

**AN ANALYSIS OF HAPTOGLOBIN GENOTYPES AND RECOVERY FROM  
ANEURYSMAL SUBARACHNOID HEMORRHAGE**

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**ABSTRACT**

**Background:** Haptoglobin (Hp) binds hemoglobin (Hgb), thereby inhibiting free radical production. It is presumed that Hp  $\alpha 2\text{-}\alpha 2$  genotype is associated with worse functional outcome after aneurysmal subarachnoid hemorrhage (aSAH) related to its isoform's weaker affinity for Hgb binding, decreased clearance of hemoglobin from the site of hemorrhage, and an associated increase in secondary injury.

**Objective:** The objective is to describe the relationship between haptoglobin genotype and mortality and gross functional outcome after aSAH.

**Methods:** A total sample of 268 subjects was narrowed down to a sample of 193 Caucasian subjects (due to differences in allele frequency distribution among races), age 18-75 with a diagnosis of aSAH, Fisher Grade  $\geq 2$ , DNA and outcome data available and without pre-existing chronic neurologic disease/deficit were enrolled into an ongoing study (NR004339). Demographic and medical condition variables were extracted from medical records. Modified Rankin Score (MRS) and Glasgow Outcome Score (GOS) were assessed at 3, 6, 12, and 24 months after hemorrhage. Data analysis included univariate analysis as well as multivariate logistic regression analysis, controlling for covariates including age, sex, and severity of hemorrhage (Fisher grade).

**Results:** The sample was primarily female (n=138; 71.5%) and Caucasian (n=237; 88.4%) with a mean age of 54.45 years. This sample was further narrowed down to include only subjects of Caucasian race due to differences in allele frequency distribution among other races previously published in literature. Haptoglobin 2-2 genotype was significantly correlated with MRS at 3 months post aSAH during univariate analysis (p=.04) and after controlling for covariates in the multivariate logistic regression analysis (p=.05). Univariate analysis produced a significant (p=.02) relationship between subjects whose genotypes yielded at least one  $\alpha$ -2 allele and development of cerebral vasospasm (CV). Subjects whose genotypes had only one  $\alpha$ -2 allele were significantly (p=.01) associated with Fisher grade. Fisher grade and Hunt and Hess score were both significantly associated with poor outcomes on MRS at all four time periods. Age was significantly (p=.01) correlated with Hp 1-1 and Hp 1-2 genotypes—specifically, these patients were younger than those with Hp 2-2 genotype. After controlling for covariates Fisher grade was the only covariate that maintained significance in predicting outcomes after aSAH at all four time periods.

**Conclusions:** Subjects whose genotypes contain at least one  $\alpha$ -2 allele more often had poor outcomes on MRS at 3 months post aSAH and were more likely to develop CV. Additionally, haptoglobin genotype can be used as predictor of gross functional outcome when measured using MRS at 3 months after aSAH. The Fisher grading scale and Hunt and Hess scoring system are both significantly useful for predicting outcomes (GOS, MRS, mortality) at all four time periods after aSAH.

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## **INTRODUCTION**

### **1.1 SUBARACHNOID HEMORRHAGE**

Subarachnoid hemorrhage (SAH), the deposition of blood into the subarachnoid space, affects approximately 30,000 Americans annually [1]. In approximately eighty percent of non-traumatic SAH cases, intracranial aneurysmal rupture is the cause (aSAH) [2]. Typically, SAH is associated with a 40-50% mortality rate [3, 4], while outcomes and complications such as rebleeding, increased intracranial pressure (ICP), reduction of cerebral blood flow, brain edema, vasospasm, hydrocephalus, and seizures in survivors are correlated with level of consciousness on admission, age, and the amount of blood visible in the cranium by CT scan [5]. These morbidities lead to poor outcome profiles. Delayed ischemia, a common result of aSAH is ischemic brain damage often secondary to cerebral vasospasm and is principally responsible for many of the cognitive and physical deficits after aSAH [6]. Kramer et al. found that patients with lower serum concentrations of hemoglobin (Hgb) had poorer outcomes than those patients with higher serum concentrations of Hgb in the first two weeks after aSAH [7]. It is hypothesized that the breakdown process of accumulated red blood cells releases Hgb into the cerebrospinal fluid (CSF) filled subarachnoid space and produces an environment that is conducive to free radical production, inflammation, and secondary injuries including cerebral vasospasm (CV) [8-14]. After aSAH, there is also nitric oxide deficiency that may promote

vasoconstriction, secondary injury and poor outcome after aSAH [15, 16]. CV is the leading cause of morbidity and mortality after aSAH, affecting approximately 50% of aSAH patients [13, 14, 17]. In those patients surviving the initial hemorrhage, physical disabilities and cognitive deficits are common, with only 20% of patients returning to their pre-aSAH functioning [17, 18].

## 1.2 HAPTOGLOBIN

Haptoglobin (Hp) is present in the human serum as an acute phase protein (APP) whose chief action is to bind hemoglobin (Hgb), therefore inhibiting its interaction with nitric oxide (NO) (which disables NO's ability to vasodilate) and preventing an inflammatory response and the oxidative activity that produces free radicals. Borsody and associates report that Hgb has a decreased ability to produce prostaglandins as a result of the Hp-Hgb binding [9]. The sequestration of free Hgb and, therefore, indirect disabling of prostaglandin synthesis inhibits platelet aggregation and fluctuations in blood pressure. These fluctuations result in instability of the vasoconstriction/vasodilation process, which often results in vasospasm [8-14]. In humans, production of the Hp protein is a result of the transcription and translation of the Hp gene on chromosome 16q22.1. [15, 19]. The Hp protein consists of a  $\alpha$ -chain and a  $\beta$ -chain, both made from the same gene, although only the  $\alpha$ -chain contains genetic variance resulting in either a  $\alpha$ -1 or  $\alpha$ -2 allele [15, 20]. These alleles are transcribed into similar, yet structurally and functionally different protein products. Genetic variability leads to three variations of the acute phase protein isoform: these are  $\alpha$ 1- $\alpha$ 1,  $\alpha$ 1- $\alpha$ 2, and  $\alpha$ 2- $\alpha$ 2. These three variations affect a human's ability to neutralize Hgb [9, 15]. The  $\alpha$ -2 isoform, in comparison to the  $\alpha$ -1 isoform, is associated with a

weaker affinity for Hgb, therefore generating poorer inhibition of free radical and prostaglandin production [9]. Additionally, literature suggests that the  $\alpha$ -2 isoform creates a larger Hp protein [9]. Due to the increased size of the Hp-Hgb complex formed with the  $\alpha$ -2 isoform, it is not cleared as easily [9]. Hp is expressed in various tissues, although most prominently in hepatic tissue [21].

Current literature establishes the Hp  $\alpha$ -2 variant as one with the weakest ability to bind Hgb and the more potent inducer of the inflammatory response, which may promote vasoconstriction and CV [9]. Since it is hypothesized that the  $\alpha$ -2 allele is less capable of binding Hgb and therefore inhibiting prostaglandin synthesis and other inflammatory effects, there appears to be an increased amount of red blood cells in the CSF filled subarachnoid space in patients with at least one  $\alpha$ -2 allele in their genotypes because the  $\alpha$ -2 protein product is not cleared as well from CSF. This produces an environment that is conducive to free radical production and inflammation, both of which potentiate CV. CV may lead to delayed cerebral ischemia (DCI) and its negative effects on physical and cognitive functioning. Rabinstein calls for a larger study that incorporates clinical measures of functional outcomes of aSAH is before Hp genotyping can be appropriated for use in clinical procedures[22]. We hypothesize that patients with a Hp  $\alpha$ 2- $\alpha$ 2 genotype will have poorer outcomes after aSAH. Additionally, we hypothesize that patients whose genotype consists of at least one  $\alpha$ -2 will have poorer outcomes than patients who do not possess the  $\alpha$ -2 allele in their genotypes and that these outcomes will exhibit a dose-response type association with the presence of the  $\alpha$ -2 allele.

### **1.3 PURPOSE**

The purpose of this study was to determine the correlation between the varying Hp genotypes and outcome from aSAH. Results of this study will serve multiple purposes such as providing insight into the physiologic mechanisms influencing recovery and outcome after aSAH and developing a genetic prognostic marker for use in the aSAH population.

### **1.4 SPECIFIC AIMS**

In summary, aSAH occurs when an aneurysm ruptures and expels blood into the subarachnoid space, leading to accumulation of this blood in the CSF. The degree to which patients suffer depends on multiple factors, including size of bleed (upon initial presentation of amount of blood on CT scan). Hp is an acute serum protein that functions mainly to bind Hgb, thereby promoting its clearance from the aSAH site in order to alleviate the negative effects of free Hgb such as inflammation and vasospasm. Hp genotype may influence clearance of the Hgb, which may lead to differing outcomes in patients. In order to accomplish our purpose, we established the following specific aims:

1. Describe the distributions of Hp genotypes in our aSAH population.
2. Describe the presence of Hp alleles among different races in our aSAH population.
3. Evaluate differences in gross functional outcomes after aSAH by Hp genotype in Caucasians.

4. Evaluate differences in gross functional outcomes after aSAH by Hp  $\alpha$ -2 allele presence in Caucasians.

### **1.5 RESEARCH QUESTIONS/HYPOTHESIS**

1. Is there a difference in gross functional outcome from aSAH based on Hp genotypes in Caucasians?
2. Is there a difference in gross functional outcome from aSAH based on Hp  $\alpha$ -2 allele presence in Caucasians?

We hypothesized that Caucasians harboring at least one Hp  $\alpha$ -2 allele will have poorer gross functional outcomes after aSAH.

## **2.0 BACKGROUND**

### **2.1 SUBARACHNOID HEMORRHAGE**

Subarachnoid hemorrhage (SAH) occurs when the space between the arachnoid and pia layers of the meninges becomes filled with extravasated blood, most commonly as a result of aneurysmal rupture. An aneurysm is a weakening and bulging of a vessel, which resembles a ballooning of the vessel. When an aneurysm ruptures, blood is released into the CSF filled subarachnoid space. This influx of blood can also result from blunt trauma that causes intracranial bleeding, however this source of SAH is associated with a different recovery profile compared to aSAH. SAH affects approximately 30,000 Americans annually and carries a mortality rate of 40-50% [1, 4]. Over half of those whom survive the initial injury go on to face complications such as increased ICP, CV, cerebral edema, seizures or stroke [5]. These complications are characterized by poor outcome profiles, and less than half of aSAH survivors resume normal pre-hemorrhage function by the first year of recovery [17].

#### **2.1.1 Demographics**

Aneurysmal SAH is more common among females with a peak incidence at 55 years of age on average [5]. Further risk factors for the development of aSAH are: the African-American race

[5, 23], cigarette smoking [24-27], hypertension [28], heavy alcohol use [4, 28, 29], and cocaine use [5]. Patients with a family history of first-degree relatives with aSAH are also at a higher risk [26, 28, 30-32]. Furthermore, patients who have a family history of aSAH are predisposed to suffering from the injury at a younger age than those who have no family history [31].

However, in the same study it was found that there is a greater genetic component for aSAH between siblings than between parents and children, although neither finding was significant [31].

### **2.1.2 Secondary injury: Cerebral Vasospasm**

CV is the leading cause of morbidity and mortality after aSAH, affecting approximately 50% of aSAH patients [13, 14, 17]. The risk of CV is directly proportional to the concentration of blood in the CSF, specifically in the subarachnoid space and ventricles [33]. The byproducts of hemoglobin breakdown also affect the regulation of vasodilatation/vasoconstriction processes of the cerebral vasculature [8-14]. CV can be defined angiographically and clinically.

Angiographic CV is defined as a narrowing of major cerebral arteries through which dye is infused [10]. Clinical symptoms may result from regional brain ischemia and include headache, increasing blood pressure, nausea, vomiting, photophobia, loss of consciousness, and/or new onset of acute focal deficit. Therefore, aSAH patients can be separated into three categories: (1) those who possess angiographic and clinical symptoms of CV (2) those who possess only signs of angiographic CV (3) those who exhibit neither clinical symptoms nor angiographic signs of CV (CV negative)[17, 34, 35]. CV occurs in two phases and in two presentations [17]. Acute CV occurs within hours of the initial hemorrhagic injury, and the delayed CV usually presents between four and twenty-one days later [17]. The delayed narrowing is associated with DCI and

the new onset of neurological deficits that can result from decreased delivery of oxygen to cerebral matter.

### **2.1.3 Outcomes**

Of all aSAH patients, approximately 30% die before reaching the hospital [2] and the one-year survival rate after suffering an aSAH is 30% [28]. Only 20% of patients will return to their full pre-aSAH physical and cognitive functional status, while the remaining patients will suffer from physical disability affected activities of everyday living, as well as cognitive deficits in areas of learning and long-term memory [2, 17, 18].

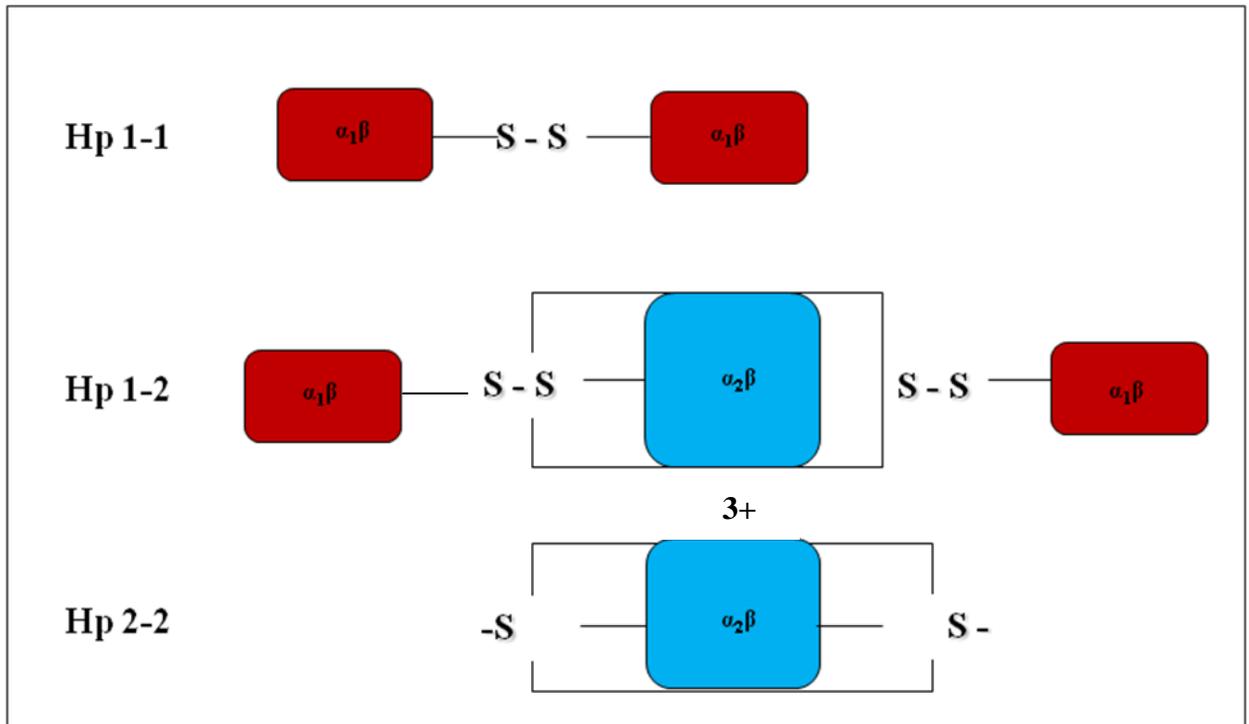
## **2.2 HAPTOGLOBIN**

### **2.2.1 Gene to Protein**

The Hp protein is coded for by a single gene on chromosome 16q22.1 and consists of two isoforms,  $\alpha$  and  $\beta$ . The  $\alpha$  isoform can produce two alleles,  $\alpha$ -1 and  $\alpha$ -2, while the  $\beta$  isoform is common to all phenotypes and exhibits no genetic variance [9, 19]. The wild type  $\alpha$ -1 allele can be further differentiated into either a  $\alpha$ -1<sup>F</sup> allele or  $\alpha$ -1<sup>S</sup> allele, which differ by their speeds of migration on an electrophoresis gel [19, 46]. The presence of a  $\alpha$ -1<sup>F</sup> allele or  $\alpha$ -1<sup>S</sup> allele results in slightly different protein structure (amino acid charge) that does not impact functioning [46]. The variant type  $\alpha$ -2 allele resulted from a crossing over of the  $\alpha$ -1<sup>F</sup> and  $\alpha$ -1<sup>S</sup> allele, and exists only in humans [15, 46]. Variability in the three genotypes,  $\alpha$ 1- $\alpha$ 1,  $\alpha$ 1- $\alpha$ 2,  $\alpha$ 2- $\alpha$ 2, leads to three possibilities for the APP phenotype: Hp 1-1, Hp 1-2, and Hp 2-2.

### 2.2.2 Protein Structure and Function

Polticelli and associates, reproduced the synthesis of the Hp protein as a single chain and the cleaving which results in a light  $\alpha$ -chain and a heavy  $\beta$ -chain[46]. Disulfide bonds link the two chains into a tetramer protein consisting of two  $\alpha/\beta$  dimers [15, 19, 46]. Therefore, the Hp 1-1 protein consists of a  $\alpha_1\beta$  dimer, the Hp 1-2 protein consists of two  $\alpha_1\beta$  units as well as at least one  $\alpha_2\beta$  unit, and the Hp 2-2 protein is a polymer consisting of at least three repeating  $\alpha_2\beta$  units (see Figure 1) [19]. It is believed that the  $\alpha$ -2 allele originated in India, while the  $\alpha$ -1 shows a stronger presence in Europe and Africa [19]. The  $\alpha$ -1 allele is less common in people of Asian descent [19].



**Figure 1.** Hp protein structure

**2.2.2.1 Haptoglobin in inflammation.** The Hp protein is a member of the family of APPs that are expressed in the liver in response to the inflammatory response in order to restore homeostasis and express their anti-inflammatory effects [19, 21]. Hp is a type 2 APP because it is activated in response to these IL-6 cytokines, the primary responders to the inflammatory response [21]. Prostaglandin synthesis is stimulated by free Hgb and this process leads to platelet aggregation and blood pressure fluctuations. Hp's binding affinity for Hgb inhibits the prostaglandin synthesis, thereby indirectly inhibiting the secondary effects.

**2.2.2.2 Haptoglobin as an anti-oxidant.** Iron can act as a catalyst for the generation of free radicals from free Hgb. Free radicals are capable of causing oxidative stress to the tissues and vasculature in the body [19]. These highly reactive species are inhibited when Hp binds Hgb, thereby Hp acts as an anti-oxidant. Hp 2-2 appears to have a decreased ability to act as an anti-oxidant, in comparison with Hp 1-1 or Hp 1-2, most likely related to its decreased affinity for hemoglobin binding [19]. Additionally, the larger Hp 2-2 protein may be unable to penetrate vasculature and tissues and is associated with decreased Hgb clearance because of its larger size.

**2.2.2.3 Haptoglobin in subarachnoid hemorrhage.** Hp's main job is to bind free hemoglobin and facilitate its clearance via the CD163 pathway and the reticuloendothelial system, specifically the spleen. The CD163 receptor is specific for the Hp:Hgb complex and is expressed only by macrophage and monocytes during inflammation [46]. The CD163 receptor binds the Hp:Hgb complex at Hp's  $\beta$ -chain and has a binding affinity for the complex twice that of the binding affinity for either Hp or Hgb individually, and the CD163 receptor exhibits the following binding affinity for Hp proteins: Hp 2-2 > Hp 1-2 > Hp 1-1 [46]. The Hp 1-1 protein is the smallest and exhibits the highest binding affinity for Hgb, unlike Hp 2-2, the largest of the three proteins

with the lowest binding affinity for Hgb [15, 19, 21]. Therefore, there may be a correlation between  $\alpha$ -2 alleles, phenotype, and outcomes in patients who suffer from injuries that result in overwhelming amounts of free Hgb in the body, such as in aSAH. Although the  $\alpha$ -1 allele promotes increased clearance of Hgb from the CSF and diminishes the negative effects of free Hgb such as inflammation and CV, the  $\alpha$ -1 allele is associated with decreased surface area for binding Hgb as compared to the  $\alpha$ -2 allele and is also associated with an increased ability to pass through the glomerulus and cause kidney damage, both due to the smaller size of the protein generated by the  $\alpha$ -1 allele [15, 21].

### 2.3 SUMMARY

In summary, aSAH occurs after an aneurysm ruptures leading to an influx of Hgb into the CSF. The accumulation of blood in the CSF can lead to inflammation and CV. Hp is an APP present in the serum of humans that is responsible for binding Hgb and inhibiting the oxidative stress that can result from its interaction with vessels. Hp genotype may influence clearance of Hgb, and therefore outcomes after aSAH.

The purpose of this study was to determine the correlation between the varying Hp genotypes and outcomes after aSAH. Results of this study will serve multiple clinical purposes such as providing insight into the physiologic mechanisms influencing recovery and outcome after aSAH and will facilitate the development of a genetic, prognostic marker(s) for use in the aSAH population. To accomplish this goal, we will address the following specific aims:

1. Describing the distributions of Hp genotypes in our aSAH population.
2. Describing the presence of Hp alleles among different races in our aSAH population.

3. Evaluating differences in gross functional outcomes after aSAH by Hp genotype in Caucasians.
4. Evaluating differences in gross functional outcomes after aSAH by Hp  $\alpha$ -2 allele presence in Caucasians.

Furthermore, we will test the following research questions:

1. Is there a difference in gross functional outcome from aSAH based on Hp genotypes in Caucasians?
2. Is there a difference in gross functional outcome from aSAH based on Hp  $\alpha$ -2 allele presence in Caucasians?

The review of literature as well as our own research questions led us to hypothesize that Caucasians harboring at least one Hp  $\alpha$ -2 allele will have poorer gross functional outcomes after aSAH.

## **3.0 METHODS**

### **3.1 DESIGN**

This retrospective study used a between-group, within-subject design to examine gross functional outcome at three, six, twelve, and twenty-four months after the aSAH in 193 individuals (with and without CV) who were classified by Hp genotype. This study was part of a larger parent study funded by the NIH grant NR004339 (Co-PI's Sherwood & Poloyac).

### **3.2 SAMPLE**

The University of Pittsburgh Medical Center admits approximately 60 patients per year, 60% women and 9% African American, for treatment of severe aSAH. These demographics are consistent with the general population of patients who are affected by aSAH. All patients admitted to the Neurovascular Intensive Care Unit were screened for eligibility for this study based on the following criteria.

Inclusion criteria:

1. 18-75 years of age
2. Diagnosis of aSAH (Hunt and Hess grade  $\geq 3$  or Fisher grade  $\geq 2$ ) verified by CT scan

3. Placement of a CSF drainage catheter within 3 days of SAH
4. Outcome data and DNA available

Exclusion criteria:

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

In order to accurately conduct this study, the parent study and any further analyses, it was necessary to exclude children because the incidence of aSAH in children is <.001% [47]. Additionally, the study excluded patients with a pre-existing, neurological disease or deficit to reduce the risk of any biased outcome assessments. Patients who had an aSAH graded as Hunt and Hess  $\geq 3$  and/or Fisher grade  $\geq 2$  have an increased risk of CV. Exclusion of patients with SAH from non-aneurysmal sources occurred because this population has a different course of recovery.

### **3.3 SETTING**

Subjects were recruited after admission to the Neurovascular Intensive Care Unit (NVICU), Presbyterian University Hospital (PUH), University of Pittsburgh Medical Center (UPMC), Pittsburgh, Pennsylvania. This 20 bed unit is specializes in acute care for patients after neurological injury.

### **3.4 RECRUITMENT**

This study was conducted as a retrospective, secondary analysis of data collected during a parent study funded by NIH/NINR RO1 NR04339 (Co-PI's Sherwood & Poloyac). Nursing staff accompanied project personnel during daily rounds on the NVICU to review potential patients for eligibility criteria. Once eligibility was established, the bedside nurse approached the patient or the patients' families and verified if the study personnel could present the study to them. If the patient/representative was willing, the nurse would introduce the study personnel, after which the study personnel would present the ongoing research study, and obtain informed consent. This study as well as the parent study were both reviewed and approved by the University of Pittsburgh Institutional Review Board. Removal of all patient identifiers from medical records and samples was done to assure subject confidentiality. Furthermore, a nonidentifying study number was provided to link the sample(s) obtained to the medical record data. The PI of this study (Kantor) received de-identified data, labeled with the study ID number only, for the purpose of statistical analysis.

### **3.5 STANDARD MEDICAL CARE**

All individuals presenting to the UPMC PUH with aSAH are admitted to the NVICU. Upon admission, a CT scan of the head is performed and Hunt and Hess grade and Fisher grade are assigned by the neurosurgeon or a neuroradiologist. To identify aneurysm presence and perform interventional procedures (such as coil embolization), cerebral angiography is conducted. Depending on the preferred treatment for the patient (as determined by the attending

neurosurgeon) either embolization or surgical clipping is performed as soon as possible. In the NVICU, monitoring of the SAH patient includes continuous arterial blood pressure (ABP), central venous pressure (CVP), pulse oximetry, respiratory rate and cardiac rate and rhythm. Mean arterial blood pressure (MAP) is maintained with anti-hypertensive or vasopressor medications. Complete neurologic exam was conducted every 1-2 hours for the first 24 hours after admission and then every 2-4 hours until discharge from the NVICU, as per clinical need. Placement of an intraventricular drain occurs if ICP for continuous monitoring and/or CSF drainage is necessary for clinical care. Temperature is monitored rectally or orally every two hours. Maintaining fluid balance is accomplished by administering fluid bolus. The head of the bed is elevated thirty degrees. Nimodipine (Nimotop) is administered at a dose of 60mg every 4 hours for 14 days as tolerated to decrease risk of CV as secondary injury. Anti-convulsants are administered to decrease risk of seizure activity after a brain injury such as SAH. Sedatives are administered as needed for agitation.

### **3.6 DATA COLLECTION**

Data regarding demographics and medical condition of subjects were extrapolated from medical records and recorded by project personnel onto data collection sheets. These sheets were electronically transmitted directly into databases using Teleform, an automated data entry and verification system. The project data was stored in a locked office in the University of Pittsburgh School of Nursing. Cerebrospinal fluid (CSF) samples were drawn at least once daily using the UPMC ventriculostomy drain bag change standard procedure. Specimens were drawn only while the drainage catheter was in place for routine medical care. CSF was sampled from the bag

during bag exchange, and distributed evenly into three-1 ml tubes and placed into a -80°C freezer located in the laboratory on the second floor of the Victoria Building, School of Nursing, University of Pittsburgh. Blood samples (3 mL) were drawn upon enrollment and deoxyribonucleic acid (DNA) extracted. Specimens were analyzed and DNA extraction and amplification (using polymerase chain reaction (PCR)) was performed by the laboratory technicians in Dr. Conley's molecular genetics laboratory under the supervision and guidance of Dr. Conley.

### **3.7 MEASUREMENT**

The study independent variable was the Hp genotype. Gross functional outcome was the dependent variable. Data on the severity of injury and demographic characteristics (age, sex, race) was collected for potential inclusion in the investigation as covariates.

#### **3.7.1 Independent variable: Haptoglobin genotyping**

The following DNA extraction and genotyping procedures were utilized to determined Hp genotype from the CSF or blood samples.

**3.7.1.1 DNA Extraction.** The CSF specimens were quick thawed in small batches. Blood specimens were processed within 48 hours of collection. DNA was extracted from blood using a simple salting out procedure as described by Miller and colleagues [48]. DNA was extracted from CSF using the Qiaamp extraction kit and provided protocols from Qiagen Corporation.

**3.7.1.2 Genotyping procedure.** Quantitative real-time polymerase chain reaction (qRT-PCR) was utilized to generate genotypes and evaluate relative copy number of the Hp  $\alpha$ -2 allele. Primers and probes were designed to multiplex amplify the region containing the duplication that identifies the Hp  $\alpha$ -2 allele as well as a region 5' to the gene as a control measurement for relative comparisons. qRT-PCR was conducted using Taqman technology using ABI7000 and SDS 2.0 software (Applied Biosystems Incorporated, Foster City, CA). Raw data was analyzed using the  $\Delta\Delta C_t$  method.

**3.7.1.3 Dichotomization of the sample.** The sample was dichotomized based on Hp  $\alpha$ -2 allele presence in two ways. First, subjects were classified into two groups: the first was a Hp  $\alpha$ -2 positive group, which included subjects with Hp 1-2 and Hp 2-2 genotype and the second was a Hp  $\alpha$ -2 negative group, which included only subjects with Hp 1-1 genotype. The second dichotomization was done to separate subjects who possessed a  $\alpha$ -1 allele in their genotypes. Therefore, we again created two groups: one group consisted of subjects with Hp 1-1 and Hp 1-2 genotypes while the other group consisted of only those subjects with Hp 2-2 genotype. However, we still used all three genotypes, Hp 1-1, Hp 1-2, Hp 2-2, in our analyses.

## **3.7.2 Dependent variable: Gross functional outcome**

Outcomes were measured using the Glasgow Outcome Score (GOS), Modified Rankin Scale (MRS), and mortality at 3, 6, 12, and 24 months after aSAH. Mortality was extracted from medical records, attending physician communication, and/or MRS. A trained Neuropsychological technician obtained all outcome data. Assessments were completed during a face-to-face interview in the outpatient Neurosurgery clinic. If the subject was unable to travel, GOS and MRS were obtained by telephone interview with the primary caregiver. As this data was completed independently of the current project, the neuropsychological technician was blinded to genotyping results.

### **3.7.2.1 Clinical outcome scales.**

**(a) Glasgow outcome scale.** The Glasgow outcome scale (GOS), not to be confused with the Glasgow coma score (GCS), is a clinical observation scale used for assessing consciousness and categorizes functional outcomes into five levels with a score of 1 meaning death and a score of 5 meaning good recovery [40](see Figure 2). GOS was dichotomized into good outcomes (scores 4, 5) and poor outcomes (scores 1-3). This scale has interrater reliability established between 68% and 95% with kappa values from .62 and .79 [41, 42]. This inter-rater reliability falls within the acceptable range when assessment guidelines are followed, interviews are structured, and examiners are trained [41].

Score	Description
1	Dead
2	Persistent vegetative state. Patient exhibits no obvious cortical function.
3	Severe disability. (Conscious but disabled). Patient depends upon others for daily support due to mental or physical disability or both
4	Moderate disability. (Disabled but independent). Patient is independent as far as daily life is concerned. The disabilities found include varying degrees of dysphasia, hemiparesis, or ataxia, as well as intellectual and memory deficits and personality changes.
5	Good recovery. Resumption of normal activities even though there may be minor neurological or psychological deficits.

**Figure 2. Glasgow outcome scale**

**(b) Modified Rankin Scale.** The Modified Rankin Scale (MRS), a seven grade clinical observation scale, categorizes functional outcomes into levels that describe disability or the level of dependence that patients exhibit during

activities of daily living. This scale ranges from a score of 0 meaning no symptoms to a score of 6 meaning death [43](see Figure 3). MRS was dichotomized into good outcomes (scores 0-3) and poor outcomes (scores 4-6). Inter-rater reliability for MRS has been established at kappa value between .25 and .71 [44, 45].

Score	Description of symptoms
0	No symptoms
1	No significant disability. Able to carry out all usual activities, despite some symptoms.
2	Slight disability. Able to look after own affairs without assistance, but unable to carry out all previous activities.
3	Moderate disability. Requires some help, but able to walk unassisted.
4	Moderately severe disability. Unable to attend to own bodily needs without assistance, and unable to walk unassisted.
5	Severe disability. Requires constant nursing care and attention, bedridden, incontinent.
6	Dead

**Figure 3. Modified Rankin Scale**

(c) **Mortality.** Mortality was defined as a MRS score of 6.

### **3.7.3 Covariate: severity of injury**

The Fisher Grade and the Hunt and Hess grading scale were used to determine severity of aSAH. The Fisher grading scale (see Figure 4) is used to appreciate the presentation of blood from aSAH on a CT scan. Although the scoring ranges from 1 (no hemorrhage evident) to 4 (any thickness with intraventricular hemorrhage or parenchymal extension), this scale is nominal because patients who are graded as a 3 using the Fisher grading scale at highest risk for developing CV and poorer outcomes after aSAH [33]. Ogilvy and associates determined that the Fisher has excellent interrater reliability with kappa value of 0.9 [49]. An association has been found between higher Fisher scores and CV after SAH [50].

<b>Fisher grade</b>	<b>CT scan findings</b>	<b>Cerebral vasospasm risk</b>
<b>1</b>	No subarachnoid blood noted	Low risk
<b>2</b>	Diffuse or vertical layers of blood <1mm thick	Moderate risk
<b>3</b>	Localized and/or vertical layers $\geq$ 1mm thick (dense blood in fissures and basal cisterns)	High risk
<b>4</b>	Intracerebral or intraventricular clot with diffuse or no subarachnoid blood (no blood in basal cisterns)	Low risk

**Figure 4. Fisher grading scale.**

The Hunt and Hess grading scale (see Figure 5) is used to classify aSAH patients based on their presenting clinical condition. The scale ranges from a score of 0 (unruptured aneurysm) and 1 (asymptomatic) to a score of 5 (coma) [51]. Oshiro et al. found the Hunt and Hess grading scale to have good inter-rater reliability a kappa value of 0.41 (p=.0005) [52]. Gruber et al. found correlation between higher Hunt and Hess and infarct related to CV [53].

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

**Figure 5. Hunt and Hess grading scale**

#### **3.7.4 Covariate: demographic characteristics**

Demographics including age, sex, and race were recorded upon entry into the study and this information was collected from the medical record.

### **3.8 DATA MANAGEMENT**

To ensure confidentiality, each subject was assigned a unique identification code upon admission to the parent studies. The unique identification code linked data were entered into SPSS and SAS databases. Demographic data and severity of injury were entered electronically. CV status and Hp genotype were hand entered and verified. Outcome data were entered and verified electronically. Demographic data, severity of injury, and Hp genotype databases were merged with outcome data (functional and neurological) at three, six, twelve, and twenty-four months after aSAH (if available).

### **3.9 DATA ANALYSIS PLAN**

All analyses were conducted using SPSS version 17.0 [SPSS Inc, Chicago, Ill.] or SAS version 9.2 [SAS Institute Inc, Cary, North Carolina].

### **3.9.1 Preliminary data analysis**

For descriptive purposes, measures of central tendency and dispersion were obtained for all variables at all time points. Exploratory data analytic techniques were completed for identification of missing data (and specific patterns) and identification of potential confounding variables. Preliminary analysis was done to [a] identify covariates(s) or predictor variables not identified a priori; [b] explore distributions of variables and [c] describe demographic and medical condition data. Although it was found that CV was significantly correlated with  $\alpha$ -2 allele presence, CV was not included in our model because it was not found to predict outcome in the univariate analyses (see results section 4.1.3). An alpha level of .05 was considered significant for all analyses.

### 3.9.2 Analysis of specific aims

1. Specific Aim #1. Describe the distributions of Hp genotypes in our aSAH population.

To address specific aim #1, we obtained frequencies and percentages of Hp genotypes.

2. Specific Aim #2. Describe the presence of Hp alleles among different races in our aSAH population.

To address specific aim #2, we obtained frequencies and percentages of Hp genotypes by race and dichotomized the sample into groups based on Hp  $\alpha$ -2 allele presence.

3. Specific Aim #3. Evaluate differences in gross functional outcomes after aSAH by Hp genotype in Caucasians.

To address specific aim #3, we compared gross functional outcome (GOS, MRS, and mortality) in different genotype groups with Chi-square analysis. After identification of covariates via univariate analysis we conducted multivariate logistic regression analysis to determine the relationship between Hp genotype and categorical outcome measures while controlling for covariates.

4. Specific Aim #4. Evaluate differences in gross functional outcomes after aSAH by Hp  $\alpha$ -2 allele presence in Caucasians.

To address specific aim #4, we compared gross functional outcome (GOS, MRS, mortality) in different genotype groups with Chi-square analysis. After identification of covariates via univariate analysis, we conducted multivariate logistic regression analysis to determine the relationship between Hp  $\alpha$ -2 allele presence and categorical outcome measures while controlling for covariates.

## **4.0 RESULTS**

### **4.1 SAMPLE DESCRIPTION**

The sample of 193 subjects included in specific aims 3 and 4 had a mean age of 54.45 years (range 18 to 75;  $SD\pm 11.1$ ). For analysis of specific aims 3 and 4, the sample was limited only to Caucasian subjects because of the differences in allele frequency distribution by race in previously published literature and insufficient number of subjects of other races in our sample. This narrowed down sample was primarily female ( $n=138$ ; 71.5%), which is representative of the general aSAH patient population. Severity of hemorrhage was assessed by the Fisher grade and ranged from 2-4 with a mode of 3 ( $n=103$ ; 53.4%) (See Table 2); clinical presentation upon admission was measured using the Hunt and Hess score and ranged from 1-5 with a mode of 3 ( $n=65$ ; 33.7%) (See Table 4). Table 1 and Table 3 represent Fisher grade and Hunt and Hess score, respectively, in the entire patient population admitted into the study before excluding all races except Caucasian.

**Table 1. Severity of injury characteristics of entire sample: Fisher grade**

<b>Fisher grade</b>	<b>Frequency</b>	<b>Percent</b>
(1): No subarachnoid blood noted	1	0.4
(2): Diffuse or Vertical Layers of Blood < 1 MM	83	31.0
(3): Localized Clot and / or Vertical Layers of Blood > 1 MM	134	50.0
(4): Intracerebral or Intraventricular Clot with Diffuse or No SAH	50	18.7
Total	268	100.0

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

<b>Fisher grade</b>	<b>Frequency</b>	<b>Percent</b>
(2): Diffuse or Vertical Layers of Blood < 1 MM	54	28.0
(3): Localized Clot and / or Vertical Layers of Blood > 1 MM	103	53.4
(4): Intracerebral or Intraventricular Clot with Diffuse or No SAH	36	18.7
Total	193	100.0

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

<b>Hunt and Hess score</b>	<b>Frequency</b>	<b>Percent</b>
(1): Asymptomatic or mild headache with slight nuchal rigidity	40	14.9
(2): No neurological deficit (other than cranial nerve palsy), moderate to severe headache, nuchal rigidity	74	27.6
(3): Mild focal deficit, lethargy or confusion	96	35.8
(4): Stupor, moderate-severe hemiparesis	43	16.0
(5): Deep coma, decerebrate rigidity, moribund appearance	15	5.6
Total	268	100.0

**Table 4. Severity of injury characteristics of Caucasian sample: Hunt and Hess grade**

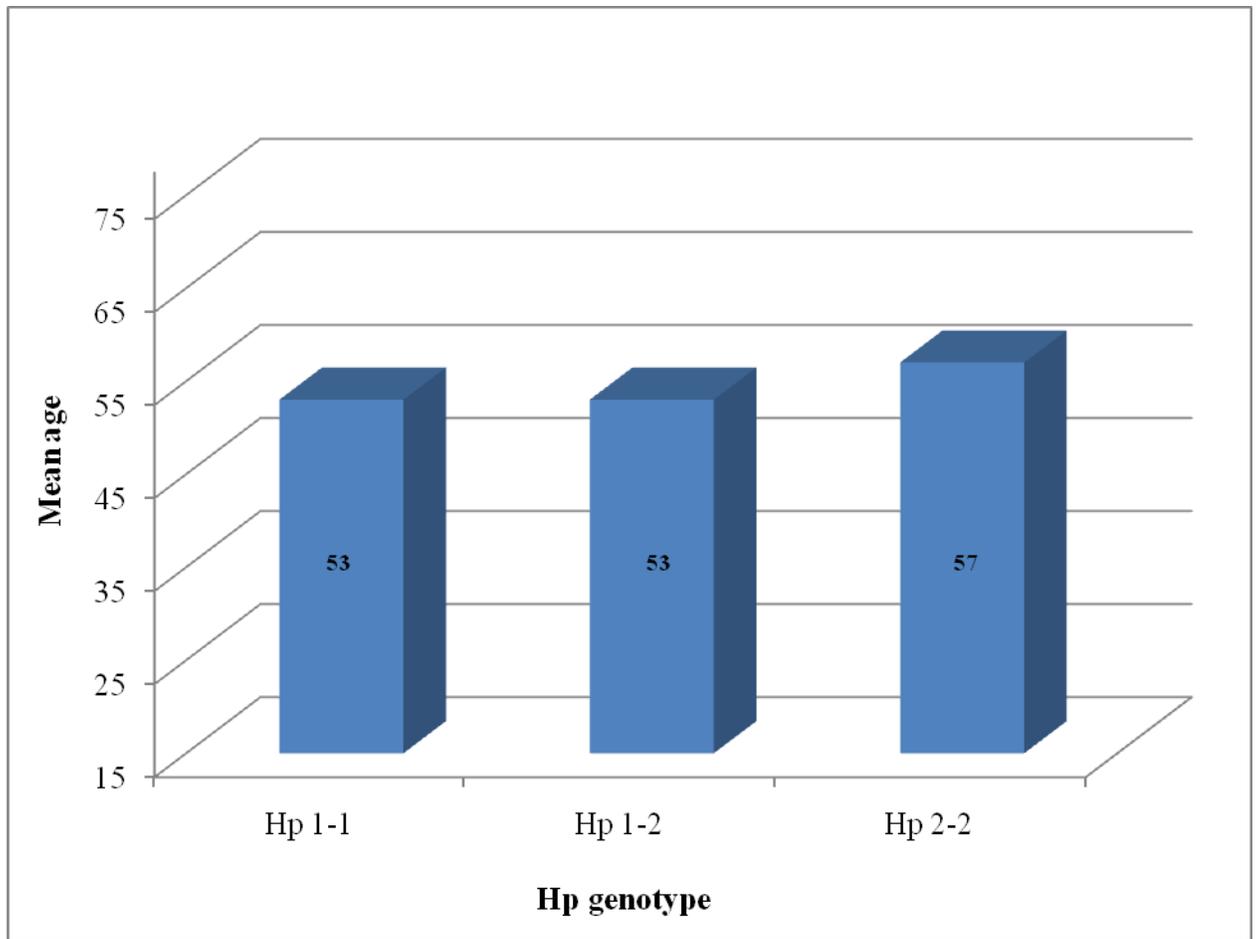
<b>Hunt and Hess score</b>	<b>Frequency</b>	<b>Percent</b>
(1): Asymptomatic or mild headache with slight nuchal rigidity	31	16.1
(2): No neurological deficit (other than cranial nerve palsy), moderate to severe headache, nuchal rigidity	56	29.0
(3): Mild focal deficit, lethargy or confusion	65	33.7
(4): Stupor, moderate-severe hemiparesis	29	15.0
(5): Deep coma, decerebrate rigidity, moribund appearance	12	6.2
Total	193	100.0

#### **4.1.1 $\alpha$ -2 allele presence and Fisher grade**

When conducting a univariate analysis of the sample, it was found that a statistically significant ( $p=.01$ ) relationship exists between Fisher grade and subjects who have a Hp genotype in which at least one copy of the  $\alpha$ -1 allele exists (Hp 1-1, Hp 1-2). Subjects who had two  $\alpha$ -1 alleles most often had a Fisher grade of 2, while those who harbored only one  $\alpha$ -1 allele (Hp 1-2) more often had a Fisher grade of 3.

#### **4.1.2 $\alpha$ -2 allele presence and age.**

During the univariate analysis we found that age is significantly ( $p=.01$ ) correlated with genotypes in that individuals with at least one copy of the  $\alpha$ -1 allele (Hp1-1 and Hp1-2) were younger. In other words, there was an increased frequency of the Hp  $\alpha$ 2-  $\alpha$ 2 genotype presentation in the older subjects within our sample. Figure 6 (below) shows the distribution of Hp genotype by mean age of the Caucasian sample.



**Figure 6. Hp genotype by mean age in the Caucasian population**

#### **4.1.3 $\alpha$ -2 allele presence and CV**

Subjects whose genotypes contained at least one  $\alpha$ -2 allele (Hp 1-2 and Hp 2-2) were significantly ( $p=.02$ ) more likely to develop CV. We selected subjects who exhibited moderate or severe angiographic vasospasm for inclusion into the positive CV category.

## 4.2 VARIABLES

### 4.2.1 Independent variable: Haptoglobin genotype

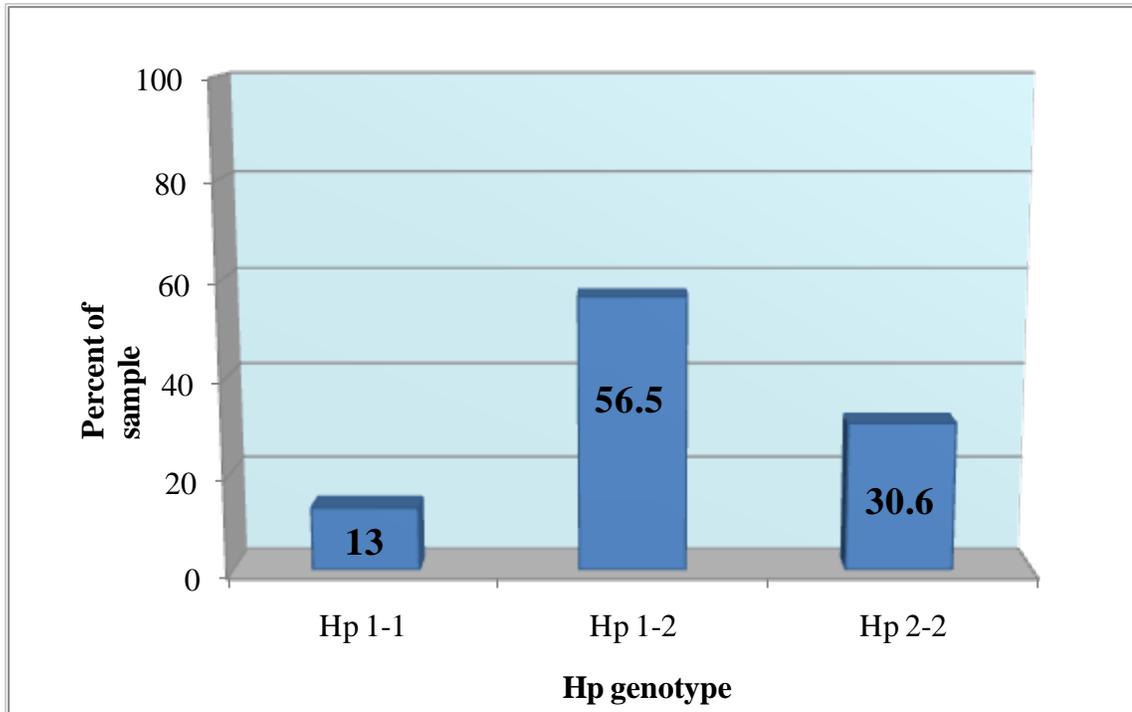
In this sample 168 subjects (87.0%) had a  $\alpha$ -2 allele present (see Figure 9). By comparing Figure 9 and Figure 10, disparities are seen between  $\alpha$ -2 allele presence in Caucasians versus  $\alpha$ -2 allele presence in African Americans. The sample was dichotomized for analysis based on the presence of the  $\alpha$ -2 allele. There were no significant differences between individuals in categories of sex or Hunt and Hess score neither based on Hp genotype nor on  $\alpha$ -2 allele presence. Refer to Table 6 for comparison of demographic and severity of hemorrhage scores by the presence/absence of a  $\alpha$ -2 allele in the Hp genotype in the Caucasian sample. Figure 7 provides the genotype distribution of the Caucasian sample. Figure 8 depicts the distribution of the Hp genotype in the African American population to show that the African American population has more subjects with Hp 1-1 genotype and less with Hp 2-2 genotype than the Caucasian population does. See Table 5 for a comparison of genotype distribution by race in the population of this study.

**Table 5. Demographic information and severity of injury characteristics of the entire sample by Hp genotype**

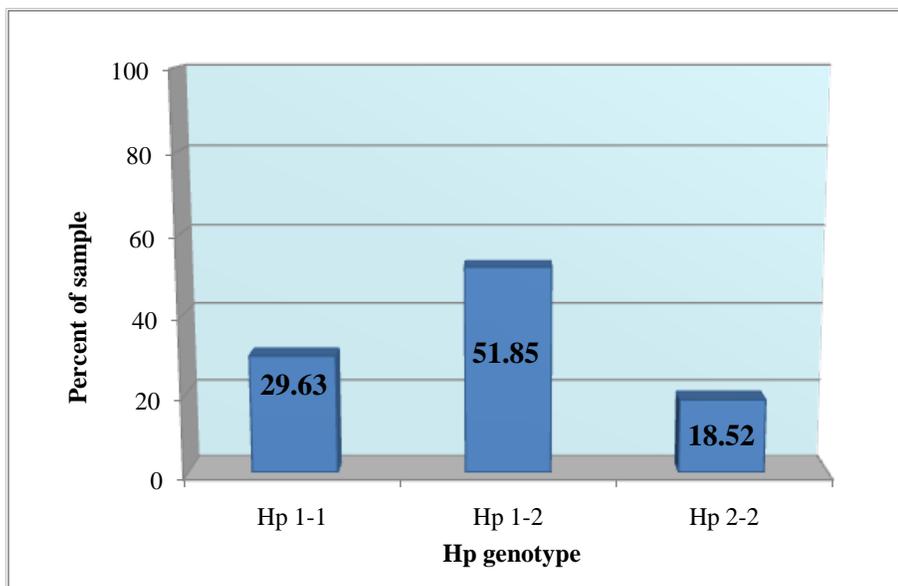
	<b>Caucasian</b>	<b>African American</b>	<b>Hispanic</b>	<b>Asian/Pacific Islander</b>
	<b>n</b>	<b>n</b>	<b>n</b>	<b>n</b>
	<b>(%)</b>	<b>(%)</b>	<b>(%)</b>	<b>(%)</b>
<b>Hp 1-1</b>	27 (11.39)	8 (29.63)	1 (33.33)	0 (0.00)
<b>Hp 1-2</b>	131 (55.27)	14 (51.85)	1 (33.33)	0 (0.00)
<b>Hp 2-2</b>	79 (33.33)	5 (18.52)	1 (33.33)	1 (100)
<b>Total</b>	237	27	3	1
<b>(N=268)</b>	(88.4)	(16.07)	(53.40)	(33.70)

**Table 6. Hp genotype distribution by race of the Caucasian patient population**

	<b>Age</b>	<b>Gender: female</b>	<b>Fisher grade</b>	<b>Hunt and Hess grade</b>
	<b>Mean</b>	<b>n</b>	<b>Mode</b>	<b>Mode</b>
	<b>(SD)</b>	<b>(%)</b>	<b>(%)</b>	<b>(%)</b>
	p=.01	p=1.00	p=.05	p=.40
	F= 6.63	$\chi^2=.99$	$\chi^2=.06$	$\chi^2=.41$
<b>Hp 1-1</b> <b>(n= 25)</b>	52.96 (10.86)	17 (68.00)	2/3 (40.00/40.00)	2 (44.00)
<b>Hp 1-2</b> <b>(n=109 )</b>	53.26 (11.11)	78 (71.60)	3 (51.40)	3 (36.70)
<b>Hp 2-2</b> <b>(n= 59)</b>	57.27 (10.96)	43 (72.90)	3 (62.70)	2/3 (25.40/25.40)
<b>Total</b> <b>(N=193)</b>	54.45 (11.14)	138 (71.5)	3 (53.40)	3 (33.70)



**Figure 7. Distribution of the Caucasian sample by Hp genotype**



**Figure 8. Distribution of the African American sample by Hp genotype**

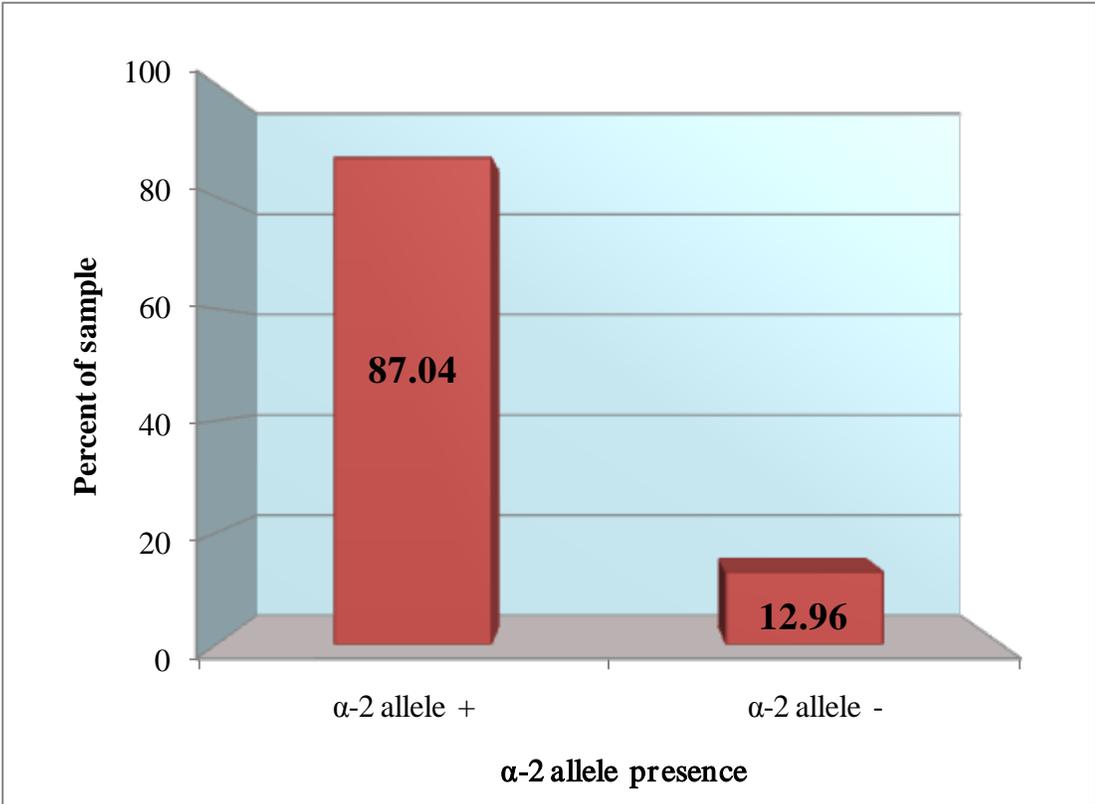
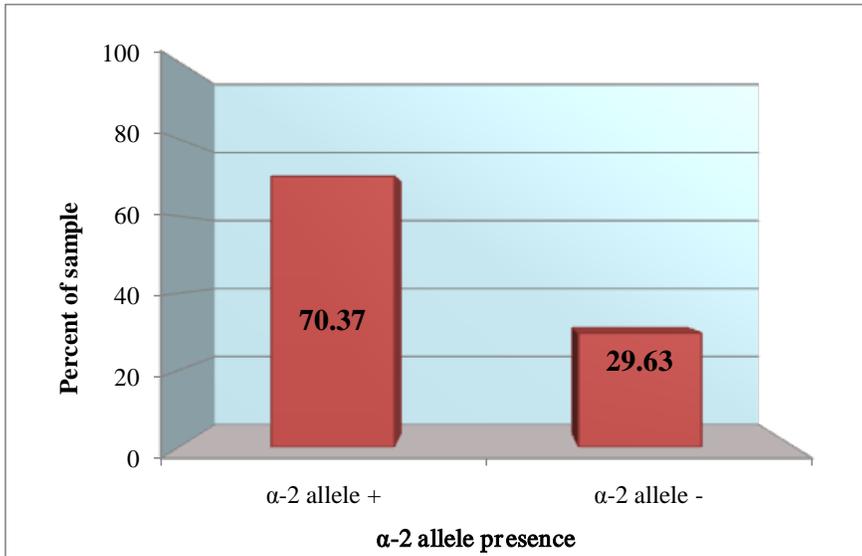


Figure 9.  $\alpha$ -2 allele presence in the Caucasian sample



**Figure 10.  $\alpha$ -2 allele presence in the African American sample**

#### **4.2.2 Dependent variable: gross functional outcome**

The dependent variable, gross functional outcome (assessed using GOS, MRS, and mortality) was analyzed at four time periods: 3, 6, 12, and 24 months after aSAH. At 3 months post aSAH there were 193 subjects with outcome data available; at 6 months post aSAH this number decreased to 160 subjects for whom outcome data was collected; at 12 months post aSAH there remained 146 subjects with outcome data available; and at 24 months post aSAH there were 121 remaining subjects for whom outcome data was successfully obtained.

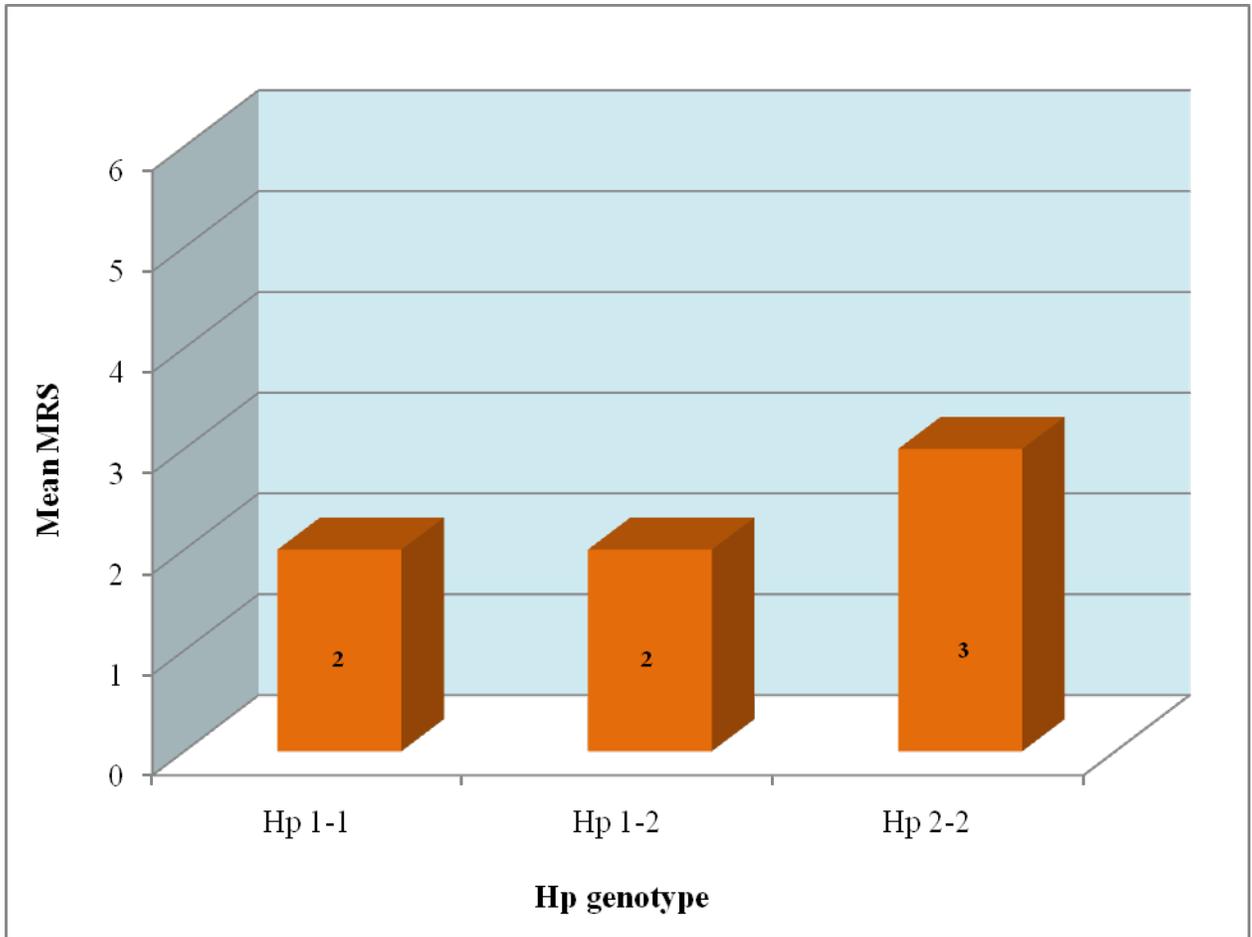
## 4.3 RESEARCH QUESTIONS

### 4.3.1 Research question #1

1. Is there a difference in gross functional outcome from aSAH based on Hp genotypes?

#### 4.3.1.1 Univariate analysis.

**(a) Hp genotype and MRS.** At 3 months after aSAH it was found that the association between Hp genotype and MRS was statistically significant ( $p=.04$ ). Specifically, individuals with Hp 1-1 genotype less often had poor outcomes on the MRS at 3 months post aSAH. Figure 11 below demonstrates the dose-response type association between Hp genotype and mean MRS.



**Figure 11. Hp genotype and mean MRS**

**(b) Hp genotype and GOS.** Hp genotype did not show a significant relationship with GOS at any of the three time periods. Refer to Table 7 below for p values.

**Table 7. Hp genotype and GOS**

	3 months	6 months	12 months	24 months
GOS	p=.30	p=.43	p=.38	p=.34

(c) **Hp genotype and mortality.** Similar to the relationship between Hp genotype and GOS, mortality was not significantly associated with Hp genotype at any time period assessed. Table 8 displays p values for this analysis.

**Table 8. Hp genotype and mortality**

	3 months	6 months	12 months	24 months
Mortality	p=.41	p=.43	p=.39	p=.34

(d) **Fisher grade and MRS.** Significance was detected in the correlation between Fisher grade and MRS at all four time periods. See Table 9 for obtained results for all time periods. Individuals with Fisher grade of 2 and 3 more often had poor outcomes on the MRS.

(e) **Hunt and Hess score and MRS.** Similar to the association between Fisher grade and MRS, there was a significant relationship between the Hunt and Hess score and MRS throughout all time periods following aSAH. See Table 9 for clarification based on time period. Subjects with at least one  $\alpha$ -2 allele

in their genotypes more often received a score of 3 on Hunt and Hess had poor outcomes on the MRS.

**Table 9. Statistical significance (p) of univariate analysis of the relationship between MRS and Hunt and Hess score and Fisher grade**

	3 months	6 months	12 months	24 months
Hunt and Hess	p=<.0001	p=<.0001	p=<.0001	p=.0003
Fisher	p=<.0001	p=<.0001	p=.003	p=.03

#### 4.3.1.2 Multivariate Logistic Regression Analysis.

**(a) Gross functional outcome and Hp genotype.** At 3 months after aSAH it was found that the association between Hp genotype and MRS was statistically significant (p=.05). Specifically, individuals with Hp 2-2 genotype more often had poor outcomes on the MRS at 3 months post aSAH when controlling for covariates. See Table 11 for the multivariate logistic regression analysis of the significant relationship between Hp genotype and MRS at all four time periods. Refer to Figure 12 for mortality by Hp genotype.

**(b) Gross functional outcome and Fisher grade.** After controlling for covariates (age, sex, Fisher grade) all predictors of outcome dropped out of significance in determining gross functional outcome (GOS, MRS, mortality)

except for the Fisher grade. See Table 10 for results obtained from analysis by time period.

**Table 10. Statistical significance (p) of multivariate logistic regression analysis of the relationship between gross functional outcome and Fisher grade**

Measure of gross functional outcome		MRS (p)				GOS (p)				Mortality (p)			
		3	6	12	24	3	6	12	24	3	6	12	24
Predictor													
Fisher grade	2	<.0001	.0004	.004	.04	.001	.001	.0003	.001	.002	.001	.0003	.001
	3	.07	.03	.07	.03	.008	.002	.002	.006	.01	.002	.002	.006

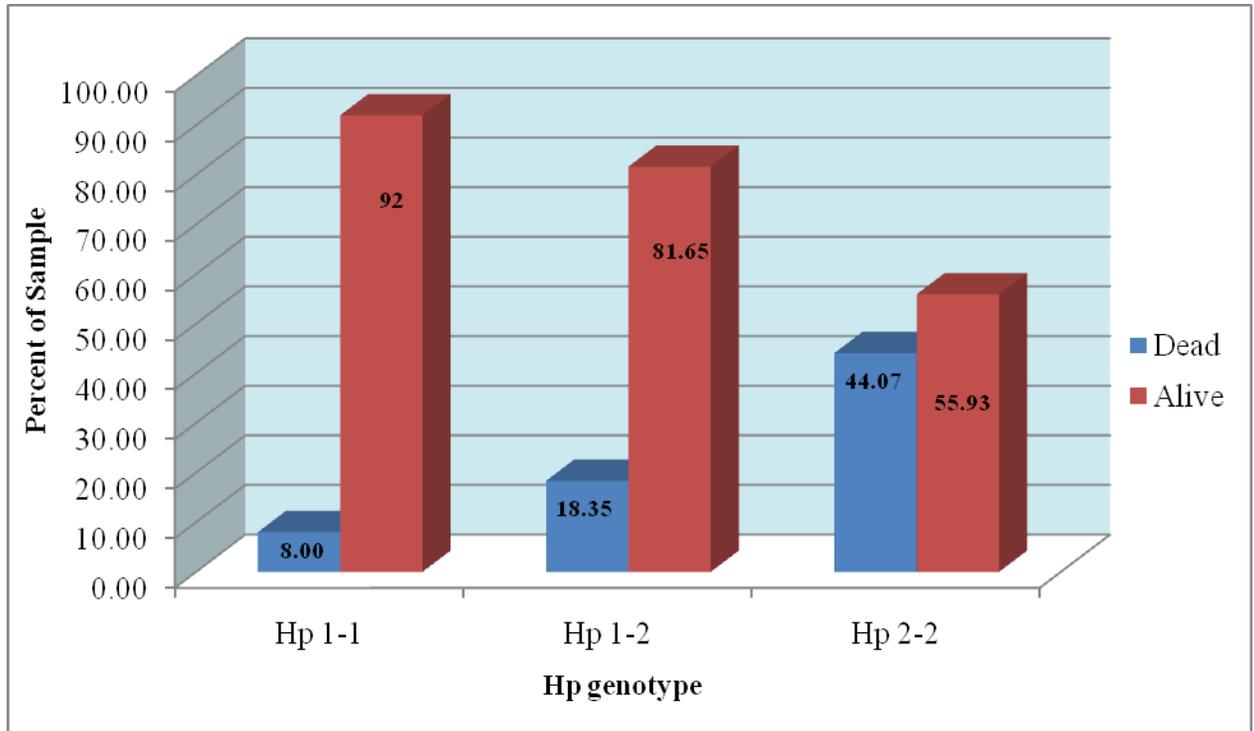
**Table 11. Statistical significance (p) of multivariate logistic regression analysis of the relationship between gross functional outcome and Hp genotype**

	MRS			
	(p)			
	3	6	12	24
<b>Hp genotype 2-2</b>	.05	.29	.35	.15

(c) **Age, sex, and gross functional outcome.** Age and sex lacked significant relationships with any of the measures of gross functional outcome (GOS, MRS, mortality) at all four time periods. See Table 12 below for clarification.

**Table 12. Statistical significance (p) of multivariate regression analysis of the relationship between age, sex, and gross functional outcome (MRS, GOS, mortality)**

Measure of gross functional outcome	MRS (p)				GOS (p)				Mortality (p)			
	3	6	12	24	3	6	12	24	3	6	12	24
<b>Age</b>	.71	.78	.96	.59	.32	.77	.66	.59	.16	.77	.66	.59
<b>Sex</b>	.61	.18	.58	.28	.98	.37	.674	.51	.71	.37	.74	.51



**Figure 12. Mortality of sample by Hp genotype**

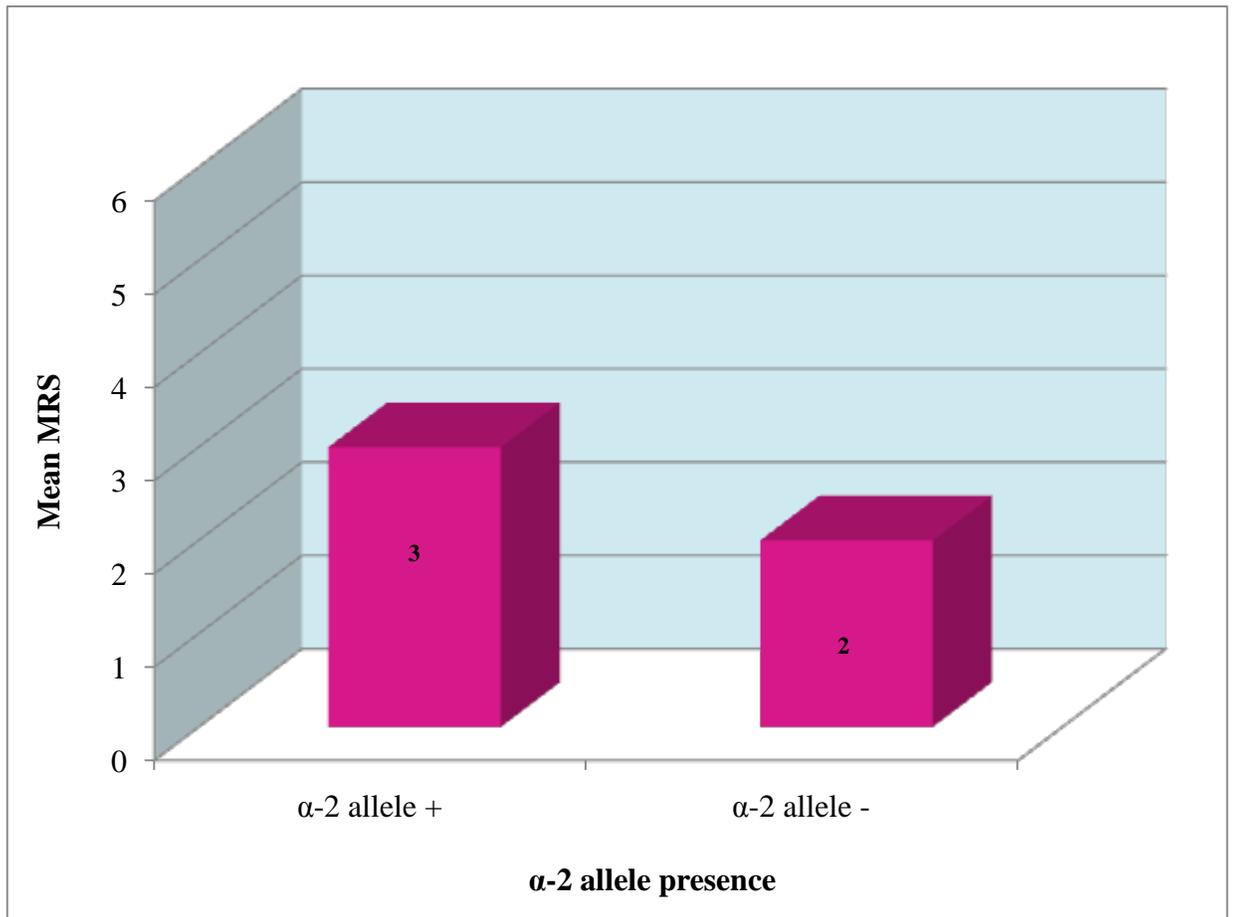
#### 4.3.2 Research question # 2

2. Is there a difference in gross functional outcome from aSAH based on  $\alpha$ -2 allele presence?

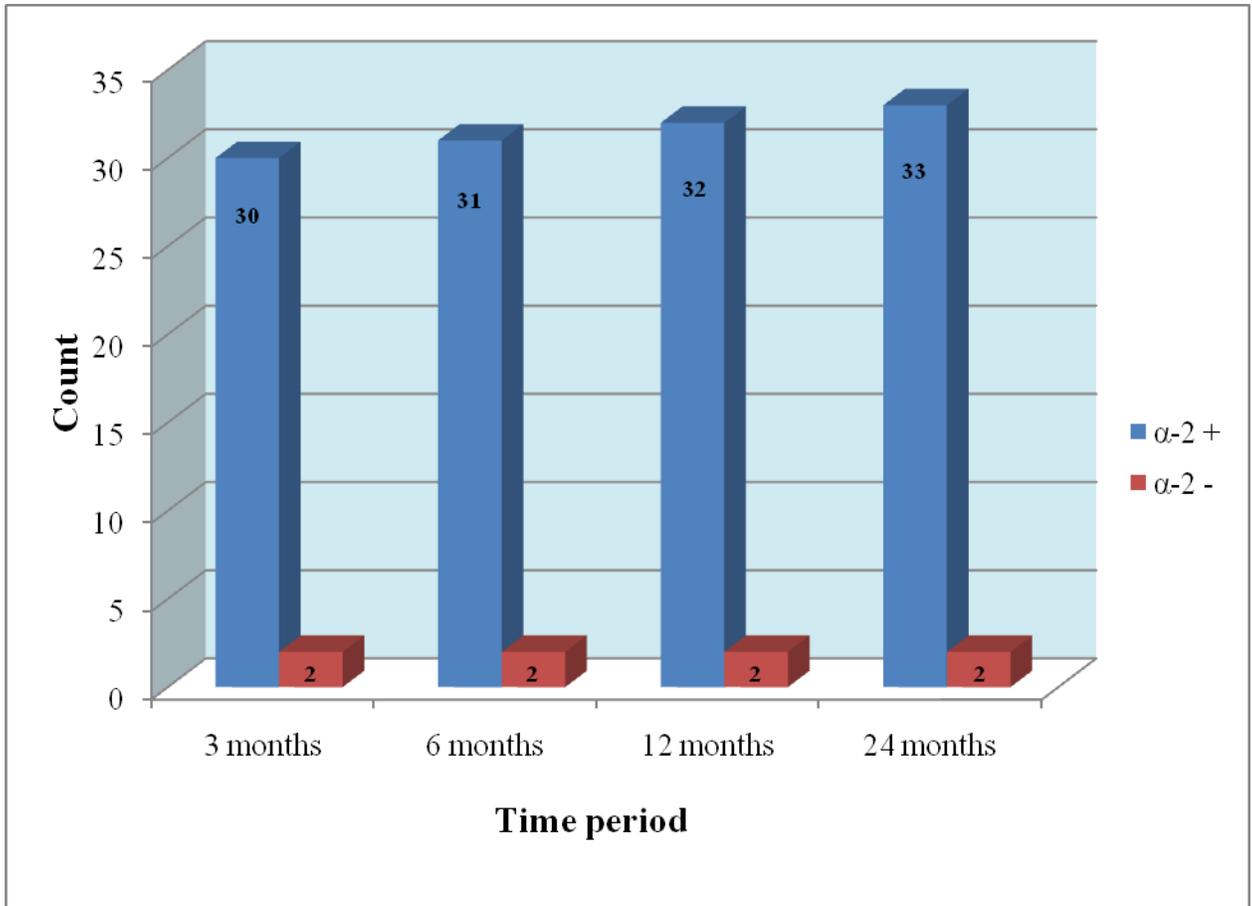
##### 4.3.2.1 Univariate analysis.

(a)  **$\alpha$ -2 allele presence and MRS.** There was a trend towards significance that was detected in the relationship between subjects having at least one copy of  $\alpha$ -2 allele in the Hp genotype (Hp 1-2 and Hp 2-2) and MRS at 3 months post

aSAH ( $p=.06$ ). We also found that the relationship between genotypes in which there existed at least one  $\alpha$ -1 allele (Hp 1-1, Hp 1-2) and MRS also trended towards significance ( $p=.06$ ). Figure 13 shows the distribution of subjects by  $\alpha$ -2 allele presence versus mean MRS. Also, see Figure 14 for mortality by presence of an  $\alpha$ -2 allele through every time period.



**Figure 13. Distribution of subjects by  $\alpha$ -2 allele presence versus mean MRS**



**Figure 14. Mortality of sample by time period and presence of  $\alpha$ -2 allele**

## 5.0 DISCUSSION

This study had the following major findings:

1. Subjects with at least one Hp  $\alpha$ -2 allele were older.
2. Subjects with at least one Hp  $\alpha$ -2 allele were more likely to have poorer outcomes.

This study examined the relationship between Hp genotype and gross functional outcome (GOS, MRS, mortality) after aSAH. We hypothesized that patients with a Hp 2-2 genotype will have poorer outcomes after aSAH. Additionally, we hypothesized that patients whose genotype consists of at least one  $\alpha$ -2 will have poorer outcomes than patients who do not possess the  $\alpha$ -2 allele in their genotypes and that these outcomes will exhibit a dose-response association with the  $\alpha$ -2 allele. We described the distributions of genotypes and alleles for Hp among subjects in our study. Additionally, we described the distribution of Hp allele frequencies among different races in our patient population. Evaluation of differences in gross functional outcomes after aSAH was done by Hp genotype and by  $\alpha$ -2 allele presence in Caucasians. Results of this study serve multiple purposes such as providing insight into the physiologic mechanisms influencing recovery and outcome after aSAH and developing a genetic marker for use in the aSAH population.

## 5.1 HAPTOGLOBIN GENOTYPE AND ALPHA-2 ALLELE PRESENCE

Hp is an APP present in the human serum and is responsible for binding Hgb, therefore inhibiting its interaction with NO and preventing an inflammatory response and the oxidative stress that results from free radical production. Borsody and associates report that Hgb has a decreased ability to produce prostaglandins as a result of the Hp-Hgb binding [9]. Langlois and Delanghe, and Kasvosve et al. report that Hp is generally present in serum levels in the following order of concentration: Hp 1-1 > Hp 1-2 > Hp 2-2 [20, 54]. However, Caucasians in our study exhibited the following Hp genotype distribution: Hp 1-2 > Hp 2-2 > Hp 1-1. Borsody and collaborators report a similar distribution of Hp genotype (Hp 1-2 > Hp 2-2 > Hp 1-1), although their sample size was only 32 patients [9]. There were several other similarities between the study conducted by Borsody and associates and ours, including an imbalance of race and sex, with Caucasians and females making up the majority of the samples [9]. Borsody et al. report an overrepresentation of Caucasians in the groups whose genotypes contained at least one  $\alpha$ -2 allele [9]. Additionally, Borsody and associates report a predominance of the female sex in their sample (21 of 32 patients), consistent with our findings and other investigations of aSAH [9]. The reason for this may be coinciding with knowledge presented previously in aSAH literature regarding an increased risk of aSAH in women [5]. In our study, we had an imbalance in the distribution of race in general, with the majority of the patient population being Caucasian (237; 88.4%). Therefore, due to differences in allele frequency distribution based on different ethnic populations in previously published literature, we limited our study sample to only Caucasian subjects in an attempt to control for population stratification when analyzing the distribution of genotypes in correlation with gross functional outcome.

In our sample of Caucasians, 168 subjects (87.0%) possessed at least one  $\alpha$ -2 allele in their genotypes. This statistic coincides with the report by Teye et al., who report that the Hp  $\alpha$ -2 allele was the most represented Hp allele in all three populations, but was significantly higher in those of Chinese ethnicity [55]. It is believed in literature that the  $\alpha$ -1 allele is less common in people of Asian descent [19]. The  $\alpha$ -2 allele is associated with a weaker affinity for Hgb, therefore struggling to inhibit free radical production, oxidative stress, and CV, which is associated with DCI and negative impacts on physical and cognitive functioning [9]. It is believed that a relationship may exist between the  $\alpha$ -2 allele's inability to bind Hgb and an increased amount of red blood cells in the CSF filled subarachnoid space in patients with at least one  $\alpha$ -2 allele in their genotype. Therefore, there may be a correlation between presence of at least one  $\alpha$ -2 allele and poorer outcomes in patients who suffer from injuries that result in overwhelming amounts of free Hgb in the body, such as in aSAH. In our study there were no significant differences between individuals in categories sex or Hunt and Hess score neither based on Hp genotype nor on  $\alpha$ -2 allele presence.

## 5.2 HAPTOGLOBIN GENOTYPE AND AGE

We found that age is significantly correlated with genotype in individuals with at least one copy of the  $\alpha$ -1 allele (Hp1-1 and Hp1-2). There was an increased frequency of the  $\alpha$ -2 allele presentation in the genotypes of older subjects within our sample. It is unknown why this increase in frequency was present in our patient population, however, it may be possible that due to the Hp  $\alpha$ -1 allele's associations with other diseases—such as infection, coronary artery disease, and liver disease [56]—subjects with the  $\alpha$ -1 allele in their genotypes have faced

mortality in larger numbers than those with only one  $\alpha$ -1 allele or those without an  $\alpha$ -1 allele in their genotypes. Additionally, the increased age of the subjects with an  $\alpha$ -2 allele in their genotypes may play a role in the poorer outcomes post aSAH since we found that those subjects who are older more often experienced poor outcomes.

### **5.3 HAPTOGLOBIN GENOTYPE AND GROSS FUNCTIONAL OUTCOME**

In our study we found that the association between Hp genotype and MRS was statistically significant at three months post aSAH. Specifically, individuals with a Hp 1-1 genotype less often had poor outcomes on the MRS at this time period. Additionally, we detected a significant relationship between Fisher grade and MRS at all four time periods, showing that individuals with Fisher grade of 2 or 3 more often had poor outcomes on the MRS. Similar to the association between Fisher grade and MRS, there was a significant relationship between the Hunt and Hess score upon admission and MRS throughout all time periods following aSAH. Subjects with at least one  $\alpha$ -2 allele in their genotypes more often had a score of 3 on Hunt and Hess had poor outcomes on the MRS. After controlling for covariates (age, sex, Fisher grade) all predictors of outcome dropped out of significance in determining gross functional outcome (GOS, MRS, mortality) except for the Fisher grade. These findings support the literature and the continued use of the Fisher grade and Hunt and Hess score as clinical measures of prognosis [33, 49, 51-53].

Although Borsody et al. found that individuals with an  $\alpha$ -2 allele in their genotypes experienced worse outcomes (such as CV) in their study [9], Rabinstein calls for a larger study that incorporates clinical measures of functional outcomes of aSAH is before Hp genotyping can

be appropriated for use in clinical procedures [22]. Kramer et al. found that subjects with lower serum Hgb concentrations post aSAH experienced poorer outcomes [7]. This brings light upon the importance of Hp in Hgb binding. One would think that patients with one or more  $\alpha$ -2 alleles would experience poorer outcomes related to the Hp  $\alpha$ -2 isoform's decreased affinity for Hgb binding. This would allow for more Hgb to persist in the CSF for a longer time, with decreased serum Hgb levels, and possibly leading to secondary injuries such as CV. Fisher found that the risk of CV is directly proportional to the concentration of blood in the CSF, specifically in the subarachnoid space and ventricles [33].

#### **5.4 ALPHA-2 ALLELE PRESENCE AND GROSS FUNCTIONAL OUTCOME**

We found that a significant relationship exists between Fisher grade and subjects who have a Hp genotype in which at least one copy of the  $\alpha$ -1 allele exists (Hp 1-1, Hp 1-2). Additionally, our results show that individuals with at least one copy of the  $\alpha$ -1 allele in their genotypes are more likely to receive a Fisher grade of 3. It is possible that although this Fisher grade of 3 may predict CV, the  $\alpha$ -1 allele or the Hp 1-1 genotype could serve as protection from developing CV. Previously it was stated in literature that, although the Fisher grading scale goes from 1 to 4, a grade of 3 is most associated with poorer outcomes after aSAH [33]. However, in our study we found that a Fisher grade of 2 is 15 times more likely to be associated with poorer outcomes than a Fisher grade of 4. Surprisingly, a Fisher grade of 3 was only 2 times more likely to experience poorer outcomes than a Fisher grade of 4. This suggests that a Fisher grade of 2 should be considered just as, if not more, significant in predicting outcomes in patients that are admitted for aSAH. Also, it is possible that the Fisher grade truly is ordinal and not nominal like previously

thought. Furthermore, if a Fisher grade of 2 may be more harmful than a Fisher grade of 3 in predicting long-term gross functional outcome, it is possible that having a Hp 1-1 genotype could be protective against risk of vasospasm and poor outcomes after aSAH.

Borsody et al. found that 20 of their 23 subjects with at least one  $\alpha$ -2 allele developed CV compared to only 2 of 9 patients with the Hp 1-1 genotype [9]. Similarly, Chaichana et al. gathered that Hp 2-2 blood injected into mice led to an increased occurrence of CV when compared to mice injected with Hp 1-1 blood [17]. To support this literature we found similar results showing that subjects in our study whose genotypes contained at least one  $\alpha$ -2 allele were significantly more likely to develop CV at 3 months post aSAH. Additionally there was a trend towards significance that was detected in the relationship between subjects with genotypes having at least one copy of  $\alpha$ -2 allele in the Hp genotype (Hp 1-2 and Hp 2-2) and MRS at 3 months post aSAH. Our findings support the idea that CV could be associated with  $\alpha$ -2 allele presence because of the  $\alpha$ -2 allele's impaired ability to clear Hgb from CSF after aSAH [9].

## 5.5 CONCLUSIONS

The most crucial finding of our study is that Hp genotype is significantly associated with gross functional outcome when utilizing MRS to assess at three months post aSAH. Additionally,  $\alpha$ -2 allele presence is significantly correlated with CV after aSAH. Furthermore, the finding of a relationship between Hp  $\alpha$ -2 allele presence and MRS at three months post aSAH lends to the importance of this study and the thought of the possible correlation between  $\alpha$ -2 allele presence and poor outcomes after aSAH. Additionally, this study validated the use of the Fisher grading scale and the Hunt and Hess score in clinical application for the prediction of prognosis in this

patient population. Our study's sample size was large and showed these associations, which is a vital step in the movement towards using  $\alpha$ -2 allele presence and Hp genotype as biomarkers for aSAH patients [22].

## 5.6 IMPLICATIONS FOR FUTURE RESEARCH

Previous literature has shown that the  $\alpha$ -2 allele is associated with poorer binding affinity for Hgb, leading researchers to postulate the outcomes of this decreased binding. It is thought that the decreased Hp:Hgb binding leads to an increased level of Hgb in the CSF after aSAH. This presence of Hgb is thought to lead to free radical and prostaglandin production, inflammation, and oxidative stress. Our results show that the  $\alpha$ -2 allele is associated with CV after aSAH, and that Hp genotype is associated with gross functional outcome (MRS). It may be useful in future research studies to avoid controlling for Fisher grade in analyses because of its correlation with a Hp 1-1 genotype. It is possible that although these subjects experience a Fisher grade of 3 and a large bleed more often, there are some protective mechanisms in patients with a Hp 1-1 genotype that prevent the poor outcomes and/or CV more efficiently than in subjects with one or more  $\alpha$ -2 alleles experience.

Due to the differences in allele frequency distribution among African Americans and Asians/Pacific Islanders (previously published in literature) as well as a small number of subjects in the Asian/Pacific Islander and Hispanic categories in our patient population, we were unable to evaluate gross functional outcomes among these races. Future research should analyze the associations between Hp genotype in these races in hopes of determining whether or not certain populations, such as Asians, will suffer from increased morbidity after aSAH related to the

increased likeliness of the  $\alpha$ -2 allele being present in their genotypes. However, before the  $\alpha$ -2 allele should be evaluated as a possible biomarker for aSAH patients or as a risk factor for development of CV and poor outcomes after the initial injury, more research is needed to evaluate mechanisms before one should suggest Hp genotyping for clinical use. After these mechanisms are explored and understood, it is possible that the use of the  $\alpha$ -2 allele as a biomarker could serve to predict the occurrence of CV, since it is currently largely misunderstood and unpredictable. Rabinstein concludes that the ability to detect risk of CV after SAH could improve treatment and prognosis in these patients [22]. We agree that many treatments for aSAH and CV involve unfavorable side effects and the possible future identification of patients who are most at risk for CV would allow for the administration of these agents only to those patients in whom they are truly warranted [22].

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