surge of extracellular Ca^{++}, but also to a potential secondary surge from the endoplasmic reticulum (Ohkubo, Mitsuda et al. 2001).

6.8. IMPLICATIONS FOR FUTURE RESEARCH

This study found a decreased incidence of the APOE ε4 allele in this SAH population. Given the current literature, further work needs to be done to identify distribution of the APOE genotypes in this population and clarify the reasons for lower incidence of APOE ε4 allele frequency in some populations.

While apoE protein level is implicated in vascular reactivity, there appear to be different mechanisms with different influences- i.e. via intracellular Ca^{++} regulation versus increased NO

production. While this project was not specifically designed to determine the influence of different mechanisms on vascular reactivity, further work into the influence of these two contradictory mechanisms would make a valuable contribution.

This project examined CSF Ca^{++} and apoE protein levels after SAH. Future studies further examining the role of apoE and Ca^{++} at the cellular level are needed. While some work has been done to identify the role of apoE in intracellular Ca^{++} maintenance, indicating that apoE4 isoform is linked with increase intracellular Ca^{++} uptake, the influence of this relationship on response to injury and disease is still not well defined. Future work examining the linkage between apoE and Ca^{++} uptake into cells would be of value in determining influences on vascular reactivity as well as neurotoxicity.

ApoE protein, and specifically the apoE4 isoform, induce neurotoxicity via Ca^{++} homeostasis pathways. While the apoE4 is associated with an increased Ca^{++} influx into the cell, there is still some ambiguity as to the source of this Ca^{++}– extracellular versus intracellular and if intracellular from which organelle.

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