ENDOTHELIN-1 POLYMORPHISMS AND ENDOTHELIN-1 CEREBROSPINAL FLUID PROTEIN EXPRESSION AND THEIR RELATIONSHIPS TO CEREBRAL VASOSPASM AND LONG TERM OUTCOMES IN INDIVIDUALS FOLLOWING ANEURYSMAL SUBARACHNOID HEMORRHAGE

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Endothelin-1 Polymorphisms and Endothelin-1 Cerebrospinal Fluid Protein Expression and their Relationships to Cerebral Vasospasm and Long Term Outcomes in Individuals Following Aneurysmal Subarachnoid Hemorrhage

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Aneurysmal subarachnoid hemorrhage (SAH) is a devastating disease that affects approximately 25,000 people a year in the United States. Cerebral vasospasm (CV) is a common complication after SAH. In addition SAH patients have poor long term outcomes, with 40-50% of patients suffering severe neurological disabilities. The most vital step in preventing CV and poor long term outcomes is identifying patients at increased risk of these poor outcomes. Endothelin-1 (ET-1) is a potent vasoconstrictor that may play a role in the pathogenesis of CV. Genetic variation within the ET-1 gene may also account for some of the variance observed in the outcomes of SAH patients. The purpose of this study was to examine the effects of ET-1 CSF protein expression, and ET-1 SNPs on CV in individuals suffering from SAH. In addition, the relationship between long-term outcomes, ET-1 SNPs, and ET-1 CSF protein expression in patients with SAH was evaluated. This study included individuals ages 18-75 with a diagnosis of aneurysmal SAH. CSF samples were collected from a drainage catheter. ET-1 levels CSF were measured using chemiluminescent ELISA kits. Genotyping was performed using TaqMan® allele discrimination assays. Individuals with CV had average CSF ET-1 elimination rates (7.94±6.47pg/hr) that were increased in the 72 hours before angiography when compared to individuals without CV (4.35±3.02 pg/hr). Of the 9 ET-1 SNP's investigated, the variant allele of 1 SNP (rs2070699) was associated with the development of CV. The odds ratio of the

heterozygous genotype compared to the homozygous wild-type genotype was 2.970 with a 95% confidence interval of 0.998 to 8.836. The odds ratio for the homozygous variant genotype compared to the homozygous wild-type genotype was 8.356 with a 95% confidence interval of 2.032 to 34.371. No relationships were found between ET-1 SNPs and long-term outcomes. In addition a predictive model with CSF ET-1 levels and ET-1 SNPs had no significant relationships with long-term outcomes. This study supports the use of ET-1 levels and ET-1 genotypes as predictors of CV, but not of long term outcomes.

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1.0 PROPOSAL INTRODUCTION

There are approximately 25,000 cases of subarachnoid hemorrhage (SAH) in the United States each year (Longstreth, Koepsell, Yerby, & van Belle, 1985) most of which occur in women at an average age of 55 years old (Sobey & Faraci, 1998). Ninety five percent of cases of spontaneous SAH result from ruptured cerebral aneurysms (aSAH) (Sobey & Faraci, 1998). Patients who survive the initial hemorrhage and subsequent surgical or radiological intervention then face the potential of developing cerebral vasospasm (CV). Cerebral vasospasm can result in diminished blood flow to cerebral tissue and subsequent ischemia and is the most common cause of mortality and morbidity after aSAH, occurring in 40-60% of individuals recovering from aSAH (Schievink, Schaid, Michels, & Piepgras, 1995). The most vital step in preventing CV is identifying patients at increased risk of CV. Once these patients are identified, closer monitoring and more aggressive interventions may be implemented. While numerous causal factors of CV have been investigated (Cohen, Vanhoutte, Cohen, & Vanhoutte, 1995; Hatake et al., 1992; Kasuya et al., 1995; P. Kim, Schini, Sundt, & Vanhoutte, 1992; Onoue et al., 1995; Sobey, Heistad, & Faraci, 1996; Yamamoto et al., 1997), the search for a single factor has been disappointing; a complex multifactorial etiology is most probable (Hamann & Schimrigk, 1995).

Researchers have suggested that endothelin-1 (ET-1), a potent vasoconstrictor produced by the endothelial cells of the vasculature (H. Suzuki, Sato, Suzuki, Oka et al., 1990), may play a role in the etiology of CV (Mascia, Fedorko, Stewart, Mohamed, terBrugge, Ranieri, Wallace et

al., 2001; Seifert, Loffler, Zimmermann, Roux, & Stolke, 1995; Yamaji et al., 1990; Yanagisawa, Kurihara, Kimura, Tomobe, Kobayashi, Mitsui, Yazaki, Goto, Masaki et al., 1988). A functional polymorphism (Lys198Asn) of the gene encoding preproendothelin-1 has shown differences in vasculature reactivity (Iglarz et al., 2002). Preproendothlein-1 is a precursor to ET-1; therefore a polymorphism that changes the preproendothlin-1 protein had a direct affect on the ET-1 protein. The difference in vasculature reactivity caused by this polymorphism may have an effect on the presence of or severity of CV thereby influencing long-term outcomes. Along with this functional polymorphism, other polymorphisms in the gene for ET-1 may influence CV and long-term outcomes. By investigating the tagging single nucleotide polymorphisms (tSNP's) of the ET-1 gene all of the genetic variation within the ET-1 gene will be evaluated in this analysis. Few studies have investigated genetic influences on development of CV, yet genetic polymorphisms that effect ET-1 may have an influence on CV after aSAH and therefore influence long-term outcomes.

1.1 PURPOSE

The purpose of this study was to examine the effects of ET-1 cerebrospinal fluid (CSF) protein expression, tSNP's for ET-1 and Lys198Asn polymorphism on CV in individuals suffering from aSAH. In addition, the relationship between long term outcomes, ET-1 polymorphisms, and ET-1 CSF protein expression in patients with aSAH was evaluated.

1.2 SPECIFIC AIMS

The specific aims were:

1) Describe the distribution of Lys198Asn and tSNP of the ET-1 gene in individuals recovering from aSAH.
2) Describe the relationship between the ET-1 polymorphisms and CV after aSAH.
3) Describe the relationship between the ET-1 polymorphisms and long term outcomes after aSAH.
4) Describe the CSF ET-1 protein expression for the 3 days prior to CV measurement.
5) Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, of individuals with and without CV over time for the first 14 days after aSAH.
6) Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, of individuals with and without CV over time for the 3 days prior to CV measurement.
7) Describe the CSF ET-1 protein expression over the first 14 days after aSAH, in association with Lys198Asn and tSNP's, and its potential relationship with long term outcomes.

1.3 RESEARCH FRAMEWORK

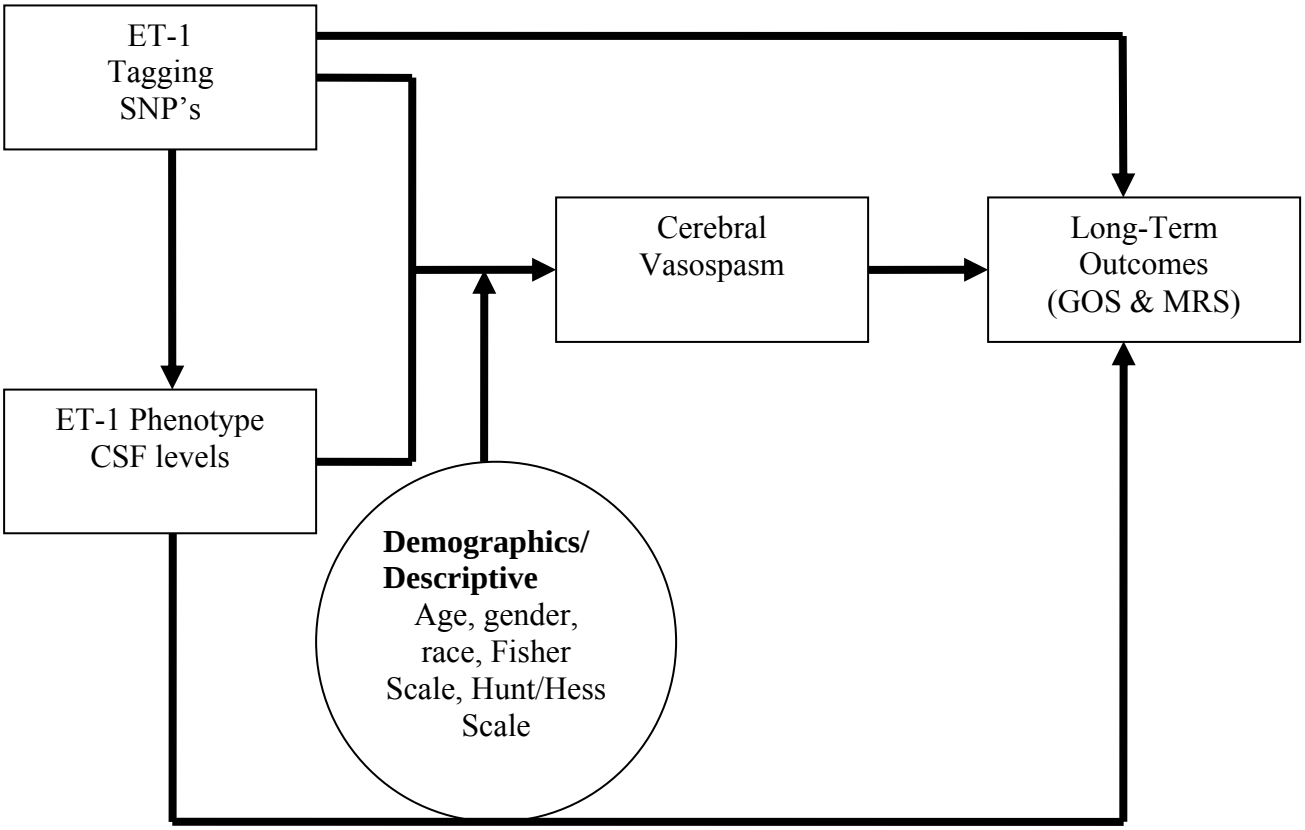

Figure 1: Diagram of research framework used for this study.

1.4 BACKGROUND AND SIGNIFICANCE

Subarachnoid hemorrhage occurs in approximately 1 out of 10,000 people in the United States each year(Longstreth et al., 1985) and is associated with a fatality rate of 30-50% (Sobey & Faraci, 1998). Those that survive the initial hemorrhage have a 40-50% chance of suffering severe long term neurological disabilities (Sobey & Faraci, 1998). A SAH is a type of hemorrhagic stroke that occurs when blood enters the subarachnoid space around the brain. The

most common cause of SAH is aneurysmal rupture of one of the major arteries of the brain (Longstreth et al., 1985). Other less common causes of SAH include rupture of an arteriovenous malformation and trauma. These causes of SAH will not be investigated in this study due to the fact that CV is not a common complication for these patients.

1.4.1 Cerebral Aneurysms

Cerebral aneurysms are abnormal focal out-pouchings of cerebral arteries. Autopsy studies have shown that cerebral aneurysms have a prevalence in the adult population between 1 and 5 percent (Wiebers et al., 2003). It has been estimated that 50 to 80 percent of all aneurysms do not rupture (Connolly & Solomon, 2004). Risk factors associated with cerebral aneurysm formation include being female, increased age, hypertension, and cigarette smoking (Schievink, 1997). Conditions associated with cerebral aneurysms include autosomal dominant polycystic kidney disease, firbromuscular dysplasia, Marfan's syndrome, and Ehlers-Danlos syndrome type IV (Vega, Kwoon, & Lavine, 2002). Numerous studies have found a strong relationship between cerebral aneurysm formation and familial relationships (De Braekeleer et al., 1996; Kassell et al., 1985; Kojima et al., 1998; Norrgard et al., 1987; Ronkainen et al., 1997; Ronkainen et al., 1993) suggesting there is a genetic predisposition to aneurysm development.

Little is known about the cause of intracranial aneurysms or the process by which they form, grow, and rupture. The most common histological finding is a decrease in the tunica media. The tunica media is the middle muscular layer of the artery. This decrease in the tunica media causes structural defects that most often occur at arterial branch points in the subarachnoid space at the base of the brain (Schievink, 1997).

1.4.2 Cerebral Vasospasm

The morbidity and mortality rate of aSAH has decreased little over the past 40 years (Solenski, Haley, Kassell, Kongable, Germanson, Truskowski, Torner et al., 1995). Surgical methods have been developed improving survival rates of the initial injury. While surgery decreased the risk of aneurysmal rebleed, the frequency of secondary injuries became evident and survival from aSAH did not improve. In great part due to the frequency of CV (Solenski, Haley, Kassell, Kongable, Germanson, Truskowski, Torner et al., 1995). Cerebral vasospasm can lead to delayed cerebral ischemia, furthering neurological damage, and often leads to ischemic stroke. Worse outcomes have been associated with the presence of CV (Goto & Yamagata, 2006; Ratsep & Asser, 2001). Cerebral vasospasm has been correlated with a 1.5 to 3 fold increase in mortality in the first 2 weeks after aSAH and occurs in 30-70% of all patients with aSAH (Treggiari-Venzi, Suter, Romand et al., 2001). Cerebral vasospasm occurs with a peak incidence 3 to 12 days after the initial aSAH (Newell, Eskridge, Mayberg, Grady, Winn et al., 1989). Angiographic CV, a decrease of the lumen of the cerebral vasculature of >25% as measured using cerebral angiogram, occurs in up to 70% of patients with aSAH (Dorsch, 2002). If the angiographic CV is severe enough to cause ischemia the patient will develop symptomatic CV (vessel narrowing on angiography followed by changes in clinical neurological status) which affects nearly one third of the patients. Without appropriate treatment CV can result in death in 30% and permanent disability in 34% of patients (Dorsch, 2002). There are few accurate predictors of CV including amount and distribution of blood on CT scan (categorized by Fisher grade), age, hypertension, cigarette smoking, and cocaine use (Conway & Tamargo, 2001; Qureshi et al., 2001). However, these risk factors do not accurately predict the occurrence of CV. The neuroscience nurse can play a critical role in preventing ischemic damage secondary to CV. Frequent neurological

assessment performed by nurses in the critical care setting often identifies the development of symptomatic CV (Kosty, 2005). Further, nursing management of patients recovering from aSAH is aimed at preventing secondary brain injury due to CV (Littlejohns & Bader, 2005). Genetic susceptibility may play a role in better identifying patients at a high risk of CV. Once these risks are identified, closer monitoring and more aggressive interventions may be considered only for those at increased risk.

The underlying pathophysiologic mechanism for CV remains unknown. Researchers have reported that the breakdown of blood in the subarachnoid space corresponds to the occurrence of CV such that increased blood in the subarachnoid space is associated with a greater development and severity of CV (Treggiari-Venzi, Suter, Romand et al., 2001). The physiological phenomena supporting this association, however, are unclear. One potential mechanism for development of CV after aSAH involves ET-1.

1.4.3 Endothelin-1 (ET-1)

Endothelin-1 is a long lasting vasoconstrictor that is produced by the endothelial cells of the vasculature (Petzold, Einhaupl, Dirnagl, & Dreier, 2003; H. Suzuki, Sato, Suzuki, Oka et al., 1990). The actions of ET-1 are balanced by the actions of nitric oxide in normal physiology and account for normal vascular tone. Nitric oxide is a short acting but powerful vasodilator produced by nitric oxide synthases produced by endothelial and other cells (Sobey & Faraci, 1998). Nitric Oxide also inhibits the synthesis of ET-1 (Boulanger & Luscher, 1990). When ET-1 is released it binds to the ETA receptors on the vasculature smooth muscle. The ETA receptor is coupled with a G-protein which releases a second messenger, inositol triphosphate (IP3). The release of the second messenger stimulates the sarcoplasmic reticulum to release

calcium eliciting vasoconstriction (Salom, Torregrosa, & Alborch, 1995; Zimmermann & Seifert, 1998). In addition ET-1 also increases calcium entry through voltage gated calcium channels (Weir, 1995). Recent animal studies have shown that ET-1 concentration is directly related to ischemia caused by vasoconstriction of the cerebral vasculature (C. Y. Kim et al., 2003; Petzold, Einhaupl, Dirnagl, Dreier et al., 2003). Petzold and associates investigated cerebral blood flow on rats using a laser-Doppler. Rats had a cranial window surgically implanted so that ET-1 and other chemicals could be infused directly on the vasculature of the brain. The infusing of ET-1 caused a decrease in cerebral blood flow. When ET-1 was combined with oxyhemoglobin the cerebral blood flow further decreased.

Suzuki and associate reported increased levels of ET-1 in CSF of aSAH patients (H. Suzuki, Sato, Suzuki, Takekoshi et al., 1990). Recent studies showed that CSF ET-1 levels are associated with the development of CV after aSAH (Kessler, Pacheco, Lozzi, de Araujo, Onishi et al., 2005), thus supporting the role of ET-1 in the pathogenesis of CV. Kessler and associates collected CSF samples from 30 patients diagnosed with aSAH. The collections of these samples varied and were all collected within the first 23 days after aneurysm rupture. They also collected CSF samples from 10 healthy subjects. There were significantly higher ET-1 CSF concentrations in the aSAH group when compared to the control group. This group found an increase in ET-1 CSF concentrations until the 6th day with a progressive decrease afterward, this was similar to what Suzuki and associates (1990) found. Finally, Kessler and associates found significantly higher ET-1 CSF concentrations in subjects with CV compared to subjects without CV.

What remains unclear is the temporal relationship of ET-1 CSF levels to CV. Mascia et al. investigated the temporal relationship between ET-1 and CV(Mascia, Fedorko, Stewart,

Mohamed, terBrugge, Ranieri, & Wallace, 2001). Mascia and associates collected CSF samples from 20 subjects diagnosed with aSAH on day 1, day 4, and day 7 after aneurysm rupture. The subjects then had an angiography performed on day 7 to measure CV. These subjects were then divided into 4 groups: Clinical CV, Angiographic CV, no CV, and poor neurological condition without CV. When groups were compared on day 1 and day 4 there were no significant differences. However, on day 7 the clinical CV and poor neurological condition groups had significantly higher ET-1 concentrations when compared to the no CV and angiographic CV groups. Because there were no differences between groups on day 1 and 4 the authors believe that ET-1 may not be an accurate predictor of CV. The authors expected an increase in ET-1 concentrations to occur before the actual CV occurred. These findings led the authors to believe that ET-1 concentration may be related to the degree of neuronal damage rather than CV. However, the collection of CSF may not have occurred frequently enough to show an increase in concentration before the occurrence of CV.

1.4.4 Preproendothelin-1 Polymorphism

One genetic polymorphism associated with variation in vascular reactivity is a polymorphism of preproendothelin-1 (RS5370) (Iglarz et al., 2002). Endothelin-1 is derived from preproendothelin-1. The preproendothelin-1 gene is located on chromosome 6. The polymorphism of the gene coding for preproendothelin-1 is described as a G-to-T transversion at position 5665 affecting the 61st nucleotide of exon 5, which predicts a Lys/Asn change at codon 198 (Iglarz et al., 2002). A G- to-T transversion is a point mutation in which a purine (G-Gaunine) is replaced by a pyrimidine (T-Thymine) in the DNA sequence. This particular change in the preproendothelin-1 gene changes the peptide sequence in the mature protein. In this case a

lysine (Lys) is replaced by an asparagine (Asn), leading to a structurally and functionally different preproendothelin-1 isoform, which leads to a functionally different ET-1 isoform. The variant ET-1 isoform (coded for by the T allele) is associated with an increased responsiveness in human mammary artery ring segments (Iglarz et al., 2002). Human mammary artery was removed from patients during coronary artery bypass surgery and later exposed to different concentrations of ET-1. It took lower concentrations of ET-1 to cause contraction in vessels of individuals with a T allele. The ET-1 that was exposed to the artery segments was an ET-1 that was not polymorphism specific. It was ET-1 purchased from a pharmaceutical lab. Therefore, it is not clear if the artery segments had different responsiveness due to the ET-1 polymorphism directly, or if the polymorphism caused a change in the ET-1 receptors that influenced responsiveness. This is the first study to show a difference in vessel reactivity due to the preproET-1 polymorphism. This increased vessel reactivity may increase the risk of CV in patients recovering from aSAH. Ultimately affecting long term outcomes in these patients.

Lys198Asn has been investigated in other diseases and phenotypes. Significant associations with Lys198Asn have been found in the following phenotypes: urinary albumin excretion (Pinto-Sietsma et al., 2003), glomerular filtration rate (Pinto-Sietsma et al., 2003) and blood pressure in women (Funalot et al., 2004). In all cases the functional relevance of the associations are not clear. Non-significant associations have been found in the following diseases or phenotypes: blood pressure (Dong, Wang, Zhu, Treiber, & Snieder, 2004), left ventricular mass index(Dong et al., 2004), ET-1 level in vitro(Tanaka, Kamide, Takiuchi, Kawano, & Miyata, 2004), and cerebral small vessel disease(Gormley, Bevan, Hassan, & Markus, 2005). The differences in association between Lsy198Asn and blood pressure may be due to the methods and populations used by the two studies. Funalot and associates (2004)

found an association between Lys198ASN and systolic and mean blood pressure in older (Mean age 65) women. While Dong and associates (2004) investigated this association in a younger population (Mean age 12) over a 15 year period.

1.4.5 Endothelin-1 System Tagging SNP's

The majority of genetic variation among people can be characterized by single nucleotide polymorphisms (SNP). These polymorphisms are mutations that occur at single nucleotide positions. It has been estimated that there are about 7 million common SNP's in the human genome (Botstein & Risch, 2003; Kruglyak & Nickerson, 2001). There may be hundreds of SNP's associated with an individual gene such as the ET-1 gene. Genotyping each of these numerous SNP's is time consuming and not cost effective. Tagging SNP's allow researchers to genotype a single nucleotide polymorphism that represents a larger region of the gene. Therefore it is possible to identify genetic variation without genotyping every SNP in a chromosomal region.

By exploring tagging SNP's, the proposed study examined how genetic differences found in the ET-1 gene, may be used as an indicator of which patients are at a higher risk of vasospasm and subsequent worse outcomes. By exploring all of the tagging SNP's of ET-1 all genetic variance can be accounted for in the ET-1 gene, including potentially functional polymorphisms. At the present time there are no studies published that investigate ET-1 tSNP's or ET-1 functional SNP's and their association with CV, or outcomes following aSAH. The novelty of this study is that it will explore gene expression (ET-1 levels) in the CSF of individuals with and without CV after SAH in light of genetic polymorphisms.

This study will advance nursing research in human genetics, as well as potentially affect nursing interventions after aSAH by helping to identify and focus resources on patients at higher risk of CV and poor long term outcomes. The results of this study will also promote the integration of nursing process into the delivery of genomics-based healthcare services. Additionally, this study may lead to improvement in cost effectiveness. The patients that are at a lower risk of CV may be able to have less costly tests, interventions, and a shorter ICU or hospital stay thereby decreasing the overall cost of the hospital stay. Finally, the results of this study will add to the literature on mechanism of CV and encourage development of interventions aimed at decreasing CV after aSAH and improving long term outcomes.

1.5 PRELIMINARY STUDIES

1.5.1 Preliminary Study #1

Preproendothelin-1 Polymorphism (Lys198Asn) and its Relationship to Long Term Outcomes after Subarachnoid Hemorrhage: A Pilot Study

Purpose: The purpose of this study was to examine the Lys198Asn and its relationship to long-term outcomes after aSAH.

Methods:

- aSAH patients
  - Age 21-75
  - Fisher Grade >1
  - No history of preexisting neurological diseases

  - DNA samples were collected as part of a parent study

- Genotyping
  - An ABI Prism® 7000 Sequence Detection System was used.
  - TaqMan® assays were used for allelic discrimination.
- Long Term Outcomes
  - Modified Rankin Scale
  - Glasgow Outcome Score
  - Collected at 3 months and 6 months after aneurysm rupture

Results: Genotype data collected on 228 subjects. The frequency of genotypes associated with the Lys198Asn included: homozygous GG wild type n=150 (66%), heterozygous GT n=65 (28%), and homozygous TT n=13(6%). The distribution of alleles within this study had a higher percentage of variant alleles than would be expected when compared to the normal population. Of the 228 that were genotyped 3 month outcome data was collected on 187 (72%) individuals 41 had missing 3 month outcomes. There were 168 (74%) individuals with 6 month outcomes data and 60 with missing 6 month outcomes data.

Conclusions: There were no significant relationships found between Lys198Asn and long term outcomes.

1.5.2 Preliminary Study #2

Preproendothelin-1 Polymorphism (Lys198Asn) and its Relationship to Cerebral Vasospasm after Subarachnoid Hemorrhage: A Pilot Study.

Purpose: The purpose of this study is to examine different genotypes of the Lys198Asn in individuals with and without CV after aSAH.

Methods:

- aSAH patients
  - Age 21-75
  - Fisher Grade >1
  - No history of preexisting neurological diseases
  - DNA samples were collected as part of a parent study
- Genotyping
  - An ABI Prism® 7000 Sequence Detection System was used.
  - TaqMan® assays were used for allelic discrimination.
- CV determination
  - Angiography performed day 3 to day 14
  - Angiography coded by Neurosurgeon

Results: Genotype data was collected on 138 subjects. The frequency of genotypes for Lys198Asn included: homozygous GG wild type n=96 (70%), heterozygous GT n=34 (24%), and homozygous TT n=8 (6%). The distribution of alleles within this study had a higher percentage of variant alleles than would be expected when compared to the normal population. Of the 159 subjects 76 (48%) suffered from CV.

Conclusion: There were no significant relationships found between the preproendothelin-1 polymorphism and CV.

1.5.3 Additional Experiences

In addition to the two formal studies listed above, I was able to have adequate funds that allowed me to genotype 8 tSNPs of endothelin-1. One tSNP was associated with CV and three of the tSNP's are associated with long term outcomes. This data were presented at an international conference in February of 2008.

When I began the doctoral program I was a bedside nurse on a 10 bed neurovascular ICU. My experiences there included working with patients with large ischemic and hemorrhagic strokes. In addition to this I took care of aSAH patients and was able to observe these patients in a clinical setting.

Since that time I have become a graduate student researcher (GSR) on a grant investigated aSAH patients. Experiences include:

- Consenting patients and their families.
- Collecting urine, blood, and CSF samples and processing them for later protein analysis.
- Collection of demographic and clinical data.
- Endothelin-1, Nitric Oxide, and 20-HETE analysis of CSF and Plasma.
- Database Management.
- Performance of Transcranial Doppler.
- Data Analysis
- Writing for scientific journals
- Presentation of research findings

1.6 RESEARCH DESIGN AND METHODS

1.6.1 Design

This study was a longitudinal descriptive correlational study. Demographic, angiographic, outcome data, blood and CSF samples were previously collected as part of two ongoing research studies (R01-NR04339, PI Dr. Leslie Hoffman and an unfunded study, PI Dr. Amin Kassam) being conducted at the University of Pittsburgh School of Nursing. The subjects from these parent studies consented for the use of all data, their genetic material, and biological samples in further studies of aSAH.

1.6.2 Clinical Setting

Patients were admitted to the Neurovascular intensive care unit (NICU) at the University of Pittsburgh Medical Center (UPMC), Pittsburgh Pennsylvania. This is a 20 bed intensive care unit that specializes in the care of individuals with acute neurological injuries.

1.6.3 Sample

The population presenting to the University of Pittsburgh Medical Center for treatment of aSAH is approximately 65% women and 9% African American, which is consistent with the general SAH population in the Pittsburgh area. Patients were enrolled in the parent studies within 48 hours of admission. Informed consent was obtained based on an Institutional Review Board accepted protocol.

The inclusion criteria for the parent studies were as follows: 1) 21 to 75 years of age. 2) diagnosis of an aSAH confirmed by CT scan (Fisher grade ≥ 2) and angiogram. Exclusion criteria include: 1) SAH due to trauma, mycotic aneurysm rupture, arterial-venous malformation rupture, or from an unknown source and 2) pre-existing neurological disease or injury. The inclusion criteria for this study included the criteria above with one additional criterion: a DNA sample must be available for genotyping. There were no additional exclusion criteria for this study when compared to the parent study.

Adults 21 and older were chosen because the incidence of aSAH in children is <0.001% (Anderson, Jamrozik, Broadhurst, & Stewart-Wynne, 1994). To prevent the influence of comorbidities on our sample the maximum age for the study was 75 years. Patients with a diagnosis of aSAH were graded as a Fisher grade 2 or greater. Fisher grade 0 is a patient without a ruptured aneurysm, and a Fisher grade 1 has no blood present on a CT scan. Since blood load is associated with CV, patients with increased risk of CV are graded 2 and above. Patients with SAH from non-aneurysmal sources have a decreased risk of CV, receive different medical treatment, and have a different course of recovery and therefore will not be included in this study. To avoid confounding our results, patients were also excluded from this study if they have a pre-existing neurological disease or injury.

1.6.4 Recruitment

Subjects were recruited as part of two ongoing research studies (R01-NR04339, PI Dr. Leslie Hoffman and an unfunded study, PI Dr. Amin Kassam) being conducted at the University of Pittsburgh School of Nursing. The parent study personnel would make daily rounds in the NVICU with the nursing staff. Potential patients with a diagnosis of SAH were reviewed for

parent study eligibility. Once it was established that the patient was eligible, the bedside nurse would ask the permission of the patient or family to be approached by the study personnel. With the verbal permission of the family or patient the project personnel would present the study and obtain informed consent. Both of the parent studies were approved by the institutional review board. The subjects from these parent studies consented for the use of all data, their genetic material, and biological samples in further studies of aSAH. Therefore, there is no additional recruitment for this proposed study.

1.6.5 Standard of Care

All individuals presenting to the University of Pittsburgh Medical Center (UPMC) Presbyterian/Shadyside were admitted to the Neurovascular ICU (NVICU). Upon admission all patients received a computed tomography (CT) scan of the head to confirm SAH. Fisher Grade and Hunt and Hess grade were assigned by a neurosurgeon upon admission. Cerebral Angiography was then performed to identify the source of the SAH. Upon confirmation of aSAH the attending neurosurgeon decided if the aneurysm would be embolized using platinum coils or clipped surgically. All patients receive central venous catheters. Patient monitoring included central venous pressure, arterial blood pressure, pulse oximetry, respiratory rate, cardiac rate, and cardiac rhythm. An extraventricular drain (EVD) or lumber drain was placed if it was necessary to treat for hydrocephalus or increased intracranial pressure. All EVD's monitored intracrainial pressure (ICP) continuously. Patients were intubated and ventilated as needed. Temperature was monitored every two hours by oral or rectal measurement. Complete neurological exams were performed by registered nurses every two hours. All patients receive triple 'H' therapy. Triple 'H' therapy consists of hypertension, which is accomplished by

increasing the patient's blood pressure to higher than normal values. The second 'H' is hypervolemia. The patient receives large amounts of IV fluids, which also helps to keep the blood pressure elevated and the cerebral vessels open. The goal for hypervolemia is a CVP greater than 8 mmHg. The final 'H' is hemodilution. By receiving large amounts of fluids the patient's blood is hemodiluted, which allows the blood to flow easier through the cerebral vasculature. The goal for hemodilution is a hematocrit of 30% to 40%. Sixty mg of nimodipine (Nimotop) was given every 4 hours for the first 21 days after aneurysm rupture. Nimotipine is a calcium channel blocker that is the only FDA approved medication for the prevention of CV. Recently, magnesium sulfate has been show to help prevent cerebral vasospasm (Chia, Hughes, & Morgan, 2002). Therefore all patients receive 12 grams a day of magnesium sulfate by continuous infusing for the first 12 days. In the event of clinical or angiographic vasospasm, patients are treated by increasing their systolic blood pressure to a minimum of 180 mmHg. This is accomplished by infusion of a vasopressor. Norepinephrine is the most common vasopressor used at UPMC. Intra-arterial papaverine and balloon angioplasty are endovascular treatments that may be used at UPMC at the discretion of the attending neurosurgeon. Anti-convulsants were administered for all patients. Sedatives were administered as needed for patient agitation.

1.6.6 Research Setting

1.6.6.1 Genetic Laboratory

All additional genetic analysis occurred at the University of Pittsburgh School of Nursing. Dr. Yvette Conley has a molecular genetics laboratory located in the Victoria Building at the University of Pittsburgh School of Nursing. This laboratory is a 3200 square foot facility divided into three sections, a culture room facility, a pre-PCR room and a post-PCR/equipment

room. Major equipment available in the laboratory include an ABI377 automated sequencer/genotyper with all of the necessary computer equipment and software for analysis of data, the WAVE® Nucleic Acid Fragment Analysis System from Transgenomic to perform dHPLC, an ABI7000 for TaqMan® real time PCR and allele discrimination assays, a Turner Designs Luminometer, various horizontal/vertical electrophoresis units and power supplies, several SSCP apparatus, a gel documentation system, centrifuges, a spectrophotometer, a cold room for DNA storage, ultra-low freezers, culture room equipment, and several 96-well thermal cyclers. The laboratory is completely OSHA compliant and is up to date for all required inspections. Three technicians with advanced degrees are employed full time in the laboratory.

1.6.6.2 Specimen Laboratory

In addition to the genetics laboratory, Dr Poloyac has a biological specimen laboratory at the University of Pittsburgh School of Pharmacy. The research in Dr. Poloyac's laboratory utilizes contemporary methods of analytical chemistry coupled with molecular biology in the pursuit of generating new knowledge about the pathogenesis of neurotraumatic disease. The laboratory is completely OSHA compliant and is up to date for all required inspections. Two technicians with advanced degrees are employed full time in the laboratory.

1.6.7 Procedures for Data Collection

Demographic data was collected from the parent study. Gender, race, and age were collected from medical. 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 project personnel of one of the parent studies. Data that used for this study was extracted from the

databases of the parent study by one of the study personnel. All data that is needed for this study was requested from the study personnel in writing. A new database for this study was constructed using all of the requested data from the parent studies. In order to assure subject confidentiality, all identifiers were removed from the data. A non-identifying study number was given to link the parent study data and biological samples for this study.

1.6.8 DNA Specimens

Subjects had 10cc of whole blood drawn on day one of enrollment into the parent studies. The tube of blood was delivered to Dr. Conley's laboratory with no personal identifiers, only the study participant's identification number, date of sample collection, and time of sample collection. The sample was logged in and centrifuged, the white cells removed, and DNA extracted from the white cells using a simple salting out procedure (Miller, Dykes, & Polesky). All DNA is stored in 1X TE buffer at 4°C.

1.6.9 Genotyping

Genotyping was performed as follows: An ABI Prism® 7000 Sequence Detection System was used to conduct allele discrimination assays using TaqMan® assays. This is a highly automated, high throughput genotyping method for SNPs. Primers flank each polymorphism and PCR is conducted using subject DNA. The main difference between TaqMan® PCR and traditional PCR is the use of a probe that is labeled with a reporter and a quencher dye that recognizes a specific allele of a SNP. Each allele of a SNP has its own probe with its own reporter dye. If the subject's DNA is homozygous for one allele of the SNP, only the probe for that allele will

hybridize and only the reporter dye for that allele will be liberated and measured by the ABI Prism® 7000 Sequence Detection System. A heterozygote will have both probes hybridize and both reporter dyes measured. Using this method, each SNP can be genotyped accurately and quickly. For the currently chosen SNP, TaqMan® assays on demand are already available through ABI. 200ng of subject DNA was utilized for this assay and more than enough DNA was available to conduct this assay.

Tagging SNP's of ET-1 and Lys198Asn were classified into one of three genotypes. These genotypes were homozygous for the wild type, homozygous for the variant, or heterozygous.

1.6.10 Cerebrospinal Fluid (CSF) Specimens

Cerebrospinal fluid (CSF) specimens were obtained twice daily from either extraventricular drains (EVD) or via lumbar drains (LDs). These drains were placed as part of medical care for the treatment of aSAH. The drains were placed using sterile techniques by trained neurosurgeons. EVDs were placed into the lateral ventricle and the lumbar drains were placed into the subarachnoid space of the lumbar spine between L-3 and L-5. To obtain the fresh samples, specimens were collected using sterile technique directly from the drainage catheter at the site closest to the patient as per institutional policy and then immediately frozen in cryogenic vials to –20oC in a laboratory across from the clinical unit. At the end of 14 days when all collections were completed the specimens were stored in –80oC freezers in the School of Nursing until analysis.

1.6.11 ET-1 CSF Analysis

The CSF samples were assayed for ET-1 using the ELISA kit (Human Endothelin-1) from R&D Systems (Minneapolis, MN) and according to manufacturer's instructions. This assay employs the quantitative sandwich enzyme immunoassay technique with chemiluminesent detection. The standard curve was modified from the R&D recommendations to include 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, & 12.5 pg/ml standards. Precision and accuracy of the modified standard curve range was evaluated and the coefficient of variation was less than 15 % over the range of standards utilized. The intra-assay and inter-assay coefficients of variation were 3.4% and 8.9% respectively. This range of standards was used to cover the range of concentrations observed in the CSF of SAH patients. A microplate luminometer (Model # 1420-012 Victor-Wallac, Finland) is used to measure the intensity of the light emitted. Many of the CSF samples have been analyzed as part of the parent study. The analyses have been performed by the laboratory technicians of Dr. Poloyac's laboratory or by me.

1.6.12 Cerebral Vasospasm

Cerebral vasospasm was diagnosed by identification of narrowing of cerebral vessels as documented on four vessel cerebral angiography. Cerebral angiography was conducted as standard of care on admission, as postoperative follow-up, or in the event of a neurological deterioration. The angiographic studies were interpreted and graded by neuro-radiologist and neurosurgeons at UPMC for evidence and degree of vasospasm. Coding of CV measured by cerebral angiography for our group has an inter-rater reliability kappa of 0.81. The degree of vasospasm was represented as the percentage of narrowing in the cerebral vessels as follows:

none to minimal, 0 to 25% narrowing; moderate, 26-75% narrowing; severe, 76-99% narrowing. Patients with either moderate or severe vasospasm were considered positive for cerebral vasospasm. Ten percent of angiograms will be independently scored by a second neurosurgeon or neuroradiologist to assess inter-rater reliability. Mismatch between exams were discussed at a team conference and a consensus as to vasospasm status was reached.

1.6.13 Long Term Outcomes

The dependent variables in this study were long term outcome scores on the MRS and GOS. These scores were collected as part of the parent study at 3 months and 6 months after aneurysm rupture. The GOS, a clinical observation scale, categorizes functional outcomes into five levels ranging from 1 to 5 with 1 indicating death and 5 indicating good recovery (Jennett & Jennett, 1976) (See Appendix A). Inter-rater reliability has been reported from 68 – 95% with kappa values from .62 to .79 (Gennarelli, Spielman, Langfitt, Gildenberg, Harrington, Jane, Marshall, Miller, Pitts et al., 1982) and correlates well with severity of illness. The GOS has wide acceptance and established validity (Jennett, Bond, Jennett, & Bond, 1975; Maas, Braakman, Schouten, Minderhoud, van Zomeren et al., 1983). Inter-rater reliability was within the acceptable range when there is adherence to assessment guidelines, administration of a structured interview, and training of examiners (Wilson, Pettigrew, Teasdale et al., 1998). Of the subjects enrolled in the parent study 79% had GOS measured at 3 months, 74% have 6 month measurements.

The Modified Rankin Scale is a measure of functional recovery and incorporates mental and physical adaptations to the neurological deficits following a neurological injury. It is easy to use and has been widely adopted for use in stroke trials. The MRS score ranges from 0 (no

symptoms at all) to 6 (death) (See Appendix B). Inter-observer kappa were .56 and weighted kappa were .91 (Sulter, Steen, De Keyser et al., 1999). Of the subjects enrolled in the parent study 79% had MRS measured at 3 months, 74% have 6 month measurements.

1.6.14 Severity of Injury

Severity of injury was measured by the Hunt & Hess (Appendix C) and size of hemorrhage was measured by the Fisher Grade score (Appendix D). Both of these scores were collected from medical records as part of the parent study. The Hunt and Hess grading system is based on clinical symptoms at the time of admission, with scores ranging from zero (0), indicating no clinical symptoms, to five (5), indicating a comatose state (Hunt, 1983). The Hunt and Hess scale inter-rater reliability is acceptable with a kappa value of 0.41 (p=.0005) (Oshiro, Walter, Piantadosi, Witham, & Tamargo, 1997). Hunt and Hess scores have also been correlated with infarct related to CV (Gruber et al., 1998).

The Fisher scoring system is a tool that measures the size of hemorrhage 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 inter-rater reliability with kappa value of 0.9 (Ogilvy & Carter, 1998). Higher Fisher scores have been correlated with CV after SAH (Lasner & Raps, 1997; McGirt et al., 2003).

1.6.15 Data Management

Covariates, CV status, and severity of injury measures were collected and entered into the existing database using an automated data entry and verification system (Teleform). Data was stored in password protected computers and locked offices of research associates of the parent study. The new genotype data from this proposed project was stored in a database within a password protected computer with no other personal identifiers.

1.6.16 Preliminary Data Analysis Plan

Initially data was screened for missing data, outliers, and possible confounding factors and to assess whether underlying statistical assumptions for the planned analysis are satisfied. All attempts were made to retrieve missing data from the parent study databases or medical records of the subjects. Appropriate descriptive statistics (based on the level of measurement and the empirical data distribution) were computed for all variables and were summarized for the entire sample and based on CV, long term outcomes, tSNP's genotypes and their combination.

1.6.17 Justification of Sample Size for specific aims

Specific Aim #1: Describe the distribution of Lys198Asn and tSNP of the ET-1 gene in individuals recovering from aSAH. There were 282 subjects with DNA collected for analysis. There was no effect size associated with this aim since it will describe the distribution of the genotypes.

Specific Aim #2: Describe the relationship between the ET-1 polymorphisms and CV after aSAH. An estimated sample size of 108 achieves 80% power to detect an effect size of 0.300 with a significance level of α=.05. At this time there are 144 subjects with angiographic CV status. A sample of 144 will achieve a 91% power with an effect size of 0.30 with a significance level of α=.05.

Specific Aim #3: Describe the relationship between the ET-1 polymorphisms and long term outcomes after aSAH. An estimated sample size of 167 achieves 80% power to detect an effect size of 0.500 with a significance level of α=.05. At this time there are 213 subjects with long term outcomes measurement. A sample of 213 will achieve an 87% power with an effect size of 0.30 with a significance level of α=.05.

Specific Aim #4: Describe the CSF ET-1 protein expression for the 3 days prior to CV measurement. An estimated sample size of 150 will achieve 80% power at significance level of α=.05. There are approximately 40 patients with ET-1 protein levels measured for the 3 days prior to CV. A sample of 40 will achieve a 20% power with a significance level of α=.05.

Specific Aim #5: Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, of individuals with and without CV over time for the first 14 days after aSAH. An estimated sample size of 150 will achieve 80% power at significance level of α=.05. There are approximately 40 patients with ET-1 protein levels and genotyping for the 3 days prior to CV. A sample of 40 will achieve a 20% power with a significance level of α=.05.

Specific Aim #6: Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, of individuals with and without CV over time for the 3 days prior to CV measurement. An estimated sample size of 150 will achieve 80% power at significance level of

α=.05. There are approximately 40 patients with ET-1 protein levels and genotyping for the 3 days prior to CV. A sample of 40 will achieve a 20% power with a significance level of α=.05.

Specific Aim #7: Describe the CSF ET-1 protein expression over the first 14 days after aSAH, in association with Lys198Asn and tSNP's, and its potential relationship with long term outcomes. An estimated sample size of 150 will achieve 80% power at significance level of α=.05. There are approximately 100 patients with ET-1 protein levels and long term outcomes measures. A sample of 100 will achieve a 33% power with a significance level of α=.05.

1.6.18 Data analysis for specific aims

Specific Aim #1: Describe the distribution of Lys198Asn and tSNP of the ET-1 gene in individuals recovering from aSAH. Frequency counts and marginal percentages were used to describe the frequency distribution for functional SNP and tSNP's of individuals recovering from SAH. Point and interval estimates (95% confidence intervals) for the prevalence of the functional SNP and tSNP will also be obtained.

Specific Aim #2: Describe the relationship between the ET-1 polymorphisms and CV after aSAH. Chi Square analysis will be used to investigate the relationship between ET-1 polymorphisms and CV with a significance level of α=.05. The null hypothesis is that the probability of having vasospasm is equal for each genotype (homozygous wild, homozygous variant, and heterozygous). A 2x3 chi square will be used in this analysis. There will be two levels of CV (CV positive and CV negative) and three levels of genotyping.

Specific Aim #3: Describe the relationship between the ET-1 polymorphisms and long term outcomes after aSAH. Chi Square analysis will be used to investigate the relationship between ET-1 polymorphisms and long term outcomes with a significance level of α=.05. The

null hypothesis for the long term outcome MRS is that the probability of having a MRS score is equal for each. For the long term outcome MRS a 3x7 chi square analysis will be used. There will be three levels of genotyping and 7 levels of MRS measurement. The null hypothesis for the long term outcome GOS is that the probabilities of having a GOS score is equal for each genotype. For the long term outcome GOS a 3x5 chi square analysis will be used. There will be three levels of genotyping and 5 levels of MRS measurement.

Specific Aim #4: Describe the CSF ET-1 protein expression for the 3 days prior to CV measurement. Logistic regression will be used to compare daily ET-1 CSF levels in patients with and without CV while controlling for the influence of covariates (i.e. race, age, Fisher grade). This analysis will examine ET-1 CSF levels over time. Logistic regression will be able to estimate the probability of the presence or absence of CV based on CSF ET-1 protein levels while controlling for the covariates.

Specific Aim #5: Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, of individuals with and without CV over time for the first 14 days after aSAH. Trajectory analysis will be used to dichotomize subjects into high or low ET-1 levels and then logistic regression will be used to compare the ET-1 CSF levels of subjects with and without CV while controlling for the ET-1 genotypes and covariates (i.e. age, race, Hunt & Hess, and Fisher). Trajectory analysis will plot the trajectory of ET-1 CSF levels for the first 14 days. These trajectories will be plotted based on CV status (CV negative and CV positive. The trajectories are then evaluated for differences at the significance level of α=.05.

Specific Aim #6: Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, of individuals with and without CV over time for the 3 days prior to CV measurement. Logistic regression will be used to compare daily ET-1 CSF levels in patients

with and without CV while controlling for the ET-1 genotypes and the influence of covariates (i.e. race, age, Fisher grade). This analysis will examine ET-1 CSF levels over time. Logistic regression will be able to estimate the probability of the presence or absence of CV based on CSF ET-1 protein levels while taking into account the genotype and controlling for the covariates.

Specific Aim #7: Describe the CSF ET-1 protein expression over the first 14 days after aSAH, in association with Lys198Asn and tSNP's, and its potential relationship with long term outcomes. Trajectory analysis will be used to dichotomize subjects into high or low ET-1 levels and then logistic regression will be used to compare the ET-1 CSF levels of subjects related to their long term outcomes while controlling for the ET-1 genotypes and covariates (i.e. age, race, Hunt & Hess, and Fisher). Trajectory analysis will plot the trajectory of ET-1 CSF levels for the first 14 days. These trajectories will be plotted based on their long term outcome status. The trajectory groups will have 5 groups for the GOS analysis and 7 groups for the MRS analysis. The trajectories are then evaluated for differences at the significance level of α=.05.

1.7 LIMITATIONS

This study has limitations worth mentioning. All data was collected as part of parent studies. Therefore, any additional demographic data cannot be collected if it was not collected as part of the parent studies. Additionally, statistical methods were chosen based on their ability to inform of possible associations in this study. The findings of this study are limited to the aSAH population and cannot be generalized to all causes of SAH.

1.8 PUBLICATIONS

The following is a published abstract directly relevant to this research proposal, although cerebral vasospasm is a major contributor to ischemia and poor outcomes following aneurysmal subarachnoid hemorrhage (aSAH); little is known regarding its etiology. It has been suggested that endothelin-1 (ET-1), a potent vasoconstrictor, is related to the formation of CV. However, ET-1's temporal role in CV has not been well established, decreasing clinicians' ability to use it as a potential biomarker of CV. We hypothesis that cerebrospinal fluid (CSF) ET-1 levels after aSAH would be higher in pts with CV. Subjects (N=40), were prospectively recruited for an on-going NIH funded study [90% White (n=36); 72.5% female (n=29)]. The inclusion criteria were patients age 18-75, with a diagnosis of aneurysmal SAH (Fisher grade ≥2), access to CSF, and a cerebral angiogram. Persons with a history of a neurologic disease were excluded. Presence/absence of CV was determined by cerebral angiogram independently read by a neuroradiologist. CSF samples were drawn twice a day for 72 hours before angiogram. CSF ET-1 levels were quantified using ELISA and Area Under the Curve (AUC) was calculated for 24, 48, and 72 hours before angiogram. Graphic analysis, descriptive statistics, t-tests, and ANOVA were used to analyze relationships. Of the 40 subjects, 16 developed CV within 14 days. Mean 72 hour AUC for the entire sample was 42.07. Mean 72 hour AUC was higher in the CV present group (M=50.9 vs. 36.2; F=3.172 P=.036). There was no significant difference in AUC at 24 and 48 hours prior to CV. The observed elevation in ET-1 CSF concentrations provides clinical evidence that ET-1 may be involved in the pathogenesis of CV in patients after SAH (Gallek et al., 2006).

The following abstract was submitted to the Society of Critical Care Medicine and is directly relevant to this research proposal. Cerebral Vasospasm (CV) is a major contributor to

ischemia and poor outcomes following aneurysmal subarachnoid hemorrhage (aSAH), little is known regarding its etiology. It has been suggested that endothelin-1 (ET-1), a potent vasoconstrictor, is related to the formation of CV. Genetic variations of ET-1 may influence the occurrence of CV or long term outcomes. The purpose of this study was to investigate ET-1 tagging SNP's (tSNP's) relationship to CV and to long term outcomes. Subjects (N=229), were prospectively recruited for an on-going NIH funded study [100% Caucasian; 72% female (n=165)]. The inclusion criteria were patients age 18-75, with a diagnosis of aneurysmal SAH (Fisher grade ≥2), and access to DNA. Persons with a history of a neurologic disease were excluded. Presence/absence of CV was determined by cerebral angiogram independently read by a neurosurgeon. Genotyping of 7 tSNP's and 1 functional SNP was performed using an ABI Prism® 7000 Sequence Detection System and TaqMan® assays. This is a highly automated, high throughput genotyping method for SNP's. Modified Rankin Score (MRS) was collected at 6 months post aneurysm rupture. These scores were collected by a trained neuropsychological technician. Graphic analysis, descriptive statistics, and chi-square were used to analyze relationships. Of the 229 subjects, 144 had cerebral angiograms, and 67 developed CV within 14 days. Of the 8 SNP's that were investigated, RS2070699 showed a significant relationship with CV (p=0.010). RS1476046, RS2071943 and RS2070699 both showed a significant relationship with MRS measured at 6 months (p=0.38, p=0.045 and p=0.006 respectively). No other SNP's showed significant relationships with CV or MRS. By investigating all of the tagging SNP's involved with ET-1 all of the genetic variation within the ET-1 gene is accounted for in this analysis. The variant allele of RS2070699 is associated with CV and with a worse MRS at 6 months after injury. The variant allele of RS1476046 and RS2071943 are associated with a better MRS at 6 months after injury The significant relationship found between a tSNP and CV

or long term outcomes is the first step that may allow clinicians to screen for SNP's that are associated with the development of CV. This screening will allow clinicians to focus resources on patients with increased risk of CV and worse outcomes.

1.9 HUMAN SUBJECTS

1.9.1 Human Subjects Involvement and Characteristics

All subjects were recruited as part of a parent study that investigates biomarkers of acute and long term recovery of aSAH patients. There were approximately 280 subjects with DNA available for genotyping. Of those 250 subjects approximately 150 subjects have angiographic CV data available for analysis. There was also a subset of approximately 210 subjects with long term outcome data available for analysis. Inclusion criteria for this study were: 1) Male or female, 2) 18-75 years of age, 3) A DNA sample must be available for genotyping. Exclusion criteria are: 1) SAH due to trauma, mycotic aneurysm, arterial-venous malformation, or from an unknown source and 2) pre-existing neurological disease or injury. No exclusion criteria were based on race, gender or ethnicity. Children below the age of 18 account for a low incidence of aSAH (<.001) and were excluded from this study. No special classes of subjects (e.g., fetuses, neonates, pregnant women, children, prisoners, institutionalized individuals or others) who may be considered vulnerable populations were recruited into the parent study or this study.

1.9.2 Sources of Data

Demographic data was collected as part of the parent studies. The parent studies collected baseline data, age, gender, race, past medical history, Fisher Grade and Hunt & Hess Score from the medical records. No additional demographic data were collected as a part of this study.

All biological and DNA specimens were collected as part of the parent studies and are labeled with the unique study identification number, and date and time drawn. Subjects had 10cc of whole blood drawn on day one of enrollment into the parent studies. The tube of blood was delivered to Dr. Conley's laboratory with no personal identifiers. The sample was logged in and centrifuged, the white cells removed, and DNA extracted from the white cells using a simple salting out procedure(Miller et al.). All DNA is stored in 1X TE buffer at 4°C. Cerebrospinal fluid (CSF) specimens were obtained twice daily from either extraventricular drains (EVD) or via lumbar drains (LDs). All samples were then immediately frozen in cryogenic vials to –20oC. At the end of 14 days when all collections were completed the specimens were stored in –80oC freezers until analysis. The ET-1 CSF level analysis took place in Dr. Poloyac's tissue and animal laboratory located in the School of Pharmacy. No additional specimens were collected as part of this study.

Outcome data was also collected as part of the parent studies at the 3 months and after aneurysm rupture. No additional outcome data were collected as part of this study. All data is stored with the subject's unique identification number.

1.9.3 Recruitment and Retention

There were no special recruitment or retention plans for this study. Since this was a study that analyses samples and data that were previously collected the investigator had no contact with potential or current subjects.

1.9.4 Potential Risks to the Participants

There were no physical risks to the research subjects due to this study's analysis of existing samples. However, there are potential anticipated risks to confidentiality. All samples and previously collected data were labeled with the parent study's identification number. The master list that links the subjects name with the study identification number is kept by the parent study's principal investigator. All data from the parent study was stored in a locked file cabinet or on a password protected computer. A new computerized database was started for the collection of the genotype data. This database was maintained by the principal investigator and the faculty mentor to ensure correct data entry and minimal risk to subject confidentiality.

1.9.5 Procedures to Minimize Risks

To protect the subjects from the risk to confidentiality the following procedures were used. All samples and subjects were identified by unique identification numbers. The master list of the subject's identification was maintained by the principal investigators of the parent study. All data collected were maintained on password protected computers.

1.9.6 Importance of Knowledge to be Gained

The risks to subjects were limited to breach of confidentiality. The importance of the knowledge to be gained is significant. This study will advance nursing research in human genetics. The results of this study may also affect nursing interventions in the care of patients after aSAH by helping nurses to identify and focus resources on patients at higher risk of CV and poor long-term outcomes. The results of this study will also promote the integration of nursing process into the delivery of genomics-based healthcare services. Finally, the results of this study will add to the literature and encourage development of interventions aimed at decreasing CV after SAH and improving long term outcomes with minimal risk to the subjects.

2.0 SUMMARY OF STUDY

The study followed the terms of the proposal except for the statistical analysis used to analyze the specific aims. Each specific aim will be restated and the statistical analysis that was used will be described in detail. Of the 7specific aims, 5 are included in the two manuscripts within this document. The two specific aims that were not included in the manuscripts will also be addressed below.

Specific Aim #1: Describe the distribution of Lys198Asn and tSNP of the ET-1 gene in individuals recovering from aSAH. This specific aim was addressed in the manuscript entitled 'Endtothelin-1 Tagging SNPs and Their Relationship to Outcomes Following Aneurysmal Subarachnoid Hemorrhage'.

Specific Aim #2: Describe the relationship between the ET-1 polymorphisms and CV after aSAH. The chi-square analysis was not used as stated in the proposal. Binary logistic regression was used as a predictive model of CV. The individuals SNP's genotypes were used as the predictor. Fisher grade and age were controlled for in this model. This specific aim was addressed in the manuscript entitled 'Endothelin-1 Tagging SNPs and Their Relationship to Outcomes Following Aneurysmal Subarachnoid Hemorrhage'.

Specific Aim #3: Describe the relationship between the ET-1 polymorphisms and long term outcomes after aSAH. Multinomial logistic regression replaced the chi-square analysis that was originally proposed. The genotypes were used as the predictor, and the model controlled for

age and Hunt& Hess grade. This specific aim was addressed in the manuscript entitled 'Endothelin-1 Tagging SNPs and Their Relationship to Outcomes Following Aneurysmal Subarachnoid Hemorrhage'.

Specific Aim #4: Describe the CSF ET-1 protein expression for the 3 days prior to CV measurement. This specific aim is addressed in the manuscript entitled 'Endothelin-1 Cerebrospinal Fluid Levels are Associated with the Development of Cerebral Vasospasm in Aneurysmal Subarachnoid Hemorrhage Patients'.

Specific Aim #5: Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, of individuals with and without CV over time for the first 14 days after aSAH. Trajectory analysis was used to dichotomize the subjects into high or low ET-1 CSF level groups based on their ET-1 CSF levels for the first 14 days after aneurysm rupture. Binary logistic regression was used to determine the relationship between the ET-1 level, ET-1 genotypes, and CV. CV was the outcome variable in this model. Age and Fisher grade were controlled for in the model. ET-1 level groups and genotypes were the predictors in this model.

Results: A total of 62 subjects were included in this analysis. These subjects were all Caucasian, predominantly female (n=47, 75.8%), and had a mean age of 55.18 ±10.49 years old. Twenty-seven (43.5%) of the 62 subjects were CV positive. ET-1 level groups and ET-1 genotypes were not significant predictors of CV in this model.

Conclusion: The lack of a significant finding is not surprising in this case. The ET-1 level groups were based on the ET-1 levels measured over a 14 day period. In the attached manuscript it is reported that a difference in ET-1 CSF levels are seen in the 72 hours before CV occurs. The other 11 days worth of ET-1 levels may not have any influence on CV occurrence.

Therefore, this analysis even with the genotype data included did not find any significant differences.

Specific Aim #6: Describe the CSF ET-1 protein expression, in relation to Lys198Asn and tSNP's, for the 3 days prior to CV measurement. This specific aim is addressed in the manuscript entitled 'Endothelin-1 Tagging SNPs and Their Relationship to Outcomes Following Aneurysmal Subarachnoid Hemorrhage'.

Specific Aim #7: Describe the CSF ET-1 protein expression over the first 14 days after aSAH, in association with Lys198Asn and tSNP's, and its potential relationship with long term outcomes. Trajectory analysis was used to dichotomize the subjects into high or low ET-1 CSF level groups based on their ET-1 CSF levels for the first 14 days after aneurysm rupture. Binary logistic regression was used to determine the relationship between the ET-1 level, ET-1 genotypes, and long term outcomes. MRS or GOS at 3 or 6 months were the outcome variables in this model. Age and Hunt & Hess grade were controlled for in the model. ET-1 level groups and genotypes were the predictors in this model.

Results: There were 74 subjects with outcomes measured at 3 months. These subjects were Caucasian, the majority were female 52 (70.3%), and had a mean age of 55.55 ± 9.90 years old. There were also 64 subjects that had outcomes measured at 6 months. All were Caucasian, 47 (73.4%) were female, and the mean age was 57.14±9.66 years old. ET-1 level groups and genotypes were not significant predictors in this model.

Conclusion: The lack of significant findings may have two possible causes. The first is that ET-1 levels and ET-1 genotypes do not have a major role in long-term outcomes. The second explanation is that the long-term outcome measures used here are not specific enough to detect outcomes changes influenced by ET-1 CSF levels or ET-1 genotypes. The GOS and MRS

are measure of global functional outcomes. It may be more appropriate to investigate specific neuropsychological outcomes to elucidate differences in long-term outcomes.

This study supports the use of ET-1 for use as a protein biomarker and a genetic biomarker in the prediction of CV following aneurysmal SAH. The ET-1 CSF levels 72 hours before angiography are significantly higher in the CV positive group when compared to the CV negative group. The SNP rs2070699's variant allele was also a predictor of the occurrence of CV. This relationship was a dose dependent relationship. An individual with no variant allele, homozygous wild type, had the lowest odds of having CV; a heterozygous individual had almost 3 times the odds of having CV. Finally, a homozygous variant individual (two copies of the variant allele) had 8 times the odds of having CV when compared to the homozygous wild-type individual.

The investigation of long-term outcomes and ET-1 levels/ET-1 SNPs produced no significant findings. While this is disappointing, it can be explained in one of two ways. The first is that ET-1 levels and ET-1 genotypes do not have a major role in long-term outcomes. The second explanation is that the long-term outcome measures used here are not specific enough to detect outcome changes influenced by ET-1 CSF levels or ET-1 genotypes.

The strengths of this study are numerous, 1) angiography was used as a definitive diagnosis of CV. Therefore, the chance of misdiagnosis is all but eliminated. 2) It is the first study to date that controls the CSF drainage volume in relation to ET-1 concentration. This may have made our study more sensitive to changes in ET-1 levels in the CSF. 3) This is the first study to date that investigates ET-1 SNPs in the SAH population.

There are limitations to this study. The subset of patients with ET-1 CSF levels may have been in a more critical state during their hospitalization. Our samples of CSF were taken

from patient with CSF drainage catheters. To receive a catheter the patient must be more critically ill than someone without. The subset of patients with angiographies would also have been in a more critical state. Repeat angiographies are typically performed for a change in neurologic exam. Therefore, this would also skew the sample to include patients in a more critical state. The patients included in the analysis of the ET-1 SNPs were limited to Caucasians. This is due to the fact that distribution of alleles and tagging SNPs themselves are different between races. The small number of minorities presenting to the UPMC Presbyterian hospital with a diagnosis of SAH did not allow for proper analysis of genotypes in minorities. Therefore, the results from this study will not apply to minorities.

Future studies would include continuing the above study with larger sample numbers, especially the subset of ET-1 CSF subjects. In addition larger minority numbers would be needed to investigate the genetic relationship of ET-1 and outcomes following SAH. While 1 ET-1 tagging SNP was associated with CV, the specific cause of this association was not elucidated. Further studies are needed to investigate the area of the ET-1 gene that the tagging SNP represents. This investigation would focus on finding functional SNPs that would change the protein structure of ET-1 thereby causing a change in ET-1 function. Additional work exploring the relationship between ET-1 genotypes and/or phenotype and outcome while controlling for angiographic CV may also show that these are significant predictors of outcome in subgroups of individuals with or without CV. Finally, other possible biomarkers of CV and long-term outcomes need to be investigated in a similar fashion as above. By exploring biomarkers it is the aim to predict and prevent poor outcome following SAH.

3.0 MANUSCRIPT 1: ENDOTHELIN-1 CEREBROSPINAL FLUID LEVELS ARE ASSOCIATED WITH THE DEVELOPMENT OF CEREBRAL VASOSPASM IN ANEURYSMAL SUBARACHNOID HEMORRHAGE PATIENTS

3.1 LETTER TO EDITOR

April 30, 2008

Vladimir Hachinski, MD
Editor
Stroke Editorial Office,
University of Western Ontario Research Park
100 Collip Circle, Suite 116
London, ON, Canada N6G 4X8

Dear Dr. Hachinski:

Please find enclosed the manuscript entitled "Endothelin-1 Cerebrospinal Fluid Levels are Associated with the Development of Cerebral Vasospasm in Aneurysmal Subarachnoid Hemorrhage Patients." that we would like for you to consider for publication in Stroke. The findings reported in this manuscript demonstrate in a large patient population that there is a significant temporal relationship between CSF endothelin-1 concentrations and the subsequent development of vasospasm as definitively determined by angiography. These findings provide much needed clarity as to the role of endothelin-1 in relation to the development of cerebral vasospasm and also represent one of the largest studies in this patient population. The authors have reviewed this manuscript and appreciate the time and effort committed by all for peer review. The content of this manuscript has not been published before and is not being considered for publication elsewhere. We look forward to future correspondence. All communication can be addressed to the following:

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Sincerely,

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3.2 MANUSCRIPT

Endothelin-1 Cerebrospinal Fluid Levels are Associated with the Development of Cerebral Vasospasm in Aneurysmal Subarachnoid Hemorrhage Patients

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Conflicts of Interest and Disclosures:

There are no conflicts of interest or disclosures associated with this manuscript.

Endothelin-1 Cerebrospinal Fluid Levels are Associated with the Development of Cerebral Vasospasm in Aneurysmal Subarachnoid Hemorrhage Patients

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Key Words: Cerebral Vasospasm, Endothelin, Subarachnoid Hemorrhage

Word Count: 4,274

3.2.1 Abstract

Background and Purpose: Aneurysmal subarachnoid hemorrhage (SAH) is a devastating disease that affects approximately 25,000 people a year in the United States. Cerebral vasospasm (CV) is a common complication after SAH that results from narrowing of the cerebral arteries. The underlying mechanism of CV remains unclear. Endothelin-1 (ET-1) is a vasoconstrictor that may play a role in the pathogenesis of CV. At this time no clear temporal relationship has been established between ET-1 levels and CV. The purpose of this study was to examine changes in volume controlled ET-1 levels in CSF in the time before angiographic measurement of CV, and to investigate the relationship between CSF ET-1 and CV in SAH patients. Methods: Forty patients with SAH were recruited as part of this study. Cerebrospinal fluid drainage volume was controlled for in these samples and expressed as elimination rate (pg/hr). Cerebral vasospasm was determined by angiography as part of medical care. ET-1 levels were measured using chemiluminescent ELISA kits. Results: The average ET-1 elimination rate for the 72 hours before angiography was 4.35±3.02 pg/hr for the CV negative group and 7.94±6.47pg/hr for the CV positive group. The ET-1 elimination rate for the 72 hours before angiography was a significant predictor of CV in this model when controlling for age and Fisher grade ($p=0.026$). Conclusions: This study showed a temporal relationship between ET-1 levels 72 hours before CV measurement and the development of CV after SAH. This study supports the potential use of ET-1 as an index of CV development.

3.2.2 Introduction

Subarachnoid hemorrhage (SAH) occurs in approximately 1 out of 10,000 people in the United States each year (Longstreth et al., 1985), accounting for 10% of the stroke population. It is associated with a mortality rate of 30-50% (Sobey & Faraci, 1998), most often originating as an aneurysmal rupture of vessels of the circle of Willis (Longstreth et al., 1985). Of those that survive the initial hemorrhage, 40-50% will suffer severe long term neurological disabilities (Sobey & Faraci, 1998). These disabilities may include cognitive dysfunction and memory loss with an estimated cost of over $10,000 per person per year for long-term care (Lee et al., 2007) alone.

Despite advances in neurosurgical treatments, the morbidity and mortality rate of SAH has not significantly improved over the past 40 years (Solenski, Haley, Kassell, Kongable, Germanson, Truskowski, & Torner, 1995). Surgical methods of aneurysm securement have improved survival after the initial injury, yet these techniques do not appear to diminish the serious adverse consequences of cerebral vasospasm (CV), leaving long term survival rates relatively unchanged (Solenski, Haley, Kassell, Kongable, Germanson, Truskowski, & Torner, 1995). Cerebral vasospasm is a narrowing of the cerebral arteries that can result in delayed cerebral ischemia, further neurological damage, and stroke. Cerebral vasospasm has a peak incidence of 3 to 12 days after the initial SAH (Newell, Eskridge, Mayberg, Grady, & Winn, 1989). Angiographic CV occurs in up to 70% of patients with SAH (Dorsch, 2002) and symptomatic vasospasm (angiographic vasospasm with an associated change in neurological assessment) affects nearly one third of all patients. Without appropriate treatment CV can result in death in 30% of patients and permanent disability in 34% of patients (Dorsch, 2002), thereby serving as a major complication affecting the morbidity and mortality of SAH. CV has been

correlated with a 1.5 to 3 fold increase in mortality in the first 2 weeks after SAH and occurs in 30-70% of all patients with a SAH (Treggiari-Venzi, Suter, & Romand, 2001).

While the underlying mechanism for CV remains unknown, individuals who have an increased amount of blood in the subarachnoid space have a greater risk of developing CV and the CV is likely to be more severe (Treggiari-Venzi, Suter, & Romand, 2001). The cause of CV is likely to be multifactorial in nature, such that the balance between normal auto-regulatory controls results in the production of vasoconstrictive mediators. Although the predominant mediators of CV remain obscure, several vasoactive metabolites have been shown to be present in the CSF of patients after SAH. These mediators include endothelin-1 (Mima et al., 1989; Petzold, Einhaupl, Dirnagl, & Dreier, 2003; Seifert et al., 1995; H. Suzuki, Sato, Suzuki, Oka et al., 1990), 20 HETE (Cambj-Sapunar, Yu, Harder, & Roman, 2003; Poloyac, Reynolds, Yonas, & Kerr, 2005), vasopressin (Mather, Ang, & Jenkins, 1981; Nishihashi, Trandafir, Wang, Ji, & Kurahashi, 2005), proinflammatory cytokines (Kwon & Jeon, 2001), angiotensin, and adhesion molecules (Lin et al., 2005; Lin et al., 2007) which have been investigated in clinical or animal models. One of these mediators with moderate support is endothelin-1 (ET-1). ET-1 is a long lasting and potent vasoconstrictor (H. Suzuki, Sato, Suzuki, Takekoshi et al., 1990; Yamaji et al., 1990). It directly binds to receptors on vascular smooth muscle cells leading to calcium mobilization and smooth muscle contraction (Davie et al., 2002) thereby influencing vasomotor tone. Vascular smooth muscle contraction is observed at ET-1 concentrations as low as 3 X 10 -11 mol/L (Hirata et al., 1989).

Several groups have investigated the role of ET-1 in CV. Initial studies showed an increase in the ET-1 concentrations in the CSF of patients with SAH compared to controls without SAH (H. Suzuki, Sato, Suzuki, Takekoshi et al., 1990). Subsequent clinical studies demonstrated a

correlation of increased ET-1 production with the presence of symptomatic vasospasm (Fassbender et al., 2000; Kastner et al., 2005; Kessler, Pacheco, Lozzi, de Araujo, & Onishi, 2005; Mascia, Fedorko, Stewart, Mohamed, terBrugge, Ranieri, & Wallace, 2001; Seifert et al., 1995; K. Suzuki et al., 2000). However, a clear temporal relationship between increased ET-1 in the CSF and occurrence of CV has yet to be reported. The use of ET-1 levels as a biomarker of CV has remained elusive due to several factors which include 1) different means of defining CV through the literature 2) investigation of ET-1 levels in relationship to aneurysm rupture rather than relationship to occurrence of CV, 3) lack of control for the volume of CSF drained during normal medical treatment, and 4) lack of sensitivity of the assays used. Therefore, the purpose of this study was to examine changes in volume controlled ET-1 levels in CSF in the time before angiographic measurement of CV, and to investigate relationship between CSF ET-1 and CV in SAH patients.

3.2.3 Subjects and Methods

Patients

Following approval of the institutional review board, patients were recruited for an ongoing NIH funded study (R01-NR004339) and informed consent was obtained from the patient or proxy. Inclusion criteria for the parent study were: adult patients (ages 18-75 years) with the diagnosis of SAH from a ruptured cerebral aneurysm verified by Digital Subtraction Angiography (DSA) or CT angiography (CTA) with a Fisher Grade > 1. The first forty patients from the parent study with both cerebrospinal fluid drains (via an external ventricular device catheter (EVD) or lumbar drain (LD)) and cerebral angiograms during the first 14 days after admission were included in this analysis. Patients were excluded from this study and the parent study if they had a

preexisting neurological disorder or if their SAH resulted from a mycotic aneurysm, arterio-venous malformation, trauma, or an unknown source. Patients were admitted to the Neurovascular intensive care unit (NV-ICU) at the University of Pittsburgh Medical Center (UPMC) from July 2003 to August 2006. All patients received standard nursing and medical care in the NV-ICU including triple-H therapy (hypertension, hemodilution, and hypervolemia), nimodipine, and early surgical or endovascular intervention.

Demographic information

Age in years, race, sex, Fisher grade and Hunt and Hess grade were extrapolated from the medical record. Fisher grade and Hunt and Hess grade were assigned by the admitting neurosurgeon.

CSF specimens

Cerebrospinal fluid (CSF) specimens were obtained twice daily from EVDs or LDs placed by trained neurosurgeons. EVDs were placed into a lateral ventricle. To obtain samples from the lateral ventricle, specimens were collected using sterile technique, drawn directly from the drainage catheter at the site closest to the patient and then immediately frozen in cryogenic vials at –20oC. After 14 days, all collections were completed and the specimens were stored at -80^{o}C until analysis. The total CSF volume drained since the last sample collection was also recorded at the time of sample collection.

Cerebral Vasospasm

Cerebral vasospasm was diagnosed by measuring the diameter of cerebral vessels as documented on cerebral angiography. Cerebral angiography was conducted as standard of care on admission, as postoperative follow-up care, and/or in the event of a neurological deterioration. The angiographic studies were interpreted and graded by neuro-radiologists and neurosurgeons at

UPMC for evidence and degree of vasospasm. The degree of vasospasm was represented as the percentage of narrowing in the cerebral vessels as follows: CV negative was 0 to 25% narrowing and CV positive was 26-99% narrowing.

Measurement of ET-1

CSF samples were assayed for ET-1 using the QuantiGlo® Human Endothelin-1 ELISA kit from R&D Systems (Minneopolis, MN, USA). This assay employs the quantitative sandwich enzyme immunoassay technique with chemiluminesent detection. Chemiluminescent kits were required in this analysis because typical colorimetric kits lacked sufficient sensitivity to allow for quantitative CSF ET-1 concentration measurements. The standard curve was modified from the R&D recommendations to include 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, & 12.5 pg/ml standards. Precision and accuracy of the modified standard curve range was evaluated with intra-assay and inter-assay coefficients of variation. The intra-assay coefficients of variation were 14.1%, 14.0%, and 13.2% for the 0.195, 1.56, and 12.5 pg/ml standards respectively. The inter-assay coefficients of variation were 9.57%, 12.67%, and 9.0% for the 0.195, 1.56, and 12.5 pg/ml standards respectively. This range of standards was used to cover the range of concentrations observed in the CSF of SAH patients. A microplate luminometer (Model # 1420-012 Victor-Wallac, Finland) was used to measure the intensity of the light emitted.

Statistical Analysis

SPSS version 14.0 was used for all analyses. Values are presented in the tables and text as mean ± SD. ET-1 protein elimination rate was calculated in a similar manner to Shore et al (2004), with the following formula:

$$\text{Elimination Rate (pg/hr)} = \frac{\text{Concentration of Sample (pg/ml) X CSF Drainage (ml)}}{\text{Time Period (hr)}}$$

The average elimination rate was calculated for 24 hours, 48 hours, and 72 hours before angiography. The subjects were divided into vasospasm positive and vasospasm negative groups as previously defined. Differences between vasospasm positive and vasospasm negative groups were investigated using chi-square analysis for categorical variables, and independent t-test for continuous variables. Binary logistic regression was used to evaluate the association between average ET-1 elimination rate at 72 hours before, 48 hours before, and 24 hours before angiography and 72 hours after angiography, with the outcome measure of CV controlling for age and Fisher grade. A significant difference was defined as $p \leq 0.05$.

3.2.4 Results

Forty subjects with CSF ET-1measurements, demographic information, and angiographic data were analyzed. These subjects were predominantly female (n=28; 70.0%) and Caucasian (n=36; 90.0%). The average subject age was 54.8 ± 10.3 years old. In this sample 16 (40%) patients developed CV. Demographic information in relationship to vasospasm is presented in Table 1. There were no significant differences in demographic characteristics between the two groups. There was an average of 5.35 CSF samples collected per subject. Graphs of representative ET-1 elimination rates versus time profile for 4 subjects are presented in Figure 2.

Table 1: Demographics (n=40)

		CV Negative	CV Positive	p value
Sex	Male	8 (20.0%)	4 (10.0%)	0.729
	Female	16 (40.0%)	12 (30.0%)	
Age	Median	55.0	53.0	
	Mean (SD)	56.3 (9.2)	52.6 (11.7)	0.272
Race	African-American	2 (5.0%)	2 (5.0%)	0.715
	Caucasian	22 (55.0%)	14 (35.0%)	
Hunt &Hess Grade	1	0 (0%)	0 (0%)	0.437
	2	5 (12.5%)	2 (5.0%)	
	3	8 (20.0%)	9 (22.5%)	
	4	9 (22.5%)	3 (7.5%)	
	5	2 (5.0%)	2 (5.0%)	
Fisher Grade	1	0 (0%)	0 (0%)	0.382
	2	8 (20.0%)	2 (5.0%)	
	3	11 (27.5%)	9 (22.5%)	
	4	5 (12.5%)	5 (12.5%)	

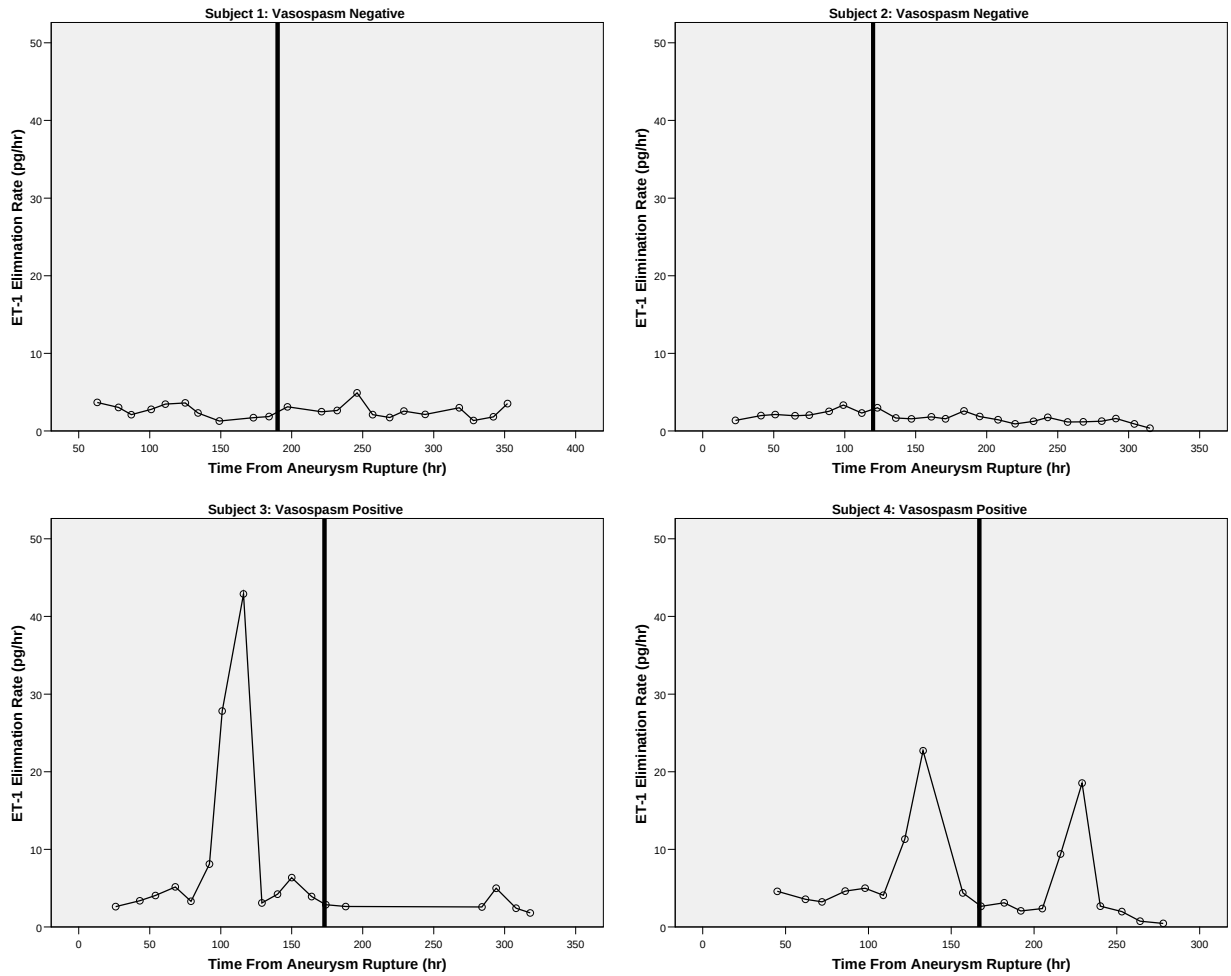

Figure 2: ET-1 elimination rate profiles of four subjects. The vertical line represents angiographic measurement of cerebral vasospasm. Subjects 1 and 2 were cerebral vasospasm negative. Subjects 3 and 4 were cerebral vasospasm positive.

The average ET-1 concentrations for the 24 hours before angiography for the CV negative and positive groups were 0.53±0.23 pg/ml and 0.58±0.47 pg/ml respectively. The 48 hours before angiography had ET-1 concentration levels of 0.54±0.29 pg/ml for the CV negative group and 0.59±0.63 pg/ml for the CV positive group. The average ET-1 concentration for the 72 hours before angiography was 0.60±0.39 pg/ml for the CV negative group and 0.67±0.63 pg/ml for the CV positive group.

The average ET-1 elimination rates for the 24 hours before angiography for the CV negative and positive groups (3.91±2.71 pg/hr versus 6.01±4.84 pg/hr) or for the 48 hours before angiography for the CV negative and positive groups (3.79±2.49 pg/hr versus 6.73±5.91 pg/hr) were not significant predictors of CV, but the data showed trends towards significance (p=.054) at 48 hours before angiography (Table 2). The average ET-1 elimination rate for the 72 hours before angiography was 4.35±3.02 pg/hr for the CV negative group and 7.94±6.47pg/hr for the CV positive group (Figure 3). The 72 hour ET-1 elimination rate was a significant predictor of CV in this model when controlling for age and Fisher grade. The average ET-1 elimination rate 72 hours after angiography showed no significant difference between the CV negative group (3.65±2.26 pg/hr) and the CV positive group (4.91±3.69 pg/hr).

Table 2: Results from Binary Logistic Regression for CV Positive Verses CV Negative Groups

Average Elimination Rate	Odds Ratio (with 95% CI)	SE	p
72 Hours before Angiography	1.244 (1.027 to 1.508)	0.098	0.026*
48 Hours before Angiography	1.225 (0.996 to 1.506)	0.105	0.054
24 Hours before Angiography	1.190 (0.959 to 1.476)	0.110	0.114
72 Hours after Angiography	1.183 (0.912 to 1.534)	0.133	0.205

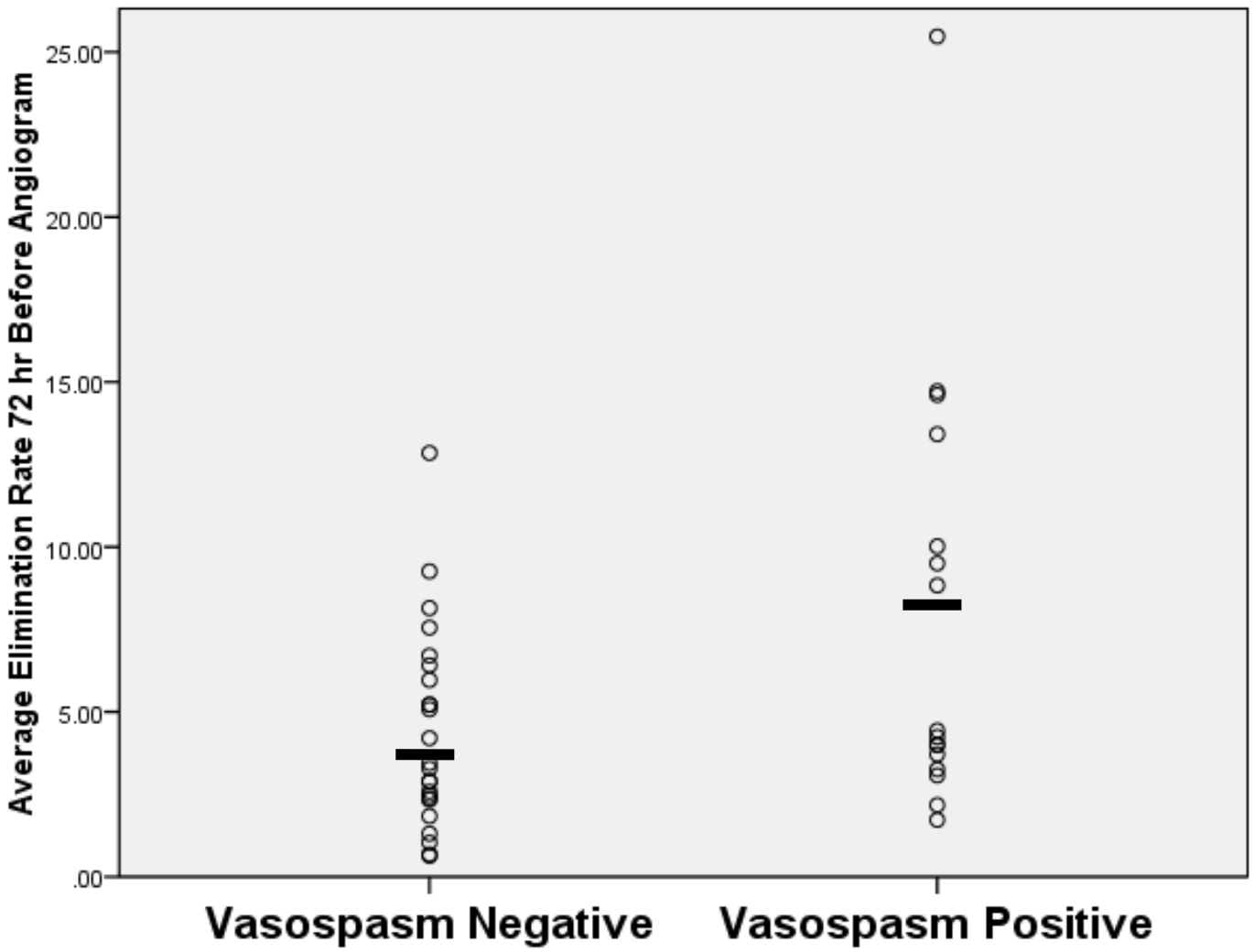

Figure 3: Average ET-1 elimination rate (pg/hr) 72 hours before angiographic measurement of vasospasm. The horizontal bar indicates the mean of each group.

3.2.5 Discussion

The results from this study demonstrate that the average ET-1 elimination rate for the 72 hours before measurement of CV by angiography is a significant predictor of CV, while controlling for age and Fisher Grade (p=0.026). The odds ratio of 1.244 indicates that for every increase of 1

pg/hr in the average elimination rate 72 hours before angiography the odds of having vasospasm is 1.2 times greater while controlling for age and Fisher grade. A strong trend toward significance was found for the average ET-1 elimination rate for the 48 hours before angiography ($p=0.054$), whereas there was no significant difference in ET-1 elimination rate observed 72 hours after angiography. In addition, the concentration of ET-1 in the CSF of SAH patients with CV was 2.69 X 10-13 mol/L. Although this concentration is below the concentration of 3 X 10 - 11 mol/L which has been shown to constrict vascular beds (Hirata et al., 1989), it is expected that the concentrations at the site of the vascular beds would be greater than the diluted concentration observed in the CSF sampling site. Based on this data, we conclude that there is a temporal relationship between ET-1 elimination rate and the formation of CV, with elevated ET-1 elimination rates observed in the CSF 72 hours prior to angiography.

Our observation that ET-1 is elevated in the CSF of SAH patients is consistent with prior studies. Specifically, our observations concur with Kessler and associates findings of an increase in ET-1 levels in the CSF of patients with CV (Kessler, Pacheco, Lozzi, de Araujo, & Onishi, 2005). In contrast Mascia et al. concluded that there was no temporal relationship of ET-1 levels in the CSF to CV, and that ET-1 levels were indicative of neuronal damage (Mascia, Fedorko, Stewart, Mohamed, terBrugge, Ranieri, & Wallace, 2001). This difference in our findings may be attributed to numerous reasons. First, our study was the first study that controls for the CSF drainage volume by investigating ET-1 elimination rate. All of our subjects had continuous passive CSF drainage. By investigating ET-1 elimination, we were able to control for subjects that drained variable amounts of CSF. Therefore, the previously mentioned studies may have been insensitive to differences caused by not controlling for the volume of CSF.

Second, we used angiography as a definitive diagnosis of CV as compared to others who used transcranial Doppler ultrasonography. While transcranial Doppler ultrasonography is an important tool in the diagnosis of vasospasm, there are instances where transcranial Doppler ultrasonography values may yield false negative or false positive results. A diagnosis of CV by transcranial Doppler ultrasonography must also be accompanied by a change in the patient's neurological exam. However, there are cases where a subject may have an increase in transcranial Doppler ultrasonography velocities and not have a change in their neurological exam or an increase in velocity and neurological change from reasons other than CV (such as fever). In these cases, the subjects may be assigned incorrectly into a CV negative group when in fact there was some sort of compensation for the CV even though they were not showing any signs of neurological deterioration. Subjects may incorrectly be assigned into CV positive groups when there change in neurological assessment may have another underlying cause such as fever. By using angiography to measure CV we have all but eliminated the chance of misdiagnosis.

Third, we performed our analysis relative to the time of CV measurement by angiography. Previous studies have based their analysis on the time from aneurysmal rupture. Because of the long period of time in which CV may occur (day 3 to day 14 post rupture) we changed the perspective of our study. By changing the approach to analysis to consider time from CSF sampling and angiography for CV verification, we believe that we have better elucidated the temporal relationship of ET-1 levels in the CSF relative to the development of CV, thereby characterizing a novel temporal relationship between ET-1 and CV.

Fourth, we utilized a chemiluminescent ELISA method for detection of ET-1 concentrations in the CSF. This detection method was necessary because colorimetric kits lacked adequate sensitivity to detect ET-1 concentrations in the CSF of our patient population.

Furthermore, we modified the calibration curves to allow for appropriate validation of these assays for the measurement of CSF ET-1 concentrations with the majority of sample concentrations quantitated from the middle of the calibration curve. Validation of this method allowed for analytical quantitation of ET-1 concentrations in the CSF. Prior studies employing colorimetric assay methods would be expected to have adequate sensitivity to measure peak ET-1 CSF concentrations, but would likely lack the sensitivity to quantitate low levels of ET-1 in non-spasm patients.

Finally, this study collected more CSF samples at numerous time points than previous studies. We were able to collect CSF every twelve hours and had an average of 5.35 samples per subject. This collection process may have made this study more sensitive to changes in the ET-1 levels of CSF.

However, there are limitations to this study. The first is the subset of SAH patients. This study examined patients that had access to CSF samples (EVD or LD). Patients with a drainage system for CSF are traditionally in a more critical state than patients without a drain, as was the case with this study's subjects, demonstrated by Hunt and Hess Scores (Table 1). However, it was not ethically sound to access patients without drains by performing lumbar punctures every twelve hours or to place drains in patients that did not have a clinical need for these invasive catheters. Additionally, of the subjects presenting to the UPMC Presbyterian Health System for care after aneurysmal SAH, on average, 75% have a CSF drainage device placed during their care. Due to the small number of patients who do not receive such a device, we have captured the majority of patients at risk for CV with our current study criteria. We were able to only include patients that also had a cerebral angiography as a measure of the presence or absence of CV. The majority of this study's patients had a clinical need for an angiography (i.e. change in

level of consciousness). By including patients that have a need for angiography the sample is skewed towards subjects that are in a more critical state. Keeping in mind that up to 70% of patients with SAH have angiographic CV, but only 33% of patients have symptomatic CV(Dorsch, 2002) (angiographic CV with a neurological change), there is a large percentage of patients that may have CV that would not have an angiography for this study.

The third limitation of this study was the relatively small number of patients in our sample. Our sample size is limited by two major factors. One, our patients need to have an EVD or LD to collect the ET-1 levels. Not all patients clinically have a need for an EVD or LD, therefore that portion of the population was not included in this study. In addition our sample size is limited by follow up angiography. As stated above, the majority of angiograms are performed on patients that have a clinical neurological deterioration. Future work should include a larger sample size to give more power to such a study. We were however able to identify significant differences in ET-1 elimination 72 hours before angiographic cerebral vasospasm suggesting that the effect size was large enough to accommodate the small sample size.

In summary, we found the average ET-1 elimination rate for the 72 hour period before angiogram to be predictive of CV when controlling for age and Fisher grade. This suggests that an average increased exposure time to ET-1 increases the likelihood of CV development, suggesting a possible mechanism of CV, and that CSF ET-1 level may be used to predict patients at risk for CV.

4.0 MANUSCRIPT 2: ENDTOTHELIN-1 TAGGING SNPS AND THEIR RELATIONSHIP TO OUTCOMES FOLLOWING ANEURYSMAL SUBARACHNOID HEMORRHAGE

4.1 INTRODUCTION

There are approximately 25,000 cases of subarachnoid hemorrhage (SAH) in the United States each year (Longstreth et al., 1985) most of which occur in women at an average age of 55 years old (Sobey & Faraci, 1998). Ninety five percent of cases of spontaneous SAH result from ruptured cerebral aneurysms (aSAH) (Sobey & Faraci, 1998). The morbidity and mortality rate of aSAH has decreased little over the past 40 years (Solenski, Haley, Kassell, Kongable, Germanson, Truskowski, Torner et al., 1995) despite new surgical methods that have been developed to improve survival rates of the initial injury. While surgery decreased the risk of aneurysmal rebleed, the frequency of secondary injuries became evident and survival from aSAH did not improve. In great part due to the frequency of cerebral vasospasm (CV) (Solenski, Haley, Kassell, Kongable, Germanson, Truskowski, Torner et al., 1995).

Cerebral vasospasm a decrease in diameter of the lumen of cerebral blood vessels, can lead to delayed cerebral ischemia, furthering neurological damage, and possibly ischemic stroke. Worse outcomes have been associated with the presence of CV (Goto & Yamagata, 2006; Ratsep & Asser, 2001). Cerebral vasospasm has been correlated with a 1.5 to 3 fold increase in mortality

in the first 2 weeks after aSAH (Treggiari-Venzi, Suter, Romand et al., 2001). Cerebral vasospasm occurs with a peak incidence 3 to 12 days after the initial aSAH (Newell, Eskridge, Mayberg, Grady, Winn et al., 1989). Cerebral vasospasm characterized by angiography is defined as angiographic CV, and may occur in up to 70% of patients with aSAH (Dorsch, 2002).

There are few accurate predictors of CV including amount and distribution of blood on CT scan (categorized by Fisher grade), age, hypertension, cigarette smoking, and cocaine use (Conway & Tamargo, 2001; Qureshi et al., 2001). However, these risk factors do not accurately predict the occurrence of CV. While numerous causal factors of CV have been investigated (Cohen et al., 1995; Hatake et al., 1992; Kasuya et al., 1995; P. Kim et al., 1992; Onoue et al., 1995; Sobey et al., 1996; Yamamoto et al., 1997), the search for a single factor has been disappointing; a complex multifactorial etiology is most probable (Hamann & Schimrigk, 1995). In addition, long term recovery from aSAH is a complex process with few known prognostic factors including severity of hemorrhage, clinical exam upon admission, age, and presence or absence of cerebral vasospasm. Of those that survive the initial hemorrhage, 40-50% will suffer severe long term neurological disabilities (Sobey & Faraci, 1998). These disabilities may include cognitive dysfunction or physical dysfunction with an estimated cost of over $10,000 per person per year for long-term care alone (Lee et al., 2007).

Genetic susceptibility may play a role in better identifying patients at a high risk of poor outcomes. Once these risks are identified, closer monitoring and more aggressive interventions may be considered. Genes predicting poor outcome also provide information about pathways involved in outcomes and may give rise to potential interventions to prevent and/or treat these poor outcomes.

Researchers have suggested that endothelin-1 (ET-1), a potent vasoconstrictor produced by the endothelial cells of the vasculature (H. Suzuki, Sato, Suzuki, Oka et al., 1990; Yanagisawa, Kurihara, Kimura, Tomobe, Kobayashi, Mitsui, Yazaki, Goto, & Masaki, 1988), may play a role in the etiology of CV (Mascia, Fedorko, Stewart, Mohamed, terBrugge, Ranieri, Wallace et al., 2001; Seifert et al., 1995; Yamaji et al., 1990; Yanagisawa, Kurihara, Kimura, Tomobe, Kobayashi, Mitsui, Yazaki, Goto, Masaki et al., 1988). Suzuki and associates reported increased levels of ET-1 in cerebrospinal fluid (CSF) of aSAH patients (H. Suzuki, Sato, Suzuki, Takekoshi et al., 1990). Recent studies showed that increased CSF ET-1 levels are associated with the development of CV after aSAH (Kessler, Pacheco, Lozzi, de Araujo, Onishi et al., 2005), thus supporting the role of ET-1 in the pathogenesis of CV. A temporal relationship has also been described with increased CSF ET-1 levels occurring 72 hours before CV (Gallek et al., 2008).

ET-1 polymorphisms have been investigated in numerous vascular conditions. Conditions that have been investigated include essential hypertension (Stevens & Brown, 1995), preeclamptic pregnancy (Barden et al., 2001), cerebral small vessel disease (Gormley et al., 2005), and arterial stiffness (Iemitsu et al., 2006), to name a few. A functional polymorphism (Lys198Asn) of the gene encoding ET-1 has shown differences in vasculature reactivity (Iglarz et al., 2002). The difference in vasculature reactivity caused by this polymorphism may have an effect on the presence of or severity of CV thereby influencing long-term outcomes. Along with this known functional polymorphism, other polymorphisms in the gene for ET-1 may influence CV and long-term outcomes.

The majority of genetic variation among people can be characterized by single nucleotide polymorphisms (SNPs). These polymorphisms result in a single nucleotide being changed at a

frequency high enough to be recognized in the population. It has been estimated that there are about 7 million common SNPs in the human genome (Botstein & Risch, 2003; Kruglyak & Nickerson, 2001). There may be hundreds of SNPs associated with an individual gene such as the ET-1 gene. Genotyping each of these numerous SNPs is time consuming and not cost effective. Tagging SNPs allow researchers to genotype a single nucleotide polymorphism that represents a larger region of the gene. Therefore it is possible to account for genetic variation within a gene without genotyping every SNP in that chromosomal region. By exploring all of the tagging SNPs of ET-1 all genetic variance can be accounted for in the ET-1 gene, including potentially functional polymorphisms. To date there are no studies published that investigate ET-1 tagging SNPs or ET-1 functional SNPs and their association with CV, or outcomes following aSAH. Additionally no studies have explored protein expression (ET-1 levels) in the Cerebrospinal Fluid (CSF) of individuals with and without CV after aSAH in light of genetic polymorphisms.

The purpose of this study is to describe the distribution of the Lys198Asn polymorphism and tagging SNPs of the ET-1 gene in individuals recovering from aSAH. This study will also investigate the relationship between the ET-1 genotypes and outcomes, including CV, global functional outcomes measured at 3 months, and global functional outcomes measured at 6 months after aSAH. Finally this study will investigate, in a subset of the sample, the relationship of ET-1 CSF levels and ET-1 polymorphisms on CV following aSAH.

4.2 SUBJECTS AND METHODS

Patients

Following approval of the institutional review board and after obtaining informed consent from the patient or next of kin 250 patients were recruited as part of an ongoing NIH funded study (RO1NR004339). Inclusion criteria were: adult patients (ages 18-75 years) with the diagnosis of aSAH verified by Digital Subtraction Angiography (DSA) or CT angiography (CTA) with a Fisher Grade > 1. All patients also had a blood or CSF sample to obtain a deoxyribonucleic acid (DNA) sample for analysis. Finally, all subjects in the sample were Caucasian. This was because the genotype distributions of the ET-1 SNPs were different between the different races. By excluding minorities we were able to prevent false associations in our finding that may have been caused by these distribution differences.

Patients were excluded from the study if they had a preexisting neurological disorder. Patients were admitted to the Neurovascular intensive care unit (NV-ICU) at the University of Pittsburgh Medical Center (UPMC) from September 2000 to April 2006. All patients received standard nursing and medical care in the NV-ICU including triple-H therapy (hypertension, hypervolemia, and hemodilution), nimodipine, and early surgical or endovascular intervention. This sample of 250 subjects will be used to describe the distribution of ET-1 polymorphisms in the aSAH population.

A subset of 96 subjects will be used to investigate the relationships between ET-1 polymorphisms and outcomes following aSAH. These subjects had the same inclusion and exclusion criteria mentioned above. In addition these subjects had a cerebral angiogram to rule out CV during their first 14 days after aneurysm rupture and global functional outcomes measured at 3 months and 6 months after aneurysm rupture.

Finally, to assess the relationship between ET-1 CSF levels, ET-1 polymorphisms, and CV a subset of 32 subjects will be investigated. These 36 subjects had external ventricular drain catheters (EVDs) present as a part of their treatment for aSAH and had a cerebral angiogram to rule out CV during the first 14 days after admission. All previously defined exclusion and inclusion criteria applied to this subset of patients.

Demographic information

Age in years, race, sex, Fisher grade and Hunt and Hess grade were extrapolated from the medical record. Fisher grade and Hunt and Hess grade were assigned by the admitting neurosurgeon.

Cerebral Vasospasm

Cerebral vasospasm was verified by visualization of narrowing of cerebral vessels as documented on cerebral angiography. Cerebral angiography was conducted in the event of a neurological deterioration. The angiographic studies were interpreted and graded by neuro-radiologists and neurosurgeons at UPMC for evidence and degree of vasospasm. The degree of vasospasm was represented as the percentage of narrowing in the cerebral vessels as follows: 0 to 25% narrowing was coded as CV negative while >25% narrowing was coded as CV positive.

Global Functional Outcomes

These scores were collected at 3 months and 6 months after aneurysm rupture. The GOS, a clinical observation scale, categorizes functional outcomes into five levels ranging from 1 to 5 with 1 indicating death and 5 indicating good recovery (Jennett, 1976). Inter-rater reliability has been reported from 68 – 95% with kappa values from 0.62 to 0.79 (Gennarelli, Spielman, Langfitt, Gildenberg, Harrington, Jane, Marshall, Miller, & Pitts, 1982) and correlates well with severity of illness. The GOS has wide acceptance and established validity (Jennett & Bond,

1975; Maas, Braakman, Schouten, Minderhoud, & van Zomeren, 1983). Inter-rater reliability is within the acceptable range when there is adherence to assessment guidelines, administration of a structured interview, and training of examiners (Wilson, Pettigrew, & Teasdale, 1998). The Modified Rankin Scale is a measure of functional recovery and incorporates mental and physical adaptations to the neurological deficits following a neurological injury. It is easy to use and has been widely adopted for use in stroke trials. The MRS score ranges from 0 (no symptoms at all) to 6 (death). Inter-observer kappa were 0.56 and weighted kappa were 0.91 (Sulter, Steen, & De Keyser, 1999). Both global functional outcome scores were collected by a trained neuropsychological technician who was blinded to genotype.

DNA Specimens

Subjects had 10cc of whole blood drawn. The tube of blood was delivered to the genetics laboratory with no personal identifiers, labeled only with the study participant's identification number, date, and time of sample collection. The sample was logged in and centrifuged, the white cells removed, and DNA extracted from the white cells using a simple salting out procedure (Miller, Dykes, & Polesky, 1988). All DNA was stored in 1X TE buffer at 4°C until genotyping was performed.

Genotyping

Genotyping was performed as follows: An ABI Prism® 7000 Sequence Detection System was used to conduct allele discrimination assays using TaqMan® allele discrimination assays. This is a highly automated, high throughput genotyping method for SNPs. Primers flank each polymorphism and polymerase chain reaction (PCR) is conducted using subject DNA. The main difference between TaqMan® PCR and traditional PCR is the use of a probe that is labeled with a reporter and a quencher dye that recognizes a specific allele of a SNP. Each allele of a SNP

has its own probe with its own reporter dye. If the subject's DNA is homozygous for one allele of the SNP, only the probe for that allele will hybridize and only the reporter dye for that allele will be liberated and measured by the ABI Prism® 7000 Sequence Detection System. A heterozygote had both probes hybridize and both reporter dyes measured. Using this method, each SNP was genotyped accurately and quickly. All SNPs in this study were available as TaqMan® assays available through ABI. Tagging SNPs of ET-1 and Lys198Asn were classified into one of three genotypes. These genotypes were homozygous for the wild type, homozygous for the variant, or heterozygous. For the final analysis that assesses the relationship between ET-1 CSF levels, ET-1 polymorphisms, and CV the genotypes were categorized as variant positive or variant negative. Variant positive genotypes were either homozygous variant or heterozygous. Variant negative genotypes were homozygous wild type.

Cerebrospinal Fluid Specimens for ET-1 Level Evaluation

Cerebrospinal fluid specimens were obtained twice daily from indwelling external ventricular devices (EVDs) or lumbar drains (LDs). To obtain samples, specimens were collected from the drainage catheter at the site closest to the patient and then immediately frozen in cryogenic vials to –20oC. At the end of 14 days when all collections were completed the specimens were stored in –80oC freezers until analysis. The total CSF volume drained since the last sample collection was also recorded at the time of sample collection.

Measurement of ET-1

The CSF samples were assayed for ET-1 using the ELISA kit QuantiGlo® Human Endothelin-1 from R&D Systems. This assay employs the quantitative sandwich enzyme immunoassay technique with chemiluminesent detection. Chemiluminescent kits were required in this analysis because typical colorimetric kits lacked sufficient sensitivity to allow for CSF ET-1

concentration measurements. The standard curve was modified from the R&D recommendations to include 0.195, 0.39, 0.78, 1.56, 3.13, 6.25, & 12.5 pg/ml standards. Precision and accuracy of the modified standard curve range was evaluated with intra-assay and inter-assay coefficients of variation. The intra-assay coefficients of variation were 14.1% 14.0% and 13.2% for the standards of 0.195, 1.56, and 12.5 pg/ml respectively. The inter-assay coefficients of variation were 9.57%, 12.67%, and 9.0% for the standards of 0.195, 1.56, and 12.5 pg/ml respectively. This range of standards was used to cover the range of concentrations observed in the CSF of SAH patients. A microplate luminometer (Model # 1420-012 Victor-Wallac, Finland) was used to measure the intensity of the light emitted.

Statistical Analysis

SPSS version 16.0 was used for all analyses. Values are presented in the tables and text as mean ± SD. Differences in demographics based on CV status, outcome scores, and genotypes were investigated using chi-square or fisher analysis for categorical variables, and independent t-test for continuous variables.

Binary logistic regression was used to evaluate the relationship between genotype and CV controlling for age and Fisher grade. A significant difference was defined as a $p \leq 0.05$. Outcomes at 3 months and at 6 months were evaluated using multinomial logistic regression. In this analysis age, Hunt and Hess grade, and CV status were controlled for in the model. A significant difference was defined as a $p \leq 0.05$.

For the evaluation of the relationship of ET-1 CSF levels and ET-1 genotypes on CV following aSAH the ET-1 protein elimination rate was calculated in a similar manner to Shore et al (2004), the formula is as follows:

$$\text{Elimination Rate (pg/hr)} = \frac{\text{Concentration of Sample (pg/ml) X CSF Drainage (ml)}}{\text{Time Period (hr)}}$$

This calculation allows the researcher to control for the CSF volume that was drained from the CSF catheters. The average elimination rate was calculated for 24 hours, 48 hours, 72 hours before angiography, and 72 hours after angiography. The subjects were divided into CV positive and CV negative groups as previously defined. Binary logistic regression was used to evaluate the association between average ET-1 elimination rates at 72 hours before, 48 hours before, 24 hours before and 72 hours after angiography and the outcome measure of CV controlling for age and Fisher grade at the first step and individual polymorphism genotypes controlled for at the second step. A significant difference was defined as a $p \leq 0.05$.

4.3 RESULTS

There were 250 subjects with genotype data and demographic information. These subjects were predominantly female (n=174; 69.9%) and all were Caucasian. The mean subject age was 53.84 ± 11.2 years old. All Et-1 genotypes were in Hardy Weinberg equilibrium. There were no significant differences in genotype distribution of the ET-1 SNPs for this sample when compared to the general population.

There were 96 subjects with genotype data, demographic information, and angiographic measurement of vasospasm, 3 months and 6 month global functional outcome scores. The subjects were predominantly female (n=73; 76%) and all subjects were Caucasian. The mean subject age was 55.60 ±10.28 years old. Demographic information in relationship to vasospasm

is presented in Table 3. There were no significant differences in demographics between the two groups. There were also no differences in demographics when comparing genotype groups within the polymorphisms.

Table 3: Demographics of Subjects with Outcomes Measurement Categorized by CV (n=96)

		CV Negative	CV Positive	p value
Sex	Male	16 (69.6%)	7 (30.4%)	0.100
	Female	36 (49.3%)	37 (50.7%)	
Age	Mean (SD)	56.2 (9.6)	54.9 (11.1)	0.545
Hunt &Hess	1	7 (7.3%)	4 (4.2%)	0.480
Grade	2	20 (20.8%)	11 (11.5%)	
	3	16 (16.7%)	16 (16.7%)	
	4	6 (6.3%)	9 (9.4%)	
	5	3 (3.1%)	4 (4.2%)	
Fisher	1	0 (0%)	0 (0%)	0.406
Grade	2	14 (14.6%)	7 (7.3%)	
	3	28 (29.2%)	26 (27.1%)	
	4	10 (10.4%)	11 (11.5%)	

Of the 9 ET-1 SNPs investigated 1 ET-1 SNPs had genotypes that were predictive of CV when controlling for age and Fisher grade (Table 4). This tagging SNP was rs2070699 (p=0.012, Table 5). The odds ratio of the heterozygous genotype compared to the homozygous wild-type genotype was 2.970 with a 95% confidence interval of 0.998 to 8.836. The odds ratio

for the homozygous variant genotype compared to the homozygous wild-type genotype was 8.356 with a 95% confidence interval of 2.032 to 34.371. See Figure 4 for distribution of rs2070699's genotypes by CV status. The ET-1 SNP's genotypes were not a significant predictor of long term outcomes when controlling for age, Hunt and Hess grade, and CV.

Table 4: Logistic Regression Analysis of Individual SNPs of the ET-1 Gene controlling for Fisher Grade and Age (* indicates significance at $p<0.05$)

SNP	Wald X2	df	Sig
rs1800541	0.701	2	0.704
rs3087459	0.527	2	0.768
rs2070699	8.782	2	0.012*
rs1476046	1.472	2	0.479
rs5369	0.546	2	0.761
rs1626492	0.632	2	0.729
rs6912834	1.786	2	0.409
rs2071943	1.360	2	0.507
rs5370	0.781	2	0.677

Table 5: Logistic Regression Analysis of rs2070699.

Predictor	Odds Ratio	Confidence Interval
Constant	0.298	
Age	0.989	0.946 to 1.034
Fisher Grade 2	1.0	
Fisher Grade 3	2.119	0.671 to 6.692
Fisher Grade 4	2.855	0.735 to 11.094
RS2070699 Homozygous Wild Type	1.0	
Heterozygous	2.970	0.998 to 8.836
Homozygous Variant	8.356	2.032 to 34.371

Test	X2	df	p
Goodness-of-fit test			
Hosmer & Lemeshow	3.035	8	0.932

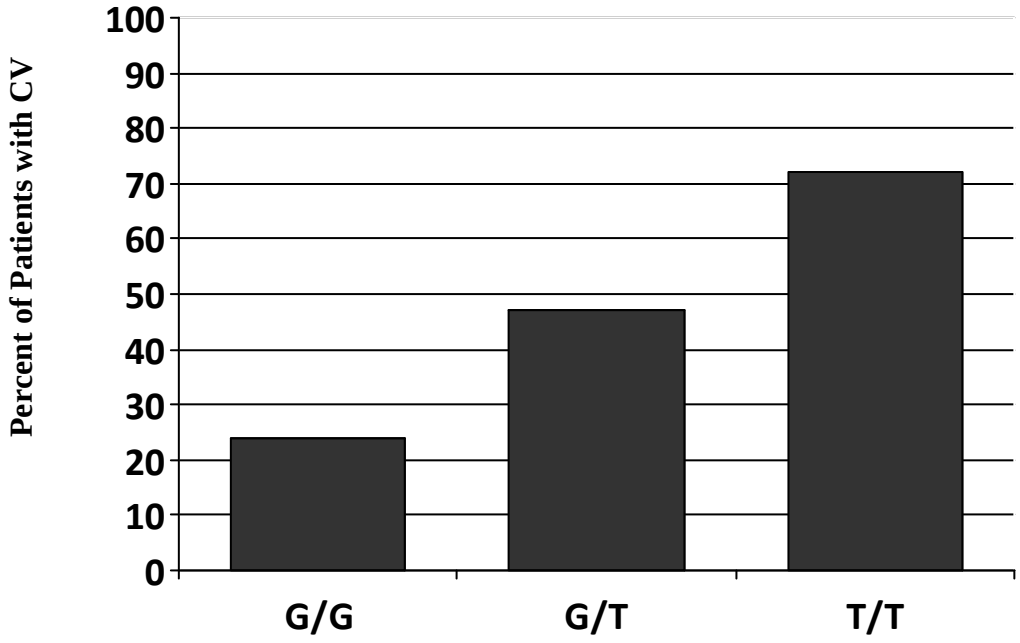

Figure 4: Percentage of subjects with CV categorized by genotype of rs2070699.

There were 32 subjects with ET-1 CSF measurements, demographic information, genotype information and angiographic data available. These subjects were predominantly female (n=20; 62.5%) and all were Caucasian (n=32; 100 %). The average subject age was 56.34 ± 10.64 years old with a median age of 56.5 years old. There were no significant differences in demographics between the CV positive and CV negative groups.

The average ET-1 elimination rate for the 24 hours before angiography for the CV negative and positive groups (3.80 ±2.82 pg/hr versus 6.72±5.71 pg/hr) or for the 48 hours before angiography for the CV negative and positive groups (3.72±2.53 pg/hr versus 7.15±6.75 pg/hr) were not significant predictors. The average ET-1 elimination rate for the 72 hours before angiography was 4.02±2.53 pg/hr for the CV negative group and 9.35±7.28 pg/hr for the CV positive group (Figure 5). The 72 hour ET-1 elimination rate was a significant predictor in this

model when controlling for age and Fisher grade (p=0.022), which supports findings from an earlier paper (Gallek et al., 2008). The average ET-1 elimination rate 72 hours after angiography showed no difference between the CV negative group (3.80±2.33 pg/hr) and the CV positive group (5.22±4.30 pg/hr). However, when ET-1 levels and ET-1 genotypes are included in the model, there is no significant interaction between the two variables.

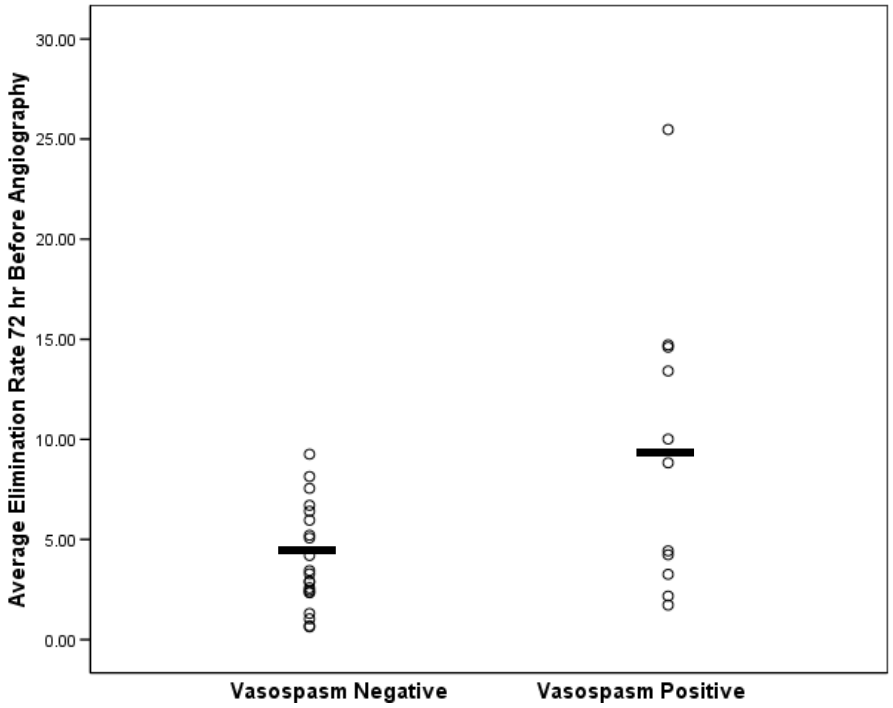

Figure 5: Average ET-1 elimination rate (pg/hr) 72 hours before angiographic measurement of vasospasm. The horizontal bar indicates the mean of each group.

4.4 DISCUSSION

The distribution of the genotypes for the sample of 250 Caucasian subjects was not significantly different when compared to the HAP-MAP database of the general Caucasian population. From this finding we conclude that ET-1 genetic variation does not play a role in aneurysm formation, aneurysm development, or rupture. If ET-1 was involved with aneurysm formation or rupture, we would have expected the genotype distribution to be different in this diseased sample when compared to the general population.

The genotype distribution of the subset of 96 subjects with angiographic and global functional outcomes also has a similar distribution when compared to the general population. The functional SNP (Lys198Asn) had no association with CV after aSAH. Therefore, the difference in vascular response to ET-1 as reported by Iglarz et al (2002) does not appear to play a part in the etiology of CV. This difference in vascular response reported previously may not play a role in CV due to the aSAH disease process and other influences following aSAH. The tagging SNP whose genotypes were associated with CV was rs2070699. The presence of the variant allele of this SNP was associated with an increased occurrence of CV in a dose response manner. A subject with a heterozygous genotype was 2.97 times more likely to have CV when compared to a subject with a homozygous wild type genotype. While a subject with the homozygous variant genotype was at the highest risk of have CV. These subjects were 8.36 times more likely to have CV when compared to a subject with the homozygous variant genotype. These genotype associations support that ET-1 plays a role in the development of CV after aSAH and that the gene product associated with the variant allele of the rs2070699 SNP may lead to more potent vasoconstriction.

While the SNP rs2070699 is within an intron of the ET-1 gene and has no direct effect on the mature protein, the region of the ET-1 gene that it represents has SNPs that may affect the mature ET-1 protein. Based on the website http://hapmap.org/ the associated block of base pairs is located on Chromosome 6: position 12400758 to 12403973. Within this block of genetic information is the SNP rs35104761 which causes a deletion of a base pair and a frameshift mutation. This frameshift mutation may significantly change amino acids of the mature protein or may cause the translation of the protein to prematurely stop. The SNP rs35104761 is a possible candidate for the differences in occurrence of vasospasm accounted for by the SNP rs2070699.

While there were no significant associations found between ET-1 SNPs and global functional outcomes at 3 and 6 months, these findings are still of interest. Long term recovery from aSAH is very complex and multifactorial. There are numerous genes that are involved in recovery and cell repair. Our findings lead to the conclusion that ET-1 is most likely not a major direct contributor to long term recovery after aSAH. However, ET-1 mediates CV which in the long run will affect long term outcomes.

While ET-1 average elimination rate at 72 hours before angiography is a significant predictor in our model ($p=0.022$) with higher ET-1 elimination rates being predictive of CV. There appears to be no interaction between the ET-1 elimination rate and ET-1 genotype in this model. This may be due to the small number of subjects within this sample. By having an underpowered test we were unable to elucidate the association between the ET-1 genotypes and the ET-1 levels.

Limitations to this study include the lack of minorities in our sample. Minority subjects are not presented in this study because the size of the minority sample that is available to us

would make it a very under powered analysis. The minority population is small in the Pittsburgh region and because of that our minority sample is less than 10% of the overall aSAH population. In the future as we are able to recruit a larger number of minorities we will be able to report the genetic characteristics of the ET-1 SNPs for this aSAH population.

The second limitation is that we were able to only include patients that had a cerebral angiography as a measure of the presence or absence of CV. The majority of this study's patients had a clinical need for an angiography (i.e. change in level of consciousness). By including patients that have a need for angiography the sample is skewed towards subjects that are in a more critical state. Those patients that were in a less critical state may have differences in their genotype distributions when compared to the more critical patients. These differences in genotype distribution may account for differences in the overall state of the patients. In addition we were only able to include patients that had follow up visits with the neuropsychological technician. In the overall study population 21% of patients were not available for 3 month follow up while 24% of patients were not available for 6 months follow up. Therefore, any subjects that were unable or unwilling to participate in the follow up testing would not be included in this sample.

The third limitation of this study was the relatively small number of patients in our sample that investigated the relationships between ET-1 SNP's, ET-1 levels and CV. Our sample size is limited by two major factors. One, our patients need to have an EVD or LD to collect the ET-1 levels. Not all patients clinically have a need for an EVD or LD, therefore that portion of the population was not included in this study. In addition our sample size is limited by follow up angiography. As stated above, the majority of angiograms are performed on patients that have a clinical neurological deterioration. Patients with clinical neurologic deterioration and

EVD's are more critically ill. As such our findings may not be representative of the general aSAH population. Future work should include a larger sample size to give more power to such a study. We were however able to identify significant differences in ET-1 elimination 72 hours before angiographic cerebral vasospasm suggesting that the effect size was large enough to accommodate the small sample size.

In summary, we found that the variant allele of tagging SNP RS2070699 was associated with an increased incidence of CV following aSAH. This suggests that ET-1 does play a role in the development of CV after aSAH. Further investigation needs to continue to find the functional SNP that is within the region of the ET-1 gene represented by that tagging SNP.

APPENDIX A

GLASGOW OUTCOME SCORE

___1. Dead

___2. Vegetative State
Unable to interact with environment; unresponsive. Patients who show no evidence of meaningful responsiveness. Patients who obey even simple commands, or who utter any words, are assigned to the better category of severe disability. Vegetative patients breathe spontaneously, have periods of spontaneous eye-opening when they may follow moving objects with their eyes, show reflex responses in their limbs (to postural or painful stimuli), and they may swallow food placed in their mouths. This non-sentient state must be distinguished from other conditions of wakeful, reduced responsiveness--such as the locked-in syndrome, akinetic mutism and total global aphasia.

___3. Severe Disability
Able to follow commands/ unable to live independently. This indicates that a patient is conscious but needs the assistance of another person for some activities of daily living every day. This may range from continuous total dependency (for feeding and washing) to the need for assistance with only one activity--such as dressing, getting out of bed or moving about the house, or going outside to a shop. Often dependency is due to a combination of physical and mental disability--because when physical disability is severe after head injury there is almost always considerable mental deficit. The patient cannot be left overnight because they would be unable to plan their meals or to deal with callers, or any domestic crisis which might arise. The severely disabled are described by the phrase "conscious but dependent."

___4. Moderate Disability
Able to live independently; unable to return to work or school. These patients may be summarized as "independent but disabled," but it is perhaps the least easily described category of survivor. Such a patient is able to look after himself at home, to get out and about to the shops and to travel by public transport. However, some previous activities, either at work or in social

life, are now no longer possible by reason of either physical or mental deficit. Some patients in this category are able to return to certain kinds of work, even to their own job, if this happens not to involve a high level of performance in the area of their major deficit.

___5. Good Recovery
Able to return to work or school. This indicates the capacity to resume normal occupational and social activities, although there may be minor physical or mental deficits. However, for various reasons, the patient may not have resumed all his previous activities, and in particular may not be working.

APPENDIX B

MODIFIED RANKIN SCORE

SCORE	DESCRIPTION
0	No symptoms at all
1	No significant disability despite symptoms; able to carry out all usual duties and activities
2	Slight disability; unable to carry out all previous activities, but able to look after own affairs without assistance
3	Moderate disability; requiring some help, but able to walk without assistance
4	Moderately severe disability; unable to walk without assistance and unable to attend to own bodily needs without assistance
5	Severe disability; bedridden, incontinent and requiring constant nursing care and attention
6	Dead

APPENDIX C

THE HUNT AND HESS GRADING SCALE

Hunt and Hess Classification

Grade 0-	Unruptured Aneurysm
Grade 1-	Asymptomatic or mild headache with slight nuchal rigidity.
Grade 2-	Cranial nerve palsy, moderate to severe headache, nuchal rigidity
Grade 3-	Mild focal deficit, lethargy or confusion
Grade 4-	Stupor, moderate to severe hemiparesis
Grade 5-	Deep coma, decerebrate rigidity, moribund appearance

APPENDIX D

THE FISHER GRADING SCALE

Fisher Grading Scale

Grade 1-	No blood detected on CT scan
Grade 2-	Diffuse deposition or thin layer with all vertical layers of blood less than 1 mm thick
Grade 3-	Localized clots and/or vertical layers of blood 1mm or greater in thickness
Grade 4-	Intracerebral or intraventricular clot with diffuse or no subarachnoid hemorrhage

APPENDIX E

IRB APPROVAL LETTER

University of Pittsburgh

Institutional Review Board

3500 Fifth Avenue
Ground Level
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

TO: MATTHEW GALLEK
FROM: SUE BEERS PHD, Vice Chair
DATE: 7/19/2007
IRB#: PRO07060091
SUBJECT: Investigation of Endothelin System Polymorphisms in subjects with Aneurysmal Subarachnoid Hemorrhage

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The above-referenced project has been reviewed by the Institutional Review Board. Based on the information provided, this project meets all the necessary criteria for an exemption, and is hereby designated as "exempt" under section 45 CFR 46.101(b)(4).

Please note the following information:

- If any modifications are made to this project, please contact the IRB Office to ensure it continues to meet the exempt category.
- Upon completion of your project, be sure to finalize the project by submitting a termination request.

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

BIBLIOGRAPHY

Anderson, C. S., Jamrozik, K. D., Broadhurst, R. J., & Stewart-Wynne, E. G. (1994). Predicting survival for 1 year among different subtypes of stroke. Results from the Perth Community Stroke Study.[see comment]. *Stroke, 25*(10), 1935-1944.

Barden, A. E., Herbison, C. E., Beilin, L. J., Michael, C. A., Walters, B. N., & Van Bockxmeer, F. M. (2001). Association between the endothelin-1 gene Lys198Asn polymorphism blood pressure and plasma endothelin-1 levels in normal and pre-eclamptic pregnancy. [Research Support, Non-U.S. Gov't]. *Journal of Hypertension, 19*(10), 1775-1782.

Botstein, D., & Risch, N. (2003). Discovering genotypes underlying human phenotypes: past successes for mendelian disease, future approaches for complex disease. *Nature Genetics, 33 Suppl*, 228-237.

Boulanger, C., & Luscher, T. F. (1990). Release of endothelin from the porcine aorta. Inhibition by endothelium-derived nitric oxide. *Journal of Clinical Investigation, 85*(2), 587-590.

Cambj-Sapunar, L., Yu, M., Harder, D. R., & Roman, R. J. (2003). Contribution of 5-hydroxytryptamine1B receptors and 20-hydroxyeiscosatetraenoic acid to fall in cerebral blood flow after subarachnoid hemorrhage. *Stroke, 34*(5), 1269-1275.

Chia, R. Y., Hughes, R. S., & Morgan, M. K. (2002). Magnesium: a useful adjunct in the prevention of cerebral vasospasm following aneurysmal subarachnoid haemorrhage. *Journal of Clinical Neuroscience, 9*(3), 279-281.

Cohen, R. A., Vanhoutte, P. M., Cohen, R. A., & Vanhoutte, P. M. (1995). Endothelium-dependent hyperpolarization. Beyond nitric oxide and cyclic GMP. [Review]. *Circulation, 92*(11), 3337-3349.

Connolly, E. S., & Solomon, R. A. (2004). Management of unruptured aneurysms. In P. D. Le Roux, H. R. Winn & D. W. Newell (Eds.), *Management of cerebral aneurysms* (pp. 271-285). Philadelphia: Saunders.

Conway, J. E., & Tamargo, R. J. (2001). Cocaine use is an independent risk factor for cerebral vasospasm after aneurysmal subarachnoid hemorrhage.[see comment]. *Stroke, 32*(10), 2338-2343.

Davie, N., Haleen, S. J., Upton, P. D., Polak, J. M., Yacoub, M. H., Morrell, N. W., et al. (2002). ET(A) and ET(B) receptors modulate the proliferation of human pulmonary artery smooth muscle cells. *American Journal of Respiratory & Critical Care Medicine, 165*(3), 398-405.

De Braekeleer, M., Perusse, L., Cantin, L., Bouchard, J. M., Mathieu, J., De Braekeleer, M., et al. (1996). A study of inbreeding and kinship in intracranial aneurysms in the Saguenay Lac-Saint-Jean region (Quebec, Canada). *Annals of Human Genetics, 60*(Pt 2), 99-104.

Dong, Y., Wang, X., Zhu, H., Treiber, F. A., & Snieder, H. (2004). Endothelin-1 gene and progression of blood pressure and left ventricular mass: longitudinal findings in youth. *Hypertension, 44*(6), 884-890.

Dorsch, N. W. (2002). Therapeutic approaches to vasospasm in subarachnoid hemorrhage. [Review]. *Current Opinion in Critical Care, 8*(2), 128-133.

Fassbender, K., Hodapp, B., Rossol, S., Bertsch, T., Schmeck, J., Schutt, S., et al. (2000). Endothelin-1 in subarachnoid hemorrhage: An acute-phase reactant produced by cerebrospinal fluid leukocytes. *Stroke, 31*(12), 2971-2975.

Funalot, B., Courbon, D., Brousseau, T., Poirier, O., Berr, C., Cambien, F., et al. (2004). Genes encoding endothelin-converting enzyme-1 and endothelin-1 interact to influence blood pressure in women: the EVA study.[see comment]. *Journal of Hypertension, 22*(4), 739-743.

Gallek, M., Sherwood, P., Crago, E., Alexander, S., Hoffman, L., Horowitz, M., et al. (2006). Endothelin-1 Cerebrospinal Fluid Levels are Associated with Development of Cerebral Vasospasm in Aneurysmal Subarachnoid Hemorrhage Patients. *Critical Care Medicine, 32*(12 suppl), A80.

Gallek, M. J., Crago, E. A., Klamerus, M., Ren, D., Alexander, S. A., Sherwood, P. R., et al. (2008). Endothelin-1 cerebrospinal fluid levels are associated with the development of cerebral vasospasm in aneurysmal subarachnoid hemorrhage patients. Submitted to *Stroke*.

Gennarelli, T. A., Spielman, G. M., Langfitt, T. W., Gildenberg, P. L., Harrington, T., Jane, J. A., et al. (1982). Influence of the type of intracranial lesion on outcome from severe head injury. *Journal of Neurosurgery, 56*(1), 26-32.

Gennarelli, T. A., Spielman, G. M., Langfitt, T. W., Gildenberg, P. L., Harrington, T., Jane, J. A., et al. (1982). Influence of the type of intracranial lesion on outcome from severe head injury. *Journal of Neurosurgery, 56*(1), 26-32.

Gormley, K., Bevan, S., Hassan, A., & Markus, H. S. (2005). Polymorphisms in genes of the endothelin system and cerebral small-vessel disease. *Stroke, 36*(8), 1656-1660.

Goto, Y., & Yamagata, S. (2006). [Deteriorating factors on the outcome in patients with fair grade of subarachnoid hemorrhage (WFNS grade I and II)]. *No Shinkei Geka - Neurological Surgery, 34*(6), 577-582.

Gruber, A., Ungersbock, K., Reinprecht, A., Czech, T., Gross, C., Bednar, M., et al. (1998). Evaluation of cerebral vasospasm after early surgical and endovascular treatment of ruptured intracranial aneurysms.[see comment]. *Neurosurgery, 42*(2), 258-267; discussion 267-258.

Hamann, G. F., & Schimrigk, K. (1995). What is the relevance of the endothelins in subarachnoid haemorrhage? [Letter]. *Journal of Neurology, Neurosurgery & Psychiatry, 58*(3), 392.

Hatake, K., Wakabayashi, I., Kakishita, E., Hishida, S., Hatake, K., Wakabayashi, I., et al. (1992). Impairment of endothelium-dependent relaxation in human basilar artery after subarachnoid hemorrhage. *Stroke, 23*(8), 1111-1116; discussion 1116-1117.

Hirata, Y., Yoshimi, H., Emori, T., Shichiri, M., Marumo, F., Watanabe, T. X., et al. (1989). Receptor binding activity and cytosolic free calcium response by synthetic endothelin analogs in cultured rat vascular smooth muscle cells. *Biochemical & Biophysical Research Communications, 160*(1), 228-234.

Hunt, W. E. (1983). Clinical assessment of SAH. *Journal of Neurosurgery, 59*(3), 550-551.

Iemitsu, M., Maeda, S., Otsuki, T., Sugawara, J., Tanabe, T., Jesmin, S., et al. (2006). Polymorphism in endothelin-related genes limits exercise-induced decreases in arterial stiffness in older subjects. [Comparative Study
Research Support, Non-U.S. Gov't]. *Hypertension, 47*(5), 928-936.
Iglarz, M., Benessiano, J., Philip, I., Vuillaumier-Barrot, S., Lasocki, S., Hvass, U., et al. (2002). Preproendothelin-1 gene polymorphism is related to a change in vascular reactivity in the human mammary artery in vitro. *Hypertension, 39*(2), 209-213.
Jennett, B. (1976). Outcome after severe head injury: definitions and predictions. *Medical Journal of Australia, 2*(13), 475-477.
Jennett, B., & Bond, M. (1975). Assessment of outcome after severe brain damage. *Lancet, 1*(7905), 480-484.
Jennett, B., Bond, M., Jennett, B., & Bond, M. (1975). Assessment of outcome after severe brain damage. *Lancet, 1*(7905), 480-484.
Jennett, B., & Jennett, B. (1976). Outcome after severe head injury: definitions and predictions. *Medical Journal of Australia, 2*(13), 475-477.
Kassell, N. F., Sasaki, T., Colohan, A. R., Nazar, G., Kassell, N. F., Sasaki, T., et al. (1985). Cerebral vasospasm following aneurysmal subarachnoid hemorrhage.[see comment]. [Review]. *Stroke, 16*(4), 562-572.
Kastner, S., Oertel, M. F., Scharbrodt, W., Krause, M., Boker, D. K., & Deinsberger, W. (2005). Endothelin-1 in plasma, cisternal CSF and microdialysate following aneurysmal SAH. *Acta Neurochirurgica, 147*(12), 1271-1279; discussion 1279.
Kasuya, H., Weir, B. K., Nakane, M., Pollock, J. S., Johns, L., Marton, L. S., et al. (1995). Nitric oxide synthase and guanylate cyclase levels in canine basilar artery after subarachnoid hemorrhage. *Journal of Neurosurgery, 82*(2), 250-255.
Kessler, I. M., Pacheco, Y. G., Lozzi, S. P., de Araujo, A. S., Jr., & Onishi, F. J. (2005). Endothelin-1 levels in plasma and cerebrospinal fluid of patients with cerebral vasospasm after aneurysmal subarachnoid hemorrhage. [Clinical Trial
Controlled Clinical Trial]. *Surgical Neurology, 64 Suppl 1*, S1:2-5; discussion S1:5.
Kessler, I. M., Pacheco, Y. G., Lozzi, S. P., de Araujo, A. S., Jr., Onishi, F. J., de Mello, P. A., et al. (2005). Endothelin-1 levels in plasma and cerebrospinal fluid of patients with cerebral vasospasm after aneurysmal subarachnoid hemorrhage. [Clinical Trial
Controlled Clinical Trial]. *Surgical Neurology, 64 Suppl 1*, S1:2-5; discussion S1:5.
Kim, C. Y., Paek, S. H., Seo, B. G., Kim, J. H., Han, D. H., Kim, C. Y., et al. (2003). Changes in vascular responses of the basilar artery to acetylcholine and endothelin-1 in an experimental rabbit vasospasm model. *Acta Neurochirurgica, 145*(7), 571-577.
Kim, P., Schini, V. B., Sundt, T. M., Jr., & Vanhoutte, P. M. (1992). Reduced production of cGMP underlies the loss of endothelium-dependent relaxations in the canine basilar artery after subarachnoid hemorrhage. *Circulation Research, 70*(2), 248-256.
Kojima, M., Nagasawa, S., Lee, Y. E., Takeichi, Y., Tsuda, E., Mabuchi, N., et al. (1998). Asymptomatic familial cerebral aneurysms. *Neurosurgery, 43*(4), 776-781.
Kosty, T. (2005). Cerebral vasospasm after subarachnoid hemorrhage: an update. *Critical Care Nursing Quarterly, 28*(2), 122-134.
Kruglyak, L., & Nickerson, D. A. (2001). Variation is the spice of life. *Nature Genetics, 27*(3), 234-236.

Kwon, K. Y., & Jeon, B. C. (2001). Cytokine levels in cerebrospinal fluid and delayed ischemic deficits in patients with aneurysmal subarachnoid hemorrhage. *J Korean Med Sci, 16*(6), 774-780.

Lasner, T. M., & Raps, E. C. (1997). Clinical evaluation and management of aneurysmal subarachnoid hemorrhage. *Neuroimaging Clinics of North America, 7*(4), 669-678.

Lee, W. C., Christensen, M. C., Joshi, A. V., Pashos, C. L., Lee, W. C., Christensen, M. C., et al. (2007). Long-term cost of stroke subtypes among Medicare beneficiaries. [Comparative Study

Research Support, Non-U.S. Gov't]. *Cerebrovascular Diseases, 23*(1), 57-65.

Lin, C. L., Dumont, A. S., Calisaneller, T., Kwan, A. L., Hwong, S. L., Lee, K. S., et al. (2005). Monoclonal antibody against E selectin attenuates subarachnoid hemorrhage-induced cerebral vasospasm. [Research Support, Non-U.S. Gov't]. *Surgical Neurology, 64*(3), 201-205; discussion 205-206.

Lin, C. L., Kwan, A. L., Dumont, A. S., Su, Y. F., Kassell, N. F., Wang, C. J., et al. (2007). Attenuation of experimental subarachnoid hemorrhage-induced increases in circulating intercellular adhesion molecule-1 and cerebral vasospasm by the endothelin-converting enzyme inhibitor CGS 26303. [Research Support, Non-U.S. Gov't]. *Journal of Neurosurgery, 106*(3), 442-448.

Littlejohns, L., & Bader, M. K. (2005). Prevention of secondary brain injury: targeting technology. [Case Reports

Review]. *AACN Clinical Issues, 16*(4), 501-514.

Longstreth, W. T., Jr., Koepsell, T. D., Yerby, M. S., & van Belle, G. (1985). Risk factors for subarachnoid hemorrhage. [Review]. *Stroke, 16*(3), 377-385.

Maas, A. I., Braakman, R., Schouten, H. J., Minderhoud, J. M., & van Zomeren, A. H. (1983). Agreement between physicians on assessment of outcome following severe head injury. *Journal of Neurosurgery, 58*(3), 321-325.

Maas, A. I., Braakman, R., Schouten, H. J., Minderhoud, J. M., van Zomeren, A. H., Maas, A. I., et al. (1983). Agreement between physicians on assessment of outcome following severe head injury. *Journal of Neurosurgery, 58*(3), 321-325.

Mascia, L., Fedorko, L., Stewart, D. J., Mohamed, F., terBrugge, K., Ranieri, V. M., et al. (2001). Temporal relationship between endothelin-1 concentrations and cerebral vasospasm in patients with aneurysmal subarachnoid hemorrhage. [Clinical Trial]. *Stroke, 32*(5), 1185-1190.

Mascia, L., Fedorko, L., Stewart, D. J., Mohamed, F., terBrugge, K., Ranieri, V. M., et al. (2001). Temporal relationship between endothelin-1 concentrations and cerebral vasospasm in patients with aneurysmal subarachnoid hemorrhage. [Clinical Trial]. *Stroke, 32*(5), 1185-1190.

Mather, H. M., Ang, V., & Jenkins, J. S. (1981). Vasopressin in plasma and CSF of patients with subarachnoid haemorrhage. *Journal of Neurology, Neurosurgery & Psychiatry, 44*(3), 216-219.

McGirt, M. J., Mavropoulos, J. C., McGirt, L. Y., Alexander, M. J., Friedman, A. H., Laskowitz, D. T., et al. (2003). Leukocytosis as an independent risk factor for cerebral vasospasm following aneurysmal subarachnoid hemorrhage. *Journal of Neurosurgery, 98*(6), 1222-1226.

Miller, S. A., Dykes, D. D., & Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. 1215, 1988 Feb 1211.

Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research, 16*(3), 1215.

Mima, T., Yanagisawa, M., Shigeno, T., Saito, A., Goto, K., Takakura, K., et al. (1989). Endothelin acts in feline and canine cerebral arteries from the adventitial side. *Stroke, 20*(11), 1553-1556.

Newell, D. W., Eskridge, J. M., Mayberg, M. R., Grady, M. S., & Winn, H. R. (1989). Angioplasty for the treatment of symptomatic vasospasm following subarachnoid hemorrhage. *Journal of Neurosurgery, 71*(5 Pt 1), 654-660.

Newell, D. W., Eskridge, J. M., Mayberg, M. R., Grady, M. S., Winn, H. R., Newell, D. W., et al. (1989). Angioplasty for the treatment of symptomatic vasospasm following subarachnoid hemorrhage. *Journal of Neurosurgery, 71*(5 Pt 1), 654-660.

Nishihashi, T., Trandafir, C. C., Wang, A., Ji, X., & Kurahashi, K. (2005). Enhanced reactivity to vasopressin in rat basilar arteries during vasospasm after subarachnoid hemorrhage. *European Journal of Pharmacology, 513*(1-2), 93-100.

Norrgard, O., Angquist, K. A., Fodstad, H., Forsell, A., Lindberg, M., Norrgard, O., et al. (1987). Intracranial aneurysms and heredity. *Neurosurgery, 20*(2), 236-239.

Ogilvy, C. S., & Carter, B. S. (1998). A proposed comprehensive grading system to predict outcome for surgical management of intracranial aneurysms. *Neurosurgery, 42*(5), 959-968; discussion 968-970.

Onoue, H., Kaito, N., Akiyama, M., Tomii, M., Tokudome, S., Abe, T., et al. (1995). Altered reactivity of human cerebral arteries after subarachnoid hemorrhage. *Journal of Neurosurgery, 83*(3), 510-515.

Oshiro, E. M., Walter, K. A., Piantadosi, S., Witham, T. F., & Tamargo, R. J. (1997). A new subarachnoid hemorrhage grading system based on the Glasgow Coma Scale: a comparison with the Hunt and Hess and World Federation of Neurological Surgeons Scales in a clinical series. *Neurosurgery, 41*(1), 140-147; discussion 147-148.

Petzold, G. C., Einhaupl, K. M., Dirnagl, U., & Dreier, J. P. (2003). Ischemia triggered by spreading neuronal activation is induced by endothelin-1 and hemoglobin in the subarachnoid space. *Annals of Neurology, 54*(5), 591-598.

Petzold, G. C., Einhaupl, K. M., Dirnagl, U., Dreier, J. P., Petzold, G. C., Einhaupl, K. M., et al. (2003). Ischemia triggered by spreading neuronal activation is induced by endothelin-1 and hemoglobin in the subarachnoid space. *Annals of Neurology, 54*(5), 591-598.

Pinto-Sietsma, S.-J., Herrmann, S.-M., Schmidt-Petersen, K., Niu, T., Hillege, H. L., Janssen, W. M. T., et al. (2003). Role of the endothelin-1 gene locus for renal impairment in the general nondiabetic population. *Journal of the American Society of Nephrology, 14*(10), 2596-2602.

Poloyac, S. M., Reynolds, R. B., Yonas, H., & Kerr, M. E. (2005). Identification and quantification of the hydroxyeicosatetraenoic acids, 20-HETE and 12-HETE, in the cerebrospinal fluid after subarachnoid hemorrhage. [Validation Studies]. *Journal of Neuroscience Methods, 144*(2), 257-263.

Qureshi, A. I., Suri, M. F., Yahia, A. M., Suarez, J. I., Guterman, L. R., Hopkins, L. N., et al. (2001). Risk factors for subarachnoid hemorrhage. *Neurosurgery, 49*(3), 607-612; discussion 612-603.

Ratsep, T., & Asser, T. (2001). Cerebral hemodynamic impairment after aneurysmal subarachnoid hemorrhage as evaluated using transcranial doppler ultrasonography:

relationship to delayed cerebral ischemia and clinical outcome. *Journal of Neurosurgery, 95*(3), 393-401.
Ronkainen, A., Hernesniemi, J., Puranen, M., Niemitukia, L., Vanninen, R., Ryynanen, M., et al. (1997). Familial intracranial aneurysms.[see comment]. *Lancet, 349*(9049), 380-384.
Ronkainen, A., Hernesniemi, J., Ryynanen, M., Ronkainen, A., Hernesniemi, J., & Ryynanen, M. (1993). Familial subarachnoid hemorrhage in east Finland, 1977-1990.[see comment]. [Review]. *Neurosurgery, 33*(5), 787-796; discussion 796-797.
Salom, J. B., Torregrosa, G., & Alborch, E. (1995). Endothelins and the cerebral circulation. *Cerebrovascular & Brain Metabolism Reviews, 7*(2), 131-152.
Schievink, W. I. (1997). Intracranial aneurysms.[see comment][erratum appears in N Engl J Med 1997 Apr 24;336(17):1267]. *New England Journal of Medicine, 336*(1), 28-40.
Schievink, W. I., Schaid, D. J., Michels, V. V., & Piepgras, D. G. (1995). Familial aneurysmal subarachnoid hemorrhage: a community-based study. *Journal of Neurosurgery, 83*(3), 426-429.
Seifert, V., Loffler, B. M., Zimmermann, M., Roux, S., & Stolke, D. (1995). Endothelin concentrations in patients with aneurysmal subarachnoid hemorrhage. Correlation with cerebral vasospasm, delayed ischemic neurological deficits, and volume of hematoma. *Journal of Neurosurgery, 82*(1), 55-62.
Sobey, C. G., & Faraci, F. M. (1998). Subarachnoid haemorrhage: what happens to the cerebral arteries? [Review]. *Clinical & Experimental Pharmacology & Physiology, 25*(11), 867-876.
Sobey, C. G., Heistad, D. D., & Faraci, F. M. (1996). Effect of subarachnoid hemorrhage on dilatation of rat basilar artery in vivo. *American Journal of Physiology, 271*(1 Pt 2), H126-132.
Solenski, N. J., Haley, E. C., Jr., Kassell, N. F., Kongable, G., Germanson, T., Truskowski, L., et al. (1995). Medical complications of aneurysmal subarachnoid hemorrhage: a report of the multicenter, cooperative aneurysm study. Participants of the Multicenter Cooperative Aneurysm Study.[see comment]. [Clinical Trial
Multicenter Study
Randomized Controlled Trial]. *Critical Care Medicine, 23*(6), 1007-1017.
Solenski, N. J., Haley, E. C., Jr., Kassell, N. F., Kongable, G., Germanson, T., Truskowski, L., et al. (1995). Medical complications of aneurysmal subarachnoid hemorrhage: a report of the multicenter, cooperative aneurysm study. Participants of the Multicenter Cooperative Aneurysm Study.[see comment]. [Clinical Trial
Multicenter Study
Randomized Controlled Trial]. *Critical Care Medicine, 23*(6), 1007-1017.
Stevens, P. A., & Brown, M. J. (1995). Genetic variability of the ET-1 and the ETA receptor genes in essential hypertension. [Research Support, Non-U.S. Gov't]. *Journal of Cardiovascular Pharmacology, 26 Suppl 3*, S9-12.
Sulter, G., Steen, C., & De Keyser, J. (1999). Use of the Barthel index and modified Rankin scale in acute stroke trials. *Stroke, 30*(8), 1538-1541.
Sulter, G., Steen, C., De Keyser, J., Sulter, G., Steen, C., & De Keyser, J. (1999). Use of the Barthel index and modified Rankin scale in acute stroke trials. *Stroke, 30*(8), 1538-1541.
Suzuki, H., Sato, S., Suzuki, Y., Oka, M., Tsuchiya, T., Iino, I., et al. (1990). Endothelin immunoreactivity in cerebrospinal fluid of patients with subarachnoid haemorrhage. *Annals of Medicine, 22*(4), 233-236.

Suzuki, H., Sato, S., Suzuki, Y., Takekoshi, K., Ishihara, N., & Shimoda, S. (1990). Increased endothelin concentration in CSF from patients with subarachnoid hemorrhage. *Acta Neurologica Scandinavica, 81*(6), 553-554.

Suzuki, K., Meguro, K., Sakurai, T., Saitoh, Y., Takeuchi, S., & Nose, T. (2000). Endothelin-1 concentration increases in the cerebrospinal fluid in cerebral vasospasm caused by subarachnoid hemorrhage. *Surgical Neurology, 53*(2), 131-135.

Tanaka, C., Kamide, K., Takiuchi, S., Kawano, Y., & Miyata, T. (2004). Evaluation of the Lys198Asn and -134delA genetic polymorphisms of the endothelin-1 gene. *Hypertension Research - Clinical & Experimental, 27*(5), 367-371.

Treggiari-Venzi, M. M., Suter, P. M., & Romand, J. A. (2001). Review of medical prevention of vasospasm after aneurysmal subarachnoid hemorrhage: a problem of neurointensive care. [Review]. *Neurosurgery, 48*(2), 249-261; discussion 261-242.

Treggiari-Venzi, M. M., Suter, P. M., Romand, J. A., Treggiari-Venzi, M. M., Suter, P. M., & Romand, J. A. (2001). Review of medical prevention of vasospasm after aneurysmal subarachnoid hemorrhage: a problem of neurointensive care. [Review]. *Neurosurgery, 48*(2), 249-261; discussion 261-242.

Vega, C., Kwoon, J. V., & Lavine, S. D. (2002). Intracranial aneurysms: current evidence and clinical practice.[see comment]. *American Family Physician, 66*(4), 601-608.

Weir, B. (1995). The pathophysiology of cerebral vasospasm. *British Journal of Neurosurgery, 9*(3), 375-390.

Wiebers, D. O., Whisnant, J. P., Huston, J., 3rd, Meissner, I., Brown, R. D., Jr., Piepgras, D. G., et al. (2003). Unruptured intracranial aneurysms: natural history, clinical outcome, and risks of surgical and endovascular treatment.[see comment]. *Lancet, 362*(9378), 103-110.

Wilson, J. T., Pettigrew, L. E., & Teasdale, G. M. (1998). Structured interviews for the Glasgow Outcome Scale and the extended Glasgow Outcome Scale: guidelines for their use. *Journal of Neurotrauma, 15*(8), 573-585.

Wilson, J. T., Pettigrew, L. E., Teasdale, G. M., Wilson, J. T., Pettigrew, L. E., & Teasdale, G. M. (1998). Structured interviews for the Glasgow Outcome Scale and the extended Glasgow Outcome Scale: guidelines for their use. *Journal of Neurotrauma, 15*(8), 573-585.

Yamaji, T., Johshita, H., Ishibashi, M., Takaku, F., Ohno, H., Suzuki, N., et al. (1990). Endothelin family in human plasma and cerebrospinal fluid. *Journal of Clinical Endocrinology & Metabolism, 71*(6), 1611-1615.

Yamamoto, S., Nishizawa, S., Yokoyama, T., Ryu, H., Uemura, K., Yamamoto, S., et al. (1997). Subarachnoid hemorrhage impairs cerebral blood flow response to nitric oxide but not to cyclic GMP in large cerebral arteries. *Brain Research, 757*(1), 1-9.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., et al. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells.[see comment]. *Nature, 332*(6163), 411-415.

Yanagisawa, M., Kurihara, H., Kimura, S., Tomobe, Y., Kobayashi, M., Mitsui, Y., et al. (1988). A novel potent vasoconstrictor peptide produced by vascular endothelial cells.[see comment]. *Nature, 332*(6163), 411-415.

Zimmermann, M., & Seifert, V. (1998). Endothelin and subarachnoid hemorrhage: an overview. [Review]. *Neurosurgery, 43*(4), 863-875; discussion 875-866.