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THE EFFECT OF HAPTOGLOBIN PHENOTYPES ON MARKERS OF NEUROINFLAMMATION IN PATIENTS WITH ANEURYSMAL SUBARACHNOID HEMORRHAGE

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DISSERTATION

A dissertation submitted in partial fulfillment of the

requirements for the degree of Doctor of Philosophy in the College of Medicine

at the University of Kentucky

By

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2020

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ABSTRACT OF DISSERTATION

THE EFFECT OF HAPTOGLOBIN PHENOTYPES ON MARKERS OF NEUROINFLAMMATION IN PATIENTS WITH ANEURYSMAL SUBARACHNOID HEMORRHAGE

Aneurysmal subarachnoid hemorrhage (aSAH) is a severe form of hemorrhagic stroke resulting from the spontaneous rupture of an intracranial aneurysm and release of oxygenated blood into the subarachnoid space. In the United States alone, aSAH occurs in approximately 30,000 patients each year and occurs, primarily, in otherwise healthy adults, frequently without warning. Sadly, approximately 40% of patients with aSAH will die in the first 30 days. Of those who survive, only about 75% will be able to live semi-independently again and only about 60% will be able to return to work.

Delayed brain injuries (DBI), which may affect up to 70% of aSAH patients, are an important cause of these devastating outcomes. DBI include delayed cerebral vasospasm (DCV) and delayed cerebral ischemia (DCI). Despite intensive research, effective preventative and treatment therapies for DBI have not been identified, in part due an incomplete understanding of their pathophysiology. Although a number of mechanisms have been proposed, a strong correlation between DBI and inflammatory mediators, including selectins, integrins, and proinflammatory cytokines, such as IL-1β, IL-6, IL-8, TNF-α and MCP-1, has been most frequently observed, suggesting an important role of inflammation in the development of DBI.

Because intracranial blood is a key feature of aSAH, haptoglobin (Hp) has emerged as a potential patient-specific modifier of DBI risk. Hp is a naturally occurring acute phase reactant that binds to free hemoglobin (Hb) molecules to facilitate Hb metabolism by circulating macrophages. In humans, there are 3 different Hp phenotypes, Hp1-1, Hp1-2 and Hp2-2. Recent clinical observations suggest that different Hp phenotypes affect the incidence of DBI after aSAH. While the mechanism for this observation remains unknown, one potential explanation is that Hp may alter the neuroinflammatory response, reflected in plasma, CSF, or parenchymal concentrations of pro-inflammatory cytokines. Macrophage CD163 binds Hp-Hb complexes and a free-floating version of CD163, termed soluble CD163 (sCD163), can also be isolated from plasma and other body tissues in response to stress and as a result of inflammatory injury. The relationship between sCD163 and different Hp phenotypes is unknown but sCD163 may also have an important role in modulating DBI risk after aSAH.

The objective of this dissertation, therefore, is to investigate the hypothesize that the different haptoglobin phenotypes modify the risk of DBI through a differential effect on sCD163 and inflammatory cytokines. To achieve this objective, three specific aims were completed and are described in detail in this dissertation. First, a prospectively

collected aSAH biobank was established at the University of Kentucky. This biobank was necessary to facilitate current and future observational aSAH studies. Second, using data and patient samples from the established biobank, plasma and CSF concentrations of 3 different neuroinflammatory cytokines (IL-1β, IL-6, and TNF-α) were determined at pre-specified time points and compared against Hp phenotype, clinical outcome, and DBI diagnoses. Finally, using data and patient samples from the established biobank, plasma and CSF concentrations of sCD163 was determined at pre-specified time points and were also compared against Hp phenotype, clinical outcome, and DBI diagnoses.

The proposed biobank successfully recruited 25 patients over a 2-year period. Of these patients, 3 (12%) were Hp1-1 phenotype, 14 (56%) were Hp1-2 phenotype, and 8 (32%) were Hp2-2 phenotype. There were no statistically significant differences in plasma or CSF concentrations of any of the measured cytokines at any time point when subjects were compared by their DCV status, DCI status, Hp phenotype, Hp class, or a combination of Hp class and DCV or DCI. While there were significant differences in plasma sCD163 concentration when subjects were compared by a combination of Hp class and DCV, there were no other statistically significant differences in plasma or CSF concentration of sCD163 at any time point when subjects were compared by their DCV status, DCI status, Hp phenotype, Hp class, or a combination of Hp class and DCV or DCI. These findings are most likely related to the relatively small number of patients included in these analyses. Future studies should include more patients to better evaluate the roles that the different Hp phenotypes play in the development of DBI after aSAH.

KEYWORDS: Aneurysmal Subarachnoid Hemorrhage, Delayed Cerebral Vasospasm, Delayed Cerebral Ischemia, Haptoglobin, Neuroinflammation

Kevin W. Hatton, M.D.

October 29, 2020

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October 29, 2020

DEDICATION

To Adrienne, my wife and life partner. I can never thank you enough for allowing me to bind my life to yours. You are wonderful and beautiful…a continued mystery and blessing to me every day.

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First and foremost, I would like to acknowledge God. He has given me life, an inquisitive mind, and the opportunity to follow the path He has placed before me. Most importantly, He has saved me from my sins and I work daily to become a more perfect example of his spirit in me.

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Finally, I wish to thank the patients and their families who agreed to be a part of these studies. Their strength and bravery throughout the process remains an important reason that I started this project and have continued it to this point (and beyond).

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CHAPTER 1: INTRODUCTION AND LITERAURE REVIEW

1.1 Introduction

Aneurysmal subarachnoid hemorrhage (aSAH) is a severe form of hemorrhagic stroke resulting from the sudden, spontaneous rupture of an intracranial arterial aneurysm. aSAH results in significant morbidity, disability, and mortality through combined effects of acute brain injuries and delayed brain injuries (DBI). Haptoglobin (Hp), a naturally-occurring protein that binds free hemoglobin exists in multiple forms due to chemical differences in hereditary phenotypes. Recently, different Hp phenotypes have been correlated to different risks of DBI in patients with aSAH. Although various pathophysiologic models have been proposed to explain the development of DBI, none have successfully incorporated the observed differences in DBI between patients with different Hp phenotypes. I, therefore, hypothesize that the different haptoglobin phenotypes modify the risk of DBI through a differential effect on soluble CD163 (sCD163) or inflammatory cytokines, including Interleukin 1-beta (IL-1β), Interleukin 6 (IL-6) and Tumor Necrosis Factor-alpha (TNF-α). This dissertation describes a series of projects designed to evaluate this hypothesis.

First, this introductory chapter provides an overview of the key features of aSAH, describes the importance of delayed brain injuries (DBI) on clinical outcomes after aSAH, and details the previously-proposed pathophysiologic models of DBI. This chapter then focuses on haptoglobin (Hp) as a potential modifier in DBI by describing the Hp molecule and its role in scavenging free hemoglobin (Hb) and details the potential

effect that different haptoglobin (Hp) phenotypes have on DBI and clinical outcomes. This chapter concludes with a discussion of potential mechanisms, including sCD163 and the inflammatory cytokines that might explain how the different Hp phenotypes affect the inflammation-mediated model of DBI.

1.2 Overview of Aneurysmal Subarachnoid Hemorrhage

The rupture of a spontaneously-occurring aneurysm and the subsequent development of aSAH is an unexpected and frequently catastrophic event for patients and their families. Because most patients do not know that they even have an aneurysm, the sudden, unexpected consequences of its rupture are even more physically and emotionally scarring. Aneurysm rupture and aSAH result in a range of clinical outcomes from minimal neurocognitive impairment to severe, lifelong neurocognitive disability or death.

1.2.1 Epidemiology

Intracranial aneurysms are relatively common. Based on autopsy studies, approximately 3% of the world's population or more than 200 million people will develop an intracranial aneurysm at some point in their lives.[1,2] Fortunately, not all people with aneurysms develop aSAH and the worldwide incidence of aSAH is only 700,000 cases per year (approximately 1:100,000 people per year).[3,4] In the U.S., the annual incidence of aSAH is approximately 30,000 cases per year.[4] Across the globe,

the incidence of and mortality from aSAH has decreased over the last few decades likely due to an increase in the detection and treatment of unruptured intracranial aneurysms.[5]

aSAH appears to occur most commonly in patients aged 35-60 years.[6] Hypertension, hypercholesterolemia, current tobacco use, current alcohol use, and female gender have all been mildly associated with an increased risk of aSAH.[2,7-9] In addition, there is an increased risk of aSAH in patients with a positive family history; however, no specific genetic link has been documented in these families. A few rare inherited diseases, such as autosomal-dominant polycystic kidney disease and Ehlers-Danlos Syndrome, are also associated with an increased risk of aSAH but the genetic or structural changes that link these diseases to aSAH have not been discovered. [7,10]

1.2.2 Clinical outcomes

Although most intracranial aneurysms do not rupture, when they do, the outcome is frequently disastrous. aSAH is associated with a very high mortality rate. Approximately 10-15% of aSAH patients die suddenly either immediately at the time of aneurysm rupture or before hospitalization.[11] Of those who do survive to hospitalization, another 25-30% die during their hospitalization in the first few weeks after aneurysm rupture.[12] Altogether, approximately 40% of aSAH patients die within the first 30-days.[12-14] These deaths represent the combined effects of profound brain injuries and systemic complications resulting from aSAH.[15] Unfortunately, of those aSAH patients who do survive, a significant percentage of patients have long term

disability. A recent survey of outcomes found that only 76% of aSAH survivors are ever able to live semi-independently again and only 60% of aSAH survivors are ever able to return to some form of work.[16]

1.2.3 Routine clinical care

For patients who survive, their hospital care can generally be described in three phases. In the first phase, initial triage evaluates and stabilizes cardiopulmonary function. This phase occurs in the emergency department at the time they are brought to the hospital. Endotracheal intubation and initiation of mechanical ventilation may be needed for patients who do not have adequate neurologic function to maintain a patent airway or who do not have adequate respiratory effort or function. Once this initial triage has occurred, an emergent computed tomography (CT) scan of the head is performed to evaluate the severity of subarachnoid hemorrhage and the extent of hydrocephalus. aSAH patients with significant hydrocephalus undergo emergent ventriculostomy and cerebrospinal fluid (CSF) drainage with an extraventricular drain (EVD). Elevated blood pressure should also be aggressively controlled to reduce the risk of aneurysm re-rupture. Patients are then transferred to a specialized neuroscience intensive care unit (NSICU) for additional care.

In the second phase, after the patient has been transferred to a NSICU, the focus of care turns to early aneurysm obliteration therapy and treatment of early brain injuries (EBI). Aggressive treatment of elevated blood pressure is continued and aneurysm

obliteration procedures occur within the first 48 hours after aneurysm rupture to further reduce the risk of aneurysm re-rupture. When possible, an endovascular approach to aneurysm obliteration is performed by placement of specialized catheters through the arterial system into the intracranial arteries and selective placement of thrombogenic coils inside the aneurysm. These coils induce an intense fibrotic reaction with the aneurysm that prevents future rupture. If an endovascular approach is not possible, surgical craniotomy and placement of a vascular clip across the neck of the aneurysm is performed.

In the third phase, the focus of care shifts to providing routine critical care and preventative monitoring and treatment of DBI. For most patients, aggressive control of elevated blood pressure can be loosened at this point. aSAH patients undergo frequent neurologic monitoring and daily screening for known or suspected DBI, using physical examination, transcranial Doppler ultrasonography (TCD), and other radiographic and angiographic modalities, as needed. In addition, since nimodipine therapy has been shown to improve long term neurologic recovery, it is initiated at hospital admission and continued through post-bleed day (PBD) 21. If an EVD was placed, it is typically removed between PBD10 and PBD14, as tolerated by the patient. If the EVD cannot be safely removed, a permanent ventriculoperitoneal shunt (VPS) is placed for chronic CSF drainage. When stable, the patient is transferred to a skilled nursing facility or rehabilitation center for aggressive brain rehabilitation with neurocognitive, as well as, occupational and physical therapy.

1.3 Delayed Brain Injuries after aSAH

Although there are many determinants of clinical outcome after aSAH, by far the most importance determinants of long-term outcome in aSAH patients are the direct and indirect injuries that occur to the brain. These brain injuries are categorized as either early brain injuries (EBI) or delayed brain injuries (DBI), based on their temporal onset after aneurysm rupture. EBI occur in the first 72 hours after aneurysm rupture and are caused by increased intracranial pressure, reduced cerebral blood flow or cerebral perfusion pressure, direct injury to brain parenchyma and associated structures, cytotoxic cerebral edema formation, or destruction and deterioration of the blood brain barrier.[17,18] Current treatment and research approaches for EBI aim to limit the impact on patients prior to aneurysm rupture, by improving identification of unruptured aneurysms and developing and employing more prophylactic endovascular and operative approaches to aneurysm obliteration.

DBI occur more than 72 hours after aneurysm rupture and add significant additional morbidity and mortality to aSAH patients. The most common DBI are delayed cerebral vasospasm (DCV), delayed cerebral ischemia (DCI) and delayed cerebral infarction, each of which will be discussed in more detail below. Reducing the occurrence and impact of DBI are important targets of current basic, translational, and clinical research to improve long term survival and recovery after aSAH. DBI, unlike

most EBI, are potentially modifiable by new hospital-based treatments and therapies and will be the major focus of the work described in this dissertation.

1.3.1 Delayed cerebral vasospasm

DCV is an important DBI that results in significant morbidity and mortality after aSAH.[19,20] DCV causes spastic narrowing of one or more intracranial arteries and results in reduced cerebral blood flow in arterial and capillary beds distal to the affected vessels that, if not rapidly restored, will result in cerebral infarction and permanent neurologic damage. [21,22] Based on multiple retrospective observational studies, DCV appears to occur in 30-70% of patients with aSAH, typically, 3-10 days after aneurysm rupture.[20,21,23,24] The amount of blood in the subarachnoid space, determined from admission CT scan of the head, is the most important risk factor for the development of DCV.[25] Tobacco use, hypertension and left ventricular hypertrophy are also reported risk factors for DCV.[26,27]

Because it is not known which patients will develop DCV and on what day it may occur, all aSAH patients are screened daily for up to 14 days using transcranial Doppler (TCD) ultrasonography.[28,29] TCD utilizes Doppler assessment of reflected sound waves (ultrasonography) through the cranial bones to evaluate blood flow velocity (BFV) across target arteries.[30] Although many different arteries can be assessed, the middle cerebral artery (MCA) and the internal carotid artery (ICA) are the most commonly-visualized vessels.[20,21] To differentiate between a focal increase in BFV, as may be seen with DCV,

and a global increase in BFV, as may be seen in hyperdynamic cardiac disease, the MCA/ICA ratio (known as the Lindegaard ratio) is used.[30,31] DCV is, therefore, defined by an elevated MCA BFV (flow velocity greater than 120 cm/s) and a Lindegaard ratio ≥ 3. Based on a meta-analysis of 5 trials, TCD appears to be most effective as a screening tool when BFV measurements are either very low, thereby ruling out DCV, or very high, thereby confirming DCV.[32,33] For patients who cannot undergo TCD monitoring or who have an equivocal diagnosis of DCV based on TCD, computed tomography angiography (CTA) or magnetic resonance angiography (MRA) may also be utilized as non-invasive diagnostic modalities.

When aSAH patients have positive TCD, CTA or MRA findings, invasive digital subtraction angiography (DSA) is considered the gold-standard diagnostic and treatment modality. Using DSA, highly-trained clinicians identify areas of vasospasm and employ treatments to restore blood flow. For most patients, there is no clear correlation between the vasospastic vessel and the vessel affected by aneurysm rupture.[34] Once areas of vasospasm are identified using DSA, targeted treatments, including angioplasty and intra-arterial injection of vasodilators, including verapamil, nicardipine, papaverine and milrinone, are used to restore the spastic vessel's intraluminal diameter.[35] This targeted vasodilator therapy minimizes the potential side effects, including hypotension, that may occur from systemic drug administration.[35,36] Following targeted treatment procedures, patients are monitored for additional episodes of DCV that may occur throughout the vasospasm window. If additional episodes of DCV occur, patients will undergo repeat

DSA for additional diagnostic and treatment procedures. Despite these aggressive interventions, patients with DCV may still develop permanent neurologic injury, disability, or death.

1.3.2 Delayed cerebral ischemia

Delayed cerebral ischemia (DCI) is another important DBI and is also a major contributor to long-term morbidity and mortality after aSAH. DCI may affect up to 30% of aSAH patients.[19,37] Patients with DCI typically demonstrate an acute, significant decrease in global or focal neurologic function that is not apparent immediately after aneurysm rupture and has no direct relationship to the aneurysm obliteration therapy.[38] The major risk factors for DCI include poor clinical condition on admission, large amount of intracranial blood on initial CT scan, and increasing age.[39]

DCI is distinguished from other potential causes of causes of neurologic deterioration by a lack of radiologic, encephalographic or laboratory findings at the time of neurologic deterioration. DCI is treated primarily with therapeutic hypertension and an aggressive assessment and reversal of potential causes, including DCV.[40-42] Therapeutic hypertension may require fluid administration and/or the use of systemic vasopressor therapy to increase systemic blood pressure and cerebral perfusion pressure.[41] Patients with refractory DCI, despite therapeutic hypertension and resolution of reversal of potential causes, is associated with worse long-term neurologic outcome than patients who have aSAH.[37]

1.3.3 Delayed cerebral infarction

Delayed cerebral infarction is diagnosed by either new CT or MRI findings up to 6 weeks after aSAH demonstrating abnormalities consistent with infarction that were not present on imaging up to 48 hours after aneurysm rupture.[38] Because they are not present on initial imaging, they are not considered to be related to EBI. Delayed cerebral infarction occurs in up to 20% of aSAH patients.[43,44] Both DCV and DCI have been associated with delayed cerebral infarction, although the relationship between these DBI remains poorly understood.[43-45]

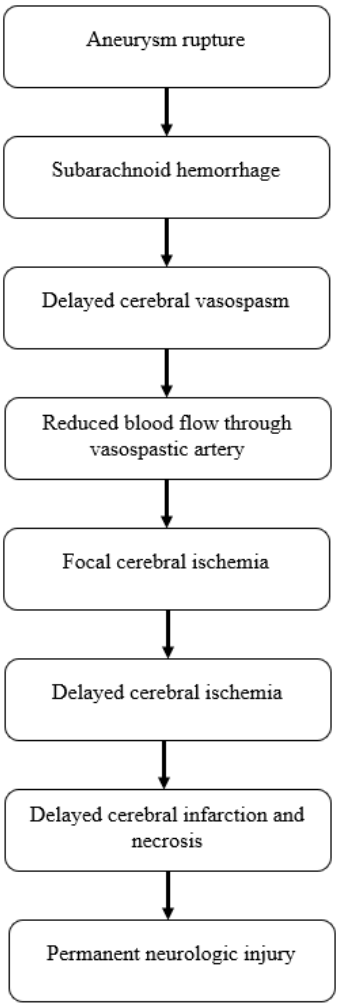
1.4 Models of Pathogenesis of Delayed Brain Injury

1.4.1 Cerebral blood flow mediated model of DBI

Despite multiple decades of research, the pathogenesis of DBI after aSAH is still not well understood. Historically, DBI were believed to occur in a sequential manner with each of the DBI leading to a more advanced or more severe type of injury as described in Figure 1.[46,47] In this model, aneurysm rupture results in DCV through one or more currently hypothesized but unproven mechanisms. Unrecognized or untreated DCV results in reduced blood flow through the spastic vessel with distal brain tissue malperfusion and DCI. If unresolved, DCI may become delayed cerebral infarction and result in permanent neurologic injury and disability. Notably, this is similar to the role that reduced blood flow appears to play in basic models of neurologic injury and cerebral infarction in acute ischemic stroke. Unfortunately, based on both clinical and

experimental reports, this sequence of events appears to be significantly over-simplified and does not accurately reflect observations from clinical reports.[45,48-50]

Figure 1: Sequential Model of DBI Pathogenesis

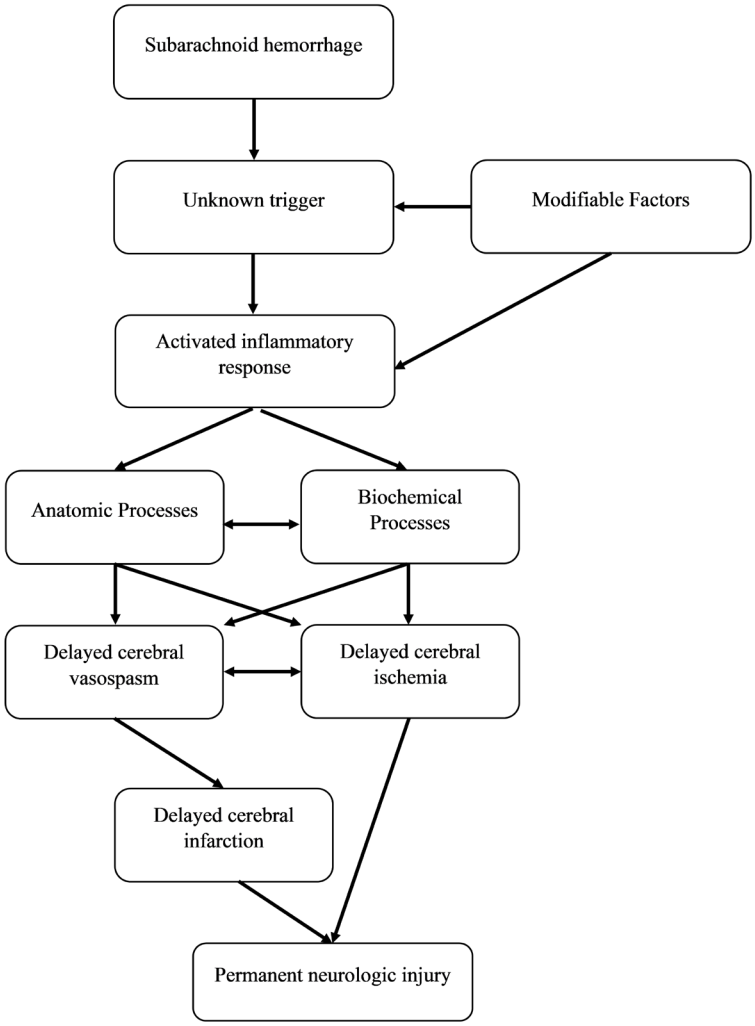


The sequential model of Delayed Brain Injury (DBI) pathogenesis is a linear progression of events from aneurysm rupture and subarachnoid hemorrhage to the clinical consequence of permanent neurologic injury.

1.4.2 Inflammation-mediated model of DBI

A more complex model of DBI would, perhaps, better describe the interplay of the various anatomical and biochemical processes that have been associated with DBI after aSAH as shown in Figure 2.[51,52] This model, developed by me from previously published work, highlights the proposed role that inflammation appears to play in driving some or all of these anatomical and biochemical processes.[53-55] In addition, this model acknowledges that there may be an as yet unknown trigger and modifiable factors that connect subarachnoid hemorrhage to the activated inflammatory response.

Figure 2: Complex Inflammation-Mediated Model of DBI Pathogenesis



The complex inflammation-mediated model of Delayed Brain Injury (DBI) pathogenesis describes the interplay of processes triggered by an activated inflammatory response that leads from subarachnoid hemorrhage to permanent neurologic injury.

In this model, activation of an acute inflammatory response plays a central role in the development of DBI after aSAH. This activated inflammatory response to subarachnoid blood or blood products has been hypothesized to directly or indirectly affect intracranial vascular tone or to activate additional pathways that stimulate additional anatomic or biochemical processes that lead to DBI.[56-58] Clinically, several observational studies have shown that aSAH patients with DBI have more symptoms of systemic inflammation (SIRS), including fever, leukocytosis, tachycardia and tachypnea than do aSAH patients without DBI.[59-61] These studies show that patients with DBI have a clinically significant inflammatory response to the development of DBI.

In addition, observational studies have attempted to define important inflammatory pathways affecting the development of DBI after aSAH through assessments of previously described cytokine concentrations in plasma and CSF after SAH.[54,56] Unfortunately, many of these studies have incomplete or conflicting results because of significant differences in DBI definitions, sampling time points, sampling procedures, and inflammatory mediator assays.[56] Despite these limitations, IL-1β, IL-6 and TNF-α have most consistently demonstrated differences in aSAH patients with and without DBI.[62-66] More detailed models of the activated inflammatory processes in DBI, using these and other inflammatory cytokines, have not been described.

1.4.3 Limitations in aSAH models research

Unfortunately, our understanding and ability to test models of DBI pathogenesis are hampered by the unpredictable nature of aneurysm development and rupture in human patients. For example, it is not possible or practical to obtain biofluid or tissue samples in the hours before aneurysm rupture because it is not known when this will occur. In addition, basic science research in aSAH is limited by the lack of an animal model that reliably generates intracranial aneurysms and aneurysm rupture.[67,68] At current, experimental SAH is produced in otherwise healthy mice by endovascular puncture of the internal carotid artery or by injection of arterial blood obtained from the femoral artery into the cisterna magna.[67-69] These models result in arterial blood in the subarachnoid space but may not produce the same anatomic, biochemical and inflammatory responses observed in human aSAH.[67,68]

1.5 Anatomic and Biochemical Processes in DBI

As has previously been described, several anatomic and biochemical processes have been associated with DBI in aSAH. These processes include endothelial cell damage and dysfunction, altered vascular smooth muscle responsiveness, excitotoxic neuronal injury, oxidative stress, apoptosis, and spreading depolarizations. It is likely that aSAH triggers more than 1 of these in individual patients and different phenotypes of injury may ultimately be caused by the combinations or inter-connectedness of these processes in their local neurovascular environment.

1.5.1 Endothelial cell damage and dysfunction

Endothelial cells (EC) line the internal surface of the intracranial vascular architecture and are responsible for maintaining the blood-brain barrier, for preventing intracranial vascular thrombus formation, and for regulating cerebral vascular tone.[70] Observational and experimental studies have shown that aSAH results in rapid EC dysfunction with resultant disruptions in the blood-brain barrier, microvascular thrombus formation and microvascular tone abnormalities.[70,71] EC damage and dysfunction appears to occur in vessels affected by aneurysm and subarachnoid hemorrhage, as well as, vessels distant from these sites of injury.[72,73] Experimental studies have shown that this blood-brain barrier breakdown occurs within a few hours of injury through combinations of both increased EC apoptosis and disruptions of EC tight junction proteins.[74-76] Likewise, abnormalities in nitric oxide (NO) production and degradation as surrogates for EC microvascular tone abnormalities have been shown to occur after aSAH.[77] While not fully understood, these abnormalities appear to be linked to increased perivascular inflammation and abnormal interactions between leukocytes and EC after aSAH.[70]

1.5.2 Altered vascular smooth muscle responsiveness

Altered intracranial microvascular smooth muscle responsiveness after aSAH results in impaired cerebral arterial autoregulation. Normally, the balance between arteriolar vasoconstrictive and vasodilatory stimuli are tightly controlled and highly responsive to changes in blood pressure to maintain a consistent cerebral blood flow

across a variety of clinical conditions.[78] Observational studies in aSAH patients, however, have demonstrated that normal autoregulatory vasodilation does not occur as a response to distal brain parenchyma hypoxia.[79,80] Likewise, experimental studies have similarly shown that altered intracranial vasoreactivity occurs in mouse models of aSAH through direct and indirect measurements of microvascular lumen diameter.[81,82] These microvascular changes in humans and experimental models (increased vasoconstriction and/or decreased vasodilation) appear to occur prior to or in the absence of the development of large vessel vasospasm.[40] Predictive models of DBI have been developed, to varying degrees of success, using assessments of altered regional vascular reactivity.[83,84] The mechanism behind these observations remains undefined, although acute inflammation and oxidative stress have both been hypothesized to play important roles.[85,86]

1.5.3 Excitotoxic neuronal injury

Neuro-excitotoxicity injury occurs as a response to a host of toxic actions of excitatory amino acids (EAA) on neurons.[87] EAA, most importantly glutamate, are the major excitatory neurotransmitters in the brain. Neurons that contain EAA play important roles in learning and memory. While generally beneficial, overactivation of these neurons or toxic release of EAA can be profoundly harmful and has been implicated in neurodegenerative and neurovascular diseases.[88] Neuronal excitotoxicity from excess glutamate results in an excessive influx of ions across the neuronal cell membrane, triggering harmful enzymatic cascades and cell death.[89,90]

Glutamate levels have been compared between aSAH patients with and without DBI. In these studies, glutamate is either measured directly in CSF or via microdialysis of brain parenchyma.[91,92] Studies have consistently demonstrated increased glutamate levels in aSAH patients with DBI compared to those without aSAH or controls.[93,94] It is not clear whether this observation is a contributor to or a consequence of DBI in aSAH. Further clinical and experimental research is needed to clarify the role that excitatory amino acids, and glutamate in particular, play in the development of DBI in aSAH patients.

1.5.4 Oxidative stress

Oxidative stress occurs when there is an imbalance in the production of free radical reactive oxygen species (ROS) production and the removal of these ROS.[95] Increased ROS may occur after aSAH due to neuronal mitochondrial oxidative stress, hemoglobin free radical generation, disrupted antioxidant protection, and activation of alternate enzymatic stress pathways.[96] In experimental and clinical studies, oxidative stress has been linked to DBI after aSAH.[96,97] In experimental aSAH, markers of oxidative stress, including elevated superoxide anion levels, are detected in the CSF of animals with vasospasm at a greater concentration than in animals without vasospasm.[98] In addition, treatment with various free radical scavengers decrease the occurrence of vasospasm in experimental animals.[96] The primary mechanism linking oxidative stress to DBI is experimental alterations in the cerebrovascular contractile responses after ROS

exposure.[99,100] It is not clear whether these experimental studies translate to aSAH and DBI in humans. Further clinical and experimental research to clarify the role of oxidative stress in human aSAH and DBI is needed.

1.5.5 Apoptosis

Apoptosis, or programmed cell death, has been shown to occur after experimental aSAH in mouse models.[101,102] Apoptosis is initiated through either the so-called intrinsic or extrinsic pathways that are each mediated by different biochemical mechanisms.[103] The intrinsic pathway is activated through mitochondrial release of apoptotic stimulator molecules; whereas, the extrinsic pathway is activated by specialized cell membrane death receptors. In experimental aSAH, activation of both intrinsic and extrinsic pathways has been described. Activation of these pathways in aSAH mouse models has also been associated with DBI.[104-106] It is not clear whether these animal studies translate to aSAH and DBI in humans. Further clinical and experimental research to clarify the role of apoptosis in human aSAH and DBI is needed.

1.5.6 Spreading depolarizations

Spreading depolarizations (SD) are spontaneous, repeating waves of neuronal cell depolarizations that occur after ischemic, hemorrhagic or traumatic injuries to the brain.[107,108] SD worsen brain injuries by dramatically increasing oxygen consumption, as well as, increasing the risk of seizure activity and cytotoxic edema formation.[109,110] In experimental animal models, SD have been observed shortly after aSAH and are

correlated with worse outcomes.[111,112] For these reasons, SD have also been associated with DCI and some small observational studies support this link.[112-114] Observational data also indicates that SD can trigger intense vasoconstriction in ischemic brain, although this appears to be reactive to ischemia, rather than causative.[115] Its role in DCV is not fully explored.[116] Additional investigations of SD in aSAH patients are limited because SD are not reliably detected using traditional transcranial electroencephalograms (EEG) due to the low amplitude, short duration and specific anatomic locations of SD.[117] In human and experimental observations, SD are studied using brain electrocorticograms derived from electrodes applied directly to the external brain surface after or during surgical craniotomy.[117,118]

1.6 Haptoglobin as a DBI Modifiable Factor

Haptoglobin (Hp) is a naturally occurring α-2 sialoglycoprotein produced in the liver.[119] Hp has a plasma half-life of approximately 5 days and is primarily responsible for binding free-floating hemoglobin (Hb) in plasma, thereby preventing systemic iron loss and reducing filtration-related kidney injury.[120,121] Hp is an acute phase reactant and its synthesis is stimulated by increased circulation of systemic proinflammatory cytokines, including IL-1β, IL-6, and TNF-α.[122] In response to these cytokines, increased Hp synthesis occurs through complex interactions between different transcriptional regulators and regions of the human haptoglobin gene.[123,124] Hp also has a role in modulating the inflammatory response to infection and injury through its effect on

macrophage CD163.[125] For these reasons, Hp may be a modifiable factor for the development of DBI, based on the inflammation-mediated model, after aSAH.

1.6.1 Haptoglobin molecular structure

Hp exists primarily as a tetramer of 2 different polypeptide subunit chains, Hpα and Hpβ. (Figure 3) These tetramers have a linear configuration, consisting of 2 internal light Hpα chains linked together by a strong disulfide bond.[126] On the outside of the molecule, heavier Hpβ chains are linked to each of the inner Hpα protein chains. The genes for both the Hpα and Hpβ chains are contiguously coded at chromosome location 16q22.[127] Once the Hp proto-protein is synthesized, the individual chains are edited and quickly polymerize to form the fully functional Hp molecule.[119]

Figure 3: Illustration of a Typical Haptoglobin Molecule

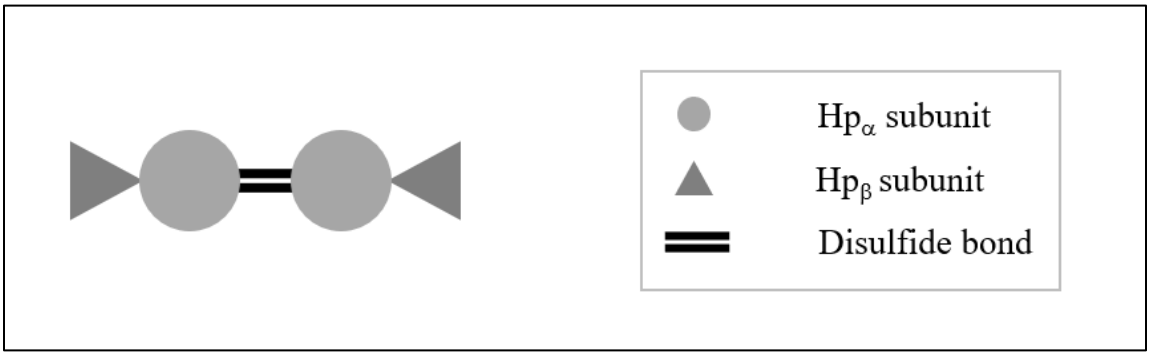


Illustration of the 2 different haptoglobin (Hp) subunits and the disulfide bond in a typical Hp molecule.

1.6.2 Haptoglobin binding to free hemoglobin

As previously discussed, the primary role of Hp is to bind to free Hb to prevent iron loss and kidney injury. To accomplish this, the heavy Hp β-chain units bind to the β-globin Hb chain through a very strong non-covalent bond in a 1:1 interaction, forming a large Hp-Hb protein complex.[128] These protein complexes are ultimately internalized and metabolized by circulating macrophages, via a process mediated through the macrophage CD163 cell-surface receptor.[129] Once internalized, lysosomal heme oxygenase-1 (HO-1) is responsible for degradation of hemoglobin into free iron, biliverdin and carbon monoxide.[130]

1.6.3 Haptoglobin scavenger receptor (CD163)

Macrophage CD163 (also sometimes known as M130, p155 or the Hb scavenger receptor) is a 130 kDa glycoprotein almost exclusively expressed on cells derived from monocytes, most commonly mature tissue macrophages, including hepatic Kupffer cells, hepatic red pulp macrophages, thymic cortical macrophages and perivascular and meningeal macrophages within the central nervous system.[131] The CD163 receptor belongs to a group of proteins known as scavenger receptor cysteine-rich (SRCR) domain-containing proteins that generally function as pattern recognition receptors for invading pathogens.[131] CD163 consists of a short 24-residue trans-membranous section, a cytoplasmic tail, and an extracellular portion that contains 9 different SRCR domains that are essential for Hp-Hb complex endocytosis and play an important role in modulating immune system activation.[132] Cell membrane CD163 expression is increased by

glucocorticoid administration and by the acute phase mediators, IL-6 and IL-10, whereas, TNF-α, interferon and LPS result in decreased CD163 expression.[133]

Hp-Hb complexes interact with CD163 on the surface of monocytes and macrophages through pH-dependent and calcium-mediated high-affinity binding.[129,134] Studies have shown that CD163 can only bind Hp and Hb when they are complexed together, suggesting that the combination, together, displays a unique epitope, allowing for CD163 binding in this specific conformation.[129,135] In addition, because Hp-Hb binding to CD163 can be prevented by specific antibodies to or by proteolytic cleavage of the SRCR domain 3, it is likely that this domain plays an important role in the complex-receptor binding mechanism.[129,136]

CD163 activation has important effects on both pro-inflammatory and anti-inflammatory processes.[125] Hp-Hb binding to CD163 increases macrophage release of the anti-inflammatory agent, IL-10.[137] IL-10, in turn, leads to increased CD163 expression, increased Hb metabolism, and down-regulation of the inflammatory response to extracellular Hb.[125] Experimental cross-linked monoclonal antibodies to CD163 result in increased macrophage release of IL-6 and GM-CSF.[138] Activation of other CD163 binding sites, as may occur with tumor necrosis factor-like weak inducer of apoptosis (TWEAK), bacterial cell membrane proteins, and viral envelope proteins, especially to CD163 domains 2 and 3 lead to increased secretion of IL-1β, IL-6 and TNF-α and decreased IL-10.[125,139,140]

1.6.4 Haptoglobin phenotypes

Many different sizes and shapes of Hp molecules can be found in human populations because three different Hp phenotypes exist in humans. These different Hp phenotypes occur because of differences coded in the Hpα subunit. These different versions of the Hpα gene allele are known as Hpα1F, Hpα1S, and Hpα2. The Hpα1F and Hpα1S alleles both code for small proteins (~86 kDa) that are differentiated by only a single amino acid, have only a single Hpβ binding site, and produce protein chains that are clinically similar. For this reason, proteins created from either the Hpα1F or the Hpα1S allele are referred to as Hpα-1. Mice and other non-human animals have only a single phenotype and produce Hp molecules made only from Hpα-1 proteins.

The Hpα2 gene allele exists only in humans and is a gene sequence that appears to be a fusion of both the Hpα1F and the Hpα1S alleles. The protein product from this allele, called Hpα-2, is significantly larger than the Hpα-1 protein and contains two Hpβ binding sites. Additional, very rare, mutations and fusions of the HPα allele have also been reported. These rare mutations have not been associated with clinical disease.

Human Hp phenotypes are defined by differences in the linked light α-chain protein products found in the final Hp molecule. When Hp molecules are formed from only Hpα-1 proteins, the Hp phenotype is known as Hp1-1. When Hp molecules are

formed from only Hpα-2 proteins, the Hp phenotype is known as Hp2-2. When Hp molecules are formed from combinations of Hpα-1 and Hpα-2 proteins, the Hp phenotype is known as Hp1-2.

Because the Hpα-2 protein is derived from 2 fused alleles, the protein has 2 distinct Hpβ binding sites, compared to the single binding site on the Hpα-1 protein. This additional binding site allows Hp molecules with one or more Hpα-2 proteins to be constructed with multiple different conformations and sizes, as seen in Figure 4. These molecular size and chemic differences allowed the different Hp phenotypes to be first discovered using gel electrophoresis of Hb-supplemented serum. [141] An ELISA test has also been developed to detect these different Hp phenotypes.

Figure 4: Illustration of Hp Phenotype Molecular Sizes and Shapes

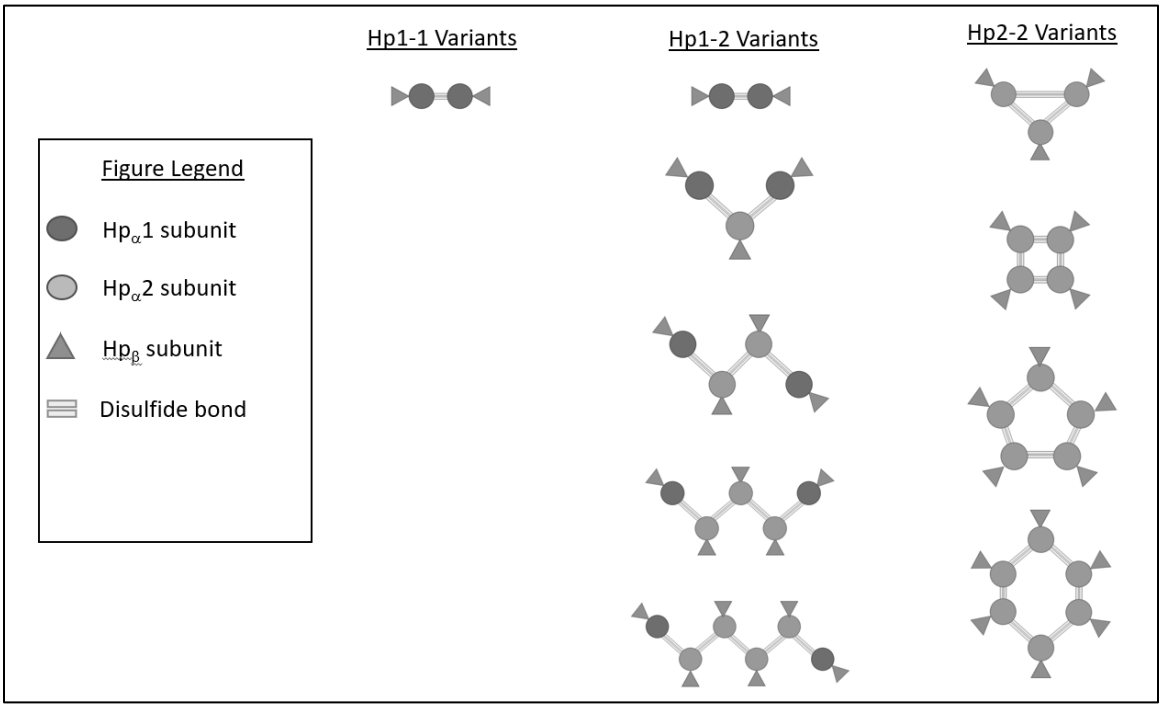


Illustration of the different haptoglobin (Hp) subunits in the Hp molecules formed by each of the Hp phenotype variants.

1.6.5 Haptoglobin phenotype frequencies

The three primary Hp phenotypes (Hp1-1, Hp1-2 and Hp2-2) exist within populations of patients at different frequencies, primarily dependent on the geographic origins of the populations.[119,142,143] The Hp2-2 phenotype occurs with the highest prevalence among native Indian and Australian Aborigines, with reported 84% and 66% penetration in these populations, respectively. [119] It is likely that the Hp$_\alpha$2 allele mutation first occurred in one of these populations and then migrated due to selection pressure throughout Asia, Europe, North American and South America. [144] Western European and North American populations are characterized by a predominance of the Hp1-2 phenotype and sub-Saharan African populations are characterized by a predominance of the Hp1-1 phenotype. Because of the "mixing pot" effect that exists in North America, the reported Hp phenotype frequencies are different between Caucasians and African-Americans with Caucasians demonstrating a ratio of 2.59 between the Hp2-2 and the Hp1-1 phenotypes whereas African-Americans demonstrated a ratio of only 1.45, primarily because of an increase in the frequency of the Hp1-1 phenotype.[119] Different ratios for Hp phenotype frequencies in specific population is likely to be additionally dependent on the local populations, migration patterns, ethnic intermixing and disease prevalence in the community.

1.6.6 Haptoglobin phenotypes and CD163 binding

Different Hp phenotypes bind CD163 with different binding affinities. CD163 has a 10-fold greater binding affinity for the Hp2-2 and Hp1-2 haptoglobin phenotypes

than for the Hp1-1 phenotype.[129] This has been hypothesized to occur because of either an increased number of binding sites or a clustering of binding sites from the multimeric variants of Hp2-2 and Hp1-2 compared to the linear Hp1-1 variant.[129,135] This phenomenon was termed "bonus effect of multivalency" and results in increased Hp-Hb uptake, as well as, secondary effects within the macrophage, including increased secretion of pro-inflammatory cytokines, including IL-1β, IL-6, IL-10 and TNF-α.[135]

1.6.7 Haptoglobin phenotypes and systemic diseases

Patients with different Hp phenotypes appear to have different outcomes in some systemic diseases.[145] Most notably, the Hp2-2 phenotype has been associated with an elevated risk of coronary artery disease (CAD) and myocardial infarction, particularly in diabetic patients.[146-148] While still under investigation, CAD appears to occur more frequently in Hp2-2 patients because of increased plasma iron accumulation, high-density lipoprotein (HDL) oxidation, and atherosclerotic plaque development.[149] The Hp2-2 phenotype has also been associated with a greater risk of hypertension, and hypertensive Hp2-2 patients are more likely to need antihypertensive medications and to require multiple drug combinations to achieve an appropriate blood pressure than are hypertensive patients with the other Hp phenotypes.[150,151] Likewise, the Hp2-2 phenotype has been associated with an increased risk of peripheral vascular disease.[152-154]

1.7 Haptoglobin Phenotypes in aSAH

Because blood containing both Hp and Hb is released into the subarachnoid space after aSAH, the interaction between Hp, Hb, and CD163 may be an important cause of DBI after aSAH. While the pathophysiologic basis for this hypothesis is not defined, observational studies in humans and transgenic mice have attempted to clarify the strength of this observation.

1.7.1 Human aSAH studies in patients with different Hp phenotypes

To date, several small human observational studies and a single meta-analysis have evaluated clinical outcomes and DBI, including DCV and DCI after aSAH among patients with different Hp phenotypes. [155-162] A summary of these published studies can be found in Table 1 below. In general, most of these studies are small (<100 patients), have variability in the prevalence of the Hp phenotypes in the target population, and poorly evaluate DCV, DCI and/or clinical outcome, using in some cases, non-standard definitions or assessments. Despite these significant limitations, the published studies suggest an association exists between the Hp phenotype and DBI, especially DCV. An association between DCI and clinical outcomes is much less clear from these studies.

Table 1: Published Observational Studies of Hp Phenotypes in aSAH.

Author	Year	# aSAH Patients	% Hp2-2 Patients	DCV	DCI	Clinical Outcome	Comment
Borsody	2006	32	34	+	NA	+	Small study. DCV defined by both TCD and DSA.
Galea	2012	30	4	NA	NA	NA	Primarily focused on biochemical endpoints.
Ohnishi	2013	95	52	+	-	-	Demonstrated significant effect of Hp phenotype on DCV
Kantor	2014	193	31	NA	NA	+	Primarily evaluated long-term clinical outcome.
LeClerc	2015	74	32	+	-	-	Complex description of DCV using unvalidated .
Murthy	2016	133	35	NA	NA	-	Evaluated infarction rather than DCV or DCI.
Kim	2018	87	45	+	+	NA	DCV defined only by DSA.

To clarify and evaluate the strength of the association between Hp and DBI in aSAH patients, a meta-analysis of the 6 published studies was published in 2017.[161] The authors of the meta-analysis categorized the results of studies into short-term outcomes, including DCV and DCI during hospitalization, and long-term outcomes, including modified Rankin Scale (mRS) and Glasgow Outcome Score (GOS) between 1-3 months after aSAH. Based on the 553 patients across these studies, the authors found an association between Hp phenotype and both short-term and long-term outcomes. In their data, the Hp2-2 phenotype was associated with more DCV and DCI when compared to Hp1-1, Hp1-2, as well as, the combined Hp1-1 and Hp1-2 cohorts.

1.7.2 Experimental SAH studies in transgenic mice with different Hp phenotypes

Because mice and other non-human animals have only the Hp1-1 phenotype, evaluations of DBI after experimental aSAH, including DCV and DCI in Hp2-2 animals utilize transgenic models. To facilitate this type of research, a mouse Hp2 gene allele was created by Levy and colleagues from a duplication of the wild-type Hp1 genetic code for exons 3 and 4, similar to the type of event that likely created the Hp2 gene allele in humans.[163,164] In subsequent validation studies, the Hp molecules from transgenic Hp2-2 mice have been shown to have a similar size and shape to human Hp2-2 molecules and the serum concentration of Hp has been shown to be similar between the wild-type Hp1-1 mice and the transgenic Hp2-2 mice.[119,164]

To date, 5 studies, all from the same lab, have evaluated the effect of DBI in this transgenic mouse model. In these studies, DCV was evaluated by measuring basilar artery lumen patency after experimental SAH. This data was then reported as a percent change in patency compared with control animals where a lower percentage was equivalent to more DCV. DCI was evaluated using a simple, objective scale of posturing, grooming and ambulation 24-hours after experimental SAH.[165] This data was reported as a score from 0-3 where a lower score was equivalent to more DCI.

Chaichana and colleagues published the first study specifically designed to measures differences in lumen patency and activity level between Hp1-1 mice and Hp2-2 mice. In this study, Hp2-2 mice had significantly more lumen patency change (more DCV) and worse activity level (more DCI).[165] Subsequent studies primarily evaluated potential DBI therapies, including a glutathione peroxidase mimetic, a NO donor in an experimental polymer, L-citrulline and S-4-CPG.[166-169] While these studies demonstrated, to varying degrees, that lumen patency was worse in the transgenic Hp2-2 animals, there was also a concerning variability in the reported lumen patency in the Hp1-1 groups (82.3% to 71.5%) that was not present in the Hp2-2 groups. Despite this concern, the experimental DBI therapies were all able to restore lumen patency. Activity level data was inconsistent across the studies and the experimental DBI therapies, except systemic L-Citrulline, were not able to restore activity levels after SAH.

1.8 Soluble CD163 (sCD163)

Based on these human and animal studies, it seems plausible that different Hp phenotypes may affect the inflammatory response after aSAH through activation of the CD163. In addition, different Hp phenotypes may also affect the inflammatory response through differential activation of a related molecule, soluble CD163 (sCD163). sCD163 is a free-floating form of macrophage CD163 found in plasma and other body fluids.[170,171] It is a large molecule (945 amino acids), containing portions of all nine of the CD163 extracellular domains.[172] While multiple avenues of research are ongoing, the physiologic and pathologic roles of sCD163 are not fully known and its effect on systemic and neurologic diseases remain poorly defined.

sCD163 is formed from enzymatic cleavage of the extracellular portions of the CD163 protein from the trans-membranous portions by the ADAM17/TACE enzyme.[173] ADAM17/TACE resides in the macrophage membrane and becomes activated by various physiologic and pathologic stimuli.[174] When activated, ADAM17/TACE results in the cleavage and release of more than 40 membrane-bound proprotein substrates, including sCD163.[174,175] Increased sCD163 release by ADAM17/TACE is stimulated by Toll-like receptor (TLR) activation and other inflammatory mediators, oxidative stress, and thrombin.[176-179] For this reason, sCD163 has been hypothesized to have a modulator role in acute and chronic systemic inflammation.[175]

1.8.1 sCD163 in systemic diseases

sCD163 has been investigated in various diseases associated with deranged inflammation, including sepsis, hemophagocytic syndrome, liver failure, and disseminated intravascular coagulation (DIC). In experimental sepsis models, plasma sCD163 concentration rapidly rises after infectious or inflammatory insult occurs.[180] Clinical studies of bacteremia and septic shock in humans have demonstrated that a significantly elevated sCD163 concentration is associated with a worse prognosis than only a moderate increase in concentration.[181,182] Likewise, in hemophagocytic syndrome, a disease associated with macrophage hyperactivity and inappropriate macrophage endocytosis of normal blood cells, very high sCD163 concentrations occur early in disease and resolve as a marker of response to disease treatment.[183,184] sCD163 is also significantly increased in patients with active liver disease such as cirrhosis and hepatitis.[185] In these conditions, a significant increase in sCD163 concentration is also correlated with a worse prognosis.[186,187] Finally, in DIC, sCD163 concentration are also significantly increased and can be correlated with abnormalities in D-dimer concentrations.[179]

1.8.2 sCD163 in neuroinflammatory diseases

Studies of plasma and CSF sCD163 concentrations in neuroinflammatory diseases are less well described. To date, sCD163 has been investigated in multiple sclerosis, infectious encephalopathy, and chronic inflammatory demyelinating polyneuropathy. The majority of these investigations are small, single-center evaluations of multiple

biomarkers of disease or disease progression in a known population.[188-190] In each of these diseases, increased concentrations of plasma and/or CSF are indicative of disease and/or its progression.

1.8.3 sCD163 in aSAH and other types of hemorrhagic stroke

sCD163 has been investigated in both intracerebral hemorrhage (ICH) and subarachnoid hemorrhage. ICH is similar to aSAH but results in bleeding within the brain tissue itself and is associated with hypertension, head injury, and the use of anticoagulation therapies, such as warfarin, clopidogrel, and direct oral anticoagulants (DOAC).[191-193]

A recent observational study grouped 54 ICH patients according to their plasma sCD163 concentration determined within 24 hours of injury.[194] Despite having similar hematoma size at admission, patients with a lower initial sCD163 concentration had a higher hematoma volume at PBD14 and a slower calculated hematoma absorption rate than did patients with a higher initial sCD163 concentration. These patients also had worse recovery at PBD14, PBD30, and PBD90 day. The authors concluded that sCD163 may have an important role in the absorption of blood after ICH, thereby affecting overall recovery and prognosis.

In a second study of 51 ICH patients, serum and CSF sCD163 concentrations were obtained at prespecified time points up to PBD10.[195] In this population, the serum

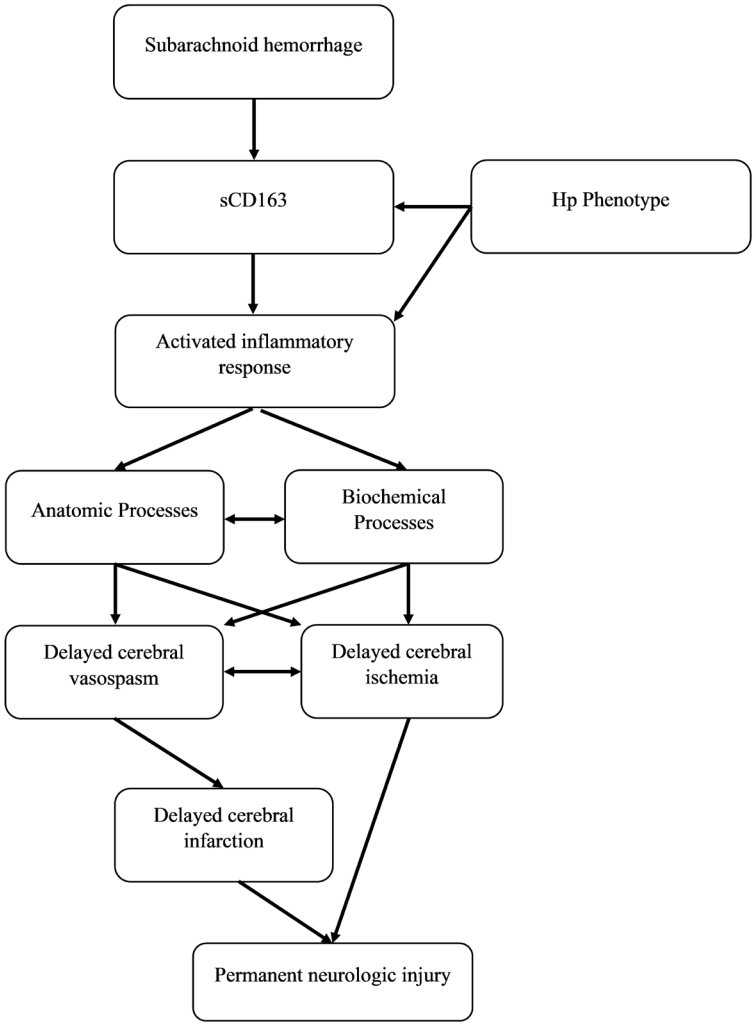
sCD163 concentration remained relatively stable from PBD1 through PBD8 before significantly increasing on PBD10; however, the CSF sCD163 concentration had significant variability at all time points and no specific pattern was identified. Patients with lower serum sCD163 concentrations at PBD2 had greater hematoma volume and were more likely to have perihematomal edema. There were no differences in CSF sCD163 concentrations between patients with different perihematomal volume or edema. The authors also concluded that sCD163 may have an important role in the absorption of intracerebral blood and further hypothesized that altered neuroinflammation as a consequence of sCD163 may play a role in the observed differences in perihematomal edema.

Only a single study has evaluated sCD163 concentrations in aSAH.[156] In this study, serum and CSF concentrations of sCD163 were analyzed from 30 aSAH patients and 20 control patients. Unfortunately, neither the serum or CSF sampling time points were described. In their analysis, there was no difference in serum sCD163 concentrations between the control group and patients with aSAH, whereas, the CSF sCD163 concentration was greater in aSAH patients than in control patients. The authors hypothesized that the normal Hp-scavenging mechanism was rapidly overwhelmed during aSAH, causing rapid release of sCD163, from macrophage cells.

1.9 Role of Haptoglobin and sCD163 in Inflammation-Mediated DBI Model

Based on these observational and experimental studies, Hp appears to play an important role in the inflammation-mediated model of DBI. I propose that, in this model, Hp is a modifier factor that can either drive the development of DBI through an effect on the linking trigger between subarachnoid hemorrhage and the activated inflammatory response or through a direct effect on inflammatory response itself. (Figure 5) In this updated model, Hp phenotype is the key driver for this interaction and incorporates the observed differences in DBI outcomes for the different Hp phenotypes. Based on this updated model, the different Hp phenotypes should also demonstrate different inflammatory responses, as well as, different rates of DCV and DCI after aSAH.

Figure 5: Updated Inflammation-Mediated Model of DBI



An updated version of the complex inflammation-mediated model of Delayed Brain Injury (DBI) that incorporates soluble CD163 (sCD163) and haptoglobin (Hp) phenotype as important modifiers of the activated inflammatory response.

Likewise, sCD163 may play an important role in this DBI model. I propose that, in this model, sCD163 is the linking trigger between subarachnoid hemorrhage and the activated inflammatory response. (Figure 1.5) Based on this updated model, changes in sCD163 should be associated with differences in the activated inflammatory response, as well as, different rates of DCV and DCI after aSAH. Importantly, sCD163 activity may also be modifiable by Hp through differential binding and secondary activation by the different Hp phenotypes, further integrating both sCD163 and Hp into this complex, inflammation-mediated model of DBI.

1.10 Conclusions

This chapter introduced the key features of aSAH, described the importance of delayed brain injuries (DBI) and their potential pathophysiologic mechanisms, and detailed the potential effect that different haptoglobin (Hp) phenotypes have on DBI and clinical outcomes. In addition, this chapter concluded with a discussion of the potential mechanisms that might link the Hp phenotypes and sCD163 to the development of DBI and long-term clinical outcomes through an inflammation-mediated model. The following chapters detail my approach to evaluating these mechanisms through the development of a prospectively-collected aSAH biobank and the use of this biobank to evaluate the effect of different Hp phenotypes on systemic and CSF inflammatory cytokines and sCD163 at multiple time points.

CHAPTER 2: DEVELOPMENT AND RESULTS OF ANEURYSMAL SUBARACHNOID HEMORRHAGE BIOBANK

2.1 Introduction

This chapter describes the successful development of a single-center, prospective aSAH biobank at the University of Kentucky. This biobank is needed to facilitate future research because the rate of annual rate of hospitalization for aSAH is relatively low, the potential data points, including demographic, disease-specific, and clinical outcome data, are not routinely collected in the EMR or other medical records, and biofluid sampling is not routinely performed at pre-specified time points. This chapter will discuss the development of protocols for screening and recruitment of subjects, definitions of clinical variables and outcomes, and protocols for biofluid sampling, preparation, and storage. This chapter also reports the results of these screening and recruitment protocols and important demographic and clinical outcome data generated from subjects recruited to the biobank over a 2-year period.

2.2 Methods

2.2.1 Study design

To facilitate current and future research in aSAH, a prospectively collected biobank of aSAH patients admitted to the University of Kentucky Chandler Medical Center was designed. This biobank includes demographic data, disease-specific data, clinical outcome data, as well as, plasma and CSF biofluids. All study procedures for

this biobank were approved by the University of Kentucky Institutional Review Board (IRB) prior to subject enrollment and data collection. Access to the biobank data and biofluid samples is limited to study-related personnel but deidentified data may be available for future IRB-approved research with principal investigator (PI) approval.

2.2.2 Study population

All adult patients (age ≥ 18 years) admitted to the University of Kentucky Chandler Medical Center between July 1, 2018 and June 30, 2020 with angiographically-proven aneurysmal subarachnoid hemorrhage who were likely to survive to, at least, PBD14 and had an EVD placed were considered for biobank inclusion.

Patients were excluded from this biobank project if consent was not obtained by PBD3, if they received blood or blood product transfusion prior to PBD3 sampling, if they died prior to PBD3 or were unlikely to survive to PBD14, as determined by their neurosurgical or neurocritical care teams, had angiographic evidence of vasospasm on or before PBD3, had pre-existing neurologic injury that impaired recovery or survival, or had pre-existing systemic or neurologic inflammatory disease.

2.2.3 Initial screening procedure

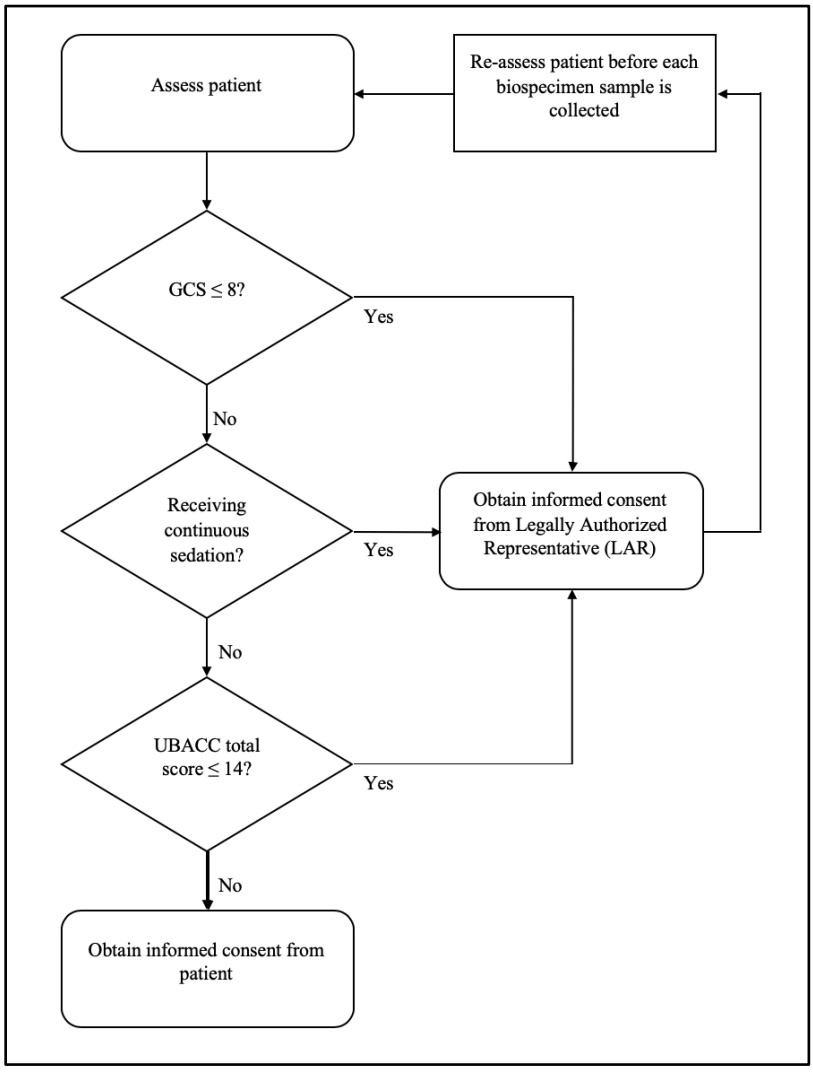
To enroll subjects in this biobank, all SAH patients were evaluated for the defined screening criteria prior to being approached for consent by biobank personnel. These screening criteria were implemented to reduce the potential emotional or psychological

burden of research participation for patients and/or their families who would not, ultimately, be enrolled in the biobank. Patients and/or their LAR were not approached for consent if they had a non-survivable neurologic injury, if no EVD was placed by PBD3, or if no aneurysm was identified on radiographic or angiographic testing by PBD3. This information was obtained from the neurosurgery or neurocritical care teams at the time of biobank team notification.

2.2.4 Consent of impaired subjects procedure

All aSAH patients who met screening criteria were then approached for informed consent. Because aSAH patients may have impaired consciousness and/or thinking processes that can interfere with their decision-making capacity, special procedures were used to obtain informed consent. (Figure 6) For patients with a GCS ≤ 8 or for patients with a GCS > 8 who were receiving continuous infusions of sedative medications that could not be temporarily discontinued, the LAR was approached for informed consent. For patients with a GCS > 8 who were receiving continuous infusions of sedative medications that could be temporarily discontinued, sedation was discontinued, and the patient was monitored until they were awake. At that point, the University of California, San Diego Brief Assessment of Capacity to Consent (UBACC) tool was used to evaluate the capacity to provide consent.[196] For patients with a GCS > 8 not receiving continuous infusions of sedation medications, the UBACC tool was used to evaluate the capacity to provide consent.

Figure 6: Process to Obtain Informed Consent for Biobank Subjects



A flow diagram of the process used to obtain informed consent for the biobank. The process is designed to assess the decision-making capability of the patient using a combination of Glasgow Coma Scale (GCS) and the University of California San Diego Brief Assessment of Capacity to Consent (UBACC), a validated research tool.

The UBACC tool is a 10-item scale, utilizing a set of standardized questions, with a focus on evaluating whether the patient has an understanding and an appreciation of the information concerning the research protocol.[196] The response to each question is scored on a scale from 0-2, based on the appropriateness of the patient's response to a set of standardized questions. The scores from each question are then totaled. A total score ≤ 14 is consistent with impaired decision-making capacity. In this project, the LAR was approached for informed consent if the patient's UBACC score was less than or equal to 14; whereas, the patient was approached for informed consent if their UBACC score was greater than 14, the patient was approached for informed consent. All decision-making capacity assessments using the UBACC tool were performed by a single researcher. The instrument used for UBACC assessments can be found in Appendix 1.

If the LAR was approached for initial consent due to impaired decision-making capacity, the patient was re-assessed for improvement in decision-making capacity prior to each biospecimen collection point using the process described above and illustrated in Figure 6. If, at any point, the patient achieved a UBACC score > 14, the patient was approached for informed consent for the research protocol prior to biospecimen collection. Patient refusal of informed consent terminated the patient's participation in the research protocol.

2.2.5 Clinical care of research subjects

Subjects enrolled in the aSAH Biobank received routine standard of care diagnostic and therapeutic modalities. There were no changes to their care, other than blood and CSF sampling, related to their participation in this biobank. For all subjects, blood pressure was controlled with nicardipine to target systolic blood pressure (SBP) ≤ 140 mmHg prior to aneurysm obliteration. After obliteration, mean arterial pressure (MAP) was maintained within a range of 60-100 mmHg with SBP goal relaxed to ≤ 200 mmHg, unless otherwise indicated. Aneurysm obliteration therapy was performed within 24 hours of hospitalization. All subjects received nimodipine therapy for 21 days underwent daily TCD scanning for at least the first 14 days after aneurysm rupture, unless they were discharged from the hospital prior to this time point.

Patients with symptomatic or suspected DCV underwent DSA and intra-arterial injection of verapamil at the discretion of the attending or interventional neurosurgeon. Patients with DCI were treated with therapeutic hypertension (MAP goal >100 mmHg) and, if symptoms did not resolve, DSA with intra-arterial injection of verapamil within vasospastic vessels. Patients with resistant DCV or DCI underwent periodic DSA and high-dose milrinone infusion until neurologic status improved or PBD14, whichever occurred earlier. Deviations from these practices were dictated by the attending neurosurgical or neurocritical care teams based on the individual patient outcomes and clinical course.

2.2.6 Demographic and disease-specific data collection

Following informed consent, baseline and demographic data was collected from the patient, the patient's family, and the electronic medical record (EMR). All data was recorded directly into an IRB-approved online database. Instruments used for this data collection can be found in Appendix 2.

Disease severity for aSAH patients was collected using both the Hunt & Hess scale (HH) or the World Federation of Neurologic Scale (WFNS).[197-200] These disease severity grading systems are most commonly associated with hospital and/or 30-day mortality.[15] The HH score was determined at the time of hospital admission from observed clinical features. (Table 2) When possible, patients were scored prior to administration of sedation or before procedures such as endotracheal intubation are performed. If patients were already intubated or were receiving sedation, sedation was held until a neurologic examination could be performed.

Table 2: Hunt & Hess Score

Score	Description
1	Mild headache
2	Moderate-severe headache, nuchal rigidity, or cranial nerve deficit
3	Drowsiness, confusion, focal neurologic deficit
4	Stupor or severe hemiparesis
5	Coma or decerebrate posturing

The World Federation of Neurological Surgeons (WFNS) scale score was also collected at the time of hospital admission.[200] The WNFS scale was developed to incorporate more objective features, such as the Glasgow Coma Scale (GCS) score and the absence or presence of motor deficits, into severity grading. (Table 3) Like the procedure for HH scoring, WFNS scoring was performed prior to sedation and/or intubation, when possible, and after sedation was temporarily held if the patient was intubated and sedated prior to hospitalization.

Table 3: World Federation of Neurological Surgeons (WFNS) Score

Score	Glasgow Coma Scale (GCS) Score	Motor Deficit
1	15	Absent
2	13 – 14	Absent
3	13 – 14	Present
4	7 – 12	Absent or Present
5	3 – 6	Absent or Present
6	Death	NA

Based on associated mortality rates, for both HH and WFNS, scores from 0-3 were considered “favorable” or and scores 4-6 were considered “poor”.[15]

The Modified Fischer Scale score was also collected for each patient from initial computed tomography (CT) scan of the head. (Table 4) The mFS score is correlated with risk of DCV. While, mFS scores 0-3 are associated with only a slight increase in the risk of DCV, mFS score =4 is associated with significantly higher risk (OR=2.2, 95% CI 1.4-3.5).[25] For this reason, mFS scores of 0-3 were considered "favorable" and mFS scores of 4 were considered "poor".

Table 4: Modified Fischer Scale Score

Score	SAH (Focal or Diffuse)	IVH
0	No SAH	Absent
1	Thin SAH layer	Absent
2	Thin SAH layer	Present
3	Thick SAH layer	Absent
4	Thick SAH layer	Present

2.2.7 Determination of delayed brain injury diagnoses

The presence or absence of DCV was determined from TCD data from PBD3 through PBD14. The left and right middle cerebral artery (MCA) and internal carotid artery (ICA) blood flow velocities were recorded daily from the EMR. Subjects were considered to have DCV if they had either: (1) an MCA mean flow velocity more than 120 cm/sec and an MCA/ICA velocity ratio greater than 3.0 or (2) angiographic evidence of intracranial arterial vasospasm documented in the EMR. The presence or absence of

DCV was recorded on the PBD30 case report form. Missing values, as may occur from patient or technical factors reducing the ability to visualize either the MCA or ICA arteries with TCD, were treated as negative DCV results.

The presence or absence of DCI was determined from Glasgow Coma Scale (GCS) data available in the EMR on PBD3 through PBD14. Patients with a decrease in their GCS of more than 2 points during a 24-hour period that could not be attributed to other medical sources such as sedation, infection or seizures were considered to have DCI. The presence or absence of DCI was recorded on the PBD30 case report form. Missing data were treated as negative DCI results.

The presence or absence of delayed cerebral infarction was determined from CT and/or MRI scans available in the EMR on PBD3 through PBD90. Patients with evidence of new cerebral infarction, as reported by a neuroradiologist, that were not present on scans performed prior to PBD3 were considered to have delayed cerebral infarction. The presence or absence of delayed cerebral infarction was recorded at the PBD90 follow-up form. Missing data were treated as negative delayed cerebral infarction results.

2.2.8 Clinical outcome and disability scoring

All patients were evaluated for survival at the time of hospital discharge. In addition, all patients were evaluated for survival, as well as, continued neurologic

disability on about PBD30 and PBD90 using the modified Rankin scale (mRS) and the Glasgow Outcome Scale Extended (GOSE). The scale scores for all research subjects were obtained by the PI using standard scripts. Scale scores for research subjects still admitted to the hospital at the time of examination were obtained at the bedside from the subject and/or the LAR. Scale scores for research subjects not admitted to the hospital was obtained via telephone conversation with the subject and/or the LAR. Telephone contact was attempted daily for 3 days and then at 1 week and 2 weeks after both PBD30 and PBD90, until contact with the patient or LAR was made. If no contact was made after these attempts, the patient was considered lost to follow-up. No contact was attempted for subjects who had previously died. These subjects were given scale scores of mRS=6 and GOSE=1 at PBD30 and/or PBD90. mRS scores 0-3 were considered "favorable" and mRS scores 4-6 were considered "poor"; whereas, GOSE scores 5-8 were considered "favorable" and GOSE scores 1-4 were considered "poor".

2.2.9 Biofluid sample preparation and storage

All biofluids (blood and cerebrospinal fluid) were collected at pre-defined specific time points by a trained member of the research team. Biofluids were collected on PBD3, PBD5, PBD7, and PBD10 using IRB-approved protocols. All biofluid processing procedures were completed within 30 minutes of sampling. Sampling and process procedures that were completed in more than 30 minutes were marked as protocol violations.

Blood samples were collected directly from research subjects via an indwelling arterial catheter into 10 mL plasma tubes with 18 mg K2 EDTA (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood samples were collected according to local hospital protocols to reduce the risk of infectious or mechanical complications. The 10 mL blood sample was then transported on ice to the research laboratory and spun in a refrigerated centrifuge (Allegra 6KR Centrifuge, Beckman Coulter Life Sciences, Indianapolis, IN) at 1200 x g for 10 minutes to separate the plasma from the red blood cells, leukocytes and platelets. The plasma portion was then pipetted into 6 separate 250 mcl aliquots and stored in a -80 C freezer until needed for analysis. Blood sample waste was destroyed per an approved biosafety protocol.

CSF samples were collected directly from research subjects via an indwelling extraventricular drain (EVD) into 15 ml polypropylene conical tubes. CSF samples (3 mL) were collected according to local hospital protocols to reduce the risk of infectious or mechanical EVD complications. CSF samples were transported on ice to the laboratory and spun in a refrigerated centrifuge (Allegra 6KR Centrifuge, Beckman Coulter Life Sciences, Indianapolis, IN) at 1200 x g for 10 minutes to separate CSF from red blood cells, leukocytes, platelets and other debris. CSF was then pipetted into 6 separate 250 mcl aliquots and stored in a -80 freezer until needed for analysis. CSF sample waste was destroyed per an approved biosafety protocol.

2.2.10 Haptoglobin phenotype analysis

After all subjects had been recruited, the haptoglobin phenotype (Hp1-1, Hp1-2 or Hp2-2) was determined for each research subject using a commercial ELISA kit from Savyon Diagnostics (Technion, Israel). (Catalog #A-710-01M, Lot # 710-A0151500, Exp. 1/2021) by the University of Kentucky Center for Clinical and Translational Science (CCTS) Biomarker Analysis Laboratory (BAL). This Hp phenotype ELISA test utilizes a monoclonal antibody (mAb) against the Hp molecule, utilizing the varying number of binding sites, to differentiate between the Hp phenotypes.[201] The specific steps used by the UK CCTS BAL in this analysis are described in Appendix 3. Subjects were defined as either Hp1-1, Hp1-2 or Hp2-2, based on the results of the ELISA. Because a recent meta-analysis demonstrated that Hp1-1 and Hp1-2 patients had similar rates of DBI compared to Hp2-2 patients, subjects were further defined as either Hp1 class (subjects with Hp1-1 or Hp1-2 phenotypes) or Hp2 class (subjects with Hp2-2 phenotype).[161]

2.2.11 Data management plan

A data management plan was created and approved by the IRB prior to study-related procedures. All research data was entered directly into an online, browser-based research database (REDCap) by IRB-approved study personnel. REDCap utilizes user authentication and role-based security, electronic case report forms (CRFs), real-time data validation, data integrity checks, centralized data backup, and data export options to common statistical packages to facilitate data collection, storage and dissemination across many different clinical and research projects.[202,203] The REDCap database was

developed at Vanderbilt University, was first launched in 2004, and has gained widespread support since that time, in large part through its use in an evolving consortium of academic institutions, including more than 3200 organizations in more than 120 countries.[204]

All subject data was collected at the time of consent, as well as at 7 specific study visits (PBD3, PBD5, PBD7, PBD10, PBD14, PBD30 and PBD90). Online CRFs were completed at each visit Appendix 2. Results of Hp phenotype analysis for each subject were also entered into the REDCap database. Data retrieval from the EMR and entry into the database was verified on a weekly basis by the PI. All data errors were immediately corrected in the online database by the PI.

2.2.12 Safety management plan

A subject safety management plan was created and approved by the IRB prior to study-related procedures. The major risks to subjects in this study were related to CSF sampling, blood sampling and breaches of confidentiality. To minimize risks from CSF and blood sampling, these procedures were performed under sterile conditions, according to pre-specified protocols, as previously described in this chapter. To minimize risks from breaches of confidentiality, subjects were identified only with the use of a study identification number for study-related data collection.

All research subjects were monitored for the maintenance of safety by the PI. Subjects with known or suspected adverse events (AE) or series adverse events (SAE) were evaluated, documented and reported to the IRB per standard research policies. The PI and clinical co-investigators reviewed the AE/SAE reports after the first 10 patients completed the research protocol to evaluate patient safety, data accuracy and protocol compliance. Because there were no AE/SAE to report at that time, interim data safety analysis was performed after every additional 20 patients completed the research procedures. Reports of the interim data safety analyses were submitted to the IRB on an annual basis.

2.2.13 Statistical analysis plan

A statistical analysis plan was created in consultation with a statistician from the University of Kentucky Applied Statistics Lab. All categorical and ordinal data were reported as count and percentage. Continuous data were first analyzed for normal distribution. Non-normal, continuous data distributions were described by median and interquartile range (IQR). Normal, continuous data distributions were described by mean and standard deviation (SD). For tables and analyses with mixed normal and non-normal data distributions, data were described by median and IQR.

For hypothesis testing involving categorical or ordinal data, the Fischer's Exact or Pearson Chi-Squared tests were used, as appropriate. For hypothesis testing involving non-normal, continuous data, the independent samples median test was used. For

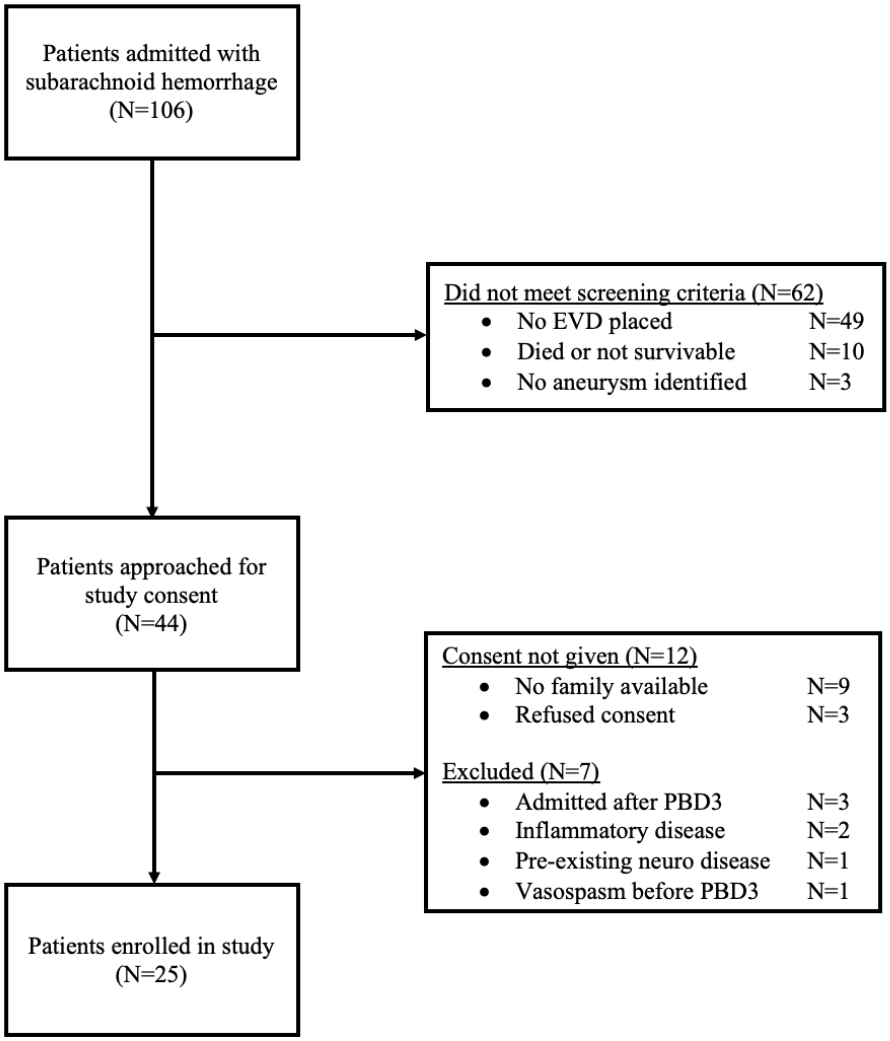
hypothesis testing involving normal, continuous data, the unpaired, two-tailed, t-test was used. For all hypothesis testing, a p-value <0.05 was considered significant. All statistical analyses were completed with IBM SPSS version 25 (IBM Corp, Armonk, NY).

2.3 Results

2.3.1 Study recruitment

From July 1, 2018 – June 30, 2020, 106 patients were admitted to the University of Kentucky Chandler Medical Center with subarachnoid hemorrhage. Of these, 44 patients (41.5%) met the initial screening criteria and were approached for study consent. Informed consent was obtained from 32 patients (30.2%). Of these, 25 patients (23.6%) were recruited to participate in the biobank during the target study period. The causes for screening failures, consenting failures, and subject exclusions during the study period are described in Figure 7. "No EVD placed" was the most frequent cause of failed screening, resulting in 79.0% of all failed screening occurrences. "No family available" was the most frequent cause of failure to obtain consent, resulting in 75% of all consenting failures. "Admitted after PBD3" was the most frequent cause of subject exclusion, resulting in 42.9% of all exclusions.

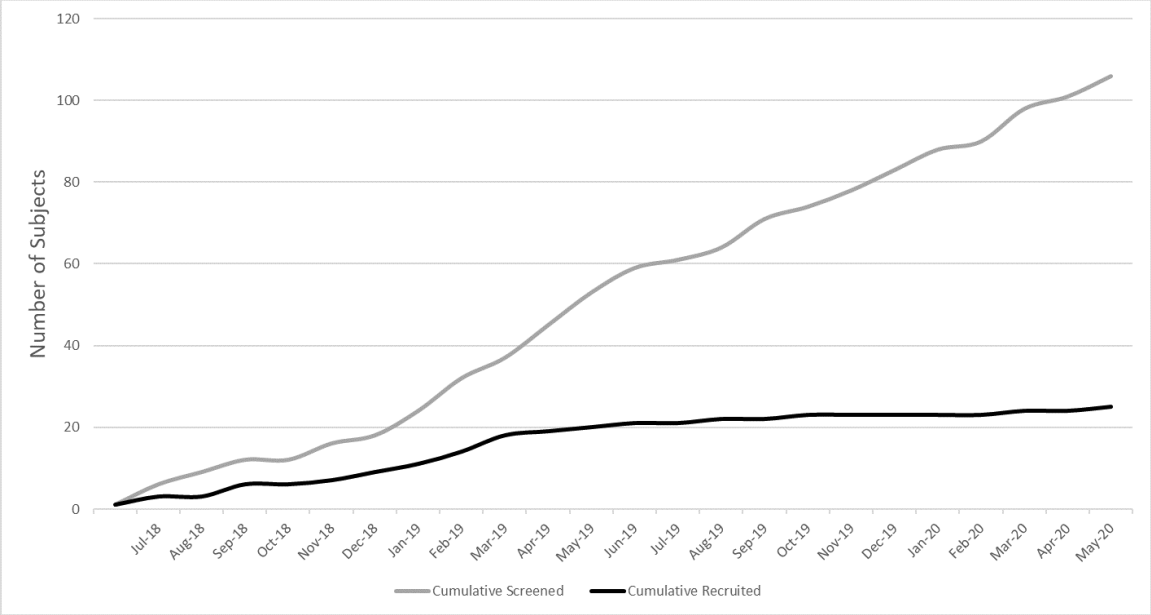
Figure 7: Flow Diagram of Screening and Recruitment to Biobank



Flow diagram describes the subject screening and recruitment success of biobank project. Only 25 patients admitted with aneurysmal subarachnoid hemorrhage were ultimately enrolled in the project. The major causes of screening failure were “No extraventricular drain (EVD) placed”, “Patient died or not survivable”, and “No family available”.

During the study period, the overall frequency of SAH admissions remained relatively stable over the 2 years with 53 patients admitted in year 1 and 53 admitted in year 2. Of these, 20 patients (80%) were enrolled in this biobank in year 1 and 5 patients (20%) were enrolled in this biobank in year 2. (Figure 8) Over the 2-year period, there were approximately 3.4 patients admitted per month; however, the subject enrollment rate significantly decreased from 1.7 cases per month in year 1 to 0.42 patients per month in year 2. Table 2.1 describes the differences in the rates of screening failures exclusions between the year 1 and year 2. There were no statistically significant differences in the causes of recruitment failure between the 2 years.

Figure 8: Biobank Screening and Recruitment During Study Period



Line graphs of the number of cumulative subjects screened and recruited per month over the 2-year study period. The number of cumulative subjects screened was nearly linear over this time period; whereas, the number of subjects recruited did not significantly increase after March 2019. This resulted in fewer enrolled research subjects than expected at the end of the study period.

Table 5: Causes of Biobank Recruitment Failure

	Year 1 (N=34)	Year 2 (N=47)	p-value
No EVD Placed	22 (64.7%)	27 (57.4%)	0.51
Not Survivable	3 (8.8%)	7 (14.9%)	0.41
No Aneurysm	1 (2.9%)	2 (4.3%)	0.76
LAR Not Available	3 (8.8%)	6 (12.8%)	0.58
Refused Consent	3 (3.3%)	0 (0.0%)	NA
Admitted after PBD3	0 (0.0%)	3 (6.4%)	NA
Pre-existing Inflammatory Disease	1 (2.9%)	1 (2.1%)	0.82
Pre-existing Neurologic Disease	1 (2.9%)	0 (0.0%)	NA
Vasospasm before PBD3	0 (0.0%)	1 (2.1%)	NA

Of note, due to COVID-19, a severe respiratory system disease caused by infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the patient visitation policy at the University of Kentucky Chandler Medical Center was restricted on March 19, 2020. At that time, visitation was limited to 1 family member per patient. On March 24, 2020, a "no visitation" policy was implemented across the hospital until May 18, 2020, a period of 55 days (approximately 7.5% of the total study period). During this period, 13 patients were admitted to the University of Kentucky Chandler Medical Center with SAH. Of these patients, 7 did not meet screening criteria and 5 met screening criteria but were not enrolled in the study because the LAR could not be approached for consent. These 5 patients accounted for a significant percent (62.5%) of all patients who were not enrolled because the LAR could not be approached for consent. During this period, 1 patient met screening criteria and was enrolled in the study. This patient was able to provide their own written documentation of informed consent.

2.3.2 Demographic and delayed brain injury analysis

Baseline demographic and disease-specific data for all subjects in the biobank is summarized in Table 6.

Table 6: Overall Biobank Subject Demographic and Disease-Specific Data

	Results N=25
Age (years)	63 (49-66)
Gender (male)	10 (40%)
Height (cm)	167.6 (162.5 – 177.8)
Weight (kg)	93.5 (70.0 – 113.6)
Body Mass Index (kg/m^2)	33.2 (26.6 – 37.1)
Favorable HH Score (1-3)	22 (88%)
Favorable WFNS Score (1-3)	17 (68%)
Favorable MFS (3)	9 (36%)
Aneurysm Location (Anterior)	16 (64%)
Obliteration Therapy (Coiled)	24 (96%)

Eleven patients (44%) in the biobank developed DCV after aSAH. Baseline demographic and disease-specific data for subjects in the biobank by their DCV status are summarized in Table 7. There were no statistically significant differences in these data between subjects with and subjects without DCV in this biobank.

Table 7: Biobank Subject Demographic and Disease-Specific Data by DCV Status

	No DCV (N=14)	DCV (N=11)	p-value
Age (years)	65 (53-73)	53 (46-63)	0.09
Gender (male)	7 (50.0%)	3 (27.3%)	0.41
Height (cm)	168.9 (162.5 – 177.8)	167.6 (162.5 – 170.1)	0.93
Weight (kg)	85 (69.2 – 111.3)	106.9 (86.1 – 113.4)	0.21
Body Mass Index (kg/m^2)	30.4 (26.5 – 33.9)	37.1 (30.8 – 40.4)	0.08
Favorable HH Score (1-3)	14 (92.9%)	9 (81.8%)	0.56
Favorable WFNS Score (1-3)	10 (71.4%)	7 (63.6%)	1.00
Favorable MFS (3)	9 (64.3%)	5 (45.5%)	0.44
Aneurysm Location (Anterior)	8 (57.1%)	8 (72.7%)	0.68
Obliteration Therapy (Coiled)	14 (100.0%)	10 (90.9%)	0.44

Seven patients (28%) in the biobank developed DCI after aSAH. Baseline demographic and disease-specific data for subjects in the biobank by their DCI status are summarized in Table 8. There were no statistically significant differences in these data between subjects with and subjects without DCI in this biobank.

Table 8: Biobank Subject Demographic and Disease-Specific Data by DCI Status

	No DCI (N=18)	DCI (N=7)	p-value
Age (years)	63 (49.8 – 68.5)	54 (50.5 – 67)	0.95
Gender (male)	6 (31.6%)	4 (57.1%)	0.38
Height (cm)	166.4 (162.5 – 177.8)	167.6 (165.1 – 170.1)	0.90
Weight (kg)	89.8 (68.9 – 111.9)	107.2 (81.6 – 112.9)	0.40
Body Mass Index (kg/m^2)	31.6 (26.5 – 34.2)	37.1 (29.9 – 38.4)	0.23
Good Hunt Hess Score (1-3)	6 (31.6%)	6 (85.7%)	0.03
Good WFNS Score (1-3)	5 (26.3%)	5 (71.4%)	0.08
Good Modified Fischer Score (4)	4 (21.1%)	5 (71.4%)	0.06
Aneurysm Location (Anterior)	4 (21.1%)	5 (71.4%)	0.06
Obliteration Therapy (Coiled)	7 (36.8%)	6 (85.7%)	0.07

Only 2 patients (8%) developed delayed cerebral infarction after aSAH. Both patients were relatively young, aged 46 and 54. One patient was male, and one patient was female. Their Hunt and Hess Scores were 4 and 3, their WFNS scores were 5 and 2, and their modified Fischer scores were 4 and 4, respectively. Both patients had anterior aneurysms that were obliterated with endovascular (coiling) therapy. Both patients had DCV and one of the patients also had DCI.

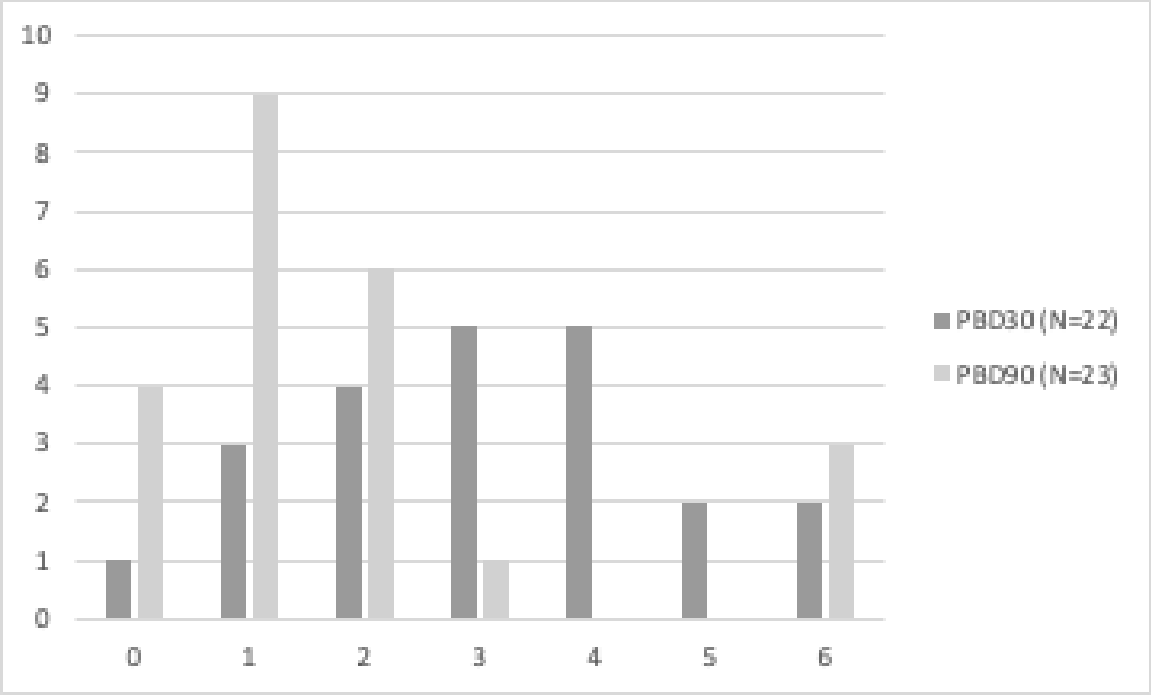
2.3.3 Clinical outcome and disability scoring analysis

Of the 25 subjects recruited to the study, 23 subjects (92%) survived to discharge and 2 subjects (8%) died prior to hospital discharge. One patient died after PBD14 due to withdrawal of life-supporting care after spontaneous rupture of a second aneurysm. The other patient died after PBD10 due to withdrawal of life-supporting care after repeated episodes of DCV and continued poor neurologic status.

Of the 23 subjects who survived to hospital discharge, disability scores were obtained from 22 subjects (88%) at PBD30. Three subjects (12%) that were discharged alive could not be contacted at PBD30 follow-up. At PBD90 follow-up, disability scores were obtained from 23 subjects (92%). All subjects who could not be contacted at PBD30 follow-up were contacted at PBD90 and 2 (8%) subjects that were contacted at PBD30 follow-up could not be contacted at PBD90 follow-up. Unfortunately, one subject died at an outside hospital between PBD30 and PBD90 due to pulmonary embolism.

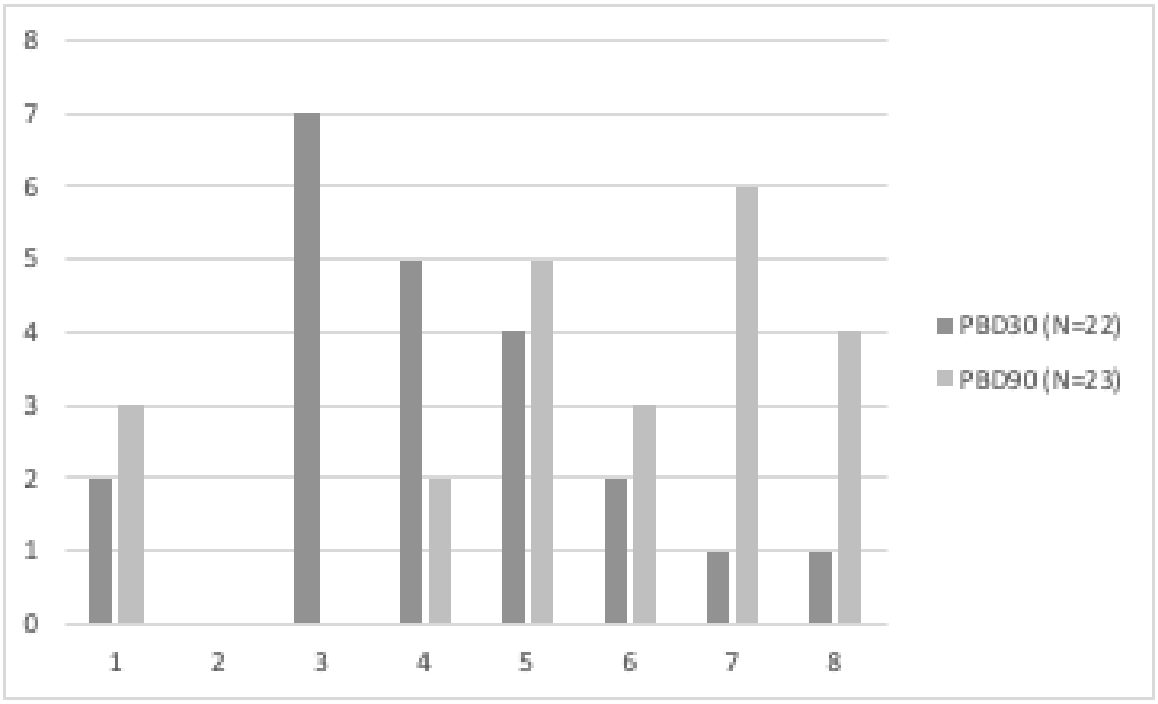
The results of mRS and GOSE scale scoring at PBD30 and PBD90 are shown in Figure 9 and Figure 10, respectively. There were significantly more subjects with good mRS scores (0-3) at PBD90 than at PBD30 (20 (87.0%) vs. 13 (59.1%), p=0.034). Likewise, there were significantly more subjects with good GOSE scores (5-8) at PBD90 than at PBD30 (18 (78.3%) vs. 8 (36.4%), p=0.004).

Figure 9: Biobank Subjects mRS Scores at PBD30 and PBD90



Graphs of the number of subjects with each modified Rankin Scale (mRS) score at post-bleed day 30 (PBD30) and post-bleed day 90 (PBD90). There were more subjects with scores 0-2 at PBD90 than at BPD30, suggesting that more subjects had better recovery, as determined by mRS, at the later time period than at the earlier time point.

Figure 10: Biobank Subjects GOSE Scores at PBD30 and PBD90



Graphs of the number of subjects with each Glasgow Outcome Scale Extended (GOSE) score at post-bleed day 30 (PBD30) and post-bleed day 90 (PBD90). There were more subjects with scores 5-8 at PBD90 than at BPD30, suggesting that more subjects had better recovery, as determined by GOSE, at the later time period than at the earlier time point.

Comparisons of the number and percentages of biobank subjects with and without DCV and DCI with good mRS and GOSE scores at PBD30 and PBD90 are described in Table 9 and Table 10, respectively. When comparing subjects with and without DCV, there was a greater percentage of subjects without DCV and good mRS scores at both PBD30 and PBD90, although these differences were not significant. Likewise, while there was a greater percentage of subjects without DCV and good GOSE scores at both PBD30 and PBD90, these differences were also not significant. When comparing subjects with and without DCI, there were significantly more subjects without DCI who had good mRS scores than there were subject with DCI at PBD30; however, this difference was not seen at PBD90. There were also no significant differences in the percentage of subjects with good GOSE scores between subjects with and without DCI at both PBD30 and PBD90.

Table 9: Biobank Subject mRS and GOSE Scores by DCV Status

	No DCV (N=14)	DCV (N=11)	p-value
Good mRS-PBD30 (Scores = 0-3)	8 (57.1%)	5 (45.4%)	0.193
Good mRS-PBD90 (Scores = 0-3)	12 (85.7%)	8 (72.7%)	0.385
Good GOSE-PBD30 (Scores = 5-8)	6 (42.9%)	2 (18.2%)	0.076
Good GOSE-PBD90 (5cores = 5-8)	11 (78.6%)	8 (72.7%)	0.772

Table 10: Biobank Subject mRS and GOSE Scores by DCI Status

	No DCI (N=18)	DCI (N=7)	p-value
Good mRS-PBD30 (Scores 0-3)	11 (73.3%)	2 (28.6%)	0.047
Good mRS-PBD90 (Scores 0-3)	15 (88.2%)	5 (83.3%)	0.759
Good GOSE-PBD30 (Scores 5-8)	7 (46.7%)	1 (14.3%)	0.141
Good GOSE-PBD90 (Scores 5-8)	15 (88.2%)	4 (66.7%)	0.231

2.3.4 Biofluid sample collection & preparation

Biofluid samples, including plasma and CSF, were collected from all 25 research subjects on PBD3, PBD5, and PBD7. For 1 subject, plasma and CSF samples were not obtained on PBD10 because the subject had already been discharged alive from the hospital. For 1 additional subject, CSF samples alone were not obtained on PBD10 because the LAR refused sampling due to significantly increased subject mental confusion and agitation prior to sampling.

Biofluid preparation was completed according to the described protocol for all samples. There were no samples that required more than 30 minutes to complete. After

biofluid preparation, 594 plasma samples and 588 CSF samples were added to the biobank. All samples were stored at -80C until used.

2.3.5 Haptoglobin phenotype analysis

The haptoglobin phenotype was determined by ELISA for all 25 patients using the described protocol. All validation criteria were successfully met for these samples. The OD results from the supplied control samples, the calculated optical density (OD) range criteria, and the mean measured OD for each phenotype are described in Table 11.

Table 11: Optical Density (OD) Results from Hp Phenotype Analysis

	Control OD measurement	Calculated OD range for Hp phenotype	Mean (SD) measured OD of biobank samples
Blank	0.053 (0.002)	NA	NA
Hp1-1	0.063 (0.010)	<0.2	0.105 (0.082)
Hp1-2	0.482 (0.011)	0.2 – 0.936	0.570 (0.135)
Hp2-2	1.561 (0.006)	>0.936	2.231 (0.366)

There were 3 subjects (12%) identified as Hp1-1, 14 subjects (56%) identified as Hp1-2, and 8 subjects (32%) identified as Hp2-2 from this analysis. An analysis of demographic and disease-specific data, as well as, DBI diagnoses based on Hp Phenotype are found in Table 12. There were no statistically significant differences in demographic or disease-specific data or DBI diagnoses among subjects with different Hp phenotypes.

Table 12: Biobank Subject Data by Hp Phenotype

	Hp1-1 (N=3)	Hp1-2 (N=14)	Hp2-2 (N=8)	p-value
Age (years)	49 (48 – 51)	63 (49 – 67)	66 (53 – 77)	0.476
Gender (male)	2 (66.7%)	6 (42.9%)	2 (25.0%)	0.430
Height (cm)	170.1 (167.6 – 177.8)	167.6 (162.5 – 173.9)	163.9 (161.2 – 177.8)	0.589
Weight (kg)	93.5 (85.3 – 122.8)	92.0 (73.5 – 107.1)	100.2 (65.9 – 115.0)	0.823
Body Mass Index (kg/m^2)	34.3 (30.5 – 39.3)	31.6 (28.1 – 34.0)	34.8 (24.9 – 38.4)	0.787
Favorable HH Score (0-3)	2 (66.7%)	11 (78.6%)	7 (87.5%)	0.729
Favorable WFNS Score (1-3)	2 (66.7%)	10 (71.4%)	5 (62.5%)	0.910
Modified Fischer Score (3)	1 (33.3%)	7 (50.0%)	3 (37.5%)	0.787
Aneurysm Location	3	8	5	NA

(Anterior)	(100.0%)	(57.1%)	(62.5%)	
Obliteration Therapy (Coiled)	3 (100.0%)	14 (100.0%)	7 (87.5%)	NA
DCV (Yes)	2 (66.7%)	7 (50.0%)	2 (25.0%)	0.367
DCI (Yes)	1 (33.3%)	4 (28.6%)	2 (25.0%)	0.961
Cerebral Infarction (Yes)	1 (33.3%)	1 (7.1%)	0 (0.0%)	NA

There were 17 subjects (68%) identified as Hp1 and 8 subjects (32%) identified as Hp2 in this research study. An analysis of demographic and disease-specific data, as well as, DBI diagnoses based on Hp Class are found in Table 13. There were no statistically significant differences in demographic or disease-specific data or DBI diagnoses between subjects in different Hp classes.

Table 13: Biobank Subject Data by Hp Class

	Hp1 N=18	Hp2 N=7	p-value
Age (years)	59 (48.3 – 66.5)	63 (52.5 – 74)	0.76
Gender (male)	8 (44.4%)	2 (28.6%)	0.66
Height (cm)	167.6 (162.5 – 173.9)	165.1 (162.6 – 177.8)	0.93
Weight (kg)	89.8 (71.8 – 107.1)	114.1 (76.1 – 115.1)	0.43
Body Mass Index (kg/m^2)	31.6 (26.9 – 34.2)	36.4 (29.1 – 39.7)	0.47
Favorable HH Score (0-3)	16 (88.9%)	6 (85.7%)	1.00
Favorable WFNS Score (0-3)	12 (66.7%)	5 (71.4%)	1.00
Modified Fischer Score (4)	10 (55.6%)	4 (57.1%)	1.00
Aneurysm Location (Anterior)	11 (61.1%)	5 (71.4%)	1.00
Obliteration Therapy (Coiled)	18 (100.0%)	6 (85.7%)	0.28
Delayed Cerebral Vasospasm	9 (50%)	2 (28.6%)	0.41

Delayed Cerebral Ischemia	5 (27.8%)	2 (28.6%)	1.00
Delayed Cerebral Infarction	2 (11.1%)	0 (0.0%)	1.00

2.3.6 Subject safety analysis

Subject safety data was recorded for all 25 subjects enrolled in this study. One subject (4%) developed bacteremia approximately 1 week after the final plasma sample. This is consistent with the historical rate of bacteremia after aSAH at the University of Kentucky. This infection was treated successfully with antibiotics and there were no apparent sequelae from this infection. After review with the attending neurosurgeons, this infection was not thought to be related to study sampling procedures.

Two subjects (8%) developed ventriculitis during study involvement. This is consistent with the historical rate of ventriculitis after aSAH at the University of Kentucky. These infections were treated successfully with antibiotics and there were no apparently sequelae from these infections. After review with the attending neurosurgeon, neither infection was thought to be related to study sampling procedures.

Three additional subjects (12%) required replacement of their EVD during study involvement. This is consistent with the historical rate of EVD replacement after aSAH at the University of Kentucky. After review with the attending neurosurgeons, none of

these cases was deemed to be related to CSF sampling for this study as all three patients had significant hemorrhage burden, likely resulting in catheter or drainage tubing thrombosis.

There were no known breaches of confidentiality or other safety concerns during this study period.

2.4 Conclusions

This chapter described the successful development of a prospective aSAH biobank at the University of Kentucky Chandler Medical Center. Over 2 years of subject recruitment, 25 subjects were enrolled in the biobank. Demographic, clinical and outcome data, as well as, biofluid samples at multiple time points were obtained from each patient and are available, upon request, for future research. This biobank will allow future research to investigate causes, interactions and outcomes of DBI in aSAH patients. Two of these studies are described in the following chapters.

CHAPTER 3: INFLAMMATORY CYTOKINE ANALYSIS IN PATIENTS WITH ANEURYSMAL SUBARACHNOID HEMORRHAGE

3.1 Introduction

This chapter focuses on an analysis of plasma and CSF concentrations of 3 inflammatory cytokines, IL-1β, IL-6 and TNF-α, at multiple time points in patients with aneurysmal subarachnoid hemorrhage (aSAH). Previous studies have demonstrated a relationship between these cytokines and the risk of delayed brain injuries (DBI) after aSAH. I have, therefore, hypothesized that these inflammatory cytokines are central in the immune-mediated model of DBI after aSAH. To investigate this hypothesis, the aims of this study, therefore, were to measure plasma and cerebrospinal fluid (CSF) IL-1β, IL-6 and TNF-α concentrations at multiple time points after aSAH and evaluate whether there was a correlation between the concentrations of these inflammatory cytokines and the incidence of delayed brain injuries (DBI) among aSAH subjects with different Hp phenotypes.

3.2 Methods

3.2.1 Study design

This was a prospective cohort study using prospectively collected clinical data and biofluid samples from a single-center aSAH biobank at the University of Kentucky Chandler Medical Center, as well as, control subjects from the University of Kentucky CCTS Research Registry and Specimen Bank.

3.2.2 Study population

Demographic, disease-specific and outcome data, as well as, biofluid samples from post-bleed day 3 (PBD3), PBD5, PBD7 and PBD10 were included from all subjects in the aSAH biobank. The details of the collection, preparation and storage of the data and samples in this biobank are described in Chapter 2. Biobank subjects were excluded if there was missing demographic or disease-specific data, if the presence or absence of DBI diagnoses were not defined, or if biofluid samples from PBD3, PBD5, or PBD7 were not drawn or were missing.

Control samples were obtained from The University of Kentucky Center for Clinical and Translation Science Research Registry and Specimen Bank. This specimen bank contains samples from more than 34,000 patients and includes more than 1400 plasma and 80 CSF samples. Samples in this registry were obtained, according to an IRB-approved protocol, and subjects provided informed consent for their involvement in this specimen bank. For this study, biofluid samples were obtained from all patients in this registry who had both blood and CSF samples obtained within 24 hours of each other. Biofluid samples were not obtained from patients with hemorrhagic stroke, ventriculitis, meningitis, and human immunodeficiency virus (HIV), hepatitis B, and hepatitis C infections.

3.2.3 Biofluid cytokine analysis

After IRB approval, plasma and CSF samples from all 25 subjects in the aSAH biobank and 9 control subjects were identified for cytokine analysis. Samples from PBD3, PBD5, PBD7 and PBD10 were selected for subjects in the aSAH biobank, when available. Quantitative cytokine analysis (IL-6, IL-1β, and TNF-α) was performed by the University of Kentucky Center for Clinical and Translation Research (CCTS) Bioanalysis Lab (BAL) using a commercial kit (V-PLEX Human Proinflammatory Panel I) manufactured by Meso Scale Diagnostics (Rockville, MD) (Catalog # K15052D, Lot # K0081080, Exp 1/31/2021; Lot # K0031225, Exp 3/31/2021; Lot # K0031225, Exp 3/31/2021; Lot # K0081189, Exp 3/31/2021). This kit contains 96-well multi-spot ELISA plates, control and calibrator samples for target cytokines, and other miscellaneous supplies needed to complete the analysis. This kit was chosen because it has been validated in both plasma and CSF and has a high reported accuracy for the target concentrations of these cytokines.[205,206]

Cytokine concentrations were determined using the MESO Quickplex SQ 120 Multiplex plate reader (Meso Scale Diagnostics, Rockville, MD). Standard curves for each cytokine were constructed using a log-log curve-fit from calibrator samples. Target cytokine concentrations were measured twice for each sample and the results were averaged to determine the calculated final concentration. For cytokine concentrations that were below the lower limit of detection (LLOD) or those with no-reading, the LLOD was used as the measured cytokine concentration. For cytokine concentrations that were

above the upper limit of detection (ULOD), the ULOD was used as the cytokine concentration.

3.2.4 Data management plan

A data management plan was created and approved by the IRB prior to study-related procedures. All biobank data was copied from the biobank's online REDCap database directly into a separate REDCap database for this analysis. Results of the cytokine analyses for each subject were entered into this separate REDCap database. All cytokine data entry was verified by the PI. All data errors were immediately corrected in the online database by the PI.

3.2.5 Statistical analysis plan

A statistical analysis plan was created in consultation with a statistician from the University of Kentucky Applied Statistics Lab. All categorical and ordinal data are reported as count and percentage. Continuous data distributions that met the assumptions for normalized data are described by mean and standard deviation (SD). Continuous data distributions that did not meet the assumptions for normalized data are reported as median and interquartile range (IQR). For tables and analyses with mixed normal and non-normal data distributions, all data are described by median and IQR.

The Fischer's Exact or Pearson Chi-Squared tests were used, as appropriate, for hypothesis testing involving categorical or ordinal data. The two-tailed t-test, the

Independent Samples Mann-Whitney U Test, or the Independent Samples Kruskal-Wallis Test was used for hypothesis testing involving normal and non-normal continuous data. respectively. For all hypothesis testing, a p-value <0.05 was considered significant. All statistical analyses were completed with IBM SPSS version 25 (IBM Corp, Armonk, NY).

3.3 Results

3.3.1 Study recruitment

After IRB approval, demographic, disease-specific and outcome data, as well as, biofluid samples from 24 subjects in the UK aSAH Biobank were used in this analysis. One subject in the biobank was excluded after cytokine analysis and the subject's data was not included in demographic and inflammatory cytokine analysis. This subject was excluded because he had ventriculitis diagnosed after consent and initial sampling procedures that may have resulted in increased CSF and/or plasma cytokine concentrations.

In addition, there were 9 patients in The University of Kentucky Center for Clinical and Translation Science Research Registry and Specimen Bank that met inclusion and exclusion criteria as previously outlined. Frozen plasma and CSF samples from these patients were used as controls in this analysis.

3.3.2 Demographic, delayed brain injury, and clinical outcome analysis

Baseline demographic and disease-specific data for all subjects in the biobank is summarized in Table 14.

Table 14: Subject Demographic and Disease-Specific Data for Cytokine Analyses

	Results N=24
Age (years)	63 (51 – 70)
Gender (male)	9 (37.5%)
Height (cm)	167.6 (162.5 – 175.9)
Weight (kg)	89.9 (69.7 – 113.7)
Body Mass Index (kg/m^2)	33.5 (26.6 – 37.4)
Good Hunt Hess Score (1-3)	20 (87.5%)
Good WFNS Score (1-3)	17 (70.8%)
Good Modified Fischer Score (1-3)	10 (41.7%)
Aneurysm Location (Anterior)	15 (62.5%)
Obliteration Therapy (Coiled)	23 (95.8%)

Ten subjects (42%) developed DCV, 7 subjects (29.2%) developed DCI, and 2 subjects (8.3%) developed delayed cerebral infarction after aSAH. There were no statistically significant differences in demographic and disease-specific data between subjects with and without DCV or DCI.

Of the 24 study subjects, 22 subjects (92%) survived to hospital discharge. One patient died after PBD14 due to withdrawal of life-supporting care after spontaneous

rupture of a second aneurysm and 1 patient died after PBD10 due to withdrawal of life-supporting care after repeated episodes of DCV and continued poor neurologic status.

3.3.3 Inflammatory cytokine analysis

The results of plasma and CSF inflammatory cytokine analyses for the 9 control subjects are described in Table 15. Table 16 and Table 17 describe the results of plasma and CSF inflammatory cytokine analyses, respectively, for study subjects by PBD. An analysis of the differences between cytokine concentrations in control and study subjects demonstrated that at all time points, the median plasma IL-1β concentration of the research subjects was significantly less than the median of the control subjects, the CSF IL-1β concentration of the research subjects was significantly greater than in the control subjects, the plasma IL-6 concentration was not significantly different from the control subjects, the CSF IL-6 concentration of the research subjects was significantly greater than in control subjects, the plasma TNF-α concentration of the research subjects was significantly less than in the control subjects, and the CSF TNF-α concentration of the research subjects was significantly greater than in the control subjects.

Table 15: Results of Inflammatory Cytokine Analysis for Control Subjects

	Plasma (N=9)	CSF (N=9)
IL-1β (pcg/mL)	0.80 (0.48 – 2.14)	0.02 (0.01 – 0.04)
IL-6 (pcg/mL)	4.97 (3.35 – 9.71)	2.75 (2.45 – 9.66)
TNF-α (pcg/mL)	3.55 (2.93 – 6.44)	0.33 (0.31 – 0.35)

Table 16: Results of Plasma Inflammatory Cytokine Analysis for all Study Subjects

	PBD3 (N=24)	PBD5 (N=24)	PBD7 (N=24)	PBD10 (N=22)
IL-1β (pcg/mL)	0.08 (0.05 – 0.17)	0.13 (0.07 – 0.19)	0.13 (0.05 – 0.16)	0.12 (0.08 – 0.14)
IL-6 (pcg/mL)	7.87 (3.02 – 12.36)	5.70 (3.46 – 11.22)	4.28 (2.86 – 7.59)	3.77 (2.54 – 9.20)
TNF-α (pcg/mL)	2.06 (1.49 – 2.57)	2.50 (2.12 – 2.64)	2.40 (2.13 – 2.64)	2.56 (2.29 – 3.10)

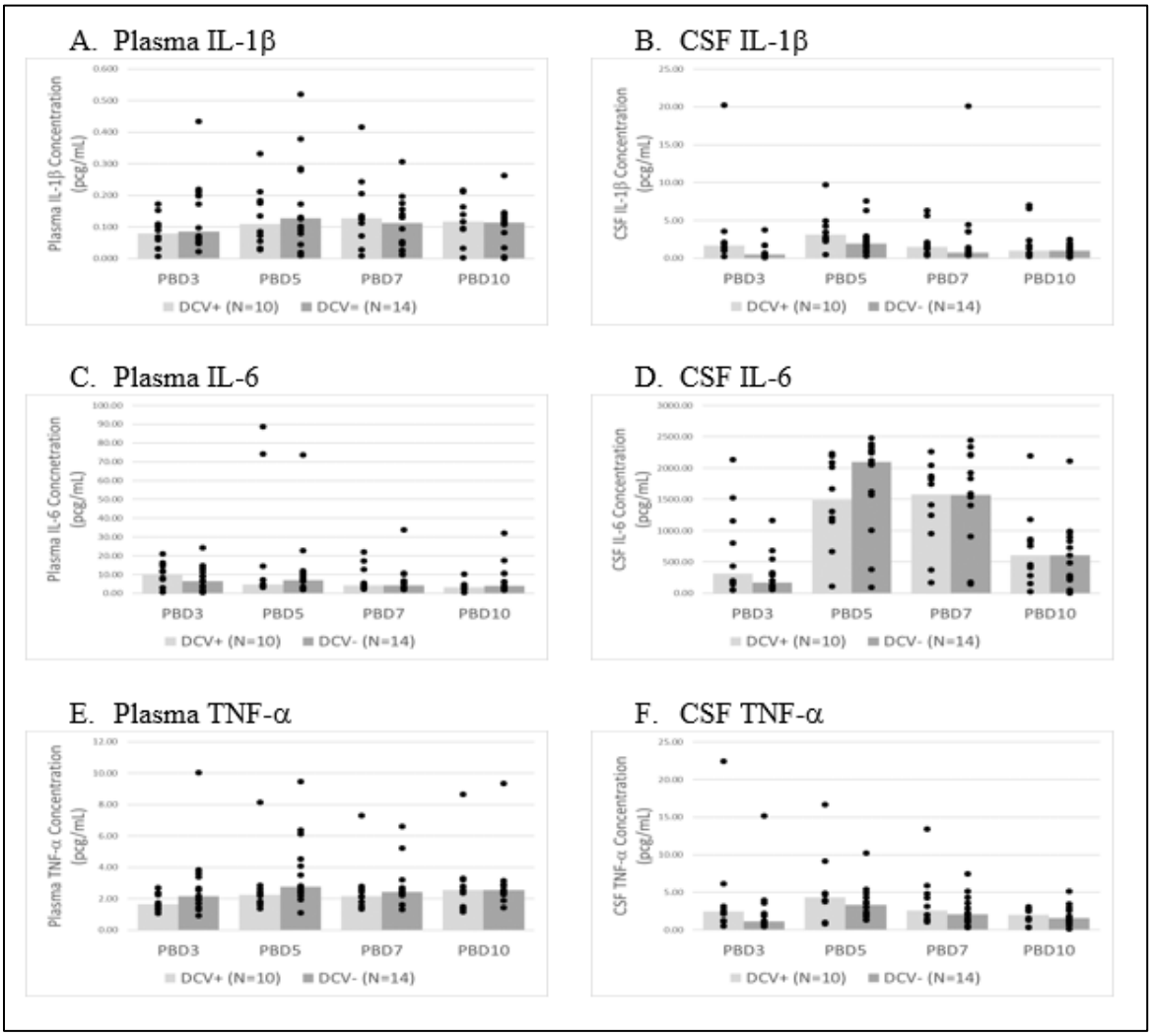
Table 17: Results of CSF Inflammatory Cytokine Analysis for all Study Subjects

	PBD3 (N=24)	PBD5 (N=24)	PBD7 (N=24)	PBD10 (N=23)
IL-1β (pcg/mL)	0.83 (0.31-1.81)	2.50 (1.66 – 3.43)	1.18 (0.57 – 2.45)	0.98 (0.52 – 1.69)
IL-6 (pcg/mL)	203.18 (103.21 – 578.85)	2033.34 (1189.13 – 2232.33)	1569.99 (945.52 – 1952.51)	610.74 (270.84 – 880.28)
TNF-α (pcg/mL)	1.60 (0.87 – 3.25)	3.76 (2.08 – 4.92)	2.10 (1.45 – 4.31)	1.60 (1.24 – 2.83)

3.3.4 Inflammatory cytokine analysis by delayed brain injury status

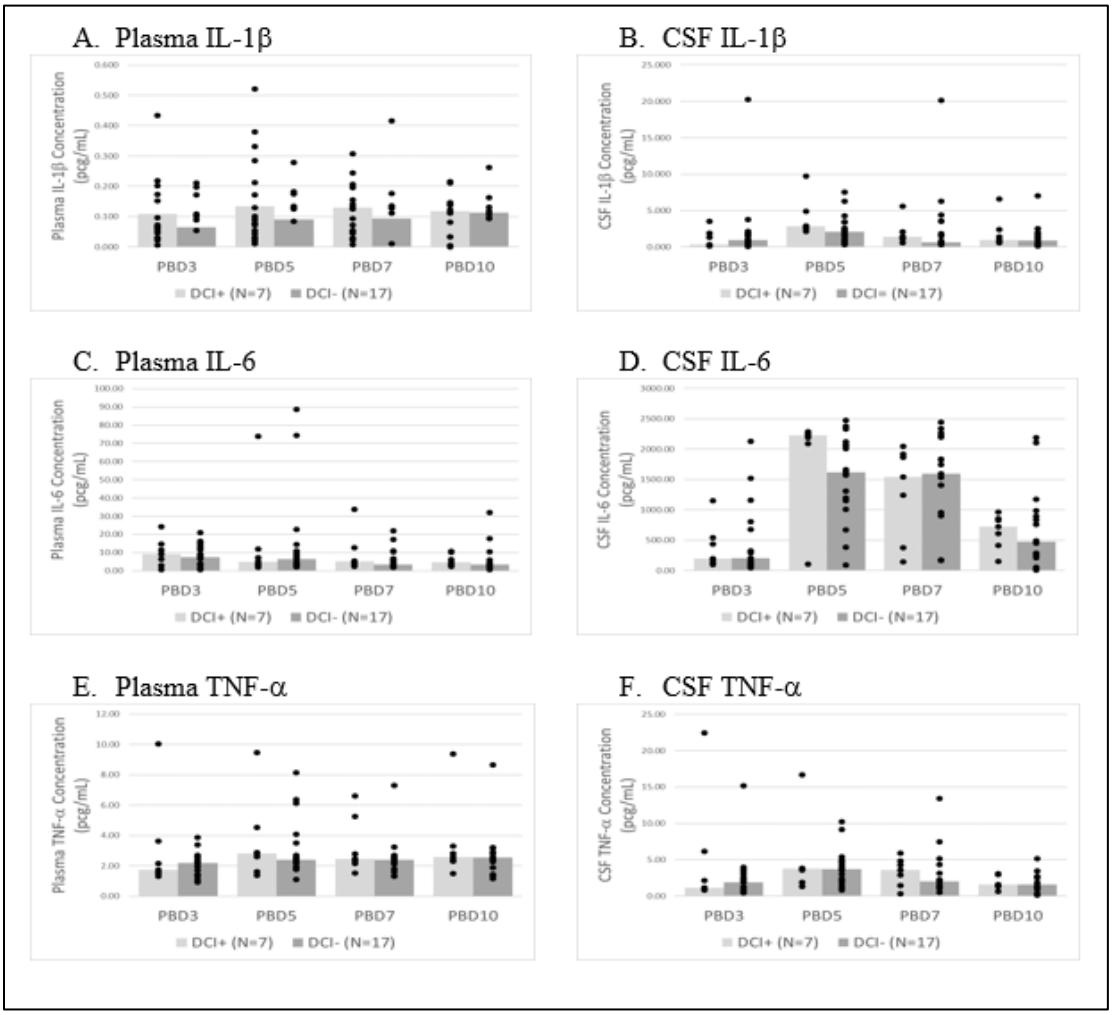
The results of plasma and CSF inflammatory cytokine analysis of study subjects, categorized by DCV and DCI status are shown in Figures 11 and 12, respectively. There were no statistically significant differences in either plasma or CSF IL-1β, IL-6 or TNF-α concentrations between study subjects with and without DCV or DCI at any time point.

Figure 11: Plasma and CSF Cytokine Concentrations by DCV Status



In this experiment, cytokine concentrations in plasma and cerebrospinal fluid (CSF) were determined at multiple pre-defined time points by ELISA from 24 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their delayed cerebral vasospasm (DCV) status. There were no statistically significant differences at any time points in any of the cytokine concentrations in plasma or CSF between subjects with and without DCV.

Figure 12: Plasma and CSF Cytokine Concentrations by DCI Status



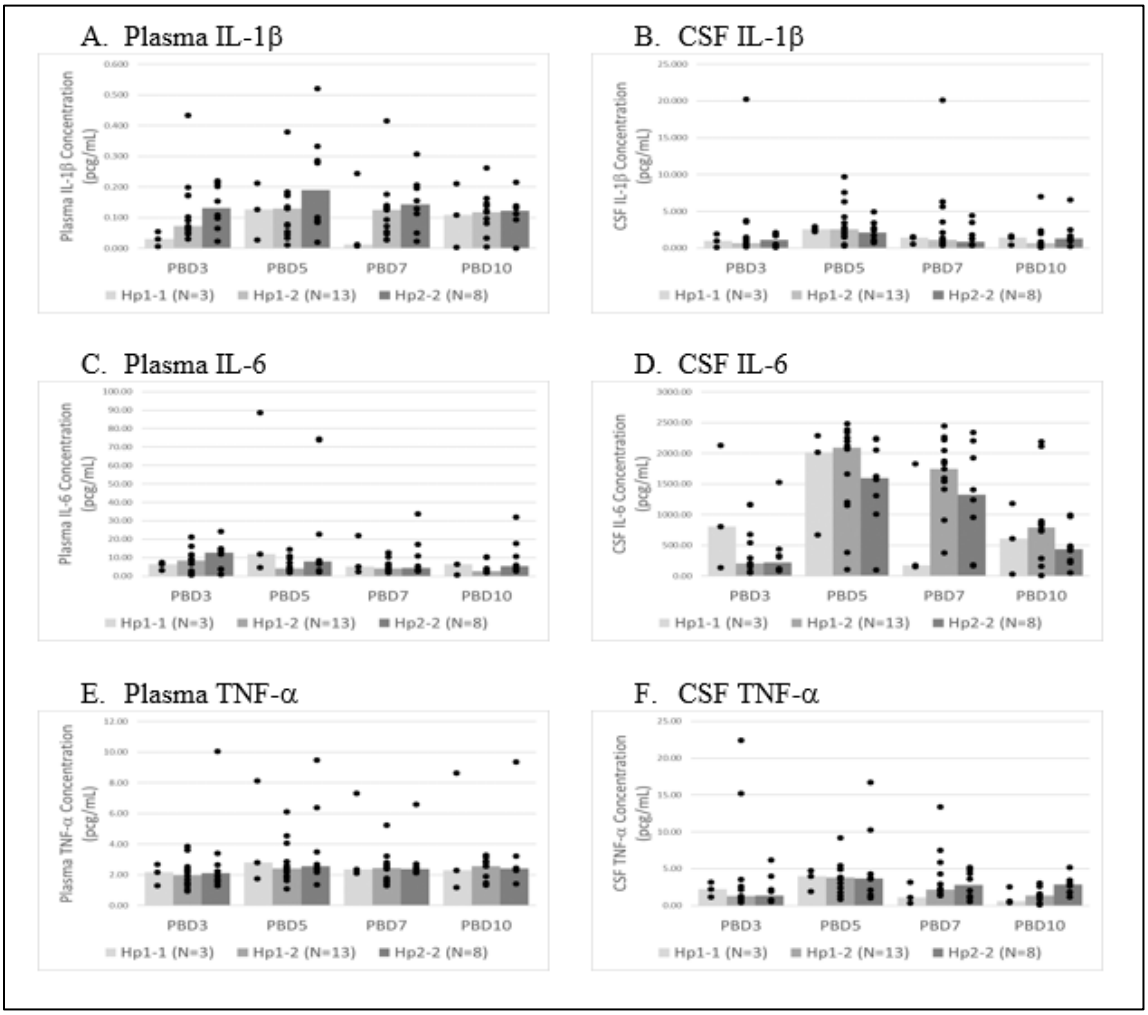
In this experiment, cytokine concentrations in plasma and cerebrospinal fluid (CSF) were determined at multiple pre-defined time points by ELISA from 24 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their delayed cerebral ischemia (DCI) status. There were no statistically significant differences at any time points in any of the cytokine concentrations in plasma or CSF between subjects with and without DCI.

3.3.5 Inflammatory cytokine analysis by haptoglobin phenotype

The results of plasma and CSF inflammatory cytokine analysis of study subjects, categorized by Hp phenotype and Hp class are shown in Figures 13 and 14, respectively. There were no statistically significant differences in either plasma or CSF IL-1β, IL-6 or TNF-α concentrations between study subjects with different Hp phenotype or Hp class at any time point.

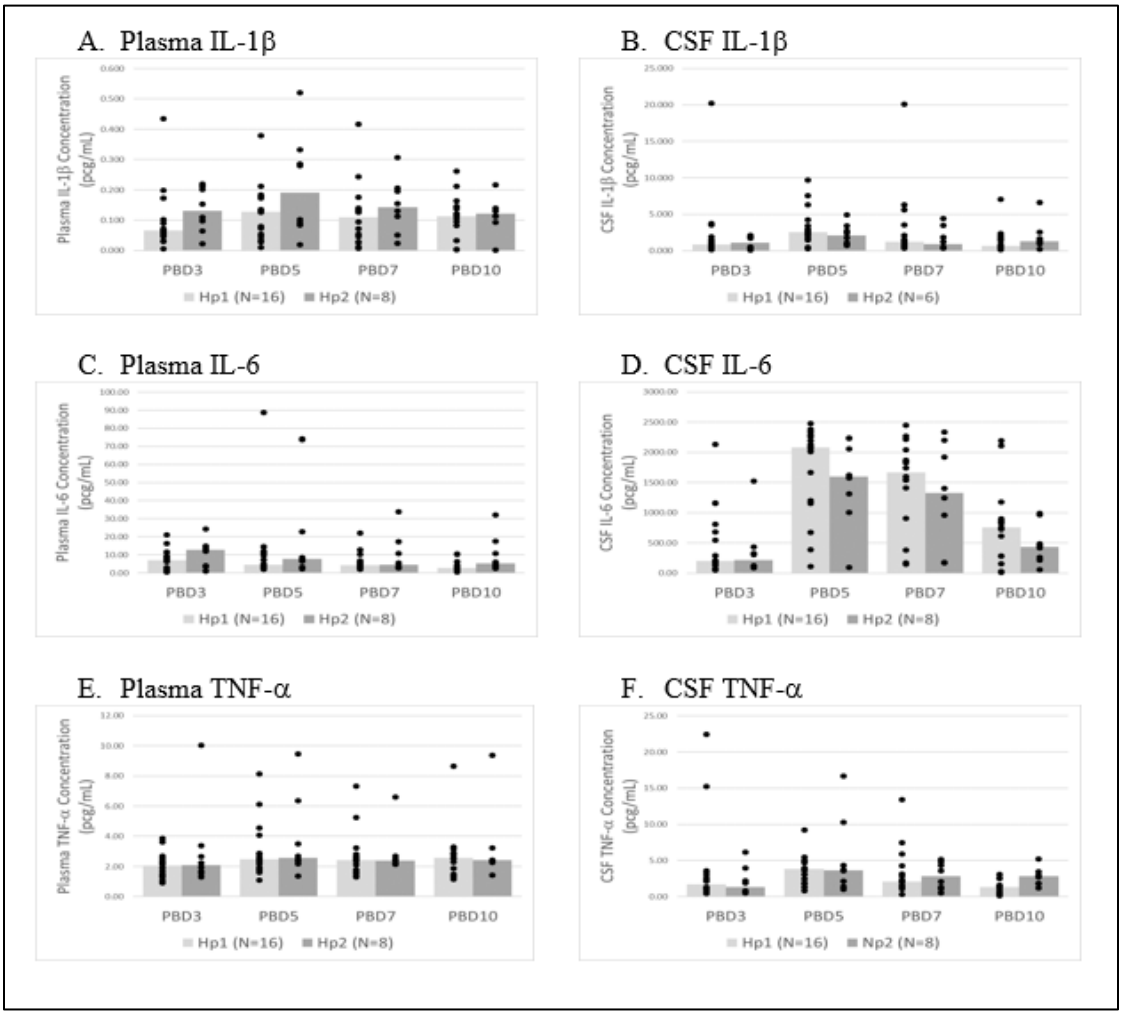
The results of plasma and CSF inflammatory cytokine analysis of study subjects, categorized by both Hp class and DCV and DCI status are shown in Figures 15 and 16, respectively. There were no statistically significant differences in either plasma or CSF IL-1β, IL-6 or TNF-α concentrations between these groupings of study subjects at any time point.

Figure 13: Plasma and CSF Cytokine Concentrations by Hp Phenotype



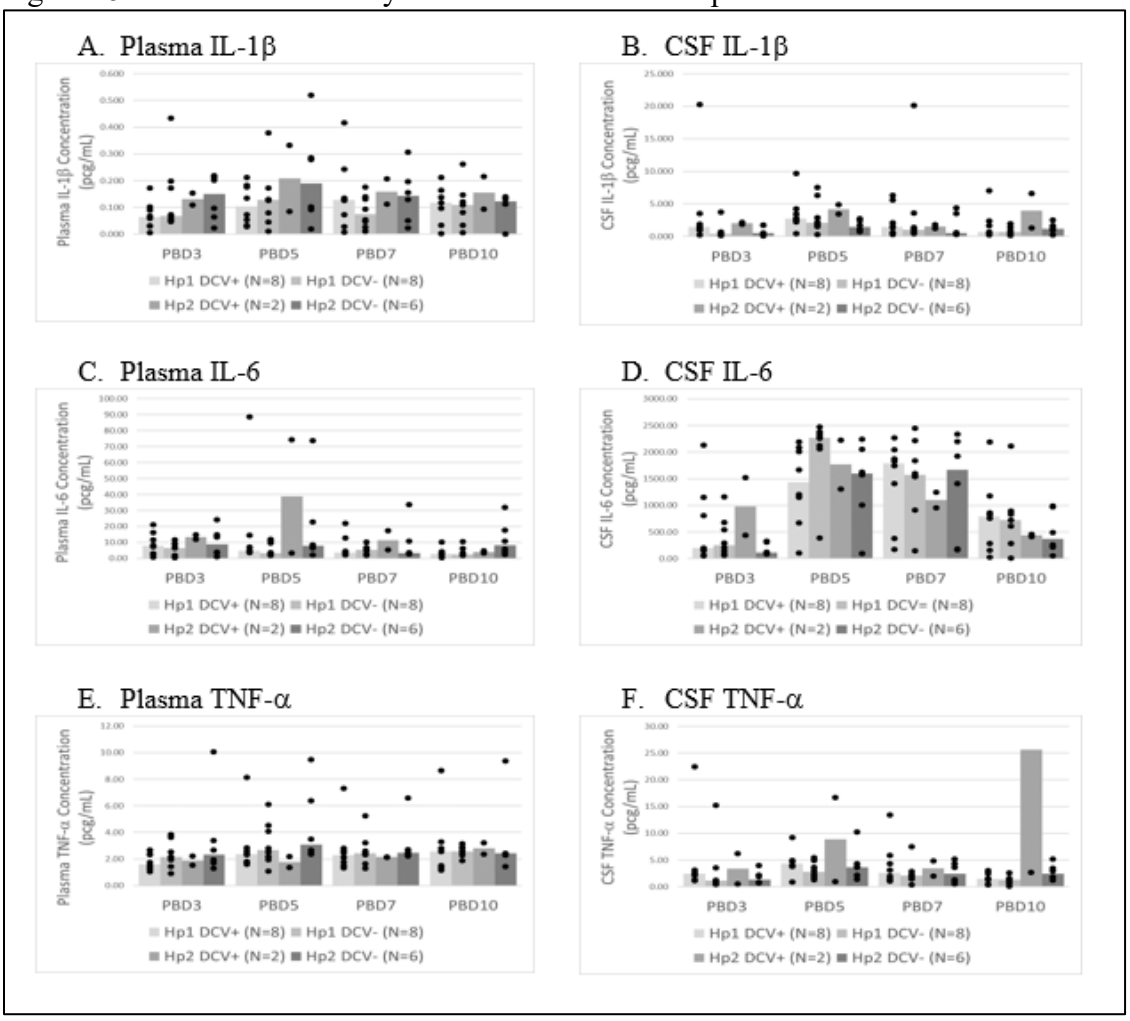
In this experiment, cytokine concentrations in plasma and cerebrospinal fluid (CSF) were determined at multiple pre-defined time points by ELISA from 24 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their haptoglobin (Hp) phenotype. There were no statistically significant differences at any time points in any of the cytokine concentrations in plasma or CSF between subjects based on their Hp phenotype.

Figure 14: Plasma and CSF Cytokine Concentrations by Hp Class



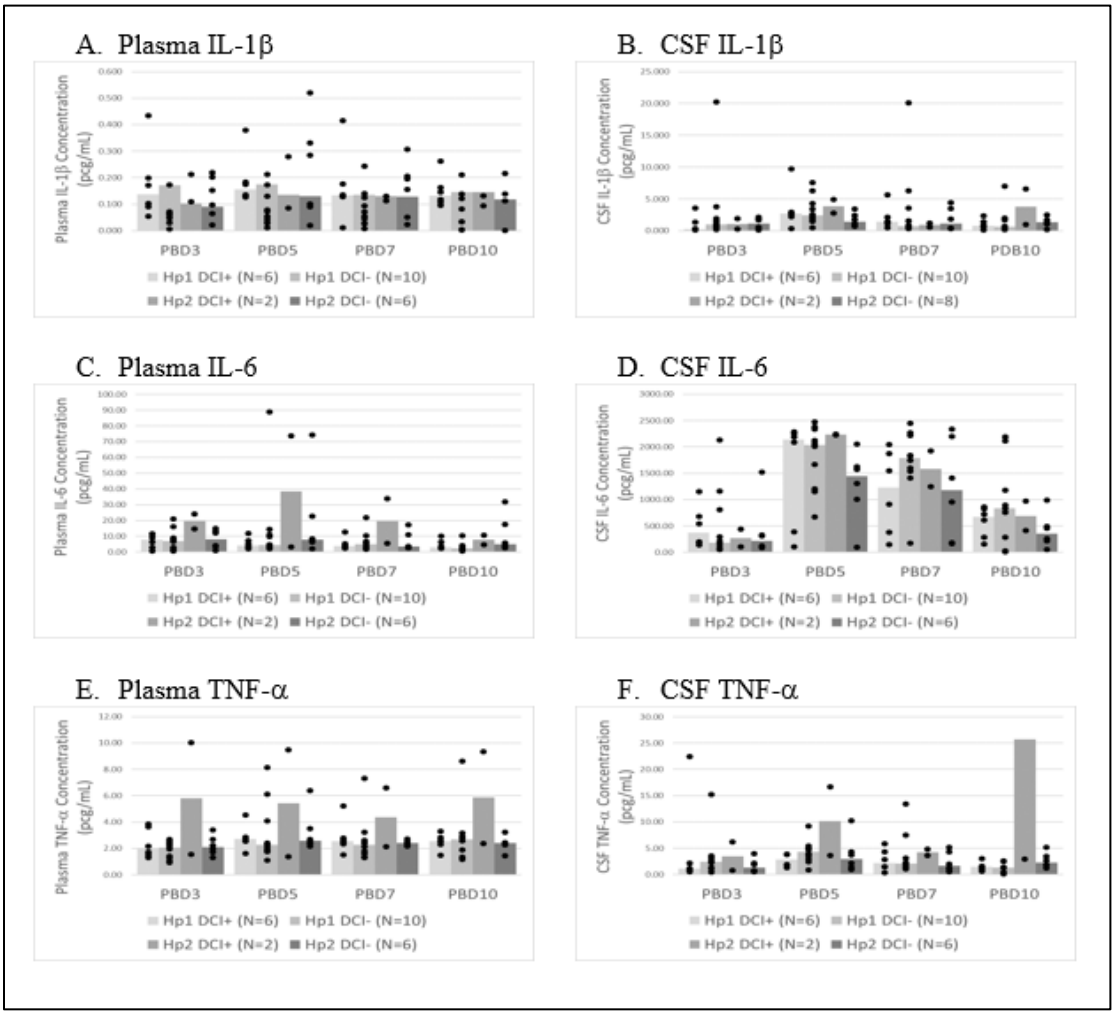
In this experiment, cytokine concentrations in plasma and cerebrospinal fluid (CSF) were determined at multiple pre-defined time points by ELISA from 24 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their haptoglobin (Hp) class. There were no statistically significant differences at any time points in any of the cytokine concentrations in plasma or CSF between subjects based on their Hp class.

Figure 15: Plasma and CSF Cytokine Concentrations Hp Class and DCV Status



In this experiment, cytokine concentrations in plasma and cerebrospinal fluid (CSF) were determined at multiple pre-defined time points by ELISA from 24 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by a combination of their haptoglobin (Hp) class and delayed cerebral vasospasm (DCV) status. There were no statistically significant differences at any time points in any of the cytokine concentrations in plasma or CSF between subjects in different combinations of Hp class and DCV.

Figure 16: Median Cytokine Concentrations by Hp Class and DCI Status



In this experiment, cytokine concentrations in plasma and cerebrospinal fluid (CSF) were determined at multiple pre-defined time points by ELISA from 24 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by a combination of their haptoglobin (Hp) class and delayed cerebral ischemia (DCI) status. There were no statistically significant differences at any time points in any of the cytokine concentrations in plasma or CSF between subjects in different combinations of Hp class and DCI.

3.4 Conclusions

This chapter described the successful analysis of inflammatory cytokines from plasma and CSF samples in aSAH subjects recruited to a biobank. This analysis was performed to evaluate the effect that different Hp phenotypes have on key inflammatory cytokines in patients with DBI after aSAH. Unfortunately, there were no significant differences detected in these inflammatory cytokines with regard to DBI, Hp phenotypes, or combinations of these outcomes. The implications, limitations and a description of potential future directions for future research from this analysis will be further discussed in Chapter 5.

CHAPTER 4: SOLUBLE CD163 (sCD163) ANALYSIS IN PATIENTS WITH ANEURYSMAL SUBARACHNOID HEMORRHAGE

4.1 Introduction

This chapter describes an analysis of soluble CD163 (sCD163) concentrations, in both plasma and cerebrospinal fluid (CSF) at multiple, pre-defined time points in patients with aneurysmal subarachnoid hemorrhage (aSAH). sCD163 is a soluble form of the macrophage CD163 receptor that is created from enzymatic cleavage of a portion of the macrophage CD163 receptor from the macrophage cell membrane by ADAM17/TACE.[172,173] sCD163 has been associated with several systemic diseases associated with deranged inflammation, including sepsis, hemophagocytic syndrome, liver failure, and disseminated intravascular coagulation (DIC).[179-184] In these conditions, a significant increase in sCD163 concentration is also correlated with a worse prognosis.[185-187] sCD163 has also been investigated in several neuroinflammatory diseases, including multiple sclerosis, infectious encephalopathy, and chronic inflammatory demyelinating polyneuropathy.[188-190] Because of theses associations to systemic and neuroinflammatory disease, I have hypothesized that sCD163 is an important modifier in the immune-mediated model of DBI after aSAH. To investigate this hypothesis, the aims of this study, therefore, were to measure plasma and cerebrospinal fluid (CSF) sCD163 concentrations at multiple time points after aSAH and evaluate whether there was a correlation between elevations in sCD163 and the incidence of delayed brain injuries (DBI) among aSAH subjects with different Hp phenotypes.

4.2 Methods

4.2.1 Study design

This was a cohort study using prospectively collected clinical data and biofluid samples from a single-center aSAH biobank at the University of Kentucky Chandler Medical Center, as well as, control subjects from the University of Kentucky CCTS Research Registry and Specimen Bank.

4.2.2 Study population

Demographic, disease-specific and outcome data, as well as, biofluid samples from post-bleed day 3 (PBD3), PBD5, PBD7 and PBD10 were included from all subjects in the aSAH biobank. The details of the collection, preparation and storage of the data and samples in this biobank are described in Chapter 2. For this study, biobank subjects were excluded if there was missing demographic or disease-specific data, if the presence or absence of DBI diagnoses were not defined, or if biofluid samples from either (post-bleed day) PBD3, PBD5, or PBD7 were not drawn or were missing.

Control samples were obtained from The University of Kentucky Center for Clinical and Translation Science Research Registry and Specimen Bank similar to the previous description in Chapter 3.

4.2.3 Biofluid sCD163 analysis

After IRB approval, plasma and CSF samples from all subjects in the aSAH biobank and 9 control subjects were identified for sCD163 concentration analysis. Samples from PBD3, PBD5, PBD7 and PBD10 were selected for subjects in the aSAH biobank, when available. sCD163 analysis was performed by the University of Kentucky Center for Clinical and Translation Research (CCTS) Bioanalysis Lab (BAL) using a commercial kit manufactured by R&D Systems (Minneapolis, MN) (Catalog # DC1630, Lot # P242744, Exp. 10/29/2021). This kit contains 96-well multi-spot ELISA plates, control and calibrator samples for sCD163, and other miscellaneous supplies needed to complete the analysis. This kit was chosen because it has been validated in both plasma and CSF and has a high reported accuracy for the target concentrations of these cytokines.[175,207]

sCD163 concentrations were determined from the measured optical density (OD) of prepared samples using a microplate reader set to 450 nm with wavelength correction set to 540nm. A standard curve using a log-log curve-fit from calibrator samples was then constructed and the OD of each sample was correlated to sCD163 concentration. sCD163 concentrations were measured twice for each sample and the results were averaged to determine the calculated final concentration. For sCD163 concentrations that were below the lower limit of detection (LLOD) or those with no-reading, the LLOD was used as the measured sCD163 concentration. For sCD163 concentrations that were above the upper limit of detection (ULOD), the ULOD was used as the sCD163 concentration.

4.2.4 Data management plan

A data management plan was created and approved by the IRB prior to study-related procedures, similar to the plan described in Chapter 3.

4.2.5 Statistical analysis plan

A statistical analysis plan was created in consultation with a statistician from the University of Kentucky Applied Statistics Lab, similar to the plan described in Chapter 3.

4.3 Results

4.3.1 Study recruitment

After IRB approval, all 25 subjects in the aSAH biobank were included in this study. Demographic, disease-specific and clinical data, as well as, biofluid samples from all 25 subjects in the UK aSAH Biobank were used in this analysis. Also, as described in Chapter 3, there were 9 control subjects that met the inclusion and exclusion criteria. Frozen plasma and CSF samples from these subjects were obtained and used in this study.

4.3.2 Demographic, delayed brain injury, and clinical outcome analysis

Baseline demographic and disease-specific data for all subjects in the biobank is summarized in Table 18.

Table 18: Subject Demographic and Disease-Specific Data for sCD163 Analyses

	Results N=25
Age (years)	63 (49-66)
Gender (male)	10 (40%)
Height (cm)	167.6 (162.5 – 177.8)
Weight (kg)	93.5 (70.0 – 113.6)
Body Mass Index (kg/m^2)	33.2 (26.6 – 37.1)
Good Hunt Hess Score (0-3)	22 (88%)
Good WFNS Score (0-3)	17 (68%)
Good Fischer Score (1-3)	9 (36%)
Aneurysm Location (Anterior)	16 (64%)
Obliteration Therapy (Coiled)	24 (96%)

Eleven subjects (44%) developed DCV, 7 subjects (28%) developed DCI, and 2 subjects (8%) developed Delayed Cerebral Infarction. There were no statistically significant differences in demographic and disease-specific data between subjects with and subjects without any of these DBI.

Of the 25 study subjects, 23 subjects (92%) survived to discharge and 2 subjects (8%) died prior to hospital discharge. One patient died after PBD10 due to withdrawal of life-supporting care after repeated episodes of DCV and continued poor neurologic

status and the other subject died after PBD14 due to withdrawal of life-supporting care after spontaneous rupture of a second aneurysm.

4.3.3 sCD163 concentration analysis

The sCD163 concentration analysis was completed for all 25 subjects. sCD163 concentrations were determined for 108 plasma samples (99 subject samples and 9 control samples) and 107 CSF samples (98 subject samples and 9 control samples). Plasma samples were not available for 1 study subject at PBD10 and CSF samples were not available for 2 study subjects at PBD10. The median and IQR plasma and CSF concentrations for the entire group of aSAH subjects were 509.3 pcg/mL (395.1 – 702.1) and 347.9 pcg/mL (211.9 – 513.1), respectively. The median and IQR of the plasma and CSF sCD163 concentrations for the 9 control subjects were 686.0 pcg/mL (570.2 – 923.9) and 56.2 pcg/mL (45.2 – 78.0), respectively. Table 19 describes the median and IQR for the plasma and CSF sCD163 concentrations in aSAH subjects according to PBD. The median plasma sCD163 concentration of the aSAH subjects was non-significantly less than in the control subjects at all time points. The CSF sCD163 concentrations in aSAH subjects were significantly greater than in control subjects at all time points.

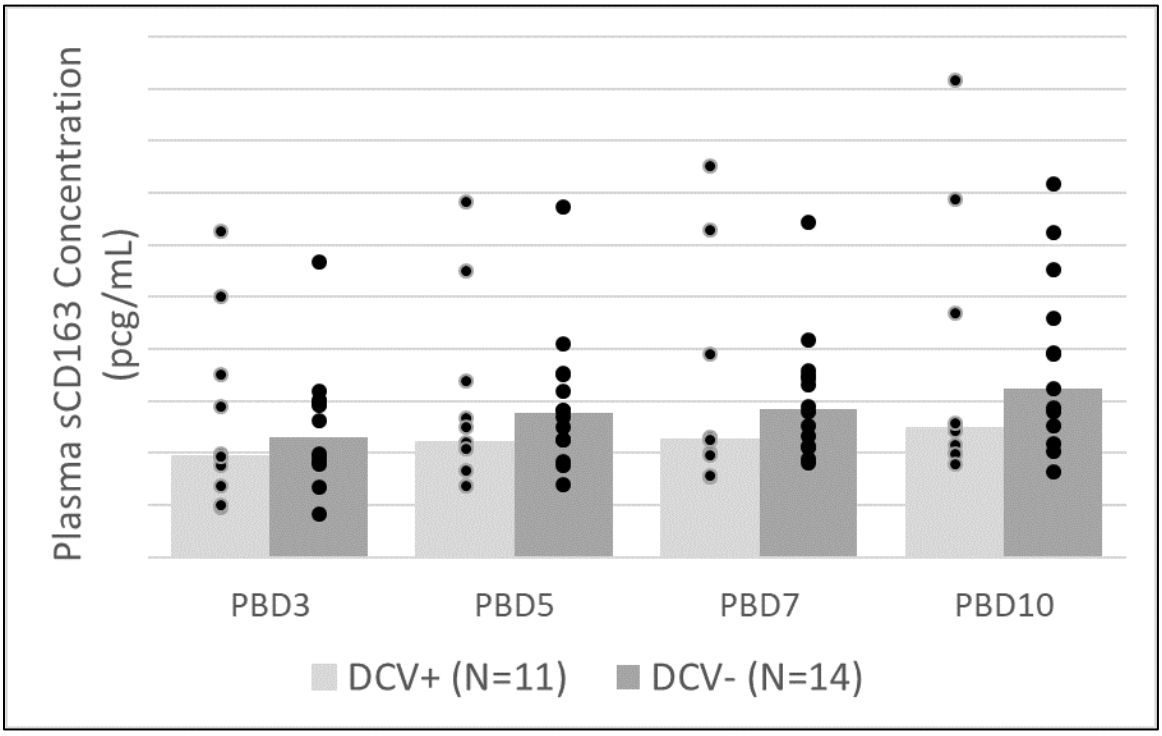
Table 19: Plasma and CSF sCD163 Concentrations in All Study Subjects

	Plasma (pcg/mL)	CSF (pcg/mL)
PBD3	394.1 (358.4 – 595.8)	268.5 (143.4 – 347.9)
PBD5	500.9 (413.9 – 676.2)	456.6 (287.0 – 625.0)
PBD7	466.7 (399.9 – 696.5)	409.0 (286.4 – 537.4)
PBD10	556.4 (433.3 – 928.0)	339.1 (158.2 – 502.5)

4.3.4 sCD163 analysis by delayed brain injury status

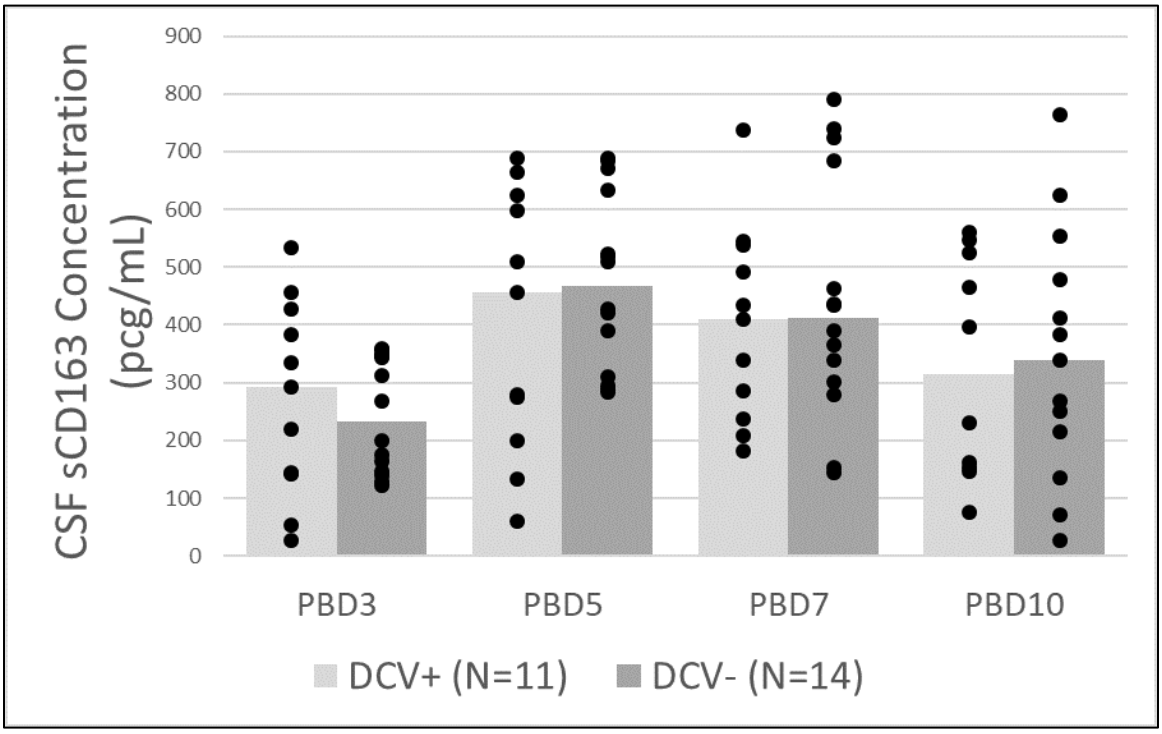
Figures 17 and 18 illustrate the plasma and CSF median sCD163 concentrations, respectively, at multiple time points according to subject DCV status. Although the median plasma sCD163 concentration was less in subjects with DCV than in those without DCV at all time points, these differences were not statistically significant. There were no significant differences in the CSF sCD163 concentration between subjects with and without DCV at any time points.

Figure 17: Plasma sCD163 in aSAH Subjects Grouped by DCV Status



In this experiment, the soluble CD163 (sCD163) concentration in plasma was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their delayed cerebral vasospasm (DCV) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. Although the median plasma sCD163 concentrations were less in subjects with DCV at all time points, these differences were not statistically significant.

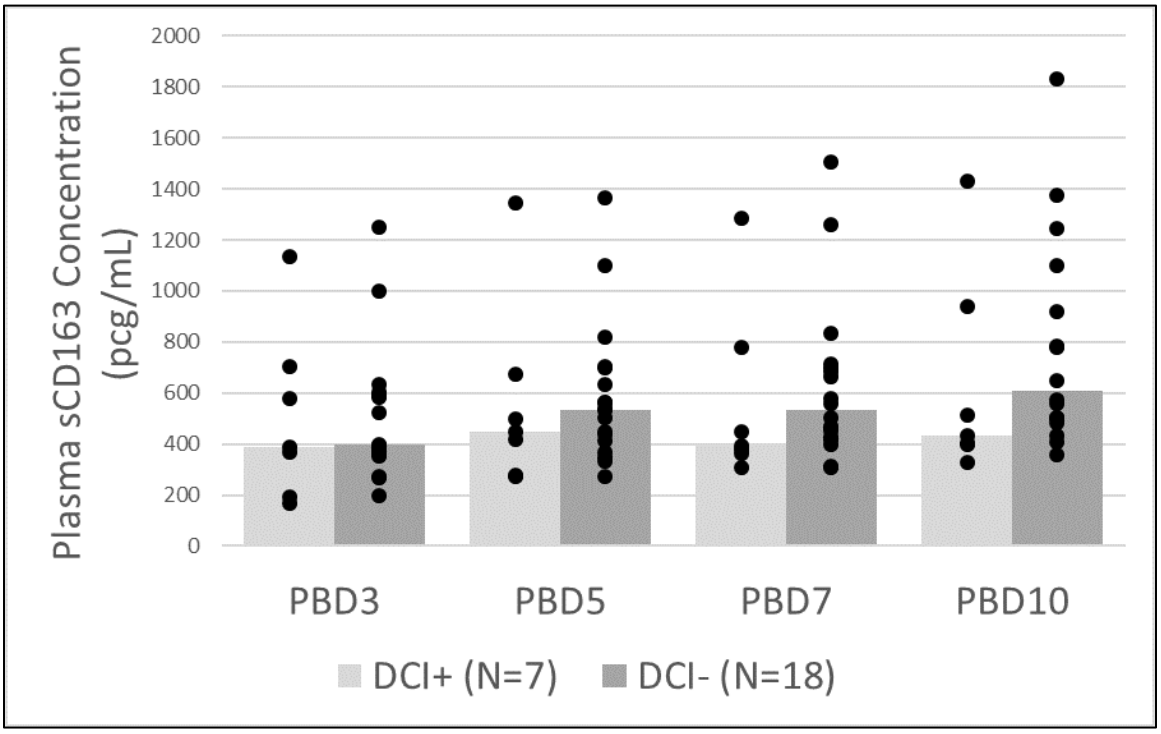
Figure 18: CSF sCD163 in aSAH Subjects Grouped by DCV Status



In this experiment, the soluble CD163 (sCD163) concentration in cerebrospinal fluid (CSF) was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their delayed cerebral vasospasm (DCV) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. There were no statistically significant differences in CSF sCD163 concentrations at any time point.

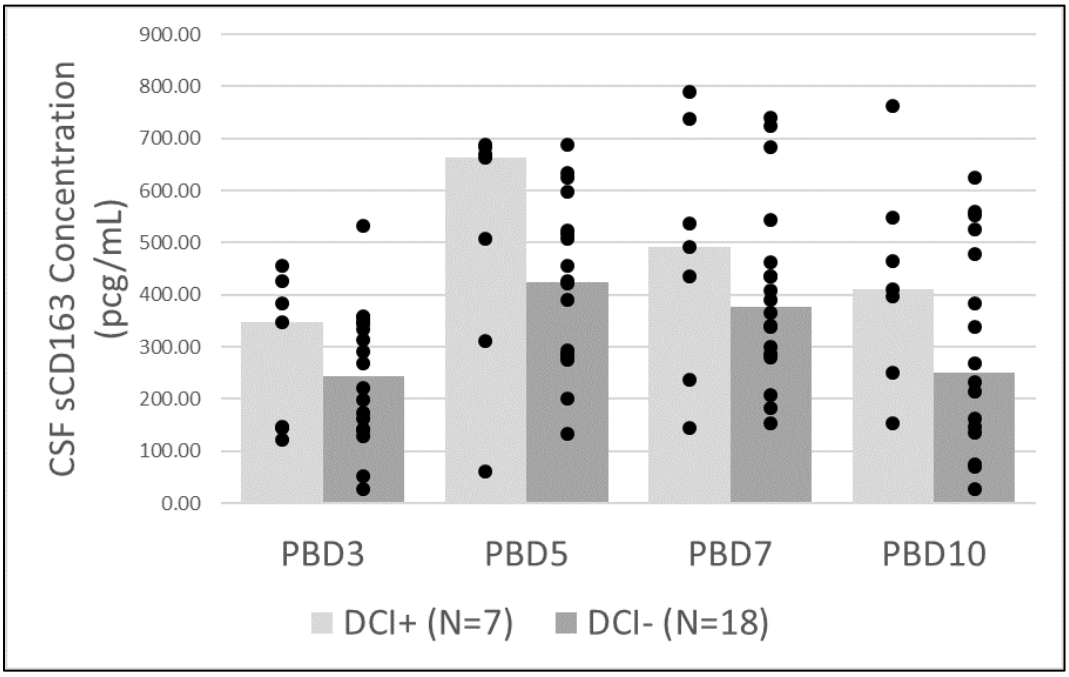
Figures 19 and 20 illustrate the median plasma and CSF sCD163 concentrations, respectively, at multiple time points according to subject DCI status. Similar to the findings with DCV, the median plasma sCD163 was less at multiple time points in subjects with DCI than in subjects without DCI, although these differences were not statistically significant. In CSF, the median sCD163 concentration was greater at all time points in subjects with DCI than in subjects without DCI; however, the differences were also not statistically significant.

Figure 19: Plasma sCD163 in aSAH Subjects Grouped by DCI Status



In this experiment, the soluble CD163 (sCD163) concentration in plasma was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their delayed cerebral ischemia (DCI) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. Although the median plasma sCD163 concentrations were less in subjects with DCI at all time points, these differences were not statistically significant.

Figure 20: CSF sCD163 in aSAH Subjects Grouped by DCI Status

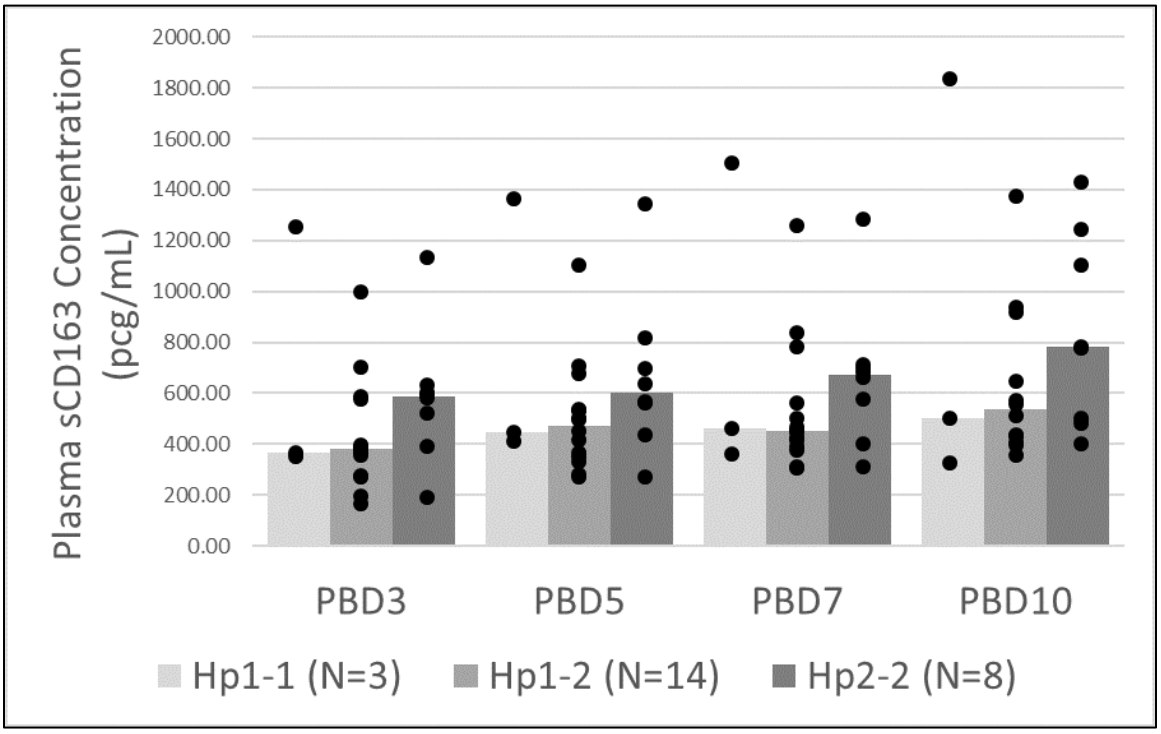


In this experiment, the soluble CD163 (sCD163) concentration in cerebrospinal fluid (CSF) was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their delayed cerebral ischemia (DCI) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. Although the median CSF sCD163 concentrations were greater in subjects with DCI at all time points, these differences were not statistically significant.

4.3.5 sCD163 analysis by haptoglobin phenotype and haptoglobin class

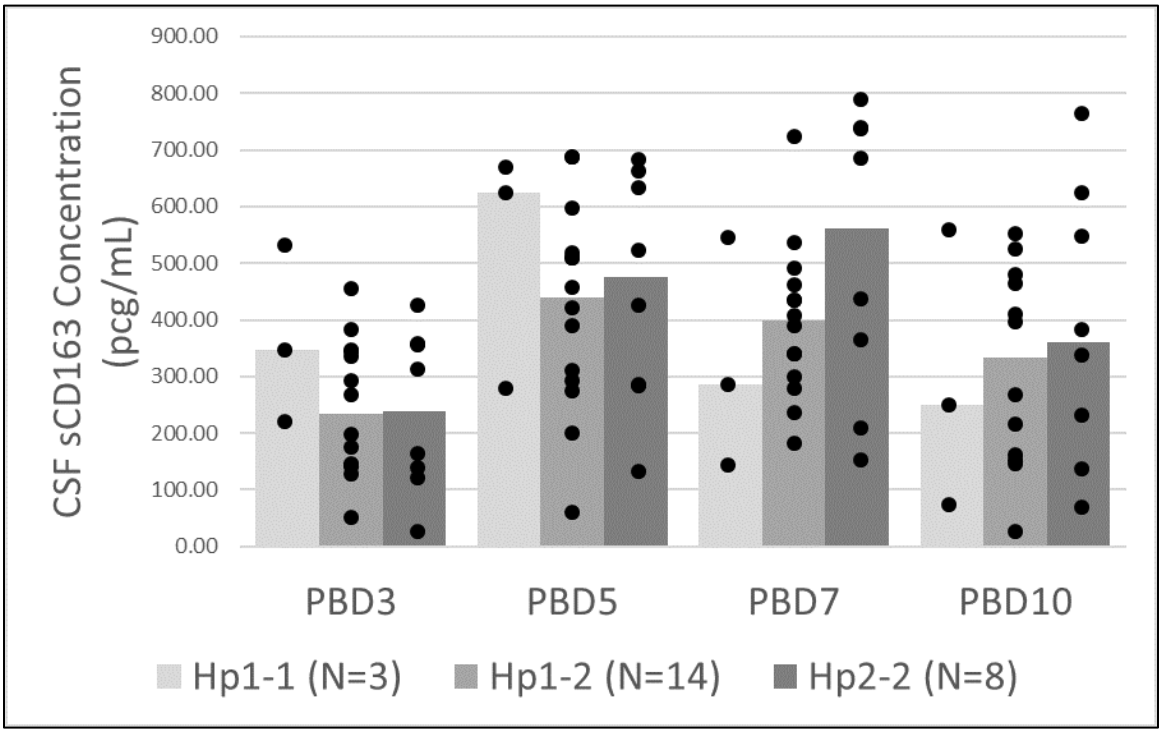
The median plasma and CSF sCD163 concentrations at PBD3, PBD5, PBD7 and PBD10 between subjects with different haptoglobin phenotype (Hp1-1, Hp1-2, and Hp2-2) are shown in Figure 21 and Figure 22, respectively. The median plasma sCD163 concentration was greater at all time points in Hp2-2 subjects than in Hp1-1 and Hp1-2 subjects; however, these differences were not statistically significant. In CSF, there was no clear association between the Hp phenotype and the median sCD163 concentrations at any time point.

Figure 21: Plasma sCD163 in aSAH Subjects Grouped by Hp Phenotype



In this experiment, the soluble CD163 (sCD163) concentration in plasma was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their haptoglobin (Hp) phenotype. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. Although the median plasma sCD163 concentrations was greatest in Hp2-2 subjects at all time points, these differences were not statistically significant.

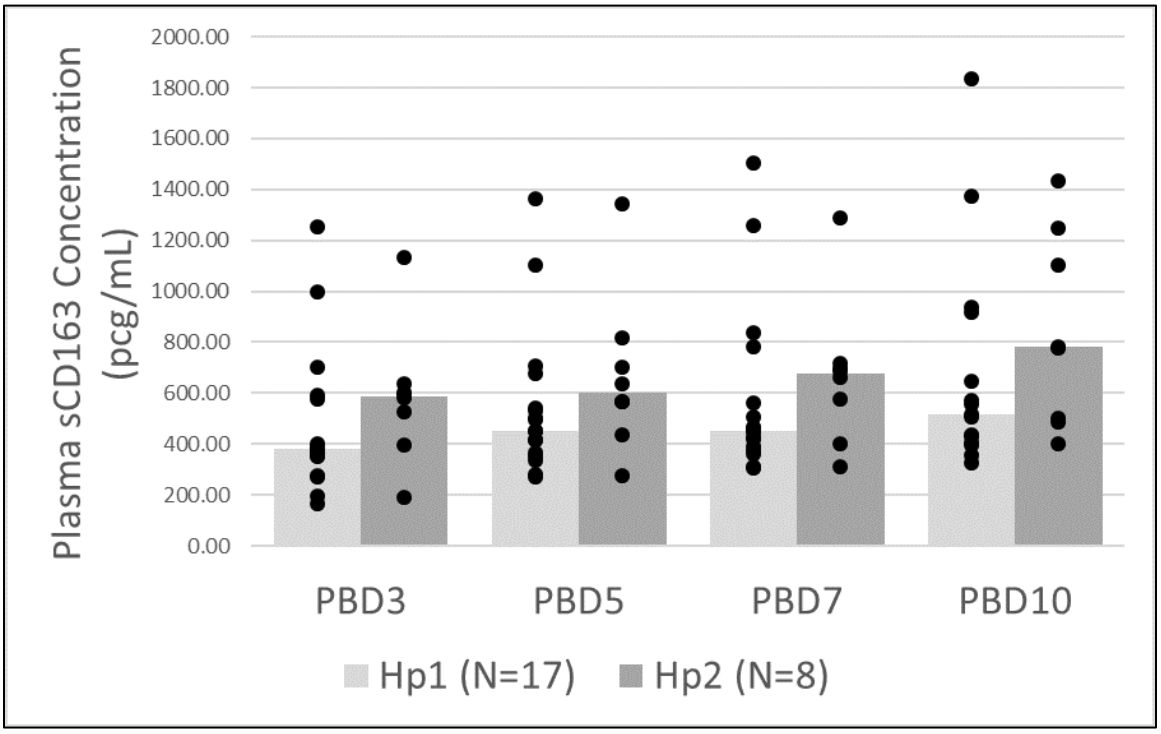
Figure 22: CSF sCD163 in aSAH Subjects Grouped by Hp Phenotype



In this experiment, the soluble CD163 (sCD163) concentration in cerebrospinal fluid (CSF) was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their haptoglobin (Hp) phenotype. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. There were no statistically significant differences in CSF sCD163 concentrations by Hp phenotype at any time point.

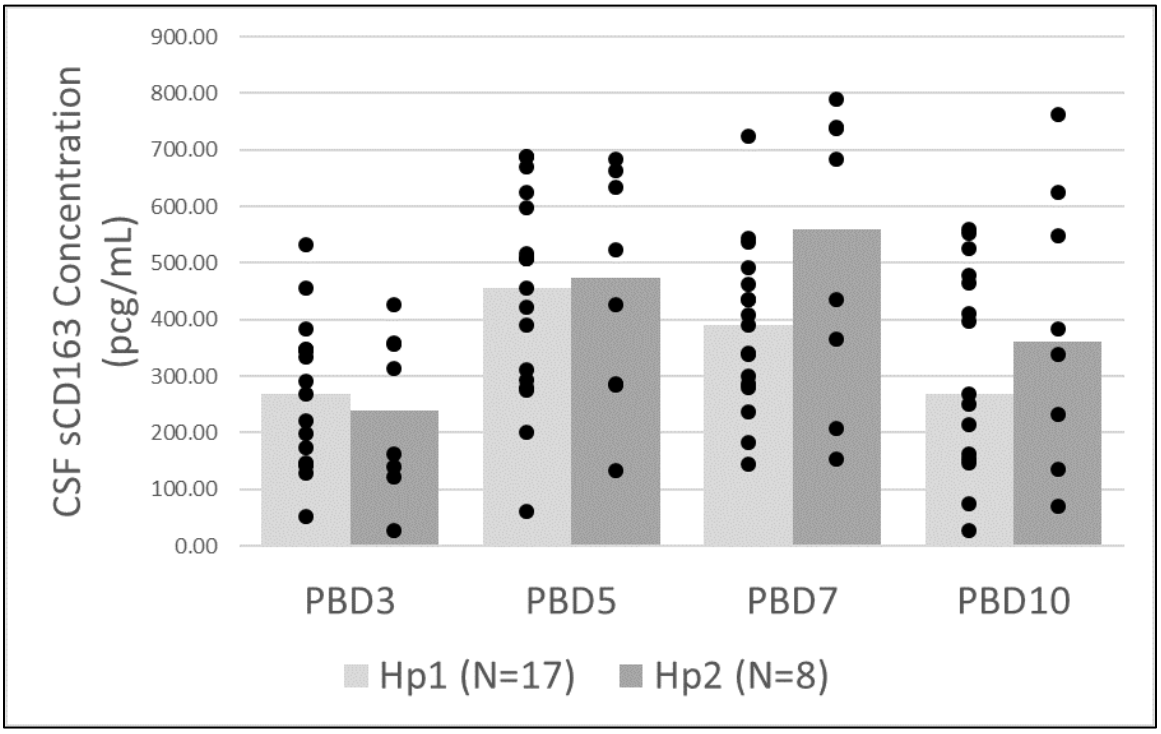
The median plasma and CSF sCD163 concentrations at PBD3, PBD5, PBD7 and PBD10 by haptoglobin class (Hp1 and Hp2) are shown in Figures 23 and 24, respectively. Similar to the findings in the Hp phenotype analysis, the median plasma sCD163 concentration was greater in the Hp2 subjects than in the Hp1 subjects at all time points; however, these differences were not statistically significant. In CSF, there were no statistically significant differences in the sCD163 concentrations between the Hp classes at any of the time points.

Figure 23: Plasma sCD163 in aSAH Subjects Grouped by Hp Class



In this experiment, the soluble CD163 (sCD163) concentration in plasma was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their haptoglobin (Hp) class. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. Although the median plasma sCD163 concentrations was greatest in Hp2 subjects at all time points, these differences were not statistically significant.

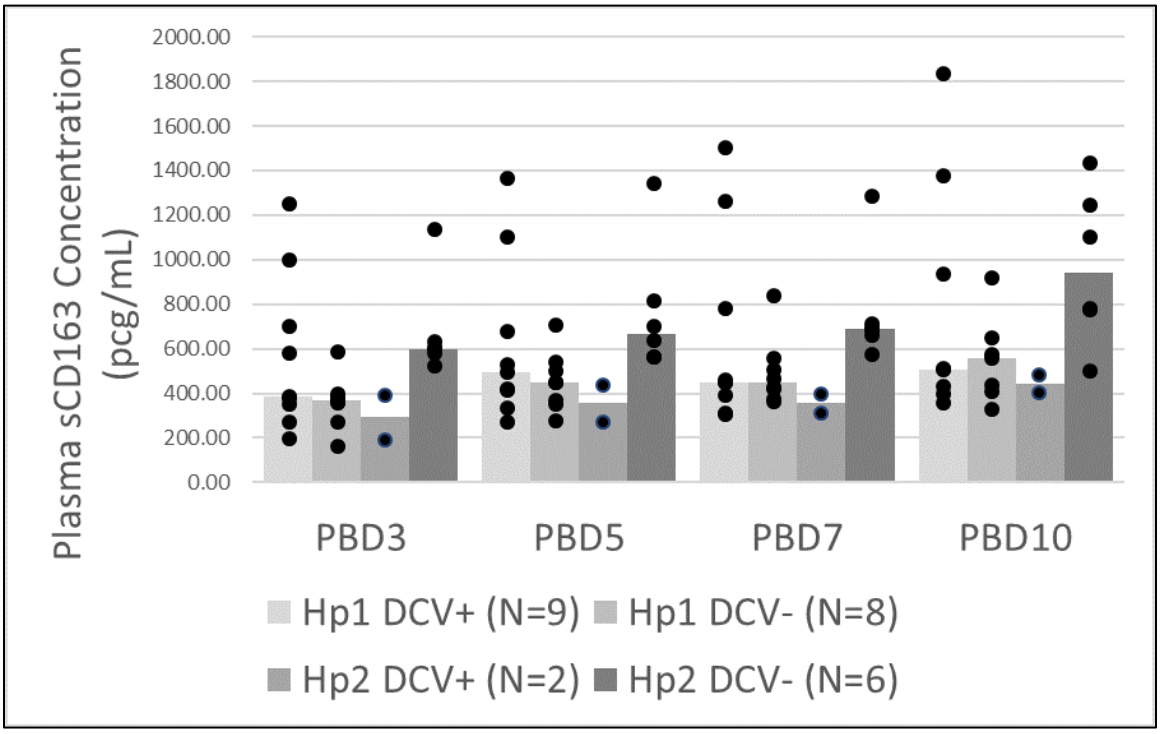
Figure 24: CSF sCD163 in aSAH Subjects Grouped by Hp Class



In this experiment, the soluble CD163 (sCD163) concentration in cerebrospinal fluid (CSF) was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by their haptoglobin (Hp) class. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. There were no statistically significant differences in sCD163 concentration by Hp class at any time point.

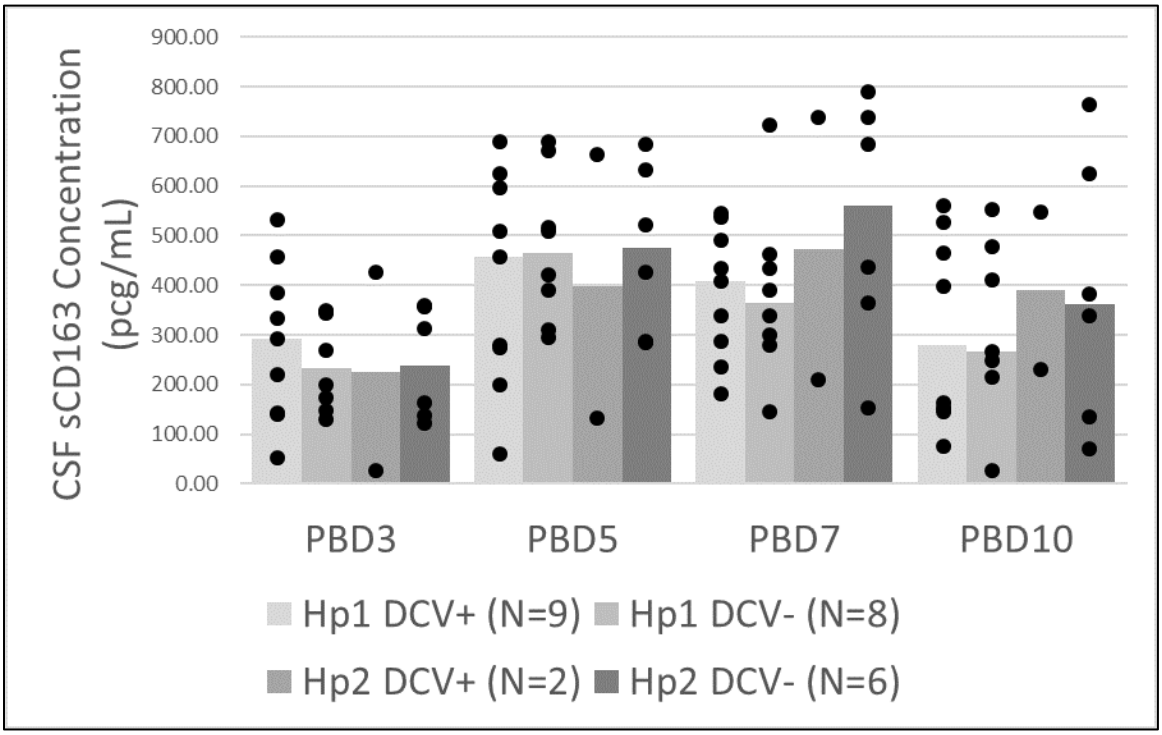
The median plasma and CSF sCD163 concentrations at PBD3, PBD5, PBD7 and PBD10 by haptoglobin class (Hp1 and Hp2) and DBI (both DCV and DCI) are shown in Figures 25-28. Although the plasma sCD163 concentration was greatest in the Hp2 subjects without DCV than in other groups, this was statistically significant compared to the Hp2 subjects with DCV at both PBD3 and PBD5. There was no trend or statistical significance between the combination of Hp class and DCV in CSF. Likewise, there were no significant trends or statistically significant differences in either the plasma or CSF sCD163 concentrations between the combination of Hp classes and DCI at all time points.

Figure 25: Plasma sCD163 in aSAH Subjects Grouped by Hp Class and DCV Status



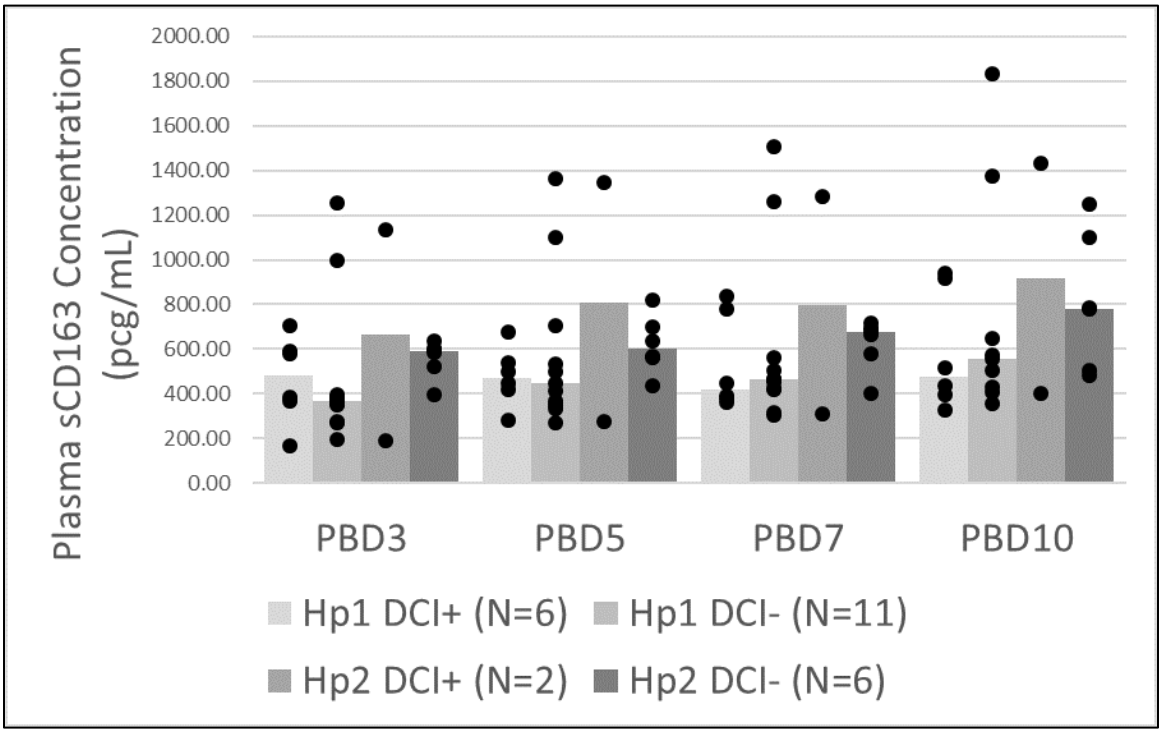
In this experiment, the soluble CD163 (sCD163) concentration in plasma was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by a combination of their haptoglobin (Hp) class and delayed cerebral vasospasm (DCV) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. There was a statistically significant difference the sCD163 concentration between Hp2 subjects with and without DCV at PBD3 and PBD5. At other time points, there is a non-significant difference between the Hp2 subjects with and without DCV. There were no differences in Hp1 subjects with and without DCV at all time points.

Figure 26: CSF sCD163 in aSAH Subjects Grouped by Hp Class and DCV Status



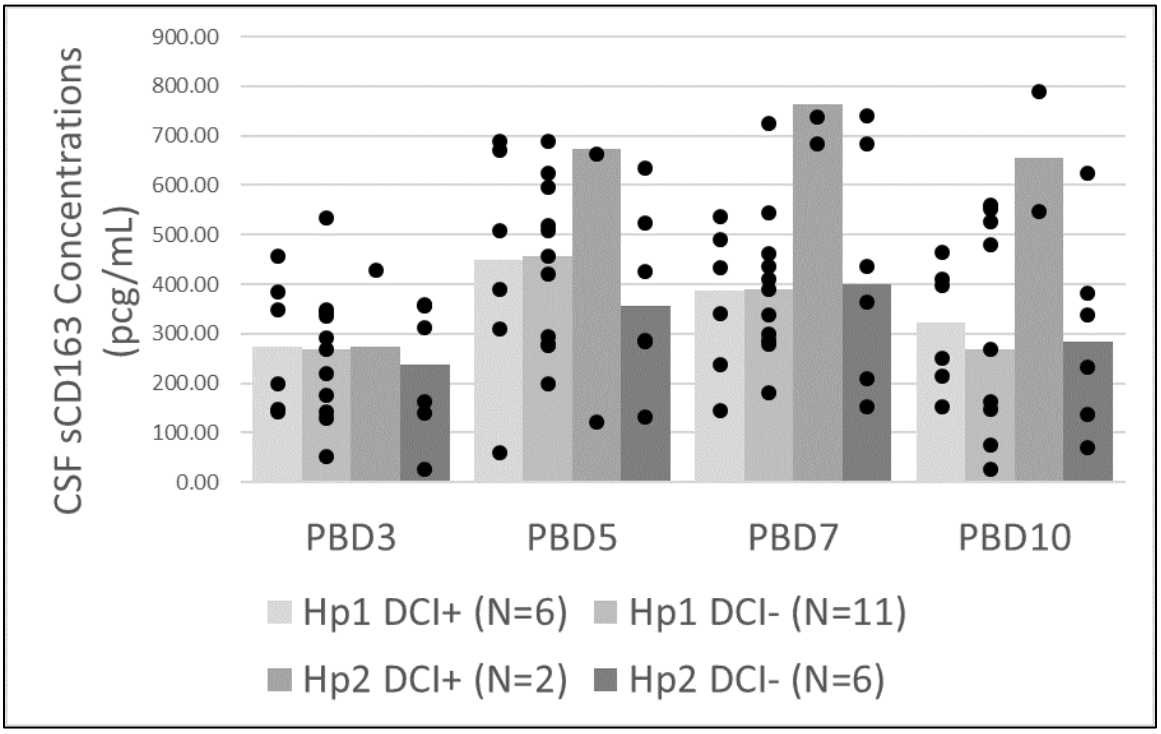
In this experiment, the soluble CD163 (sCD163) concentration in cerebrospinal fluid (CSF) was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by a combination of their haptoglobin (Hp) class and delayed cerebral vasospasm (DCV) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. There were no statistically significant differences in any of the defined groups at any time point.

Figure 27: Plasma sCD163 in aSAH Subjects Grouped by Hp Class and DCI Status



In this experiment, the soluble CD163 (sCD163) concentration in plasma was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by a combination of their haptoglobin (Hp) class and delayed cerebral ischemia (DCI) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. There were no statistically significant differences in any of the defined groups at any time point.

Figure 28: CSF sCD163 in aSAH Subjects Grouped by Hp Class and DCI Status



In this experiment, the soluble CD163 (sCD163) concentration in cerebrospinal fluid (CSF) was determined at multiple pre-defined time points by ELISA from 25 subjects with aneurysmal subarachnoid hemorrhage (aSAH) in a prospectively-collected biobank. These subjects were grouped by a combination of their haptoglobin (Hp) class and delayed cerebral ischemia (DCI) status. The bars represent the median sCD163 concentrations for each group at each time point; whereas, the individual markings represent the measured sCD163 concentrations for each subject at each time point. There were no statistically significant differences in any of the defined groups at any time point.

4.4 Conclusions

This chapter described the successful analysis of sCD163 from plasma and CSF samples in aSAH subjects recruited to a biobank. This analysis was performed to evaluate the effect that different Hp phenotypes have on sCD163 concentration to assess their influential role in the development of DBI after aSAH. Although there were few statistically significant differences in the primary analyses, there are multiple non-significant differences and trends that may warrant additional research with more patients to detect significant differences in the groups. These differences will lead to a better understanding of the potential mechanisms that explain the mediator function of Hp phenotypes and inflammation in DBI after aSAH. The implications, limitations and future directions from this analysis will be further discussed in Chapter 5.

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CHAPTER 5: CONCLUSIONS

5.1 Introduction

This chapter summarizes the key findings, implications, limitations and future direction with regard to the effects of different haptoglobin phenotypes on delayed brain injuries (DBI) after aneurysmal subarachnoid hemorrhage (aSAH). aSAH, a form of hemorrhagic stroke, is associated with significant mortality and morbidity related DBI that occur more than 72 hours after the onset of aSAH, namely delayed cerebral vasospasm (DCV) and delayed cerebral ischemia (DCI). Recently, observational clinical data have correlated the incidence and outcome of DBI with different haptoglobin (Hp) phenotypes through an unknown mechanism. For this reason, previous chapters have described the development of an aSAH biobank at the University of Kentucky to facilitate current and future observational research in aSAH (chapter 2) and, using this biobank, two different potential mechanisms, altered systemic or CSF cytokine (chapter 3) expression and differential soluble CD163 (sCD163) (chapter 4) expression have been explored.

5.2 Key Findings

5.2.1 Key findings from the development of the UK aSAH biobank

During the 2-year period from July 2018 to June 2020, the UK aSAH biobank project screened 106 patients with aSAH and successfully recruited 25 patients with radiographically-proven aSAH. Approximately 50% of these recruited subjects

developed 1 or more DBI during their hospitalization. Specifically, 45% of all subjects developed DCV, 30% developed DCI, and 10% developed delayed cerebral infarction. Despite these findings, approximately 90% of subjects had a favorable outcome at PBD90. This is significantly better than would be expected from the published literature. Plasma and CSF samples were successfully collected, processed and stored at multiple time points, according to study protocols, for all 25 subjects. There were no significant adverse events, data safety issues, or patient complaints directly related to the biobank research protocols.

In addition, the Hp phenotype and Hp class were successfully determined from stored plasma samples in all 25 subjects. There were 3 Hp1-1 patients (12%), 14 Hp1-2 patients (56%), and 8 Hp2-2 patients (32%). Likewise, there were 17 Hp1 class patients (68%) and 8 Hp2 class patients (32%). These findings are consistent with the expected prevalence of the Hp phenotypes and Hp classes based on the population demographics of Kentucky. There were no significant differences in the baseline demographics or disease-specific data between subjects with different Hp phenotypes or Hp classes. There were also no significant differences in the percentage of subjects who developed DBI between the different Hp phenotypes or Hp classes.

5.2.2 Key findings from the inflammatory cytokine analysis

Clinical data and biofluid samples from 24 subjects in the UK aSAH biobank and 9 control subjects from the UK CCTS Research Registry and Specimen bank were used

to evaluate the association of three inflammatory cytokines, IL-1β, IL6, and TNF-α, and DBI at multiple time points after aSAH. While there were significant differences in the plasma and CSF concentrations of IL-1β, IL-6 and TNF-α between study subjects and control subjects, there were no significant differences in any of the plasma or CSF inflammatory cytokine concentrations when study subjects were grouped based on either their DCV or DCI status. Likewise, there were no significant differences in any of the plasma or CSF inflammatory cytokine concentrations when study subjects were grouped based on either their Hp phenotype or Hp class. Finally, there were no significant differences in any of the plasma or CSF inflammatory cytokine concentrations when study subjects were grouped based on a combination of their Hp class and DCV status or a combination of their Hp class and DCI.

5.2.3 Key findings from the sCD163 analysis

Clinical data and biofluid samples from 25 subjects in the UK aSAH biobank and 9 control subjects from the UK CCTS Research Registry and Specimen bank were used in plasma and CSF analyses of sCD163 concentrations. The plasma sCD163 concentration in the study subjects was non-significantly lower than in the control subjects, while the CSF sCD163 concentration was significantly greater in study subjects than in the control subjects. The plasma sCD163 concentration in study subjects with DCV was non-significantly lower than the sCD163 concentration in those without DCV. Unfortunately, there were no differences in the CSF sCD163 concentration in study subjects based on their DCV status. There were no differences in either the plasma or

CSF sCD163 concentrations in study subjects based on their DCI status. The plasma concentration of sCD163 was non-significantly greater at all time points in Hp2-2 subjects than in either Hp1-1 or Hp1-2 study subjects. Similarly, the plasma sCD163 concentration was non-significantly greater in Hp2 class subjects than in Hp1 class study subjects. There were no differences in the CSF sCD163 concentration between subjects based on either Hp phenotype or class. Hp2-2 subjects without DCV had a significantly greater plasma sCD163 concentration that other subjects at PBD3 and PBD5 and non-significantly greater sCD163 concentration at other time points. Finally, there were no significant differences in either the plasma or CSF sCD163 concentrations when study subjects were grouped based a combination of their Hp class and DCI.

5.3 Implications

5.3.1 Implications of the UK aSAH biobank

There are several important implications from the UK aSAH biobank project. First, the UK aSAH biobank project demonstrated the ability to successfully recruit subjects early in their clinical course, to collect relevant clinical data from the EMR, and to obtain, process and store important biofluid samples from patients admitted to the hospital with aSAH. It was important to demonstrate this ability because enrolling and following a complex research protocol in these patients is challenging. Patients do not arrive to the hospital in a predictable fashion, in fact, they frequently arrive at night, on weekends, or other times when research staff availability may be reduced. The future development of a sustainable process to continue subject recruitment beyond this initial

2-year period will be important to facilitate future clinical and translation research in aSAH patients.

Second, the UK aSAH biobank project demonstrated that, despite using conservative estimates of hospitalization and rates of exclusion criteria in the target population, the number of subjects actually recruited during the study period was significantly lower than was predicted. In fact, only 25% of the aSAH patients admitted to the UK Chandler Medical Center were ultimately recruited to the study and, therefore, the amount of data and biofluid samples available for analyses at the end of the 2-year recruitment period was limited. This was largely due to a larger than expected number of subjects having 1 or more of the exclusion criteria. The most frequent exclusion criteria were subjects not receiving an EVD before PBD3, subjects having a non-survivable injury at the time of admission, or subjects not having family available before PBD3. Future research should take these findings into consideration when evaluating the feasibility of future research studies in this population.

Third, the UK aSAH biobank project demonstrated that that the Hp phenotype and Hp class of aSAH patients can be successfully determined from stored plasma samples, although comparing the results of these ELISA tests against the results of the more established gel electrophoresis method may also be needed. Nevertheless, the prevalence of the different Hp phenotypes in this study sample is similar to the predicted distributions based on the population estimates of Kentucky with the following

phenotype proportions: Hp1-1: 15%, Hp1-2: 55%, and Hp2-2: 30%. Likewise, the proportion of the different Hp classes appear to be similar to the predicted distributions from population estimates of Kentucky. These findings are important to allow for more accurate future predictions of research feasibility in aSAH patients based on Hp phenotype or Hp class prevalence.

5.3.2 Implications of the inflammatory cytokine analysis

The inflammatory cytokine analysis failed to provide direct evidence to support the more complex inflammation-mediated model of DBI after aSAH. In fact, there were no discernable differences in any of the inflammatory cytokines tests in plasma or CSF with regard to DCV status, DCI status, or Hp phenotype. In addition, subgroup analyses of Hp class and DBI also did not demonstrate any discernable differences in the measured cytokine concentrations.

This study may have failed to support the advanced mechanistic model of DBI for several reasons. First, there really may be no significant differences in the measured inflammatory cytokine concentrations between patients with and without DBI. This seems unlikely given the pre-existing published literature detailing cytokine concentration differences in these populations.[54,56] Second, this observation may reflect differences in patients, treatments or other conditions at UK Chandler Medical Center that affect cytokine concentrations differently from other medical centers. This also seems unlikely because the baseline patient demographics were not different between

patients with different DBI status and different Hp phenotypes and the treatments provided to patients at UK Chandler Medical Center, based on published guidelines, are similar, if not identical, to the treatments provided at other medical centers. Third, this observation may have occurred because of problems or complications resulting from either sampling/processing/storage of biofluids or from cytokine analysis. The sampling/processing/storage of plasma and CSF were performed using standard procedures, similar to the assay manufacturer recommendations for processing and storage. The assay plates and supplies themselves were all purchased at the same time and from the same manufacturing lots within 3 months of their use, were stored according to manufacturer recommendations, and none of the supplies or plates had reached their expiration dates at the time they were used. In addition, the measured concentrations of the control samples used on each plate were all consistent with the reported concentration from the manufacturer and the UK CCTS Biomarker Analysis Laboratory (BAL) was used to perform these assays and has significant previous experience with these assays. For these reasons, it seems unlikely that there were significant issues with sampling/processing/storage or analysis of these inflammatory cytokines. Fourth, there may have been important differences discovered if biofluid samples had been obtained earlier. While, the focus of this work was injury that occurred after PBD3, by not obtaining samples until this time point, significant differences that affected DBI may have occurred earlier. Finally, there may have been no detected differences in cytokine analyses simply because there were too few patients in any of the group or subgroup analyses to detect these differences in cytokine

concentrations. This seems most plausible as published studies have generally used at least twice as many patients in each group. The small number of samples may mask any potential positive findings in group and subgroup analyses with very low numbers of subjects. Repeating this analysis with significantly greater numbers may be important for future studies in this patient population.

5.3.3 Implications of the sCD163 Analysis

There are also several important implications from the sCD163 analysis project. First, the sCD163 analysis project demonstrated important differences in both plasma and CSF sCD163 concentrations in aSAH patients compared to the measured concentration in control patients. This is similar to the previous publication that compared the sCD163 concentrations in aSAH subjects and controls.[156] This suggests that there are important differences in either the rate of CD163 cleavage by ADAM17/TACE to sCD163 between aSAH and control patients or in the total number of macrophages present in blood or CSF between aSAH and control patients. In plasma, the sCD163 was non-significantly decreased in the aSAH patients compared to the control subjects. Future studies should attempt to evaluate these differences by controlling for changes in macrophage count, using a ratio of sCD163 concentration and macrophage count. A decrease in both the sCD163 concentration and the calculated ratio would suggest a decreased production in sCD163; whereas, a measured decrease in the sCD163 concentration but an increase or no change in the ratio would suggest that sCD163 was decreased because of a decrease in

the macrophage count. This novel ratio could also be used to evaluate mechanisms of increases in the measured sCD163 concentration.

In further analysis of the CSF sCD163 concentration between PBD3 and PBD10, it is important to note that the sCD163 concentration was already increased significantly elevated over the controls (approximately 5-fold) at the earliest time point (PBD3) and peaked between PBD5-7 at approximately 10 times the control concentration. This suggests that evaluations performed even earlier in the hospital course may provide additional insights into the time course of sCD163 alterations.

In addition, while there was no significant difference in the sCD163 concentrations in patients with and without DCV or DCI, there were non-significant differences in patients with and without DCV (decreased in DCV patients), as well as, those with and without DCI (increased in DCV), starting at PBD3. These findings suggest that, if sCD163 is involved in the development of DBI as defined in the mechanistic model, the pathways for DCV and DCI may be separately and differentially activated and may support sCD163 as one of the "unknown triggers" as described in the inflammation-mediated model.

It is interesting to also note that the plasma sCD163 concentration in Hp2-2 patients was greater than both the Hp1-1 and Hp1-2 patients at all time points, a finding that may also support the inflammation-mediated models of DBI as described in Figure 5.

With data from more patients, this trend may rise to the level of statistical significance. This is particularly interesting, given that there are significant differences in plasma sCD163 concentration, with the greatest difference in Hp2-2 patients. Differences in CSF sCD163 concentrations in patients with regard to Hp phenotypes are more difficult to interpret from this data; however, the peak sCD163 concentration in Hp2-2 patients is greater and occurs later (PBD7 vs PBD5) after aSAH in Hp2 patients. It is unclear what, if any significance, this delayed peak in sCD163 concentration has with regard to clinical outcomes but may warrant additional research.

Finally, while there were no significant differences in the plasma sCD163 concentrations in the combination of analysis of Hp class and DCV status, the sCD163 concentration in Hp2 patients who did not develop DCV was non-significantly greater than the other groups. This might, in part, explain why there was no apparent differences in the sCD163 concentration among patients with and without DCV. The cause of this finding is unclear and warrants additional evaluation. Differences in CSF sCD163 concentrations are difficult to assess with no clear patterns to suggest future directions of research.

In DCI, there were no significant differences in the plasma sCD163 concentrations that were not explained by differences in baseline levels. In CSF, there was a non-significant increase in the sCD163 in Hp2 patients compared to all other patients. This may be an important finding, suggesting a statistically significant

difference may be detected with more patients. These findings should be further compared to long term clinical outcomes.

5.4 Limitations

5.4.1 Limitations of the UK aSAH biobank

There were several important limitations from the UK aSAH biobank. First, only 25% of all aSAH patients admitted to the University of Kentucky Chandler Medical Center were enrolled in this study. This was significantly below the predicted enrollment rate; however, the most common reasons for subject exclusion were related to patient and disease-specific factors that would have negatively impacted completion of the research protocols if the exclusion criteria were loosened. Nearly 90% of the patients were excluded because of just three of these criteria, including "No EVD placed", "non-survivable injury" and "no family available for consent". Estimation of the rates of these exclusion criteria at the start of this project did not adequately account for the frequency of these exclusions in aSAH patients hospitalized during the enrollment period.

Second, during the study period, there was significant variability from year to year in the number of subjects recruited. In fact, significantly more subjects were enrolled the first year than the second year (N=20 versus N=5, $p<0.001$). Unfortunately, it is not clear whether the enrollment rate in the first year was increased above baseline, the enrollment rate in the second year was decreased below baseline, or both. In fact, in

an analysis of the reasons that patients were excluded, there was no one single reason to explain the significantly reduced enrollment in the second year compared to the first year. Also, while there was a negative impact in recruitment related to COVID-19 that impacted the final 4 months of the study period, it is not the primary cause of reduced recruitment as this trend existed throughout the other 8 months of the year. Recruitment of patients during the 4-month period at the rate of the previous month would have increased overall recruitment by just 2 patients.

Third, patient data and biofluid samples were not collected prior to PBD3, limiting the opportunity to assess changes in cytokines or sCD163 prior to this time point. The decision to collect biofluid samples starting at PBD3 was made for several reasons, including patient and family comfort and the impact that this has on subject recruitment. Anecdotal experience suggests that early discussion of research may cause undue anxiety in patients and their family that translates to refusal to volunteer for research. By delaying recruitment and sampling to PBD3, patients and their families were able to see some early neurologic recovery and to have time to process the consequences of aSAH. In addition, biofluid sampling was intentionally delayed to PBD3 because the overall focus of the biobank and subsequent research was to study mechanisms of DBI that start on PBD3. In the future, collecting data and biofluid samples from subjects before this time period might, however, provide additional insight into mechanisms of disease and could be used for in-patient biofluid analyses in future research.

5.4.2 Limitations of the inflammatory cytokine analysis

There were also several important limitations of the inflammatory cytokine analysis. First, as has been previously described, clinical data and biofluid samples from only 24 subjects were available. The low number of subjects likely dramatically decreased the ability to detect important differences in subgroup analyses. Similarly, there were also outlier results in many analyses, affecting the data distributions, necessitating the use of non-parametric statistical tests in essentially all of the analyses. The effect of these outliers on data distributions were compounded by the relatively low number of subjects, especially in group and subgroup analyses.

Second, the control subjects were drawn from a large pool of hospitalized patients rather than healthy research volunteers. This decision was made because lumbar puncture to obtain CSF in healthy volunteers is not benign nor is it inexpensive. While the control subjects were screened to remove patients with ventriculitis and meningitis, it is possible, and perhaps likely, that they had other systemic or neurologic diseases that necessitated CSF collection and that may have impacted the blood or CSF inflammatory cytokine concentrations. The use of healthy research volunteers without evidence of infection or inflammatory diseases as control subjects in future research may improve research comparisons with aSAH patients.

Third, although the inflammatory cytokines in this study were chosen based on previously published reports, the analysis of only 3 cytokines limited the ability to detect

potential changes in other inflammatory pathways. In future analyses, a more complex panel including more potentially active inflammatory cytokines should, perhaps, be considered. In addition, the measured concentrations of Il-1β and TNF-α were relatively low compared to the sensitivity of the assay, limiting the precision of measurement of small differences that may have existed between group and subgroups of patients. The use of more sensitive assays for these cytokines may be important in future analyses.

5.4.3 Limitations of the sCD163 analysis

As has been described above, clinical data and biofluid samples from only 25 subjects were available and as was true with the inflammatory cytokine analyses, this low number of subjects likely decreased the ability to detect important differences in group and subgroup analyses. The patient with ventriculitis that caused his exclusion in the inflammatory cytokine analysis was not excluded in this analysis because his sCD163 concentrations were not significantly different from the other study subjects. Nevertheless, the inclusion of his results in the final analyses may have negatively impacted the results. In addition, concerns about the impact of sampling, processing and storage protocols, as well as, sCD163 analysis are similar to the concerns described for the inflammatory cytokine analysis with the caveat that the UK CCTS BAL had no prior experience with the sCD163 analysis procedures.

Another potential limitation in the sCD163 analysis project is uncertainty over whether differences in sCD163 concentrations reflect a key driver of DBI or whether

these differences reflect injury caused by DBI. Because this is the first study to evaluate sCD163 in this population, additional research will be needed to clarify the potential associations that may exist between DBI, Hp phenotypes and sCD163 in aSAH patients.

5.5 Future Directions

5.5.1 Future directions from the UK aSAH biobank project

There are several potential future directions for the UK aSAH biobank project. First, because overall enrollment in the aSAH research project was too low to support high-quality research over short time periods, as demonstrated by the previously described work in the inflammatory cytokine and sCD163 analyses, continued screening and recruitment of patients to this biobank will remain important for future clinical and translational research in this population. Although time intensive, enrollment of patients over a 4-5-year period would be expected to include more than 50 patients, providing significantly more clinical data and biofluid samples for future research.

Second, in some cases, it may be beneficial to relax or revise exclusion criteria to increase overall patient enrollment in the biobank. Based on an analysis of the reasons for subject exclusions, removing the exclusion for subjects who did not receive an EVD would likely significantly increase the number of patients to recruit. In these patients, even though CSF samples would not be collected, clinical data and blood samples would still be collected, processed and stored for future research. In addition, the use of electronic forms of consent (e-consents) may also increase the likelihood that legally-

authorized representatives are able to provide written documentation of consent in the event that they are not able to be physically present at the hospital in a timely fashion.

Third, the biobank may significantly benefit from building it into a collaborative project with other local or regional medical centers to collect data and biofluids from a larger pool of patients. This would increase the opportunity to recruit patients beyond the approximately 50 patients per year currently admitted to the UK Chandler Medical Center. As a secondary benefit, expansion of the single-center biobank may also improve generalizability of the data collected by including patients from a greater variety of backgrounds and by minimizing the potential effect that treatment at any single center may have on the outcomes. In developing these collaborations, it may be possible to, first, develop partnerships with the other local hospitals, allowing current research personnel to obtain clinical data and biofluids for processing in a central laboratory at UK. Further expansion beyond the local community may be desirable and could capitalize on the pre-existing collaborations with one or more of the UK Center for Clinical and Translational Science research alliances.

Finally, regardless of biobank expansion opportunities, the biobank should develop a more formal governance structure. This governance is critical for determining fair use and compensation of clinical data and biofluid samples by future researchers. In addition, this governance is important to coordinate regulatory requirements, especially if

this project grows into a multicenter biobank. Finally, the governance is important to formally monitor patient safety, clinical data accuracy, and biofluid sample stability.

5.5.2 Future directions from the inflammatory cytokine analysis project

Because this inflammatory cytokine analysis project was an underpowered, but ultimately negative study, contradicting the published literature, it will be important to repeat this study, perhaps with additional samples from the biobank to evaluate the potential causes of this conflicting data. In addition, as has been discussed elsewhere, future studies should consider additional cytokines, chemokines and activators of inflammation that may be involved in DBI after aSAH to more fully develop the inflammation-mediated model of DBI.

5.5.3 Future directions from the sCD163 analysis project

There are several potential next steps for additional research with the sCD163 analysis project. First, the study should be repeated with more patients and across more medical centers to test whether the interesting but non-significant findings in this project might rise to a level of significance with more recruited patients across more medical centers. These interesting findings include testing differences with regard to DBI, Hp phenotypes and the combination of Hp phenotypes and DBI.

If, in future studies, these non-significant associations gain more significance, studies should test whether blood or CSF sCD163 concentration differences reflect

changes in inflammatory responses. Likewise, future studies should test the association between blood or CSF sCD163 concentrations and the individual anatomic and biochemical processes described in Figure 5. These studies will be needed to further evaluate the inflammation-mediated model of DBI.

Finally, with more patients and medical centers, it might be possible to also test the associations between blood or CSF sCD163 concentrations and long-term clinical outcomes, including recovery and disability at PBD90 or beyond. These outcomes are clinically meaningful and it may be important to establish sCD163 as a target for future therapeutics or a biochemical method to assess the effectiveness of future therapeutics.

5.6 Conclusion

This chapter detailed the key findings, implications, limitations and future directions of the development of the UK aSAH biobank, as well as, inflammatory cytokine analyses and sCD163 analyses to examine mechanisms of DBI after aSAH. Overall, this work highlights important steps taken to better understand the potential mechanisms that are triggered by aSAH and that ultimately lead to DBI, neurologic injury and permanent neurologic disability. Future research should build on this work to improve patient outcomes in this frequently-devastating disease.

APPENDICES

APPENDIX 1: UBACC SCORE WORKSHEET

Instructions: Ask potential subjects questions from the first column. Listen to responses and score responses 0-2 based on whether the answers are inappropriate (score=0), partially appropriate (score=1), or appropriate (score=2). If there is no response to the question, it should be given score=0. Scores from each question should be summed to calculate the UBACC total score.

Questions to ask:	0 – Inappropriate	1 – Partially appropriate	2 – Appropriate
What is the purpose of the study that was just explained to you?			
What makes you want to consider participating in this study?			
Do you believe this is primarily research or primarily treatment?			
Do you have to be in this study if you do not want to participate?			
If you want to withdraw from this study, will you still be able to receive regular treatment?			
If you participate in this study, what are some of the things that you will be asked to do?			
Please describe some of the risks or discomforts that people may experience if they participate in this study.			
Please describe some of the benefits of this study.			
Is it possible that being a part of this study will not have any benefit to you?			
Who will pay for your medical care if you are injured as a direct result of your participation in this study?			

Total Score: _____ (Score ≤ 14 consistent with inability to provide meaningful consent)

APPENDIX 2: CASE REPORT FORMS

Confidential

Consent, Screening and Exclusion Data

Record ID _____

Consent Information

UBACC evaluation completed?	◯ Yes ◯ No
UBACC score > 14?	◯ Yes ◯ No ((If no, LAR must provide consent))
Informed consent obtained from:	◯ Subject ◯ LAR ◯ Other
Informed consent	

Study Screening Criteria

	Yes	No
Age >18?	◯	◯
EVD placed?	◯	◯
Survival?	◯	◯
Aneurysm identified?	◯	◯

Study Exclusion Criteria

	Yes	No
Admitted after PBD3?	◯	◯
Pre-existing neurologic disease?	◯	◯
Inflammatory disease?	◯	◯
Vasospasm before PBD3?	◯	◯

Baseline Data

Record ID

_____

Baseline Subject Information

Age

_____

Gender

◯ Male
◯ Female

Height (cm)

_____
(Enter as number to 1 decimal place.)

Weight (kg)

_____
(Enter as number to 1 decimal place.)

Body Mass Index (kg/m2)

_____

Ethnic Origin

◯ American Indian/Alaskan Native
◯ Asian
◯ Black African American
◯ Hispanic/Latino
◯ Native Hawaiian/Pacific Islander
◯ White/Caucasian
◯ Other or Unknown

Baseline aSAH Scores

Hunt & Hess Score

◯ 1
◯ 2
◯ 3
◯ 4
◯ 5
◯ Unknown

WFNS Score

◯ 1
◯ 2
◯ 3
◯ 4
◯ 5
◯ Unknown

Fischer Score

◯ 1
◯ 2
◯ 3
◯ 4
◯ Unknown

Baseline Aneurysm Information

Date and Time of Aneurysm Rupture	_____
Was primary aneurysm located on angiography?	◯ Yes ◯ No
Location of Ruptured Aneurysm	◯ ACOM ◯ ACA ◯ OA ◯ MCA ◯ PCOM ◯ PCA ◯ PICA ◯ BA ◯ VA ◯ ICA ◯ Other ◯ Unknown
Type of Aneurysm Obliteration Therapy	◯ Coil ◯ Clip ◯ Other ◯ Unknown
Date and Time of Aneurysm Obliteration	_____
Are Additional Aneurysms Present?	◯ Yes ◯ No

Visit 1 - PBD3

Record ID

Blood Sampling Information

Blood Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of Blood Sampling

Blood Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #

Sample 3 ID #

Sample 4 ID #

Sample 5 ID #

Sample 6 ID #

Reason Blood Sample Not Obtained:

CSF Sampling Information

CSF Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of CSF Sampling

CSF Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #	_____
Sample 3 ID #	_____
Sample 4 ID #	_____
Sample 5 ID #	_____
Sample 6 ID #	_____
Reason CSF Sample Not Obtained	_____

Vasospasm Information

Did Patient Undergo Recent Angiography?	◯ Yes ◯ No
Date of Recent Angiography	_____
Did angio demonstrate vasospasm?	◯ Yes ◯ No

Visit 2 - PBD5

Record ID

Blood Sampling Information

Blood Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of Blood Sampling

Blood Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #

Sample 3 ID #

Sample 4 ID #

Sample 5 ID #

Sample 6 ID #

Reason Blood Sample Not Obtained:

CSF Sampling Information

CSF Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of CSF Sampling

CSF Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #	
Sample 3 ID #	
Sample 4 ID #	
Sample 5 ID #	
Sample 6 ID #	
Reason CSF Sample Not Obtained	

Vasospasm Information

Did Patient Undergo Recent Angiography?	◯ Yes ◯ No
Date of Recent Angiography	
Did angio demonstrate vasospasm?	

Visit 3 - PBD7

Record ID

Blood Sampling Information

Blood Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of Blood Sampling

Blood Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #

Sample 3 ID #

Sample 4 ID #

Sample 5 ID #

Sample 6 ID #

Reason Blood Sample Not Obtained:

CSF Sampling Information

CSF Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of CSF Sampling

CSF Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #	
Sample 3 ID #	
Sample 4 ID #	
Sample 5 ID #	
Sample 6 ID #	
Reason CSF Sample Not Obtained	

Vasospasm Information

Did Patient Undergo Recent Angiography?	◯ Yes ◯ No
Date of Recent Angiography	
Did angio demonstrate vasospasm?	

Visit 4 - PBD10

Record ID

Blood Sampling Information

Blood Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of Blood Sampling

Blood Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #

Sample 3 ID #

Sample 4 ID #

Sample 5 ID #

Sample 6 ID #

Reason Blood Sample Not Obtained:

CSF Sampling Information

CSF Obtained Per Protocol?
◯ Yes
◯ No

Date and Time of CSF Sampling

CSF Sample Obtained By:
(Enter last name only)

Sample 1 ID #

Sample 2 ID #	
Sample 3 ID #	
Sample 4 ID #	
Sample 5 ID #	
Sample 6 ID #	
Reason CSF Sample Not Obtained	

Vasospasm Information

Did Patient Undergo Recent Angiography?	◯ Yes ◯ No
Date of Recent Angiography	
Did angio demonstrate vasospasm?	

Visit 5 - TCD Data

Record ID	_____
PBD 3 TCD Performed?	◯ Yes ◯ No
PBD 3 TCD Value - Right	_____
PBD 3 TCD Ratio- Right	_____
PBD 3 TCD Value - Left	_____
PBD 3 TCD Ratio - Left	_____
PBD 4 TCD Performed?	◯ Yes ◯ No
PBD 4 TCD Value - Right	_____
PBD 4 TCD Ratio- Right	_____
PBD 4 TCD Value - Left	_____
PBD 4 TCD Ratio - Left	_____
PBD 5 TCD Performed?	◯ Yes ◯ No
PBD 5 TCD Value - Right	_____
PBD 5 TCD Ratio- Right	_____
PBD 5 TCD Value - Left	_____
PBD 5 TCD Ratio - Left	_____
PBD 6 TCD Performed?	◯ Yes ◯ No

PBD 6 TCD Value - Right	
PBD 6 TCD Ratio- Right	
PBD 6 TCD Value - Left	
PBD 6 TCD Ratio - Left	
PBD 7 TCD Performed?	◯ Yes ◯ No
PBD 7 TCD Value - Right	
PBD 7 TCD Ratio- Right	
PBD 7 TCD Value - Left	
PBD 7 TCD Ratio - Left	
PBD 8 TCD Performed?	◯ Yes ◯ No
PBD 8 TCD Value - Right	
PBD 8 TCD Ratio- Right	
PBD 8 TCD Value - Left	
PBD 8 TCD Ratio - Left	
PBD 9 TCD Performed?	◯ Yes ◯ No
PBD 9 TCD Value - Right	
PBD 9 TCD Ratio- Right	
PBD 9 TCD Value - Left	

PBD 9 TCD Ratio - Left	_____
PBD 10 TCD Performed?	◯ Yes ◯ No
PBD 10 TCD Value - Right	_____
PBD 10 TCD Ratio- Right	_____
PBD 10 TCD Value - Left	_____
PBD 10 TCD Ratio - Left	_____
PBD 11 TCD Performed?	◯ Yes ◯ No
PBD 11 TCD Value - Right	_____
PBD 11 TCD Ratio- Right	_____
PBD 11 TCD Value - Left	_____
PBD 11 TCD Ratio - Left	_____
PBD 12 TCD Performed?	◯ Yes ◯ No
PBD 12 TCD Value - Right	_____
PBD 12 TCD Ratio- Right	_____
PBD 12 TCD Value - Left	_____
PBD 12 TCD Ratio - Left	_____
PBD 13 TCD Performed?	◯ Yes ◯ No
PBD 13 TCD Value - Right	_____

PBD 13 TCD Ratio- Right	_____
PBD 13 TCD Value - Left	_____
PBD 13 TCD Ratio - Left	_____
PBD 14 TCD Performed?	◯ Yes ◯ No
PBD 14 TCD Value - Right	_____
PBD 14 TCD Ratio- Right	_____
PBD 14 TCD Value - Left	_____
PBD 14 TCD Ratio - Left	_____

Visit 6 - PBD30

Record ID _____

DCV Data

Did subject develop TCD-based DCV during hospitalization?
◯ Yes
◯ No
(TCD Ratio > 3.0)

Did subject develop angiographic vasospasm during hospitalization?
◯ Yes
◯ No

DCI Data

Did subject develop DCI during hospitalization?
◯ Yes
◯ No

Adverse Event Data

Did Subject Develop Ventriculitis?
◯ Yes
◯ No

Did Subject Develop Bacteremia?
◯ Yes
◯ No

Was EVD replaced during study period?
◯ Yes
◯ No

Was there a breach of confidentiality during study period?
◯ Yes
◯ No

Were there other study problems or safety concerns during study period?
◯ Yes
◯ No

Describe study problems or safety concerns that occurred during study period
_____

30-Day Modified Rankin Score Evaluation

Was 30-day mRS Performed?
◯ Yes
◯ No

Reason 30-day mRS NOT Performed
_____

30-Day Modified Rankin Score	◯ 0 - Completely back to normal ◯ 1 - Not normal but able to do everything as before ◯ 2 - Independent but not able to do everything as before ◯ 3 - Not independent but able to walk ◯ 4 - Not independent, not able to walk but not bedridden ◯ 5 - Bedridden ◯ 6 - Death

30-Day GOSE Evaluation

Was 30-Day GOSE Performed?	◯ Yes ◯ No
Reason 30-Day GOSE NOT Performed	_____
30-Day GOSE Score	◯ Death ◯ Vegetative State ◯ SD- (Non-vegetative but not independent at home) ◯ SD+ (Independent at home but not independent outside of the home) ◯ MD- (Independent but not able to work) ◯ MD+ (Independent, able to work, but not able to resume regular activities consistently) ◯ GR- (Independent, working, regular activities, but still not completely back to normal) ◯ GR+ (Normal)

Visit 7 - PBD90

Record ID _____

90-Day Cerebral Infarction Evaluation

Did subject have new cerebral infarction by CT scan?
- ◯ Yes
- ◯ No

90-Day Modified Rankin Score Evaluation

Was 90-day mRS Performed?
- ◯ Yes
- ◯ No

Reason 90-day mRS NOT Performed _____

90-Day Modified Rankin Score
- ◯ 0 - Completely back to normal
- ◯ 1 - Not normal but able to do everything as before
- ◯ 2 - Independent but not able to do everything as before
- ◯ 3 - Not independent but able to walk
- ◯ 4 - Not independent, not able to walk but not bedridden
- ◯ 5 - Bedridden
- ◯ 6 - Death

90-Day GOSE Evaluation

Was 90-Day GOSE Performed?
- ◯ Yes
- ◯ No

Reason 90-Day GOSE NOT Performed _____

90-Day GOSE Score
- ◯ Death
- ◯ Vegetative State
- ◯ SD- (Non-vegetative but not independent at home)
- ◯ SD+ (Independent at home but not independent outside of the home)
- ◯ MD- (Independent but not able to work)
- ◯ MD+ (Independent, able to work, but not able to resume regular activities consistently)
- ◯ GR- (Independent, working, regular activities, but still not completely back to normal)
- ◯ GR+ (Normal)

Haptoglobin Phenotype

Record ID	_____
Was haptoglobin phenotype analyzed?	◯ Yes ◯ No
What was the Hp sample 1 optical density (OD)?	_____
What was the Hp sample 2 optical density (OD)?	_____
What was the average Hp sample optical density (OD)?	_____
What was the haptoglobin phenotype?	◯ Hp1-1 ◯ Hp1-2 ◯ Hp2-2 ◯ Unknown

APPENDIX 3: HAPTOGLOBIN PHENOTYPE ANALYSIS PROTOCOL

Reagent and Sample Preparation

1. Allow all reagents, controls, and samples to reach room temperature (22-28°C), including:
    a. Concentrated wash buffer stock solution
    b. Sample dilution buffer solution (ready to use)
    c. Conjugate diluent solution (ready to use)
    d. Concentrated HRP-conjugated mAb solution
    e. TMB substrate solution (ready to use)
    f. Stop solution (ready to use)
    g. Positive controls (Hp1-1, Hp1-2, Hp2-2)
2. Prepare wash buffer solution from dilution of 50 ml wash buffer stock in 950 mL deionized water.
3. Prepare biofluid sample solutions by diluting 15 uL biofluid samples with 135 uL sample dilution buffer solution.
4. Prepare HRP-conjugated mAb solution by diluting 50 uL of concentrated HRP-conjugated solution with 9950 uL of conjugate diluent.

Biofluid Assay Protocol

1. Add 100 uL of blank control sample, positive control samples, and diluted study samples to appropriate microtiter wells.

2. Cover plate and incubate for 30 min at room temperature while shaking at approximately 750 rpm.
3. Wash plate 5 times with prepared wash buffer.
4. Add 100 uL of HRP-conjugated mAb solution to each well.
5. Cover plate and incubate for 30 min at room temperature while shaking at approximately 750 rpm.
6. Wash plate 5 times with wash buffer.
7. Add 100 uL of TMB substrate to each well.
8. Cover plate and incubate for 30 min at room temperature while shaking at approximately 750 rpm.
9. Add 100 uL of stop solution (1M H_2SO_4 solution).
10. Read optical density (OD) at 450 nm in a microtiter plate reader.
11. Calculate the OD upper limit of detection (ULOD) for Hp1-2 phenotype as the Hp2-2 OD multiplied by 0.6.
12. Interpret results from the O.D. of each sample
    a. Hp1-1 = O.D. < 2
    b. Hp1-2: O.D between 0.2 and calculated ULOD
    c. Hp2-2: OD > calculated ULOD

Test Validation Criteria

1. The O.D. of the blank should be < 0.2.
2. The O.D. of the Hp1-1 positive control should be < 0.2

3. The O.D of the Hp1-2 positive control should be between 0.2 and the calculated upper limit of detection for the Hp1-2 phenotype

APPENDIX 4: CYTOKINE ANALYSIS PROTOCOL

Reagent and Sample Preparation

1. Allow all reagents, controls, and samples to reach room temperature (22-28°C)
2. Prepare calibrator dilutions from calibrator standard using a serial 1:4 dilution technique with supplied diluent to create calibrator standard samples #1 to #8.
3. Dilute plasma samples (1:2) using supplied diluent.
4. Reconstitute control samples using supplied diluent.
5. Dilute antibody detection samples (1:50) using supplied diluent.
6. Dilute wash buffer (1:20) using deionized water.
7. Dilute read buffer (1:2) using deionized water.

Biofluid Assay Protocol

1. Wash ELISA plate 3 times with 150 uL of wash buffer.
2. Add 50 uL of prepared plasma samples, calibrators, and controls to designated wells. Seal the plate with an adhesive plate seal and incubate at room temperature for 2 hours.
3. Wash ELISA plate wells with 150 uL of wash buffer.
4. Add 25 uL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature for 2 hours.
5. Wash ELISA plate wells with 150 uL of wash buffer.
6. Add 150 uL of prepared red buffer to each well.

7. Analyze ELISA plate on appropriate MSD instrument. Determine target cytokine concentrations from results of calculated calibration curves.

APPENDIX 5: sCD163 CONCENTRATION ANALYSIS PROTOCOL

Reagent and Sample Preparation

1. Allow all reagents, controls, and samples to reach room temperature (22-28°C)
2. Prepare wash buffer solution from dilution of 20 mL of wash buffer in 480 mL of deionized water.
3. Prepare calibrator diluent solution from dilution of 20 mL of calibrator diluent in 20 mL of deionized water.
4. Prepare serial calibrator dilutions from 300 uL calibrator standard using a serial 1:2 dilution technique with 300 uL of deionized water to create calibrator standard samples #1 to #8 with concentrations ranging from 200 ng/mL to 1.56 mg/mL.
5. Prepare substrate solution by mixing color reagents A and B together (1:1) within 15 minutes of use.
6. Prepare biofluid samples (plasma or CSF) using a 1:4 dilution technique with 15 uL of biofluid and 45 uL of prepared calibrator diluent.

Biofluid Assay Protocol

1. Add 100 uL of calibrator diluent to appropriate microtiter wells.
2. Add 50 uL of prepared biofluid samples, calibrators, or controls to designated wells. Seal the plate with an adhesive plate seal and incubate at room temperature for 2 hours.

3. Aspirate liquid from each well then wash each well with 400 uL of wash buffer. Perform this step 4 times.
4. Add 200 uL of detection antibody solution to each well. Seal the plate with an adhesive plate seal and incubate at room temperature for 2 hours.
5. Aspirate liquid from each well then wash each well with 400 uL of wash buffer. Perform this step 4 times.
6. Add 200 uL of substrate solution to each well. Incubate for 30 minutes at room temperature. Protect from light.
7. Add 50 uL of stop solution to each well.
8. Determine the optical density of each well using a microplate reader set to 450 nm with wavelength correction set to 540nm.
9. Create a standard curve using a log-log curve-fit from the calibrator and control samples results.

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VITA

Education and Post-Graduate Training

08/94 – 05/98	Bachelor of Arts (cum laude) Transylvania University Lexington, KY, USA
09/98 – 05/02	Doctor of Medicine (High Distinction) University of Kentucky College of Medicine Lexington, KY, USA
07/02 – 06/06	Anesthesiology Resident (Clinical) University of Kentucky Chandler Medical Center Lexington, KY, USA
07/06 – 06/07	Anesthesiology Critical Care Medicine Fellow (Clinical) Shand's Hospital at the University of Florida Gainesville, FL, USA

Academic Positions Held

07/07 – 06/12	Assistant Clinical Professor Departments of Anesthesiology and Surgery University of Kentucky College of Medicine Lexington, KY, USA
07/12 – 06/19	Associate Clinical Professor Departments of Anesthesiology and Surgery University of Kentucky College of Medicine Lexington, KY, USA
07/19 – Present	Clinical Professor Departments of Anesthesiology and Surgery University of Kentucky College of Medicine Lexington, KY, USA

Scholastic and Professional Honors

Junior Faculty Member of the Year Department of Anesthesiology University of Kentucky College of Medicine	2010

Abraham Flexner Master Educator Award, Outstanding Teaching Contribution or Mentorship Center for Excellence in Medical Education University of Kentucky College of Medicine	2011
Presidential Citation Society for Critical Care Medicine	2014
Fellow, American College of Critical Care Medicine Society for Critical Care Medicine	2015
Presidential Citation Society for Critical Care Medicine	2016
Wethington Research Award University of Kentucky	2020

Professional Publications – Peer Reviewed

1. Dorfling J, **Hatton KW**, Hassan Z. Integrating echocardiography into human patient simulator training of anesthesiology residents utilizing a severe pulmonary embolism scenario. Simul Healthcare. 2006; 1(2): 79-83.

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13. Hayes D, **Hatton KW**, Feola DJ, Murphy BS, Mullett TW. Airway dehiscence after lung transplantation in a patient with cystic fibrosis. *Respir Care*. 2010 Dec;55(12):1746-50.

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15. Craig J, Flynn J, Hatton KW. Managing postoperative nausea and vomiting: current controversy. *Orthopedics*. 2011;34(1):28-32.

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17. Harned ME, Dority J, **Hatton KW**. Transient neurologic syndrome: a benign but confusing clinical problem. *Adv Emerg Nurs J.* 2011; 33(3):1-5.

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23. Langley T, Dority J, Fraser J, **Hatton KW**. A comprehensive onboarding and orientation plan for neurocritical care advanced practice providers. *J Neurosci N*. 2018;50(3):157-60.

24. Srour H, Pandya K, Flannery A, **Hatton K.** Enteral Guanfacine to Treat Severe Anxiety and Agitation Complicating Critical Care After Cardiac Surgery. *Semin Cardiothorac Vasc Anesth*. 2018;22(4):403-6.

25. **Hatton KW**, Bacon JD, McKinney K, Schell RM. A Novel Educational Tool to Guide Reintegration of Graduate Medical Education Trainees into Clinical and Academic Work Following Concussion. *A A Pract*. 2019;12(9):336-9.

26. Faulkner AL, Bacon JD, Fischer B, Grupke SL, **Hatton KW**. Successful Extracorporeal Membrane Oxygenation (ECMO) Use Without Systemic Anticoagulation for Acute Respiratory Distress Syndrome in a Patient with Aneurysmal Subarachnoid Hemorrhage. *Case Rep Neurol Med.* 2019; 2019: 9537453.

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choice between intravenous cocaine and a non-drug alternative in humans and rhesus monkeys. Exp Clin Psychopharmacol. 2020;28(2):169-80.

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32. Bacon JD, Slade E, Smith A, Allareddy G, Ran D, Fraser JF, **Hatton KW**. The Development and Validation of a Simple Tool for Predicting Potentially Harmful Ionizing Radiation Exposure (PHIRE) from Diagnostic Tests and Medical Procedures in Patients with Aneurysmal Subarachnoid Hemorrhage. *World Neurosurg.* Accepted for publication on 5/5/2020.

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34. Wang WX, Spring JE, Xie K Fardo DW, **Hatton KW**. A highly predictive microRNA panel for determining cerebral vasospasm risk following aneurysmal subarachnoid hemorrhage." *Biomark Res*. Submitted 09/23/2020.

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<u>Professional Publications – Non-Peer Reviewed</u>

1. **Hatton KW**, Hassan Z, Fuhrman T. Chest tubes: a review of the indications for placement and subsequent care. *NBRC Horizons* 2005; 19(4): 2-3.

2. **Hatton KW**. Con: recombinant activated Factor VII should only be used as salvage therapy after cardiac surgery. *ASCCA Interchange* 2009; 20(3): 4.

3. **Hatton KW**. Literature Review: The sequential organ failure assessment score for predicting outcome in patients with severe sepsis and evidence of hypoperfusion at the time of emergency department presentation. *ASCCA Interchange* 2009; 20(4): 9.

4. **Hatton KW**. Fellowship review III: critical care medicine fellowship at the University of Florida. *ASCCA Interchange* 2010; 21(1): 11.

5. **Hatton KW**. Literature Review: Sodium bicarbonate to prevent increases in serum creatinine after cardiac surgery: a pilot double-blind, randomized controlled trial. *ASCCA Interchange.* 2010; 21(1): 13.

6. **Hatton KW**, Dority J. Literature review: prophylactic intravenous magnesium sulfate for treatment of aneurysmal subarachnoid hemorrhage. *ASCCA Interchange.* 2010; 21(3): 14.

7. **Hatton KW.** Con: the PAC should not be abandoned for hemodynamic monitoring in septic shock! *ASCCA Interchange* 2010; 21(4):7.

8. **Hatton KW.** Literature Review: Fluids after cardiac surgery: a pilot study of the use of colloids versus crystalloids. *SOCCA Interchange* 2011; 22(2):12.

9. **Hatton KW**. Practical advances in Extracorporeal Life Support (ECLS) for acute and chronic cardiac and respiratory system disease. *SOCCA Interchange* 2013; 24(4): 3-4.

10. **Hatton KW**. A brief conversation with…Aryeh Shander. *SOCCA Interchange*. 2015; 26(2): 1-2.

11. **Hatton KW**. A brief conversation with…Philip Boysen. *SOCCA Interchange.* 2015; 26(3): 7-8.

12. **Hatton KW**, Gesin G. Drug shortages in critical care: Can good come from the bad? *SCCM Critical Connections.* 2015; 6(3):1-2.

13. Ploetz J, Moyer A, **Hatton KW**. Leveraging IT solutions to improve outcomes during critical drug shortages. *SCCM Critical Connections*. 2016.

14. **Hatton KW**. A brief conversation with…Avery Tung. *SOCCA Interchange*. 2016; 27(1): 9-10.

15. **Hatton KW**. A brief conversation with...Jacob Gutsche. *SOCCA Interchange*. 2017; 28(1): 3.

16. **Hatton KW**. A brief conversation with…David Shimabukuro. *SOCCA Interchange*. 2017; 28(2): 5-6.

17. **Hatton KW**. A brief conversation with…Roman Dudaryk. *SOCCA Interchange*. 2017; 28(3): 8-9.

18. **Hatton KW**. A brief conversation with…Todd Dorman. *SOCCA Interchange*. 2018; 29(1): 6-7.

19. **Hatton KW**. A brief conversation with…Sean Josephs. *SOCCA Interchange*. 2018; 29(2): 4-5.

20. Gesin G, Barletta JF, Brown DR, **Hatton KW**. Alternative analgesic and sedative agents in the setting of drug shortages during the COVID-19 pandemic. SCCM Critical Connections. Submitted for publication on 05/18/2020.

Professional Publications – Books Edited

1. Boling B, **Hatton K**, Hartjes T, eds. *Concepts in Critical Care*. Jones and Bartlett Learning: Burlington, MA; 2020.

Professional Publications – Book Chapters

1. **Hatton KW**, Fuhrman T. Electrolyte disorders: derangements of sodium, potassium, calcium and magnesium. In: Szalados JE, Rehm CG, eds. *Adult Multiprofessional Critical Care Review*. Mount Prospect, IL: Society of Critical Care Medicine; 2007: 283-92.

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3. **Hatton KW**, Hatton KC. Renal replacement therapy in critically ill patients. In: Rehm CG, ed. 13th Critical Care Refresher Course. Mount Prospect, IL: Society of Critical Care Medicine; 2009: 113-8.

4. **Hatton KW**, Fahy BG. Glucose control for the diabetic patient requiring cardiothoracic surgery: does it matter? In: Cohen N, ed. *Medically challenging*

patients undergoing cardiothoracic surgery. Philadelphia, PA: Lippincott, Williams and Wilkins; 2009: 109-28.

5. Flynn JD, **Hatton KW**. Perioperative management of antithrombotic therapy. In: Richardson M, Chant C, Cheng JWM, Chessman KH, Hume AL, Hutchinson LC, et al., eds. Pharmacotherapy Self-Assessment Program, 7th edition. Cardiology. Lenexa, KS: American College of Clinical Pharmacy, 2010:153-169.

6. Shannon JD, **Hatton KW**, Elamin EM. Disordered glucose metabolism. In: Gabrielli A, Layon AJ, Yu M, eds. *Civetta, Taylor, & Kirby's Manual of Critical Care.* Philadelphia, PA: Lippincott, Williams and Wilkins; 2012: 856-62.

7. **Hatton KW**. Neurologic injury and mechanical ventilation. In: Layon AJ, Gabrielli A, Friedman WA, eds. *Textbook of Neurointensive Care, 2nd ed*. London, England: Elsevier Inc; 2013: 217-240.