The Role of Estrogen (Estradiol and Estrone) on the Development of Delayed Cerebral Ischemia After Aneurysmal Subarachnoid Hemorrhage

by

Elizabeth A. Crago

BSN, California University of Pennsylvania, 1992

MSN, LaRoche College, 1998

Submitted to the Graduate Faculty of

School of Nursing in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2012

UNIVERSITY OF PITTSBURGH

School of Nursing

This dissertation was presented

by

Elizabeth A. Crago

It was defended on

April 16, 2012

and approved by

Paula R. Sherwood, RN, PhD, FAAN, Professor, School of Nursing

Samuel M. Poloyac, PharmD, PhD, Associate Professor, School of Pharmacy

Catherine Bender, RN, PhD, FAAN, Professor, School of Nursing

Jeffrey Balzer, PhD, Associate Professor, Neurological Surgery

Dianxu Ren, PhD, Assistant Professor, School of Nursing

Dissertation Advisor: Paula R. Sherwood, RN, PhD, FAAN, Professor, School of Nursing

The Role of Estrogen (Estradiol and Estrone) on the Development of Delayed Cerebral Ischemia After Aneurysmal Subarachnoid Hemorrhage

Elizabeth A. Crago, RN, MSN, PhD

University of Pittsburgh, 2012

Delayed cerebral ischemia (DCI) is a significant complication following aneurysmal subarachnoid hemorrhage (aSAH). Recent evidence has suggested that biochemical mediators alter cerebral perfusion resulting in neurological decline. Estrogens (estrone–E1 and estradiol–E2) are mediators with demonstrated neuroprotective properties that could be implicated in DCI. The impact of E1 or E2 on outcomes in humans following aSAH has been understudied. The purpose of this study was to examine the association between E1 and E2 levels and DCI following aSAH. Plasma and cerebral spinal fluid (CSF) samples collected after hemorrhage on 99 acute, adult aSAH patients admitted to the Neurovascular ICU enrolled in a NIH funded study-RO1NR004339. Three plasma and up to 5 CSF samples were selected for E1 and E2 analysis from each patient representing early(1–4), middle(4-6) and late(7-10) days after hemorrhage and were assayed using liquid chromatography-tandem mass spectrometry. DCI was operationalized as radiographic/ultrasonic evidence of impaired cerebral blood flow accompanied by neurological deterioration. Statistical analysis included detailed descriptive, group based trajectory and multiple logistic regression using SAS v9.2.

Group based trajectory identified 2 groups over time for both plasma E1 (61.4% E1-high and 38.6% E1-low) and E2 (48% E2-high and 52% E2-low) values using censored normal model. Weighted Chi Square analysis identified differences between trajectory groups by gender($p=.02$), menopause(.05), age($p<.001$) and fisher grade($p=.008$) with patients in the high

E1 group having higher severity of injury than those in the low E1 group. Likewise, patients with higher HH (E1 p=.01, E2 p=.02) and Fisher (E1 p=.008, E2 p=.08) were more likely to have higher plasma estrogen levels. The presence of DCI was also significantly associated with higher levels of plasma E1(p=.002) and E2(p=.03) and the high E1 trajectory group(p=.09). CSF was evaluated in 36 aSAH patients. Similar correlations between higher E1 and E2 CSF concentrations and severity of injury and DCI were noted. These results provide the first clinical evidence that E1 and E2 concentrations in plasma and CSF are associated with severity of injury and DCI and provide incentive for future studies to clarify the potential role of estrogen in ischemic complications after aSAH.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

"If I have seen further it is by standing on the shoulders of giants."

Sir Isaac Newton, 1675

As I reflect back on 3 decades of school and career I am amazed at the where I stand. From the time I could remember, I have always wanted to work in healthcare. Almost exactly 30 years ago I decided that medical school seemed too long and laborious and so I set off to become a nurse. Now all these years later, I stand at the precipice of a different doctoral degree and I realize that my lifetime goal has not changed. I want to make a difference in the delivery of care to patients and families. To arrive at this achievement I have relied on the direction and support of many individuals, faculty, friends, colleagues and family.

First I must thank God for providing me strength, wisdom and courage. Even when I didn't fully understand the turns in my path or the roadblocks in my way, He has guided me. I have never been so completely at His mercy. I owe an enormous debt of gratitude to the members of my dissertation committee who have been mentors, models, teachers, friends and advocates. Each one brought unique contributions to this endeavor. I especially want to thank Dr. Paula Sherwood for agreeing to be my Dissertation chair. Her tireless patience, support and guidance have allowed me to progress to this point. To Dr. Sam Poloyac for being my surrogate chair while Dr. Sherwood was in Finland fulfilling her Fulbright Scholarship obligations, for his patience in helping me to learn metabolite analysis and for his persistence with perfecting

analysis techniques. I am indebted to Dr. Jeff Balzer for providing his expert knowledge of neurological injury and cerebral blood flow and for always challenging my perspective. To Dr. Cathy Bender not only for her insight into Estrogen from years of her own research, but also her gentle guidance a mentor and nursing faculty member. And I thank Dr. Ren for his guidance during the analysis of these findings. I look forward to years of collaboration with all of you.

I would like to express my sincere appreciation to the faculty within the School of Nursing for sharing their expertise and wisdom. I would like to thank the subarachnoid hemorrhage research team at the University of Pittsburgh, both past and current, for supporting and encouraging me through this journey. Dr. Mary Kerr started this team over 10 years ago and hired me into this academic environment. I still follow the fundamentals she instilled in me years ago. And I would like to thank the members of the small molecular biomarker core at the School of Pharmacy for the patience to teach a nurse how to process and analyze biological specimens. I would like to acknowledge the funders of my research, the Neuroscience Nursing Foundation and the Nightingale Awards of Pennsylvania who supported this project.

Finally, and most importantly, I would like to thank my family. My mother as a fellow nurse and a beacon of nursing advocacy and leadership. My father, who has never doubted any of my accomplishments. And to my husband and children who have endured all of these years of school, often when they didn't understand the demands or the milestones. They have given me the opportunity to focus on my professional and academic life even when it took time away from them. I count my blessings.

1.0 INTRODUCTION

Aneurysmal subarachnoid hemorrhage (aSAH) is a sudden and devastating neurological event affecting approximately 10 in 100,000 persons annually and resulting in significant morbidity (30% of survivors) and mortality (12% pre-hospital, 40% within one month of admission) (Cahill & Zhang, 2009). Despite advances in medical and surgical management, aSAH continues to be associated with complications which necessitate vigilant monitoring and emergent interventions during the acute recovery period (Cahill & Zhang, 2009; Frontera et al.., 2009; Ostrowski, et al.., 2006; Sehba et al.., 2011; Zubkov & Rabinstein, 2009). Following hospital discharge, many patients experience long-term functional and cognitive impairment that impacts the ability of patients to resume previously held familial, social and occupational roles (Vergouwen et al.., 2009).

Unstable intracranial physiology and systemic medical complications are common after aSAH and may influence outcomes (Rose, 2011). Structural changes such as cerebral edema and hemorrhage may occur but do not adequately predict the risk of neurological decline and poor outcomes after aSAH (Cahill & Zhang, 2009; Frontera et al.., 2009). Cerebral vasospasm, or vessel narrowing, can result in regional reduction of cerebral perfusion and potential ischemia and therefore, has been a primary focus of research and treatment after aSAH (Cahill & Zhang, 2009; Frontera et al.., 2009; Keryoux & Diringer, 2007; Ostrowski, et al.., 2006 ; Rose, 2011;

Sehba et al.., 2011; Vergouwen et al.., 2011; Zubkov & Rabinstein, 2009). The diagnosis of cerebral vasospasm is often based on visualization of the macrovascular circulation using cerebral angiography which has inconsistently correlated with neurologic demise, complications or long term outcomes period (Cahill & Zhang, 2009; Frontera et al.., 2009; Ostrowski, et al.., 2006; Sehba et al.., 2011; Vergouwen et al.., 2009; Zubkov & Rabinstein, 2009).

More recently, delayed cerebral ischemia (DCI) has been implicated as a leading cause of complications after aSAH (Vergouwen et al.., 2011). DCI, which occurs in approximately 20-60% of patients who survive the initial hemorrhage, (Cahill & Zhang, 2009; Frontera et al.., 2009; Keryoux & Diringer, 2007; Ostrowski, et al.., 2006; Rose, 2011; Sehba et al.., 2011; Vergouwen et al.., 2011; Vergouwen et al.., 2009; Zubkov & Rabinstein, 2009) is thought to be the result of a mismatch between available cerebral blood flow (CBF) and metabolic demands of brain tissue, a consequence that leads to neuronal changes and neurological decline (Crago et al.., 2011; Dupont et al.., 2009; Kolias et al.., 2009; Macdonald et al.., 2008; Vergouwen et al.., 2011;). In order to provide early intervention and potentially reverse ischemia, it is important to promptly recognize and treat DCI. However, clear clinical indicators of DCI have not been identified. While an association between vasospasm, DCI, cerebral infarction and poor outcomes has been identified, each may occur independently. Of concern, reduced incidence of angiographic vasospasm has not significantly altered the development of ischemic complications or improved outcomes(Cahill & Zhang, 2009; Frontera et al.., 2009; Dupont et al.., 2009; Kolias et al.., 2009; Macdonald et al.., 2008; Ostrowski, et al.., 2006; Sehba et al.., 2011; Vergouwen et al.., 2011; Vergouwen et al.., 2009; Zubkov & Rabinstein, 2009). Further, approximately 50-70% of aSAH patients have angiographic evidence of cerebral vascular constriction

(angiographic vasospasm), but only one third experience symptoms of DCI (Crago et al.., 2011; Frontera et al., 2009).

More recent proposals have theorized that microvascular dysfunction or changes are the pathogenesis of DCI (Vergouwen et al., 2009). The origin of these microvascular alterations may result from molecular and cellular changes that alter vascular dynamics and cerebral perfusion; therefore shifts in vasoactive metabolite levels may provide a marker of ischemic complications after neurologic insults (Crago et al., 2011; Macdonald et al., 2008; Ostrowski, et al., 2006; Sehba et al., 2011). Research studies have identified an association between higher levels of endothelin (ET-1) and cerebral vasoconstriction after aSAH (Macdonald et al., 2008; Neuschmelting et al., 2009). In addition, the presence of 20-hydroxyicosatetraenoic acid (20-HETE), a potent microvascular vasoconstrictor, has been associated with the development of DCI after aSAH (Crago et al., 2011; Imig et al., 2011; Kehl et al., 2002; Roman et al., 2006). Conversely, epoxyeicosatrienoic acids (EETs) are potent vasodilators. Inhibition of the enzyme soluble epoxide hydrolase (sEH) which metabolizes EETs has been linked to reduction in cerebral infarction in experimental studies (Crago et al., 2011; Losiniecki & Zuccarello, 2008; Kehl et al., 2002; Imig et al., 2011; Roman et al., 2006). The presence of inflammatory markers has been correlated with early brain injury and poor outcomes and glutamate has long been associated with apoptosis and neuronal death (Cahill & Zhang, 2009; Frontera et al., 2012; Provencio & Vora, 2005; Sehba et al., 2012). Unfortunately, despite extensive research, the clinical utility of markers of injury or complications have not been predictive of DCI development in these susceptible patients (Cahill & Zhang, 2009; Crago et al., 2011; Sehba et al., 2012; Sehba et al., 2011). The absence of a clear clinical indicator of DCI prevents early

detection and effective management of this complication. Hence, a change in focus may be necessary to improve outcomes.

Another biomarker, estrogen, has been widely studied in neurological disorders including both experimental ischemic and hemorrhagic insults (Azcoitia et al., 2011; Carwile et al., 2009; Herson et al., 2009). Estrone (E1) is a physiologic estrogen that is the major remaining estrogen after menopause. In experimental studies E1 has been related to vascular reactivity and vasodilation through nitrous oxide pathways (Gatson et al., 2011; Rauschemberger et al., 2011, Selles et al., 2005). Estradiol (E2) is the most commonly studied neuro-steroid that alters vascular reactivity, reduces tissue damage, improves functional recovery and may stimulate repair processes (Herson et al., 2009). The absence of E2 has been linked to vasoconstriction, impaired cellular metabolism and cellular death in animal models (Brown et al., 2009; Herson et al., 2009). In humans, increased levels of E2 have been positively correlated with improved outcomes in stroke and traumatic brain injured patients (Azcoitia et al., 2011; Brown et al., 2009; Carwile et al., 2009; Herson et al., 2009; Krause et al., 2006). E2 has been associated with a number of the markers currently under investigation in SAH research. In preclinical studies, E2 suppresses soluble epoxide hydrolase (sEH) expression which reduces inflammation, suppresses glutamate excitoxicity, potentially preventing cell death, and competes with 20-HETE through known mechanical channels to restrict vasoconstriction (Behl, 2002; Ba et al., 2007; Brown et al., 2009; Fassbender et al., 2000; Rauschemberger et al., 2011; Sarkar et al., 2008; Zhang et al., 2009). Prior research examining E2 and other vasoactive metabolites has involved experimental conditions (animal models) or non SAH clinical populations, e.g., traumatic brain injury (TBI), stroke and dementia. There are substantial differences in the pathophysiological basis of injury

following aSAH compared to these other neurologic insults. No study was identified that explored whether changes in estrogen levels may play a role in reducing ischemic complications and improving outcomes in patients with aSAH. Therefore, the proposed study will address this gap in the knowledge related to the role of E1 and E2 in promoting complications such as DCI in aSAH patients.

1.1 PURPOSE

The purpose of this study was to examine the association between levels of estrogen (estradiol and estrone) and the development of DCI in patients with aSAH.

1.2 SPECIFIC AIMS

1. Examine patterns in plasma E1 and E2 levels during the 10 days following aSAH.

   H1: Distinct groups of patients will be identified with varying patterns of plasma E1 and E2 levels following aSAH.

2. Determine the relationship between plasma E1 and E2 levels and the presence or absence of DCI during the 10 days following aSAH.

   H1: After controlling for age, gender, menopausal status and severity of hemorrhage, higher levels of E1 and E2 will be associated with lower incidence of DCI.

Exploratory

3. Examine patterns in CSF E1 and E2 levels during the 10 days following aSAH.

H1: Distinct groups of patients will be identified with varying patterns of CSF E1 and E2 levels following aSAH.

4. Determine the relationship between CSF E1 and E2 levels and the presence or absence of DCI during the 10 days following aSAH.

   H1: After controlling for age, gender, menopausal status and severity of hemorrhage, higher levels of E1 and E2 will be associated with lower incidence of DCI.

5. Explore the associations among CSF levels of E1 and E2, 20-HETE and EET and the presence or absence of DCI following aSAH.

   H1: Higher levels of E1 and E2 will be associated with higher levels of EETs which will be associated with a lower incidence of DCI.

   H2: Higher levels of E1 and E2 will be associated with lower levels of 20-HETEwhich will be associated with a lower incidence of DCI.

1.3 INNOVATION AND SIGNIFICANCE

The aims of this study address a gap in knowledge related to the role of estrogens in ischemic complications such as DCI after aSAH. The proposed study has the potential to significantly advance our understanding of the mechanisms underlying the development of DCI, a major complication faced by critical care practitioners, and more accurately predict patients at high risk for poor outcomes. The study was significant because: 1) it was the first known study to describe the presence of E1 and E2 in plasma and CSF from human aSAH patients and explore the association of E1 and E2 to ischemic complications after insult. This innovative step may

lead to improved understanding of factors which promote DCI and methods to improve patient outcomes. Neither E1 nor E2 has been described in human CSF using HPLC in a moderate or large sample; 2) it utilized demographic and sample data from a well-established a SAH research program with an existing large bank of human aSAH samples from a highly productive, multidisciplinary team of critical care experts. This resource enabled concomitant evaluation of findings from bench and bedside research. 3) Finally, analysis of the role of E1 and E2 in outcomes following aSAH was conducted concurrently with analysis of other potentially related metabolites (20-HETE, EET). Assays for these biomarkers were available from the parent study, thereby adding an additional innovative path of analysis to the proposed study. The proposed study was the first study to analyze E1 and E2 levels in relation to these other metabolites in patients with aSAH. Ultimately, these new data will advance the science for patients with critical illness related to aSAH by identifying potential paths for intervention to prevent the cascade of events that produces DCI and other adverse patient outcomes.

1.4 BACKGROUND

1.4.1 Aneurysmal SAH is the cause of substantial mortality and morbidity

Aneurysmal SAH (aSAH), which accounts for 5-7% of all strokes, occurs in approximately 1 in 1000 persons annually at a mean age of 53 years and is more common in females than males (Rose, 2011).[7] In spite of treatment advances, significant improvement in morbidity and mortality has not been achieved and poor outcomes often occur in survivors (Cahill & Zhang,

2009; Diringer et al., 2011). This acute cerebrovascular event has a devastating effect on the central nervous system as well as other organs and is a frequent cause of long-term functional and cognitive disabilities (Diringer et al., 2011). At the time of aneurysm rupture, a sudden rise in intracranial pressure triggers the stress response inducing a surge of circulating mediators which, along with blood products in the subarachnoid space, result in impaired cerebral blood flow and autoregulation, disruption of the blood brain barrier and the onset of systemic complicationssuch as increased blood pressure and cardiopulmonary dysfunction (Ostrowski et al., 2006; Sehba et al., 2011). Early aneurysm treatment has reduced re-bleeding; however, patients are a risk for delayed complications that lead to cerebral ischemia up to two weeks later. This development is believed to be the primary factor in the development of subsequent complications (Diringer et al., 2011; Dupont et al., 2009).

1.4.2 Prevention of complications after aSAH remains an elusive goal

Aneurysmal SAH results when a pathological dilation of a cerebral artery ruptures releasing blood into the subarachnoid space and ventricular system. The subarachnoid blood also results in a sudden rise in intracranial pressure which triggers the stress response, induces a surge of circulating catecholamines and a subsequent release of arachidonic acid from plasma membranes (Cahill & Zhang, 2009; Ostrowski et al., 2006). Activation of the arachidonic acid cascade causes the release of inflammatory and vasoactive mediators (Cahill & Zhang, 2009; Ostrowski et al., 2006). The consequence of vasoactive mediators and the presence of blood products in the subarachnoid space result in impaired cerebral autoregulation, global ischemia, apoptosis,

necrosis and cellular death (Cahill & Zhang, 2009; Ostrowski et al., 2006; Sehba et al., 2012). The consequences of these events are disruption of the blood brain barrier and the development of cerebral edema in the early minutes and hours after aSAH (Cahill & Zhang, 2009; Ostrowski et al., 2006; Sehba et al., 2012). The pathologic effects of early brain injury have been suggested to impact mortality as well as acute and long term recovery after aSAH (Cahill & Zhang, 2009).

Historically, structural changes, primarily cerebral vasospasm, have been considered the primary cause of clinical decline after aSAH. Cerebral vasospasm is the narrowing of cerebral arteries which may or may not result in diminished perfusion to tissues distal to the affected blood vessels (Cahill & Zhang, 2009; Ostrowski et al., 2006; Sehba et al., 2012; Sehba et al., 2011). Traditionally, vasospasm has been diagnosed by visualization of the macrovascular circulation using radiologic techniques, primarily digital subtraction angiography. The risk of cerebral vasospasm peaks between 5 and 15 days after hemorrhage although it has been reported earlier and is associated with the severity of hemorrhage following aSAH (Dupont et al., 2009). Vascular constriction may occur in one or multiple arteries and ranges from mild to severe in degree of narrowing. If vasoconstriction along with impaired autoregulation result in blood flow that is below the ischemic threshold, symptoms of neurologic compromise will ensue (Keyroux & Diringer, 2007). Clinical evidence has demonstrated that up to 70% of aSAH patients have angiographic evidence of vascular constriction known as angiographic vasospasm, while most studies report only 20-30%experience symptoms of neurologic deterioration (Dankbaar et al., 2011; Frontera et al., 2009; Vergouwen et al., 2011a; Vergouwen et al., 2011b; Zubkov & Rabinstein, 2009). Basic and clinical research have focused on predicting and treating cerebral vasospasm based on the hypothesis that vasospasm leads to DCI and consequently poor

outcomes as a result of additional ischemic injury; evidence is inconclusive regarding how this finding links to DCI or recovery.

These findings have led to a reexamination of theories regarding causality and patient outcomes following aSAH. In a recent study of 413 SAH patients of whom 194 had moderate to severe vasospasm, only 43% exhibited signs of neurological worsening, 20% had cerebral infarction and 46% had a poor outcome (Vergouwen et al., 2011b). In this same cohort, path analysis revealed that only cerebral infarction had a direct effect on outcome which was independent of angiographic vasospasm. Results of the CONSCIOUS-1 clinical trial also support this incongruity; prevention of cerebral vasospasm did not result in reduction of DCI or improvement in outcomes (Vergouwen et al., 2011a). Consistent with findings of these studies, the majority of clinical evidence appears to implicate other factors in the pathogenesis of DCI including those that result in microvascular dysfunction and changes.

DCI, a concept focused on insufficient blood flow, has become the primary outcome target in aSAH studies. DCI, defined as cerebral ischemia that occurs in the days after aSAH (Vergouwen et al., 2011b), affects up to two thirds of the patients who survive aSAH and is considered the leading cause of morbidity and mortality (Frontera et al., 2009; Kolias et al., 2009). DCI, which occurs when cerebral blood flow is inadequate to meet cerebral metabolic demand, is manifested by neuronal changes and neurologic decline from inadequate blood supply to the cerebral tissue. DCI is most likely to occur 3-14 days after the initial bleeding, which may allow sufficient timing for therapeutic intervention; however clear clinical indicators of DCI have not been identified (Frontera et al., 2009; Vergouwen et al., 2011a; Vergouwen et al., 2011b; Zubkov & Rabinstein, 2009). While an association exists between vasospasm, DCI,

cerebral infarction and poor outcomes after aSAH, each has also been described independently Dankbaar et al., 2011; Vergouwen et al. 2011a).

Clinical care for aSAH patients is targeted at preventing or controlling complications with standard treatment protocols that include serial neurological exams, blood pressure control, fluid management, medications (calcium channel blockers, magnesium sulfate and statins), and HHH therapy (hypertension, hypervolemia, hemodilution) (Diringer et al., 2011; Rose, 2011; Sehba et al., 2011). Following aSAH, patients require extensive critical care resources and monitoring in an attempt to prevent and control both intracranial (cerebral vasospasm, edema, ischemia and increased intracranial pressure) and systemic complications (fever, cardiopulmonary compromise and anemia) (Diringer et al., 2011; Rose, 2011; Sehba et al., 2011). Unfortunately despite standardized practices, marked improvement in outcomes has not occurred and the rate of ischemic complications including DCI remains high (Diringer et al., 2011; Rose, 2011; Sehba et al., 2011; Vergouwen et al., 2011).

1.4.3 Potential additional explanations for this complication

The limited success of vasospasm-directed trials in reducing delayed ischemic injury or improving outcomes has led investigators to seek other explanations for ischemic complications. Injury to internal structures of the brain tissue, vasculature and cells as a consequence of the hemorrhage and the sudden rise in intracranial pressure may precipitate other mechanisms associated with early brain injury (Ostrowski et al., 2006; Sehba et al., 2011). These mechanisms include molecular, cellular, vascular, and ionic changes that effect intracranial processes and loss of cerebral autoregulation and vascular stability. In particular, biochemical changes that alter the

balance of vasoactive metabolites may influence microvascular vasosconstriction and development of ischemic complications following acute neurological insult (Ostrowski et al., 2006; Sehba et al., 2011). Nitric Oxide (NO), inflammatory markers, catecholamines, endothelin (ET-1), 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EET's) are among the metabolites that have been associated with regulation of vascular stability after aSAH.

The release of hemoglobin into the subarachnoid space creates multiple chemical changes that are implicated in reduced cerebral blood flow and ischemia (Ostrowski, 2006). Initially, hemoglobin scavenges NO, eliminating its vasodilatory effects in cerebral autoregulation (Ostrowski, 2006). Inflammatory markers are released remotely in response to both global ischemia and the release of vasoactive compounds such as platelets at the time of hemorrhage. Both parenchymal and vascular inflammation follows 48 h ours after ictus which has a compounding effect on apoptotic cell death. Elevations in inflammatory markers have been confirmed in the plasma and CSF of aSAH patients (Naredi et al., 2006; Provencio & Vora, 2005). Blood clot is also implicated in the generation of secondary spasmogens including catecholamines and 5-HT which have been implicated in vasoconstriction alone and in conjunction with other metabolites such as 20-HETE (Ostrowski, 2006). Extracellular glutamate rises dramatically after hemorrhage and is considered a robust marker of ischemia (Ostrowski et al., 2006; Sehba et al., 2011). Glutamate may also propagate from extravasated blood, further escalating its concentration and contributing to neuronal death (Ostrowski et al., 2006; Sehba et al., 2011). ET-1 is released from cerebral arteries in response to vascular injury reducing sodium/potassium ATPase activity and has been described as a potential mediator of cerebral vasospasm (Fassbender, 2000; Neuschemberger et al., 2008; Ostrowski et al., 2006; Sehba et al., 2011). Clinical research studies have found increased ET-1 levels in CSF of aSAH patients with

cerebral vasospasm and accordingly administration of an ET-1 antagonist has effectively reduced the occurrence angiographic vasospasm. Molecular, cellular, vascular, and ionic changes influence microvascular vasosconstriction and ischemic complications following acute neurological insult (Ostrowski et al., 2006; Sehba et al., 2011).

1.4.4 Estrogen may facilitate vascular stabilization

Estrogens have been widely studied in neurological disorders including both experimental ischemic and hemorrhagic insult models (Brown et al., 2009; Herson et al., 2009; Hurn & Brass, 2003; Rauschemberger et al., 2008). The observation of gender differences along with differences between pre- and post-menopausal females and outcomes in both clinical and experimental brain injury sparked much of the interest in estrogen (Brown et al., 2009; Herson et al., 2009; Hurn & Brass, 2003; Gilles & Mcarthur, 2010; Krause et al., 2006). Estrone (E1) is a physiologic estrogen that is the major remaining estrogen in the circulation after menopause. E1 is converted to estrone sulfate which serves as a reservoir for E2 conversion. In experimental studies E1 has been related to vascular reactivity and vasodilation through nitrous oxide pathways (Gatson et al., 2011; Rauschemberger et al., 2011, Rauschemberger et al., 2008; Selles et al., 2005). While the role of E1 in neurological insults has not been described, experimental vascular studies have shown thatE1 has both a positive effect on NO synthesis and COX activation (Selles et al., 2005; Rauschemberger et al., 2008) as well as having potential modulating effects on cellular endothelial growth and survival including production, proliferation, apoptosis and cell adhesion events (Rauschemberger et al., 2008).

Estradiol (E2) is a neuro-steroid that may facilitate vascular stabilization. E2 alters vascular reactivity, reduces tissue damage, improves functional recovery and may stimulate repair processes (Brown et al., 2009; Herson et al., 2009; Krause et al., 2006). In preclinical studies, female animals suffered less tissue damage than males after both ischemic and traumatic insults (Alkayed et al. 1998; Auriat et al., 2005; Gilles & Mcarthur, 2010) Of additional interest, benefit was lost in reproductively senescent or ovarectomized animals, but restored with estrogen supplementation (Gilles & Mcarthur, 2010). In animal studies, estrogen supplementation consistently improved functional outcomes after ischemic injury (Alkayed et al. 1998; Auriat et al., 2005; Brown et al., 2009; Gilles & Mcarthur, 2010; Kehl et al., 2002; Sribnick et al., 2009). In an experimental animal model, the administration of E2 after aSAH resulted in significantly less ($p<.05$) basilar artery spasm and apoptosis manifested by DNA fragmentation when measured 7 da ys after insult (Alkayed et al., 1998). Likewise, Yang reported reduction in mortality ($p=.01$) and secondary ischemic damage from improved blood flow ($p=.012$) in an aSAH animal model (Yang et al., 2001). This effect was also noted in male animals treated with E2 administration after intracranial hemorrhage ($p=.05$) (Alkayed et al., 1998).

There have been relatively few studies investigating the role of E2 in human subjects; however results have supported the hypothesis that E2 has positive effects on cerebral blood flow and improved cognitive outcomes. In a study of 24 healthy post-menopausal women, the administration of intranasal estrogen resulted in significant increases in cerebral blood flow ($p<.05$) in all regions of interest by SPECT (Kaya et al., 2008). As a p otential biomarker, increased levels of E2 have been correlated with improved outcomes in stroke (less delayed ischemia; $p<.05$) and traumatic brain injured patients as well as neuro-degenerative and aging diseases (increased white matter; $p<.002$) (Duy et al., 2007; Lang & McCullough, 2008).

It is important to note that recent studies have questioned existing scientific evidence that would lead us to believe that higher levels of E2 are beneficial to outcomes related to vascular and neurological insults. Kaufmann reported that higher admission E2 levels and to a more significant degree changes in E2 levels during critical illness were independently associated with mortality (CI 1.1) in addition to other markers of stress response (Kauffman et al., 2011). Likewise, higher plasma estradiol levels were found to be associated with higher all-cause and non-cardiovascular mortality in a study of 2078 older men referred for coronary angiography however these levels were associated with other risk factors for mortality such increased BMI (Lerchbaum et al., 2011). Finally, Wagner et al. reported significantly altered hormonal levels in acute male and female severe traumatic brain injured patients however their findings indicated acute mortality was associated with the high plasma estradiol trajectory group ($p<.01$) (Wagner et al., 2011). The results of these studies support an association between critical illness and estrogen levels and provide important considerations to consider when exploring the impact of estrogen levels on outcomes after aSAH.

Other than overall incidence of aSAH, investigation of gender influence on aSAH has been limited. One study of 163 SAH patients explored predictors of angiographic vasospasm and noted significantly less cerebral vasospasm in women ($p=.04$) in univariate analyses which remained a trend in a multivariate logistic regression model ($p=.07$) along with scales of injury, smoking ($p=.02$) and hydrocephalus ($p=.04$) (Dupont et al., 2009). A second study that reviewed over 29,000 hospital admissions related to acute brain disorders of which 1029 were diagnosed with SAH, noted that male patients had higher incidence of in hospital death (OR 1:1.61, $p=.001$), although specific sources for this finding were not explored (Macleod & Andrew, 2002). No gender differences were noted in a study by Orbo and colleagues which evaluated

cognitive impairment one year after surgical management in 44 aSAH patients (Orbo et al., 2008). Evidence that estrogen alters vascular reactivity, reduces tissue damage, improves functional recovery and may stimulate repair processes following experimental and clinical neurological insults along with differences in outcomes by gender further substantiates the need to explore the role of estrogen and DCI after aSAH.

1.4.5 Estradiol neuroprotection may be associated with other metabolites

The potential mechanisms of estradiol afforded neuroprotection have been associated with a number of the markers currently under investigation in SAH research primarily through molecular interactions that promote vasodilation. E2 inhibits ET-1production ($p<.05$) (Brown et al., 2009) which has been a targeted vasoconstrictor in clinical trials (Macdonald et al., 2008; Vergouwen et al., 2011a). E2 may help vascular stabilization by potentiating NO via eNOS and iNOS expression ($p<.01$) (Alkayed et al., 1998; Sabri et al., 2011), reducing apoptosis by blocking glutamate, and reducing inflammatory cytokines by suppressing sEH and excitatory metabolites (catecholamines) (Brown et al., 2009; Herson et al., 2009; Krause et al., 2006; Zhang etal, 2009).

Another area of influence may be within the cyp450 pathway which is activated through the arachidonic acid cascade after neurological insult (Crago et al., 2011; Imig et al., 2011; Losniecki & Zuccarello, 2008). Current evidence suggests that metabolites of arachidonic acid form products to regulate cerebral blood flow (Imig et al., 2011). The hydroxylated metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) is a vasoconstrictor while epoxygenated metabolites or epoxyeicosatrienoic acids (EET's) are vasodilators. 20-HETE is formed by enzymes of

CYP4A and 4F families via ω-hydroxylation of arachidonic acid (AA) in cerebral arteries (Rejdak et al., 2004; Roman et al., 2006). S timulated by angiotensin II, endothelin and norepinephrine; 20-HETE is a potent microvascular vasoconstrictor including cerebral vascular beds. There is also evidence that 20-HETE plays an important role as an oxygen sensor in the microcirculation. Pre-clinical and clinical evidence has implicated 20-HETE in changes in the cerebral vascular tone including the development of delayed cerebral vasospasm and ischemia (Crago et al., 2011; Roman et al., 2006). Administration of inhibitors of 20-HETE synthesis and 20-HETE antagonists have been shown to reverse delayed vasospasm and prevent acute decreases in CBF (Omura et al., 2006;Poloyac et al., 2006; Yu et al., 2004). Likewise, inhibition of 20-HETE formation was found to be neuroprotective in a temporary focal ischemia SAH animal model (Poloyac et al., 2006) a nd attenuated the post-injury CBF that typically accompanies ischemic and hemorrhagic stroke (Poloyac et al., 2005). A lthough 20-HETE is a potent microvascular constrictor, it has little or no effect on larger arteries (Roman et al., 2006). More recently, elevated levels of 20-HETE in CSF and plasma have been reported in a small number of aSAH patients with documented evidence of cerebral vasospasm and neurological deficits (Crago et al., 2011). Clinical evidence from our work showed a significant relationship between the presence of 20-HETE in cerebral spinal fluid and the presence of DCI (Crago et al., 2011). In 108 acute aSAH patients, the presence of detectable 20-HETE levels were observed in 31% of patient samples and were associated with severity of hemorrhage ($p=.04$) and DCI ($p=.016$) (Crago et al., 2011). E 2 inhibits 20-HETE pathways of vasoconstriction through known channels ($p<.001$) (Ba et al., 2007; Be, 2002; Brown et al., 2009; Rauschemberger et al., 2011; Sarkar et al., 2008; Zhang et al., 2009), diminishing t he influence of 20-HETE on vasoconstriction.

Conversely, EETs are considered sensors of neuronal activity that may enhance functional hyperemia (Illif et al., 2009; Imig et al., 2011). EETs are potent vasodilators and important regulators of vascular function that are synthesized from arachidonic acid by cytochrome P-450 epoxygenase enzymes of the CYP2C and Cyp2J families (Illif et al., 2009). In addition to their vasodilatory role, clinical studies have identified EETs as possessing anti-inflammatory, anti-thrombotic, fibrinolytic, angiogenic, anti-apoptotic and anti-oxidant properties (Illif et al., 2009). EETs are released from astrocytes and vascular endothelium in the brain in response to glutamine release. The EETs dilate cerebral arteries through activation of K^+ channels and recruit blood flow to the active areas of the brain (Illif et al., 2009; Roman et al., 2006). EETs are rapidly degraded by soluble epoxide hydrolase (sEH) into dihydroxyeicosatrienoic acids (DHETs). Likewise sEH is responsible for converting EpOMEs to DiHOME's which are also surrogate measurements for EETs (Crago et al., 2011; Illif et al., 2009; Roman et al., 2006). E2 suppresses sEH which increases the availability of EETs which have been identified as vasodilators (Zhang et al., 2009). In experimental studies inhibition of sEH has resulted in smaller infarct size after experimental stroke (Zhang et al., 2009).

The premise of this study is that these molecular alterations are thought to be a primary cause of vascular constriction in the microcirculation which may precipitate ischemia that results in prolonged and more profound consequences. Clinically small vessel ischemia may not be detected during cerebral angiograms and may be responsible for neurological decline in the absence of radiographic vasospasm (Vergouwen et al., 2009). Vascular stability after aSAH is dependent upon a balance in the brain between metabolites that cause vasoconstriction (ET-1 and 20-HETE) and those that cause vasodilation (EETs and Nitrous Oxide). If the equilibrium is

maintained after insult, there is a low risk of DCI. However if equilibrium is not maintained, the risk of DCI is increased.

The potential influence of estrogen on these more established vasoactive markers that impact cellular and vascular response after aSAH deserves further attention. Evidence of the actions of E2, and to a lesser degree E1, in animal models and research exploring its mechanisms of action in other neurologic conditions provide a strong rationale for the significance of the proposed study which will explore the relationship between plasma E2 levels and ischemic complications after acute aSAH in humans. This evidence provides support for the framework for this study which includes the potential effect E2 may have on the vascular changes that occur after aSAH depicted in Figure 1.

1.4.6 Conceptual Framework

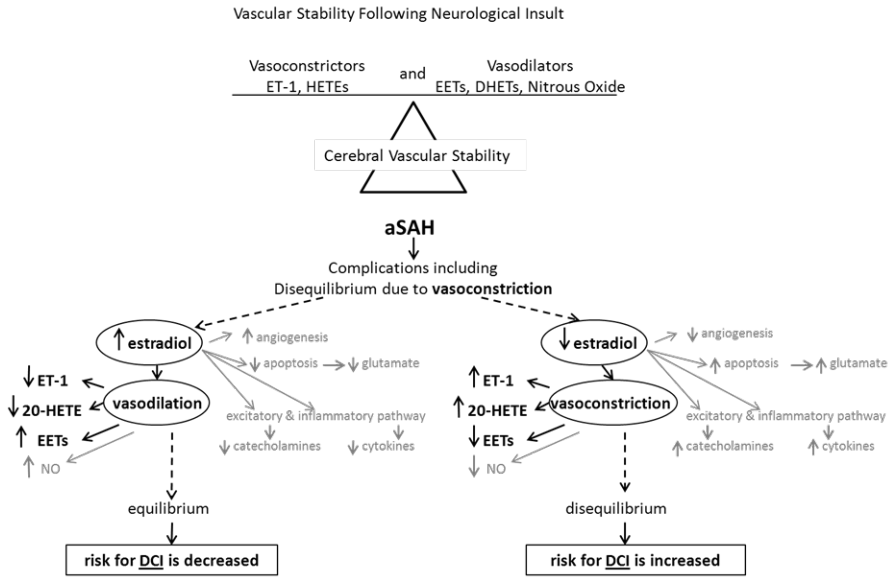


Figure 1.Conceptual Framework

1.5 RESEARCH METHODS

1.5.1 Design

This study used a prospective, longitudinal design to examine E1 and E2 levels in plasma and cerebral spinal fluid (CSF) and ischemic outcomes (DCI) in patients with acute aSAH.

1.5.2 Setting

The study was a secondary analysis of data from patients with acute aSAH admitted to the neurovascular intensive care unit at the University of Pittsburgh Medical Center (UPMC) Presbyterian Hospital who were enrolled in a NIH-funded study (R01NR004339) exploring the influence of biomarkers related to complications of aSAH. UPMC comprises a network of 20 hospitals, servicing the health needs of over 4 million people. UPMC Presbyterian is an 800-bed, designated Level I Regional Resource Trauma Center with over 24 specialty services. UPMC Presbyterian contains 10 intensive care units including a 20 bed neurovascular intensive care unit that provides care to persons with a broad spectrum of neurovascular insults; 14% of their admissions are persons with aSAH. The internationally renowned Department of Neurological Surgery, started more than 60 years ago, is one of the largest neurosurgical academic providers in the United States; more than 6,500 procedures are performed annually through the department.

1.5.3 Sample

This study used data from 99 acute aSAH patients consecutively selected from the database of the previously cited NIH study (RO1NR004339). At the time of this analysis, 350 subjects had been recruited.

1.5.3.1 Participant Recruitment for the Parent Study

Inclusion criteria for the parent study were: 1) 21-75 years; 2) verified aneurysmal SAH; and 3) Fisher grade > 1 (determined by evidence of blood on CT scan). Confirmation of aneurysm was verified by cerebral angiography or CT angiogram read and interpreted by a n eurosurgeon. Exclusion criteria were: 1) non aneurysmal SAH; and 2) history of preexisting debilitating neurologic disease and 3) mycotic aneurysm. Potential patients were identified by physician or nursing staff involved in the care of the patient and permission was established for the study team to approach the patient and/or family. Procedures, risks and benefits were explained to the patient and/or family and consent was obtained. When necessary, both surrogate and proxy consent using Pennsylvania state law guidelines were used due to the cognitive dysfunction common in acute aSAH patients. When possible, assent or patient consent was obtained; if not, consent for continued participation was obtained when the patient was able to understand and provide consent. The protocol was approved by the Institutional Review Board and informed consent was obtained from the patient or proxy prior to data collection (Appendix A). The consent allows use of samples for analyses determined to be potentially useful by the research team.

1.5.3.2 Dissertation Study

The sample for the dissertation analysis was selected from the database of the parent study in consecutive order based on availability of outcome data and biologic specimens. Only patients with a minimum of 3 available plasma and corresponding CSF samples were included. The sample was stratified to include women (both pre- and postmenopausal) and men without statistical differences in severity of injury or incidence of DCI. Females on oral contraceptives or estrogen replacement were excluded from the analysis. Age was not statistically different between males and females with the exception of pre and postmenopausal female groups.

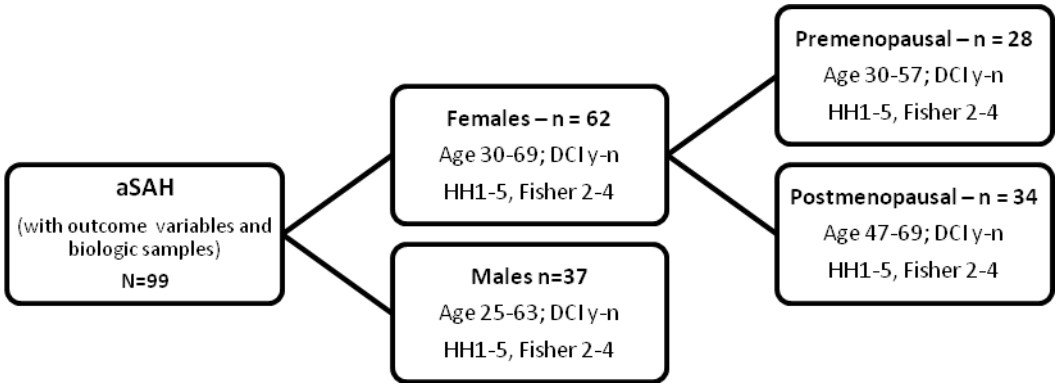


Figure 2. Study Consort

Rationale: To permit examination of the variables of interest related to the outcome of DCI while controlling for differences in age, gender, severity of injury and menopausal status.

1.5.3.3 Sample Size

Data from 99 aSAH subjects with DCI and biomarker information was used in this analysis. **Rationale**: There are no known studies that have analyzed E1 and E2 levels in human aSAH patients. To achieve study aims, it was judged necessary to control for age, gender and menopausal status as well as confounding variables related to severity of injury (HH and Fisher grade). The proposed sample size was also based on consideration of the incidence of DCI which, in a previous analysis in this sample, was approximately 50 percent. A logistic regression of the binary response variable DCI(Y) on a continuous, normally distributed E2 variable (X) with a sample size of 100 observations (patients with the dependent variable DCI) achieves 80% power at a .05 significance level to detect a change in probability (Y=1) from the value of .5 at the mean of X to .643 when X is increased to one standard deviation above the mean. This change corresponds to an odds ratio of 1.799. An adjustment was made since a multiple regression of the independent variable of interest on the other independent variables in the logistic regression obtained an R-Squared of .09 (Hseih et al., 1988). A Cox regression of the log hazard ratio on a covariate with a standard deviation of 1.23 based on a sample of 100 observations achieves 90% power at a .05 significance level to detect a regression coefficient equal to .3907 and hazard ratio of 1.48. The sample size was adjusted since a multiple regression of the variable of interest on the other covariates in the Cox regression is expected to have an R-Squared of .09. The sample size was adjusted for an anticipated event rate (DCI) of .5 (Hseih & Lavori, 2000; Schoenfeld, 1983).

1.5.4 Study Variables

1.5.4.1 Demographics (Co-variates)

Basic demographic information including severity of injury, aneurysm information, age (in years), race, gender, past medical history and menopausal status was inpatient medical records. Past medical history including menopausal status were obtained from the patient, family or medical record. Severity of injury [Hunt and Hess scores (HH) and Fisher grades] and aneurysm information were obtained from the neurosurgical notes in the patient's clinical record.

1.5.4.2 Estradiol and Estrone (Independent variables)

E1 and E2 were measured in plasma and CSF samples of each aSAH patient and analyzed as a ratio scaled, continuous variable. Each morning (8am +/- 1 hour) and evening (7pm +/- 1 hour) through 10 days after initial hemorrhage, blood and CSF were collected as part of the parent study protocol. At each time point, a blood sample was gathered in an EDTA tube and centrifuged. The plasma was aliquoted and frozen for analysis within 60 minutes of collection. CSF was withdrawn by registered nurses directly from the CSF tubing as long as the CSF access remained in place. Specimens were immediately frozen and stored at -80 degrees for batch analysis. In order to capture changes in circulating estrogen levels, at least 3 plasma samples per subject were analyzed for E1 and E2 including the initial (first) sample, a sample from day 5-day 7, and day 10 after hemorrhage. For CSF, E1 and E2 was analyzed in 3-5 samples per subject including a sample from the initial (first) sample, a sample from day 5 through day 7, and day 10 after hemorrhage. E1 and E2 were assayed at the University of Pittsburgh Small Molecule

Biomarker Core, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS is a preferred method to measure E2 among postmenopausal women given its high sensitivity at low postmenopausal E2 concentrations, which are often below the sensitivity of standard immunoassays. The UPLC-MS/MS method employs liquid-liquid extraction, derivatization and detection with a triple quad mass spectrometer. Plasma and CSF is spiked with n-butyl chloride, derivatized with dansyl chloride and transferred to autosampler glass vials for injection. E2 is eluted from a Waters Acquity UPLC BEH C18, 1.7 um, 2.1x150 mm reversed-phase column, with an acetonitrile: water (0.1% formic acid) gradient. Detection and quantitation are achieved in the positive mode with a Thermo Fisher TSQ Quantum Ultra mass spectrometer interfaced via an electrospray ionization (ESI) probe with the Waters UPLC Acquity solvent delivery system. Transitions used for analysis are 506 --> 171 for estradiol, 504 → 171 for estrone, and 511 --> 171 for the deuterated internal standard. The calibration curves, obtained from extracting known concentrations of E2 from 2-hydroxypropyl-beta-cyclodextrin (0.2% in water), range from 0.5 pg/ml (lower limit of quantitation) to 200 pg/ml. The calibration curves, obtained from extracting known concentrations of E1 from 2-hydroxypropyl-beta-cyclodextrin (0.2% in water), range from .5 pg/ml (lower limit of quantitation) to 200 pg/ml. The limit of detection is 0.5 pg/ml. All back calculations of calibrators, inter-day and intra-day precision and accuracy, and stability are within acceptable limits (<15% at all tested concentrations) (Nelsen et al., 2004).

1.5.4.3 DCI (Dependent Variable)

Measurements of DCI were evaluated from admission through the 10th day after aSAH (or discharge from the acute care setting) and operationalized as evidence of impaired cerebral blood flow (cerebral vasospasm by angiography or transcranial Doppler, cerebral ischemia or cerebral infarction via CT or MRI brain/perfusion scan) accompanied by neurological deterioration (simultaneously or within 12 hours pre or post visualization of impaired cerebral blood flow). Neurological deterioration was defined by the presence of any of the following: at least a 2 point increase in National Institute of Health Stroke Scale (NIHSS) and or decline in Glasgow coma score (GCS); deterioration in pupil reaction; nursing documentation of a focal deficit (visible weakness or difficulty speaking); or nursing documentation of a decline in level of consciousness (lethargy, agitation, confusion) not associated with fever or medication administration (such as sedatives, paralytics or anti-anxiety medications). The NIHSS, which was routinely measured twice a day by the NV-ICU nurse as part of standard of care, is widely used to quantify neurologic deficits using a 42 point scale (0=no neurologic deficits) and has shown high inter-rater reliability and criterion validity (Lyden et al., 1999). In addition, routine care for aSAH in the NV-ICU included documentation of neurological assessments (including GCS) every one to two hours. The GCS has a reported sensitivity of 79-97% and specificity of 84-97% (Prasad, 1996; Zeucher et al., 2009).

Cerebral blood flow was assessed by cerebral angiography, transcranial Doppler and CT or MR perfusion scans. Although not quantitative, cerebral angiography serves as a method for assessing blood flow and was used to evaluate the presence or absence of angiographic vasospasm. Cerebral angiograms (ordered as standard of care or in situations of potential deterioration) were examined and coded by neurosurgeons blinded to participant identity and

dichotomized as either 'negative' (0-24% narrowing of cerebral blood vessels) or 'positive' (≥25% narrowing of cerebral blood vessels). Daily transcranial Dopplers were evaluated for abnormal flows using a systolic middle cerebral artery velocity > 200 ml/sec and/or a Lindegaard ratio > 3.0 (Frontera et al., 2009; Zubkov & Rabinstein, 2009). Transcranial Doppler ultrasounds were performed daily for 14 days provide a real-time indicator of presence of vessel narrowing by using reflected gated ultrasound waves at 2MHz to determine velocity of the red blood cells within the cerebral arteries. Sensitivity of TCD ultrasound has been reported between 84% and 85.7% with specificity between 89% and 100%, data which indicates that TCDs provide a reasonable assessment of blood flow (Kirkness, 2005). Finally, head CT/MR and head CT/MR perfusion scans (ordered a patient care or follow up) were reviewed for the presence/absence of new ischemia, infarction or low blood flow (documentation of low, delayed, asymmetric or absent blood flow) in the absence of hydrocephalus or rebleed (Ito et al., 2005). The combination of these blood flow measures and neurological deterioration provide a more definitive measure of flow-metabolism mismatch. Patients were categorized as a nominally scaled binary variable "yes" or "no" for DCI based on the presence or absence of the criteria described. For time-dependent analysis all patients were be assigned a ratio-scaled time to event. In patients who developed DCI, this time corresponded to the time of DCI criteria. In patients who did not develop DCI, time to event was recorded as 340 hours, which is the end of the monitoring period for the parent study. If a patient died prior to the end of the monitoring period or DCI, time of death was used as a censoring event.

1.5.4.4 Biomarkers

20-HETE levels in CSF collected in an identical manner and at the same time points as the samples used for E2 examination were analyzed as ratio-scaled, continuous variables. 20-HETE and EETs were analyzed as a part of the parent grant using a published solid phase extraction procedure with minor modifications (Miller et al., 2009). Modifications included using a sample load of 2.0-3.0ml and reconstituting the samples in 50µl of 80:20 methanol/water. Quantitation of CYP-eicosanoids was performed using a published ultra-performance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) method (Miller et al., 2009). Briefly, liquid chromatography was performed using a UPLC BEH C18, 1.7µm (2.1 x 100mm) reversed-phase column (Waters, Milford, MA). Mass spectrometric analysis of analyte formation was performed using a TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA) triple quadrupole mass spectrometer. Quantitation of analytes by selective reaction monitoring SRM analysis was performed by monitoring their *m/z* transitions. The SRN conditions for these molecules and their retention times are shown in Table 1.

Table 1. UPLC-MS/MS Method Parameters for Analytes

Analytes	Precursor→ Product (m/z)	Collision Energy (V)	Retention Time (min)
20-HETE	319→ 245,289	18	3.48
14,15-DHETE	337→ 207	15	3.01
11,12-DHETE	337→ 167	14	3.19
9,10-EpOME	295→227	18	4.43
9,10-DiHOME	313→201	22	2.89

1.5.5 Data Collection

A total of 99 subjects with aSAH and biologic samples were selected for inclusion in this study. Convenience sampling was used to select the first males, post-menopausal females and pre-menopausal females from database of the parent study based on availability of outcome data and biologic specimens (minimum of 3 available plasma and corresponding CSF samples) without statistical differences in severity of injury or incidence of DCI. Age was not statistically different between males and females with the exception of pre and postmenopausal females. Demographic and medical condition data was extracted from the inpatient medical record. Data relating to biomarkers (with the exception of E1 and E2) was also obtained from this database. Analysis of E1 and E2 specimens was performed for the present study. All p lasma and CSF samples collected as a part of the parent study (see appendix) were analyzed using standard procedures and samples maintained in a –80^{o}C freezer.

Table 2. Collection Procedure

	Collection Day										Chart Review
	1	2	3	4	5	6	7	8	9	10	
Demographics											X
DCI											X
Plasma E1&E2	X				X					X	
CSF E1& E2	X		X		X		X		X		
CSF HETE & EET	X		X		X		X		X		

1.5.6 Data Analysis

1.5.6.1 Preliminary Analysis

All statistical analyses were preceded by detailed descriptive analysis of the data, using standard descriptive summaries (e.g., means, standard deviation, percentiles, ranges and frequencies) and graphical techniques (e.g., histograms, scatter plots). In addition, the distributions of all key variables were examined to ensure that proposed modeling techniques were suitable. Univariate analyses were used to determine associations between outcome variables and key covariates, followed by multivariable analysis. Patterns of missing data were reviewed to assess potential biases and possible model assumption violations.

1.5.6.2 Data Analysis Aims 1 and 3

The goal of Specific Aims 1and 3 was to examine patterns in plasma and CSF E1 and E2 levels during the 10 days following aSAH. Detailed descriptive statistics for E1 and E2 levels in plasma and CSF were reported first. Since there are repeated measures of E1 and E2 level for each subject over the first 10 days after SAH, group-based trajectory analysis (Nagin & Odgers, 2010; Nagin, 2005) was used to identify the distinct short-term patterns (homogeneous latent trajectory classes) of E1 and E2 from day 1 to day 10 after initial hemorrhage. Group based trajectory also controls for missing data and inconsistent data points. The different trajectory models were then tested and compared based on model selection including entropy which quantifies the uncertainty or variability in a system as well as statistical significance, the Bayesian Information Criterion (BIC), Akaike's Information Criterion (AIC) and the substantive utility of the classes (e.g.

distinctiveness of the trajectories, proportion assigned to a given class) to determine the optimal solution for the number of trajectory groups.

1.5.6.3 Data Analysis Aims 2 and 4

For Specific Aims 2 and 4, we sought to determine the relationship between plasma and CSF E1 and E2 levels and the presence or absence of DCI during the 10 days following aSAH. First, weighted and non-weighted chi-square test and/or Fisher exact test were used as appropriate to explore the association between presence of DCI and the different trajectory classes identified in specific aims 1 and 3 for E1 and E2 in the first 10 days after SAH. Then multiple logistic regression models (GENMOD and PROC MIXED, SAS 9.2) were conducted to evaluate the relationship between them controlling for possible covariates such as demographic characteristics (age, gender, menopausal status and severity of hemorrhage).

1.5.6.4 Data Analysis Aims 5

For Specific Aim 5, we explored the associations between CSF levels of E1 and E2, 20-HETE and EET and the presence or absence of DCI following aSAH. Multiple logistic regression models were conducted using SAS v9.2 to evaluate the relationship between these metabolites controlling for possible covariates such as demographic characteristics (age, gender, menopausal status and severity of hemorrhage).

1.6 HUMAN SUBJECTS

1.6.1 Human Subjects

The study was a secondary analysis of data from patients with acute aSAH admitted to the neurovascular intensive care unit at UPMC Presbyterian Hospital who were enrolled in the previously cited NIH-funded study (R01NR004339) exploring the influence of biomarkers related to complications of aSAH. In addition to CSF and plasma samples for metabolite measurement, demographic and clinical data were extracted from the research record. All study data were stored in locked filing cabinets and managed on secure, password-protected databases stored on password protected servers. All patient samples were stored in locked freezers at the University of Pittsburgh School of Nursing.

1.6.1.1 Protection of Human Subjects

The parent study, which included the use of samples for future analysis in studies performed by the research team, was approved by the Institutional Review Board of the University of Pittsburgh (Appendix A).

1.6.2 Source of Materials

Patient information including demographic, medical history, clinical information and related metabolite data were obtained from the parent study database (Appendix B). Stored frozen blood and CSF samples, collected as part of the parent study protocol, were obtained for E1 and E2 analysis.

1.6.2.1 Potential Risks

Potential risks for data collection are described in the parent study including those related to transcranial Doppler studies, CSF and blood collection and confidentiality (Appendix B). For this analysis privacy and confidentiality were assured by not providing individual identifiers.

1.6.3 Recruitment and Informed Consent

Potential patients for the parent study were identified by physician or nursing staff involved in the care of the patient and permission was established for the study team to approach the patient and/or family. Procedures, risks and benefits were explained to the patient and/or family and consent was obtained. Both surrogate and proxy consent (using Pennsylvania state law guidelines) were used due to the cognitive condition of many acute aSAH patients. When possible, assent or patient consent was obtained; if not, consent for continued participation was obtained when the patient was able to understand and provide consent. Informed consent was

obtained from the patient or proxy prior to data collection. The consent allows use of samples for future analysis in studies performed by the research team (Appendix C).

1.6.4 Protection Against Risks

Since this study is a secondary analysis of data and banked samples, there were no acute risks associated with collection of the samples. Protections against risks for data collection are described in the parent study including confidentiality, use of transcranial Doppler gel and the risk of infection and discomfort from sample collection. There was the possibility of a breach of confidential patient information, although measures to avoid this were followed. All records were kept in locked cabinets within the departments of the principal investigators of the parent study. All specimens were kept in locked freezers at the University of Pittsburgh, under the control of the principle investigator of the parent study and accessible only by the research staff. A case number is linked to the records and specimens without indicating the subject's individual identity. All analysis was done on coded samples identified in the records only by case number. The plasma and CSF samples collected for the parent study are maintained in a -80^{o}C freezer utilizing the case numbers. No results were provided directly to any participant.

1.6.5 Potential Benefits of the Proposed Research to the Subjects and Others

There were no direct benefits to subjects who were included in the study. The general benefit of this study was for future patients with aSAH by developing earlier predictors of acute complications following aSAH which may result in earlier therapeutic treatments.

1.6.6 Importance of Knowledge Gained from the Proposed Study

Data from the proposed study, which had not been explored in aSAH, will advance the state of the science for patients with hemorrhagic stroke and cerebral insult by addressing a gap in the knowledge related to the role of E2 in influencing complications such as DCI which can markedly impact patient outcomes. Ultimately advancement of our understanding of the mechanisms underlying the development of DCI may result in target therapies and more accurate identification of patients at high risk for poor outcomes.

1.6.7 Data Safety Monitoring Plan

The principal investigator of the parent study is responsible for the maintenance and protection of the grant data. The original consent and screening forms are stored in a locked cabinet, separate from data storage. All data is entered and maintained in an SPSS database (v. 19.0). Paper copies of subject data are labeled with only a unique study identifier for each subject and

kept in a locked cabinet. All electronic data is kept on a password-protected computer. Patient specimens are maintained in locked study freezers at the University of Pittsburgh School of Nursing and labeled only with unique study identifier for each subject. Data and safety monitoring related to this secondary analysis were conducted during meetings with the dissertation committee. Any adverse events would have been reported immediately to the IRB however there were no adverse events.

1.6.8 Inclusion of Women, Minorities and Children

The racial, gender and ethnic characteristics of the proposed subject population reflects the demographics of Pittsburgh and the surrounding area and/or the patient population of the University of Pittsburgh Medical Center. Patient data were not excluded based on race, ethnicity, gender or HIV status. The racial characteristics of the parent study include 88% white, 11% African American and 1% other minority subjects. Research suggests that the majority of persons who develop aSAH are female. In the parent study, 75% of the sample is female. For the purpose of this study distinct criteria were used to include equal representation of pre and post - menopausal females as well as males. There were no children involved in this study. There were no advertisements for this study.

2.0 SUMMARY OF STUDY

The purpose of this study was to examine the association between levels of estrogen (estradiol and estrone) and the development of DCI in patients with aSAH. An article describing plasma result prepared in the format for submission to Stroke (Aim 1 and 3) is appended to this chapter. A second article published in Stroke that describes the relationship between 20-HETE and DCI after aSAH is included in bibliography (Crago et al., 2011).

2.1 FINDINGS RELATED TO AIM 1

Aim 1. *Examine patterns in plasma E1 and E2 levels during the 10 days following aSAH.*

The study variables for analysis were plasma E1 and E2 levels, patient characteristics [age, race, gender, menopausal status and severity of injury (HH and fisher scores)] and ischemic outcomes including DCI, vasospasm and cerebral ischemia. Binary variables were created for HH and race to capture relationships. Both E1 and E2 data were non-normally distributed; therefore data transformation (natural logarithmic) was applied. Since few metabolite measurements were not quantifiable or below limits of detection [E1-1 sample (<1%), E2-12 samples (4%)], these measurements were assigned a number at the limit of detection. Findings related to aim 1 are presented in the manuscript.

The sample consisted of 99 aSAH subjects of which 28 were pre-menopausal females, 34 were post-menopausal females and 37 were men.(Table 3) Subjects in this analysis were also predominately Caucasian (87%, n=86) with a mean age of 50.03 years (SD=10.1). Severity of injury by Hunt and Hess ranged from 1-5 with 46% (n=45) graded as HH 3, while the average fisher score was 3 (n=45, 46%) (Table 3). Three plasma samples were selected for E1 and E2 analysis from each patient representing early (first – 4 days after hemorrhage), middle (4-6 days after hemorrhage) and late (7-10 days) after hemorrhage and were analyzed at the SMBC (small molecule biomarker core) lab at the University of Pittsburgh School of Pharmacy. E1 and E2 measurements were arranged by day (1-10) and values were log transformed for analysis. Group based trajectory analysis (SAS v9.2) was used to identify distinct populations over time for both E1 and E2 values using censored normal model. Preliminary analyses were conducted to test models from one to 4 trajectory groups. The numbers of groups for each hormone were determined by evaluating the mixed fit statistics including Entropy, BIC, and AIC. Weighted and non-weighted chi-square test and/or Fisher exact test were used to explore the association between demographic and outcome variables and the different trajectory classes for both plasma E1 and E2 in the first 10 days after SAH.

There was an estimated 61.4% (n=61) of patients in the E1 high and 38.6% (n=38) in the E1 low trajectories with a declining pattern that was significant for both groups ($p<.001$; mean low = 3.9, SD=.69; mean high= 5.12, SD=.66) (Figure 3). Weighted Chi square analysis revealed that compared with patients in the low E1 group, patients in the high E1 group were significantly more likely to be female ($p=.02$), post-menopause (.05), older ($p<.001$) and had a higher severity of injury as evidenced by Fisher grade ($p=.008$). Although it did not reach statistical significance, patients with the presence of DCI were statistically more likely to be in

the high E1 group (p=.09), however patients with DCI specifically from infarction, neurological decline associated with new CT evidence of cerebral infarction, were more likely to be in the high E1 trajectory group (p=.03) (Table 4).

An estimated 48% (n=46) of the patients were categorized in the E2 high group and 52% (n=53) in the E2 low trajectories with both groups displaying a declining pattern (p=.01) (Figure 4). Using weighted Chi square analysis patients in the high E2 trajectory group were significantly younger than those in the low group (p=.02) and were more likely to have higher Fisher grade although this was not statistically significant (p=.09). No differences were noted between E2 groups by gender, race, menopausal status or HH, and no differences were identified by E2 trajectory group and DCI (Table 4).

These findings support a relationship between severity of injury and estrogen metabolite measurements. Patients in the high E1 and E2 trajectory group were more likely to have a higher severity of injury by fisher score. E1 levels were also significantly different by age, gender and menopausal status with younger and premenopausal women more likely to be in the high trajectory group. E2 levels were also significantly different by age; however patients with high E2 trajectories were younger than patients in the low E2 trajectory group. Finally, patients who developed DCI were more likely to be in the high E1 trajectory group.

2.2 FINDINGS RELATED TO AIM 2

Aim 2. *Determine the relationship between plasmaE1 and E2 levels and the presence or absence of DCI during the 10 days following aSAH.*

The study variables for aim 2 were plasma E1 and E2 levels, patient characteristics (age, race, gender, menopausal status and severity of injury including HH and fisher scores) and DCI including individual measurements of blood flow such as cerebral vasospasm, ischemia or infarct. Similar to aim one, binary variables were created for HH and race to capture relationships. Both E1 and E2 data were non-normally distributed; therefore data transformation (natural logarithmic) was applied. As in aim 1, since few metabolite measurements were not quantifiable or below limits of detection [E1-1 sample (<1%), E2-12 samples (4%)], these measurements were assigned a number at the limit of detection. Findings related to aim 2 are reported in the manuscript.

This sample also consisted of 99 aSAH subjects with plasma samples as described in aim one (Table 3). Using log transformed E1 and E2 measurements multiple logistic regression models (GENMOD, SAS v9.2) were conducted to evaluate the relationship between E1 and E2 measurement and DCI while controlling for possible covariates such as demographic characteristics (age, gender, menopausal status and severity of hemorrhage).

Pre-menopausal aSAH patients had higher mean E1 and E2 levels than both post-menopausal females and males however this was not statistically significant. Metabolite measurements were comparable to other studies using HPLC measurements of E1 and E2 in human subjects (Haring et al., 2011; Masi et al., 2009; Naessen et al., 2010; Rothman et al., 2011; Xu et al., 2007). There was a significant association between plasma E1 and E2 ($P<.001$). Females had higher E1 concentrations than males ($p=.06$) and E1 levels were statistically different by age ($p<.001$), however this was not found for E2 ($p=.31$) (Table 5).

Higher values of plasma E1 and E2 were associated with higher severity of injury regardless of age. Patients with higher HH and Fisher were more likely to have higher plasma E1

levels in all analysis except between the groups of HH4 and HH5. Similarly, patients with higher HH and Fisher scores were more likely to have higher plasma E2 levels although this was only statistically significant between HH groups (1&2 vs 3-5) and Fisher 3 vs Fisher 4 (Table 5).

The presence of DCI was significantly associated with higher levels of both plasma E1 (p=.004) and E2 (.03). This relationship to DCI remained significant when severity of injury was added to the regression model for both E1 (p=.02) and E2 (p=.08). There was also a significant association between DCI from cerebral infarction and both plasma E1 (p<.001) or E2 (p=.05) (Table 5).

Using all variables with a maximum significance of p<.10 from the univariate analyses, generalized estimating equation (GEE) models were created to predict E1 and E2. Log plasma E1 was significantly associated with DCI (p=.02) and age (p<.001) when controlling for age, gender, HH and DCI. In a model with age, gender, Fisher and DCI, age (p<.001), Fisher (p=.05) and DCI (p=.003) were significantly associated to E1 levels. Similarly, (log) E2 was analyzed in a model of age, HH and DCI with DCI (p=.05) and age (p=.08) having an association to E2 levels. Using Fisher instead of HH in the model, DCI was significantly associated with E2 levels (p=.02).

These findings are consistent with the findings related to study Aim 1 which support a relationship between plasma E1 and E2 measurements and severity of injury. Further, a significant relationship was identified between higher levels of E1 and E2 and the presence of DCI after aSAH while controlling for severity of injury.

2.3 FINDINGS RELATED TO AIM 3

Aim 3. *Examine patterns in CSF E1 and E2 levels during the 10 days following aSAH.*

The study variables for analysis were CSF E1 and E2 levels, patient characteristics [age, race, gender, menopausal status and severity of injury (HH and fisher scores)] and ischemic outcomes including DCI, vasospasm and cerebral ischemia. Binary variables were created for HH and race to capture relationships. Both E1 and E2 CSF data were non-normally distributed, therefore data transformation (natural logarithmic) were applied.

The sample consisted of 36 a SAH subjects with CSF samples of which 11 w ere premenopausal females, 11 were post-menopausal females and 14 were men (Table 6). Subjects in this analysis were also predominately Caucasian (78%, n=28) with a mean age of 49.9 years (Table 6). Severity of injury by Hunt and Hess ranged from 1-5 with 44% graded as HH 3, while the average fisher grade was 3 (47%) (Table 6). Up to 5 CSF samples were selected for E1 and E2 analysis from each patient representing the first available sample, a sample from the 10^{th} day after hemorrhage and 1-3 samples in between. S amples were analyzed at the SMBC at the University of Pittsburgh School of Pharmacy. E 2 levels were only detected in 33% of the samples analyzed which was consistent across gender and menopausal status. E1 levels were more consistently measured and were detected in 83% of samples that were analyzed. E1 and E2 measurements were arranged by day (1-10) and values were log transformed for analysis. Group based trajectory analysis (SAS v9.2) was used to identify distinct populations over time for both E1 and E2 values using censored normal model. Preliminary analyses were conducted to test models from one to 4 trajectory groups. The number of groups for each hormone was determined by evaluating the mixed fit statistics including Entropy, BIC, and AIC. Weighted and non-

weighted chi-square test and/or Fisher exact test were used to explore the association between demographic and outcome variables and the different trajectory classes for both CSF E1 and E2 in the first 10 days after SAH.

There was an estimated 58% (n=22) of patients in the E1 low and 42% (n=14) in the E1 high trajectories with a constant pattern over time in the high group and a significantly declining pattern in the E1 low group (p=.04) (Figure 5). Using non weighted chi square, significant differences were noted between groups by HH (p=.03) and DCI (p=.06) and specifically DCI from vasospasm (p=.02) (Table 7). The relationship between group and DCI did not remain significant using weighted chi squares (p=.12) (Table 7). Patients with higher HH score, DCI and specifically DCI from vasospasm were more likely to be in the consistently high CSF E1 group. Mixed fit statistics were improved using a 3 group trajectory for E1 measurements. There were an estimated 61.3% of patients in the E1 low group, 22.4% in the E1 higher group and an estimated 16.3% in a third E1 group which declined over time (Figure 6). Using weighted and non-weighted chi-square test and/or Fisher exact test to explore the association between demographic and outcome variables higher HH was associated with the high E1 group in the 2 group trajectory model (p=.04). In the 3 trajectory classes only gender (p=.009) was significantly associated with group membership. In the 3 group analysis, only male patients were found in the third E1 group which declined over time (Table 7). No relationship was identified between E1 trajectory groups and DCI.

Trajectory analysis for E2 CSF levels resulted in relatively poor fit statistics in all attempted models which may be a result of both sample volume and low detectable levels in the samples that were quantified. In the best trajectory model resulting in 2 linear groups, there was an estimated 41% (n=10) of patients in the E2 CSF low and 58% (n=26) in the E2 CSF high

trajectories. In this model the high trajectory significantly declined over time (p=.05) while the low trajectory had a consistent pattern over time (Figure 7). Using weighted Chi Square analysis no statistical differences were identified between groups (Table 7).

These findings provide evidence that estrogen metabolites are measureable in CSF and that a relationship exists between severity of injury and estrogen metabolites in CSF. Patients in the high E1 trajectory group were more likely to have a higher severity of injury by HH score and to develop DCI.

2.4 FINDINGS RELATED TO AIM 4

Aim 4. *Determine the relationship between CSF E1 and E2 levels and the presence or absence of DCI during the 10 days following aSAH.*

The study variables for Aim 4 were CSF E1 and E2 levels, patient characteristics [age, race, gender, menopausal status and severity of injury (HH and fisher scores)] and ischemic outcomes including DCI, vasospasm and cerebral ischemia. Binary variables were created for HH and race to capture relationships. Both E1 and E2 CSF data were non-normally distributed, therefore data transformation (natural logarithmic) were applied.

As in aim 3, the sample consisted of 36 aSAH subjects with CSF samples (Table 6). Up to 5 CSF samples were selected for E1 and E2 analysis from each patient representing the first available sample, a sample from the 10^{th} day after hemorrhage and 1-3 samples in between. Samples were analyzed at the SMBC lab at the University of Pittsburgh School of Pharmacy. E2

levels were only detected in 33% of the samples analyzed which was consistent across gender and menopausal status. E1 levels were more consistently measured and were detected in 83% of samples that were analyzed. Using log transformed E1 and E2 measurements logistic regression models (PROC MIXED, SAS v9.2) were conducted to evaluate the relationship between E1 and E2 CSF measurement and DCI while controlling for possible covariates including such as demographic characteristics (age, gender, menopausal status and severity of hemorrhage). In addition, because of the level of detection of E2 in CSF, estrogen metabolites were dichotomized into groups of detectable and non-detectable concentrations. Chi-square test and/or Fisher exact test were conducted to explore the association between detectable and non-detectable levels of E1 and E2 in CSF with socio-demographic, clinical, and DCI factors.

There was a significant relationship between CSF E1 and E2 concentrations ($p<.001$). Although limited references exist, metabolite levels were comparable to other studies using HPLC measurements of CSF E1 and E2 in human subjects (Nguyen et al., 2011). Using log metabolite levels, higher CSF E1 measurements were associated with higher HH scores ($p=.03$) (Table 8). Similarly higher fisher scores were associated with higher CSF E1 concentrations, where patients graded Fisher 2 had lower concentrations of CSF E1 than patients with Fisher 4 grade ($p=.06$). Higher CSF E1 levels were also associated with DCI from vasospasm ($p=.05$) however relationships were not identified in other ischemic outcomes including DCI ($p=.24$). There were no difference noted between CSF E2 and severity of injury or DCI (Table 8). There were no identified differences between groups of detectable versus non-detectable concentrations in either CSF E1 or CSF E2 (Table 8).

Consistencies in study findings were identified between estrogen metabolites in CSF and plasma where higher levels of CSF E1 were associated with severity of injury after aSAH.

However, these findings need for additional patients and sample selection to effectively explore this aim.

2.5 FINDINGS RELATED TO AIM 5

Aim 5. *Explore the associations among CSF levels of E1 and E2, 20-HETE and EET and the presence or absence of DCI following aSAH.*

This exploratory aim used the study variables CSF E1, E2, 20HETE, 11,12 DHET and 14,15 DHET and DCI. All of the CSF measurement data were non-normally distributed; therefore data transformation (natural logarithmic) was applied. Any metabolite measurements below limits of detection were excluded from the analysis.

As in aim 3 and 4, the sample consisted of 36 aSAH subjects with CSF samples (Table 4). Up to 5 CSF samples were selected for E1 and E2 analysis from each patient representing the first available sample, a sample from the 10^{th} day after hemorrhage and 1-3 samples in between. In addition CSF 20HETE, 11,12 DiHET and 14,15 DiHET measurements at the same time points were entered into the database. All samples were analyzed at the CORE lab at the University of Pittsburgh School of Pharmacy. As described in aim 3, E2 levels were only detected in 33% of samples analyzed while E1 levels were more consistently measured and were detected in 83% of samples that were analyzed. CSF 20HETE was quantified in 122 samples (76%), while 11,12 DiHET was measured in 53% (n=86) and 14,15 DiHET in 66% (n=107). Using log transformed measurements of all CSF metabolites regression models (PROC MIXED, SAS v9.2) were

conducted to evaluate the relationship between levels of E1 and E2 with 20HETE, 11,12 DiHET and 14,15 DiHET measurements in CSF as well as DCI.

No relationships were identified between CSF E2 and 20HETE (p=.85), 11,12 DiHET (p=.3) or 14,15 DiHET (p=.63). A significant relationship was noted between CSF E1 and 20-HETE (p=.05). And although not significant, a relationship was noted between 11,12 DiHET (p=.07) and CSF E1. No relationship was found between CSF E1 and 14,15 DiHET (p=.21). The relationship between CSF E1 and DCI did not reach significance in models containing CSF 20-HETE and CSF 11,12 DiHET. A low percentage of HETE and DiHET detection in this already small sample size is a limiting factor in this analysis, however there are indications that relationships exist between these vasoactive metabolites warranting the need for additional patients and sample selection to effectively answer this aim.

2.6 RECOMMENDATIONS AND LIMITATIONS

This study only analyzed E1 and E2 levels after aSAH. Future studies should include other neurosteroid levels (progesterone, allopreganalone and testosterone) as well as growth hormone (IGF-1) and inflammatory markers (cortisol, interleukins or CRP) to provide additional information and explanation of findings. At the same time, measurement of FSH and LH could enhance the evaluation of menstrual cycle and menopause in females as well as provide documentation for hormone suppression. A second limitation is that the population of aSAH patients selected for this study was based on availability of cerebral spinal fluid. Patients who

require placement of external ventricular catheters represent a subgroup of aSAH patients who have higher severity grades and may have higher risk of complications after aSAH due to extent of injury including DCI. Measuring plasma estrogen concentrations on all aSAH patients regardless of CSF availability would capture a larger sample volume and would further evaluate the association between estrogen measurements and severity of injury since patients with less symptom burden and blood load are less likely to have CSF drainage devices.

A third limitation is the definition of DCI. In this analysis we defined DCI via components of altered blood flow in the presence of neurological deterioration. Although the definition is similar to the most recently defined criteria for DCI it does use TCD information in addition to cerebral blood flow and CT information. Not all patients in this analysis had angiographic data available, as it is performed as a result of clinical changes rather than research protocols; therefore the correlation with angiographic vasospasm is on a subset of this patient sample. Furthermore determining a clinical change on a patient with poor neurological condition from the onset of injury limits the use of neurological assessment used to determine whether the patient has symptomatic ischemia.

While there were 99 patients in the plasma analysis portion of this study only a subset (n=36) of patients with CSF were analyzed. Future studies should consider more subjects as well as more data time points. Additional samples would provide more robust trajectory analysis and with additional sample time points, estrogen levels could be analyzed by time to ischemic complications using survival analysis and CSF findings. It would also allow the addition of analytical models to explore changes in multiple metabolites measurements after aSAH in relationship to ischemic outcomes. Using more complex modeling techniques may help to promote understanding of hormone relationships to injury as well as outcomes. In the meantime,

correlations between CSF and plasma levels should be evaluated. Other considerations include survival analysis to evaluate if E1 and/or E2 concentrations are related to the time of DCI. Further, metabolite concentrations could be analyzed using ROC curves to potentially determine a threshold when E1 and/or E2 concentrations are predictive of ischemic outcomes after aSAH.

Future studies should also analyze acute and long term outcomes including mortality, functional disabilities and neuropsychological outcomes to determine if higher estrogen levels also correspond with more long term consequences. F inally, the exploration of estrogen receptors ERα and ERβ should be completed since availability of these receptors has been linked to the presence and the influence of estrogen in the central nervous system.

3.0 RESULTS MANUSCRIPT #1

3.1 ABSTRACT

Background and Purpose: Delayed cerebral ischemia (DCI) causes significant morbidity and mortality following aneurysmal subarachnoid hemorrhage (aSAH). Although the precise mechanisms are not known, recent evidence has suggested that biochemical mediators alter cerebral perfusion and are a primary mechanism for neurological decline. Estrogens (estrone – E1 and estradiol – E2) are mediators that have demonstrated neuroprotective properties that could play a role in DCI. There is a dearth of literature evaluating the impact of E1 or E2 on outcomes in humans following aSAH. The purpose of this study is to examine the association between E1 and E2 levels and DCI following aSAH.

Methods: Plasma samples were collected after hemorrhage on 99 acute, adult aSAH patients admitted to the Neurovascular ICU enrolled in a NIH funded study (RO1NR004339). Three plasma samples were selected for E1 and E2 analysis from each patient representing early (1–4), middle (4-6) and late (7-10) days after hemorrhage and were assayed using liquid chromatography-tandem mass spectrometry. DCI was operationalized as radiographic/ultrasonic evidence of impaired cerebral blood flow accompanied by neurological deterioration. Statistical analysis included detailed descriptive, group based trajectory and multiple logistic regression using SAS v9.2.

Results and Conclusions: Group based trajectory identified 2 distinct populations over time for both E1 (61.4% E1 high and 38.6% E1 low) and E2 (48% E2 high and 52% E2 low) values using censored normal model. Weighted Chi Square analysis identified differences between trajectory groups by gender (p=.02), menopause (.05), age (p<.001) and fisher grade (p=.008) with patients in the high E1 group having higher severity of injury than those in the low E1group. Likewise, patients with higher HH (E1 p=.01, E2 p=.02) and Fisher (E1 p=.008, E2 p= .08) were more likely to have higher plasma estrogen levels. The presence of DCI was also significantly associated with higher levels of plasma E1 (p=.002) and E2 (.03) as well as the high E1 trajectory group (p=.09). These results provide the first clinical evidence that E1 and E2 concentrations are associated with severity of injury and DCI and provide incentive for future studies to clarify the potential role of estrogen in ischemic complications of patients with aSAH.

3.2 INTRODUCTION

Aneurysmal subarachnoid hemorrhage (aSAH) is a sudden and devastating neurological event affecting approximately 10 in 100,000 persons annually and resulting in significant morbidity and mortality (Cahill & Zhang, 2009). Despite advances in medical and surgical management, aSAH continues to be associated with complications which necessitate vigilant monitoring and emergent interventions during the acute recovery period (Cahill & Zhang, 2009; Frontera et al., 2009; Ostrowski, et al., 2006; Sehba et al., 2011; Zubkov & Rabinstein, 2009). Following hospital discharge, many patients experience long-term functional and cognitive impairment that

impacts their ability to resume previously held familial, social and occupational roles (Vergouwen et al., 2009).

Unstable intracranial physiology and systemic medical complications are common after aSAH and have a direct impact on outcomes (Rose, 2011). Cerebral edema and hemorrhage may occur but do not adequately predict the risk of neurological decline and poor outcomes after aSAH (Cahill & Zhang, 2009; Frontera et al., 2009) Similarly, cerebral vasospasm that causes vessel narrowing can result in regional reduction of cerebral perfusion and has been a primary focus of research and treatment after aSAH (Cahill & Zhang, 2009; Frontera et al., 2009; Keyroux & Diringer, 2007; Ostrowski, et al., 2006; Rose, 2011; Sehba et al., 2011; Vergouwen et al., 2009; Zubkov & Rabinstein, 2009). The diagnosis of cerebral vasospasm is often based on visualization of the macrovascular circulation using cerebral angiography which has been inconsistently associated with neurologic decline complications and/or long term outcomes (Cahill & Zhang, 2009; Frontera et al., 2009; Ostrowski, et al., 2006; Sehba et al., 2011; Vergouwen et al., 2009; Zubkov & Rabinstein, 2009).

More recently, delayed cerebral ischemia (DCI) has been implicated as a leading cause of poor outcomes following aSAH (Vergouwen et al., 2009). DCI, which occurs in approximately 20-60% of patients who survive the initial hemorrhage (Cahill & Zhang, 2009; Frontera et al., 2009; Keyroux & Diringer, 2007; Ostrowski, et al., 2006; Rose, 2011; Sehba et al., 2011; Vergouwen et al., 2009; Zubkov & Rabinstein, 2009), is thought to be the result of a mismatch between available cerebral blood flow (CBF) and metabolic demands of brain tissue, a consequence that leads to neuronal changes and neurological decline (Crago et al., 2011; Dupont et al., 2009; Kolias et al., 2009; Macdonald et al., 2008; Vergouwen et al., 2011a). In order to provide early intervention and potentially reverse ischemia, it is important to promptly recognize

and treat DCI. However, clear clinical indicators of DCI have not been identified. While associations between vasospasm, cerebral infarction, DCI, and poor outcomes have been reported, each may occur independently. Of concern, reduced incidence of angiographic vasospasm has not significantly altered the development of ischemic complications or improved outcomes (Cahill & Zhang, 2009; Crago et al., 2011; Dupont et al., 2009; Frontera et al., 2009; Kolias et al., 2009; Macdonald et al., 2008; Ostrowski, et al., 2006; Sehba et al., 2011; Vergouwen et al., 2011a; Vergouwen et al., 2009; Zubkov & Rabinstein, 2009).

More recently, microvascular dysfunction or changes are theorized to be involved in the pathogenesis of DCI (Vergouwen et al., 2009). The origin of these microvascular alterations may result from molecular and cellular changes that alter vascular dynamics and cerebral perfusion. Therefore shifts in vasoactive metabolite levels could provide markers of ischemic complications after neurologic insults (Ostrowski et al., 2006; Sehba et al., 2011; Crago et al., 2011; Dupont et al., 2009; Losniecki & Zuccarello, 2008; Macdonald et al., 2008). Estrogen has been widely studied in neurological disorders including both experimental ischemic and hemorrhagic insults (Azcoitia et al., 2011; Carwile et al., 2009; Herson et al., 2009).

Estradiol (E2) alters vascular reactivity, reduces tissue damage, improves functional recovery and may stimulate repair processes in the CNS (Herson et al., 2009). The absence of E2 has been linked to vasoconstriction, impaired cellular metabolism and cellular death in animal models (Brown et al., 2009; Herson et al., 2009). In humans, increased levels of E2 have been positively correlated with improved outcomes in stroke and traumatic brain injured patients (Azcoitia et al., 2011; Brown et al., 2009; Carwile et al., 2009; Herson et al., 2009; Krause et al., 2006). Estrone (E1) is a physiologic estrogen that is the predominant estrogen remaining after menopause. In experimental studies E1 has been related to vascular reactivity and vasodilation

through nitrous oxide pathways (Gatson et al., 2011; Rauschemberger et al., 2011, Selles et al., 2005). It has also demonstrated neuroprotective properties in animal stroke, reperfusion injury and TBI studies (Gatson et al., 2011; Rauschemberger et al., 2011, Selles et al., 2005). More recently, higher estrogen levels have been associated with greater severity of injury and illness in patients and have been implicated as potential independent markers of poor outcomes in these populations (Lerchbaum et al., 2011; Kauffman et al., 2011; Wagner et al., 2011). No study has been found to date that explores whether changes in estrogen levels are related to reduced ischemic complications and improved outcomes in patients with aSAH. The purpose of this study was to examine patterns in plasma E1 and E2 concentrations and to determine the relationship between plasma E1 and E2 concentrations and presence or absence of DCI in the 10 days following aSAH with the hypothesis that higher estrogen levels would result in lower incidence of delayed cerebral ischemia.

3.3 METHODS

3.3.1 Patient Sample

This study was a secondary analysis of data from adult patients with acute aSAH admitted to the neurovascular intensive care unit at the University of Pittsburgh Medical Center (UPMC) Presbyterian Hospital who were enrolled in a NIH-funded study (R01NR004339) exploring the relationship between biomarkers and complications of aSAH. Inclusion criteria for the parent

study were: 1) 21-75 years; 2) verified aneurysmal SAH; and 3) Fisher grade > 1 (determined by evidence of blood on CT scan) and/or Hunt and Hess (HH) >2. Exclusion criteria were: 1) non aneurysmal SAH; 2) mycotic aneurysm or 3) history of preexisting debilitating neurologic disease. In addition, eligibility for this analysis included the availability of at least 3 plasma samples. The sample was stratified to include women (both pre- and postmenopausal) and men without statistical differences in severity of injury or incidence of DCI. Females on oral contraceptives or estrogen replacement were excluded from the analysis. The parent study was approved by the Institutional Review Board and informed consent was obtained from the patient or proxy prior to data collection.

3.3.2 Data Collection

Socio-demographic data including gender, age, race, severity of injury (Fisher Grade and Hunt & Hess (HH) Score) as well as clinical data were collected from the medical record.

3.3.3 Estrogen Measurement

In order to capture changes in circulating estrogen levels, 3 plasma samples per subject were analyzed for E1 and E2 concentrations including the initial (first) sample, a middle sample (day 5 to 7) and at days 10 after hemorrhage. Samples were collected each morning (8am +/- 1 hour) and evening (7pm +/- 1 hour) from consent through 10 days after initial hemorrhage, centrifuged

for 5 minutes at 2500rpm and aliquoted into cryovials within 60 minutes of collection. They were stored at -80°C until analysis.

E1 and E2 were assayed at the University of Pittsburgh Small Molecule Biomarker Core, using liquid chromatography-tandem mass spectrometry (LC-MS/MS). T he UPLC-MS/MS method employs liquid-liquid extraction, derivatization and detection with a t riple quad mass spectrometer. Plasma is spiked with with n-butyl chloride, derivatized with dansyl chloride and transferred to autosampler glass vials for injection. E2 is eluted from a Waters Acquity UPLC BEH C18, 1.7 um, 2.1x150 mm reversed-phase column, with an acetonitrile: water (0.1% formic acid) gradient. Detection and quantitation are achieved in the positive mode with a Thermo Fisher TSQ Quantum Ultra mass spectrometer interfaced via an electrospray ionization (ESI) probe with the Waters UPLC Acquity solvent delivery system. Transitions used for analysis are 506 --> 171 for estradiol, 504 → 171 for estrone, and 511 --> 171 for the deuterated internal standard. The calibration curves, obtained from extracting known concentrations of E1 and E2 from 2-hydroxypropyl-beta-cyclodextrin (0.2% in water), range from 0.5 pg/ml (lower limit of quantitation) to 200 pg/ ml. The limit of detection is 0.5 pg/ml. All back calculations of calibrators, inter-day and intra-day precision and accuracy, and stability are within acceptable limits (<15% at all tested concentrations) (Nelson et al., 2004).

3.3.4 DCI

DCI was defined as the evidence of impaired CBF accompanied by neurological deterioration (simultaneously or within 12 hours pre or post determination of impaired CBF). Neurological

deterioration was defined by the presence of any of the following: at least a 2 point increase in National Institute of Health Stroke Scale (NIHSS) and or decline in Glasgow coma score (GCS); deterioration in pupil reaction; nursing documentation of a focal deficit (visible weakness or difficulty speaking); or nursing documentation of a decline in level of consciousness (lethargy, agitation, confusion) not associated with fever or medication administration (such as sedatives, paralytics or anti-anxiety medications). Cerebral blood flow was assessed by cerebral angiography, transcranial Doppler (TCD) and CT or MR perfusion scans. Angiographic vasospasm was determined from cerebral angiograms read and coded by neurosurgeons blinded to participant identity and dichotomized as either 'negative' (0-24% narrowing of cerebral blood vessels) or 'positive' ($\geq$25% narrowing of cerebral blood vessels). Daily TCDs were coded as abnormal flows when there was a systolic middle cerebral artery velocity > 200 ml/sec and/or a Lindegaard ratio > 3.0. Finally, head CT/MR and head CT/MR perfusion scans were reviewed for the presence of ischemia, infarction or low blood flow in the absence of hydrocephalus or rebleed by study personnel. All patients received standard therapy for the study institution for SAH patients including strict blood pressure and central venous pressure parameters, nimodipine and triple H therapy.

3.3.5 Data Analysis

Plasma E1 and E2 measurements were analyzed in two ways. Both E1 and E2 data were not normally distributed; therefore data transformation (natural logarithmic) was applied. Since few metabolite measurements were not quantifiable or below limits of detection [E1-1 sample (<1%),

E2-12 samples (4%)], these measurements were assigned a number at the limit of detection First, group-based trajectory analysis (Nagin & Odgers, 2010) was used to identify the distinct short-term patterns (homogeneous latent trajectory classes) of E1 and E2 from day 1 to day 10 after initial hemorrhage. Group based trajectory also controls for missing data and inconsistent data points. Preliminary analyses were conducted to test models from one to 4 groups. The number of groups for each hormone were then tested and compared based on model selection such as statistical significance, entropy, the Bayesian Information Criterion (BIC), and the substantive utility of the classes (e.g. distinctiveness of the trajectories, proportion assigned to a given class) along with clinical judgment to determine the optimal solution for the number of trajectory groups. Using SAS 9.2 weighted and non-weighted chi-square test and/or Fisher exact test were used to explore the association between demographic and outcome variables and the different trajectory classes for both plasma E1 and E2 in the first 10 days after SAH.

Second, using log transformed E1 and E2 measurements, generalized estimating equation models (GENMOD, SAS v9.2) were conducted to evaluate the relationship between E1 and E2 measurement and DCI while controlling for possible covariates such as demographic characteristics (age, gender, menopausal status and severity of hemorrhage).

3.4 RESULTS

3.4.1 Patient Characteristics

The sample consisted of 99 aSAH subjects of whom 28 were pre-menopausal females, 34 were post-menopausal females and 37 were men (Table 3). Subjects in this analysis were predominately Caucasian (87%, n=86) with a mean age of 50.4 years (SD=10.1). Age was not stignficantly different between males and females with the exception of pre and postmenopausal female groups. Severity of injury by Hunt and Hess ranged from 1-5 with 46% (n=45) graded as HH 3, while the average fisher score was 3 (n=45, 46%). Of the patients with DCI (46%; n=45), a combination of vasospasm demonstrated by cerebral angiography and/or TCD along with neurological deterioration was present in 38 patients. The presence of new cerebral ischemia or infarct along with neurological decline was used in the other 7 patients with DCI. DCI could not be determined on 3 patients (3%) due to poor neurological condition which precluded the ability to derive a neurologic deterioration. They were excluded from any analysis involving DCI. (Table 3)

3.4.2 Plasma Estrogen Levels Following aSAH

E1 and E2 measurements were arranged by day (1-10) and values were log transformed for analysis. Group based trajectory analysis (SAS v9.2) was used to identify 2 distinct populations over time for both E1 and E2 values using censored normal model. There was an estimated

61.4% (n=61) patients in the E1 high and 38.6% (n=38) in the E1 low trajectories, with a significant declining pattern in both groups (p<.001; mean low = 3.9, SD=.69; mean high= 5.12, SD=.66) (Figure 3). Weighted chi square analysis revealed that compared with patients in the low E1 group, patients in the high E1 group were significantly more likely to be female (p=.02), post-menopausal (.05), older (p<.001) and have a higher severity of injury as evidenced by Fisher grade (p=.008). No differences were noted between groups by race or HH. There was an estimated 48% (n=46) patients in the E2 high and 52% (n=53) in the E2 low trajectories with both groups displaying a significant declining pattern (p=.01) (Figure 4). Using weighted chi square analysis patients in the high E2 trajectory group were significantly younger than those in the low group (p=.02) and were more likely to have a higher Fisher grade although not statistically significant (p=.09). No differences were noted between E2 groups by gender, race, menopausal status or HH (Table 4).

Using log transformed E1 and E2 measurements multiple logistic regression models (GENMOD, SAS v9.2) were conducted to evaluate the relationship between E1 and E2 and key demographic variables. Pre-menopausal aSAH patients had higher mean E1 and E2 levels than both post-menopausal females and males however these were not statistically significant (Table 5). Metabolite measurements were comparable to other studies using HPLC measurements of E1 and E2 in human subjects (Haring et al., 2011; Masi et al., 2009; Naessen et al., 2010; Rothman et al., 2011; Xu et al., 2007). There was a significant association between plasma E1 and E2 levels (P<.001). Younger patients had significantly higher levels of E1 (p<.001) but not E2 (p=.27) (Table 5).

Plasma E1 and E2 were associated with severity of injury. Patients with higher HH (HH1, HH2 and HH3 versus HH 5) and Fisher were significantly more likely to have higher plasma E1

levels. Similarly, patients with higher HH and Fisher scores were more likely to have higher plasma E2 levels between HH groups (1&2 vs 3-5) and Fisher 3 versus Fisher 4 (Table 5).

3.4.3 Plasma Estrogen Levels and Ischemic Outcomes

DCI was associated with E1 trajectory groups. Patients in the high E1 group had a trend toward a marginally significant relationship with the presence of DCI (p=.09), and patients with DCI specifically from infarction were significantly more likely to be in the high E1 trajectory group (p=.03). No differences were noted between groups by individual blood flow measurements such as vasospasm, ischemia or infarction (Table 3). No differences were noted between E2 trajectory groups and DCI or individual blood flow measurements (Table 4).

Using log transformed E1 and E2 measurements multiple logistic regression models (GENMOD, SAS v9.2) were conducted to evaluate the relationship between E1 and E2 and ischemic outcomes. The presence of DCI was significantly associated with higher levels of both plasma E1 (p=.002) and E2 (.03). Patients with DCI had a mean E1=140.4pg/ml (SD=145.2) and mean E2 = 8.7pg/ml (SD 19.3) when compared to patients without DCI with a mean E1 = 91.2 pg/ml (SD=89.5) and mean E2 = 12.9 pg/ml (SD 13.5). There was also a significant association between DCI from cerebral infarction and both plasma E1 (p<.001) and E2 (p=.05). There was no significant interaction effect between HH and DCI or age and DCI. No associations were identified between estrogen levels and individual measurements of cerebral blood flow including vasospasm, ischemia, infarction or stroke (Table 5).

Using all variables with a maximum significance of p=.10 from the univariate analyses, generalized estimating equation (GEE) models were created to predict E1 and E2. Log plasma E1 was significantly associated with DCI (p=.02) and age (p<.001) when controlling for age, gender, HH and DCI. In a model with age, gender, Fisher and DCI, age (p<.001), Fisher (p=.05) and DCI (p=.003) were significantly associated with E1levels. Similarly, (log) E2 was analyzed in a model of age, HH and DCI with DCI (p=.05) and age (p=.08) having an association with E2 levels. Using Fisher instead of HH in the model, DCI was significantly associated with E2 levels (p=.02).

3.5 DISCUSSION

This is the first known clinical evidence of plasma E1 and E2 concentrations in a large cohort of aSAH patients. In this study plasma estrogen levels were associated with severity of injury and DCI after aSAH. Using trajectory analysis, patterns were noted between higher levels of E1and higher Fisher scores. Using regression analysis, both higher E1 and E2 concentrations were significantly associated with higher HH and Fisher scores. Higher estrogen levels were also associated with delayed cerebral ischemia. Higher E1 trajectory groups and well as higher E1 and E2 levels were significantly associated with this ischemic complication.

The correlation between higher levels of estrogen and greater severity of injury are consistent with the most current literature related to estrogens and critical illness in humans. High estrogen levels, primarily E2, have been associated with greater severity of injury and poor

outcomes after traumatic brain injury (May et al., 2008; Wagner et al., 2011); sepsis, critical illness and post op coronary artery bypass patients (Lerchbaum et al., 2011; Kauffman et al., 2011; May et al., 2008). Increased hormone levels were also noted independent of age or gender (May et al., 2008). In many cases, higher estrogen levels were associated with higher markers of stress response such as cortisol, interleukins and c-reactive protein (Kauffman et al., 2011; May et al., 2008; Spratt et al., 2006; Wagner et al., 2011). In these settings, stimulation of inflammatory cytokines has been a co ntributing factor to increased estrogen levels through increased peripheral aromatization (Kauffman et al., 2011; May et al., 2008; Spratt et al., 2006; Wagner et al., 2011). Following aSAH, inflammatory mediators released in response to aneurysm rupture and the coinciding blood in the arachnoid space (Sehba et al., 2012; Frontera et al., 2012) could result in a subsequent increase in hormone production. Estrogen may be a protective response to a more severe injury which may be the reason that E1 and E2 levels are associated with a common pathologic mechanism such as inflammation that is also indicative of worse injury further compounding the elevation of E1 and E2 levels.

Animal literature has provided considerable support that estrogen possesses neuroprotective properties. In pre-clinical studies, increased estrogen levels have been associated with decreased ischemia and infarct after hemorrhagic, traumatic and ischemic neurological insults (Alkayed et al., 1998; Auriat et al., 2005; Lin et al., 2009; Shih et al., 2008; Sribnick et al., 2009 Yang et al., 2001). For this analysis, we hypothesized that increased serum estrogens after aSAH would result in lower incidence of delayed cerebral ischemia; however increase in serum estrogens did not translate into neuroprotection from delayed ischemic events. Other factors necessary for estrogen supported neuroprotection warrant explanation to investigate and substantiate these findings.

The means by which estrogen may provide neurological protection may be dependent on other factors which could be altered by neurological insult. Neuronal injuries that might impact other brain structures, specifically the pituitary and hypothalamus, could mitigate estradiol neuroprotection. Insulin growth factor (IGF-1) has been has been reported to attenuate the neuroprotective actions of estrogen (Azcoitia et al., 2011; Selvamani & Sohrabji, 2010). Aging and estrogen reduces the levels of circulating IGF-1 in young and middle-aged females, which along with an age-related decline in IGF-1 may be harmful for the injured brain. Stroke severity in older females has been associated with decreased IGF-1, while treatment with IGF-1 resulted in reduced infarct size and the development of compensatory post-stroke cellular events in an animal model (Azcoitia et al., 2011). Low IGF-1 has also been associated with increased morbidity and mortality in ischemic heart disease and stroke (Selvamani & Sohrabji, 2010). In a study of aSAH patients (n=30) IGF-1 concentrations were low following ictus and returned to baseline at 3 months (Bendel et al., 2010).

Estrogen receptors (ERs) are necessary for estradiol actions. ERα and ERβ, necessary for the inflammatory and neuroprotective responses, may be suppressed in such a manner that these mechanisms are diminished. ERα has been implicated in anti-inflammatory and protective mechanisms while ERβ may be more active in recovery after insult. Selective estrogen receptor modulators (SERMs) have been shown to reduce brain inflammation, neurodegeneration and promote positive cognitive outcomes in animal studies (Kipp et al., 2011; Yi et al., 2011).

Increased estrogen levels may be associated with enhanced clotting mechanisms that could lead to small vessel ischemia after aSAH. Animal studies suggest that major coagulation and inflammation disorders occur early after SAH and may contribute to cerebral ischemia and early brain injury (Kasius et al., 2010). Frontera reported that markers of platelet activation and

inflammation were consistently and significantly elevated in severely injured patients after aneurysm rupture and prior to cerebral vasospasm (Frontera et al., 2012).

There has been speculation that differences between human and animal models may provide some explanation for the divergence of results. First, animal studies use isolated neurological insult which does not result in the robust inflammatory response that occurs after critical insults (Kauffman et al., 2011; May et al., 2008; Spratt et al., 2006; Wagner et al., 2011). Second, peripheral estrogen aromatization activated by the stress response may occur to a greater extent in primates and humans versus rodent species (Simpson, 2004). Since the majority of preclinical studies have been done in rodent models, applicability of some animal findings to clinical outcomes in patients has been questioned (Kauffman et al., 2011; May et al., 2008; Spratt et al., 2006; Wagner et al., 2011).

The balance between estrogens and other neurosteroids such as progesterones and allopreganalone may play a role in ischemic outcomes (Azcoitia et al., 2011). Rapid changes in estrogens have been reported in other neurovascular events such as central venous thrombosis. Progesterone has become a target for clinical trials in traumatic brain injured patients (Espinoza & Wright, 2011). Finally, studies have noted increased Testosterone (T) levels after acute injury. This study did not analyze T levels however increased T levels in response to critical illness may be implicated in both inflammation and ischemia. In the study by Wagner et al., patients with severe TBI had lower Testosterone levels after injury which correlated with worse outcomes (Wagner et al., 2011).

As described, the results from the current study demonstrate a relationship between E1 and E2 levels with both DCI and injury severity. This relationship may be due to increases in E1 and E2 levels that result from inflammation or other pathogenic mechanisms of injury severity,

thereby, representing an adaptive response for neuroprotection. How E1 and E2 may be involved in the pathogenic response to injury are unknown and warrant further clinical investigation.

3.6 CONCLUSION

Our study provides preliminary evidence regarding the relationship between estrogen concentrations (E1 and E2) and injury severity as well as ischemic outcomes after aSAH. These findings are however contrary to experimental studies which have repeatedly reported neuroprotective effects of estradiol and estrone after neurological insult and ischemia. Estrogen may be a protective response to injury which may lead to addition pathologic mechanisms that impact outcomes. Certainly further work is necessary to better understand the role of estrogen in the brain and in the development of ischemic complications after aSAH in humans.

3.7 LIMITATIONS

This study only analyzed E1 and E2 levels after aSAH. Future studies including other neurosteroid levels (progesterone, allopreganalone and testosterone) as well as growth hormone (IGF-1) and inflammatory markers (cortisol, interleukins or CRP) could provide additional information and explanation for the findings. In addition, CSF was available for this sample.

Patients who require placement of external ventricular catheters represent a subgroup of aSAH patients who may have higher risk of complications after aSAH due to extent of injury including DCI. A third limitation is the definition of DCI. In this analysis we defined DCI via components of altered blood flow in the presence of neurological deterioration. Although the definition is similar to the most recently defined criteria for DCI it does use TCD information in addition to cerebral blood flow and CT information. Not all patients in this analysis had angiographic data available, as it is performed as a result of clinical changes rather than research protocols; therefore the correlation with angiographic vasospasm is on a subset of this patient sample. Furthermore determining a clinical change on a patient with poor neurological condition from the onset of injury limits the use of neurological assessment used to determine whether the patient has symptomatic ischemia.

Table 3. Demographics Plasma Estrogen Analysis N=99

	FEMALE		MALE
	Pre-menopausal (N=28)	Post-menopausal (N=34)	N=37
Age (Mean In Years)	43.54 (Sd=5.97)	59.1 (Sd=5.78)	47.5 (Sd=10.2)
Race			
Caucasian	22 (79%)	31 (91%)	33 (89%)
Other	6 (21%)	3 (9%)	4 (11%)
Hunt and Hess			
1	2 (7%)	2 (6%)	1 (3%)
2	9 (32%)	5 (15%)	11 (30%)
3	12 (43%)	17 (50%)	16 (43%)
4	4 (14%)	7 (21%)	9 (24%)
5	1 (4%)	3 (9%)	0 (0%)
Fisher			
2	14 (50%)	8 (24%)	14 (38%)
3	10 (36%)	16 (47%)	19 (51%)
4	4 (14%)	10 (29%)	4 (11%)
DCI	16 (57%)	15 (44%)	14 (38%)
DCI from vasospasm	14 (50%)	12 (35%)	12 (32%)
DCI From CT	2 (7%)	3 (9%)	2 (5%)
Vasospasm	12 (43%)	8 (24%)	10 (27%)
Infarct	11 (39%)	10 (29%)	13 (35%)
Ischemia	17 (61%)	18 (53%)	15 (41%
Samples			
E1 Detection	84 (100%)	102 (100%)	110 (97%)
E2 Detection	83 (99%)	96 (94%)	106 (95%)

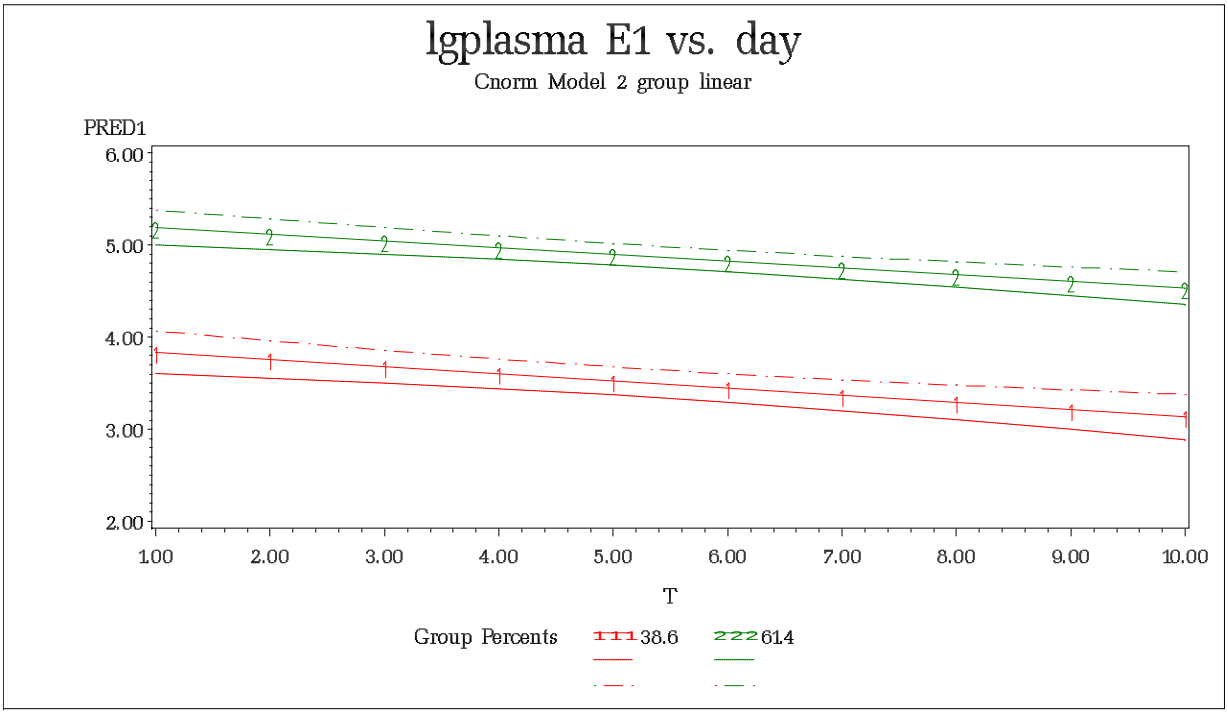


Figure 3. Plasma E1 Trajectory

Cnorm Model 2 group linear

Average probability per group

	1	2
1	0.950	0.050
2	0.034	0.966

Mixture Fit Statistics: Entropy 0.847; AIC 711.440; BIC 729.606; CAIC 736.606; ssBIC 707.499; CLC 718.390; ICL-BIC 750.556

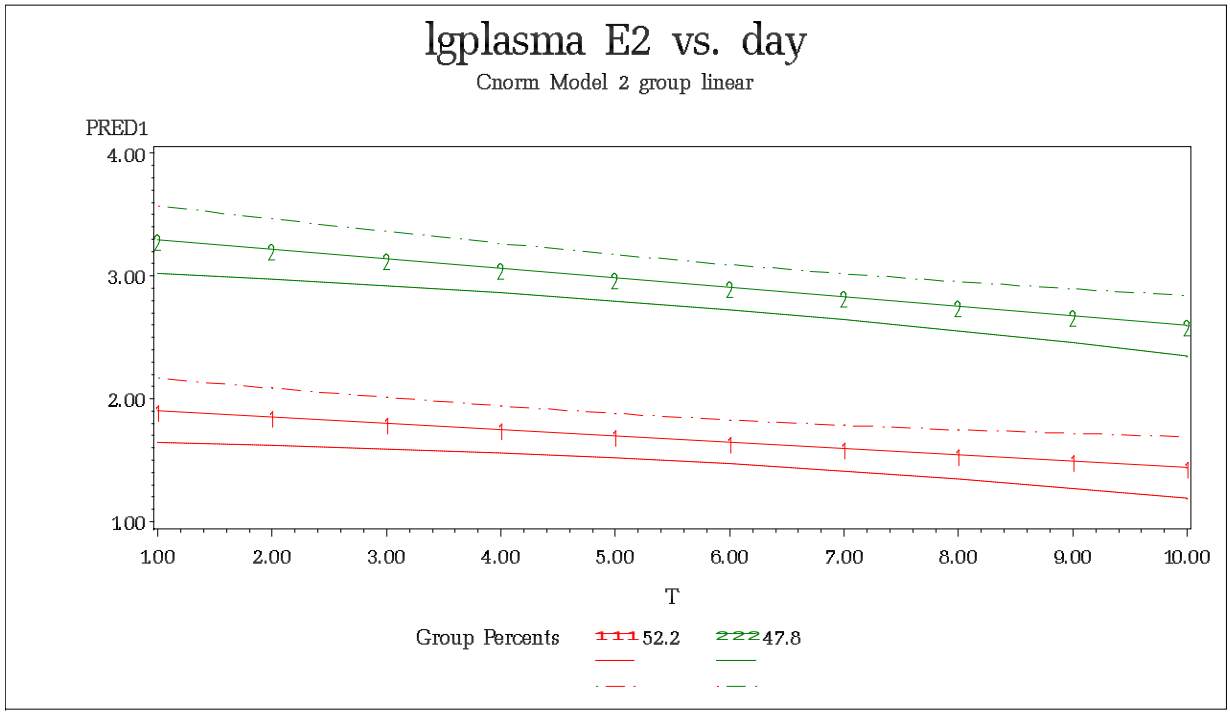


Figure 4. Plasma E2 Trajectory

Cnorm Model 2 group linear
Average probability per group

	1	2
1	0.911	0.089
2	0.073	0.927

Mixture Fit Statistics- Entropy 0.729; AIC 721.700; BIC 739.866; CAIC 746.866; ssBIC 717.759; CLC 744.842; ICL-BIC 777.008

Table 4. Trajectory analysis of (log) Plasma Estrogen measurements

	E1 group			E2 group		
	High n=61	Low n=38	*p*value	High n=46	Low n=53	*p*value
Gender			.02			.2
male	17	20		14	23	
female	44	18		32	30	
Race			.25			
Caucasian	51	35		41	45	.61
Other	10	3		5	8	
Age			<.001			.02
Hunt and Hess			.31			.86
HH 1	2	3		2	3	
HH 2	13	12		10	15	
HH 3	29	16		21	24	
HH 4	13	7		11	9	
HH 5	4	0		2	2	
Hunt and Hess 1,2	23	15	.11	12	18	.33
vs 3-5	46	15		34	35	
Fisher			.008			.09
2	24	12		17	19	
3	21	24		17	28	
4	16	2		12	6	
DCI no	14	24	.09	20	31	.13
yes	31	27		24	21	
DCI-vasospasm sources no	34	23	.78	25	32	.5
yes	24	15		20	19	
DCI-cerebral infarction no	0	27	.03	28	35	.14
yes	7	36		5	2	
Vasospasm no	24	6	.27	17	13	.79
yes	20	10		16	14	
Ischemia no	32	17	.41	25	24	.39
yes	29	21		21	29	
Infarct no	39	26	.74	29	36	.66
yes	22	12		17	17	
Menopause			.05			.32
pre-menopause	19	9		16	12	
post-menopause	25	9		16	18	
male	17	20		14	23	

Table 5. Univariate analysis of (log) Plasma Estrogen measurements adjusting for time

	E1			E2		
	Mean	Std dev	*p*value	Mean	Std dev	*p*value
Gender			.06			.71
male	4.2	.14		2.24	.14	
female	4.52	.1		2.31	.1	
Race			.66			.99
Caucasian	4.38	.09		2.28	.09	
Other	4.48	.2		2.28	.24	
Age			<.001			.31
Hunt and Hess			<.001			.02
HH 1	3.82	.34	.003	1.93	.32	.14
HH 2	4.15	.16	<.001	1.99	.19	.04
HH 3	4.39	.12	.004	2.35	.11	.55
HH 4	4.7	.17	.25	2.49	.18	.91
HH 5	4.95	.16		2.46	.16	
Hunt and Hess 1,2	4.09	.15	.014	1.99	.17	.02
vs 3-5	4.51	.1		2.4	.1	
Fisher			.13			.19
2	4.36	.14	.008	2.23	.16	.08
3	4.22	.12	<.001	2.21	.12	.02
4	4.86	.13		2.55	.11	
DCI no	4.18	.11	.004	2.11	.11	.03
yes	4.64	.13		2.47	.13	
DCI-vasospasm sources no	4.32	.11	.33	2.2	.11	.16
yes	4.49	.13		2.43	.14	
DCI-cerebral infarction no	4.35	.11	<.001	2.27	.11	.05
yes	5.16	.18		2.7	.2	
Vasospasm no	4.64	.13	.77	2.45	.14	.98
yes	4.58	.15		2.44	.14	
Ischemia no	4.46	.11	.42	2.34	.13	.52
yes	4.33	.12		2.23	.11	
Infarct no	4.36	.1	.52	2.27	.11	.8
yes	4.47	.14		2.31	.13	
Menopause			.17			.33
pre-menopause	4.45	.13		2.44	.14	
post-menopause	4.58	.14		2.18	.15	
male	4.2	.14		2.24	.14	
E1/E2						<.001

Appendix A

IRB APPROVAL

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: Paula Sherwood PHD

From: Sue Beers PHD, Vice Chair

Date: 9/19/2011

IRB#: MOD021039-03 / IRB021039

Subject: Detection of Physiologic Predictors of Complications and Outcomes Following SAH

The University of Pittsburgh Institutional Review Board reviewed and approved the requested modifications by expedited review procedure authorized under 45 CFR 46.110 and 21 CFR 56.110.

Modification Approval Date: 9/19/2011
Expiration Date: 5/9/2012

For studies being conducted in UPMC facilities, no clinical activities that are impacted by the modifications can be undertaken by investigators until they have received approval from the UPMC Fiscal Review Office.

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. The IRB Reference Manual (Chapter 3, Section 3.3) describes the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least one month prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

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

Appendix B

PARENT STUDY POLICY AND PROCEDURE MANUAL

Policy & Procedure Manual

Determining Genetic and Biomarker Predictors of DCI and Long Term Outcomes after aSAH

Principal Investigators:

Paula Sherwood, RN, PhD, CNRN
Assistant Professor
School of Nursing; School of Medicine
University of Pittsburgh
336 Victoria Bldg, 3500 Victoria Street
Pittsburgh, Pennsylvania 15260

Samuel Poloyac, PharmD, PhD
Associate Professor
School of Pharmacy
University of Pittsburgh
801 Salk Hall, 3501 Terrace St.
Pittsburgh, PA 15261

Purpose of Manual

INTRODUCTION AND INTENDED USE

This policy and procedure manual is to be used for the grant Determining Genetic Biomarker Predictors of DCI and Long Term Outcomes after aSAH. This manual includes the official policies and procedures under which the protocols of the study are evaluated. Please become familiar with the components and use it often as a reference and guide. You are responsible to abide by these policies.

TOPIC:

CONFIDENTIALITY

POLICY:

All participants will be afforded maximum confidentiality regarding their participation in the project. The health status of the patient is not to be disclosed to anyone by any project staff member as mandated by the Health Insurance Portability and Accountability Act.

PROCEDURE:

1. Any and all email exchanges concerning a specific participant **must not** include the participant's name or any other identifiable information. Participant's are to be identified only by their study ID number (e.g. 0012P).

2. When telephoning participants at home or work, do not assume that the participant has shared with any person that the care recipient has cancer. Identify yourself by name, and state "I am calling on behalf of Subarachnoid Hemorrhage Project at the University of Pittsburgh. May I speak with _____"?

3. If you need to leave a message with a person, on an answering machine or voice mail, leave the following message: "This is _____ calling on behalf of the Subarachnoid Hemorrhage Project from the University of Pittsburgh. I am trying to reach (state participant's name) and may be reached at _____ (leave your work telephone number and days and times in which you are available to receive their return call).

4. Do not telephone participants at their work site unless they have explicitly identified this as an option for contacting them.

5. Project staff members are **not** to enter into any discussions or share information regarding any participant outside of the project area.

6. Project staff members are **not** to enter into any discussions regarding any participants with anyone except the Principal Investigator.

7. Identifiable data will be shared only with those individuals who are directly involved in the project (Investigators, Recruiters, Interviewers), or when the patient has given expressed written permission for such disclosure.

TOPIC:

REQUIRED TRAININGS FOR ALL RESEARCH TEAM MEMBERS

POLICY:

All research team members must complete the following trainings prior to taking part in human subjects research.

PROCEDURE:

1. The following trainings may be accessed by going to https://cme.hs.pitt.edu in order to complete each module.
    a. Research Integrity (no recertification necessary)
    b. Human Subjects Protection B-Social and Behavioral (no recertification necessary)
    c. Conflict of Interest (no recertification necessary)
    d. HIPPAA Researchers Privacy Requirements (no recertification necessary)
    e. Bloodborne Pathogens (Annual recertification required-Staff and students must complete training "live", but annual retraining can be via the online module.)
    f. Chemical Hygiene (Recertification required every 3 years)

If sending emails regarding participants, never use identifying information. Use study ID only to identify participants. You must include the following at the end of the email…

This email and any files transmitted with it are confidential and intended solely for the use of the individual or entity to whom they are addressed. If you have received this email in error please notify the system manager. This message contains confidential information and is intended only for the individual named. If you are not the named addressee you should not disseminate, distribute or copy this e-mail. Please notify the sender immediately by e-mail if you have received this e-mail by mistake and delete this e-mail from your system. If you are not the intended recipient you are notified that disclosing, copying, distributing or taking any action in reliance on the contents of this information is strictly prohibited.

TOPIC:

SCREENING AND ENROLLMENT

POLICY:

The original consent form for each patient must be kept on file in a secure location in the project office at the University of Pittsburgh.

PROCEDURE:

Patient Recruitment and consent

a. Screen patients by checking a list of patients with SAH admitted to NICU via MARS sent to investigators and project personnel on email every morning.

b. The investigator or project director (PD) will verify the diagnosis and determine whether the patient meets inclusion and exclusion criteria for the study.

c. The inclusion criteria are: 18 - 75 years of age and
admitted to the NICU following a severe SAH as indicated using severity of SAH scales: Hunt and Hess grade $\geq$3 OR a Fisher grade $\geq$ 2

i. The exclusion criteria are:
1) any other pre-existing chronic neurologic diseases or deficits, and
2) SAH resulting from a traumatic injury or mycotic aneurysm.

i. Call Jeff Balzer's office if the patient is eligible, and let them know there is a possibility that there may be a patient

a. The patient (family member or legal representative) who meets the study criteria will be approached by the investigators or project director to obtain the consent for participation in the study. CONSENTS ARE LOCATED:
behind the unit secretary's desk on 4F in the small drawer by the EMTEC computer. Also, there are copies in a folder marked "consents" in project director's office in the top drawer of the file cabinet. In addition, the most current version of both consents is saved on the hard drive of the SAH computer in the SAH file marked "consents".

i. If family gives consent, give the family a copy of the signed consent

ii. An attending physician who is also an investigator on this study must also sign as investigator. This is can be done later, as long as a witness has signed the consent in addition to the patient or proxy prior to hook-up.

iii. Make a copy of the consent using the fax on the unit.

iv. Place a copy of the consent on the chart and sign and date the chart

v. Place a copy of the consent in the data collection chart kept in the locked file cabinet in project director's office.

TOPIC:

SAH Study Set up Checklist

POLICY:

PROCEDURE:

_____ Obtain consent from patient or family

_____ Complete screening form

_____ Set up chart

_____ Print time from injury sheet and place in chart

_____ Set up boxes and lab forms in lab
___(If CSF) 1 large box - Bag CSF, 3 small boxes - 1-20-HETE, 1-EET and 1 - ET1
___3 small boxes for plasma samples - 1-20-HETE, 1-EET and 1-ET1

_____ Get labs
___1 green top tube (serum for studies) see lab procedure
___4 purple top tubes - 3 send to genetics lab at SON, 1 serum studies (see lab procedure)

_____ Enter patient in TCD machine and do first TCD

_____ Document TCD's and first check on bedside chart

_____ Complete ED form

_____ Complete medical history form

_____ Complete any information on Co-existing form

_____ Make copy of consent for patient and/or family

_____ Mark patient on board in lab and update "next up" ID number

TOPIC:

DAILY BEDSIDE INSTRUCTIONS

POLICY:

PROCEDURE:

1. Check with RN prior to beginning
2. Introduce yourself to patient
3. Collect blood samples (see blood collection procedure). Store immediately
4. Collect CSF sample (see collection procedure). Store immediately
5. Collect TCD (am only)
6. To complete forms in folder:

a. Flip to the TFI printout to figure out the number of hours from insult.

b. Fill in the velocities for each vessel in the appropriate boxes of the "Whole TCD" form.

b. Place the TCD printouts in the bedside chart.

c. Write any comments about the check on the daily notes form. Please be sure to note the following:

1. Document any testing the patient has gone to since last check.
2. Document any clinical complications that have occurred since the last check on the "co-existing medical condition form"
3. Any sedatives or paralytics.
5. Any abnormal symptoms or neurological changes as noted by you or the bedside nurse.
6. Any difficulty with the check.
7. Anything special you had to do, tips for the next check.

7. Check with the RN if he or she has been charting stroke scale in EMTEK

10. Document the NIHSS in the front sheet in the blue unit folder
11. Verify in the EMTEC system that these are entered and being documented.

8. Put all unnecessary equipment away.
9. Check with RN for any questions.
10. Inform RN of when next visit will be.

TOPIC:

Blood Collection

POLICY:

Blood will be collected twice daily (morning and evening).

PROCEDURE:

1. Get supplies for lab draw
    a. Get tubes needed for blood collection
        i. 1 green top for HETE and EETs
        ii. 1 purple top for ET-1
        iii. 3 purple for genetics (first draw only)
    b. Get syringes and needles necessary to draw labs

2. Draw blood from patient
    a. Arterial line
        i. You will need a needless vacutainer and the specimen tubes.
    b. Central line
        i. You will need alcohol preps, a syringe and needle for waste, syringes and needles for the specimen draw and a NSS flush
    c. Peripheral IV
        i. You will need a tourniquet, alcohol prep, at least a 3cc syringe with needle for waste, syringes and needles for the specimen draw and a NSS flush
    d. Peripheral stick
        i. You will need a vacutainer and vacutainer needle, a tourniquet, alcohol prep, 2X2, tape and lab draw tubes.

3. Bedside nurse
    a. Ask nurse to draw necessary blood sample – 7cc for each green tube, 4cc for each purple top tube. Give the nurse any needed syringes to draw the specimen with.
        i. Place blood sample into tubes
        ii. Place green and purple tops on ice and take to lab for processing – see lab-processing procedure

TOPIC:
Blood Processing- 20-HETE, EET and ET-1 studies

POLICY:
Process plasma for 20-HETE, EET and ET-1 studies

PROCEDURE:

1. Obtain 1 green top and 1 purple top tube and fill with blood (about 10ml) from patient.
2. Mark with ID and time
3. Place on ice
4. Spin tubes at 2500 rpm for 5 minutes in centrifuge. (Be sure that tubes are balanced in the centrifuge).
5. While tubes are spinning, prepare tubes for plasma to be aliquot into. Label with ID number, date and time.
   2cc for 20-HETE
   2cc for EET
   2cc tube from purple tube for ET
6. Remove tubes from centrifuge. Open vacuum tubes carefully and remove plasma with a transfer pipette.
7. Place in labeled plasma tubes.
   1.5cc from green tube in 20-HETE tube (fill to 2cc if enough)
   1.5cc from green tube in EET tube
   1.5cc from purple tube in ET tube
   *Do this for all tubes – be sure to use a fresh pipette for each patient to avoid cross contamination.
8. Dispose of all contaminated pipettes and lab tube in biohazardous trash.
9. Cap plasma tubes and place in marked boxes in –20 freezer.
   *Be sure that the correct specimens go in the correct boxes. Remember: there will be more than one box for each patient.

TOPIC:
Blood Processing-genetics

POLICY:
Process plasma for genetics

PROCEDURE:

1. Login specimens
2. Spin tubes at 2500 rpm for 5 minutes in centrifuge. (Be sure that tubes are balanced in the centrifuge).
3. While tubes are spinning, prepare tubes for plasma to be aliquot into. Label with ID number, date and time.
   Plasma 2cc tube (for UPMC lab),
   1cc tube for (Sam P. lab) and a
   1-2cc tube for any remaining plasma for our study lab.
4. Prepare solution for genetics – for each specimen: 50ml of ammonium chloride + 5ml ammonium bicarbonate. These are found in the refrigerator. You can make a large quantity of solution based on how many samples you have for example: 3 specimens you would need 150ml ammonium chloride and 15ml ammonium bicarb. Place all this in a large beaker.
5. Pour about 45ml into a 50ml conical tube that is labeled with the ID number assigned to the patient. Excess solution can be disposed of down the sink with plenty of water.
6. Remove tubes from centrifuge. Open vacuum tubes carefully and remove plasma with a transfer pipette.
7. Place in labeled plasma tubes. Do this for all tubes – be sure to use a fresh pipette for each patient to avoid cross contamination.
8. Remove buffy coat with out taking too many red cells. Make sure to get the entire buffy coat. Place this in the 50ml conical tube with lysis solution. If you should happen to overflow the 50ml tube, carefully split into 2 tubes – each marked with the ID number. Do this for each patient lab tube.
9. Place conical tube aside for 20 minutes.
10. Dispose of all contaminated pipettes in biohazardous trash and recap lab tube and place in biohazardous trash.
11. Cap plasma tubes and place in marked boxes in –20 freezer. Be sure that the correct specimens go in the correct boxes. Remember there will be more than one box for each patient.
12. After 20 minutes, place the 50ml conical tubes into the centrifuge and spin for 20 minutes at 2500 rpms. (be sure that the centrifuge is balanced)
13. Remove tubes from centrifuge and take to sink. With water running off to the side, decant supernatant into the sink without losing the pellet. If the pellet starts to move, then leave a bit of liquid in the tube. If the pellet doesn't move, then drain on paper towel next to the sink. Place towel in biohazardous waste and clean counter with 10% Clorox solution.
14. Resuspend pellets in 1ml of freezing solution and place in –20 freezer.

TOPIC:
CSF Collection

POLICY:
Fresh and bagged CSF will be collected twice daily (morning and evening).

PROCEDURE:

FRESH CSF

7. Fresh cerebrospinal fluid will be collected every 12 hours (while available) for analysis
   - A. Gather Equipment
     1. Clean non-sterile gloves
     2. Sterile Gloves
     3. Mask
     4. Sterile Towel
     5. 1-pkg 2x2
     6. 3cc syringe
     7. 23g needle (small sub Q needle)
     8. Betadine Solution (not 2x2 swab)
   - B. Wash Hands
   - C. Put on clean gloves, scrub CSF access port for 3 minutes with 2x2 gauze SOAKED in betadine solution allow solution to dry
   - D. Wash Hands
   - E. Prepare syringe and sterile gloves for access
   - F. Put on mask and sterile gloves
   - G. Twist on syringe to access port and SLOWLY withdraw 3cc of CSF
   - H. Wash Hands
   - I. Place fresh CSF on ice until aliquoted and placed in freezer

BAG CSF

1. The Cerebrospinal fluid drained into the drainage bag will be collected every 12 hours (while available) for analysis.
2. For SAH studies you will need approximately 30cc of CSF (if available) which will fill 9-3cc tubes. (NOTE: if the patient has small amount of CSF. Aliquot available csf into 1cc tubes)
3. The following procedure taken from UPMC policy and procedure manual will be used for changing the bag from an EVD or LD
   11. Gather equipments:
       10. Sterile gloves
       11. Mask
       12. Sterile towel
       13. New bag
       14. 1-pkg 4x4
       15. Betadine solution
   11. Prepare 4x4 soaked with betadine solution & open new bag
   12. Close clam/stopcock most proximal to the catheter
   13. Close the clamp most distal to the catheter (just before the drainage bag)
   14. Place disconnection site on sterile towel
   15. Put on sterile gloves and mask
   16. Scrub disconnection site with betadine for two minutes, allow to dry
   17. Disconnect old bag and replace with new
   18. Open all closed clamps

Aliquot CSF in the following manner:

- A. Gather Equipment
  1. Clean non-sterile gloves
  2. 30cc syringe

3. 9-3cc tubes
4. Blue tube rack
5. Labels

B. Put on clean gloves
C. Gently agitate the CSF bag and pull 30cc from the bag with a syringe
D. Discard unused CSF/bag in red garbage
E. Place tubes in the blue tube rack 9-3ccml tubes for bag CSF and 1-2cc and 3 1cc for fresh CSF.
F. Fill tubes with CSF - DO NOT OVERFILL and place lids on
G. NOTE - if the patient has only small amounts of CSF - aliquot CSF into 1ml tube
H. Label tubes with patient ID, Date, time and specimen CSF for bag CSF and CSF-F for fresh CSF
I. Place in appropriate boxes in the -20C freezer

- Boxes are kept in the cupboard below the -20 □ C freezer
- Exterior of box should be labeled with 'SAH,' and study number.
- The first 1ml specimen for each patient should be placed into the small specimen box labeled "SAH Genotyping" kept on the bottom shelf of the -20□C freezer in the PUH lab.
- Specimens should be entered in the box in the following manner:
    1. The first specimen of the box should go into any corner
    2. Specimens should then be entered from left to right, back to front.
    3. Large (3 ml) vials should be deposited first, then small (1 ml) vials (if any) for each time point.
    4. Once box fills, mark it 'box 1,' and begin a second box marked 'box 2.'

- Blue tube rack should be:
    1. Rinsed with 'DNA OFF' (kept beside and under the sink in the 4th floor PUH study lab)
    2. Soaked for a minimum of 20 minutes
    3. Rinsed with water
    4. Dried with paper towels

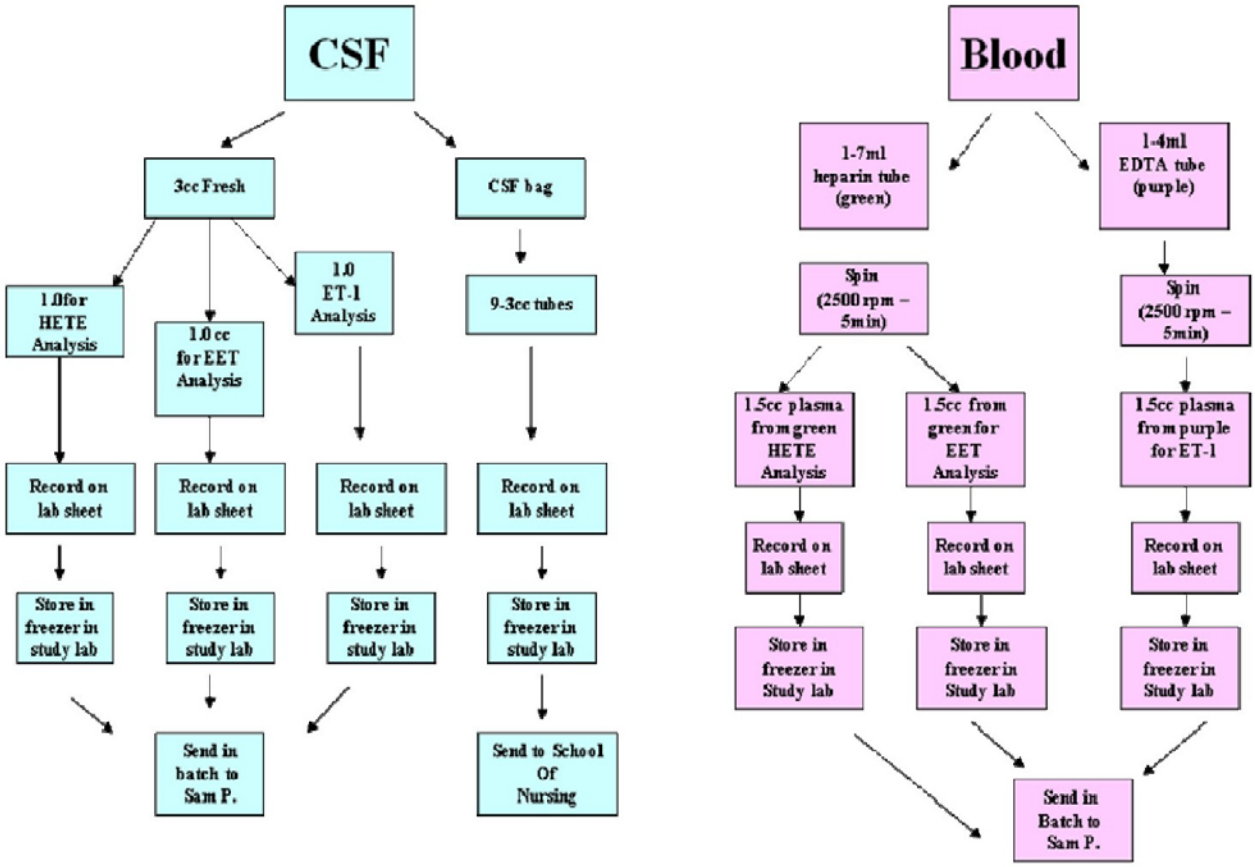
- Work area should be cleaned with diluted bleach solution

- Upon D/C from study,
    1. Specimen boxes should be transferred to the -80 □ C freezer (labeled SAH) on the 2nd floor Victoria bldg lab. Keys to this lab are kept on the 'research team key ring'.
    2. IDENT, date, time, and number of specimens should be taken from the specimen documentation sheet and entered into specimen tracking database

TOPIC:
CSF/Blood Collection Flowchart

POLICY:
CSF and Blood will be collected and sorted by the following flowchart.

PROCEDURE:



•First draw blood– draw (3) purple tubes for genetics – label and send to Dr. Couley's genetics lab

•First draw csf-1cc for genetics – place in SAH first sample box

TOPIC:
Quality Assurance

POLICY:
Quality Assurance will be monitored periodically to ensure accuracy of data collection procedures.

PROCEDURE:

1. Purpose: To evaluate accuracy of clinical specimen collection and documentation
   Target: Review five timepoints per subject; five subject charts per quarter; 20 subject charts per year
   Goal: Less than 5% erroneous/missing data
   a. Blood
      a. Verify time/date on data collection sheet matches time/date on specimen
      b. Verify ID number on box matches ID number on sample
   b. CSF
      a. Verify time/date on data collection sheet matches time/date on specimen
      b. Verify ID number on box matches number on sample

2. Purpose: To evaluate completeness of signatures on consent forms
   Target: Review five subject charts per quarter (Charts must be at least 6 months past consent date); 20 subject charts per year
   Goal: Less than 5% erroneous/missing data
   a. Review and verify consent is present and in correct chart
   b. Review and verify signature at the time of consent (representative or subject)
   c. Review and verify signature of subject if representative gave original consent
   d. Review and verify date & time with signature
   e. Review and verify physician cosigned consent

3. Purpose: To evaluate completeness of inpatient subject chart and bedside chart
   Target: Review five timepoints per subject; five subject charts per quarter; 20 subject charts per year
   Goal: Less than 5% erroneous/missing data
   a. Verify copy of consent form or consent note/sticker is in the progress section of subjects inpatient chart
   b. Evaluate whether standing orders for SAH were used
   c. If standing orders were not used, verify pregnancy test (if applicable) was ordered
   d. Verify NIHSS documentation (at least every 24hrs)
   e. Verify that daily transcranial doppler was obtained
   f. Evaluate completeness of TCD teleform
   g. Evaluate TCDs were printed out
   h. Evaluate accuracy between date/time of TCD teleform and TCD printout

4. Purpose: To monitor nosocomial infection related to CSF sampling
   Goal: Five people per quarter of a year; 20 people per year
   a. Verify CSF culture was obtained
   b. Document organism and verify date and time of positive culture
   c. Document research staff involved in specimen collection at the time of the positive culture

Appendix C

PARENT STUDY CONSENT

University of Pittsburgh

School of Nursing
Acute and Tertiary Care Department
336 Victoria Bldg, 3500 Victoria St
Pittsburgh, PA 15261 Phone: 412-624-4722

CONSENT TO ACT AS A SUBJECT IN A CLINICAL STUDY

TITLE: Detection of Physiologic Predictors of Complications and Outcomes Following SAH

PRINCIPAL INVESTIGATOR:
Paula Sherwood RN, PhD, Assoc Prof, Univ of Pgh, School of Nursing, Pgh, PA 15261(412)624-4802

CO-INVESTIGATORS:
Sam Poloyac,PharmD,PhD, Assoc Prof Univ.of Pgh School of Pharm, Pgh PA (412)624-4595
Michael B. Horowitz, MD, Professor, Dept. of Neurosurgery, UPMC, Pgh, PA (412)647-3685
Brian Jankowitz, MD, Asst. Prof, Dept. of Neurosurgery, UPMC, Pgh, PA (412)647-3685
Paul Gardner, MD, Asst Prof Dept Neurosurgery, UPMC, Pgh, PA (412)647-3685
Marilyn Hravnak, RN PhD, Assoc Prof, Univ of Pgh, Sch of Nursing, Pgh PA (412)624-3796
Elizabeth Crago, RN MSN, Research Assoc, Univ of Pgh, Sch of Nursing, Pgh, PA (412)352-3060
Sheila Alexander, RN PhD, Asst. Prof, Univ of Pgh, Sch of Nursing, Pgh PA (412) 624-3831
Patricia Colorito, RN Research Asst, Univ of Pgh, Sch of Nursing, Pgh, PA (412)352-3060
Khalil Yousef RN MSN Grad Stud Researcher, U of Pgh, Sch of Nursing, Pgh PA (412)352-3060
Marci Nilsen RN PhDc, Grad Stud Researcher, Univ of Pgh Sch of Nsg, Pgh,PA (412)624-3060

SOURCE OF SUPPORT: NIH

Why is this research being done?
The purpose of this research study is to identify early warning signs of decreased blood flow in your heart or brain following a subarachnoid hemorrhage (bleeding in your brain).

Who is being asked to take part in this study?
You have been invited to participate in this research study because you are between 21 and 75 years of age and have been admitted to the intensive care unit for treatment of a subarachnoid hemorrhage (bleeding into the brain), a condition that puts you at risk for low blood supply to the brain. You will be one of 564 patients who will be studied. You understand that you will be excluded from some of the studies if you are a woman who is pregnant.

What procedures will be performed for research purposes?
If you decide to take part in this research study, you will undergo the following procedures that are not part of your standard medical care.

Screening procedures: No additional procedures beyond standard medical care will be necessary to determine if you are eligible to take part in this research study. Screening based on standard medical information will be completed (including a urine or blood pregnancy test).

Experimental procedures: If you qualify to take part in this research study, you will undergo the following procedures:

1. Daily collection of body fluids for analysis. Samples of your blood, fluid in your brain [cerebrospinal fluid (or CSF)] and urine will be tested for substances that may tell us about the blood flow in your heart and brain.
   a. In subjects with a tube inserted to measure the pressure in the brain and/or to remove fluid, a minimum of one teaspoon of this fluid (CSF) that would normally be discarded, will be collected every 12 hours for this project for up to 14 days or hospital discharge, whichever comes first. This fluid will only be collected while the catheter (a thin flexible tube) is in place. If the catheter is removed, the collection of fluid will end. This fluid will be used to identify specific markers that may be related to decreased blood flow to the brain. This fluid will be collected in addition to any fluid that is collected for testing as a part of your routine medical care.
   b. A blood sample (approximately two tablespoons) will be collected each day from a catheter or tube placed in a blood vessel. If a catheter is not available, a blood sample will be obtained by inserting a needle into your arm. This blood will be used to identify specific markers that may be related to changes in blood flow to your heart and brain.
   c. Urine samples will be collected within each 24-hour period. Most subjects have a Foley catheter (a tube in the bladder) that drains urine into a bag that is normally discarded. Urine will be collected from this bag or from the bedpan/urinal you use.

2. The blood flow to your brain will be monitored each day at the bedside up to the 14th day after admission or discharge from the hospital.
   a. The first test is called a transcranial Doppler. A Doppler ultrasound test uses sound waves to evaluate blood as it flows through a blood vessel in the brain. A small amount of gel and a handheld instrument (transducer) are placed lightly on the head. The sound waves are converted to pictures and numbers that show the blood flow. This test will take approximately 20 minutes each day.

3. We will monitor your heart daily through the fifth day after your admission.
   a. This first test is a holter monitor. We will place this monitor on your chest. This monitor will continuously record your heart activity. It will be used in addition to the normal heart monitoring that is used in the intensive care unit.
   b. The second test is called an echocardiogram. An echocardiogram uses sound waves to see a picture of your heart. A small amount of gel and a handheld instrument (transducer) are placed lightly on the chest. The sound waves are converted to pictures and numbers that show the heart's function. This test will take approximately 20 minutes each day.
   c. The third test is an electrocardiogram (ECG). An electrocardiogram measures the electrical signals that control the rhythm of your heartbeat. It will be daily through the 5th day and

once on the 14th day or prior to your discharge from the hospital. It will take approximately 5 minutes.

4. We will monitor you up to 14 days after admission (or until the day of discharge if it occurs before 14 days). If the study personnel observe a significant change, your physician will be notified.

Monitoring/Follow-up Procedures

Procedures performed to evaluate the safety and effectiveness of experimental procedures are called monitoring or follow-up procedures. For this research study, the monitoring/follow-up procedures include:

1. Review and collection of a health history and information from your medical record.
2. If you consented to collection of genetics specimens for the study 'Biological Risk Factor Analysis for Cerebral Aneurysms DNA Analysis of Blood and Cerebrospinal Fluid', your health history and clinical information from this hospital stay along with follow up information will be used together with your genetic information to evaluate aneurysms and subarachnoid hemorrhage (bleeding in the brain). Since this is being done for research your individual genetic (DNA) results will not be available.
3. After discharge from the hospital, you may be asked to visit your doctor at the clinic between 1-3 months and again at 12 months for a follow up exam. We will perform an outcome evaluation with you at the same visit. This visit will take approximately 1-2 hours. During the visit, trained research study personnel will ask you questions to determine how well you are able to think and recall information as well as how well you can perform normal daily activities. If your are unable to keep either of these appointments, a member of the research team may come to visit you or you will receive a telephone call in order to ask you questions regarding how well you are able to participate in activities of daily living. It will take approximately 1-2 hours for the visit or 30 minutes to complete the phone call. We will call you at 24, 36, 48 and 60 months to ask you the same questions described above. Your participation in the study will end after your 6th (60 month) outcome evaluation.

What are the possible risks, side effects and discomforts of this research study?

1. As with any investigational study, there may be adverse events or side effects that are currently unknown and it is possible that certain of these unknown risks could be permanent, serious or life-threatening
2. Removal of CSF from a catheter drain is standard of care; however there can be a risk of infection. Infection from removal of CSF from the drainage bag is considered rare (infection occurs <1% or < 1 out of 100 patients).
3. To minimize discomfort every attempt will be made to draw the blood sample from a catheter or tube placed in a blood vessel as a part of normal care. If this tube is not available, one risk of participating in the study is the discomfort, soreness, bruising, and rarely infection associated with blood sampling. This risk is considered rare (occurs in < 1% or < 1 out of 100 patients).
4. EKG leads and ultrasound gel may cause skin irritation, redness and chafing, in addition the ultrasound gel may feel cool and sticky. These risks are considered rare (occurs in <1% or <1 out of 100 persons). To minimize these risks, the ultrasound gel will be washed off with soap and water immediately after the Doppler study and/or echocardiogram are completed. In addition, if you have any reaction to the patches or gel, they will be discontinued.

5. There is a rare risk of a breach of confidentiality. In very rare cases, people associated with this research study may inadvertently see your identifiable research results. We will do everything in our power to prevent this from happening by keeping all research records in locked files and identify all specimens and medical information by a research record number, rather than by your name or identifying numbers. The link of this information will be kept secure by the study investigators.

To minimize these risks, trained study and hospital personnel will obtain the samples and perform the procedures in this protocol according to hospital and research standards.

What are the possible benefits from taking part in this research study?
There may be no direct benefit from participating in this study. The general benefit will be for future patients with subarachnoid hemorrhage from cerebral aneurysms.

If I agree to take part in this research study, will I be told of any new risks that may be found in the course of the study?
You or your representative will be promptly notified if any new information develops during the conduct of this research study which may cause you to change your mind about continuing to participate.

Will I or my insurance provider be charged for the costs of any procedures performed as a part of this research study?
You and/or your insurer will not be billed for research only services. These studies include the additional blood, CSF and urine testing for markers of injury to your heart or brain; daily transcranial Doppler monitoring, ECGs and echocardiograms done as a part of the research protocol; and electrodes and monitoring equipment described in this protocol.

Although we may review the results, you and/or your insurer will be billed for standard of care treatments and procedures ordered by your attending physicians such as monitoring in the intensive care unit, angiograms, CTs, CTAs, ECGs, echocardiograms and baseline lab testing that may be initiated upon admission to the hospital, deemed necessary to your daily care or at times of changes in you clinical conditions. Transcranial Doppler studies and angiograms may also be ordered as standard of care for these purposes. You will also remain responsible for any applicable copays, coinsurances and deductibles.

Will I be paid if I take part in this research study?
You will be paid $50.00 as well as reimbursement for travel following each outcome visit. You will be asked to sign a form at the time of the outcome visit to verify that you have received this payment.

Who will know about my participation in this research study?
Records pertaining to your involvement in this research study will be stored in locked file cabinets within the departments of the principal investigators. Your biologic samples will be kept in locked freezers at the University of Pittsburgh under the control of the principal investigator and accessible only by the research staff. To protect your confidentiality, all personal identifiers (i.e., name, social security number, birthdate) will be removed (de-identified) and replaced with a specific code number. The information linking these code numbers to the corresponding subjects' identities will be kept in a separate, secure location. The

investigators on this study will keep the samples indefinitely. Your biologic samples may be given to investigators outside of UPMC or may be utilized in future studies.

Any information about you obtained from this research will be kept as confidential (private) as possible. You will not be identified by name in any publication of research results unless you sign a separate form giving your permission (release). In unusual cases, your research records may be released. If you choose to withdraw from the study, your samples will be destroyed.

Will this research study involve the use or disclosure of my identifiable medical record information?

This research study will involve the recording of current and/or future identifiable medical information from your hospital and/or other health care provider (e.g., physician office) records. The information that will be recorded will be limited to information concerning your past medical history and your hospital stay related to your aneurysm or surgical procedure. This information will be used for the purpose of analyzing subarachnoid hemorrhage from cerebral aneurysms.

Who will have access to identifiable information related to my participation in this research study?

In addition to the investigators listed on the first page of this authorization (consent) form and their research staff, the following individuals will or may have access to identifiable information (which may include your identifiable medical record information) related to your participation in this research study: Authorized representatives of the University of Pittsburgh Research Conduct and Compliance Office may review your identifiable research information (which may include your identifiable medical record information) for the purpose of monitoring the appropriate conduct of this research study.

Authorized representatives of the University of Pittsburgh Medical Center (UPMC) or other affiliated health care providers may have access to identifiable information (which may include your identifiable medical record information) related to your participation in this research study for the purpose of (1) fulfilling orders, made by the investigators, for hospital and health care services (e.g., laboratory tests, diagnostic procedures) associated with research study participation; (2) addressing correct payment for tests and procedures ordered by the investigators; and /or (3) for internal hospital operations (i.e. quality assurance).

Other members of the subarachnoid hemorrhage research team may have access to data or samples collected as part of this research study in order to complete specimen testing and data analysis. We will be happy to provide you with a list of these members at your request.

May I have access to my medical record information that results from my participation in this research study?

In accordance with the UPMC Notices of Privacy Practices document that you have been provided, you are permitted access to information contained within your medical records filed with your health care provider. Information related to research procedures, specifically biomarker information will not be shared since they are experimental, not performed at the time of your hospitalization and are not run in a clinically certified laboratory.

What additional confidentiality (privacy) protections are provided by a federal Confidentiality Certificate?"

To further help to protect your privacy, the investigators have obtained a Confidentiality Certificate from the U.S. Department of Health and Human Services (DHHS). With this federal Certificate, the investigators cannot be forced (for example, by court order) to disclose information that may identify you in any federal, state, or local court; administrative; legislative; or other proceeding. Disclosure will be necessary, however, upon the request of the DHHS (for example, for audit or program evaluation purposes).

You should understand that this federal Certificate does not prevent you or a member of your family from voluntarily releasing information about yourself or your involvement in this research study. Note, however, that if an insurer or employer learns about your study participation and obtains your consent to receive your identifiable research information, then the investigators may not use the Certificate to withhold this information from the insurer or employer. This means that you or your family must also actively protect your privacy. Finally, you should also understand that this federal Certificate does not prevent investigators from taking steps, including reporting to appropriate authorities, to prevent serious harm to yourself or others.

Is my participation in this research study voluntary?

Your participation in this research study, to include the use and disclose of your identifiable information for the purposes described above, is completely voluntary. (Note, however, that if you do not provide your consent for the use and disclosure of your identifiable information for the purposes described above, you will not be allowed, in general, to participate in the research study.) Whether or not you provide your consent for participation in this research study will have no affect on your current or future relationship with the University of Pittsburgh. Whether or not you provide your consent for participation in this research study will have no affect on your current or future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

Your doctor may be an investigator in this research study, and as an investigator, is interested both in your medical care and in the conduct of this research. Before agreeing to participate in this research study or at any time during your study participation, you may discuss your care with another doctor who is in no way associated with this research study. You are not under any obligation to participate in any research study offered by your doctor.

May I withdraw, at a future date, my consent for participation in this research study?

You may withdraw, at any time, your consent for participation in this research study, to include the use and disclosure of your identifiable information for the purposes described above. (Note, however, that if you withdraw your consent for the use and disclosure of your identifiable information for the purposes described above, you will also be withdrawn, in general, form further participation in this research study). Any identifiable research or medical record information recorded for, or resulting from your participation in this research study prior to the date that you formally withdrew your consent may continue to be used and disclosed by the investigators for the purposes described above. Should you decide to withdraw, you may choose to have your specimens returned to you or destroyed

To formally withdraw your consent for participation in this research study you should provide a written and dated notice of this decision to the principal investigator of this research study at the address listed on the first page of this form.

Your decision to withdraw your consent for participation in this research study will have no affect on your current or future relationship with the University of Pittsburgh. Your decision to withdraw your consent for participation in this research study will have no affect on your current for future medical care at a UPMC hospital or affiliated health care provider or your current or future relationship with a health care insurance provider.

Who will pay if I am injured as a result of taking part in this research study?
University of Pittsburgh researchers and their associates who provide services at the UPMC recognize the importance of your voluntary participation in their research studies. These individuals and their staffs will make reasonable effort to minimize, control, and treat any injuries that may arise as a result of this research. If you believe that you are injured as the result of the research procedures being performed, please contact immediately the Principal Investigator or one of the co-investigators listed on the first page of this form.

Emergency medical treatment for injuries solely and directly relating to your participation in this research will be provided to you by hospitals of the UPMC. It is possible that the UPMC may bill your insurance provider for the costs of this emergency treatment. Any co payments or deductibles will remain the responsibility of the insured party. If your research-related injury requires medical care beyond this emergency treatment, you will be responsible for the costs of this follow-up care unless otherwise specifically stated below. There is no plan for monetary compensation. You do not, however, waive any legal rights by signing this form.

****

VOLUNTARY CONSENT:
All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this research study during the course of this study, and that such future questions will be answered by the researchers listed on the first page of this form. Any questions I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-866-212-2668). By signing this form, I agree to participate in this research study. A copy of this consent form will be given to me.

_____ _____
Participant's Signature Date

_____
Participant's Name (Print)

CERTIFICATION of INFORMED CONSENT
I certify that I have explained the nature and purpose of this research study to the above named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise.

_____ _____
Printed Name of Person Obtaining Consent Role in Research Study

_____ _____
Signature of Person Obtaining Consent Date

_____
Participant's Name (Print)

is unable to provide direct consent for study participation because

_____

Therefore by signing this form, I give my consent for his/her participation in this research study.

_____ _____
Representative's Name (Print) Representative's Relationship to Participant

_____ _____
Representative's Signature Date/Time

Verification of Explanation
I certify that I have carefully explained the purpose and nature of this research study to the above named participant in appropriate language. He/she has had an opportunity to discuss it with me in detail. I have answered all his/her questions and he/she has provided affirmative agreement (i.e., assent) to participate in this study.

_____ _____
Investigator's Signature Date

CONSENT FOR CONTINUED RESEARCH PARTICIPATION:

I understand that I am currently participating in a research study. I further understand that consent for my participation in this research study was initially obtained from my authorized representative as a result of my inability to provide direct consent at the time that this initial consent was requested. I have now recovered to the point where it is felt that I am able to provide direct consent for continued participation in this research study.
All of the above has been explained to me and all of my current questions have been answered. I understand that I am encouraged to ask questions about any aspect of this study and that future questions will be answered by the researchers listed on the first page of this form. I also understand that any questions I have about my rights as a research participant will be answered by the Human Subject Protection Advocate of the IRB Office, University of Pittsburgh (1-888-212-2668). By signing below, I agree to continue my participation in this research study. A copy of this consent from will be given to me.

_____ _____
Participant's Signature Date

CERTIFICATION of INFORMED CONSENT
I certify that I have explained the nature and purpose of this research study to the above named individual(s), and I have discussed the potential benefits and possible risks of study participation. Any questions the individual(s) have about this study have been answered, and we will always be available to address future questions as they arise.

_____ _____
Printed Name of Person Obtaining Consent Role in Research Study

_____ _____
Signature of Person Obtaining Consent Date

Appendix D

CSF TABLES AND FIGURES

D.1 CSF TABLE 6

Table 6. Demographics CSF Estrogen Analysis N=36

	Female		Male
	Pre-menopausal (N=11)	Post-menopausal (N=11)	N=14
Age (Mean In Years)	43.36 (Sd=6.23)	60.91 (Sd=4.83)	46.4 (Sd=9.68)
Race			
Caucasian	8 (73%)	10 (91%)	10 (71%)
Other	3 (27%)	1 (9%)	4 (29%)
Hunt And Hess			
1	2 (18%)	0	1 (7%)
2	3 (27%)	1 (9%)	2 (14%)
3	3 (27%)	5 (46%)	8 (57%)
4	2 (18%)	3 (27%)	3 (21%)
5	1 (9%)	2 (18%)	0 (0%)
Fisher			
2	5 (45.5%)	1 (9%)	4 (29%)
3	5 (45.5%)	4 (36%)	8 (57%)
4	1 (9%)	6 (55%)	2 (14%)
DCI	7 (64%)	5 (45.5%)	5 (36%)
DCI from vasospasm	7 (64%)	3 (27%)	4 (29%)
DCI From CT	0	1 (9%)	1 (7%)
Vasospasm	7 (64%)	5 (45.5%)	4 (29%)
Infarct	6 (54.5%)	4 (36%)	4 (29%)
Ischemia	8 (73%)	5 (45.5%)	5 (36)%
Samples			
E1 Detection	37 (76%)	47 (94%)	50 (81%)
E2 Detection	16 (33%)	16 (50%)	21 (34%)

D.2 CSF FIGURE 1

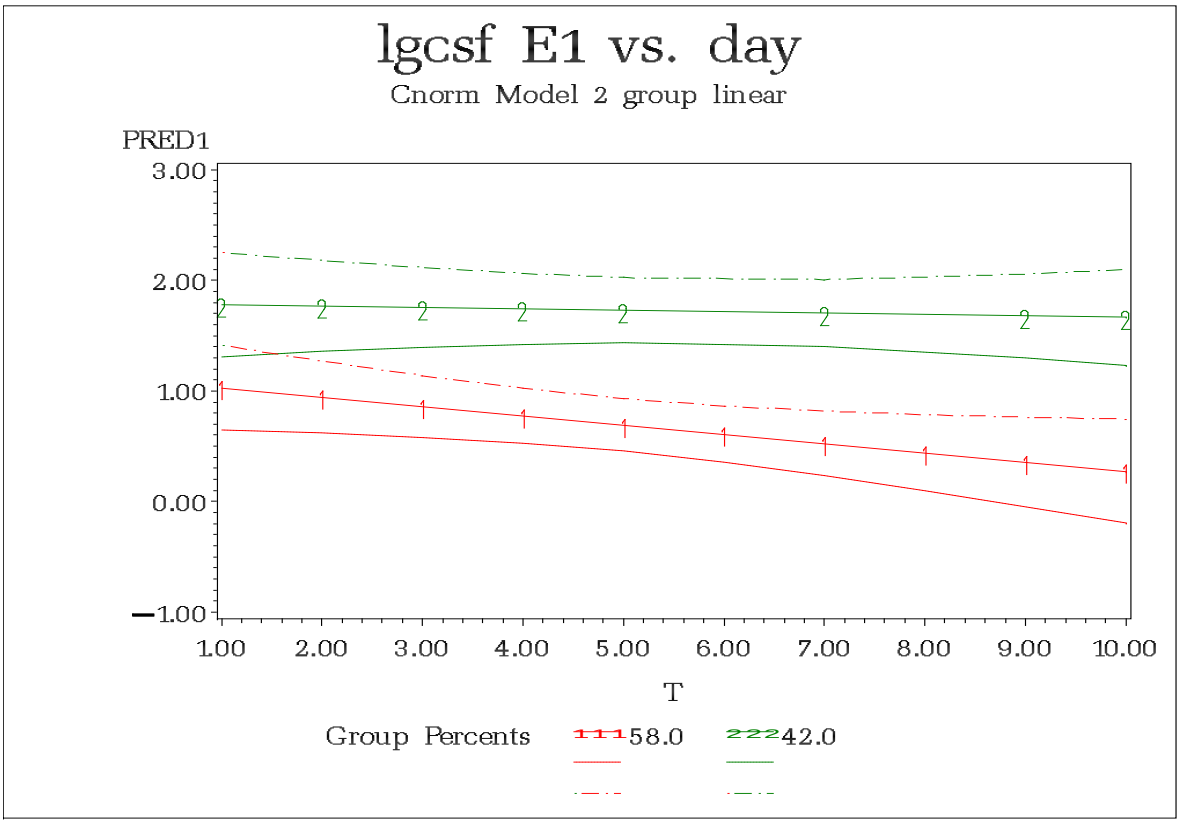


Figure 5. CSF E1 2-Group Trajectory

lgcsf E1 vs. day cnorm model 2 group linear
Average probability per group

	1	2
1	0.881	0.119
2	0.112	0.888

Mixture fit statistics - entropy 0.637, AIC 359.780, BIC 370.865, CAIC 377.865, SSBIC 348.997, CLC 363.886, ICL-BIC 388.970

D.3 CSF FIGURE 2

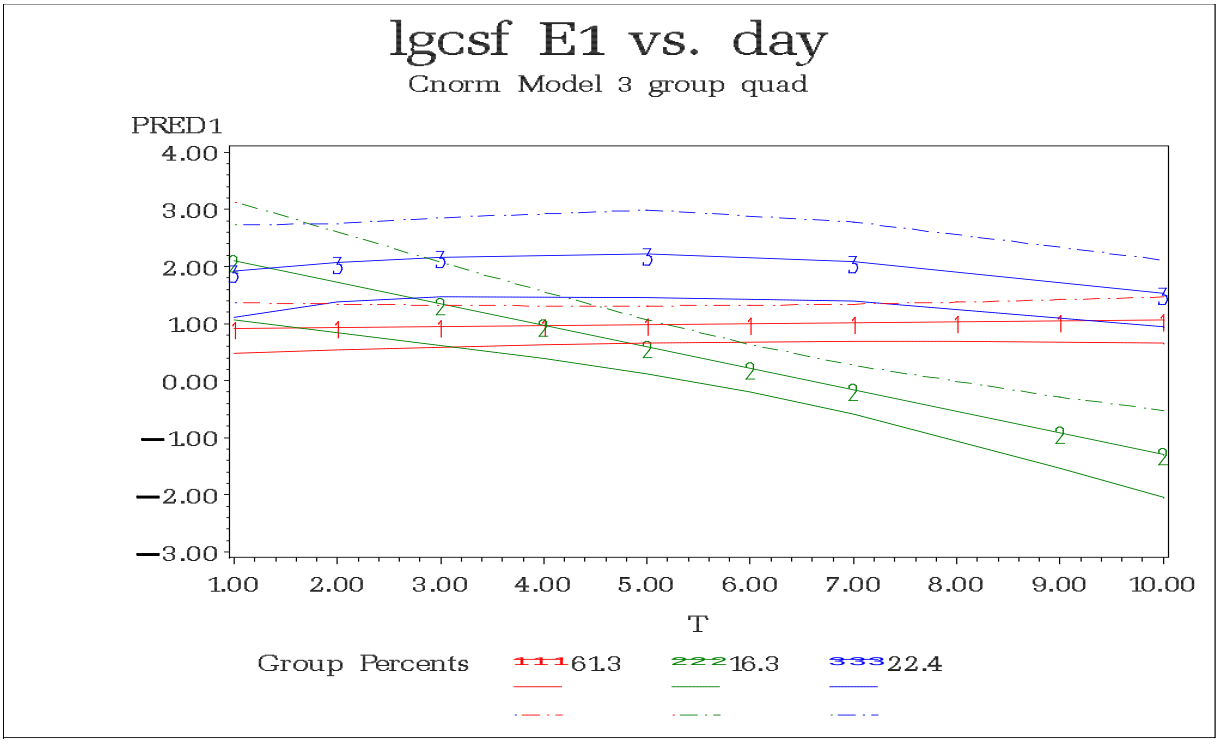


Figure 6. CSF E1 3-Group Trajectory

lgcsf e1 vs. day
cnorm model 3 group quad
Average probability per group

	1	2	3
1	0.877	0.055	0.068
2	0.138	0.862	0.000
3	0.072	0.023	0.905

Mixture fit statistics - Entropy 0.727, AIC 355.220, BIC 375.806, CAIC 388.806, SSBIC 335.194, CLC 350.784, ICL-BIC 397.370

D.4 CSF FIGURE 3

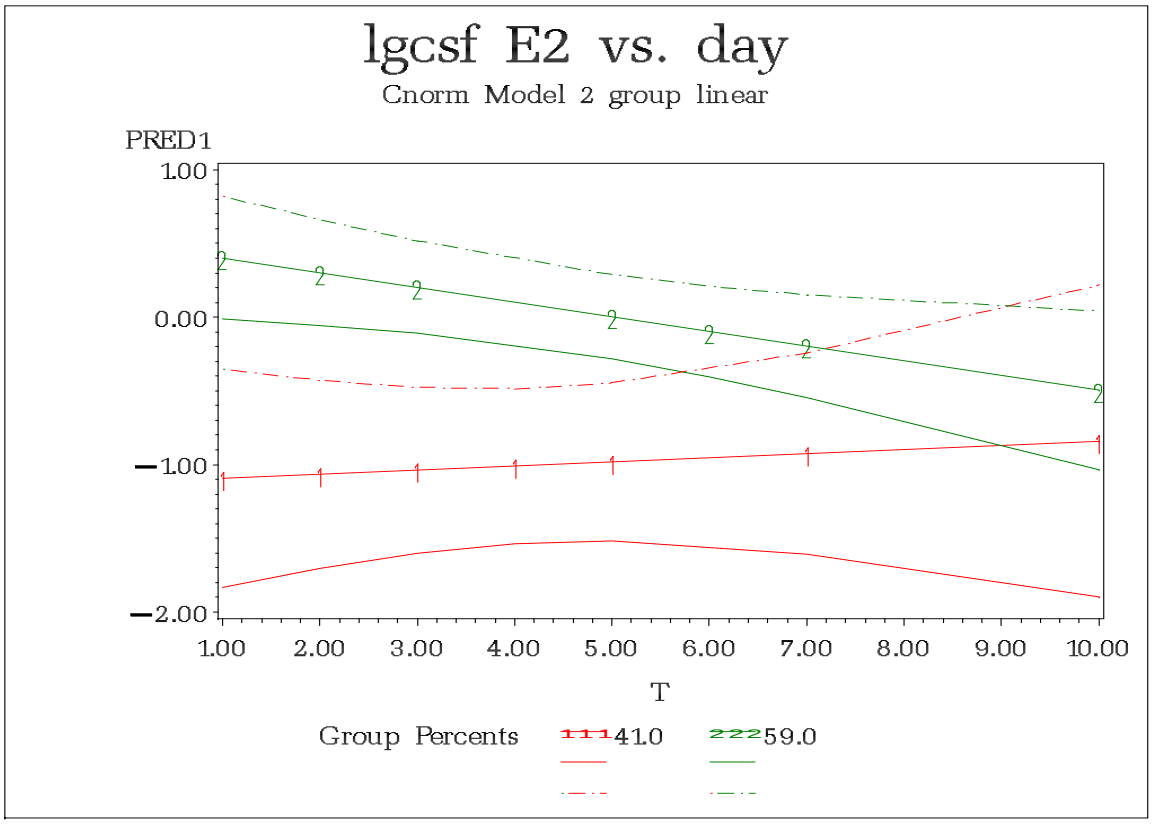


Figure 7. CSF E2 Trajectory

lgcsf E2 vs. day
Cnorm Model 2 group linear
Average probability per group

	1	2
1	0.877	0.123
2	0.223	0.777

Mixture Fit Statistics - Entropy 0.425, AIC 142.160, BIC 153.245, CAIC 160.245, ssBIC 131.377, CLC 156.835, ICL-BIC 181.919

D.5 CSF TABLE 7: TRAJECTORY ANALYSIS OF (LOG) CSF ESTROGEN MEASUREMENTS

Table 7. Trajectory Analysis of (log) of CSF estrogen measurements

	E1							E2		
	2 group			3 group				2 group		
	High n=14	Low n=22	*p*value	High n=7	Low n=24	Decline n=5	*p* value	High n=26	Low n=10	*p* value
Gender			.6				.009			.23
male	6	8		3	6	5		12	2	
female	8	14		4	18	0		14	8	
Race			.28				.2			.5
Caucasian	12	16		7	18	3		21	7	
Other	2	6		0	6	2		5	3	
Age			.11				.65			.46
Hunt and Hess			.04				.69			.35
HH 1	0	3		0	2	1		1	2	
HH 2	1	5		0	5	1		5	1	
HH 3	5	11		3	11	2		13	3	
HH 4	5	3		3	4	1		6	2	
HH 5	3	0		1	2	0		1	2	
Hunt and Hess 1,2	1	8	.03	0	7	2	.2	6	3	.74
vs 3-5	13	14		7	17	3		20	7	
Fisher			.22				.46			.18
2	2	8		0	8	2		6	4	
3	7	10		5	0	2		15	2	
4	5	4		2	6	1		5	4	
DCI no	4	14	.12	3	12	3	.98	13	5	.93
yes	9	8		3	12	2		12	5	
DCI-vasospasm sources no	3	16	.02	2	13	4	.37	15	4	.35

Trajectory Analysis of (log) of CSF estrogen measurements (continued)

	E1							E2		
	2 group			3 group				2 group		
	High n=14	Low n=22	*p* value	High n=7	Low n=24	Decline n=5	*p* value	High n=26	Low n=10	*p* value
yes	9	6		4	10	1		9	6	
DCI-cerebral infarction no	5	16	.42	3	15	3	.4	16	5	.45
yes	0	2		0	1	1		2	0	
Vasospasm no	4	6	.43	2	6	2	.47	8	2	.53
yes	10	6		5	10	1		11	5	
Ischemia no	6	12	.66	3	13	2	.7	14	4	.5
yes	8	10		4	11	3		12	6	
Infarct no	7	15	.47	4	16	2	.52	18	4	.15
yes	7	7		3	8	3		8	6	
Menopause			.83				.04			
pre-menopause	4	7		1	10	0		6	5	
post-menopause	4	7		3	8	0		8	3	
male	6	8		3	6	5		12	2	

D.9 CSF TABLE 8: UNIVARIATE ANALYSIS OF (LOG) CSF ESTROGEN MEASUREMENTS

Table 8. Univariate analysis of (log) CSF estrogen measurements

	E1						E2					
	Log measurements			Detection			Log measurements			Detection		
	Mean	Std dev	*p* value	Det n=33	Ndet n=3	*p* value	Mean	Std dev	*p* value	Det n=24	Ndet n=12	*p* value
Gender			.67			.84			.44			.33
male	.96	.23		13	1		-.14	.28		8	6	
female	1.1	.18		20	2		-.38	.2		16	6	
Race			.09						.39			.78
Caucasian	1.1	.16		25	3		-.26	.18		19	9	
Other	.67	.26		8	0		-.58	.36		5	3	
Age			.08			.66			.79			.34
Hunt and Hess			.03			.39			.92			.28
HH 1	.33	.38	.03	3	0		-.67	.61	.9	2	1	
HH 2	.74	.3	.01	6	0		-.09	.41	.45	4	2	
HH 3	.97	.2	.2	13	3		-.25	.29	.33	8	8	
HH 4	1.5	.24	.97	8	0		-.29	.29	.61	7	1	
HH 5	1.5	.38		3	0		-.57	.49		3	0	
Hunt and Hess 1,2	.58	.24	.02	9	0	.3	-.27	.32	.87	6	3	1
vs 3-5	1.2	.16		24	3		-.33	.2		18	9	
Fisher			.12			.16			.96			.69
2	.66	.24	.06	10	0		-.39	.34	.93	6	4	
3	1.2	.21	.67	14	3		-.28	.23	.85	11	6	

Univariate analysis of (log) CSF estrogen measurements (continued)

		E1						E2					
		Log measurements			Detection			Log measurements			Detection		
		Mean	Std dev	*p* value	Det n=33	Ndet n=3	*p* value	Mean	Std dev	*p* value	Det n=24	Ndet n=12	*p* value
	yes	1.1	.2		16	1		-.19	.23		13	4	
DCI-vasospasm	no	.7	.18	.05	17	2	.69	-.29	.27	.79	10	9	.1
	yes	1.2	.2		14	1		-.38	.23		12	3	
DCI-cerebral infarct	no	.74	.18	.84	19	2	.65	-.21	.23	.22	13	8	.74
	yes	.64	.44		2	0		.76	.74		1	1	
Vasospasm	no	1.1	.27	.55	2	2	.29	-.1	.29	.55	8	2	.77
	yes	1.3	.24		8	1		-.3	.23		12	4	
Ischemia	no	.99	.2	.77	17	1	.55	-.22	.24	.56	12	6	1
	yes	1.1	.2		16	2		-.38	.22		12	6	
Infarct	no	1.0	.18	.88	19	3	.15	-.27	.21	.68	14	8	.63
	yes	1.1	.22		14	0		-.39	.25		10	4	
Menopause				.78		.3				.71			
	pre-menopause	.97	.25	.98	9	2		-.43	.25	.42	9	2	
	post-menopause	1.2	.24	.52	11	0		-.3	.32	61	7	4	
	male	.96	.23		13	1		-.12	.29		8	6	
E1/E2				<.001									

BIBLIOGRAPHY

Alkayed, N.J., Harukuni, I., Kimes, A.S., London, E.D., Traystman, R.J., Hurn, P.D. (1998). Gender-linked brain injury in experimental stroke. *Stroke*, 29(1), 159-166.

Auriat, A., Plahta, W.C., McGie, S.C., Yan R., Colbourne, F. (2005). 17β-estradiol pretreatment reduces bleeding and brain injury after intracerebral hemorrhagic stroke in male rats. *Journal of Cerebral Blood Flow and Metabolism*, 25(2), 247-256.

Azcoitia, I., Arevalo, M.A., De Nicola, A.F., Garci-Segura, L.M. (2011). Neuroprotective actions of estradiol revisited. *Trends in Endocrinology and Metabolism*, 22(12), 467-473.

Ba, Z.F., Lu, A., Shimizu, T., Szalay, L., Schwacha, M.G., Rue, L.W.3rd, Bland, K.I., Chaudry, I. H. (2007). 17β-estradiol modulates vasoconstriction induced by endothelin-1 following trauma-hemorrhage. *Am J Physiol Heart Circ Physiol*, 292(1), 245-250.

Behl, C. (2002). Oestrogen as a neuroprotective hormone. *Nature Reviews*, 3(6), 433-442.

Bendel, S., Koivisto, T., Ryynanen, O., Ruokonen, E., Romppanen, J., Kiviniemi, V., Uusar, A. (2010). Insulin like growth factor-1 in acute subarachnoid hemorrhage: a p rospective cohort study. *Critical Care*, 14(2), R75.

Brown, C.M., Suzuki, S., Jelks, K.A., Wise, P.M. (2009). Estradiol is a potent protective, restorative, and trophic factor after brain injury. *SeminReprod Med*, 27(3), 240-249.

Cahill, J., Zhang, J. H. (2009). Subarachnoid hemorrhage: is it time for new direction? *Stroke*, 40(Suppl 1), S86-87.

Carwile, E., Wagner, A.K., Crago, E., Alexander, S.A. (2009). Estrogen and stroke: a review of the current literature. *Journal of Neuroscience Nursing*, 41(1), 18-25.

Crago, E.A., Thampatty, B.P., Sherwood, P.R., Kuo, C.J., Bender, C., Balzer, J., Horowitz, M., Poloyac, S.M. (2011). Cerebrospinal Fluid 20-HETE is associated with delayed cerebral ischemia and poor outcomes after aneurysmal subarachnoid hemorrhage. *Stroke*, 42 (7), 1872-1877.

Dankbaar, J.W., de Rooij, N.K., Smit, E.J., Velthuis, B.K., Frijns, C.J., Rinkel, G.J., van der Schaar, I.C. (2011). Changes in cerebral perfusion around the time of delayed cerebral ischemia in subarachnoid hemorrhage patients. Cerebrovascular Disease, 32(2), 133-140.

Diringer, M.N., Bleck, T.P., Hemphill, J.3rd, Menon, D., Shutter, L., Vespa, P., Bruder, N., Connolly, E.s. Jr, Citerio, G., Gress, D., Hanggi, D., Hoh, B.L., Lanzino, G., Le Roux, P., Rabinstein, A., Schmutzhard, E., Stocchetti, N., Suarez, J.I., Treggiari, M., Tseng, M.Y., Vergouwen M.D., Wolf, S., Zipfel, G; Neurocritical Care Society.(2011). Critical care management of patients following aneurysmal subarachnoid hemorrhage: recommendations from the Neurocritical Care Society's multidisciplinary consensus conference. Neurocritical Care, 15(2), 211-240.

Dupont, S.A., Wijkicks, E.F., Lanzino, G., Rabinstein, A.A. (2010). Aneurysmal subarachnoid hemorrhage: an overview for the practicing neurologist. *Seminars in Neurology*, 30(5), 545-554.

Dupont, S.A., Wijkicks, E.F., Manno, E.M., Lanzino, G., Rabinstein, A.A. (2009). Prediction of angiographic vasospasm after aneurysmal subarchnoid hemorrhage: Value of the Hijdra Sum Scoring System. *NeuroCritical Care*, 11, 172-176.

Espinoza, T.R., Wright, D.W. (2011). The role of progesterone in traumatic brain injury. *Journal of Head Trauma Rehabilitation*, 26(6), 497-499.

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.

Frontera, J.A., Aledort, L., Gordon, E., Ergorova, N., Moyle, H., Patel, A., Bederson, J.B., Sehba, F. (2012). Early platelet activation, inflammation and acute brain injury after subarachnoid hemorrhage: A pilot study. *Journal of Thrombosis and Haemostasis*, 10(4), 711-3.

Frontera, J.A., Fernandez, A., Schmidt, J.M., Claassen, J., Wartenberg, K.E, Badjatia, N., Connolly, E.S., Mayer, S.A. (2009). Defining vasospasm after subarachnoid hemorrhage; what is the most clinically relevant definition? *Stroke*, 40, 1963-1968.

Gatson, J., Liu, M.M., Abdelfattah, K., Wigginton, J.G., Smith, S., Wof, S.E., Simpkins, J.W., Minei, J.P. (2012). Estrone is Neuroprotective in Rats Following Traumatic Brain Injury. *Journal of Neurotrauma*, epub ahead of print Mar 21.

Gilles, G.E., Mcarthur, S. (2010). Estrogen actions in the brain and the basis for differential action in men and women: a case for sex-specific medicines. *PharmacologicalReviews*, 62(2), 155-198.

Ha, D.M., Xu, J., Janowsky, J.S. (2007). Preliminary evidence that long-term estrogen use reduces white matter loss in aging. *Neurobiol Aging*, 28(12), 1936-1940.

Haring, R., Travison, T.G., Bhasin, S., Vasan, R.S., Wallaschofski, H., Davda, M.N., Coviello, A., Murabito, J.M. (2011). Relation between sex hormone concentrations, peripheral arterial disease, and change in ankle-brachial index: Findings from the Framingham heart study. *Journal of Clinical Endocrinology Metabolism*, 96(12), 3724-3732.

Herson, P.S., Koerner, I.P., Hurn, P.D. (2009). Sex, sex steroids and brain injury. *Seminars in Reproductive Medicine*, 27(3), 229-239.

Hsieh, F.Y., Block, D.A., Larsen, M.D. (1988). A simple method of sample size calculation for linear and logistic regression. *Statistics in Medicine*, 17(14), 1623-1634.

Hsieh, F.Y., Lavori, P.W. (2000). Sample – size calculations for the Cox proportional hazards regression model with nonbinary covariates. *Controlled Clinical Trials*, 21(6), 552-560.

Hurn, P.D., Brass, L.M. (2003). Estrogen and stroke: a balanced analysis. *Stroke*, 34(2), 338-341.

Illiff, J.J., Wang, R., Zeldin, D.C., Alkayed, N.J. (2009). Epoxyeicosanoids as mediators of neurogenic vasodilation in cerebral vessels. *Am J Physiol Heart CircPhysiol*, 296(5), H1352-1363.

Imig, J.D., Simpkins, A.N., Renic, M., Harder, D.R. (2011). Cytochrome P450 eicosanoids and cerebral vascular function. *Expert Reviews*, 13 (e7), 1-15.

Ito H, Kanno I, Fukuda H. (2005). Human cerebral circulation: positron emission tomography studies. *Annals of Nuclear Medicine,* 19(2), 65-74.

Kasius, K.M., Frjns, C.J., Algra, A., Rinkel, G.J. (2010). Association of platelet and leukocyte counts with delayed cerebral ischemia in aneurysmal subarachnoid hemorrhage. *Cerebrovascular Disease*, 29(6), 576-583.

Kauffman, R.M., Norris, P.R., Jenkins, J.M., Dupont, W.D., Torres, R.E., Blume, J.D., Dossett, L.A., Hranjec, T., Sawyer, R.G., May, A.K. (2011). Trends in estradiol during critical illness are associated with mortality independent of admission estradiol. *J Am CollSurg*, 212(4), 703-713.

Kauffman, R.M., Norris, P.R., Jenkins, J.M., Dupont, W.D., Torres, R.E., Blume, J.D., Dossett, LA.., Hranjec, T., Sawyer, R.G., May, A.K. (2010). Trends in estradiol during critical illness are associated with mortality independent of admission estradiol. *Journal of the American College of Surgeons*, 212(4), 703-713.

Kaya, E., Sahin, F.K., Koken, G., Kose, M., Cevrioglu, A.S. (2008). Acute effect of intranasal estrogen on cerebral and cerebellar perfusion in postmenopausal women. *Maturitas,* 59(1), 72-82.

Kehl, F., Cambj-Sapunar, L., Maier, K.G., Miyata, N., Kametani, S., Okamoto, H., Hudetz, A.G., Schulte, M.L., Zagorac, D., Harder, D.R., Roman, R.J. (2002). 20-HETE contributes to the acute fall in cerebral blood flow after subarachnoid hemorrhage in the rat. *American Journal of Physiology Heart Circulation Physiology*, 282(4), 1556-65.

Keyroux, S.G., Diringer, M.N. (2007). Clinical review: Prevention and therapy of vasospasm in subarachnoid hemorrhage. *Critical Care*, 11(4), 220-230.

Kirkness, C.J. (2005). Cerebral blood flow monitoring in clinical practice. *AACN Clinical Issues in Advanced Practice in Critical Care*, 16(4), 476-487.

Kipp, M., Berger, K., Clarner, T., Dang, J., Beyer, C. (2011). Sex steroids control neuroinflammatory processes in the brain: relevance for acute ischaemia and egenerative demyelination. *Journal of Neuroendocrinology*, 24(1), 62-70.

Koerner, I.P., Zhang, W., Cheng, J., Parker, S., Hurn, P.D., Alkayed, N.J. (2008).Soluble epoxide hydrolase: regulation by estrogen and role in the inflammatory response to cerebral ischemia. *Frontiers of Bioscience*, 13, 2833-2841.

Kolias, A.G.,Sen, J., Belli, A.(2009). Pathogenesis of cerebral vasospasm following aneurysmal subarachnoid hemorrhage: putative mechanisms and novel approaches. *Journal of Neuroscience Research*, 87 (1), 1-11.

Krause, D.N., Duckles, S.P., Pelligrino, D.A. (2006). Influence of sex steroid hormones on cerebrovascular function. *Journal of Applied Physiology*, 101(4), 1252-1261.

Lang, J.T., McCullough. L.D. (2008). Pathways to ischemic neuronal cell death: are sex differences relevant? *Journal of Translational Medicine*, 23, 6-33.

Lerchbaum, E., Pilz, S., Grammer, T.B., Boehm, B.O., Marz, W., Obermayer-Pietsch, B. (2011). High estradiol levels are associated with increased mortality in older men referred to coronary angiography. *ExpEndocrinol Diabetes*, 119(8), 490-496.

Lin, C.L., Dumont, A.S., Su, Y.F., Tsai, Y.J., Huang, J.H., Chang, K.P., Howng, S.L., Kwan, A.L., Kassell, N.F., Kao, C.H. (2009). Attenuation of cerebral vasospasm and secondary injury by 17 beta estradiol following experimental subarachnoid hemorrhage. *Journal of Neurosurgery,* 110(3), 457-461.

Losiniecki, A., Zuccarello, M. (2008). Subarachnoid hemorrhage: effect on cerebral blood flow and cerebral metabolism. *Frontier of Bioscience*, 1(13), 1845-56.

Lyden, P., Lu, M., Jackson, C., Marler, J., Kothari, R., Brott, T., Zivin, J. (1999). Underlying Structure of the National Institutes of Health Stroke Scale: Results of a Factor Analysis. *Stroke*, 30(11), 2347-2354.

MacDonald, R.L., Kassel, N.F., Mayer, S., Ruefenacht, D., Schmiedek, P., Weidauer, S., Frey, A., Roux, S., Pasqualin, A.: CONSCIOUS-1 Investigators. (2008). Clazosentan to overcome neurological ischemia and infarction occurring after subarachnoid hemorrhage (CONSCIOUS-1): randomized, double-blind, placebo-controlled phase 2 dose-finding trial. *Stroke*, 39(11), 3015-21.

Macleod, M.R., Andrew, P.J. (2002). Effect of deprivation and gender on the incidence and management of acute brain disorders. *Intensive Care Medicine*, 28(12), 1729-1734.

May, A.K., Dossett, L.A., Norris, P.R., Hansen, E.N., Dorsett, R.C., Popovsky, K.A., Sawyer, R.G. (2008). Estradiol is associated with mortality in critically ill trauma and surgical patients. *Critical Care Medicine*, 36,(1), 62-69.

Masi, C.M., Hawkley, L.C., Xu, X., Veenstra, T.D., Cacioppo, J.T. (2009). Serum estrogen metabolites and systolic blood pressure among middle-aged and older women and men. *American Journal of Hypertension*, 22(11), 1148-1153.

Miller, T.M., Donnelly, M.K., Crago, E.A., Roman, D.M., Sherwood, P.R., Horowitz, M.B., Poloyac, S.M. (2009). Rapid, simultaneous quantitation of mono and deoxygenated metabolites of arachidonic acid in human CSF and rat brain. *Journal of Chromatography B*, 877(31), 3991-4000.

Naessen, R., Sjogren, U., Bergquist, J., Larsson, M., Lind, L., Kushnir, M.M. (2010). Endogenous steroids measured by high-specificity liquid chromatography-tandem mass spectrometry and prevalent cardiovascular disease in 70-year-old men and women. *Journal of Clinical Endocrinology and Metabolism*, 95(4), 1889-1897.

Nagin, D. S. (2005). *Group-based modeling of development*. Cambridge, Massachusetts: Harvard University Press.

Nagin, D.S., Odgers, C.L. (2010). Group-based trajectory modeling in clinical research. *Annual Review Clinical Psychology*, 6, 109-38.

Naredi, S., Lamber, G., Friberg, P., Zall, S., Eden, E., Rydenhag, B., Tylman, M., Bengtsson, A. (2006). Sympathetic activation and inflammatory response in patients with subarachnoid haemorrhage. *Intensive Care Medicine*, 32(12), 1955-1961.

Nelson, R.E., Grebe, S.K., O'Hare, D.J., Singh, R.J. (2004). Liquid Chromatography-Tandem Mass Spectrometry Assay for Simultaneous Measurement of Estradiol and Estrone in Human Plasma. *Clinical Chemistry,* 50(2), 373-384.

Neuschmelting, V., Marbacher, S., Fathi, A.R., Jakob, S.M..,Fandino, J. (2009). Elevated level of endothelin-1 in cerebrospinal fluid and lack of nitric oxide in basilar arterial plasma associated with cerebral vasospasm after subarachnoid haemorrhage in rabbits. *ActaNeurochiga*, 151(7), 795-801.

Nguyen, H.P., Li, L., Gatson, J.W., Maass, D., Wigginton, J.G., Simpkins, J.W., Schug, K.A. (2011). Simultaneous quantification of four native estrogen hormones at trace levels in human cerebrospinal fluid using liquid chromatography-tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, 54, 830-837.

Omura, T., Tanaka, Y., Miyata, N., Doizumi, C., Sakurai, T., Fukasawa, M., Hachiuma, K., Minagawa, T., Susumu, T., Yoshida, S., Nakaike, S., Okuyama, S., Harder, D.R., Roman, R.J. (2006). Effects of a new inhibitor of the synthesis of 20-HETE on cerebral ischemia reperfusion injury. *Stroke*, 37,1307-1313.

Orbo, M., Waterloo, K., Egge, A., Isaken, J., Ingebrigtsen, T., Romner, B. (2008). Predictors of cognitive impairment one year after surgery for aneurysmal subarachnoid hemorrhage. *Journal of Neurology*, 255(11), 1770-1776.

Ostrowski, R.P., Colohan, A.R., Zhang, J.H. (2006). Molecular mechanisms of early brain injury after subarachnoid hemorrhage. *Neurological Research*, 28, 399-414.

Poloyac, S., Zhang, Y., Bies, R., Kochanek, P., Graham, S. (2006). Protective effect of the 20-HETE inhibitor HET0016 on brain damage after temporary focal ischemia. *Journal of Cerebral Blood Flow Metabolism*, 26,1551-61.

Poloyac, S., Reynold, R., Yonas, H., Kerr, M. (2005). Identification and quantification of the hyeroxyeicosatetraenoic acids, 20-HETE and 12-HETE, in the cerebrospinal fluid after subarachnoid hemorrhage. *Journal of Neuroscience Methods*, 144,257-263.

Prasad, K. (1996). The Glasgow coma scale: A critical appraisal of its clinimetric properties. *Journal of Clinical Epidemiology*, 49 (7), 755-763.

Provencio, J.J., Vora, N. (2005). Subarachnoid hemorrhage and inflammation: Bench to bedside and back. *Seminars in Neurology*, 25 (4), 435-444.

Rauschemberger, M.B., Sandoval, M.J., Massheimer, V.L. (2011). Cellular and molecular actions displayed by estrone on vascular endothelium. *Molecular and Cellular Endocrinology,* 339(1-2), 136-143.

Rauschemberger, M.B., Selles, J., Massheimer, V. (2008). The direct action of estrone on vascular tissue involves genomic and non-genomic actions. *Life Sciences*, 82(1-2), 115-123.

Rejdak, K., Petzold, A., Sharpe, M.A., Kay, A.D., Kerr, M., Keir, G., Thompson, E.J., Giovannoni, G. (2004). Cerebrospinal fluid nitrate/nitrate correlated with oxyhemoglobin and outcome in patients with subarachnoid hemorrhage. *Journal of Neurological Science*, 219(1-2), 71-6.

Roman, R.J., Renci, M., Dunn, K.M., Takeuchi, K., Hacein-Bey, L. (2006). Evidence that 20-HETE contributes to the development of acute and delayed cerebral vasospasm. *Neurological Research*, 28(7), 738-49.

Rose, M.J. (2011). Aneurysmal subarachnoid hemorrhage: an update on the medical complications and treatments strategies see in these patients. *Current Opinion in Anesthesiology*, 24 (5), 500-7.

Rothman, M.S., Carlson, N.E., Xu, M., Wang, C., Swerdloff, R., Lee, P., Goh, V.H., Ridgway, E.C., Wierman, M.E. (2011). Reexamination of testosterone, dihydrotestosterone, estradiol and estrone levels across the menstrual cycle and in postmenopausal women measured by liquid chromatography-tandem mass spectrometry. *Steroids*, 76, 177-182.

Sabri, M., Ai, J., Knight, B., Tariq, A., Jeon, H., Shang, X., Marsden, P.A., Loch Macdonald, R. (2011). Uncoupling endothelial nitric oxide synthase after experimental subarachnoid hemorrhage. *Journal of Cerebral Blood Flow and Metabolism*, 31(1), 190-199.

Sarkar, S.N., Huang, R.Q., Logan, S.M., Yi, K.D., Dillon, G.H., Simpkins, J.W. (2008). Estrogens directly potentiate neuronal L-type Ca $^{2+}$ channels. *PNAS*, 105 (39), 15148-15153.

Schoenfeld, D. A. (1983). Sample – size formula for the proportional – hazards regression model. *Biometrics*, 39(2). 499-503.

Sehba, F.A., Hou, J., Pluta, R.M., Zhang, J.H. (2012). The importance of early brain injury after subarachnoid hemorrhage. *Progress in Neurobiology*, 97(1) 14-37.

Sehba, F.A., Pluta, R.M., Zhang, J.H. (2011). Metamorphosis of subarachnoid hemorrhage research: from delayed vasospasm to early brain injury. *Molecular Neurobiology*, 43, 27-40.

Selles, J., Polini, N., Alvarez, C., Massheimer, V. (2005). Novel action of estrone on vascular tissue:regulation of NOS and COX activity. *Steroids*, 70(4), 251-256.

Selvamani, A., Sohrabji, F. (2010). The neurotoxic effects of estrogen on ischemic stroke in older female rats is associated with age-dependent loss of insulin-like growth factor-1. *The Journal of Neuroscience*, 30(20), 6852-6861.

Shih, H.C., Lin, C.L., Wu, S.C, Kwan, A.L, Hong, Y.R., Howng, S. L. (2008). Upregulation of estrogen receptor α and mediation of 17β-estradiol vasoprotective effects via estrogen receptor α in basilar arteries in rats after experimental subarachnoid hemorrhage. *Journal of Neurosurgery*, 109(1), 92-99.

Simpson, E.R. (2004). Aromatase: biologic relevance of tissue-specific expression. *Seminar in Reproductive Medicine*, 22(1), 11-23.

Spratt, D.J., Morton, J.R., Kramer, R.S., Mayo, S.W., Longcope, C., Vary, C.P. (2006). Increases in serum estrogen levels during major illness are caused by increased peripheral aromatization. *American Journal of Physiologic Endocrinology and Metabolism*, 291(3), E631-638.

Sribnick, E.A., Del Re, A.M., Ray, S.K., Woodward, J.J., Banik, N. L. (2009) Estrogen attenuates glutamate-induced cell death by inhibiting Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels. *Journal of Brain Research*, 1276, 159-170.

Vergouwen, M.D.; Participants in the International Multi-disciplinary Consensus Conference on the Critical Care Management of Subarachnoid Hemorrhage. (2011a). Vasospasm versus delayed cerebral ischemia as an outcome event in clinical trials and observational studies. *NeuroCritical Care*, 15(2), 308-311.

Vergouwen, M.D., Ilodigwe, D., Macdonald, R.L. (2011b). Cerebral infarction after subarachnoid hemorrhage contributes to poor outcome by vasospasm-dependent and independent effects. *Stroke*, 42(4), 924-929.

Vergouwen, M.D., Vermeulen, M. Roos, Y.B. (2009). Delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage: is angiographic vasospasm an epiphenomenon? *Stroke*, 40(2), e39.

Wagner, A.K., McCullough, E.H., Niyonkuru, C., Ozawa, H., Loucks, T.L., Dobos, J.A., Brett, C.A., Santarsieri, M., Dixon, C.E., Berga, S.L., Fabio, A. (2011). Acute serum hormone levels: characterization and prognosis after severe traumatic brain injury. *Journal of Neurotrauma*, 28(6), 871-888.

Yang, S.H., He, Z., Wu, S.S., He, Y.J., Cutright, J., Millard, W.J., Day, A.L., Simpkins, J.W. (2001). 17β-estradiol can reduce secondary ischemic damage and mortality of subarachnoid hemorrhage. *Journal of Cerebral blood Flow and Metabolism*, 21(2), 174-181.

Xu, X., Roman, J.M., Issaq, H.J, Keefer, L.K., Veenstra, T.D., Ziegler, R.G. (2007). Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography-tandem mass spectrometry. *Analytical Chemistry*, 79(20), 7813-7821.

Yi, K.D., Perez, E., Yang, S., Liu, R., Covey, D.F., Simpkins, J.W. (2011). The assessment of non-feminizing estrogens for use in neuroprotection. *Brain Research*, 1379, 61-70.

Yu, M., Cambj-Sapunar, L., Kehl ,F., Maier, K., Takeuchi, K., Miyata, N., Ishimoto, T., Reddy, L.M., Falck, J.R., Gebremedhin, D., Harder, D.R., Roman, R.J. (2004). Effects of a 20-HETE antagonist and agonists on cerebral vascular tone. *European Journal of Pharmacology*, 486 (3), 297-306.

Zhang, W., Iliff, J.J., Campbell, C.J., Wang, R.K., Hurn, P.D., Alkayed, N.J. (2009). Role of soluble epoxide hydrolase in the sex-specific vascular response to cerebral ischemia. *Journal of Cerebral Blood Flow and Metabolism*, 29(8), 1475-1481.

Zubkov, A.Y., Rabinstein, A.A. (2009). Medical management of cerebral vasospasm: present and future. *Neurological Research,* 31, 626-631.

Zuercher, M., Ummenhofer, W., Baltussen, A., Walder, B. (2009). The use of Glasgow Coma Scale in injury assessment: A critical review. *Brain Injury*, 23 (5), 371-384.